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Molecular detection and characterization of tick-borne pathogens of domestic animals in Malawi

（マラウィの家畜におけるマダニ媒介性病原体の分子学的解析）

Elisha CHATANGA

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Abbreviations

°C	degrees celsius
µL	microliter
6-FAM	flouresence labelling dye
AMOVA	analysis of molecular variance
AU-CTTBD	African Union centre for ticks and tick-borne diseases
bp	base pair
BTH	*Babesia*, *Theileria* and *Hepatozoon*
$CD8^+$/ CLT	cytotoxic T lymphocytes
CSO	Central Statistical Office
DAHLD	Department of Animal Health and Livestock Development
DDBJ	DNA Data Bank of Japan
Df	degrees of freedom
DISTMAT	distance matrix
DNA	deoxyribonucleic acid
DnaSP	DNA sequence polymorphism
dNTP	deoxyribose nucleotide triphosphate
ECF	East Coast fever
EDTA	ethylene diaminete traacetic acid
EHR	*Ehrlichia*
F	forward
F_{CT}	fixed indice of variation among groups of populations
F_{SC}	fixed indice of variation among populations within groups
F_{ST}	Wright's fixation index
FTA	Flinders technology association
GAVmed	The global alliance for livestock veterinary medicines
gltA	citrate synthase gene
GoM	Government of the republic of Malawi
groEL	heat shock protein gene
GTR	general time reversible
H_e	estimated heterozygosity
I_AS	standardized index of association
ITM	infection and treatment method
kD	kilo Dalton

LD	linkage disequilibrium
LE	linkage equilibrium
LSPCA	Lilongwe society for the protection and care of animals
LUANAR	Lilongwe University of Agriculture and Natural Resources
MC	Muguga cocktail vaccine
MEGA	molecular evolutionary genetics analysis
MHC	major histocompatibility complex
MJ	median joining
ML	maximum likelihood
MLG	multilocus genotyping
MS	minisatellite
ms	microsatellite
MW	Malawi
NC	negative control
NCO	no co-infection observed
NSO	National Statistical Office
OIE	World Organisation for Animals Health
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
R	reverse
rDNA	ribosomal RNA gene
RLB	reverse line blot
RNA	ribonucleic acid
SD	standard deviation
SNP	single nucleotide polymorphism
TBDs	tick-borne diseases
TBPs	tick-borne pathogens
Tp1	*Theileria parva* CTL antigen gene 1
*Tp*2	*Theileria parva* CTL antigen gene 2
UV	ultra violet
Var.	variant
VD	mismatch variance

Notes

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GENERAL INTRODUCTION

Tick-borne pathogens (TBPs) belonging to the genera *Anaplasma*, *Babesia*, *Ehrlichia*, *Hepatozoon* and *Theileria* remain a major challenge to the health of domestic animals due to the impact of the tick-borne diseases (TBDs) they cause in these animals worldwide. Malawi is located in the tropics in southern Africa within latitudes 9° and 18° S and longitudes 32° to 36° E, covering an area of 118,484 km^2. It is bordered by Mozambique to the south east, Tanzania to the north and Zambia to the west. The country is divided into three geographical regions, northern, central and southern and has a climate that favours the thriving of the vector ticks.

Malawi cattle population is estimated at 1,884,803 heads of which the indigenous Malawi zebu accounts for 91.2% (1,719,641) while the exotic and crossbred cattle account for the remaining 8.8% (165,862) as of 2020. The population of goats, sheep, pigs, chickens and dogs is estimated at 10,073,975, 356,258, 8,362,551, 171,408,370 and 508,934, respectively (DAHLD, 2020). With an estimated human population of 17,563,749 as of 2018 (NSO, 2018), Malawi has one of the lowest cattle population per capita in Africa. Approximately 71% of the total pure and crossbred dairy cattle in Malawi are found in the southern region which is considered to be non-endemic to East Coast fever (ECF) (DAHLD, 2006). The endemic status of ECF has hampered the establishment of the dairy industry in the central and northern regions of the country (Chinombo et al., 1988; Lawrence et al., 1996).

More than 20 tick species belonging to six genera; *Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Ornithodoros* and *Rhipicephalus* have been reported to infest domestic animals in Malawi (Walker et al., 2003; Pegram et al., 1987; Berggren, 1978). These are *Amblyomma variegatum*, *Haemaphysalis elliptica* formerly called *Haemaphysalis leachi*, *Hyalomma marginatum rufipes*, *Hyalomma truncatum*, *Ixodes cavipalpus*, *Ornithodoros moubata*, *Rhipicephalus appendiculatus*, *Rhipicephalus compositus*, *Rhipicephalus decoloratus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus kochi*, *Rhipicephalus longus*, *Rhipicephalus lunulatus*, *Rhipicephalus maculatus*, *Rhipicephalus masseyi*, *Rhipicephalus microplus*, *Rhipicephalus pravus*, *Rhipicephalus punctatus*, *Rhipicephalus reichenowi*, *Rhipicephalus sanguineus*, *Rhipicephalus simus*, *Rhipicephalus sulcatus*, *Rhipicephalus tricuspis* and *Rhipicephalus turanicus*.

Ticks transmit a lot of pathogens to both humans and animals. These include protozoal (babesiosis/ redwater, hepatozoonosis and theileriosis) (de la Fuente et al., 2008), viral (African swine fever), bacterial (African tick bite fever, anaplasmosis/gall sickness and ehrlichiosis/heartwater) pathogens. Tick bites cause toxic reactions (Mans et al., 2014), allergic responses (Van Nunen, 2015) and lethal paralysis (Lysyk and Majak, 2003). Furthermore, the inflicted wounds on animals may act as portal of entry for secondary microbial infections such as dermatophilosis in cattle caused by *Dermatophilus congolensis* which is associated with bites of the tropical bont tick *Amblyomma variegatum* (Levin, 2020), and diminish the value of livestock by damaging their hides (Levin, 2020).

African swine fever virus (ASFV), which is transmited by the tampan tick *Ornithodoros moubata*, causes a lot of outbreaks every year among pigs in Malawi and is economically the most important viral TBDs in Malawi (Haresnape, 1984, Haresnape et al., 1985, 1987). Crimean Congo haemorrhagic fever (CCHF), which is transmitted by the ticks of the genus *Hyalomma*, is an important zoonotic viral infection that has been reported in 30 African countries including Malawi (Ergonul, 2012; Chitanga et al., 2014; Phonera et al., 2021).

Anaplasmosis (gall sickness) in cattle is predominantly caused by *Anaplasma marginale*, which is widespread on the African continent. The *Anaplasma* species that have been reported to infect cattle in southern Africa are *Anaplasma marginale*, *Anaplasma bovis*, and *Anaplasma centrale* (Tembo et al., 2018). Other *Anaplasma* species which have been reported to infect cattle include *Anaplasma platys*-like and *Anaplasma phagocytophilum* (Ogata et al., 2021; Peters et al., 2020. Ben Said et al., 2017). In sheep and goats, anaplasmosis is caused by *Anaplasma ovis* although it is generally asymptomatic in immunocompetent animals. Dogs are infected with *Anaplasma platys* and *Anaplasma phagocytophilum* which causes canine cyclic thrombocytopenia and canine granulocytic anaplasmosis, respectively (Sykes and Foley, 2014; Wang et al. 2019; Guzman et al. 2020).

Ehrlichiosis (heart water) in cattle is mainly caused by *Ehrlichia ruminantium*. Other *Ehrlichia* species such as *Ehrlichia minasensis* (Peters et al., 2020) and *Ehrlichia* sp. Omatjenne (Tembo et al., 2018) have been reported in cattle although their effect on animal health has not yet been fully explored. Ovine ehrlichiosis is also caused by *Ehrlichia ruminantium* (Deem, 2008), while canine ehrlichiosis is caused by *Ehrlichia canis* (Qiu et al., 2018). Other *Ehrlichia* species that infect domestic animals include *Ehrlichia chaffeensis*, *Ehrlichia ewingii* and *Ehrlichia muris* (Qurollo et al. 2014).

Babesiosis in cattle is mainly caused by *Babesia bigemina*, *Babesia bovis* (Jacob et al., 2020) and *Babesia* sp. Mymegnsingh (Sivakumar et al., 2018). Other *Babesia* species that infect cattle are *Babesia divergens*, *Babesia major*, *Babesia occultans* and *Babesia argentina* (Jacob et al., 2020). Ovine babesiosis is mainly caused by *Babesia ovis*. Other *Babesia* species that infect sheep and goats are *Babesia crassa, Babesia foliata, Babesia motasi, Babesia taylori, Babesia* sp. Xinjiang and *Babesia* sp. Mymegnsingh (Sivakumar et al., 2018; Hashemi-Fesharki and Uilenberg, 1981). Canine babesiosis is caused by *Babesia canis*, *Babesia gibsoni*, *Babesia rossi* and *Babesia vogeli* (Irwin, 2009; Matjila et al., 2008; Qiu et al., 2018). In southern Africa, *Babesia rossi* is more commonly diagnosed than *Babesia vogeli* (Qiu et al. 2018; Matjila et al. 2008).

ECF in southern Africa is mainly caused by *Theileria parva* (Oura et al., 2003, 2007) while, in North Africa, southern Europe and Asia, it is caused by *Theileria annulata* (tropical theileriosis) (Morrison, 2015). *Theileria orientalis, Theileria taurotragi, Theileria mutans, Theileria sergenti, Theileria buffeli* and *Theileria velifera*, which are considered less or non-pathogenic, have also been reported to infect cattle (Morrison, 2015). Ovine theileriosis is predominantly caused by *Theileria lestoquardi*, *Theileria luwenshuni* and *Theileria uilenbergi* (Yin et al., 2007; Ahmed et al., 2006). The non-pathogenic species that have been

reported in sheep and goats are *Theileria ovis*, *Theileria separata*, *Theileria recondita* and *Theileria* sp. OT1 and OT3 (Jianxung and Yin, 1997).

Hepatozoonosis has been widely described in dogs where its causative agents are *Hepatozoon canis*, and *Hepatozoon americanum*. *Hepatozoon felis* has also been documented to cause clinical disease in cats. *Hepatozoon canis* infection in dogs has been reported in Zambia (Qiu et al., 2018).

The economic impact of ticks and TBDs resulting from morbidity, mortality, chemotherapy and the cost of control measures such as acaricide application has been investigated in other sub-Saharan African countries. In Tanzania, the annual losses due to ticks and TBDs was estimated at US$ 364 million (Kivaria, 2006), theileriosis accounted for 68% of these losses. In Uganda, the economic impact of TBDs in one district only around Lake Mburo National Park was estimated at US$ 308,144 (Ocaido et al., 2008). In Zambia, it was estimated that the effective control of ECF would cost an estimated annual amount of US$25.9 per animal (Minjauw et al., 1999), with an estimated cattle population of 3,654,668 (CSO, 2021), and this translates into US$ 95 million per annum. Those studies show that TBDs have a great economic impact in sub-Saharan Africa where the public funding for livestock production is usually shrinking compared to crop production which enjoys massive subsidies (GoM, 2021). However, in Malawi, the economic impact of ticks and TBDs has not yet been investigated.

Humans are also negatively impacted by ticks and zoonotic TBPs in southern Africa. *Anaplasma platys*, *Borrelia*–like organism causing human borreliosis and *Rickettsia africae* causing African tick bite fever (ATBF) have been documented in southern Africa (Qiu et al., 2019, 2018; Vlahakis et al., 2018; Beati et al., 2012). Serological survey has confimed the presence of CCHF virus (CCHFV) in cattle in Malawi, which also infect humans (Phonera et al., 2021).

The wildlife have also been implicated in the epidemiology of ticks and TBDs in southern Africa. In Zambia, Squarre et al. (2021) reported the infection of wildlife with *Babesia*, *Theileria*, *Hepatozoon* and *Colpodella* species. The African buffalo (*Syncerus caffer*) has been reported to be involved in the epidemiology of *Theileria parva* which is transmitted to cattle at the human, wildlife and livestock interface and is usually more pathogenic in cattle resulting in a fatal outcome (Morrison et al., 2020; Kock et al., 2014). The change in human behavior due to increasing human population where humans and domestic animals encroach protected wildlife reserves increases exposure to TBPs of wildlife. Although Malawi is well endowed with vast wildlife reserves throughout the country. There has been no attempt to investigate the epidemiology of ticks and TBPs in the wildlife.

The control of ticks and TBDs in Africa is mainly based on tick control by either intensive acaricide application or livestock movement control. This use of acaricides faces a lot of challenges due to increase in reports of tick resistance to acaricides, the contamination of the environment and the unsustainability of the costs incurred (George et al., 2004, Lawrence et al., 1996). The other approach is to manage the pathogens within the host through methods such as immunisation and chemotherapy (Di Giulio et al., 2009; Lawrence et al., 1996). Chemotherapy has proven to be expensive and unsustainable among smallholder livestock

farmers (Chinombo et al., 1988). Those studies and reports show that there is a need to develop other novel methods to control tick and TBDs in Africa.

In Malawi, the control of ticks and TBDs was mainly based on intensive acaricide application which was implemented by government from 1920-1996 (Lawrence et al., 1996; Norman and Soldan , 1994), through operating dip tanks countrywide where smallholder farmers would dip their animals weekly free of charge. When the government stopped offering dipping services in 1996, farmers have failed to operate the diptanks which has now resulted in high tick infestation in domestic animals as mainly commercial farms have the capacity to dip their animals to control ticks (DAHLD, 2006). Majority of the cattle in Malawi are Malawi zebu owned by smallholder farmers who keep them under extensive grazing in communal grazing lands. This allows mixing of cattle from different herds and increases exposure to ticks and TBPs.

Currently, immunization of cattle using the Muguga cocktail (MC) live vaccine (composed of three stocks of *Theileria* strains namely; Muguga, Kiambu-5 and Serengeti transformed) is used to control ECF in Malawi. However, concerns over the MC vaccine components undergoing expansion in vaccinated animals, the carrier status of vaccinated animals which continue to infect ticks and the ability of vaccine strain being transmitted to unvaccinated cattle by ticks have affected the adoption of MC vaccine (Geysen et al., 1999; De Deken et al., 2007; Oura et al., 2007). There is now a general interest in investigating the potential of using sub-unit vaccine. The genes that encode the *Theileria parva Tp1* and *Tp2* antigens are the current candidate of the antigens for sub-unit vaccine (Taracha et al., 1995; Graham et al., 2006, 2007, 2008; MacHugh et al., 2009).and they have also been used to characterize the strains of *Theileria parva* in eastern and southern African countries (Mwega et al., 2015; Salih et al., 2017). However, due to the limited coverage of the *Theileria parva* chromosome region by *Tp1* and *Tp2* genes, satellite markers which cover all the four chromosomes of *Theileria parva* have been designed (Oura et al., 2003, 2005; Katzer et al., 2006, 2010) and are currently being used to study the population structure of *Theileria parva* in endemic countries to investigate the relatedness of the local strains to the MC vaccine which is currently used in eastern and southern African countries (Muleya et al., 2012; Salih et al., 2018; Lubembe et al., 2020).

The diagnosis of TBPs in Malawi is mainly based on basic parasitological blood smear examination. With the availability of new and more sensitive molecular methods, it is important to improve the diagnostic methods of TBPs in Malawi especially at the national reference laboratory (Central Veterinary Laboratory; CVL) in Lilongwe. The molecular techniques are more accurate and have higher sensitivity when compared to blood smear examination. However, molecular methods required expensive equipment when compared to the basic blood smear examination.

Although the tick species that have been reported in Malawi are known vectors of a widerange of pathogens of domestic animals, there has been no attempt to detect and characterize the TBPs of domestic animals in Malawi. While the neighbouring countries of Malawi including Mozambique (Martins et al., 2008, 2010), Tanzania (Ringo et al., 2019, 2020) and Zambia (Tembo et al., 2018; Simuunza et al., 2011; Makala et al., 2003) have made much progress in molecular studies on TBPs in domestic animals, Malawi

has lagged behind. Thus, there was a great need to carry out an investigation on the TBPs of domestic animals in Malawi using molecular techniques.

In the first chapter, molecular survey of *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species infecting cattle in Malawi was conducted using conventional PCR methods and sequencing. Due to the economic importance of ECF in Malawi, in the second chapter, the study narrowed down on the characterization of the *Theileria parva* by investigating the genetic diversity and sequence polymorphism of two genes (*Tp1* and *Tp2*) that encode *Theileria parva* antigens recognized by bovine $CD8^+$ T cells and are the current candidate of the antigens for sub-unit vaccine was conducted. Thereafter, in the third chapter, the study further investigated the genetic composition of *Theileria parva* in Malawi by targeting the markers that cover all the four chromosomes of *Theileria parva* by carrying out population structure analysis using nine satellite markers. In the fourth chapter, the study was extended to the TBPs of sheep and goats as other domestic animals may act as source of infection to cattle. In this chapter, the molecular detection and characterization of TBPs of sheep and goats in Malawi was conducted. Finally in the fifth chapter, the study was furthermore extended to investigate the TBPs of dogs due to their close contact to both humans and other domectic animals. In this chapter, the molecular identification and characterization of tick-borne haemoparasites and *Anaplasmataceae* of dogs in Malawi was also conducted. The sampled animals did not show any clinical signs during sampling except dogs in chapter V and the clinical information on dogs has been provided.

Chapter I

Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens of cattle in Malawi

1. Introduction

Bovine anaplasmosis (gall sickness) is caused by *Anaplasma marginale* infection, and the clinical disease is characterized by anaemia, icterus, fever, weight loss, abortion, lethargy and in severe cases may be fatal (Kocan et al., 2003). The occurrence of clinical disease is dependent on the age of the animal, as susceptibility increases with age. Calves under 6 months of age are generally less susceptible (Kocan et al., 2003; Richey and Palmer, 1990). In Malawi, cases of bovine anaplasmosis have been reported but its causative agents have not been characterized. *Anaplasma marginale* is transmitted by the vector ticks *Amblyomma variegatum*, *Rhipicephalus decoloratus* and *Rhipicephalus evertsi evertsi* (Walker et al., 2003). Cattle may also be infected with *Anaplasma centrale* and *Anaplasma bovis* formerly referred as *Ehrlichia bovis* (Dumler et al., 2001), which cause benign anaplasmosis. *Anaplasma platys*-like infection in cattle has also been reported in Bolivia (Ogata et al., 2021), Kenya (Peters et al., 2020), and Tunisia (Ben Said et al., 2017) although its clinical impact on cattle has not yet been fully explored.

Bovine ehrlichiosis (heartwater) is caused by *Ehrlichia ruminantium* formerly known as *Cowdria ruminantium* in Africa. The clinical disease is characterized by non-specific signs such as fever, anorexia, aggressiveness and sudden death (Allsopp, 2015). The vector ticks of *Ehrlichia ruminantium* in southern Africa are *Amblyomma hebraeum* and *Amblyomma variegatum* (Walker et al., 2003). Other uncharacterized *Ehrlichia* species closely related to *Ehrlichia minasensis* but separated from the pathogenic *Ehrlichia ruminantium* have been reported to infect cattle in Kenya (Peters et al., 2019, 2020).

Bovine babesiosis (red water) which is caused by *Babesia bovis* and *Babesia bigemina* has also been reported in Malawi (Lawrence et al., 1996). The newly described *Babesia* sp. Mymegnsingh has also been documented to cause clinical disease in cattle in Argentina, Phillipines, Sri Lanka and Vietnam (Sivakumar et al., 2018, 2020). The clinical disease is characterized by high fever, haemoglobinuria, dark coloured urine, anorexia, lethargy and neurological signs which usually result in fatal outcome (Uilenberg, 1995). The vector ticks for *Babesia bigemina* and *Babesia bovis* in southern Africa are *Rhipicephalus microplus*, *Rhipicephalus decoloratus* and *Rhipicephalus evertsi evertsi* (Walker et al., 2003). Other *Babesia* species that have been reported to cause clinical disease in cattle are *Babesia divergens*, *Babesia major*, *Babesia occultans*, and *Babesia argentina* (Jacob et al., 2020).

ECF (one of bovine theilerioses) is caused by *Theileria parva* and is considered the most economically important tick-borne disease in Africa (Lubembe et al., 2020). Calves of indigenous zebu cattle below the age of 6 months are highly susceptible to the clinical disease (Moll et al., 1984, 1986). The disease is severe in exotic and crossbred cattle with morbidity and mortality rates ranging from 80-100% (Oura et al., 2007). The clinical disease is characterized by anaemia, icterus, tachypnea, tachycardia, lethargy, froth coming out of the mouth, anorexia, fever, abortion and enlarged superficial lymph nodes (Nene et al., 2015). *Theileria parva* is transmitted by the brown ear tick *Rhipicephalus appendiculatus* (Walker et al., 2013). Other *Theileria* species that infect cattle but cause benign theileriosis in southern Africa are *Theileria mutans*, *Theileria taurotragi* and *Theileria velifera* (Ringo et al., 2020; Tembo et al., 2018). The pathogenic

Theileia species of cattle reported elsewhere include *Theileria annulata* (OIE, 2014) and *Theileria orientalis* (Gebrekidan et al., 2016).

This study aimed to provide molecular epidemiological data on the current prevalence and characterization of TBPs infecting cattle in Malawi. This epidemiological data is a pre-requisite for the development of novel control measures and monitoring current measures against tick and TBPs in Malawi to achieve the national goal of Malawi being self-sufficient in safe locally produced livestock and livestock products.

2. Materials and methods

2.1 Study site and sample collection

Cattle blood samples (*n* = 619) were collected from four districts in the central region and one district in the southern region of Malawi (Figure I-1) from February 2018 to March 2019 during the rainy seasons (November to April) from apparently healthy animals. In Kasungu district (*n* = 199), samples were collected from Chulu Extension Planning Area (EPA) (12° 49′ 03″ S, 33° 18′ 10 ″E) (*n* = 62), Lisasadzi EPA (13° 16′ 21″ S, 33° 08′ 11″ E) (*n* = 72), and Chipala EPA (13° 07′ 03″ S, 33° 19′ 07″ E) (*n* = 65). In Nkhotakota district (*n* = 185), samples were collected from Mphonde EPA (12° 48' 19" S, 34° 11' 27" E) (*n* = 84), and Linga EPA (12° 56′ 09″ S, 34° 13′ 36″ E) (*n* = 101). In Lilongwe district (*n* = 156), samples were collected from Katete farm (14° 01′ 24″ S, 33° 45′ 17″ E) (*n* = 62), and LUANAR student farm (14º 17' 96" S, 33º 77' 83" E,) (*n* = 51). In Mchinji district (*n* = 94), all samples were collected from Likasi farm (14º 02' 43" S; 33º 17' 05" E). In the southern region, the samples were collected from Chiradzulu district at Mikolongwe farm (15º 51' 49" S; 35º 12' 30" E) (*n* = 28).

The animals investigated in Kasungu and Nkhotakota were Malawi Zebu cattle that were managed under extensive grazing in communal grazing lands. In this management system, animals from different smallholder farms were mixed freely and there was no ECF vaccination or dipping history to control ticks. The animals investigated at Katete farm in Lilongwe were Holstein Friesian which were kept under a semi-intensive management with no contact with other herds. They were dipped weekly during rainy season and fortnightly during dry season to control ticks and MC vaccine was also used to control ECF in some animals. A total of 30 of the sampled animals at Katete farm were vaccinated with Muguga cocktail vaccine 2 years and 3 months prior to sampling period while the remainder (*n* = 32) were unvaccinated but these animals had been co-grazing for more than 2 years. At Likasi, LUANAR and Mikolongwe farms both exotic dairy breed Holstein Friesian and local Malawi zebu were kept. The animals were dipped fortnightly and MC vaccine was used to immunize the exotic breeds but not the local Malawi zebu. Approximately 5 ml of whole blood was collected by venipuncture of the external jugular vein after disinfection of the puncture site with methylated spirit cotton swab into ethylene diaminete traacetic acid (EDTA) vacutainer tube.

2.2 DNA extraction

DNA was extracted from 200 µl of whole blood using the Quick Gene DNA whole blood kit S (DB-S) (Kurabo Industries Ltd, Osaka, Japan) according to the manufacturer's recommendations. The extracted DNA was stored at -20°C until required for use.

2.3 Polymerase chain reaction

The screening for *Anaplasma* and *Ehrlichia* species was done using EHR polymerase chain reaction (PCR) assay targeting the V1 hypervariable region of the 16S small subunit ribosomal RNA gene (rDNA) as described by Parola et al. (2000). All PCR reactions were conducted in a 25 µl reaction mixture containing 0.5 µl of Tks Gflex DNA Polymerase 1.25 units/ µl (TaKaRa Bio Inc., Shiga, Japan), 12.5 µl of 2× Gflex PCR Buffer (Mg^{2+}, dNTP plus), 200 nM of each primer, 1.0 µl of template DNA and molecular grade water. The characterization of *Anaplasmataceae* was done using the citrate synthase gene (*gltA*) and heat shock protein gene (*groEL*) nested PCR assays as previously described (Rar et al., 2011; Liz et al., 2000; Grofton et al., 2006; Inokuma et al., 2005). The *groEL* gene secondary PCR assays has two sets: one that is specific for the genus *Anaplasma* and another that is specific for the genus *Ehrlichia*. This allows detection of mixed infection of *Anaplasma* and *Ehrlichia* spp. in a sample.

The screening for *Babesia* and *Theileria* species was done using reverse line blot (RLB) PCR assay targeting the V4 hypervariable region of the 18S rDNA (Gubbels et al., 1999). To further characterize the piroplasms almost the full length of the 18S rDNA gene was amplified using nested PCR assays targeting *Babesia*, *Theileria* and *Hepatozoon* (BTH) species as previously described (Masatani et al., 2017). The reaction mixtures were set as described above, while the cycling conditions for both PCR assays were set as previously described (Qiu et al., 2018). Mixed signals were observed on Sanger sequences of few selected samples due to co-infection among the piroplasms. Then a multiplex PCR assay for *Babesia bigemina, Babesia bovis, Theileria mutans, Theileria parva, Theileria taurotragi* and *Theileria velifera* that could discriminate theese pathogen to species level was designed. The first BTH PCR product that was diluted 100 fold was used as DNA template for the BTH multiplex PCR assay. The primers, their annealing temperatures and the expected amplicon sizes are listed in Table I-1.

2.4 Cloning

Due to high levels of mixed infections observed in the piroplasms based on the 18S BTH multiplex PCR assay and mixed signals in few sequences based on 2nd BTH PCR products, 15 samples were selected for cloning to obtain single colonies for sequencing. The high-fidelity PCR enzyme, KOD-Plus-Neo Ver.-2 DNA polymerase (Toyobo, Osaka, Japan), was used. The reaction mixture and the reaction conditions were set as previously described (Qiu et al., 2018). The cloning PCR product was A-tailed using 10 × A-attachment Mix (Toyobo) and then cloned into a T-vector pMD20 (TaKaRa Bio Inc.).

2.5 Sequencing

All samples that were positive on 2nd *groEL* gene PCR (n = 110) were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, CA, USA) and sequenced. Thereafter, based on the species identified from the obtained *groEL* gene sequences, five randomly selected samples per *Anaplasma*

and *Ehrlichia* species from the 2nd *gltA* gene PCR for sequencing. Approximately, 20 colonies were obtained from each sample after cloning, and the colony PCR products were purified by using a NucleoSpin Gel and PCR Clean-Up Kit (Takara Bio Inc.). Sequencing was done in both directions using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and ABI genetic analyzer 3500xl (Applied Biosystems, CA, USA). Sequence data editing was conducted using ATGC software (GENETYX Corporation, Tokyo, JP) by trimming the primers annealing sites and the consensus sequences were extracted for phylogenetic analysis. From the colony PCR products, if three sequences from the same sample were identical they were considered to be genuine and were retained for further analysis. The sequences generated in this study were submitted to the DNA Data Bank of Japan (DDBJ) (*http://www.ddbj.nig.ac.jp/*).

2.6 Statistical analysis

The significance of co-infections was examined by statistical modeling using generalized linear model (Glm) using glm function in R version 4.1.2. Thereafter, the odds ratios were calculated based on modeling results using oddsration function in R which were eventually used for calculation of the correlation of co-infection using kendall method cor function in stats package in R software (*http://www.rstudio.com/*). The "Holm" method was used for adjusting the p-value over mutiple testing (*https://www.jstor.org/stable/2346101*). The $p < 0.05$ was considered significant.

2.7 Phylogenetic analysis

Alignments of the consensus nucleotide sequences generated from the amplified DNA fragments were done using ClustalW in Molecular Evolutionary Genetics Analysis (MEGA version 7) (Kumar et al. 2016). To further characterize the sequences obtained in this study, maximum likelihood (ML) phylogenetic trees were constructed using MEGA version 7 software (*https://doi.org/10.1093/molbev/msw054*) using the Tamura 2 parameter model with other sequences deposited in the GenBank.

3. Results

3.1 Polymerase chain reaction and sequencing

The screening for *Anaplasmataceae* using *groEL* gene nested PCR assay showed that 57.6% (110/191) were positive. The sequencing of all 2nd *groEL* gene PCR positive samples (n = 110) showed that cattle in Malawi were infected with *Anaplasma bovis* 5/191 (2.6%), *Anaplasma marginale* 47/191 (24.6%), *Anaplasma platys*-like 26/191 (13.6%), uncharacterized *Anaplasma* sp. 27/191 (14.1%) and uncharacterized *Ehrlichia* sp. 31/191 (16.2%) as shown in Table I-2.

The screening for piroplasms using BTH nested PCR assay showed that 153/191 (80.1%) were positive. Then, 15 randomly selected samples were sequenced using 2nd BTH PCR product to obtain almost full length 18S rDNA sequences for phylogenetic analysis. Mixed signals were observed in the sequences obtained due to mixed infection and a multiplex PCR was developed to identify the pathogens in mixed infections. Further screening for the piroplasms from the samples that were positive on the 2nd BTH PCR using multiplex PCR assay showed that the examined animals were infected with *Babesia bigemina* 5/191

(2.6%), *Theileria mutans* 141/ 191 (73.8%), *Theileria parva* 63/191 (33.0%), *Theileria taurotragi* 24/191 (12.6%) and *Theileria velifera* 102/191 (53.4%), as shown in Table I-2. Fifteen random samples were used for cloning to obtain single colonies for sequencing. To make sure that the obtained sequences were not due to error in sequencing, the sequence was considered genuine if sequences obtained from at least 3 colonies were identical.

3.2 Infection status of tick-borne pathogens in cattle

Overall, 177 cattle were infected with at least one TBP, representing 92.7% positive rate. The co-infections rate observed was 79.6% (152/191), and the number of pathogens co-infecting a sample ranged from two to six (Table I-3). Triple co-infection had the highest frequency at 39.3% (n = 75), followed by double co-infection at 28.3% (n = 54). Sextuple co-infection had the least frequency at 0.5% (n = 1). LUANAR farm had the highest co-infection rate at 91%, followed by Likasi, Mikolongwe, Nkhotakota, Kasungu and Katete at 85%, 79%, 78%, 77% and 61%, respectively (Table I-3). The summary of the pathogen combinations per sampling site has been provided in Table I-4. The double infections, combination with the highest frequency was *Theileria mutans* + *Theileria velifera* at 12.0% (n = 23). In triple infections, the highest frequency was observed in the combination *Theileria mutans* + *Theileria parva* + *Theileria velifera* at 7.9% (n = 15). For quadruple infections the highest frequency was observed in the combination *Anaplasma marginale* + *Theileria mutans* + *Theileria parva* + *Theileria velifera* at 2.6% (n = 5). Statistical analysis showed both positive and negative correlations between the pathogens observed in co-infections in this study. A strong positive correlation that was statistically significant was observed between uncharacterized *Anaplasma* species infection with *Anaplasma bovis* (p = < 0.001) and *Anaplasma platys*-like (p = 0.026). This study also showed statistically significant correlation between infection with *Theileria mutans* and *Theileria taurotragi* (p = 0.01) and *Theileria mutans* with *Theileria velifera* (p = < 0.001). However, there was no any statistically significant correlation between *Theileria parva* infection with *Theileria mutans* (p = 0.232), *Theileria taurotragi* (p = 0.778) and *Theileria velifera* (p = 0.444) (Table I-5).

3.3 Phylogenetic analysis

To determine the phylogenical relationship of the detected pathogens with those reported elsewhere phylogenetic trees were constructed as shown in Figures I-2, I-3 and I-4 for 18S rDNA of *Babesia* and *Theileria* species, *gltA* gene of *Anaplasma* and *Ehrlichia* species and *groEL* gene of *Anaplasma* and *Ehrlichia* species, respectively. For *Babesia bigemina*, *Theileria mutans*, *Theileria parva*, *Theileria taurotragi*, *Theileria velifera* and *Theileria* sp. Merck, Sharp & Dome (MSD)-like strain we obtained 1, 2, 2, 1, 1, and 1 consensus sequences, respectively which were included in the phylogentic tree. The *Babesia* and *Theileria* sequences obtained in this study clustered with homologous sequences available in the GenBank (Figure I-2). There was divergence in the clustering of *Theileria mutans* sequences obtained in this study, indicating that there are at least two strains of *Theileria mutans* circulating in cattle in Malawi. *Theileria parva* sequences obtained in this study clustered with sequences of MC vaccine strains (Muguga, Kiambu-5 and Serengeti transformed sequence IDs MG952923, MG952924 and MG952925, respectively), even from non-vaccinated cattle.

In the *groEL* gene tree, 1, 3, 4 and 7 sequences of *Anaplasma bovis*, *Anaplasma marginale*, *Anaplasma platys*-like and uncharacterized *Ehrlichia* species obtained in this study were included. While for the *gltA* tree, 1, 2, 1 and 10 sequences for *Anaplasma bovis*, *Anaplasma marginale*, *Anaplasma platys*-like and uncharacterized *Ehrlichia* species obtained in this study were included. The *Anaplasma marginale* sequences obtained in this study clustered with other homologous sequences registered in the GenBank. The *Anaplasma bovis* sequences obtained from all the five samples were identical and shared 100% identity with the sequence of *Anaplasma bovis* (MH255914) reported from cattle in China. The phylogenetic trees of both the *gltA* and *groEL* genes showed that the *Anaplasma platys*-like sequences obtained in this study clustered with other *Anaplasma platys*-like sequences from ruminants and associated ticks but separated from those reported from dogs or dog ticks (Figures I-3 and I-4). The obtained sequences of *Ehrlichia* species showed high degree of divergence as evidenced by the branching of their clade. Some sequences had 98-100% identity with sequence of *Ehrlichia minasensis* (JX629807) isolated from *Rhipicephalus microplus* tick in Brazil. However, the sequences of *Ehrlichia* species clustered separately from those of *Ehrlichia ruminantium* (Figures I-3 and I-4).

4. Discussion

Tick-borne *Anaplasmataceae* and piroplasms in cattle have been investigated using molecular techniques in the neighboring countries of Malawi, such as Mozambique (Martins et al., 2010, 2008; Alfredo et al. 2005), Tanzania (Ringo et al., 2020, 2018; Swai et al., 2009), and Zambia (Tembo et al., 2018; Simuunza et al., 2011; Makala et al., 2003). However, in Malawi, the epidemiological data on TBPs of cattle is mainly based on basic blood smear and serological examinations. This is the first study that has investigated tick-borne *Anaplasmataceae* and piroplasms in cattle in Malawi using molecular techniques. Sequence analysis has shown that cattle in Malawi were infected with *Anaplasma bovis*, *Anaplasma marginale*, *Anaplasma platys*-like, uncharacteised *Anaplasma* species, uncharacterized *Ehrlichia* species, *Babesia bigemina*, *Theileria mutans*, *Theileria parva*, *Theileria taurotragi, Theileria velifera* and *Theileria* sp. MSD-like strain.

Anaplasma marginale which causes bovine anaplasmosis was detected in samples from all the six sampling sites. The observed prevalence of 24.7% is comparable to 25.7% reported in Zambia (Tembo et al., 2018). However, it is higher than 10.2%, 7.4% and 18.0% reported in Tanzania (Ringo et al., 2020, 2018; Swai et al., 2007) and 9.4% reported in Mozambique (Martins et al., 2010). The *Anaplasma bovis* was detected at a prevalence of 2.9%. Although it is considered non-pathogenic, it has been reported to cause mild disease in calves (Park et al., 2018), and could be fatal when there is co-infection with other TBPs (Jurković et al., 2020).

Anaplasma platys-like infection in cattle with a prevalence of 13.6% is being reported for the first time in southern Africa. *Anaplasma platys* is known to be pathogenic in dogs where it cause infectious cyclic thrombocytopenia in dogs. The clinical disease in dogs is characterized by lethargy, anorexia, fever, weight

loss and bleeding disorder (Skyes & Foley, 2014). The prevalence of 13.6% reported herein is higher than 3.5% reported in Tunisia in North Africa (Ben Said et al., 2017). However, it is lower than 44.7% reported in Kenya in east Africa (Peters et al., 2020). This finding suggests that either the pathogen is expanding its geographical distribution or that it was previously neglected. The phylogenetic trees of the *gltA* and *groEL* genes showed that *Anaplasma platys*-like isolates from cattle, water buffalo and associated ticks clustered separately from the isolates from dogs and associated ticks as previously reported (Ogata et al., 2021). This shows that the strains circulating in ruminants are different from those reported in dogs.

In this study, *Ehrlichia* species have been detected at a prevalence of 16.2%. All sequences obtained were closely related to sequence of *Ehrlichia minasensis* (JX629807) isolated from *Rhipicephalus microplus* tick which was previously reported to be closely related to *Ehrlichia canis* (Cabezas-Cruz et al., 2012). This finding is also in accordance with the findings of another study conducted in Kenya by Peters et al. (2020), which showed that all sequences of *Ehrlichia* species detected were closely related to *Ehrlichia minasensis*.

However, *Ehrlichia ruminantium* which causes bovine ehrlichiosis (heart water) was not detected in this study, although it has been reported with prevalence ranging from 0.33% to 45.3% in neighboring Zambia (Tembo et al., 2018; Simmunza et al., 2011). The failure to detect the pathogen in this study may be either due to the predisposition of *Ehrlichia ruminantium* to infect vascular endothelial cells but not circulating cells which limits rickettsemia and chances of being detected in peripheral blood (Deem, 2008). Furthermore, the pathogenicity of the strains circulating in the animals may be another determinant as the more pathogenic easily invade more cells which increases the rickettsemia and chances of being detected unlike the less pathogenic strains.

The prevalence of *Babesia bigemina* of 2.6% reported in this study is within the same range as 3.3% (Tembo et al., 2018) and 5.5% (Simuunza et al., 2011) reported in Zambia and 5.1% (Ringo et al., 2020) reported in Tanzania. However, in this study, *Babesia bovis* which is the most pathogenic *Babesia* species was not detected in the examined animals. This is in contrast to 7.7% (Tembo et al., 2018) and 15.5% (Simuunza et al., 2011) reported in Zambia and 4.5% reported in Tanzania (Ringo et al., 2020). The negative results of *Babesia bovis* may be due to low prevalence of the pathogen in the sampled areas or due to small sample size used in this study.

Theileria parva causing ECF was one of the pathogens reported with the highest frequency in this study. The detection of *Theileria parva* at all the six sampling sites including the previously non-endemic region indicates that the pathogen has expanded its distribution in Malawi. The prevalence in this study (33.0 %) is comparable to 34.4% and 41.2% reported in Tanzania (Ringo et al., 2020; 2018) but higher than 24.4% and 0.3% reported in Zambia (Simuunza et al., 2011; Tembo et al., 2018). This indicates that *Theileria parva* is expanding its geographical distribution in Malawi. However, because in some sampling sites, in the central region the MC vaccine is used to control ECF some positive samples may be due to the vaccinations. The phylogenetic analysis also showed that the sequences of *Theileria parva* from Malawi clustered together with sequences obtained from the components of the MC vaccine (Muguga, Kiambu-5 and Serengeti transformed) deposited in GenBank even those from animals without vaccination history. Furthermore, the

obtained sequences of *Theileria parva* clustered with the sequence of Katete vaccine strain from Zambia. Since the stocks of MC vaccine are originally from Kenya and Tanzania. This study has shown that the strains of *Theileria parva* circulating in cattle in eastern and southern Africa are closely related to one another and the vaccine strains.

There was high infection rates of *Theileria mutans* and *Theileria velifera* at 73.8% and 53.4%, respectively. These rates are higher than 38.1% and 3.4% reported in Tanzania (Ringo et al., 2020) and 54.5% and 51.5% in Zambia (Tembo et al., 2018), respectively. *Theileria mutans* has been reported to be the most prevalent TBP in both Tanzania (Ringo et al., 2020, 2018) and Zambia (Tembo et al., 2018; Simuunza et al., 2011). *Theileria mutans* sequences obtained in this study clustered in two clades, one with *Theileria mutans* (AF078815) isolated from cattle in South Africa and another with *Theileria* sp. MSD like (AF078816), a strain of *Theileria mutans* (Chaisi et al., 2013) reported from cattle in South Africa. This shows that there are at least two strains of *Theileria mutans* circulating in cattle in Malawi. The finding of closely related strain reported in South Africa may suggest that most strain circulating in cattle in southern Africa are closely related. Although *Theileria mutans* infection in cattle is considered less pathogenic, it has been reported that when it occurs in mixed infection with *Theileria parva,* it increases the pathogenicity of *Theileria parva*.

Theileria taurotragi although it is considered less pathogenic in cattle, cases of clinical disease do occur and have been reported to present with clinical signs like those of ECF. The phylogenetic tree of the 18S rDNA support the close relatedness with *Theileria parva* as they cluster in the same clade. The vector tick for both *Theileria parva* and *Theileria taurotragi* is the brown ear tick *Rhipicephalus appendiculatus* (Walker et al., 20003; Ringo et al., 2020). The positive detection rate of 12.6% reported in this study is lower than 30.9% reported in Tanzania (Ringo et al., 2020) and 27.5% reported in Zambia (Simuunza et al., 2011). However, it is comparable to 11.4% reported by another study in Zambia (Tembo et al., 2018).

The detection of co-infections in 152 animals (79.6%) with both *Anaplasmataceae* and piroplasms is an important finding as it complicated the proper diagnosis and treatment of the animals that show clinical disease as some combinations need different treatment regimen. The co-infection rate reported in this study was higher than those reported in neigbouring Tanzania of 36.4% (Ringo et al., 2020) but lower than those reported in Zambia of 52.9% and 75.6% by Simuunza et al. (2011) and Tembo et al. (2018), respectively. Mixed infections with TBPs that are individually less pathogenic than other species within the same genus have been reported to cause clinical disease (Jurković et al., 2020). However, another study has suggested that this may be beneficial to the animals as the less pathogenic species may help to generate immunity against the more pathogenic species by heterologous protection (Woolhouse et al., 2015). In this study, although there were many combinations of mixed infections, only four combinations were statistically significant suggesting that these pathogens have high likelihood of being detected when there is co-infection. *Theileria mutans* and *Theileria velifera* co-infection correlation in cattle was also reported in Zambia (Tembo et al., 2018). However, in this study, there was no any co-infection correlation between *Theileria parva* and

the causative agents of benign theilerioses (*Theileria mutans*, *Theileria taurotragi* and *Theileria velifera*) in this study as also previously reported in Zambia (Tembo et al., 2018).

This finding shows that the challenge to TBPs is widespread in the southern Africa and collaborative efforts to develop novel methods to control ticks and TBDs are warranted.

5. Summary

This study has provided evidence of high prevalence of tick-borne pathogens in cattle in Malawi using molecular techniques. *Anaplasma bovis*, *Anaplasma marginale*, *Anaplasma platys*-like, uncharacterized *Anaplasma* species, *Babesia bigemina*, uncharacterized *Ehrlichia* species, *Theileria mutans*, *Theileria parva*, *Theileria taurotragi* and *Theileria velifera* have been reported in this study. The failure to detect other pathogens may not necessarily mean the absence of the pathogens but the small sample size used in this study may have reduced their chances of being detected and the limited geographical area investigated may be another contributing factor. Further studies with national wide coverage are recommended to generate a countrywide profile of tick-borne pathogens in Malawi. The newly developed multiplex polymerase chain reaction assay for the detection of *Babesia* and *Theileria* pathogens to species level would be a useful resource in the diagnosis and control of tick-borne piroplasms especially when co-infections are highly prevalent. The high prevalence of tick-borne pathogens even in farms where dipping is done to control ticks warrants the need to conceive novel tick control methods.

List of tables

Table I-1. List of primers used to detect and characterize tick-borne pathogens infecting cattle in Malawi.

Primer name	Primer sequence (5′ to 3′)	Target gene/ organism	PCR type	Amplicon size (bp)	Annealing temperature (°C)	Reference
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA/ *Babesia*, *Theileria* and *Hepatozoon*	1st PCR	1400 - 1600	55	Masatani et al., 2017
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG		2nd PCR	1400 - 1600		
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
BTH_Bbig_ F	AGCCTTGGTAATGGTTAATAG	18S rDNA/ *Babesia*, and *Theileria*	Multiplex PCR	848	57	This study
BTH_Bbov_ F3	TGTCCTTTCTTGATTCTCTGGGTAG			469		
BTH_Tmut_ F	GGCCCTTGCCTTGAATACTTTAG			915		
BTH_Tpar_F	CTCTGCATGTGGCTTATTTCGG			995		
BTH_Ttau_F	TGCTAAATAGGGTACGGGAGC			330		
BTH_Tveli_F	AGGCTTTTGCCGTCCCGTGATCGC			310		
BTH_ NEW_R	TCCGAATAATTCACCGGATCAC					
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* gene/ *Anaplasmataceae*	1st PCR	1300	54	Rar et al., 2011
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* gene/ *Anaplasma*	2nd PCR	1256	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* gene/ *Ehrlichia*	2nd PCR	1100	50	Gofton et al., 2016
groEL_rev2	GCCGACTTTTAGTACAGCAA					
F4b	CCAGGCTTTATGTCAACTGC	*gltA* gene/ *Anaplasmataceae*	1st PCR	800	55	Inokuma et al., 2005
R1b	CGATGACCAAAACCCAT					
EHR-CS136F	TTYATGTCYACTGCTGCKTG	*gltA* gene/ *Anaplasma* and *Ehrlichia*	2nd PCR	650	55	Inokuma et al., 2005
EHR-CS778R	GCNCCMCCATGMGCTGG					

PCR = Polymerase chain reaction; F = Forward; R = Reverse

Table I-2. Positive detection rates of tick-borne pathogens in cattle in Malawi.

Pathogen	Study site						Overall
	Kasungu	Nkhotakota	Katete	Likasi	LUANAR	Mikolongwe	
	n = 26	*n* = 37	*n* = 36	*n* = 34	*n* =33	*n* = 28	*n* = 191
Anaplasma bovis	0	0	0	0	2 (6%)	3 (11%)	5 (2.9%)
Anaplasma marginale	5 (19%)	6 (16%)	7 (19%)	11 (32%)	6 (18%)	12 (43%)	47 (24.6%)
Anaplasma platys	2 (8%)	13 (35%)	4 (11%)	6 (18%)	0	1 (4%)	26 (13.6%)
Anaplasma sp.	7 (27%)	4 (11%)	5 (14%)	4 (11%)	3 (9%)	4 (14%)	27 (14.1%)
Ehrlichia sp.	12 (46%)	5 (14%)	2 (6%)	6 (17%)	1 (3%)	5 (18%)	31 (16.2%)
Babesia bigemina	1 (4%)	2 (5%)	1 (3%)	2 (6%)	0	0	6 (3.1%)
Babesia bovis	0	0	0	0	0	0	0
Theileria mutans	19 (73%)	28 (77%)	22 (61%)	26 (76%)	28 (85%)	18 (64%)	141 (73.8%)
Theileria parva	9 (35%)	10 (27%)	17 (47%)	9 (26%)	11 (33%)	7 (25%)	63 (33.0%)
Theileria taurotragi	4 (15%)	2 (5%)	6 (17%)	3 (9%)	3 (9%)	6 (21%)	24 (12.6%)
Theileria velifera	14 (54%)	19 (51%)	13 (36%)	19 (56%)	25 (76%)	12 (43%)	102 (53.4%)

n = number of samples, the number of samples positive and percentage in parenthesis based on the total sample size of 191.

Table I-3. Number of tick-borne pathogens co-infections observed in cattle in Malawi.

Number of pathogens observed in co-infection	Study site						Overall
	Kasungu	Nkhotakota	Katete	Likasi	LUANAR	Mikolongwe	
	n = 26	*n* = 37	*n* = 36	*n* = 34	*n* =33	*n* = 28	*n* = 191
2	7 (27%)	14 (38%)	10 (28%)	8 (24%)	13 (39%)	2 (7%)	54 (28.3%)
3	12 (46%)	13 (35%)	7(19%)	17 (50%)	13 (39%)	13 (46%)	75 (39.3%)
4	1 (4%)	1 (3%)	5 (14%)	4 (12%)	1 (3%)	6 (21%)	18 (9.4%)
5	0	1 (3%)	0	0	2 (6%)	1 (4%)	4 (2.1%)
6	0	0	0	0	1 (3%)	0	1 (0.5%)
Total	20 (77%)	29 (78%)	22 (61%)	29 (85%)	30 (91%)	22 (79%)	152 (79.6%)

n = number of samples, number of samples positive and percentage in parenthesis based on the total sample size of 191.

Table I-4. Pathogens combination and frequency of co-infections observed in cattle in Malawi.

Double infections	Study site						Overall *n* = 191
	Kasungu *n* = 26	Nkhotakota *n* = 37	Katete *n* = 36	Likasi *n* = 34	LUANAR *n* =33	Mikolongwe *n* = 28	
Anaplasma marginale + *Theileria mutans*	0	1 (3%)	1 (3%)	1 (3%)	0	0	3 (1.6%)
Anaplasma marginale + *Theileria parva*	0	1 (3%)	1 (3%)	0	0	0	2 (1.0%)
Anaplasma marginale + *Theileria velifera*	0	0	1 (3%)	0	1 (3%)	0	2 (1.0%)
Anaplasma platys + *Theileria parva*	0	2 (5%)	2 (6%)	2 (6%)	1 (3%)	0	7 (1.6%)
Anaplasma platys + *Theileria velifera*	0	0	0	1 (3%)	0	0	1 (0.5%)
Anaplasma sp. + *Theileria mutans*	1 (4%)	0	0	0	0	0	1 (0.5%)
Ehrlichia sp. + *Theileria mutans*	0	1 (3%)	0	1 (3%)	0	1 (4%)	3 (1.6%)
Ehrlichia sp. + *Theileria parva*	1 (4%)	1 (3%)	0	0	0	0	2 (1.0%)
Ehrlichia sp. + *Theileria velifera*	0	1 (3%)	0	0	0	0	1 (0.5%)
Theileria mutans + *Theileria parva*	1 (4%)	3 (8%)	4 (11%)	1 (3%)	0	0	9 (4.7%)
Theileria mutans + *Theileria velifera*	4 (15%)	4 (11%)	1 (3%)	2 (6%)	11 (33%)	1 (4%)	23 (12.0%)
Sub-total	7 (27%)	14 (38%)	10 (28%)	8 (24%)	13 (39%)	2 (7%)	54 (28.3%)
Triple infections							
Anaplasma bovis + *Theileria mutans* + *Theileria velifera*	0	0	0	0	1 (3%)	1 (4%)	2 (1.0%)
Anaplasma marginale + *Theileria mutans* + *Theileria parva*	1 (4%)	0	2 (6%)	0	1 (3%)	0	4 (2.1%)
Anaplasma marginale + *Theileria mutans* + *Theileriatautotragi*	0	1 (3%)	0	0	0	1 (4%)	2 (1.0%)
An, marginale + *Theileria mutans* + *Theileria velifera*	1 (4%)	1 (3%)	0	0	0	1 (4%)	3 (1.6%)
Anaplasma platys + *Anaplasma* sp. + *Theileria mutans*	0	1 (3%)	0	6 (18%)	3 (9%)	3 (11%)	13 (6.8%)
Anaplasma platys + *Anaplasma* sp. + *Theileria velifera*	1 (4%)	0	0	0	0	0	1 (0.5%)
Anaplasma platys + *Theileriamutans* + *Theileria parva*	1 (4%)	0	0	0	0	0	1 (0.5%)
Anaplasma platys + *Theileria mutans* + *Theileria tautotragi*	1 (4%)	0	1 (3%)	0	0	0	2 (1.0%)
Anaplasma platys + *Theileria mutans* + *Theileria velifera*	0	2 (5%)	0	0	0	0	2 (1.0%)
Anaplasma sp. + *Theileria mutans* + *Theileria tautotragi*	0	6 (16%)	0	3 (9%)	0	0	9 (4.7%)
Anaplasma sp. + *Theileria mutans* + *Theileria velifera*	1 (4%)	0	0	2 (6%)	0	0	3 (1.6%)

Table I-4 cont'd

Ehrlichia sp. + *Theileria mutans* + *Theileria parva*	2 (8%)	0	0	1 (3%)	0	0	3 (1.6%)
Ehrlichia sp. + *Theileria mutans* + *Theileria velifera*	1 (4%)	0	0	0	0	0	1 (0.5%)
Babesia bigemina + *Theileria mutans* + *Theileria velifera*	1 (4%)	2 (5%)	0	2 (6%)	2 (6%)	6 (22%)	13 (6.8%)
Theileriamutans + *Theileria parva* + *Theileria tautotragi*	1 (4%)	0	0	0	0	0	1 (0.5%)
Theileria mutans + *Theileria parva* + *Theileria velifera*	1 (4%)	0	4 (11%)	3 (9%)	6 (18%)	1 (4%)	15 (7.9%)
Sub-total	12 (46%)	13 (35%)	7 (19%)	17 (50%)	13 (39%)	13 (46%)	75 (39.3%)
quadruple infection							
Anaplasma bovis + *Anaplasma marginale* + *Theileria mutans* + *Theileria tautotragi*	0	0	0	0	0	1(4%)	1 (0.5%)
Anaplasma marginale + *Babesia bigemina* + *Theileriamutans* + *Theileria velifera*	0	0	0	1 (3%)	0	0	1 (0.5%)
Anaplasma marginale + *Theileria mutans* + *Theileria parva* + *Theileria tautotragi*	0	0	1 (3%)	0	1 (3%)	2 (7%)	4 (2.1%)
Anaplasma marginale + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	0	2 (6%)	3 (9%)	0	0	5 (2.6%)
Anaplasma platys + *Theileria mutans* + *Theileria parva* + *Theileria tautotragi*	0	0	1 (3%)	0	0	0	1 (0.5%)
Anaplasma platys + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	1 (3%)	1 (3%)	0	0	0	2 (1.0%)
Anaplasma sp. + *Ehrlichia* sp. + *Theileria mutans* + *Theileria velifera*	0	0	0	0	0	1 (4%)	1 (0.5%)
Anaplasma sp. + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	1 (4%)	0	0	0	0	1 (4%)	2 (1.0%)
Ehrlichia sp. + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	0	0	0	0	1 (4%)	1 (0.5%)
Sub-total	1 (4%)	1 (3%)	5 (14%)	4 (12%)	1 (3%)	6 (21%)	18 (9.4%)
Quintuple infection							
Anaplasma Platys + *Babesia bigemina* + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	1 (3%)	0	0	0	0	1 (0.5%)
Anaplasma platys + *Anaplasma* sp. + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	0	0	0	1 (3%)	0	1 (0.5%)

Table I-4 cont'd

Anaplasma marginale + *Anaplasma platys* + *Anaplasma* sp. + *Theileria mutans* + *Theileria velifera*	0	0	0	0	1 (3%)	0	1 (0.5%)
Anaplasma bovis + *Anaplasma platys* + *Anaplasma* sp. + *Theileria mutans* + *Theileria velifera*	0	0	0	0	0	1 (4%)	1 (0.5%)
Sub-total	0	1 (3%)	0	0	2 (6%)	1 (4%)	4 (2.1%)
Sextuple infection							
Anaplasma bovis + *Anaplasma platys* + *Anaplasma* sp. + *Theileria mutans* + *Theileria parva* + *Theileria velifera*	0	0	0	0	1 (3%)	0	1 (0.5%)

n = number of samples, number of samples positive and percentage in parenthesis based on the total sample size of 191.

Table I-5. Association between co-infections of tick-borne pathogens observed in cattle in Malawi.

	Anaplasma marginale	*Anaplasma platys*-like	*Anaplasma* sp.	*Ehrlichia* sp.	*Babesia bigemina*	*Theileria mutans*	*Theileria parva*	*Theileria taurotragi*	*Theileria velifera*
Anaplasma bovis	C: 1, (0.5) r = -0.008 95% CI: -0.15-0.134 *p* = 0.914	C: 2, (1.0) r = 0.106 95% CI: -0.037-0.244 *p* = 0.146	C: 3, (1.6) r = 0.294 95% CI: 0.159-0.419 *p* = < 0.001*	NCO	NCO	C: 5, (2.6) r = 0.079 95% CI: -0.064-0.219 *p* = 0.277	C: 1, (0.5) r = -0.047 95% CI: -0.188-0.096 *p* = 0.519	C: 1, (0.5) r = 0.034 95% CI: -0.109-0.175 *p* = 0.645	C: 3, (1.6) r = 0.025 95% CI: -0.117-0.166 *p* = 0.73
Anaplasma marginale		NCO	C: 1, (0.5) r = -0.122 95% CI: -0.259-0.021 *p* = 0.094	NCO	C: 1, (0.5) r = 0.011 95% CI: -0.132-0.152 *p* = 0.884	C: 34, 17.8) r = -0.035 95% CI: -0.176-0.107 *p* = 0.63	C: 12, (6.3) r = -0.029 95% CI: -0.17-0.114 *p* = 0.693	C: 7, (3.7) r = 0.056 95% CI: -0.086-0.197 *p* = 0.439	C: 21, (11.0) r = -0.025 95% CI: -0.166-0.117 *p* = 0.731
Anaplasma platys-like			C: 6, (3.1) r = 0.162 95% CI: 0.02-0.297 *p* = 0.026*	NCO	C: 1, (0.5) r = 0.035 95% CI: -0.108-0.176 *p* = 0.633	C: 26, (13.6) r = 0.031 95% CI: -0.112-0.172 *p* = 0.674	C: 12, (6.3) r = 0.049 95% CI: -0.094-0.189 *p* = 0.505	C: 4, (2.1) r = -0.002 95% CI: -0.144- 0.14 *p* = 0.973	C: 20, (10.5) r = 0.136 95% CI: -0.007-0.272 *p* = 0.061
Anaplasma sp.				1, (0.5) r = -0.074 95% CI: -0.214-0.069 *p* = 0.309	NCO	C: 16, (8.4) r = 0.104 95% CI: -0.039-0.242 *p* = 0.154	C: 4, (2.1) r = -0.066 95% CI: -0.266-0.077 *p* = 0.364	C: 4, (2.1) r = 0.097 95% CI: -0.046-0.235 *p* = 0.183	C: 9, (4.7) r = 0.004 95% CI: -0.138-0.146 *p* = 0.96
Ehrlichia sp.					NCO	C: 19, (9.9) r = -0.073 95% CI: -0.213-0.069 *p* = 0.313	C: 6, (3.1) r = -0.129 95% CI: -0.266-0.013 *p* = 0.076	C: 3, (1.6) r = -0.024 95% CI: -0.165-0.119 *p* = 0.744	C: 14, (7.3) r = -0.004 95% CI: -0.146-0.138 *p* = 0.955

Table I-5 cont'd

Babesia bigemina	C: 3, (1.6) r = -0.023 95% CI: -0.164-0.119 *p* = 0.752	C: 1, (0.5) r = -0.026 95% CI: -0.168-0.116 *p* = 0.717	NCO	C: 3, (1.6) r = 0.066 95% CI: -0.076-0.206 *p* = 0.362
Theileria mutans		C: 57, (29.8) r = 0.087 95% CI: 0.056-0.0.226 *p* = 0.232	C: 25, (13.1) r = 0.187 95% CI: 0.046-0.32 *p* = 0.01∗	C: 96, (50.2) r = 0.344 95% CI: 0.213-0.464 *p* = <0.001∗
Theileria parva			9, (4.7) r = 0.02 95% CI: -0.122-0.162 *p* = 0.778	31, (16.2) r = -0.056 95% CI: -0.196-0.087 *p* = 0.444
Theileria taurotragi				NCO

C = Number of co-infections and percent related to the total cattle population examined (191 specimens). NCO = no co-infection observed. R = correlation coefficient of infections between pathogens in cattle in Malawi. 95% CI = 95% confidence intervals for R. ∗ A *p*-value of ≤0.05 was considered to be significant.

List of figures

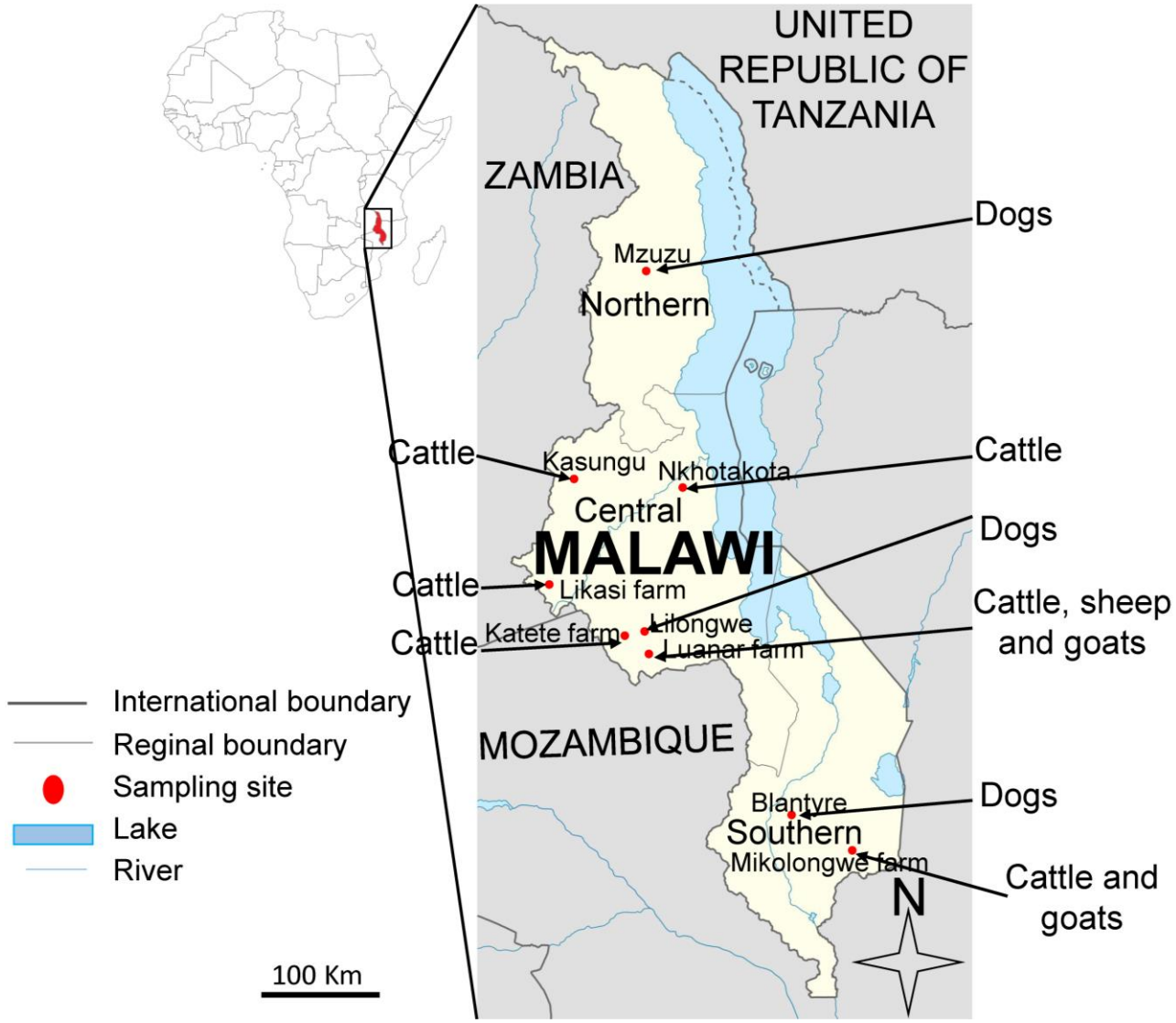

Figure I-1. Map of Malawi showing the sample collection sites and animal species sampled.

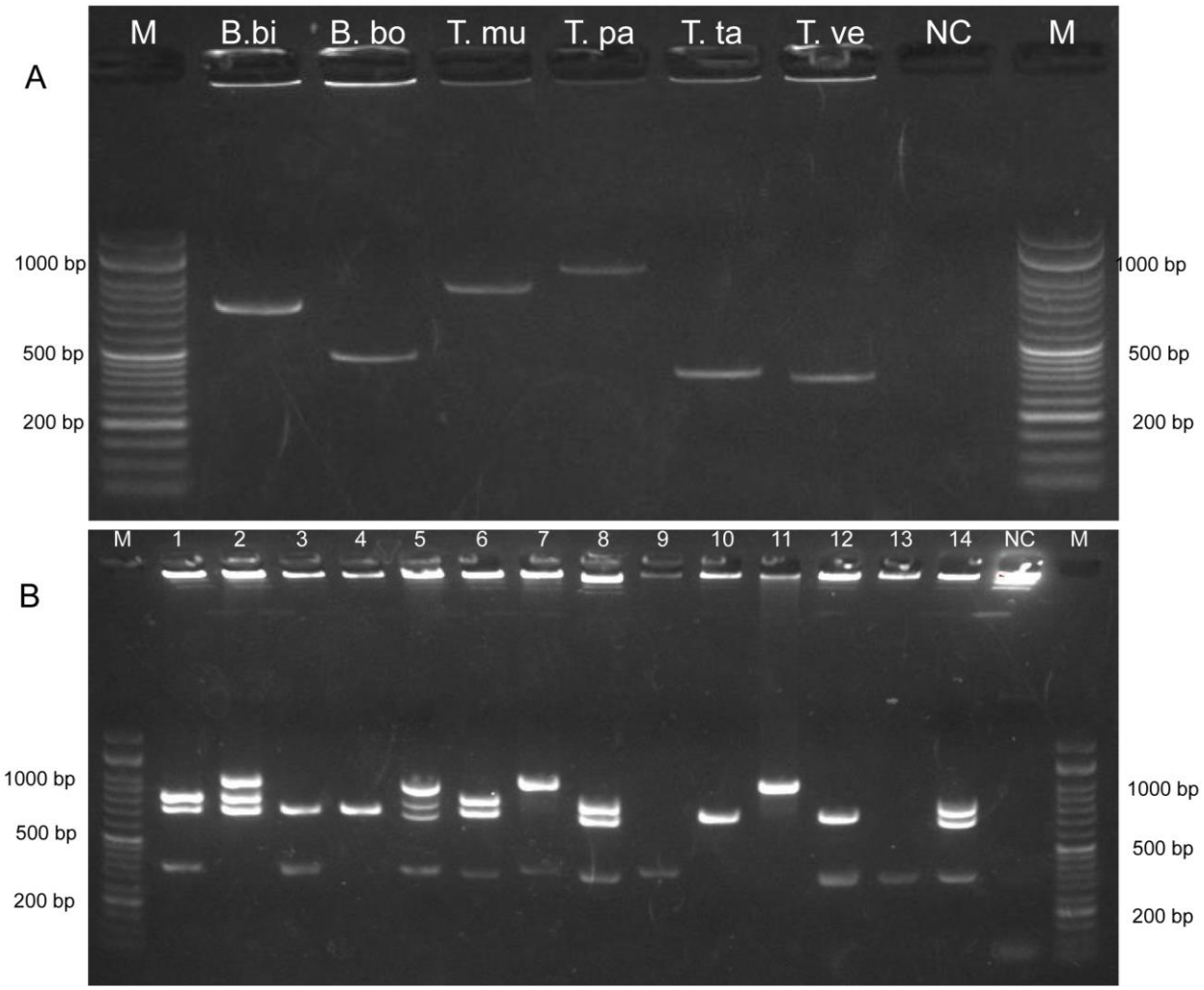

Figure I-2. Representative gel electrophoresis images of the designed multiplex PCR. A: M = 50 bp marker; B.bi = *Babesia bigemina*, B.bo = *Babesia bovis*, T.mu = *Theileria mutans*, T.pa = *Theileria parva*, T.ta = *Theileria tautotragi*, T.ve = *Theileria velifera*, NC = negative control.
B: Detection of mixed infection in cattle samples, M = 50 bp marker; lanes 1-13 samples, NC = negative control.

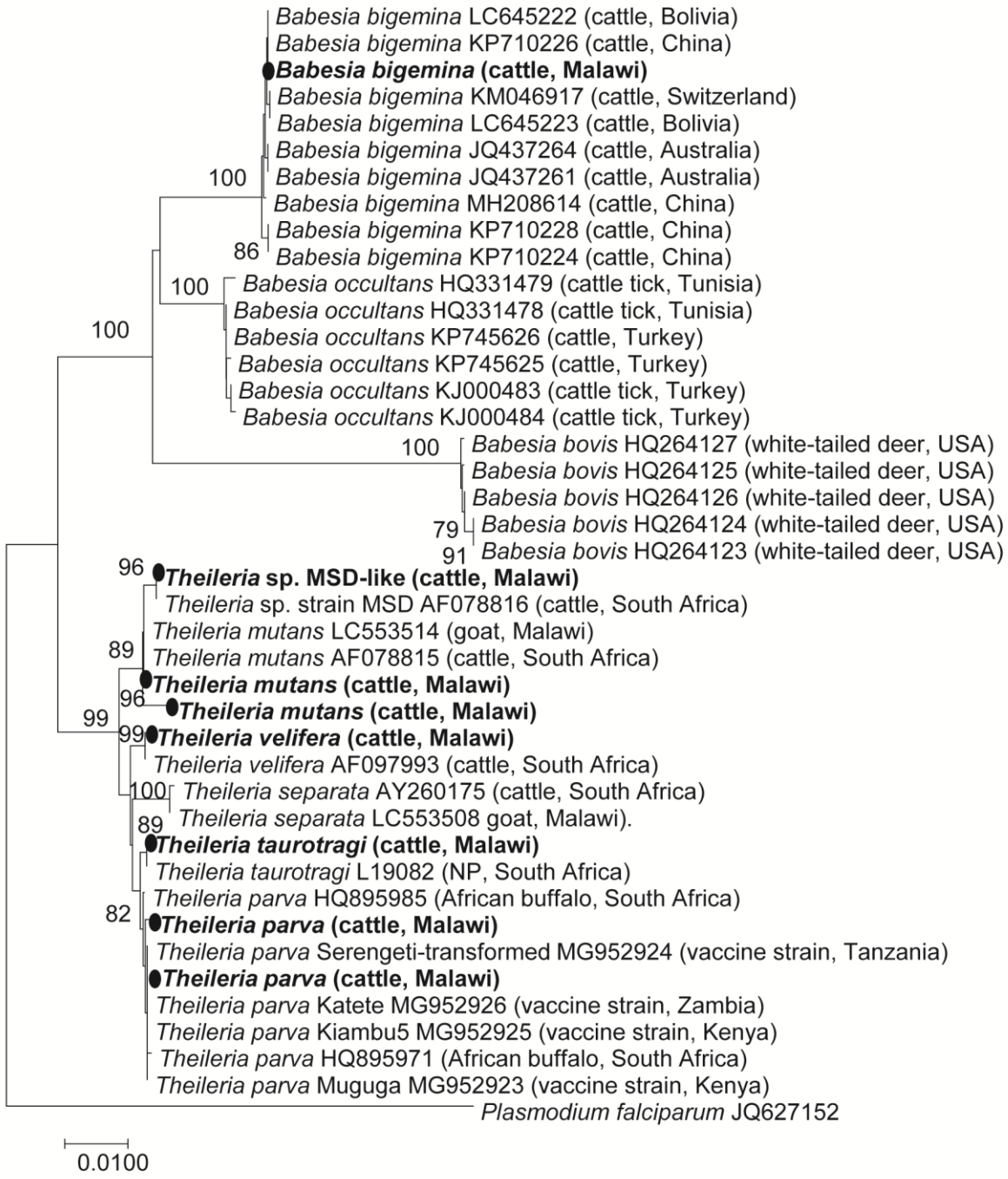

Figure I-3. The maximum likelihood phylogenetic tree of the species of *Babesia* and *Theileria* detected in cattle and vaccine strain(s) in Malawi and other countries. The analysis was based on partial sequences of 18S rDNA (1400-1500 bp) which was constructed using the Kimura 2 parameter model. *Plasmodium falciparum* was used as outgroup. All bootstrap values > 75% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.10 is the genetic distance of each sequence from the root of the tree.

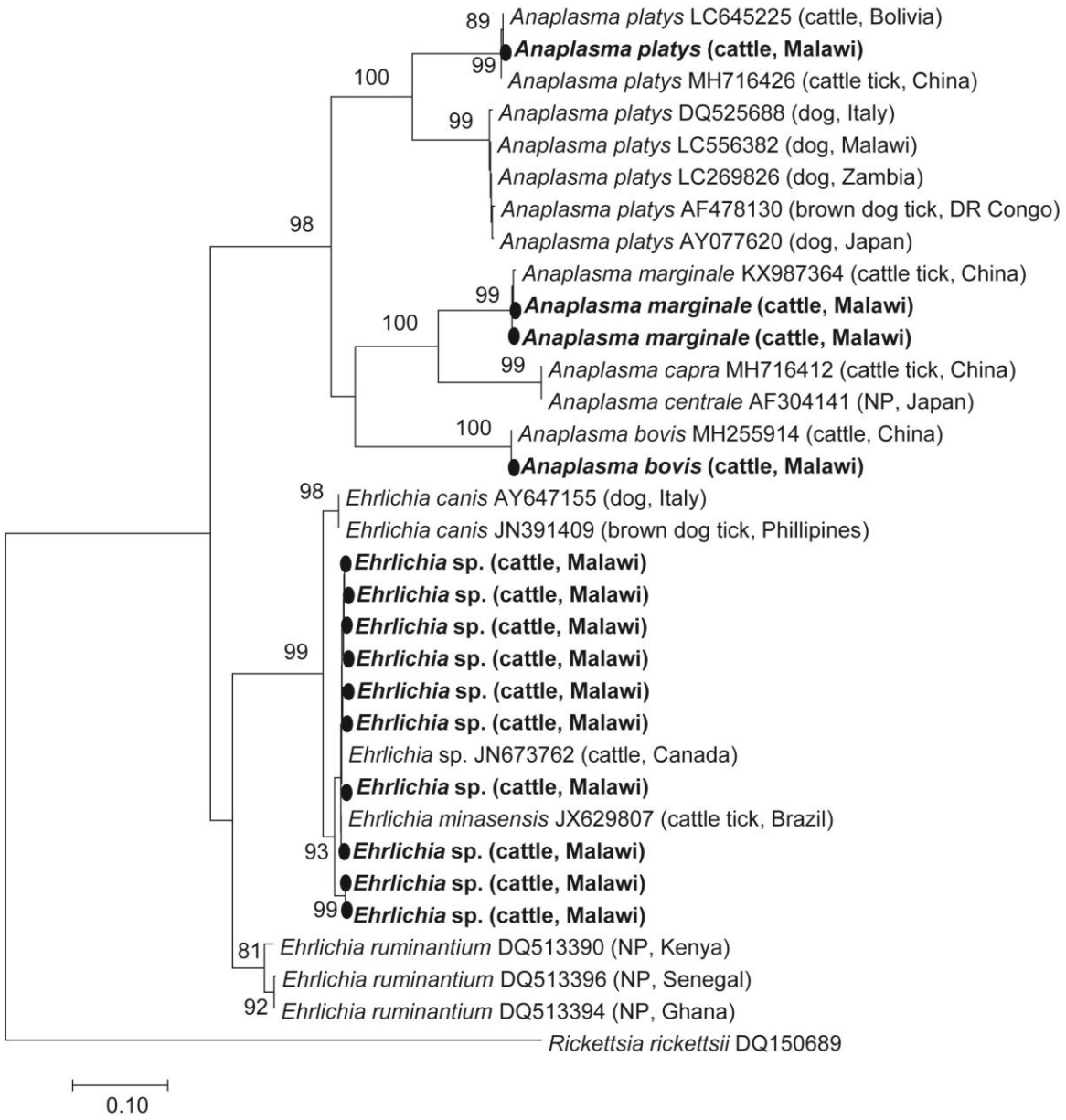

Figure I-4. The maximum likelihood phylogenetic tree of the species of *Anaplasma* and *Ehrlichia* detected in cattle. The analysis was based on partial sequences of *gltA* gene which was constructed using the Tamura 2 parameter model. *Rickettsia rickettsii* was used as outgroup. All bootstrap values > 75% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.10 is the genetic distance of each sequence from the root of the tree.

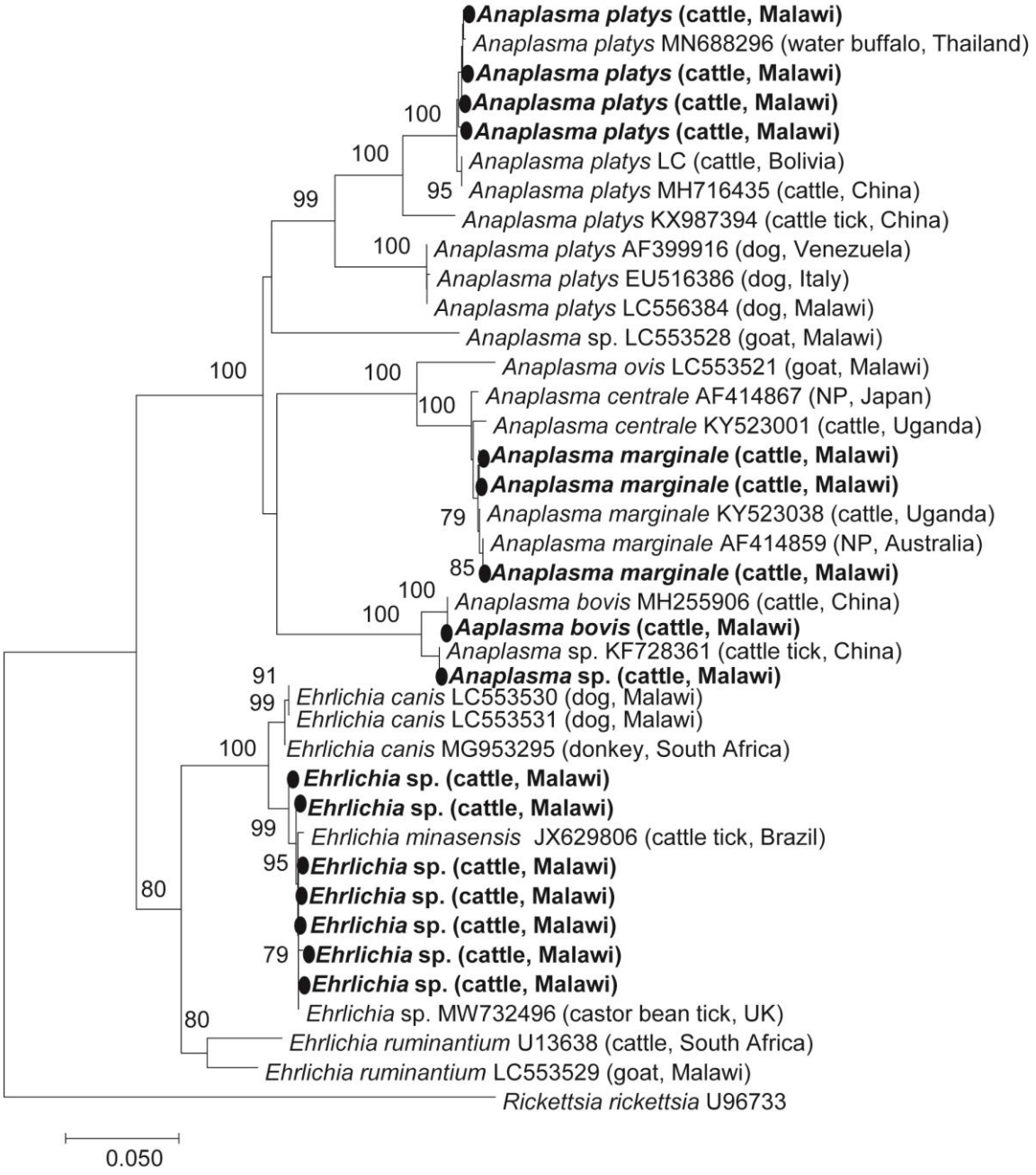

Figure I-5. The maximum likelihood phylogenetic tree of the *Anaplasma* and *Ehrlichia* species detected in cattle in Malawi. The analysis was based on partial sequences of *groEL* gene (1100 bp) which was constructed using the Tamura 2 parameter model. *Rickettsia rickettsii* was used as outgroup. All bootstrap values > 80% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The host and country of origin are indicated in parenthesis, NP means not provided. The bar 0.050 is the genetic distance of each sequence from the root of the tree.

Chapter II

Genetic diversity and sequence polymorphism of two genes encoding *Theileria parva* antigens recognised by $CD8^+$ T cells derived from vaccinated and unvaccinated cattle in Malawi

1. Introduction

Theileria parva, which is transmitted by the brown ear tick (*Rhipicephalus appendiculatus*), infects the T and B lymphocytes of cattle (Geysen et al., 1999), and it exhibits a complex life cycle (Nene et al., 2015) involving two stages, i.e., the schizont stage, which is responsible for the pathology of the disease and the blood stage, which is infective to the vector tick (Geysen et al., 1999; Morrison, 2009). In lymphocytes, the parasite develops into a schizont through the process of schizogony in which the infected cells divide into a clonally proliferating lymphoblast (Geysen et al., 1999). During the pathogenesis of the disease, the parasite changes numerous signaling pathways of the infected host lymphocytes, causing the transformation of the infected host cells into a cancer-like phenomenon and eventually the infected host cells disseminate throughout the body of the animal. The clinical disease is charactersed by the enlargement of superficial lymph nodes, pulmonary oedema and an eventual fatal fever (Geysen et al., 1999; Morrison, 2009; Norval et al., 1992; Dobbelaere and Heussler, 1999; Tretina et al., 2015). ECF remains a major acute and usually lethal disease of cattle present in 13 countries in eastern, central and southern Africa including the Comoro Islands (Norval et al., 1992; De Deken et al., 2007; Malak et al., 2012) and now South Sudan (Malak et al., 2012; Hoogstraal, 1956) in the north and Cameroon in the west (Silatsa et al., 2020).

Exotic and cross-breed cattle (*Bos taurus*) are more susceptible to ECF than the indigenous zebu cattle (*Bos indicus*) (Guilbride and Opwata, 1963). However, indigenous calves below 6 months of age are also highly susceptible (Moll et al., 1984, 1986; Malak et al., 2012; Oura et al., 2007). Serious losses have been reported even among adult indigenous zebu cattle which are normally resistance to clinical disease (Oura et al., 2007; Chinombo et al., 1988; Young, 1981). This occurs when endemic stability defined as an epidemiological state of a population in which clinical disease is scarce despite high levels of infection is disturbed (Young, 1981, Moll et al., 1984, 1986). ECF is responsible for about half of all calf deaths in pastoral herds in endemic countries (GAVmed, 2010). It is estimated that 1 million cattle are killed by ECF annually with a projected annual loss of US$300 million and some 25 million cattle are at risk of which 21 million are under small holder farmers (GALVmed, 2010). In Malawi, it was reported that 66% of each year's calf crop from indigenous Malawi zebu died before two years of age and most of these deaths were attributed to ECF (Knudsen, 1971). This disease is therefore a major limiting factor for the development of the livestock industry in the affected countries (Muleya et al., 2012). This highlights the need to prioritize the management and control of ECF in Malawi to achieve the vision of the country to be self-sufficient in safe locally produced livestock and livestock products (DAHLD, 2006).

Despite TBDs being widespread in all the three geographical regions (northern, central and southern) of Malawi, only few studies have been done. Among these studies or reports (Chinombo et al., 1988; Musisi et al., 1996), ECF was a single major cause of cattle mortality in central and northern regions of Malawi. However, sporadic cases attributed to illegal movement of animals from endemic areas have also been reported in southern region (Chinombo et al., 1988). The control of TBDs in Malawi mainly has focused on vector control by acaricide application administered through community cattle dips, livestock movement controls, chemotherapy (Chinombo et al., 1988; Musisi et al., 1996) and immunisation with the Muguga Cocktail (MC) vaccine consisting of Muguga, Kiambu 5 and Serengeti-transformed strains in the central and northern regions

predominantly in the exotic and crossbred cattle (Chinombo et al., 1988; Musisi et al., 1996). The vaccine is sourced from African Union Centre for Ticks and Tick-borne Diseases (AU-CTTBD), Lilongwe, Malawi (Perry, 2016). Currently, dipping occur predominantly in commercial farms keeping exotic and cross-bred cattle where it is done weekly in the rainy season and fortnightly during the dry season and not in the local Malawi zebu (DAHLD, 2006). Treatment with buparvaquone is done in both exotic and local Malawi zebu cattle when clinical disease occur (DAHLD, 2006).

Determinination of the suitable live parasite vaccination cocktails should be based on the strain specificity in a particular geographical location (Geysen et al., 1999; Salih et al., 2017). In the follow-up studies to evaluate the impact of MC in southern province of Zambia after introduction of MC vaccine from 1985 until 1992, possible clonal expansion of one of the components of a non-Zambian MC trivalent vaccine was observed (Geysen et al., 1999). Due to this observation, it was recommended that MC should not be used in the province instead a local strain approach should be used. This resulted in the use of *Theileria parva* Chitongo strain isolated from the same province to immunize cattle in the southern province of Zambia (Perry, 2016). The transmission of Kiambu 5 vaccine strain from vaccinated to unvaccinated cattle through ticks if the animals co-graze for more than 1 year has been demonstrated (Oura et al., 2007). These observations emphasizes the need to conduct follow-up studies where the "Infection and Treatment" method with cocktail vaccine is being used. This provides indisputable epidemiological data based on which effective control measures can be conceived on whether to use a 'local' strain approach, in which a broadly protective local stock of *Theileria parva* is used; or a 'cocktail' approach, where a combination of stocks is used to provide broad immunity over most of the ECF endemic region (Geysen et al., 1999).

Genes that encode for the *Theileria parva Tp1* and *Tp2* antigens have been demonstrated to be highly dominant targets of the $CD8^+$ T cell response in cattle which provide protection for *Theileria parva* in about 30% of immunised cattle. The immune response in immunised cattle is specific for major histocompatibility complex (MHC) class I gene products of the A18 and A10 haplotypes for *Tp1* and *Tp2*, respectively (MacHugh et al., 2009). There is a profound immunodominant response to *Tp1* and *Tp2* in cattle of these MHC class I haplotypes to *Theileria parva*, and this is a major determinant of the parasite strain specificity of the response and hence the immune protection (Taracha et al., 1995). These genes have also been used characterize the strains of *Theileria parva* and to study the nature and selective pressures driving diversity in antigens that induce T cell responses in cattle (Taracha et al., 1995; Graham et al., 2006, 2007, 2008). Thus, the *Tp1* and *Tp2* genes are good genetic markers to evaluate the impact of the vaccination.

This study intends to evaluate the endemicity and genetic diversity of *Theileria parva* in Malawi through sequence and amino acid variant analysis of *Theileria parva* $CD8^+$ T cell antigens *Tp1* and *Tp2*. The study also intends to assess the similarity of the field parasite population to the MC vaccine components. This information will cover the knowledge gap that has existed since Malawi started using the MC to control ECF in 1984 and help monitor the impact of this control method using molecular techniques.

2. Materials and Methods.

2.1 Study site, sampling and DNA extraction

Cattle sampling was done at three sampling sites in Kasungu, Nkhotakota and Katete as shown in Figure I-1. A total of 446 cattle blood samples, from Kasungu (*n* = 199), Nkhotakota (*n* = 185) and Katete (*n* = 62) were employed in this study. The sampling and DNA extraction was done as described in the previous chapter.

2.2 Polymerase chain reaction and Sequencing

Molecular detection of *Theileria parva* was done by nested PCR assays targeting the *Theileria parva*-specific 104-kD antigen (*p104*) gene (Skilton et al., 2002; Odongo et al., 2010). Primary PCR reactions were conducted in a 25 µL using Tks Gflex DNA polymerase and the reaction mixture and PCR conditions were set as described in chapter I, with minor modifications, where 1.0 µL was used as the DNA template. The primers used in this study, their annealing temperatures and expected product size are listed in Table II-1. The secondary PCR was set as the primary PCR except that the reaction volume was adjusted to 10 µL and 1.0 µL of 10-fold diluted primary PCR product which was used as the DNA template and water. The amplicons were electrophoresed in a 1.5% agarose gel stained with Gel-Red (Biotium, Hayward, CA, USA) and visualized under UV light.

PCR-positive samples were further used to amplify two *Theileria parva* $CD8^+$ antigen genes (*Tp1* and *Tp2*) using newly designed semi-nested PCR assays. To improve the sensitivity of the assays two inner primers were designed for semi-nested PCRs for both *Tp1* and *Tp2* genes. The primary PCRs were set as described by Pelle et al. (2011) to amplify a 432 bp and 525 bp of *Tp1* and *Tp2* genes, respectively. All PCRs were conducted in a 10 µl reaction mixture using Tks Gflex DNA polymerase. The amplicons were electrophoresed in 1.5 % agarose gel stained with Gel-Red and visualized under UV light.

The purification of positive PCR products, sequencing and sequence analysis was done as described in chapter I. The sequences obtained in this study were submitted to DDBJ under accession numbers: LC522067 to LC522072 for *Tp1* and LC522073 to LC522085 for *Tp2*.

2.3 Data analysis

Alignments of the consensus nucleotide sequences, generated from the amplified DNA fragments, were created using ClustalW in MEGA which was also used to translate the aligned nucleotide sequences into amino acid sequences.

The genetic distances (expressed in terms of the number of differences per 100 bases or amino acids, including length polymorphisms) between every pair of sequences in a multiple alignment were generated using the DISTMAT program accessible at *http://emboss.sourceforge.net/* (Kimura, 1980). Estimates of DNA polymorphism, π, determined as the average number of nucleotide differences per site, were obtained with DnaSP v.6 *http://www.ub.edu/dnasp* (Rozas et al., 2017). Analysis of molecular variance (AMOVA) was performed using 'Genalex6' *https://doi.org/10.1093/bioinformatics/bts460* (Peakall et al., 2006, 2012) in order to investigate the distribution of genetic variation among allelic sequences and to determine the level of population differentiation.

To assess the similarity between *Theileria parva* haplotypes found in Malawi with those of the MC strains, ML phylogenetic analysis and median joining (MJ) network incorporating the Malawi *Tp1* and *Tp2* haplotypes and those from the components of the MC live vaccine strains, respectively, was constructed using MEGA and NETWORK version 10.0.1.1 *http://fluxus-engineering.com/* (Bandelt et al., 1999), respectively. Chi-square statistics was used to determine the correlation between *Theileria parva* positive detection rate with the sampling site, vaccination status, age and sex of the animals.

3. Results

3.1 *Theileria parva* screening

The screening of cattle blood samples (n = 446) using the *p104* gene primers showed *Theileria parva* positive detection rate of 54.5% (243/446) from the three districts as shown in Table II-2. All the 62 samples used to compare the positive rate of vaccinated and unvaccinated cattle were obtained from Katete farm in Lilongwe. There was significantly higher infection rate in vaccinated animals (73.3%, X^2 = 4.34, df = 1, p = 0. 037227), whereas 46.9% of unvaccinated cattle were positive for *Theileria parva* within Lilongwe where MC vaccine was used. There was also a positive association between age of the animals and *Theileria parva* infection rate (X^2 = 15.9271, df = 2, p = 0.000348). There was higher detection rate in adult than calves which may be due to longer exposure to vector ticks in adult animals as compared to calves. There was also a positive correlation between the sampling site and *Theileria parva* positive detection rate (X^2 = 19.7667, df = 2, p = 0.000051). However, with regard to sex, there was no statistically significant correlation with *Theileria parva* positive detection rate (X^2 = 0.3698, df = 1, p = 0.543121). Similarly, there was no statistically significant correlation between breed of cattle with *Theileria parva* positive detection rate (X^2 = 0.7831, df = 1, p = 0.376194)

3.2 Sequencing analysis

A total of 243 *Theileria parva* samples (22 vaccinated and 221 unvaccinated) from three districts in central Malawi were used to study sequence polymorphisms in the partial sequences of *Tp1* and *Tp2* genes. Forteen positions (3.65%) in *Tp1* and 156 positions (42.05%) in *Tp2* were shown to be polymorphic plus a deletion of 12 nucleotides (5' TCTGCACCTCCT 3') corresponding to four amino acid residues: serine, alanine, proline, proline (SAPP) in *Tp1* (Figure II-1) gave rise to 6 and 10 amino acid variants in *Tp1* and *Tp2*, respectively. The naming of the nucleotide alleles and the translated amino acid variants follows the nomenclature by previous studies that described nucleotide alleles 1 to 49 and amino acid variants 1 to 34 for *Tp1* and nucleotide alleles 1 to 63 and amino acid variants 1 to 59 for *Tp2* (19, 27, 34). This study detected a total of 4 and 10 novel nucleotide alleles, respectively, for *Tp1* and *Tp2*, leading to 4 and 7 novel antigen variants for *Tp1* and *Tp2*, respectively. These novel alleles or variants continued the numbering sequence from the ones indicated above.

3.3 *Tp1* gene

The partial sequences of the *Tp1* gene encoding 128 amino acids were obtained from 223 *Theileria parva* positive samples. A total of 20 samples were not successfully amplified in *Tp1* gene. The alleles were recognized by single nucleotide polymorphisms (SNPs) at 14 nucleotide positions (3.65%) and a short deletion

of 12 nucleotides, resulting in 6 nucleotide alleles (Figure II-2). The nucleotide polymorphism (π) in this region was 1.02%. Tp1 allele number 1, which is present in the *Theileria parva* Muguga, Kiambu 5 and Serengeti-transformed reference sequence was represented in 139 of the 223 samples (62.33%). There were 6 distinct amino acid variants, resulting from amino acid changes at 11 residues (Figure II-4). Comparison of the *Theileria parva* Muguga $CD8^+$ T cell epitope sequence (VGYPKVKEE**ML**) located within the sequenced region of *Tp1* revealed two epitope variants ending with **-ML** and **-II**. Majority of the samples analyzed (212 out of 223; 95.07%) displayed the `-ML' epitope sequence variant present in *Theileria parva* Muguga reference isolate. The other variant (-II) was observed in 11 samples (4.89%) obtained from Kasungu and Nkhotakota sampling sites. Two of the 6 alleles identified in *Tp1* gene (allele IDs 1 and 5) were reported previously (Pelle et al., 2011), while 4 (allele IDs 43 to allele 46) are reported here for the first time. Regarding the amino acid variants, two (variant-1 and variant-10) were reported previously (Pelle et al., 2011), while 4 (variant-35 to variant-38) are reported here for the first time.

3.4 *Tp2* gene

The partial sequences of the *Tp2* gene encoding 161 amino acids were obtained from 190 *Theileria parva*-positive samples. A total of 53 samples were not successfully amplified for *Tp2* gene. SNPs in this region were found at 156 positions (42.05%), resulting in 13 nucleotide alleles (Figure II-3). The nucleotide polymorphism (π) observed in the sequenced region was 3.05%. *Tp2* allele 1, which is present in *Theileria parva* Muguga and Serengeti-transformed reference sequences, was observed in 118 out of the 190 samples (62.11%). While, *Tp2* allele 2 present in *Theileria parva* Kiambu 5 reference sequence was observed in 22 out of 190 samples (11.58%). There were 10 amino acid variants resulting from amino acid changes at 76 positions (Figure II-4). The 190 *Tp2* sequences revealed several variants for each of the six epitopes mapped in this region that ranged from four variants for epitope number 1 to three for epitope numbers 2, 3 and 6 and two variants at epitope 4 and 5 (Table II-3). Among the 13 nucleotide alleles identified in the *Tp2* gene, three (allele IDs 1, 2 and 3) were reported previously (Pelle et al., 2011), while the remaining 10 (allele IDs 64 to 73) are reported here for the first time. Likewise, from the 10 amino acid variants identified, three (variant IDs 1, 2 and 3) were reported previously (Pelle et al., 2011), while the remaining 7 variants (variants-60 to variant-66) are reported here for the first time.

3.5 Phylogenetic analysis of *Tp1* and *Tp2* sequences from *Theileria parva* in Malawi

The sequence diversity and similarity, observed in *Tp1* and *Tp2*, was examined further by generating ML trees for both loci that were rooted using the orthologous nucleotide sequences of *Theileria annulata* (GenBank accession numbers TA17450 and TA19865 for *Tp1* and *Tp2* genes, respectively). In *Tp1*, allele 1 found in the reference *Theileria parva* Muguga, Kiambu 5 and Serengeti-transformed had majotity of the sequences 139 out of 223 (62.33%) including the sequences obtained from the vaccinated animals. This allele also clustered together with eight relatively similar *Tp1* alleles with majority of the samples that originated from all three sampling sites (Figure II-5). However, some sequences obtained from Kasungu and Nkhotakota clustered together with those of *Theileria parva* isolated from buffaloes in Kenya. Phylogenetic analysis

involving the 13 *Tp2* alleles showed that the obtained sequences clustered into different clades with majority of the sequences clustering together with *Theileria parva* Muguga, Serengeti-transformed in *Tp2* allele 1 while *Theileria parva* Kiambu 5 was in Tp2 allele 2 with other 22 sequences. Two alleles clustered together with sequences isolated from buffaloes in Kenya as also observed in *Tp1*. While other two alleles clustered together with those of Zambian Chitongo strain. The samples that did not cluster together with the reference Muguga isolate were from Kasungu and Nkhotakota. However, sequences identical to the one present in *Theileria parva* Kiambu 5 were only found in Lilongwe where *Theileria parva* MC vaccine was used in both the vaccinated and unvaccinated cattle (Figure II-6).

The analysis of genetic diversity in *Tp1* and *Tp2*, using AMOVA revealed that in *Tp1* sequences, 50.5% of the variation occurred within populations, while 43.03% of the variation was attributable to differences between populations (Table II-4). When the *Tp2* locus was subjected to AMOVA, the result indicated that, 77.16% of the variation was within populations, while 20.06% was due to differences between populations (Table II-5).

The MJ network of *Theileria parva* haplotypes from Malawi using *Tp1* and *Tp2* sequence alignments for individual samples generated in this study were visually checked and concatenated to generate a combined data matrix (*Tp1 + Tp2*) in order to optimize the network signal as shown in Figure II-7. Samples with missing data for either of the loci were not included in the concatenated dataset.

Haplotype H1, was represented by majority of the sequences including *Theileria parva* Muguga and Serengeti-transformed and included samples from vaccinated cattle in Lilongwe and unvaccinated cattle from Kasungu and Nkhotakota. Whereas the other haplotypes (haplotype IDs: H2, H4, H5, H6, H7, H18 and H23) were separated from H1 by mutations at two or three positions. However, only 5 haplotypes (haplotype IDs: H3, H9, H17, H19 and H24) were disconnected from haplotype H1 by median vectors while the rest 20 haplotypes were not disconnected by median vector meaning that they share a common ancestor. Haplotype H6, which includes *Theileria parva* Kiambu 5 strain, clustered with sequences from both the vaccinated and unvaccinated cattle in Lilongwe. Sequences from the vaccinated animals were also observed in haplotypes H1, H18 and H20 which were separated from haplotype H6 by mutations at 2 to 3 positions. The composition of the sequences in each haplotype in the network are provided in table II-6.

4. Discussion

The overall *Theileria parva* positive rate of 54.5% (243/446) observed in this study using the nested *p104* PCR assay is comparable to those reported from the neighboring countries such as Tanzania and Zambia, where overall positive rates were 62%, and 54.9% respectively (Mwega et al., 2014; Muleya et al., 2012). Since the sampled animals were apparently healthy, this may suggest that the endemic stability against ECF in these southern African countries is well established in the local zebu cattle. Furthermore, the absence of clinical disease despite the presence of infection in the vaccinated exotic breed cattle may suggest that the MC vaccine worked as it was designed to prevent the occurrence of clinical disease but not infection (Oura et al., 2007; Chinombo et al., 1988).

Tp1 and *Tp2* genes sequence analysis has been used to investigate the genetic diversity of *Thileria parva* in Kenya (Pelle et al., 2011), South Sudan (Salih et al., 2017), Tanzania (Mwega et al., 2014; Kerario et al., 2019), DR Congo and Burundi (Amzati et al., 2019). These studies have shown that the *Tp1* gene is more conserved and has limited genetic diversity when compared with *Tp2* which is highly polymorphic, which is in accordance with the results obtained in this study. It has also been reported that *Theileria parva* parasites isolated from buffaloes have a higher genetic diversity when compared with cattle-derived parasites (Pelle et al., 2011; Kerario et al., 2019. Furthermore, these studies have also shown that there is widespread polymorphism even within the epitope coding regions in both antigens. This observation is supported by the identification of new epitopes that have not been reported previously whenever a new study was conducted (Salih et al., 2017, Kerario et al., 2019; Amzati et al., 2019). However, it has also shown that the majority of parasites in cattle share epitopes that are present in the *Theileria parva* Muguga strain, a component of the MC vaccine even in populations where the MC vaccine has not been deployed (Salih et al., 2017, Kerario et al., 2019; Amzati et al., 2019). This is also supported by the results of the current study and warrants the deployment of the MC live vaccine in these ECF endemic countries as the most effective way to control the disease. This observation may also help to explain why the MC live vaccine has been able to confer protection to immunized cattle over a wider geographical area.

This polymorphism in the nucleotide sequences may also help to explain the difficulties in designing primers to amplify all samples that were positive by the *Theileria parva* species-specific PCR, as also observed in Kenya (Pelle et al., 2011) and South Sudan (Salih et al., 2017). Similarly, the nucleotide polymorphisms (μ) of 1.02% in *Tp1* and 3.05% in *Tp2* also support previous studies that there is higher polymorphism in *Tp2* than in *Tp1*. This also translated into a higher level of polymorphism in amino acid residues of the *Tp2* gene, as the alignment of the amino acid sequences showed that 77/156 (49.36%) residues were variable, while 51.64% were conserved. However, in *Tp1*, 116/128 (90.6%) of the residues were conserved, while 9.4% were variable. The failure to sequence a considerable number of the *Tp2* gene may not provide a complete genetic diversity profile, which may result in underestimation as also reported by Salih et al. (2017). In this study, new epitope variants within the six *Tp2* mapped epitopes were identified than those reported previously (Salih et al., 2017; Pelle et al., 2011; Amzati et al., 2019; Kerario et al., 2019) Thus, this finding may also provide further evidence that there is genetic diversity in *Theileria parva* circulating in cattle in Malawi.

The phylogenetic analysis revealed that some of the sequences obtained in Kasungu and Nkhotakota were closely related to the buffalo type reported in Kenya (Pelle et al., 2011). Sibeko et al. (2010) reported that *Theileria parva lawrencei* (buffalo sp.) transmission to cattle can occur in the presence of vector ticks where contact with infected buffaloes or ticks is made susceptible in cattle. The sampling sites in the Kasungu and Nkhotakota districts were less than 5 km from the Kasungu National Park and Nkhotakota Wildlife Reserve, respectively, where buffaloes are present as such this may support the hyponthesis that some of these parasites may have been transmitted to cattle from buffaloes as the vector tick *Rhipicephalus appendiculatus* is present. However, *Theileria parva* has not been isolated from buffaloes in Kasungu and Nkhotakota wildlife reserves as no study has been conducted involving the samples from buffaloes. Thus, there is a need to conduct similar studies using samples collected from buffaloes in these areas. This may help to answer if *Theileria parva*

lawrencei is present in Malawi. Information from such molecular epidemiological studies will provide indisputable data based on which effective control strategies can be conceived.

In this study, most of the sequences obtained in both *Tp1* and *Tp2* genes were identical to the MC vaccine strains in the unvaccinated cattle. This observation may imply that the vaccine components are expanding into the unvaccinated cattle or that one of the local strains with similar genotypes is undergoing expansion. This may also be supported by a previous finding that the *Theileria parva* Kasowa strain isolated from the Karonga district in northern Malawi was antigenically similar to the *Theileria parva* Muguga strain (Musisi et al., 1996). However, the sequences identical to Kiambu 5 were only obtained in Lilongwe, where animals were vaccinated with MC at 27 months before the sampling period, in both the vaccinated and the unvaccinated. This demonstrates the transmission of the Kiambu 5 vaccine strain to unvaccinated cattle (Oura et al., 2007; Chinombo et al., 1988). It has also been documented that the *Theileria parva* Marikebuni vaccine strain can also be transmitted to unvaccinated cattle (Bishop et al., 2020a). Despite the MC being made up of three strains, sequences related to the Serengeti-transformed were not detected in this study. This was also observed in the previous reports, where researchers found only Muguga and Kiambu 5 but not Serengeti-transformed strains after the experimental inoculation of the MC against Friesian and Zebu cattle (Oura et al., 2007; Chinombo et al., 1988). The transmission of a vaccine strain to unvaccinated cattle provides evidence that the MC live vaccine may also be contributing to the genetic diversity of *Theileria parva* in cattle in Malawi. However, the absence of pre-vaccination data makes it difficult to confirm this hypothesis. Nonetheless, the significance of this observation is that the MC may have resulted in the induction of the immune response in the unvaccinated animals that get infected with the vaccine strains, which is beneficial in the short-term but its long-term impact cannot be theoretically predicted. The absence of complications resulting from the presence of vaccine strains in cattle in Malawi may suggest that there is cross-protective immunity between the vaccine strains and the local *Theileria parva* strain circulating in cattle in Malawi, which is advantageous.

In this study, it was observed that the vector tick can transmit the vaccine stocks from the vaccinated to the unvaccinated cattle when they mix during grazing. Thus, vaccination of animals with the MC in non-endemic areas is not recommended as such a vaccination can result in disease outbreak in naïve animals, as in the case of the Comoros Islands, in which the introduction of the MC-vaccinated cattle from Tanzania preceded the first outbreak of ECF in naive cattle (De Deken et al., 2007). In Malawi, where the Southern region is considered non-endemic to ECF, it is not recommended to introduce animals that have been immunized with the MC vaccine as this may result in an introduction of the MC vaccine stocks which can be virulent in naïve animals. As the vaccinated animals become asymptomatic carriers, they can be a potential source of parasites in the areas where the vaccinated animals are introduced (De Deken et al., 2007; Geysen et al., 1999). It is recommended that other control measures such as restriction of animal movement should also be taken together with the MC immunization.

Monitoring of the local genotypes of *Theileria parva* can help to know if any of the local genotypes are undergoing expansion or not. Such information will provide valuable knowledge for the control of ECF. Monitoring the MC sporozoite live vaccine using molecular tools when deployed is important as it provides epidemiological data based on which effective control strategies of ECF in endemic areas can be made such

as whether to continue with the MC approach or to use a local strain approach, as in the case of southern Zambia, where there were complications following the introduction of the MC vaccine (Geysen et al., 1999). Similarly, longitudinal studies, unlike cross-sectional studies, can provide more insight into the impact of the infection and treatment method (ITM) in the control of ECF in endemic countries, like the one done in Tanzania (Gwakisa et al., 2020). These studies can also help to address some concerns associated with the long-term use of the MC (Gwakisa et al., 2020; Bishop et al., 2020b). Thus, there is a need to carry out longitudinal studies in Malawi to have a complete profile and impact of the MC on the population genetics of *Theileria parva*.

This study has also revealed that *Theileria parva* in Malawi is diverse due to the presence of both MC-related and non-related *Tp1* and *Tp2* sequences. Considering that the majority of the sequences generated in this study were MC-related, this finding may justify the continuous use of the MC in the sampled districts in the central region of Malawi, especially among the exotic and the crossbreed cattle which are highly susceptible to clinical disease. However, despite the observed endemic stability in the Malawi zebu cattle, especially the adult cattle, it may be necessary to immunize calves which easily succumb to ECF when infected by *Theileria parva* (Chinombo et al., 1988; Knudsen, 1971). However, since the *Tp1* and *Tp2* antigens' immune response is dependent on the phenotype of MHC class 1 haplotypes, further studies are required to determine these phenotypes in the Malawi zebu cattle population. Similarly, since the samples were only collected in a limited geographical area in central Malawi, these results may not fully reflect the genetic profile of *Theileria parva* in Malawi. Furthermore, since the identified new epitope variants whose impact cannot be theoretically predicted, it is recommended that a national survey be conducted with additional molecular tools that can help to provide more information on the *Theileria parva* genetic profile and the impact of the MC approach in the prevention of ECF in Malawi. Border districts, due to the illegal international livestock movement, and national parks with buffaloes are vital to determine the full genetic profile of *Theileria parva* in Malawi and the contribution of wildlife to the epidemiology of ECF in Malawi. Continuous monitoring of the use of the MC is a pre-requisite to the understanding of the parasite population flow in the country.

5. Summary

The study in this chapter has provided molecular evidence that there is genetic diversity in *Theileria parva* in Malawi. It has also provided evidence that is in accordance with other studies, that there is a transmission of vaccine strains to unvaccinated cattle, as such a vaccination may be contributing to the genetic diversity of *Theileria parva* in Malawi. The finding of the sequences that are closely related to those isolated from buffaloes also emphasizes the need to sample buffaloes to know the role that wildlife may play in the epidemiology of *Theileria parva* in Malawi. Thus, this study has provided information that can help in the control of ECF in Malawi and other endemic countries as well as those countries that are at risk of introduction of the disease.

Table II-1. Polymerase chain reaction primers used for *Theileria parva* screening and *Tp1* and *Tp2* amplification and sequencing.

Primer name	Primer sequence (5′ to 3′)	Target gene/ (PCR type)	Amplicon size (bp)	Annealing temperature (°C)	Reference
IL 3231	ATTTAAGGAACCTGACGTGACTGC	*Theileria parva p104* gene (PCR)	496	55	Skilton et al., 2002
IL 755	TAAGATGCCGACTATTAATGACACC				
IL4234	GGCCAAGGTCTCCTTCAGAATACG	*Theileria parva p104* gene (nested PCR)	277	55	Odongo et al., 2010
IL3232	TGGGTGTGTTTCCTCGTCATCTGC				
Tp1 F outer	ATGGCCACTTCAATTGCATTTGCC	*Tp1* gene (PCR)	432	50	Pelle et al., 2011
Tp1 R outer	TAAATGAAATATTTATGAGCTTC				
Tp2 F outer	ATGAAATTGGCCGCCAGATTA	*Tp2* gene (PCR)	525	50	Pelle et al., 2011
Tp2 R outer	CTATGAAGTGCCGGAGGCTTC				
Tp1 Inner F1	CCGCKGATCCTGGATTCT	*Tp1* gene (semi-nested PCR)	419	55	
Tp1 R outer	TAAATGAAATATTTATGAGCTTC				
Tp1 Inner F2	CATTTGCCGCKGATCCTG	*Tp1* gene alternative (semi-nested PCR)	413	55	The outer primers: Pelle et al., 2011
Tp1 R outer	TAAATGAAATATTTATGAGCTTC				
Tp2 Inner F	CCGCCAGATTAATTAGCCTTT	*Tp2* gene (semi-nested PCR)	511	57	Inner primers: This study
Tp2 R outer	CTATGAAGTGCCGGAGGCTTC				
Tp2 F outer	ATGAAATTGGCCGCCAGATTA	*Tp2* gene alternative (semi-nested PCR)	505	57	
Tp2 Inner R	CCGGAGGCTTCTCCTTTTT				

F: forward; R: Reverse; PCR: Polymerase chain reaction

Table II-2. *Theileria parva* detection rates in cattle in Malawi with regard to host attributes.

Attribute	Number of cattle	Number of *Theileria parva* positive (%)	*p*-value
Origin			0.000051*
Kasungu	199	128 (64.3%)	
Nkhotakota	185	78 (42.2%)	
Lilongwe	62	37 (59.7%)	
Age			0.000348*
Calves (< 3months)	12	3 (25.0%)	
Weaners (3months–1 year)	83	32 (40.0%)	
Adults (>1years)	351	208 (59.3%)	
Breed			0.376194
Holstein Friesian	62	37 (59.7%)	
Malawi zebu	384	206 (53.6%)	
Sex			0.543121
Male	132	69 (52.3%)	
Female	314	174 (55.4%)	
Vaccination status			0. 037227*
Vaccinated	30	22 (73.3)	
Non-vaccinated	32	15 (46.9)	

Chi-square analysis determined the association, and *p*-values are shown. * $p < 0.05$ was considered to be significant.

Table II-3. *Tp2* Cytotoxic T lymphocytes epitope variants obtained in cattle in Malawi.

Epitope 1 ($Tp2_{27-37}$) (4 variants)	Epitope 2 ($Tp2_{40-48}$) (3 variants)	Epitope 3 ($Tp2_{49-59}$) (3 variants)	Epitope 4 ($Tp2_{96-104}$) (2 variants)	Epitope 5 ($Tp2_{98-106}$) (2 variants)	Epitope 6 ($Tp2_{138-147}$) (3 variants)
SHEELKKLGML (1,60,63,64,65,66)	**DGFDRDALF** (1,2,60,62,63,64)	**KSSHGMGKVGK** (1,2,60,62,63,64)	**FAQSLVCVL** (1,2,60,62,63,64,65,66)	**QSLVCVLMK** (1,2,60,62,63,64,65,66)	**KTSIPNPCKW** (1,2,60,62,64,65,66)
SDDELDTLGML (3,61)	PDLDKNRLF (3,61)	LTSHGMGRIGR (3,61)	FAASIKCVA (3,61)	ASIKCVAQY (3,61)	KPSVPNPCDW (3,61)
SDEELNKLGML (2)	*DGLNKDALF* (65,66)	*KTSKGMTEVGK* (65,66)			*KTSILNPCKW (63)*
SREKLKKLGML (62)					

Epitope variants were identified using the reference amino acid positions (Figure II-4). Numbers in brackets following the epitope sequences correspond to amino acid variants carrying the epitopes (Figure II-2). Epitope variants described for the first time are in italic and those found in Muguga cocktail vaccine stocks are in bold. *Tp2* antigen variants var-1 and var-2 are found in Muguga (identical with Serengeti-transformed) and Kiambu5 stocks, respectively. Var-1 to var-66, antigen variant names

Table II-4. *Tp1* analysis of molecular variation of cattle in Malawi.

Source of variation	*df*	sum of squares	Variance components	Percentage of variation
Among groups	1	49.554	4.13637 Va	43.03
Among populations within groups	2	93.706	0.62209 Vb	6.47
Within populations	224	1087.612	4.85541 Vc	50.50
Totals	227	1230.873	9.61387	
Fixed Indices				
F_{SC}	0.11357			
F_{ST}	0.49496			
F_{CT}	0.43025			

df = degress of freedom,

F_{SC} = variation among populations within groups,

F_{ST} = measure of population differentiation due to genetic structure,

F_{CT} = Variation among groups of populations.

Table II-5. *Tp2* analysis of molecular variation of cattle in Malawi.

Source of variation	*df*	sum of squares	Variance components	Percentage of variation
Among groups	1	39.384	1.90318 Va	20.06
Among populations within groups	2	40.925	0.26387 Vb	2.78
Within populations	183	1339.381	7.31902 Vc	77.16
Totals	186	1419.690	9.48607	
Fixed Indices				
F_{SC}	0.03480			
F_{ST}	0.22845			
F_{CT}	0.20063			

df = degress of freedom,

F_{SC} = variation among populations within groups,

F_{ST} = measure of population differentiation due to genetic structure,

F_{CT} = Variation among groups of populations.

Table II-6. The link between *Tp1* and *Tp2* sequences and the network haplotypes

Network Haplotype	*Tp1* sequence	*Tp2* sequence
H1	LC522067	LC522073
H2	LC522067	LC522079
H3	LC522068	LC522083
H4	LC522067	LC522080
H5	LC522067	LC522081
H6	LC522070	LC522074
H7	LC522067	LC522078
H8	LC522068	LC522075
H9	LC522071	LC522083
H10	LC522069	LC522076
H11	LC522069	LC522077
H12	LC522067	LC522076
H13	LC522067	LC522077
H14	LC522069	LC522080
H15	LC522070	LC522075
H16	LC522067	LC522082
H17	LC522068	LC522082
H18	LC522067	LC522075
H19	LC522068	LC522084
H20	LC522070	LC522073
H21	LC522067	LC522074
H22	LC522068	LC522073
H23	LC522067	LC522084
H24	LC522068	LC522085
H25	LC522067	LC522085

The linkage between the haplotypes of the concatenated *Tp1* and *Tp2* gene and their sequences

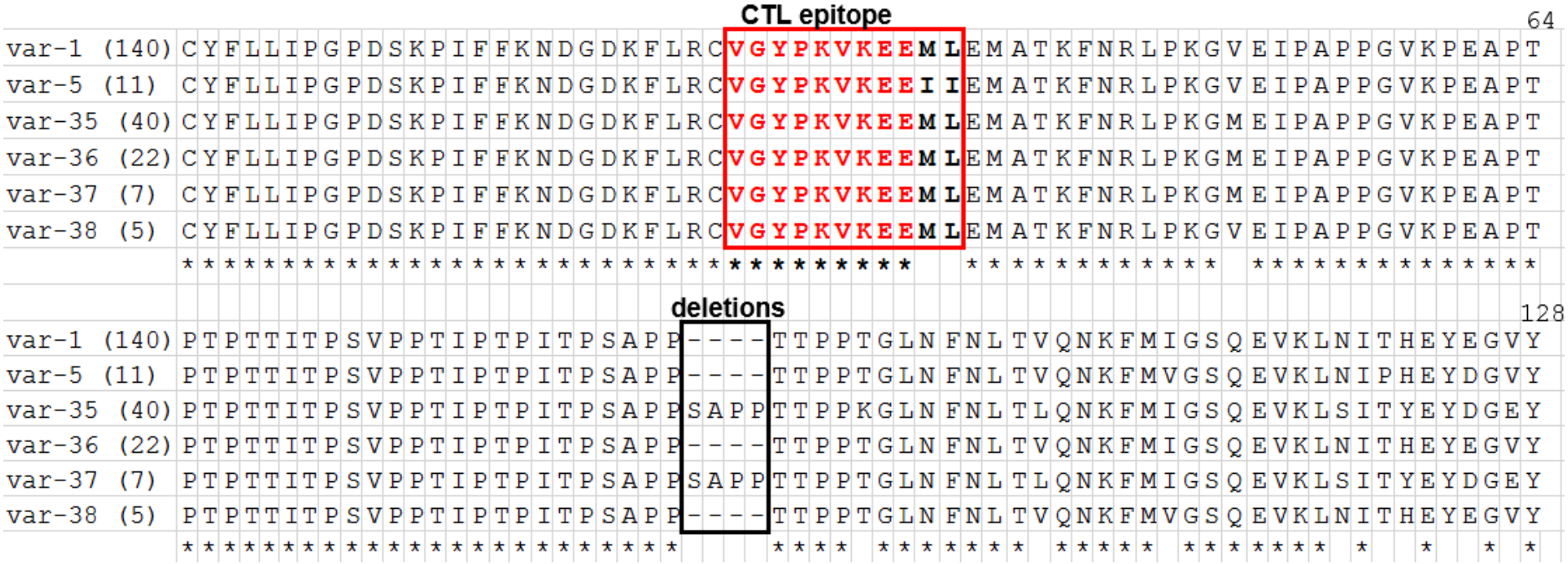

Figure II-1. Multiple amino acid sequence alignment of 6 *Tp1* amino acid variants in 223 *Theileria parva* samples obtained from cattle in Malawi. Antigen variants are named var-1 to var-38. The single letter amino acid code is used. The antigen variants nomenclature used in this study was first proposed by Pelle et al. (2011). Variants (var-1 and var-5) were first described by Pelle et al. (2011). The numbers in brackets behind variants names indicate the number of *Theileria parva* isolates represented by each variant. The single previously identified *Theileria parva* $CD8^+$ T cell target epitope is bolded and red boxed. The conserved amino acid residues in the CTL epitope are coloured in red. Conserved amino acid residues are denoted by (*) below the alignment, and dashes (–) denote deletion region. *Tp1* antigen variant var-1 is found in the three Muguga vaccine stocks (Muguga, Kiambu5 and Serengeti-transformed). Corresponding gene alleles are presented in figure II-3.

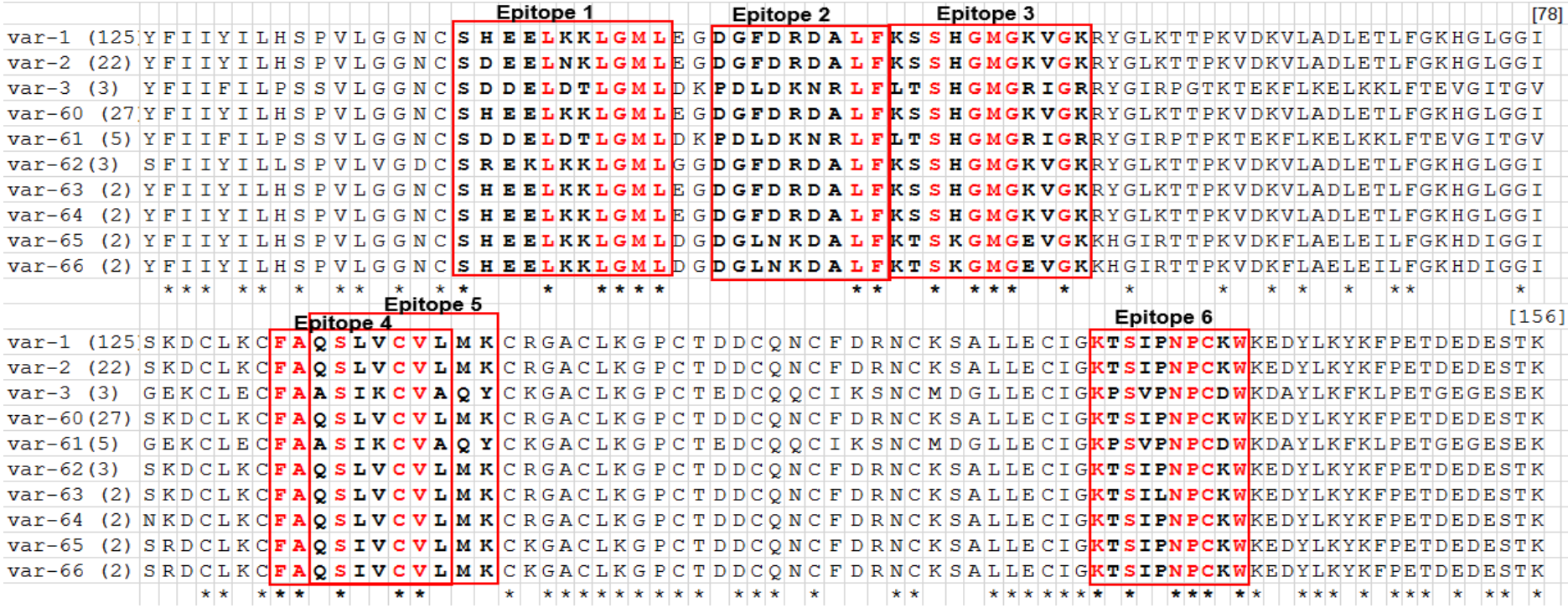

Figure II-2. Multiple amino acid sequence alignment of 10 *Tp2* antigen variants in 190 *Theileria parva* samples obtained from cattle in Malawi. Amino acids are denoted by the single-letter code. Variants var-1 to var-66 are named. The antigen variants nomenclature used in this study was first proposed by Pelle et al. (2011). Antigen variants var-1, var-2 and var- 3 were described in Pelle et al. (2011). The numbers in brackets behind variants names indicate the number of *Theileria parva* samples represented by each variant. The six previously described epitopes (epitope 1 to 6) that are the target of the bovine $CD8^+$ T cells immune responses are bolded and red boxed. The conserved amino acid residues in the epitopes are coloured in red. The star (*) below the alignment indicates positions of conserved amino acid residues. *Tp2* Antigen variants var-1 is found in two *Theileria parva* Muguga vaccine stocks (Muguga and Serengeti-transformed) while *Tp2* antigen variant var-2 is found in Kiambu5 vaccine stock. Corresponding gene alleles are presented in figure II-4.

Figure II-3. Multiple sequence alignment of *Tp1* alleles obtained in this study from cattle from Malawi. The naming of the alleles follows the nomenclature by Pelle et al. (2011). Alleles 1 and 5 were first described by Pelle et al. (2011). The epitope coding region are boxed in red. The number in parenthesis is the number of sequences obtained in the allele.

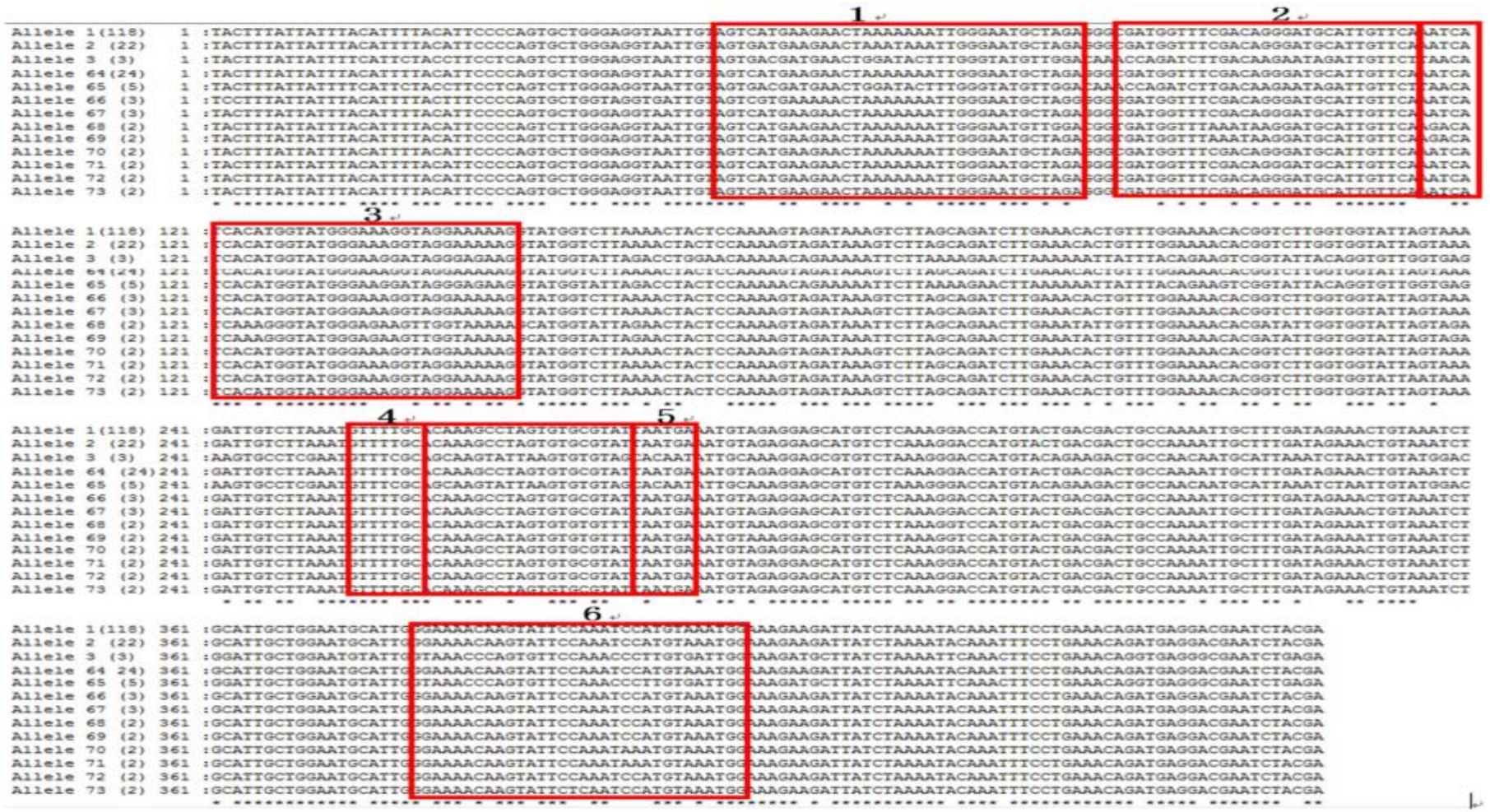

Figure II-4. Multiple sequence alignment of *Tp2* alleles obtained in this study from cattle from Malawi. The naming of the alleles follows the nomenclature by Pelle et al. (2011). Alleles 1 and 2 and 3 were first described by Pelle et al. (2011). The epitope coding region are boxed in red. The number in parenthesis is the number of sequences obtained in the allele.

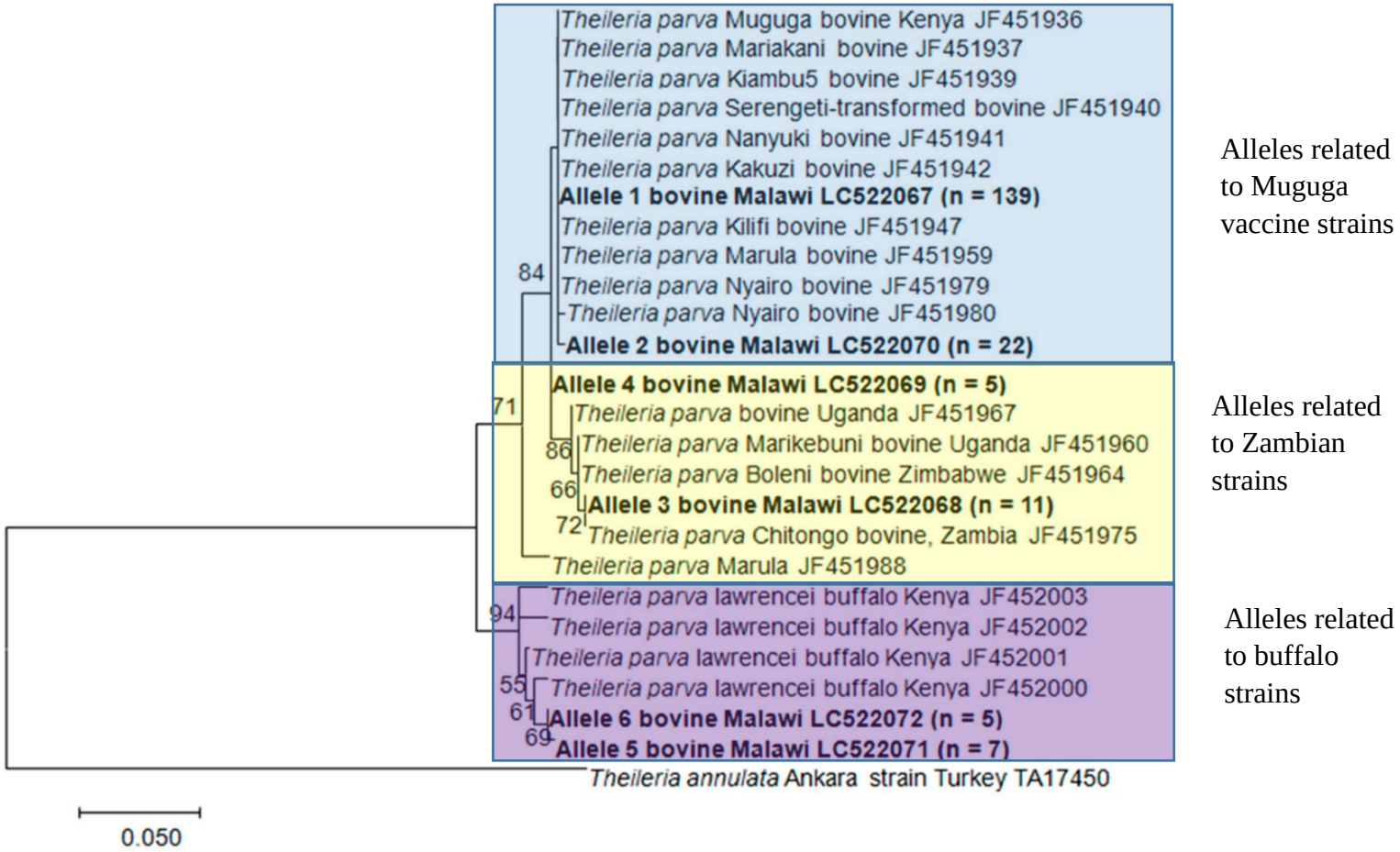

Figure II-5. The maximum likelihood tree of the *Tp1* gene sequences indicating phylogenetic relationships among the cattle-derived *Theileria parva* isolates. The partial sequences obtained in this study are in bold and the number in parenthesis is the number of sequences obtained in the allele. The sequence of the *Theileria annulata Tp1* homologue (TA17450) was used to root the tree. Bootstrap values >50% are shown above the branches.

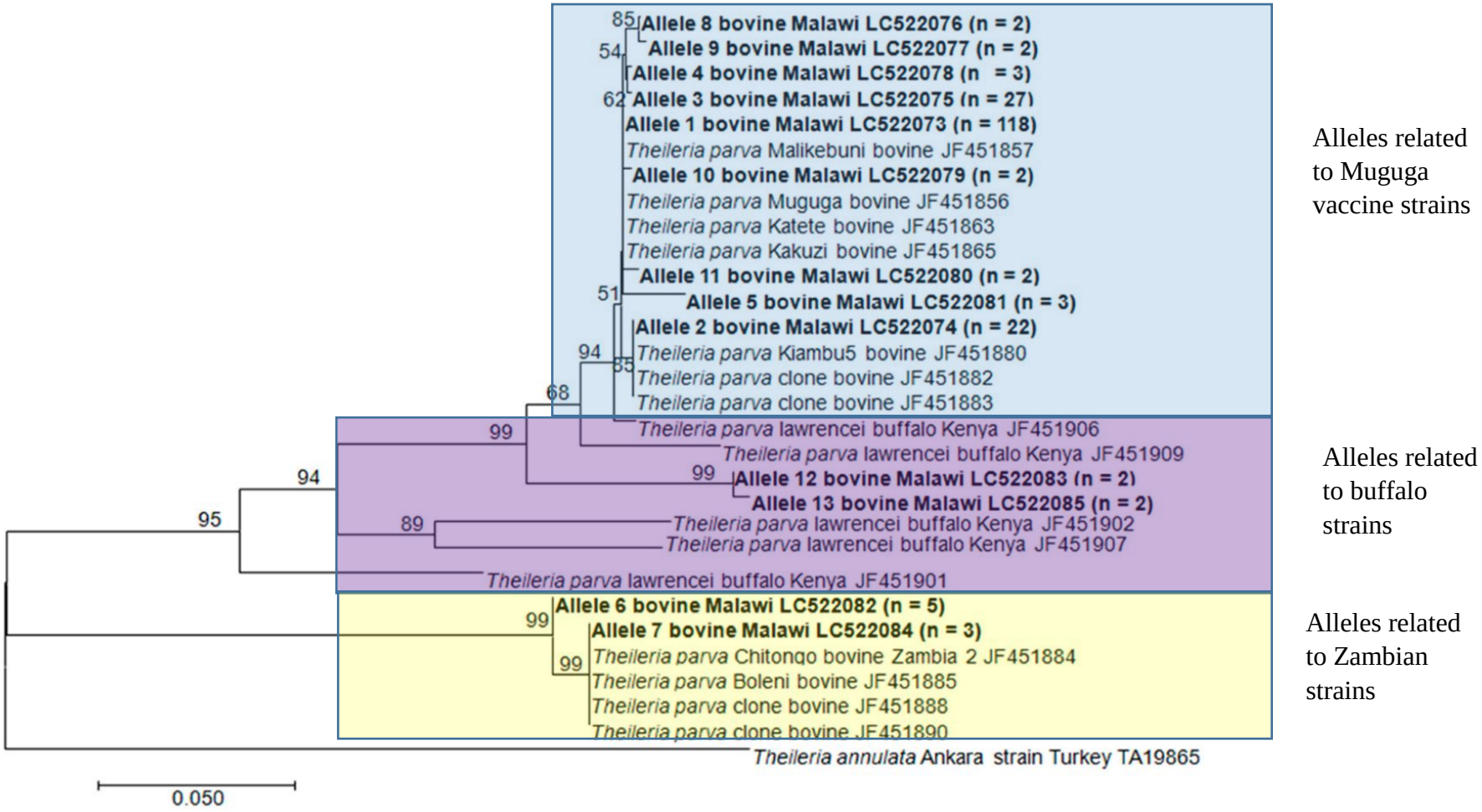

Figure II-6. The maximum likelihood tree of the *Tp2* gene sequences indicating phylogenetic relationships among the cattle-derived *Theileria parva* isolates. The partial sequences obtained in this study are in bold and the number in parenthesis is the number of sequences obtained in the allele. The sequence of the *Theileria annulata Tp2* homologue (TA19865) was used to root the tree. Bootstrap values >50% are shown above branches.

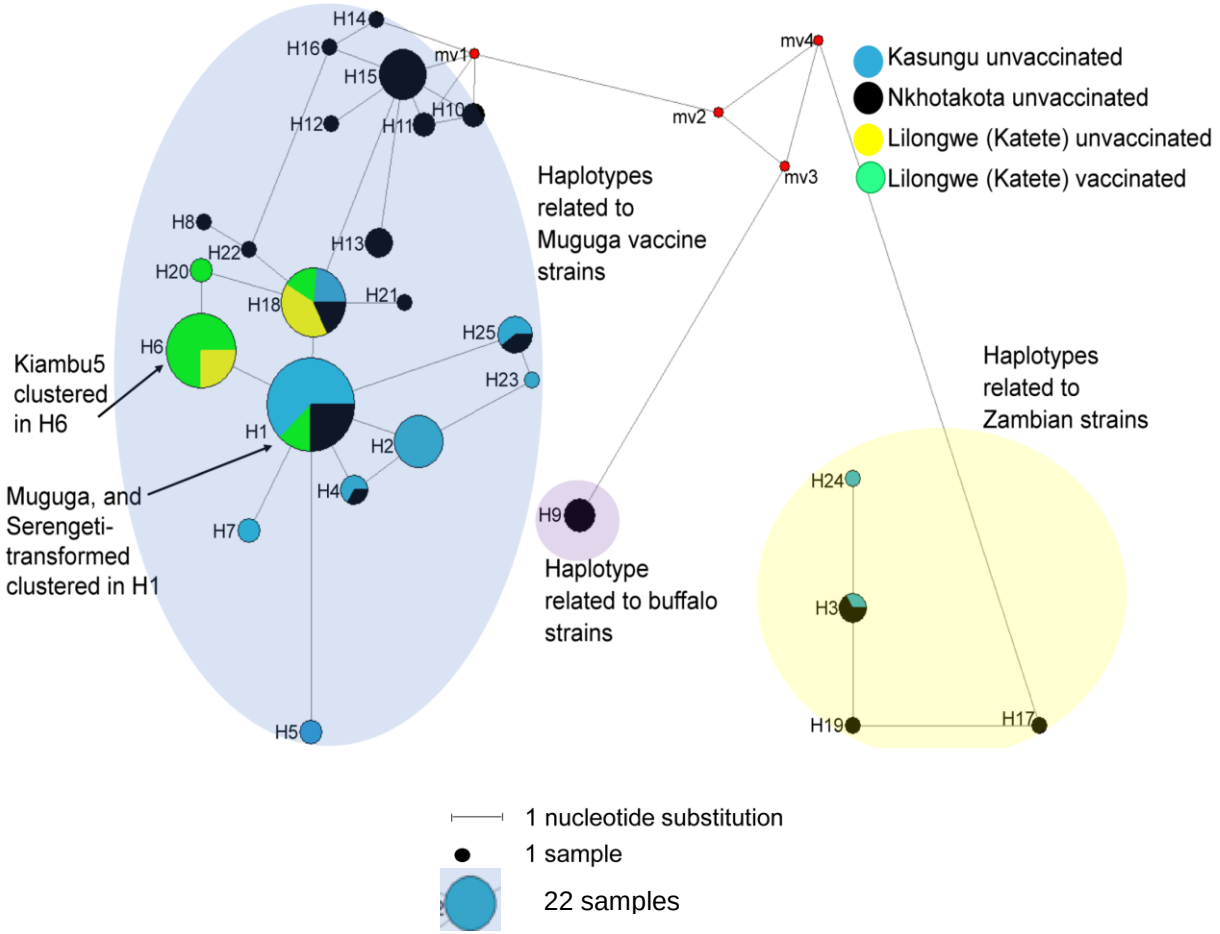

Figure II-7. Median joining network of concatenated (*Tp1 + Tp2*) nucleotide sequences using samples that have sequences in both loci. *Theileria parva* Muguga, and Serengeti-transformed sequences clustered in H1 while Kiambu-5 sequences clustered in H6. The size of the circle is proportional to the haplotype frequencies. The origin of each haplotype is colour coded.

Chapter III

Genotyping of *Theileria parva* populations in vaccinated and non-vaccinated cattle in Malawi

1. Introduction

Theileria parva, is the most important tick-borne pathogen of cattle on the African continent (Lubembe et al., 2020). It is estimated that almost half of the cattle population on the African continent are at risk of infection with *Theileria parva* (Lubembe et al., 2020). Although adult indigenous cattle in Africa rarely develop clinical diseases, calves under 6 months old are highly susceptible (Perry & Young, 1995; Moll, 1984, 1986). However, in the exotic and crossbred cattle, cases fatality rate may be as high as 80-100% and this has negatively impacted the development of the dairy industry in the endemic countries (Perry & Young, 1995).

The control of ECF in Malawi is mainly based on tick control through acaricide application, livestock movement restriction where cattle from endemic northern and central regions are not allowed to go to the non-endemic southern region. Chemotherapy using buparvaquone is used to manage clinical cases in the endemic areas and immunisation of cattle using the Muguga cocktail live vaccine. The infection and treatment method (ITM) which involves inoculating the animal with a dose of the Muguga cocktail vaccine and silmutaneous treatment with long acting teteracycline is currently in use in the northern and central regions where the disease is endemic (DAHLD, 2006). However, in the southern region which has been considered non-endemic, the vaccine has not been deployed although sporadic cases of ECF have been reported (Chinombo et al., 1988). The study in chapter II showed extensive genetic diversity of *Theileria parva* in cattle in Malawi from the central region based on the two genes, *Tp1* and *Tp2,* encoding *Theileria parva* antigens recognised by bovine $CD8^+$ T cells. However, since these genes cover a limited region of the *Theileria parva* genome, there was a need to employ markers that cover the whole genome for comprehensive characterization (Oura et al., 2007). The whole genome sequencing of *Theileria parva* by Gardner et al. (2005) and the identification of *Theileria parva* specific minisatellite and microsatellite markers which were first described and applied by Oura et al. (2003, 2005) and Katzer et al. (2006, 2010) have provided good markers for the genotyping and characterization of the population structure of *Theileria parva*.

However, the deployment of vaccine that will be effect against the *Theileria parva* strains circulating in cattle requires genotypic and population genetics data in the endemic countries. Despite *Theileria parva* being widely spread in central, eastern and southern Africa, there is limited information on the parasite genotypes and population structure available in southern Africa. The information on *Theileria parva* parasites from cattle available is only from Zambia (Muleya et al., 2012) and South Africa (Lubembe et al., 2020), although the disease is controlled in South Africa. Lubembe et al. (2020) investigated the genotypes of *Theileria parva* from buffaloes in Mozambique and South Africa. The knowledge about the genotypes and population structure of *Theileria parva* on the African continent is important to assess diversity of *Theileria parva* on the continent and these strains may affect the current vaccines being used to control ECF in Africa.

This study aimed at investigating the genetic population structure of *Theileria parva* among cattle in Malawi using *Theileria parva* mini- and microsatellite markers. The specific objectives were 1) to

determine if genotypes of *Theileria parva* from vaccinated cattle with *Theileria parva* Muguga cocktail vaccine will be different from those without vaccination history. 2) to determine if there is presence of sub-structuring among the *Theileria parva* isolates in Malawi and 3) to determine if the population of *Theileria parva* in Malawi is panmictic.

2. Materials and methods

2.1 Sample collection and DNA extraction

The cattle blood DNA samples from Kasungu (*n* = 20), Nkhotakota (*n* = 20) and Lilongwe (Katete farm) (*n* = 19), used in this study were obtained from the previous study in chapter II. The other samples were collected from Likasi farm (33º 17' 05" E; 14º 02' 43" S) (*n* = 92), and Lilongwe University of Agriculture and Natural Resources (LUANAR) student farm (33º 77' 83" E; 14º 17' 96" S,) (*n* = 53) in the central region and Mikolongwe farm (35º 12' 30" E; 15º 51' 49" S) (*n* = 28) in the southern region (Figure I-1).

The breeds of cattle sampled at Likasi farm, LUANAR farm and Mikolongwe farm, were Holstein Friesians, Malawi zebu and their crossbreeds. At Likasi and LUANAR farms, cattle were kept under semi-intensive management system and immunised with the Muguga cocktail vaccine and dipping is done weekly to control ticks. At Mikolongwe farm, which is located in the southern region, the sampled animals were Holstein Friesians and Malawi zebu cattle which were dipped weekly but were not vaccinated against *Theileria parva* as the farm falls in a non-endemic area. Although the farms at Likasi, LUANAR and Mikolongwe did not allow the animals to mix with other herds, they allowed introduction of Malawi zebu heifers from smallholder farmers to crossbreed with the exotic breeds.

The sampling was done as described in chapter I.

2.2 *Theileria parva* screening

Nested PCR assays targeting the *Theileria parva*-specific *p104* gene were used for the screening as described by Odongo et al. (2010). The amplification was done using Tks Gflex DNA Polymerase, the reaction mixture and PCR conditions were set as previously described in chapter II. Thereafter, 100 *Theileria parva* positive samples were randomly selected for microsatellite analysis.

2.3 PCR amplification of microsatellites

The forward primer of each pair was fluorescently labelled with either 6-FAM, VIC or PET at the 5' end. The amplifications were conducted in a 10 µL reaction mixture, containing 5.0 µL of 2×Gflex PCR Buffer (Mg^{2+}, dNTP plus) 0.2 µL of Tks Gflex DNA Polymerase, 0.5 µL of 200 nM of each primer, 10.0 ng of the DNA template. The volume was adjusted using distilled water. Negative control containing distilled water instead of DNA template was used for quality control. The cycling conditions were set with an initial denaturation at 94 °C for 3 min, followed by 45 cycles of denaturation at 98 °C for 10 sec, annealing for 30 sec, and extension at 68 °C for 1 min and final extension at 68 °C for 5 min. The amplicons were

electrophoresed in a 1.5% agarose gel stained with Gel-Red and visualized under UV light. The primers used for satellite amplification and their annealing temperatures are shown in Table III-1.

2.4 Capillary electrophoresis

The capillary electrophoresis was conducted in a reaction volume of 11.5 µL comprising of 0.5 µL 600 LIZ size standard (Applied Biosystems, CA, USA), 10.0 µL ABI HiDi formamide (Applied Biosystems, CA, USA) and 1.0 µL of 10 fold diluted microsatellite PCR product. The mixture was denatured at 95°C for 5 min and the heat shock on ice before capillary electrophoresis on an ABI 3500xl genetic analyser.

2.5 Fragment analysis

The DNA fragment sizes were analysed relative to the ROX-labelled GS 600 LIZ size standard using Gene Mapper software version 6 (Applied Biosystems, CA, USA). This facilitated the resolution of multiple products with 1 base pair (bp) difference in a single reaction. Multiple products from a single PCR reaction indicated the presence of mixed genotypes. The output data from the genetic analyser were provided as the area under the peak of each allele (quantitative measurement), with the predominant allele possessing the greatest peak area. In this way, the predominant allele at each locus was identified for each sample, and this data was combined to generate a multi-locus genotype (MLG) representing the most abundant genotype in each sample. Only the alleles with the prescribed base pair range were used to generate the MLG and samples from the same area were electrophoresed and gene scanned on the same plate.

2.6 Data analysis

An allele sharing co-efficient (Bowcock et al., 1994) in Excel microsatellite toolkit (*http://animalgenomics.ucd.i.e./sdepark/ms-toolkit/*) was used for the similarity comparison of the MLGs (Peakall and Smouse, 2006, 2012). Similarity analysis was determined by Principal coordinate analysis (PCoA). A similarity matrix was constructed and used to construct PCoA using the Excel plug-in software GenAIEx6 (*http://www.anu.edu.au/BoZo/GenAIEx/*). FSTAT computer package version 2.9.3.2 was used to calculate estimates of F statistics for population genetic analysis (*http://www2.unil.ch/popgen/softwares/fstat.htm*). LIAN (*http://adenine.biz.fh-weihenstephan.de/lian/*) was used to test the null hypothesis of linkage equilibrium by calculating a quantification of linkage equilibrium/linkage disequilibrium called the standardised index of association (I_AS) (Haubold & Hudson, 2000). The statistical independence of allele's at all pairwise combinations of loci under study characterizes linkage equilibrium (LE) and this independent assortment was initially tested by LIAN by determining the number of loci at which each pair of MLGs differs. The mismatch values from this distribution were then used to calculate the variance (VD) which was then compared to the variance expected (VE) for LE. Monte Carlo (MC) computer simulation was used to test the null hypothesis that VD = VE. The computer software calculates a 95% confidence limit L. When VD was greater than critical limit L, the null hypothesis of LE was rejected.

2.7 Multiplicity of infection

Mixed infection in the isolates were observed as shown by the presence of several alleles at a locus in 1 sample in most of the samples analysed. To determine the multiplicity of infection at the 9 loci used, the mean number of alleles in each sample was calculated. Finally, the mean for each population as well as the combined population from the index value of each were also calculated to show the overall multiplicity of infection for each population and combined population.

3. Results

3.1 Confirmation of *Theileria parva* PCR-positive samples

Overall, 41 (23.5%) of the 173 examined samples were positive on *Theileria parva* species specific PCR assays. The positive rates for Likasi, LUANAR and Mikolongwe farms were 18% (17/95), 32% (17/53), and 25% (7/28), respectively. The findings of this study confirms for the first time that the *Theileria parva* infection in cattle in the southern region using molecular technique.

3.2 Satellite marker diversity and allelic variation

The number of alleles per locus ranged from 3 in marker MS77 to 33 in marker MS14 (Table III-2), the average number of alleles observed per locus was 18.67 (Table III-3). Markers MS14, MS9, and MS19 had the most number of alleles observed at 33, 30, and 29 alleles, respectively. In this study, shared alleles were observed at all the 9 loci investigated with no locus having unique alleles for a particular population (Figure III-1). The number of shared alleles ranged from 2 at locus MS77 to 11 at locus MS19 (Figure III-1). Similarly, the analysis of genetic diversity of the 9 loci investigated showed that markers MS48 and MS77 were the least diverse in all the six populations. The populations from Kasungu and Nkhotakota showed low or no diversity at the loci MS48 and MS77 (Table III-2). Similarly, the population from LUANAR farm, showed no diversity at locus MS77, but the diversity of marker MS47 could not be determined in this population due to limited number of samples successfully amplified at this locus (n = 3) (Table III-2).

3.3 Population diversity structure

The PCoA of *Theileria parva* for the six cattle populations in Malawi and the *Theileria parva* Muguga reference strain showed that there were two clusters that were designated A and B (Figure III-2). Cluster A had majority of the samples (n = 83) from all the six sampling sites and the *Theileria parva* Muguga reference strain while cluster B, had 17 samples from Katete (n = 14) and Likasi (n = 3) in the central region. There was sub-structuring clustering in which majority of the isolates from Katete farm clustered only with three samples from Likasi (cluster B) but separate from cluster A (Figure III-2). The majority of the isolates from Malawi, apart from some from Katete and Likasi clustered together with *Theileria parva* Muguga reference strain. Interestingly, it was observed that even in populations without MC vaccination history, the isolates clustered in the same population as *Theileria parva* Muguga strain, the component of the Muguga cocktail vaccine (Figure III-2). The AMOVA showed that the genetic variation observed was mainly within populations (99%) while that due to differences between populations was only

1% (Table III-4).

The analysis of the allelic profile data used to determine the linkage of *Theileria parva* in Malawi showed that when the sub-populations were combined as a single population, the standardized index of association (I_SA) was greater than zero (0.0286). The pairwise variance (VD) was greater than the 95% critical L value (ML) which indicated that it was in linkage disequilibrium (Table III-3). However, when each sub-population was treated as a separate population, it was observed that most populations 4/6 (66.67%) had an (ISA) which was either negative or close to zero and the (VD) was less than the (ML) which provided evidence of linkage equilibrium (Table III-3). There were two populations (Katete and Likasi) which showed linkage disequilibrium when treated separately. This observation may be supported by the presence of isolates that clustered into two separate populations as shown on the PCoA (Figure III-2). When the samples were treated as two population based on the findings of the PCoA, both clusters A and B, showed linkage equilibrium (Table III-3).

To determine the differences among the sampled populations the estimated heterozygosity (H_e) and mean number of genotypes/locus were calculated for each of the six population. The estimated heterozygosity (H_e) ranged from 0.612 for Mikolongwe and LUANAR to 0.790 for Likasi (Table III-3). The mean number of genotypes/locus ranged from 3.56 to 7.22 for Mikolongwe and Likasi, respectively (Table III-3). This shows that the population at Likasi is more diverse than the other populations while the one at Mikolongwe is the least diverse. The overall estimated heterozygosity (H_e) and mean number of genotypes/locus for the combined population was 0.666 and 18.67, respectively.

To determine the degree of genetic differentiation, the Wright's F index was calculated for each of the two clusters based on the PCoA. Cluster A which had majority of the isolates had an F_{ST} value of 0.008 while cluster B with isolates from Likasi and Katete had an F_{ST} value of 0.273 (Table III-3). This finding shows significant differences between these two clusters. The combine population had an F_{ST} value of 0.105 (Table III-3).

3.4 Multiplicity of infection

Multiple genotypes of *Theileria parva* were observed in the majority of the samples regardless of the sampling site and region. The multiplicity of infection in the six sub-populations ranged from 1.14 to 1.40 for the population from Mikolongwe and Nkhotakota, respectively as estimated by the calculated mean number of genotypes/ locus (Table III-5). The standard deviation ranged from 0.21 to 0.36 for Mikolongwe and Kasungu populations, respectively. The combined population had the mean value of 1.32 and a low standard deviation of 0.29 (Table III-5).

4. Discussion

Knowledge about the genetic information and population structure of *Theileria parva* is a pre-requisite to the conception of effective control measures and monitoring of current measures (Lubembe *et al*. 2020). However, this information is not available in most southern African countries including Malawi

although it is one of the endemic regions. *Theileria parva* is the most important tick-borne pathogen in Malawi and is endemic in the central and northern regions (DAHLD, 2006). Current control measures in use include the immunisation using *Theileria parva* Muguga cocktail live sporozoite vaccine for exotic and crossbreed cattle but not for Malawi zebu in the endemic areas (Perry, 2016). To understand the genetic composition and population structure of *Theileria parva* in Malawi, nine microsatellite markers were employed to examine 100 *Theileria parva* samples from six populations; five in the central region where ECF is endemic and one in the southern region which is considered non-endemic until this study. To control spread of ECF in Malawi, movement of animals from the northern and central regions to the southern region is not allowed and vaccination with *Theileria parva* Muguga cocktail vaccine is not permitted (DAHLD, 2006).

Almost all the populations investigated had high mean genetic diversity (expected heterozygosity) that ranged from 0.612 for Mikolongwe and LUANAR to 0.790 for Likasi. The lower expected heterozygosity at Mikolongwe may be due to limited number of samples as it had the least number of samples among the populations examined. Furthermore, this observation may result from the pathogen being newly introduced in this region as reported previously in South Sudan (Salih et al., 2018; Marcelino et al., 2017). The high expected heterozygosity at Likasi farm is also supported by the PCoA results which showed that the isolates at the farm separated into two populations and majority were in population B while the rest were in population A. The high estimated heterozygosity of 0.790 at Likasi farm than the other sites may result from longer history of genetic recombination than the other populations as it was used as a trial site when the Muguga cocktail live vaccine was being developed for the eastern and southern Africa region (Lawrence et al., 1996; Dolan, 1988). The overall estimated heterozygosity of 0.670 is within the same range as 0.750 reported in Zambia (Muleya et al., 2012), 0.730 reported in South Sudan (Salih et al., 2018), and 0.790 reported in Tanzania (Rukambile et al., 2016). However, it was slightly lower than 0.910 reported in Burundi (Atuhaire et al., 2021) and 0.810 of combination of eastern and southern Africa isolates from cattle and buffaloes (Lubembe et al., 2020). This observation may be due to sampling from a wider geographical area where there was higher mean genetic diversity.

The observation of shared alleles in majority of the samples from all the sampled populations at all loci investigated shows that similar strains of *Theileria parva* are circulating among cattle in Malawi. This has also been strongly supported by the PCoA which clustered majority of the isolates in population A from all the six sampled populations and included the Muguga vaccine reference strain. However, it has also been shown that there is a separate population especially those from Katete that is different from the Muguga vaccine reference strain and majority of the samples from Malawi. This finding is in accordance with a previous study in chapter II which showed that most sequences of *Theileria parva* in Malawi based on *Tp1* and *Tp2* genes were either identical or similar to *Theileria parva* Muguga, *Theileria parva* Kiamabu-5 and *Theileria parva* Serengeti transformed, the components of Muguga cocktail vaccine. The separate population at Katete may result from it being a closed population that does not allow introduction of other *Theileria parva* populations in Malawi. The other isolates at Katete farm that clustered in population A may be due to

the immunisation with Muguga cocktail vaccine. Although the other farms also have restricted access to other animals, they allow breeding stock from smallholder farmers in Malawi into their population which is likely to explain the relatedness with those populations from smallholder farmers. The finding of mixed genotypes within a sample shows that mixed infections of *Theileria parva* are common among cattle in Malawi. This may be due to prolonged exposure to ticks and the complex life cycle to *Theileria parva* which includes sexual and asexual stages in the vector tick and host cattle, respectively (Katzer et al., 2010). The genetic recombination phenomenon of *Theileria parva* in the vector tick which it has adopted as a strategy to survive and drive genetic diversity ensures that host cattle can still be infected with new strains (Oura et al., 2003).

The AMOVA has shown that *Theileria parva* isolates in cattle in Malawi have high chance of exchanging genetic material within each population than between populations. This has been supported by high genetic variation within population (99%) than between populations (1%). This finding is also in agreement with previous studies which reported similar findings in other countries (Lubembe et al., 2020; Salih et al., 2018). As reported previously by Salih et al. (2018) in South Sudan, the findings of this study do not support the presence of any correlation between genetic structure of a population and the geographical location of the isolates.

The analysis of linkage equilibrium which showed that majority of the individual populations was in linkage equilibrium and thus in panmixia further support the results of PCoA and AMOVA that gene flow is likely to occur within a particular population. Although this finding is not in agreement with that reported in Zambia (Muleya et al., 2012), it is supported by the findings by Lubembe et al. (2020) that individual populations in southern Africa were in linkage equilibrium. When the populations were combined, linkage disequilibrium was observed. This may be due to Katete farm being a closed population that makes it difficult for random mating to occur even with other populations within a 50 km radius. When the population from Katete farm was excluded, the combined population was in linkage equilibrium even with those from Mikolongwe farm with almost 500 km distance from the other population.

The wildlife, especially the African buffalo (*Syncerus caffer*), plays an important role in the epidemiology of ECF in eastern and southern Africa (Mukolwe et al., 2020, Oura et al., 2011a, 2011b). The *Theileria parva* parasites in the buffalo have shown to be more diverse than those isolated from cattle (Pelle et al., 2011; Kerario et al., 2017). The African buffalo is a non-symptomatic carrier of *Theileria parva* that occasionally spill over to cattle at the wildlife, livestock, and human interface areas (Kock et al., 2014). The *Theileria parva* parasites from buffaloes have been reported to be more pathogenic when they infect cattle and usually have a fatal outcome (Morrison et al., 2020; Kock et al., 2014). In Zambia, Square et al. (2020), showed that the wildlife species were infected with a wide range of piroplasms of the genera *Babesia*, *Theileria*, *Hepatozoon* and *Colpodella*. Despite the presence of the African buffaloes in most wildlife reserves in Malawi, there is no any study that has been conducted to investigate their role in the epidemiology of *Theileria parva* and other TBPs.

In conclusion, this study has for the first time provided molecular evidence of the presence of *Theileria parva* infection in cattle in the southern region of Malawi. Furthermore, the findings have shown that the *Theileria parva* population in southern Malawi is closely related to the populations in the central region which is endemic to ECF. Generally, the population of *Theileria parva* in Malawi is not panmictic but majority of individual populations are in panmixia. It has also been shown that there is presence of sub-structuring among the samples investigated. It is therefore important to carry out a nationwide wide study to provide a comprehensive genetic population structure of *Theileria parva* in Malawi and to include isolates from the neighbouring countries to see the relatedness of the isolates in the region. This will help to assess the impact of the *Theileria parva* populations that are different from the *Theileria parva* MC vaccine strain on the control of ECF in Malawi in particular and southern Africa as a region in general.

5. Summary

The study in this chapter has provided molecular epidemiological data that there are two populations of *Theileria parva* strains circulating in cattle in Malawi. Furthermore, this study has shown that the transfer of *Theileria parva* is likely to occur within a population than between populations. It has also been highlighted in this study that there are no differences in the *Theileria parva* strains that were investigated in all the six populations in Malawi. The detection of *Theileria parva* infection in cattle in southern region of Malawi indicate that the parasite is expanding its geographical distribution in Malawi.

List of tables

Table III-1. The list of satellite marker primers used for population genetic analysis of *Theileria parva* in Malawi.

Marker ID	Primer name	Sequence 5'→ 3'	Chromosome	Annealing temperature (°C)	Amplicon size (bp)	Reference
ms1	ms1F	TGAGGCAGTGTAGAGCGCATAAC	1	60	235-368	Oura et al. 2003
	ms1R	AAATCCGCAACGCTATTGCCGAGG				
MS9	MS9F	CTGGTTCCTCATCTTCACACTA	3	60	230	Katzer et al., 2006
	MS9R	CTTTCCAGAACCTACAATCAC				
MS14	MS14F	ATGCCAATTCGGTAAAGGTCTCCG	2	60	360-600	Katzer et al. 2010
	MS14R	GCATATCTCAGTCAAGCCAACATC				
MS19	MS19F	CCAGACACCTCAAATCCCAAGTA	2	60	304	Oura et al. 2003
	MS19R	CCACACTGCCACCTAATACAAA				
MS39	MS39F	CCAATCAACATCAACTACTCC	4	60	263	Katzer et al. 2010
	MS39R	CGAACTCCAAACGATCTAAAC				
ms47	M47F	GTCACAAGGGAAATCATGTCACTC	1	60	398	Katzer et al. 2006
	M47R	GAGCCTTGAGTAGGTCTAAATTTG				
MS48	M48F	CTACTTCTGGATCAGGTGTGGTGG	1	60	223	Katzer et al. 2006
	M48R	GATTGAGACGATCCCGGTAGTCCT				
MS68	M68F	TCACATCGGGTAACAAGAA	1	60	469	Katzer et al. 2010
	M68R	TATTTATCGACCCCAAATCG				
MS77	MS77F	GGTAACCAACAACCACATTT	2	60	270	Katzer et al. 2010
	MS77R	TGCTTATGAACTCAATCATCTC				

ms = microsatellite, MS = minisatellite, F = forward, R = reverse, bp = base pair.

Table III-2. Allelic variation among *Theileria parva* in cattle in Malawi

	Sampling site	N	Satellite marker								
			ms1	MS9	MS14	MS19	MS39	MS47	MS48	MS68	MS77
Alleles within population	Kasungu	20	3	11	13	12	6	9	1	5	1
	Nkhotakota	20	4	7	13	12	8	8	2	5	1
	Katete	19	4	8	5	8	6	2	2	7	2
	Likasi	17	6	11	11	11	8	6	4	6	2
	LUANAR	17	4	7	10	9	3	1	4	7	1
	Mikolongwe	7	3	5	6	4	3	4	2	3	2
	Overall	100	9	30	33	29	22	20	7	15	3
Genetic diversity	Kasungu	20	0.511	0.860	0.963	0.971	0.542	0.945	0.000	0.660	0.000
	Nkhotakota	20	0.616	0.858	0.949	0.961	0.641	0.886	0.118	0.625	0.000
	Katete	19	0.654	0.808	0.405	0.669	0.758	0.154	0.425	0.857	0.118
	Likasi	17	0.794	0.949	0.941	0.952	0.848	0.893	0.467	0.848	0.400
	LUANAR	17	0.714	0.802	0.895	0.936	0.700	NA	0.495	0.818	0.000
	Mikolongwe	7	0.667	0.905	1.000	0.900	0.700	1.000	0.571	0.600	0.571

N= number of samples, ms = microsatellite, MS = minisatellite, NA = Not calculated

Table III-3. Population genetic analyses of *Theileria parva* in cattle in Malawi

Population	N	H_e	Mean number of genotypes/ locus	I_A^S	V_D	L	*p*-value	Linkage	F_{ST}
Kasungu	20	0.666	6.78	-0.0177	1.2222	1.7936	8.20×10^{-1}	LE	
Nkhotakota	20	0.6947	6.67	0.0038	1.3644	1.6290	3.00×10^{-1}	LE	
Katete	19	0.6368	4.89	0.0988	3.1507	2.4566	1.50×10^{-2}	LD	
Likasi	17	0.7900	7.22	0.0762	2.0248	1.6100	1.02×10^{-2}	LD	
LUANAR	17	0.6127	5.11	0.0082	1.2739	1.5109	3.30×10^{-1}	LE	
Mikolongwe	7	0.6127	3.56	-0.0334	1.0000	2.2000	8.70×10^{-1}	LE	
Pop A	83	0.7019	16.33	0.0034	1.4721	1.5777	3.20×10^{-1}	LE	0.008
Pop B	17	0.7019	3.89	0.0034	1.4721	1.6322	3.90×10^{-1}	LE	0.273
Combined population	100	0.666	18.67	0.0286	1.6642	1.4900	0.067	LD	0.105

N= number of samples, H_e = estimated heterozygosity, I_A^S = standard index of association, V_D = mismatch variance (linkage analysis), L = upper 95% confidence critical limits of Monte Carlo simulation, LD = linkage disequilibrium, LE = linkage equilibrium, F_{ST} = Wright's fixation index

Table III-4. The analysis of molecular variance of *Theileria parva* in Malawi.

Source	*df*	Sum of squares	Mean Sum of squares	Variance components.	% of variation
Among Populations	5	456442.9	91288.58	835.277	1%
Among Individuals	94	6002033	63851.41	0	0%
Within Individuals	100	9308005	93080.05	93080.05	99%
Total	199	15766480		93915.32	100%
	Fixed indices				
	F_{SC}	0.011			
	F_{ST}	-0.186			
	F_{CT}	-0.174			

df = degress of freedom, FSC = variation among populations within groups, FST = measure of population differentiation due to genetic structure, FCT = Variation among groups of populations.

Table III-5. Multiplicity of infection of *Theileria parva* in Malawi

Population	Number of samples	Multiplicity of infection				Total number of alleles identified on all loci
		Mean	SD	Min.	Max.	
Kasungu	20	1.34	0.36	0.78	2.22	61.00
Nkhotakota	20	1.40	0.27	0.89	1.89	60.00
Katete	19	1.31	0.33	0.44	1.88	44.00
Likasi	17	1.39	0.30	0.67	1.89	65.00
LUANAR	17	1.14	0.28	0.67	1.56	46.00
Mikolongwe	7	1.31	0.21	1.00	1.56	32.00
Overall	100	1.32	0.29	0.74	1.83	51.33

SD = standard deviation, Min. and Max. refers to the minimum and maximum, respectively for the number of alleles identified per locus per sample. The summary of the number of the alleles per locus for each population is provided in Table III-2.

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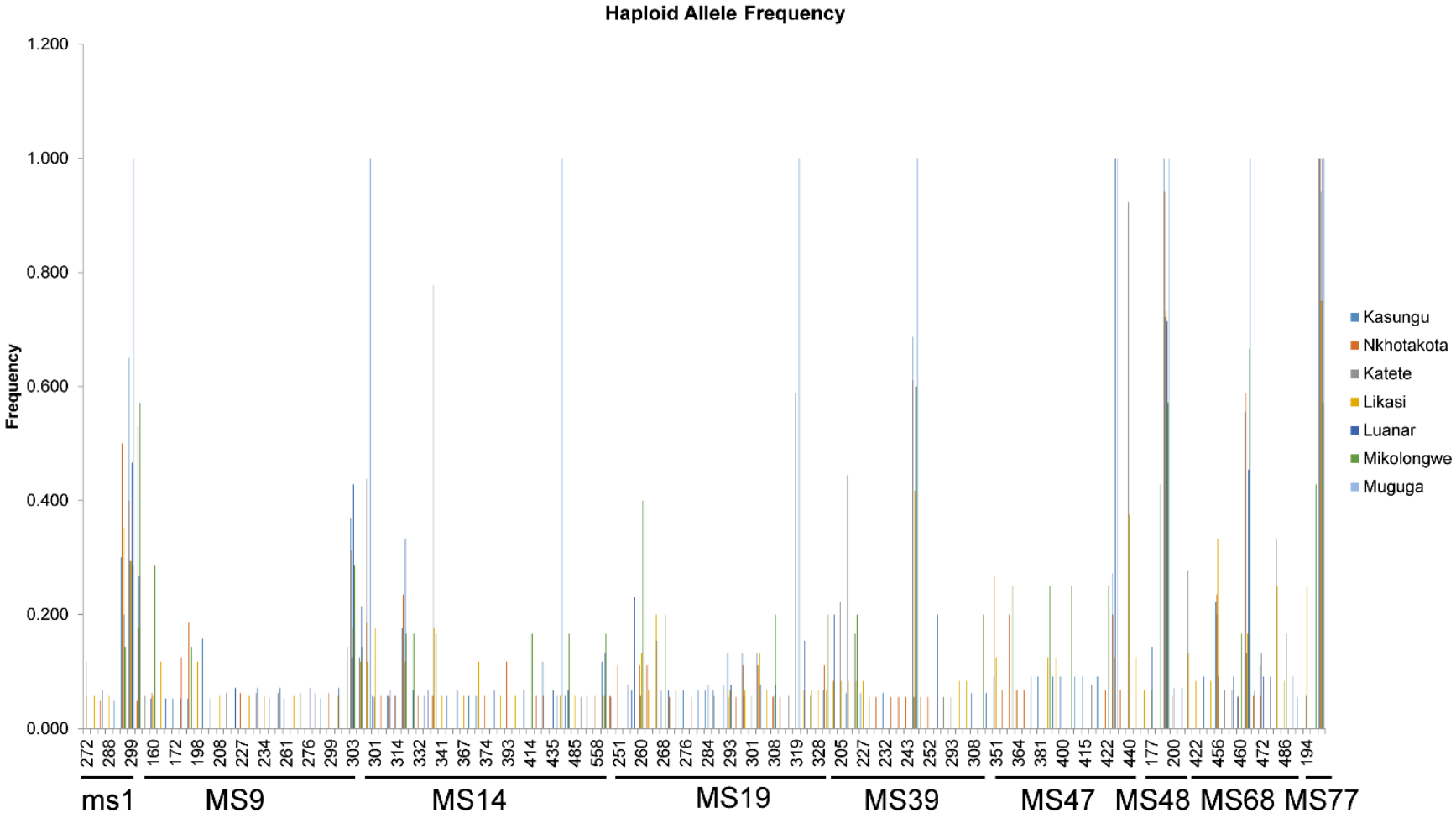

Figure III-1. The observed allele frequencies of alleles in field populations of *Theileria parva* from six populations in Malawi and the *Theileria parva* Muguga reference strain.The overall allele frequency from all six sites greater number of shared alleles and few unique qlleles. Multi-locus genotype (MLG) data was used to generate the histograms. The frequency of each predominant allele was calculated as a proportion of the total of each marker.

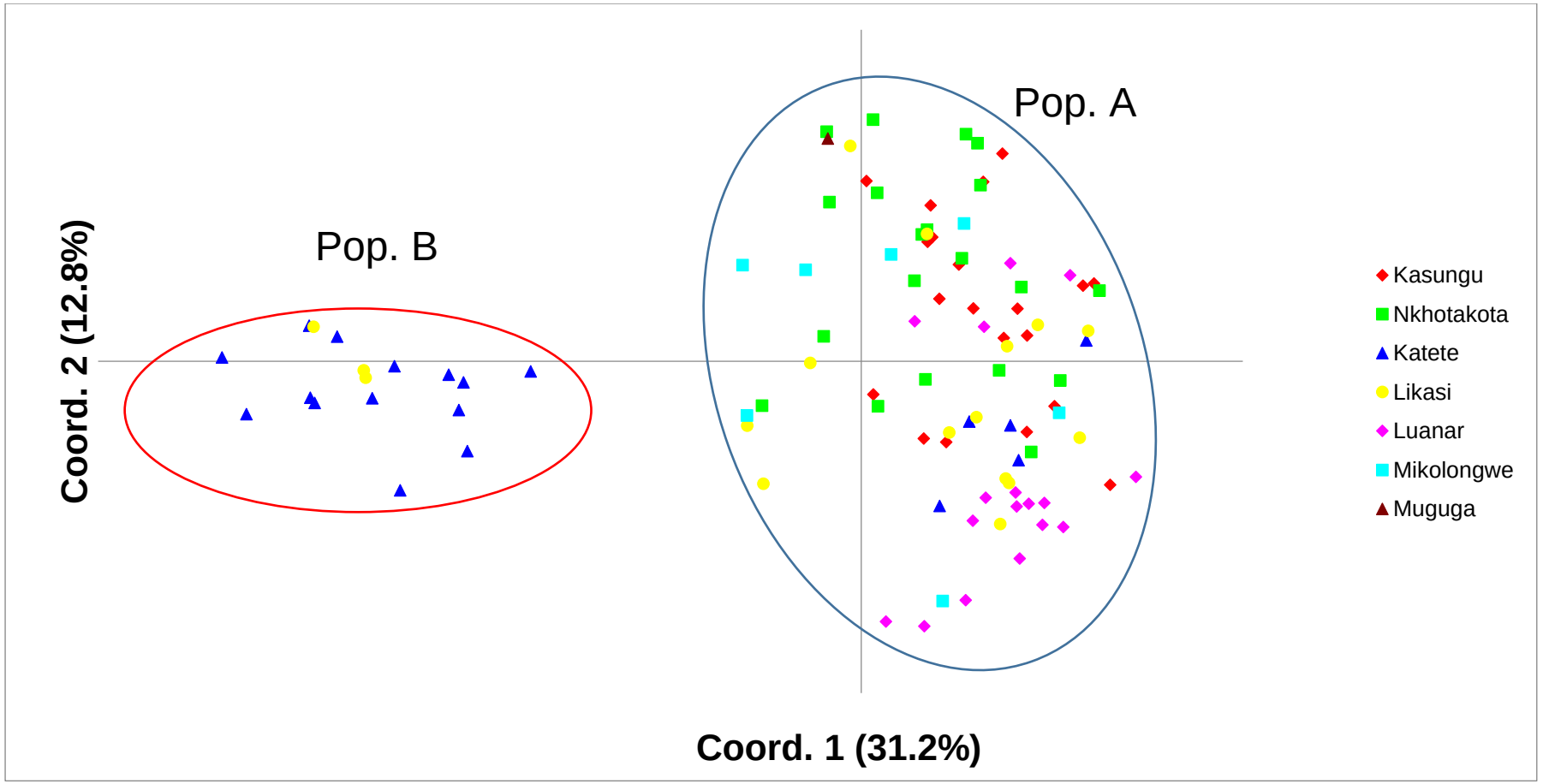

Figure III-2. Principal Coordinates Analysis (PCoA) of *Theileria parva* from six populations in Malawi and the Muguga reference strain. The proportion of variation in the population dataset explained by each axis is shown in parentheses. PCoA was performed using multi-locus genotype data from Kasungu, Nkhotakota, Likasi, Katete, LUANAR, Mikolongwe and Muguga reference strain. Sub-structuring among the populations from Katete and Likasi was observed as some isolates clustered separately in population B (Pop. B). Isolates from all the six sampling sites clustered together with *Theileria parva* Muguga referenbce strain in population A (Pop. A).

Chapter IV

Molecular identification and genetic characterization of tick-borne pathogens in sheep and goats at two farms in the central and southern regions of Malawi

1. Introduction

Anaplasmosis in small ruminants is caused by *Anaplasma ovis* which is usually subclinical but may be characterized with a low-grade fever with minimal impact on the animal wellbeing (Cabezas-Cruz et al., 2019). However, under stressful conditions and other secondary infections, clinical disease may occur (Renneker et al., 2013). Some highly pathogenic strains associated with high mortality rates of 40–50 % in small ruminants such as *Anaplasma ovis* Haibei strain have also been documented (Lu, 1997). Furthermore, *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, *Anaplasma marginale, Anaplasma centrale* and *Anaplasma bovis* which infect cattle have also been reported in sheep and goats (da Silva et al., 2018; Yousefi et al., 2017; Ben Said et al., 2015; Liu et al., 2012). However, their clinical and economical significance in small ruminants is not well understood. Ehrlichiosis in small ruminants is caused by *Ehrlichia ruminantium, Ehrlichia ovina* and *Ehrlichia* sp. Omatjenne (Bilgic et al., 2017). *Ehrlichia ruminantium* is economically the most important species in small ruminants and the one commonly diagnosed in sub-Saharan Africa (Ringo et al., 2019; Ringo et al., 2018b). It is mainly transmitted by the vector ticks *Amblyomma variegatum* and *Amblyomma hebraeum* which are widespread in Africa and southern Africa, respectively (Walker et al., 2003).

Babesiosis is principally caused by *Babesia ovis* and *Babesia motasi* (Liu et al., 2007), which are the most pathogenic species in small ruminants. Recently another pathogenic species *Babesia* sp. Mymensingh was reported in sheep and goats from Vietnam (Sivakumar et al., 2020). Other species include *Babesia taylori, Babesia foliata* and non-pathogenic *Babesia crassa* (Hashemi-Fesharki and Uilenberg, 1981). Theileriosis in small ruminants is caused by *Theileria lestoquardi, Theileria ovis, Theileria recondita, Theileria separata, Theileria luwenshuni* (*Theileria* sp. China 1), *Theileria uilenbergi* (*Theileria* sp. China 2) and *Candidatus* Theileria sp. (OT1 and OT2) (Yin et al., 2007; Luo and Yin, 1997). Among these, *Theileria lestoquardi, Theileria luwenshuni* and *Theileria uilenbergi* have been reported to be pathogenic in sheep and goats while *Theileria ovis* and *Theileria separata* are non-pathogenic (Luo and Yin, 1997; Uilenberg, 1981).

In Malawi, sheep and goats are kept in all the three geographical regions (northern, central and southern). The 2018 national housing and population census showed that 0.4 % and 17.8 % of the total 3,984,981 households own sheep and goats, respectively (NSO, 2018). Small ruminants especially goats play a vital role in improving the socioeconomic standards as proceeds from goat sells account for about 61.2 % of the total household income from livestock (Kaumbata et al., 2020). Ticks are widespread throughout the country and this pose a serious threat to livestock production (Chintsanya et al., 2004; Musisi and Kamwendo, 1996). Several tick species that are known to transmit TBPs, namely *Amblyomma variegatum, Hyalomma truncatum, Rhipicephalus appendiculatus, Rhipicephalus microplus, Rhipicephalus decoloratus, Rhipicephalus bursa, Rhipicephalus simus,* and *Rhipicephalus sanguineus sensu lato* have all been reported in Malawi (Walker et al., 2003). The most diagnosed TBDs in cattle in Malawi are theileriosis and anaplasmosis, which is based on clinical presentation of the disease, basic parasitological examination,

postmortem findings and serology (DAHLD, 2018). Despite sheep and goats being one of the important source of income and animal protein in Malawi. There is no study that has investigated infection with TBPs of the genera *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* in small ruminants in Malawi. The aim of this study was to detect and genetically characterize TBPs of the genera *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* infecting small ruminants in Malawi using molecular techniques.

2. Materials and methods

2.1 Study area and sample collection

DNA blood samples (n = 107) from goats (n = 99) and sheep (n = 8) were collected at sheep and goats at Bunda student farm of LUANAR in Lilongwe district in the central region (8 sheep and 44 goats) and Busa at Mikolongwe Veterinary Station (55 goats) in Chiradzulu district in the southern region (Figure I-1). The samples from LUANAR were from 18 Saanen goats and 26 (Local and Saanen crosses) and all 8 sheep were Blackhead Persians, while all samples from Busa farm were from boar goats. The blood collection was done asceptically as described in chapter I. The sampled animals were grouped into two categories, i.e. the young (less than 1 year old) and the adult (more than 1 year old) based on the records at the two farms. All the sampled sheep and goats were apparently healthy and did not show any clinical signs.

2.2 DNA extraction

DNA was extracted using the Quick Gene DNA whole blood kit S (DB-S) as described in chapter I.

2.3 PCR and sequencing

The screening for *Anaplasmataceae* and hemoparasites was done using EHR and RLB PCR assays, respectively as described in chapter I. The characterization for *Anaplasmataceae* was done using the *gltA* and *groESL* genes while for haemoparasites 18S rDNA using BTH PCR assays was used as described in chapter I. The major surface protein 4 (*msp4*) gene PCR was also used to further characterize *Anaplasma ovis* detected in this study as it has been reported that the *msp4* gene has high resolution in characterization of *Anaplasma ovis* than the *groEL* gene (de la Fuente et al., 2002; Selmi et al., 2019). The primers used in this study, their annealing temperatures and expected amplicon size are listed in Table IV-1. The purification of PCR amplicons, sequencing and editing of the sequences was done as described in chapter I. The sequences generated in this study were submitted to DDBJ under the accession numbers LC553508 to LC553515 for 18S rDNA, LC553516 to LC553531 for the *groEL* gene and LC553532 to LC553542 for the *msp4* gene.

2.4 Data analysis

Alignments of the consensus nucleotide sequences and construction of phylogenetic trees was done using MEGA7 asdescribed in chapter I. Chi-square statistics was used to determine the correlation between TBP detection rate with regard to study site, age, species and sex of the animals.

3. Results

3.1 Polymerase chain reaction-positive

Table IV-2 shows the PCR-positive rates for various speceies of *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria*. From the tested animals, seven sheep (88 %) and 72 goats (73 %) were infected with either *Theileria*, or *Babesia* species while for *Anaplasma* and *Ehrlichia* species it was found that eight sheep (100 %) and 74 goats (75 %) were infected with either of the pathogens. *Theileria ovis* infection rate in sheep was 88 % while it was 49 % in goats. The TBPs that were detected only in goats were *Theileria mutans* (3%), *Theileria separata* (2%), *Theileria* sp. strain MSD-like (18 %) and *Babesia gibsoni*-like strain (1%). The *Anaplasma ovis* infection rates in sheep and goats were 100 % and 62 %, respectively. An uncharacterized *Anaplasma* sp. was detected in one goat (1%), *Ehrlichia ruminantium* was detected in three sheep (38%), and one goat (1%) while *Ehrlichia canis* was only detected in two goats (2%).

Mixed infection of *Theileria* and *Anaplasma* or *Ehrlicha* were observed in 48 animals (45 %). Infection with three TBPs, *Theileria ovis, Anaplasma ovis and Ehrlichia ruminantium*, was detected in four animals (4%), while infection with two TBPs, *Theileria ovis and Anaplasma ovis*, was detected in 36 animals (34 %). A mixed infection of *Anaplasma ovis* and *Theileria* sp. strain MSD-like was detected in eight animals (7%).

3.2 Statistical analysis

There was a statistically significant correlation between TBP-positive detection rate and age. However, there were no statistically significant correlations between the TBPs positive detection rates and the breed, study site, species and sex of the animals (Table IV-3).

3.3 Phylogenetic analysis

To compare the sequences of *Babesia* and *Theileria* species from this study with those deposited in the GenBank, a ML phylogenetic tree based on almost full length of the 18S rDNA was constructed (Figure IV-1). The results showed that *Theileria* sequences clustered with *Theileria ovis* (S1, S2 and G18), *Theileria separata* (G57), *Theileria mutans* (G5) and *Theileria* sp. strain MSD-like (G9). Interestingly, one sequence of a *Babesia* strain (G50) that was closely related to *Babesia gibsoni* (JX962780) reported from a pig in China was obtained in this study (Figure IV-1).

The phylogenetic tree for the *groEL* gene showed that some of the sequences from this study clustered with of *Anaplasma ovis*, while one sequence of *Anaplasma* sp. (G29) clustered separately and had only 79% identity with *Anaplasma phagocytophilum* (HQ629909; AY529489). The obtained sequences for *Ehrlichia canis* (G46 and G77) and *Ehrlichia ruminantium* (G18) shared 100 % and 97 % identities with *Ehrlichia canis* FL strain (U96731) and *Ehrlichia ruminantium* Kiswani and Welgevonden strains (DQ647004; U13638), respectively (Figure IV-2). The phylogenetic tree of *MSP4* showed divergency of the sequences obtained in this study (Figure IV-3).

4. Discussion

Anaplasma ovis infection has been reported in several domestic and wild animal species in Africa (Ben Said et al., 2018). The overall prevalence of 65 % in this study shows high infection rate which is comparable to those reported in other sub-Saharan African countries such as 36.3 % in South Africa (Ringo et al., 2018b) and 34.2 % in Kenya (Ringo et al., 2019). This shows that *Anaplasma ovis* is an important TBP in the region especially with the common finding of mixed infections with other TBPs which enhances the occurrence of clinical disease and complicates its diagnosis and control (Bilgic et al., 2017).

Despite the sampling sites being more than 500 km apart, most of the *Anaplasma ovis* sequences obtained from the two study sites generally clustered closely in the *groEL* tree (Figure IV-2). This is suggestive that closely related strains of *Anaplasma ovis* are circulating in small ruminants in Malawi, as also reported in Kenya (Ringo et al., 2019). However, some *msp4* sequences clustered in different clades in ML tree (Figure IV-3), which may suggest the presence of divergent *Anaplasma ovis* in Malawi, as observed by Selmi et al. (2019).

The *Ehrlichia ruminantium*-positive rate of 4% is similar to that found in other studies in sub-Saharan African countries, in which generally low prevalences of 14.3 % in South Africa (Ringo et al., 2018b) and 7.9 % in Kenya (Ringo et al., 2019) were reported in small ruminants. No any clinical signs of ehrlichiosis were observed in the animals during the sampling period which is in accordance with other studies that have reported that the occurrence of clinical disease is mainly dependent on the pathogenicity of the *Ehrlichia ruminantium* strain and the breed or species of the infected animals (Ahmadu et al., 2004; Steyn and Pretorius, 2020). *Ehrlichia canis* which is a pathogen of dogs was detected in goats in this study. Infection of *Ehrlichia canis* in both domestic and wild ruminants has been documented previously (Li et al., 2016; Qiu et al., 2016; Zhang et al., 2015). This finding emphasizes the need to apply molecular techniques that can detect a wider range of pathogens when screening for TBPs as species-specific methods may result in failure to detect novel infections.

In this study, a *Babesia* strain that is closely related to *Babesia gibsoni* in goat based on the almost full-length sequence of 18S rDNA was detected. This finding supports previous studies that have reported *Babesia gibsoni* infection in non-canine species such as cattle, goat, sheep and donkey (Li et al., 2015). However, species-specific BgTRAP gene, cytochrome *B* gene and *p18* gene primers for *Babesia gibsoni* failed to amplify the sample (G50) (data not shown). This suggests that the sample (G50) may not be infected with *Babesia gibsoni* reported from dogs but a closely related *Babesia* strain. This may also suggest that small ruminants are infected with a previously uncharacterized strain of *Babesia* closely related to *Babesia gibsoni*. In this study, *Babesia ovis* and *Babesia motasi*, the most pathogenic *Babesia* species in small ruminants, were not detected.

Theileria ovis and *Theileria separata* the causative agents of theileriosis in small ruminants were detected in this study. Furthermore, *Theileria mutans* and *Theileria* sp. closely related to *Theileria* sp. strain MSD reported in cattle (Chae et al., 1999) were detected for the first time in small ruminants. *Theileria*

lestoquardi, the causative agent of malignant theileriosis in small ruminants, was not detected in this study, which might be attributed to the limited sample size employed here. A further survey with a larger sample size needs to be carried out to confirm its absence.

The observation of the infection rate being higher in older animals compared to younger animals may be due to prolonged exposure of the older animals to vector ticks. The higher prevalence of anaplasmosis and theileriosis in sheep and goats is in accordance with the reports from the department of animal health and livestock development in Malawi (DAHLD, 2018). This study has also shown that, despite the absence of the vector ticks on the sampled animals due to recent application of acaricides, the animals were still infected with TBPs. This shows that absence of the ticks on the animals may not imply that the animals are not infected with TBPs as such even those animals without tick infestation should be screened for TBPs to control the spread of the pathogens when animals are introduced in a new area.

5. Summary

In this study, the presence of *Anaplasma ovis*, uncharacterized *Anaplasma* species, *Ehrlichia canis*, *Ehrlichia ruminantium*, *Theileria ovis*, *Theileria separata*, *Theileria mutans*, *Theileria* sp. strain MSD-like, and *Babesia gibsoni*-like strain infection in sheep and goats has been confirmed using molecular techniques for the first time in Malawi. This study also reports an observation of mixed infections which may complicate the proper diagnosis and treatment of tick-borne pathogens. This information is important for the development and monitoring of the disease's diagnosis, prevention, management and evaluation of control measures. Further, the observation of new host range of some pathogens further highlights the need to conduct more studies on tick-borne pathogens in a variety of hosts in Malawi.

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Table IV-1. List of primers used detection and characterization of tick-borne pathogens of sheep and goats in Malawi.

Primer name	Primer sequence (5′ to 3′)	Target gene/ Pathogen	PCR type*	Product size (bp)	Annealing temperature (°C)	Reference
RLB_F	GAGGTAGTGACAAGAAATAACAATA	18S rDNA of *Babesia* & *Theileria*	PCR	460-540	55	Gubbels et al., 1999
RLB_R	TCTTCGATCCCCTAACTTTC					
EHR_F	GGTACCYACAGAAGAAGTCC	16S rDNA of *Anaplasmataceae*	PCR	345	61	Parola et al., 2000
EHR_R	TAGCACTCATCGTTTACAGC					
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA of *Babesia* & *Theileria*	1st PCR	1400-1600	55	Masatani et al., 2017
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	18S rDNA of *Babesia* & *Theileria*	2nd nPCR	1400-1600	55	
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
Thei 1F	AACCTGGTTGATCCTGCCAG	18S rDNA of *Theileria*	1st PCR	1412-1421	50	Heidarpour Bami et al., 2009
Thei 1R	AAACCTTGTTACGACTTCTC					
Thei 2F	TGATGTTCGTTTYTACATGG	18S rDNA of *Theileria*	2nd nPCR	1412-1421	55	
Thei 2R	CTAGGCATTCCTCGTTCACG					
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* gene of *Anaplasmataceae*	1st PCR	1300	54	Rar et al., 2010
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* gene of *Anaplasma*	2nd nPCR	1256	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* gene of *Ehrlichia*	2nd nPCR	1100	50	Gofton et al., 2016
groEL_rev2	GCCGACTTTTAGTACAGCAA					
MSP43	CCGGATCCTTAGCTGAACAGGAATCTT	*msp4* gene of *Anaplasma ovis* & *Anaplasma marginale*	PCR	851	60	de la Fuente et al., 2003
MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC					

PCR = Polymerase Chain Reaction; F = Forward; R = Reverse; bp = base pairs, * = PCR was either single (PCR) or nested (nPCR)

Table IV-2. Tick-borne pathogens detected in sheep and goats in Malawi.

Study site	Animal spp.	To	Tm	Ts	*Theileria* sp. strain MSD-like	Bg - like strain	Ao	*Anaplasma* species	Er	E c	To + Ao + Er	To+ Ao	*Theileria* sp. strain MSD-like + Ao
Bunda Student Farm	Goats (*n* = 44)	27 (61)	1 (2)	0	13 (30)	0	35 (80)	1 (2)	1 (2)	0	1 (2)	17 (39)	5 (11)
	Sheep (*n* = 8)	7 (88)	0	0	0	0	8 (100)	0	3 (38)	0	3 (38)	4 (50)	0
Busa Farm	Goats (*n* = 55)	21 (38)	2 (4)	2 (4)	5 (9)	1 (2)	26 (47)	0	0	2 (4)	0	15 (27)	3 (5)
Total		55 (51)	3 (3)	2 (2)	18 (17)	1 (1)	69 (65)	1 (1)	4 (4)	2 (2)	4 (4)	36 (34)	8 (7)

To = *Theileria ovis,* Tm = *Theileria mutans,* Ts = *Theileria separata,* Bg = *Bebesia gibsoni,* Ao = *Anaplasma ovis,* Er = *Ehrlichia ruminantium,* Ec = *Ehrlichia canis. Theileria ovis, Theileria mutans, Theileria separata* and *Babesia gibsoni*-like strain were characterized using BTH PCR while *Theileria* sp. strain MSD-like was characterized using *Theileria* genus-specific PCR assay. *Anaplasma ovis, Anaplasma* sp., *Ehrlichia ruminantium* and *Ehrlichia canis* were characterized using *groEL* PCR assay. The number of samples positive for each species and the percentages in brackets.

Table IV-3. Tick-borne pathogens detection rate in sheep and goats in Malawi based on polymerase chain reaction with regard to the host attributes.

Attribute	No. of sheep and goats	No. infected with at least one TBP (%)	*p-value*
Study site			0.192588
Bunda student farm	52	37 (71)	
Busa farm	55	45 (82)	
Age			0.000789*
< 1 year	28	15 (54)	
>1 year	79	67 (85)	
Sex			0.932563
Male	9	7 (78)	
Female	98	75 (76)	
Species			0.418544
Goats	99	74 (75)	
Sheep	8	8 (100)	
Breed			0.251801
Saanen (goats)	18	14 (78)	
Boar (goats)	55	45 (82)	
Crossbreed (goats)	26	15 (58)	
Dorper (sheep)	8	8 (100)	

PCR = Polymerase chain reaction. Chi-square analysis determined the significant difference between variables, * *p* value = <0.05 was considered significant.

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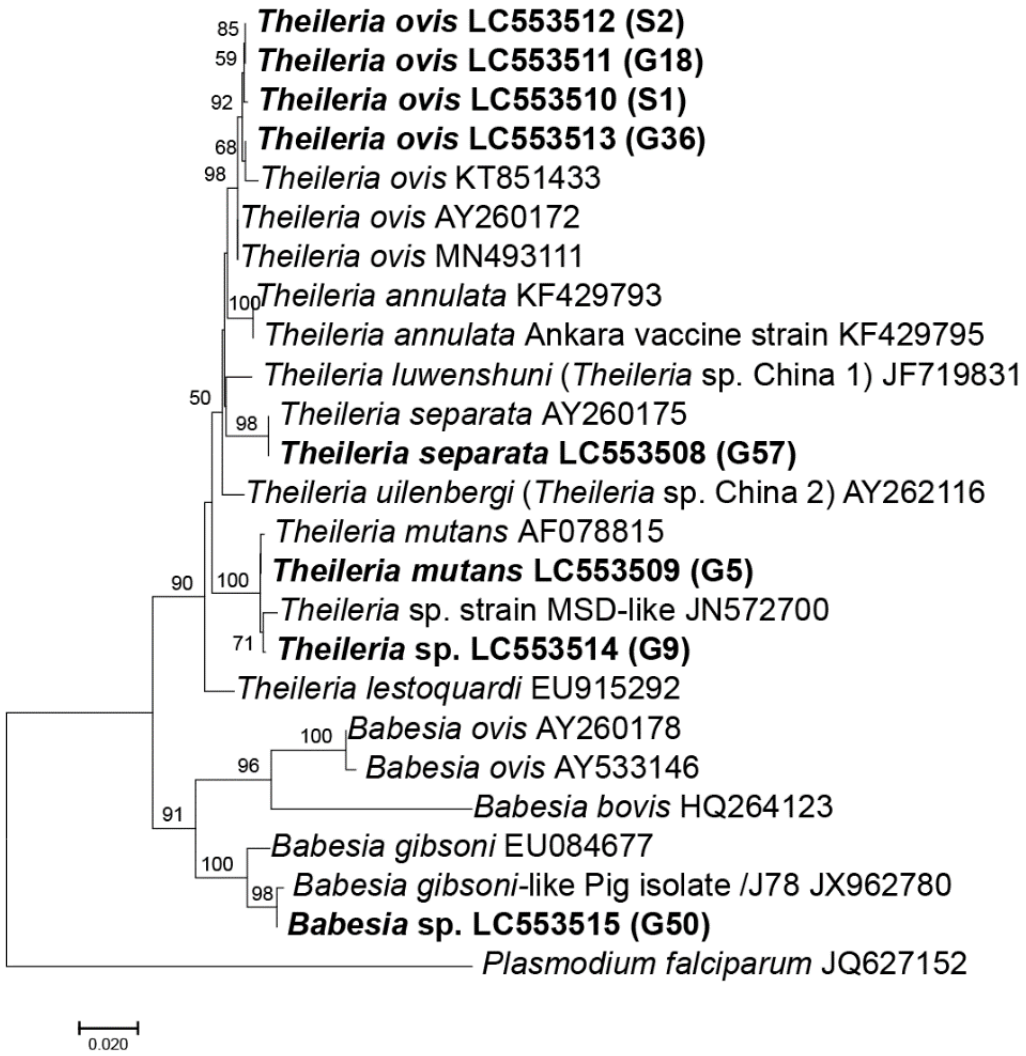

Figure IV-1. The maximum likelihood phylogenetic tree of the *Theileria* spp. and *Babesia* sp. detected in sheep and goats. The analysis was based on almost the full-length sequences of 18S rDNA (1400-1500 bp) which was constructed using the Kimura 2 parameter model. *Plasmodium falciparum* was used as outgroup. All bootstrap values > 50% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The sample IDs are in the parenthesis after accession number where S and G represent sheep and goat, respectively where the sample was collected.

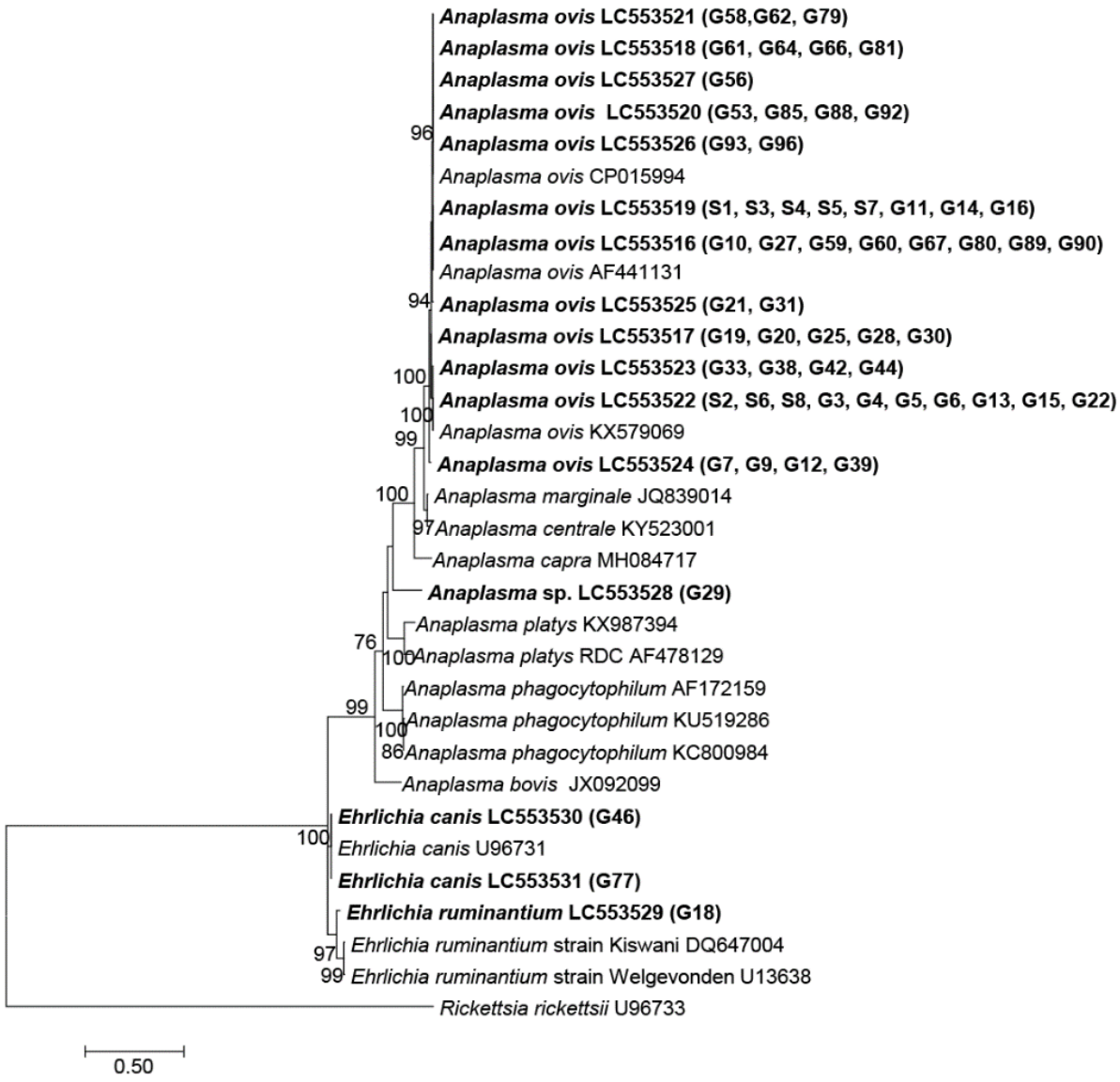

Figure IV-2. The maximum likelihood phylogenetic tree of the *Anaplasma* spp. and *Ehrlichia* spp. detected in sheep and goats. The analysis was based on partial sequences of *groEL* gene (1000-1100 bp) which was constructed using the General Time Reversible (GTR) +G model. *Rickettsia rickettsii* was used as outgroup. All bootstrap values > 50% from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The sample IDs are in the parenthesis after accession number where S and G represent sheep and goat, respectively where the sample was collected.

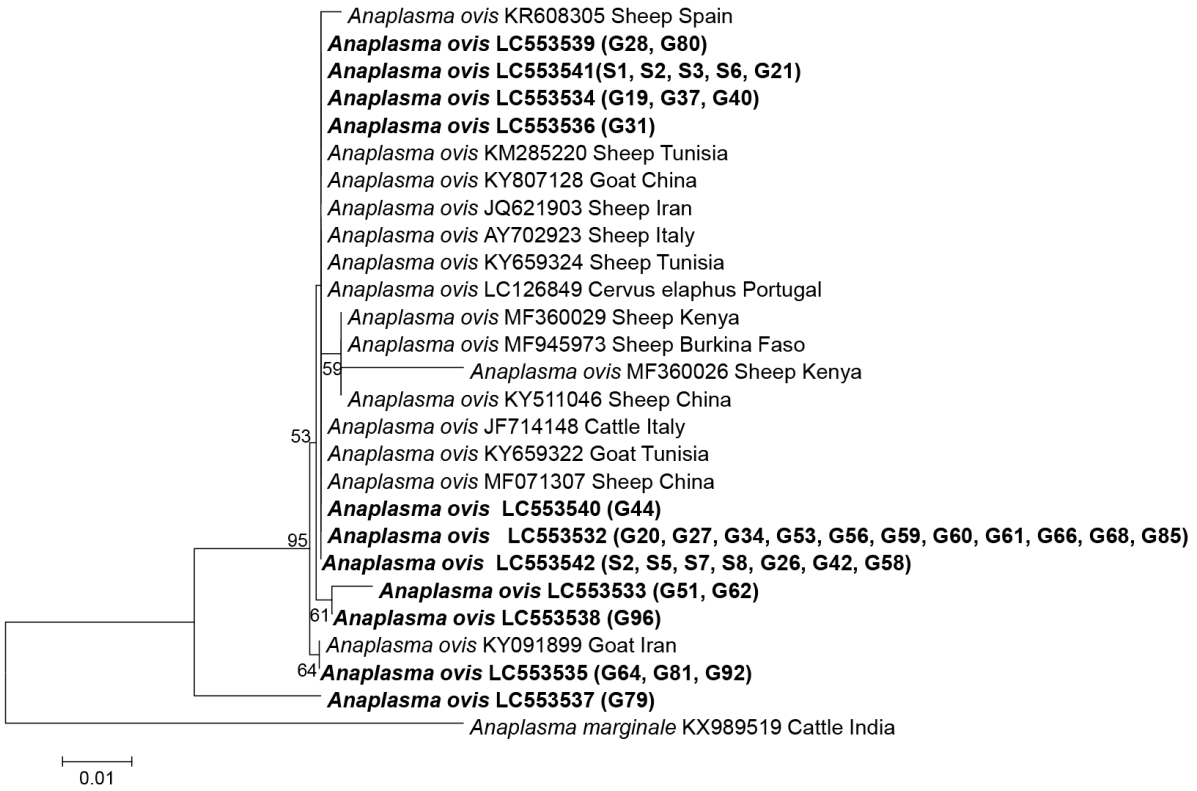

Figure IV-3. The maximum likelihood phylogenetic tree of the *Anaplasma ovis* detected in sheep and goats. The analysis was based on almost the full-length sequences of *msp4* gene (808 bp) which was constructed using the Kimura 2 parameter model. *Anaplasma marginale* was used as outgroup. All bootstrap values > 50 % from 1000 replications are shown on the interior branch nodes and the sequences obtained in this study are indicated in bold. The sample IDs are in the parenthesis after accession number where S and G represent sheep and goat, respectively where the sample was collected.

Chapter V

Molecular detection and characterization of tick-borne haemoparasites and *Anaplasmataceae* in dogs in major cities of Malawi

1. Introduction

TBPs that cause canine anaplasmosis, babesiosis, ehrlichiosis, and hepatozoonosis are some of the emerging pathogens with a worldwide distribution. Some of these pathogens affect both animal and human health sectors as some have been reported to be zoonotic (Arraga-Alvarado et al., 2014; Maggi et al., 2013; Perez et al., 2006).

Anaplasma phagocytophilum, and *Anaplasma platys* are the causative agents of canine granulocytic anaplasmosis, thrombocytotropic anaplasmosis, respectively, and are transmitted by the vector ticks, *Ixodes* spp. and *Rhipicephalus sanguineus sensu lato*, respectively (Sykes and Foley, 2014; Wang et al., 2019; Guzman et al., 2020). Infection with *Anaplasma phagocytophilum* is generally self-limiting while that of *Anaplasma platys* is considered mostly non-pathogenic (Sykes and Foley, 2014). Importantly, these pathogens have been suggested to be of public health importance as they also cause clinical disease in humans with typical clinical signs of anaplasmosis (Arraga-Alvarado et al., 2014; Maggi et al., 2013, Wang et al., 2019). In sub-Saharan Africa, *Anaplasma platys* is more commonly reported in dogs than *Anaplasma phagocytophilum* (Vlahakis et al., 2018; Qiu et al., 2018; Matei et al. 2016 Matjila et al. 2008). Other studies have reported the presence of *Anaplasma phagocytophilum*-like organisms in both dogs and ticks in South Africa (Mtshali et al., 2017; Inokuma et al., 2005). Ehrlichiosis in dogs which is caused by *Ehrlichia canis*, *Ehrlichia chaffensis*, and *Ehrlichia ewingii* is a severe chronic fatal disease characterized by fever, swollen lymph nodes, respiratory distress, weight loss, bleeding disorders and occasionally, neurological signs (Qurollo et al., 2014). In southern Africa, the major causative agent of canine ehrlichiosis is *Ehrlichia canis* (Qiu et al., 2018, Mtshali et al., 2017). A case of ehrlichiosis in human caused by *Ehrlichia canis* has been documented in Venezuela (Perez et al., 2006).

Clinical babesiosis in dogs, which is caused by *Babesia canis, Babesia conradae, Babesia gibsoni*, *Babesia negevi*, *Babesia rossi*, *Babesia vogeli* and *Babesia vulpes*, is characterized by fever, haemoglobinuria, thrombocytopenia, jaundice, anaemia and loss of function of some organs (Solano-Gallego and Baneth 2011; Baneth et al., 2019, 2020). In sub-Saharan Africa, *Babesia rossi* is more commonly diagnosed than *Babesia vogeli* (Qiu et al., 2018; Matjila et al., 2008) and is transmitted by the vector yellow dog tick *Haemaphysalis elliptica* formerly known as *Haemaphysalis leachi*. A mixed infection of *Babesia rossi* and *Hepatozoon canis* has been reported in a dog in the neighbouring Zambia (Qiu et al., 2018). Co-infections in dogs with *Hepatozoon canis* and *Babesia* species have also been reported elsewhere and has been implicated in altering the clinical presentation of the disease (Rojas et al., 2014; Cardoso et al., 2010). *Hepatozoon canis* which is transmitted by the brown dog tick *Rhipicephalus sanguineus sensu lato* infects the haemolymphatic tissues but its clinical presentation varies from being subclinical in apparently healthy dogs to severe with lethargy, fever, cachexia and pale mucous membranes in young dogs, and immunocompromised dogs (Attipa et al., 2018; Beaufils et al., 1996). The ticks *Haemaphysalis elliptica* and *Rhipicephalus sanguineus sensu lato* are the principal vectors of *Babesia* species and *Hepatozoon canis*, respectively infecting dogs in sub-Saharan Africa (Walker et al., 2003).

In Malawi, serological studies done have reported the presence of antibodies for rickettsial and protozoan TBPs in dogs (Alvasen et al., 2016; Johansson, 2015). However, no molecular study has been carried out to characterize these pathogens. This study aimed at detecting and characterising TBPs in dogs from the three major cities of Malawi using molecular tools.

2. Materials and methods

2.1 Study area

The study was conducted in the three major cities of Malawi namely; Mzuzu in the northern region, Lilongwe in the central region, and Blantyre in the southern region (Figure I-1).

2.2 Sample collection

A total of 209 dog blood samples were employed in this study. The samples were collected from dogs in the major cities in Malawi of Mzuzu (*n* = 8), Lilongwe (*n* = 197), and Blantyre (*n* = 4). Sampling was done during free rabies and neutering campaigns by the Lilongwe Society for the Protection and Care of Animals (LSPCA) and All Creatures Animal Welfare League from February 2018 to March 2019. In 46 dogs (Mzuzu, *n* = 8 Lilongwe, *n* = 34; and Blantyre, *n* = 4), 125 µl whole blood was collected onto the sample area on the Whatman FTA micro card (GE Healthcare Japan Corporation, Tokyo, Japan) from the ear vein after disinfecting the puncture site with methylated spirit cotton swab and were dry dried at room temperature. These dogs were sampled in the peri-urban areas where the campaigns were being conducted. From 163 dogs in Lilongwe, whole blood was collected into EDTA vacutainer tubes where approximately 5 ml of blood sample was drawn by venipuncture of the external jugular vein after disinfection of the venipuncture site with a methylated spirit swab. The presence of tick by checking the hair of the dogs, head, ears, toes, groin and axillar region and clinical signs on the dogs associated with TBPs such as pale mucous membranes, blood tinged urine and lymphadenopathy were noted and recorded. The collected ticks were stored in 50 ML centrifuge tubes containing dry silica.

2.3DNA extraction

DNA extraction from blood samples collected in EDTA vacutainer tubes was done using the Quick Gene DNA whole blood kit S (DB-S) as decribed in chapter I.

Extraction of DNA from blood samples collected on Whatman FTA micro card was done as per manufacturer's recommendations with minor modifications. In brief, a single 10.0 mm circle punch was made from the center of the blood spot using a sterile chisel into a sterile 1.5 ml microfuge tube instead of three 3.0 mm circles. The chisel was sterilised and cleaned before being used on a new sample by dipping in 70% ethanol and flaming it under Bunsen burner until it was growing red. Thereafter, it was also dipped in fresh 70% ethanol to cool the chisel then it was used to make punches three times on fresh sterile filter paper before being used to make a new punch on another FTA card sample. Then, 400 µl of PCR grade water was used for DNA elution instead of 100 µl of sterile water. The extracted DNA was stored at -20^{o}C until required for use.

2.4 Polymerase chain reaction

The screening for *Babesia, Theileria* and *Hepatozoon* species was done using BTH nested PCR assays as described in chapter I. The primers used in this study, their annealing temperatures and expected amplicon size are listed in Table V-1. Due to high number of positive samples, a multiplex PCR was used to identify the pathogens detected on BTH nested PCR assay to species level as described by Qiu et al. (2018). The electrophoresis and visualisation of the amplicons was done as described in chapter I. The screening for *Anaplasma* and *Ehrlichia* species was done using EHR PCR assay as described in chapter I. Further characterization of the EHR PCR positice samples was done using *gltA* and *groEL* genes.

2.5 Sequencing

Five randomly selected amplicons of 2nd BTH PCR assay for *Babesia rossi* and *Hepatozoon canis,* plus all *Babesia vogeli* amplicons, and all the amplicons of 2nd *groEL* and 2nd *gltA* PCR assays were purified using the NucleoSpin Gel and PCR Clean-Up Kit. Sequencing and sequence editing was done as described in chapter I. The sequences generated in this study were submitted to DDBJ under the accession numbers LC556376 to LC556379 for 18S rDNA, LC556380 and LC556382 for *gltA* gene and LC556383 to LC556384 for *groEL* gene.

The sequence allignments, phylogenetic trees construction was done in MEGA-7 as described in chapter I. The correlation between TBP detection rate with regard to ownership, age, breed and sex of the animals as done using Pearson's chi-square statistics.

3. Results

3.1 Positive detection rate of tick-borne pathogens in dogs.

The number of TBPs detected by 18S rDNA BTH species discriminating multiplex PCR for *Babesia rossi, Hepatozoon canis* and *Babesia vogeli* were 49 (23.6%), 40 (19.1%), and 6 (2.9%), respectively. *Anaplasmataceae gltA* and *groEL* gene PCRs detected *Anaplasma platys* in 5 (2.4%) and *Ehrlichia canis* in 8 (3.8%) (Table V-2). Overall infection with at least one TBP was detected in 93 dogs (44.5%). Mixed infections with at least two TBPs were detected in 16 dogs (7.8%) (Table V-3). The detection rate of the piroplasms in the samples collected on FTA cards and those in EDTA vacutainer tubes did not show any significant difference while for the *Anaplasmataceae* the detection rate was higher in those samples collected in EDTA tubes than those collected on FTA cards.

3.2 Statistical analysis

Due to limited number of positive samples for *Anaplasmataceae* (n =13), Pearson's chi-square statistics analysis was used to determine correction of infection with protozoan hemoparasites which showed that there was no statistically significant correlation between protozoan haemoparasites in owned dogs (43.3%) and free roaming dogs (75.0%) (X^2 = 3.1337, df = 1, p = 0.076689) (Table V-4). Similarly, there was also no statistically significant correlation between protozoan haemoparasites infection rates in young dogs (less than 1 year old) (43.5%) and adult dogs (44.9%) (X^2 = 0.0322, df = 1, p = 0.857678). However,

there was a significant correlation between the protozoan haemoparasites infection rate and the sex of the dogs where males (54.8%) were higher than females (45.2%) (X^2 = 5.3512, *df* = 1, *p* = 0.020708). There was also a strong positive correlation between the protozoan haemoparasites infection rates in local breed dogs (47.8%) and exotic breed dogs (24.1%) (X^2 = 5.6515, *df* = 1, *p* = 0.01744). There was also observed a strong correlation between protozoan haemoparasites detection rates and the presence of tick infestation on the dogs being higher when ticks were present (72.4%) than where absent (24.6%) (X^2 = 47.0294, *df* = 1, *p* = < 0.0001). Similarly, there was also a strong positive correlation between the presence of clinical signs and positive detection rate of protozoan haemoparasites in the dogs where it was very high in those dogs presented with clinical signs of pale mucous membranes and blood in urine (72.9%) than those where without clinical signs (40.0%) (X^2 = 19.9835., *df* = 1, *p* = < 0.0001).

3.3 Phylogenetic analysis

Phylogenetic analysis based on 18S rDNA showed that the sequences obtained in this study for *Babesia vogeli* LC556376 (MWD3) and LC556377 (MWD157) clustered together with *Babesia vogeli* (LC331058) reported in Zambia (Figure V-1). Similarly, the sequences of *Babesia rossi* LC556378 (MWD96) and *Hepatozoon canis* LC556379 (MWD141) from Malawi were respectively clustered together with *Babesia rossi* (LC331056 and LC331057) and *Hepatozoon canis* (LC331052, LC331054 and LC331055) reported from dogs in Zambia. Furthermore, the ML trees of both *gltA* and *groEL* genes showed that *Anaplasma platys* LC556382, LC556384 (MWD160) and *Ehrlichia canis* LC556381 (MWD45), LC556380 (MWD157) and LC5563843 (MWD139) sequences obtained in this study also were identical or almost identical to *Anaplasma platys* (AF478129, AF478130, LC373037 and LC373039) and *Ehrlichia canis* (LC373038 and LC373041) reported in other southern African countries (Figures V-2 and V-3).

4. Discussion

The TBPs that infect dogs have over the last decade attracted much attention and more studies are being conducted to understand their epidemiology and the threat they pose to both animal and human heath sectors as some are now known to be zoonotic (Arraga-Alvarado et al., 2014; Maggi et al., 2013; Perez et al., 2006). Despite the increase in the number of studies on TBPs in dogs worldwide, few studies have been conducted in resource limited countries where research priority is given to food animals than companion animals. The increase in the contact between pets and people as well as abandoned and free roaming dogs poses the risk of transmission of ticks and TBPs from animals to humans (Galay et al., 2018). The lack of epidemiological data on the occurrence of TBDs in dogs' results in misdiagnosis or delay in commencement of treatment especially when there are mixed infections.

In Malawi, two studies have been documented on the status of TBPs in dogs using commercial serological tests (Alvasen et al., 2016; Johansson, 2015), which reported seroprevalence of 12% and 22% for *Anaplasma* and *Ehrlichia*, respectively. However, serological investigations have the limitations of being less specific than molecular techniques, also the persistence of antibodies in infected animals makes it difficult to differentiate between previous infection and present infection and the inability to identify

pathogens to species level (Otranto et al., 2010). The previous studies in Malawi also just used samples collected from Lilongwe thus generating epidemiological data from a limited geographical area.

In this study, of the 209 dogs screened, 93 (44.5%) were positive with at least one TBP. This finding is comparable with the positive detection rate of 46.6% reported in domestic dogs in Zambia (Qiu et al. 2018). Overall, *Babesia rossi* was detected the most in 49 dogs (23.1%) and observed in all the three study sites despite the limited number of samples from Blantyre and Mzuzu. Whereas in Zambia, Qiu et al. (2018) reported a positive detection rate of 2.0% which is lower than that of our study. This shows that *Babesia rossi* infection in dogs in Malawi is widespread. In this study, *Hepatozoon canis* was detected in 40 dogs (19.1%) and this was the second highest detected pathogen from all the three study sites. The positive detection rate of 19.1% in this study was relatively lower when compared to 40.5% reported in Zambia (Qiu et al. 2018). The rickettsials pathogens *Anaplasma platys* and *Ehrlichia canis* had the positive detection rates of 2.4% and 3.8%, respectively. These pathogens were only detected in samples from Lilongwe, the source of majority of the samples. This shows that these tick-borne rickettsial pathogens have generally low prevalence which is also in accordance with 0.8% and 0.4 %, respectively reported in Zambia (Qiu et al., 2018) and and 1.0% prevalence of *Anaplasma platys* reported by another study in Zambia (Vlakihas et al., 2018). These rickettsial TBPs have been suggested to be of public health significance as they have been reported to infect humans as well (Wang et al., 2019; Arraga-Alvarado et al., 2014; Maggi et al., 2013; Perez et al., 2006).

The occurrence of mixed infections detected in 16 dogs (7.7%) is of great significance as mixed infections have been reported to exacerbate the clinical presentation of TBDs and complicates the diagnosis and treatment of TBDs as protozoal and rickettsial pathogens have different treatment regimen (Kordick et al., 1999; Otranto et al., 2009). The cases of mixed infections of *Anaplasma platys, Ehrlichia canis, Babesia vogeli* and *Hepatozoon canis* were usually characterized by pale mucous membranes (anaemia), peripheral lymphadenopathy, mucopurulent ocular discharges, loss of appetite, pyrexia, loss of condition, weight loss and failure to respond to treatment for single infection of either rickettsial or protozoan TBP (Attipa et al. 2017). In this study, most of the dogs that were positive for *Anaplasma*/*Ehrlichia* spp. were also positive for either of the *Babesia* species/*Hepatozoon* species. Thus, this finding necessitates the need to conduct tests for multiple pathogens when TBDs are suspected so as to avoid misdiagnosis that can lead to wrong treatment in the case of missed infection. More samples that were collected in EDTA tubes followed by DNA extracted using Quick Gene DNA whole blood kit S were positive for rickettsial pathogens than those on FTA cars (Table V-2). This may result from the differences in the DNA extraction methods as the use of proteinase K in those samples collected in EDTA tubes may have facilitated the breaking of bacterial cell wall for the recovery of DNA.

The statistical analysis showed that there was no significant correlation between household dogs and free roaming dogs from being infected with protozoan haemoparasites. The brown dog tick, *Rhipicephalus sanguineus* is endophilic which prefer indoors areas and more likely to infest household dogs

than free roaming dogs (Dantas Torres, 2010). However, free roaming dogs if infested with ticks may become a medium of transmission from one location to another (Galay et al., 2018). Young dogs (43.5%) were equally infected with protozoan haemoparasites like adult dogs (44.9%) which may be due to their close contact with adult dogs infested with ticks, their inactivity which favors attachment of ticks and susceptibility to protozoan haemoparasites due to weaker immunity (Gray et al. 2013). More male dogs (54.8%) were infected with protozoan haemoparasites than female dogs (45.2%) which is in accordance with the finding in the neighboring Zambia (Qiu et al., 2018). However, this is in contrast with previous studies (Galay et al., 2018; Konto et al., 2014) which reported higher prevalence in female dogs. In Malawi, most male dogs usually stay in groups during the breeding season where fights for females are common which increases their chances of tick infestation by coming in contact with dogs from different areas. There was also high infection rates in local breed dogs than exotic breed dogs. This may be because most of these local breed dogs were from low socio-economical background and were only presented to LSPCA or animal welfare league because of free vaccination and neutering service. Furthermore, the local breed dogs may rarely present with clinical disease than exotic breeds thereby being rarely having access to veterinary services.

The phylogenetic analysis for the *Babesia* and *Hepatozoon* 18S rDNA has shown that the *Babesia rossi*, *Babesia vogeli* and *Hepatozoon canis* reported in this study clustered in the clades with those sequences reported in Zambia. This finding suggests that similar strains are infecting dogs in these neighbouring countries. Similarly, phylogenetic trees based on the *gltA* and *groEL* genes revealed the clustering of *Anaplatysma platys* and *Ehrlichia canis* from this study with the sequences reported from Zambia and DR Congo. This observation may result from the increased movement of people and pets within the southern African region.

5. Summary

This study has shown that the TBPS that infect dogs in Malawi include *Anaplasma platys*, *Ehrlichia canis*, *Babesia rossi*, *Babesia vogeli* and *Hepatozoon canis*. Since some of the pathogens detected have been reported to be of public health significance. There is a general need to sensitise healthy parcatitioners, veterinary personnel and the general public on the importance of TBPs of both veterinary and medical importance. The observation of mixed infections highlights the need to test for multiple pathogens when tick-borne infections are suspected in both animals and humans. Finally, the study has also highlighted that there a general need to expand the geographical and host coverage in future studies on TBPs in Malawi.

List of tables

Table V-1. List of primers used the detection and characterization of tick-borne pathogens of dogs in Malawi.

Primer name	Primer sequence (5′ to 3′)	Target gene/ organism	PCR type	Amplicon size (bp)	Annealing temp. (°C)	Reference
EHR_F	GGTACCYACAGAAGAAGTCC	16S rDNA/ *Anaplasmataceae*	Single	345	61	Parola et al., 2000
EHR_R	TAGCACTCATCGTTTACAGC					
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	18S rDNA/*Babesia*, *Theileria* and *Hepatozoon*	1st PCR	1400 - 1600	55	Masatani et al., 2017
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG		2nd PCR	1400 - 1600		
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
BTH_Hc_ F	TAAGAGCTAAATTAATGATTGATAGGG		Multiplex PCR	360	55	Qiu et al., 2018
BTH_Bcv_ F	GCTTTTGGGTTTTCCCTTTT			1024		
BTH_Bcr_ F	GTATTTTTGCTTGGCGGTTT			522		
BTH_ Multi. R	CCCGTGTTGAGTCAAATTAAGC					
HS1-F	CGYCAGTGGGCTGGTAATGAA	*groEL* gene/ *Anaplasmataceae*	1st PCR	1300	54	Rar et al., 2010
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL* gene/ *Anaplasma*	2nd PCR	1256	50	Liz et al., 2000
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*groEL* gene/ *Ehrlichia*	2nd PCR	1100	50	Gofton et al., 2016
groEL_rev2	GCCGACTTTTAGTACAGCAA					
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	*gltA* gene/ *Anaplasmataceae*	1st PCR	1096	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG					
EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	*gltA* gene/ *Anaplasma* and *Ehrlichia*	2nd PCR	748	50	Loftis et al., 2015
EHRCS-879R	TIGCKCCACCATGAGCTG					
EHRCS-754F	ATGCTGATCATGARCAAAATG	*gltA* gene/ *Anaplasma* and *Ehrlichia*	2nd PCR	473	50	Loftis et al., 2015
EHRCS-1226R	CCAGTATATAAYTGACGWGGACG					

PCR = Polymerase chain reaction; F = Forward; R = Reverse

Table V-2. Tick-borne pathogens detected in dogs in Malawi by PCR and species-discriminating multiplex PCR

Location	Sampling method	Sex	Total number (n = 209)	*Anaplasmataceae* PCR positive		*Babesia* and *Hepatozoon* PCR positive		
				Anaplasma platys	*Ehrlichia canis*	*Babesia canis rossi*	*Babesia canis vogeli*	*Hepatozoon canis*
Blantyre	FTA cards	Male	2	0	0	1	0	1
		Female	2	0	0	0	0	0
Lilongwe		Male	15	0	1	1	1	1
		Female	19	1	0	3	0	2
Mzuzu		Male	4	0	0	3	0	1
		Female	4	0	0	1	1	1
Sub-totals			46	1	1	9	2	6
Blantyre	EDTA vacutainer tubes	Male	0	0	0	0	0	0
		Female	0	0	0	0	0	0
Lilongwe		Male	75	1	4	18	2	14
		Female	88	3	3	22	2	20
Mzuzu		Male	0	0	0	0	0	0
		Female	0	0	0	0	0	0
Sub-totals			163	4	7	40	4	34
Grand totals			209	5 (2.4)	8 (3.8) *	49 (23.6) *	6 (2.9) *	40 (19.1) *

The number of positive samples and percentage in brackets, * mixed infections were detected in 16 dogs.

Table V-3. The analysis of the occurrence of tick-borne pathogens in dogs in Malawi.

TBPs detected	Number of dogs (%)*
1. Infection with 1 TBP only	77 (36.8)
Babesia rossi	40 (19.1)
Hepatozoon canis	28 (13.4)
Anaplasma platys	5 (2.4)
Babesia vogeli	2 (1.0)
Ehrlichia canis	2 (1.0)
2. Infection with 2 TBPs	16 (7.7)
Babesia rossi + *Hepatozoon canis*	6 (2.9)
Babesia vogeli + *Hepatozoon canis*	3 (1.4)
Ehrlichia canis + *Hepatozoon canis*	3 (1.4)
Babesia rossi + *Ehrlichia canis*	2 (1.0)
Babesia vogeli + *Ehrlichia canis*	1 (0.5)
Babesia rossi + *Babesia vogeli*	1 (0.5)

* The number of dogs positive and the percentage in bracket is based on the total number of 209 sampled dogs

Table V-4. The occurrence of protozoan hemoparasites in dogs in Malawi with regard to host attribute.

Attribute	Number of dogs	Number (%) infected with at least 1 TBP	*p-value*
Ownership			0.076689
Owned	201	87 (43.3)	
Free roaming	8	6 (75.0)	
Age			0.857678
< 1 year	62	27 (43.5)	
≧ 1 year	147	66 (44.9)	
Sex			0.020708*
Male	96	51 (54.8)	
Female	113	42 (45.2)	
Breed			0.01744*
Local	180	86 (47.8)	
Exotic	29	7 (24.1)	
Tick infestation			< 0.00001*
Present	87	63 (72.4)	
Absent	122	30 (24.6)	
Clinical signs **			< 0.00001*
Present	24	19 (79.2)	
Absent	185	74 (40.0)	

* $p < 0.05$

** These included: pale mucous membranes, blood in urine and swollen lymph nodes.

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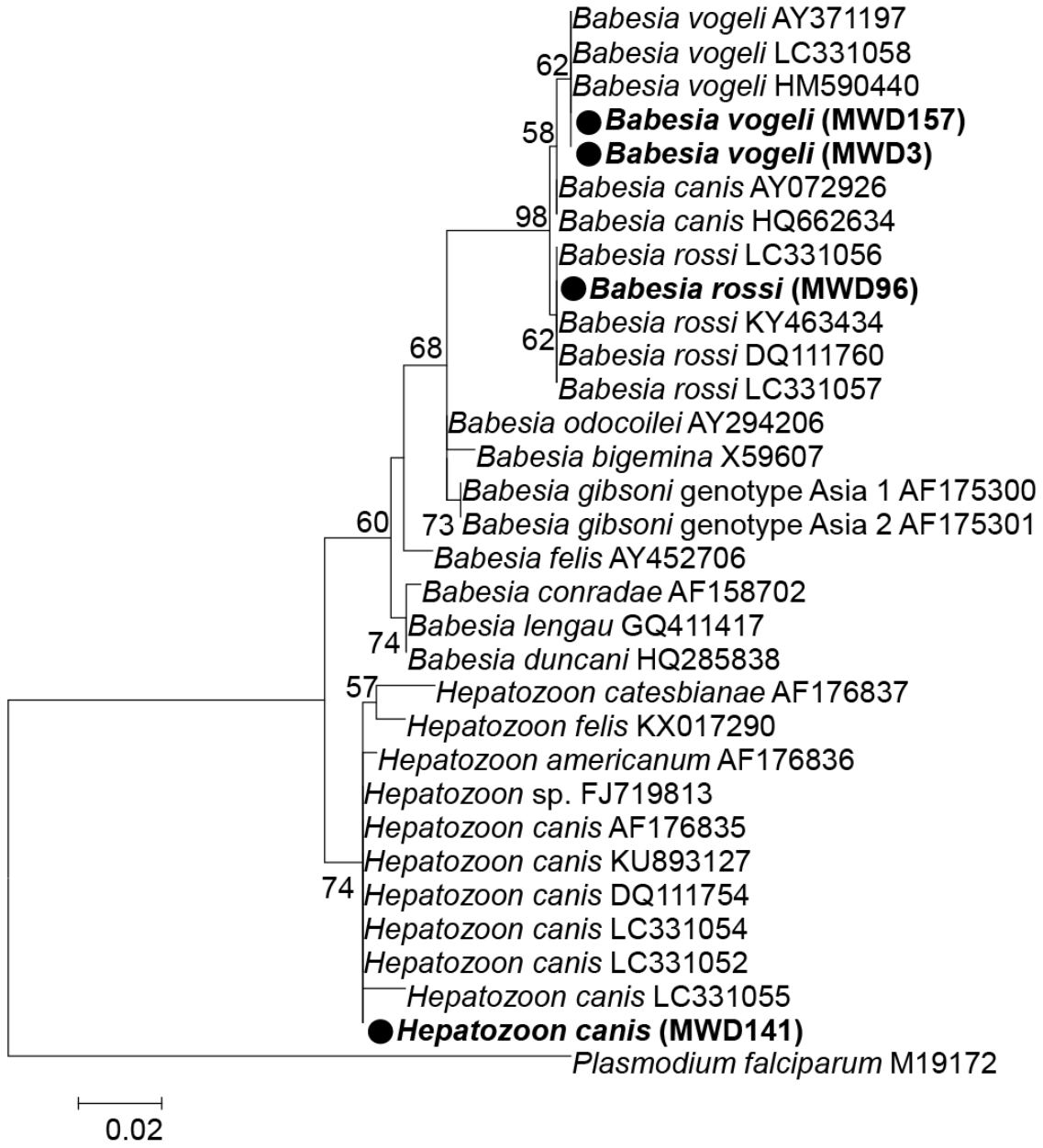

Figure V-1. Maximum likelihood phylogenetic tree based on approximately 1400-bp sequences of the 18S rDNA of *Babesia* spp. and *Hepatozoon* spp. detected in dogs using the Kimura 2 parameter model. The tree was rooted with *Plasmodium falciparum* and bootstrap values >65 from 1000 replicates are shown above the branches. Sequences obtained in this study are shown in bold.

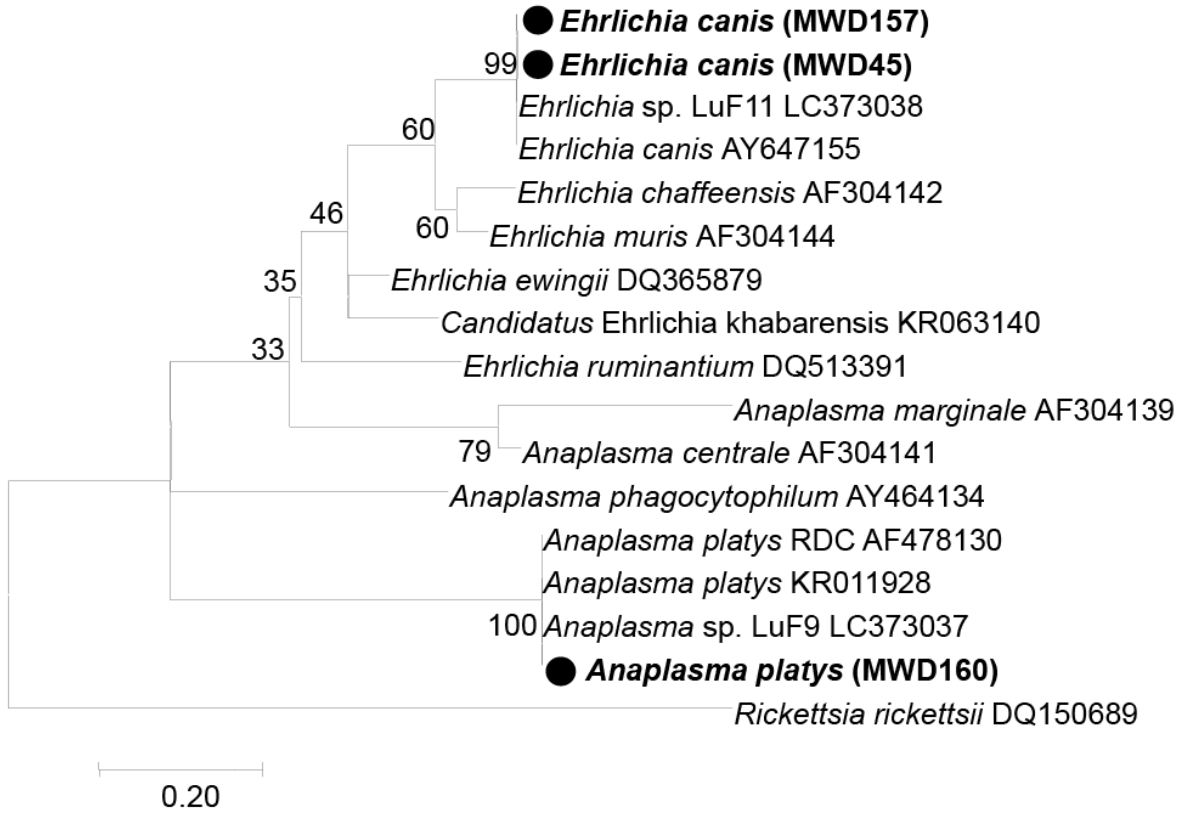

Figure V-2. A maximum likelihood phylogenetic tree based on approximately 650-bp sequences of the *gltA* genes of *Anaplasma* spp. and *Ehrlichia* spp. detected in dogs using the Kimura 2 parameter model. The tree was rooted with *Rickettsia rickettsii* and bootstrap values >65 from 1000 replicates are shown above the branches. Sequences obtained in this study are shown in bold.

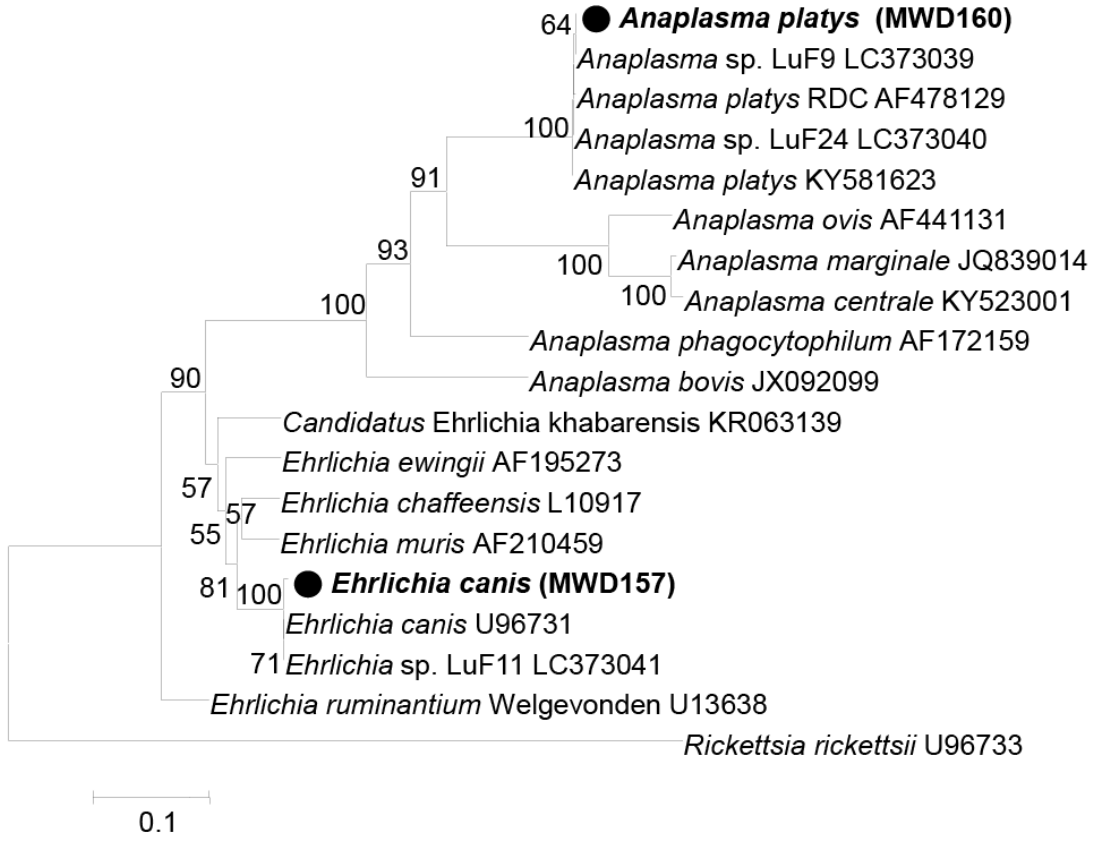

Figure V-3. A maximum likelihood phylogenetic tree based on approximately 1100-bp sequences of the *groEL* gene of *Anaplasma* spp. and *Ehrlichia* spp. detected in dogs using the Kimura 2 parameter model. The tree was rooted with *Rickettsia rickettsii* and bootstrap values >65 from 1000 replicates are shown above the branches. Sequences obtained in this study are shown in bold

GENERAL CONCLUSION

Tick-borne diseases (TBDs) remains one of the major challenges to the improvement of livestock production in the tropics where the vector ticks are widespread. The commonly diagnosed TBDs of domestic animals are anaplasmosis, babesiosis, ehrlichiosis, hepatozoonosis and theileriosis. In Malawi, although ticks that infect domestic animals have been documented for over a century, there has been no study conducted to characterize the tick-borne pathogens (TBPs) of domestic animals. This thesis aimed at detecting and characterizing the TBPs in cattle, sheep, goats and dogs in Malawi using molecular techniques.

In chapter I, 191 cattle DNA samples collected from the central and southern regions of Malawi were screened for tick-borne piroplasms and *Anaplasmataceae*. Overall, 177 (92.7%) of the samples were positive for at least one TBP species. Co-infection of different species was observed in 152 (79.6%) of the samples analysed. *Anaplasma bovis*, *Anaplasma marginale*, *Anaplasma platys*-like, *Anaplasma* species, *Babesia bigemina*, *Ehrlichia* species, *Theileria mutans*, *Theileria parva*, *Theileria taurotragi* and *Theileria velifera* were detected in cattle in Malawi for the first time using molecular techniques.

In chapter II, the genetic diversity and sequence polymorphism of two genes encoding *Theileria parva* antigens recognized by bovine $CD8^+$ T cells among vaccinated and unvaccinated cattle in Malawi was investigated. High prevalence of *Theileria parva* was reported at 54.4% in this study. Single nucleotide polymorphisms were observed at 14 positions (3.65%) in *Tp1* and 156 positions (33.12%) in *Tp2*, plus short deletions in *Tp1*, resulting in 6 and 10 amino acid variants in the *Tp1* and *Tp2* genes, respectively. Most sequences were either identical or similar to *Theileria parva* Muguga, Kiambu 5 strains and Serengeti transformed, the components of Muguga cocktail (MC) vaccine. This may suggest the possible expansion of vaccine components into unvaccinated cattle, or that a very similar genotype to vaccine components existed in Malawi. This study provides information that support the use of *Theileria parva* Muguga cocktail vaccine to control East Coast fever (ECF) in Malawi.

In chapter III, the genotyping and population structure of *Theileria parva* in Malawi was conducted using 100 *Theileria parva*–PCR positive samples. A total of 9 satellite markers were employed in this study. Linkage equilibrium in 4 of the 6 populations was observed when the populations were treated separately but, when treated as a single population there was linkage disequilibrium. There was sub-structuring among some isolates Katete and Likasi farms as shown by the principal coordinate analysis (PCoA). The analysis of molecular variance (AMOVA) showed that the differences were mainly within (99%) than between (1%) populations. Majority of the isolates clustered with the *Theileria parva* Muguga reference strain, suggesting that the isolates in Malawi are closely related to the MC vaccine component. The data support the current use of MC vaccine to control ECF.

The study in chapter II showed that there were three groups of *Theileria parva* circulating in cattle in Malawi (those related to MC vaccine strains, those related to buffalo strains and those related to Zambian strains).

The study in chapter III has shown that there are two populations (those related to MC strains and those related to Zambian strains). Those samples that clustered with buffalo strains from Nkhotakota in chapter II clustered with MC strains in chapter III. This observation may be due to low resolution power of *Tp1* and *Tp2* genes used in chapter II when compared to high resolution of satellite markers used in chapter III. Thsese findings have shown that satellite markers provide a comprehensive genetic profile as compared to *Tp1* and *Tp2* genes which cover a limited region in the *Theileria parva* genome. These findings show that *Theileria parva* in Malawi has two types of strains in which majority are closely related to MC strains and other to Zambian strains. More studies are required to ascertain the impact of the other strains on the efficacy of MC vaccine.

In chapter IV, Molecular detection and characterization of TBPs of sheep and goats in Malawi was conducted. A total of 99 goats and 8 sheep blood DNA were used in this study. Overall, 77 (72.5%) of the samples analysed were positive for at least one TBP species. *Anaplasma ovis*, *Babesia gibsoni*-like strain, *Ehrlichia ruminantium*, *Ehrlichia canis*, *Theileria ovis*, *Theileria* spp. MSD like, *Theileria mutans* and *Theileria separata* were detected in sheep and goats in Malawi for the first time using molecular techniques.

In chapter V, molecular detection and characterization of TBPs of dogs in Malawi was conducted. A total of 209 canine blood DNA samples were screened for tick-borne haemoparasites and *Anaplasmataceae*. Among the examined dogs, 93 (44.5%) were infected with at least one TBP species. This study has provided molecular evidence that dogs in Malawi are infected with *Anaplasma platys*, *Babesia rossi*, *Babesia vogeli*, *Ehrlichia canis* and *Hepatozoon canis*. Sensitization is required for veterinary practitioners, dog handlers and pet owners as the detected pathogens affect the animals' wellbeing. Further studies focusing on rural areas with limited or no access to veterinary care are required to ascertain the extent of the TBP infection in dogs.

The studies in this thesis have shown that domestic animals in Malawi are infected with a wide range of TBPs. These studies have also shown that domestic animals do not always present clinical symptoms even when infected with certain TBPs and serve as reservoirs of TBPs, which could be the source of TBP infections to other susceptible domestic animals. The molecular epidemiological data generated in these studies are important in monitoring the control measures currently in place and conceiving novel methods to control ticks and TBDs in Malawi.

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JAPANESE ABSTRACT

マダニは人や家畜の病原体を含む多様な微生物を保有している。特に *Anaplasma* 属、*Babesia* 属、*Ehrlichia* 属、*Hepatozoon* 属、*Theileria* 属に属するダニ媒介性病原体は、熱帯地域における重要なダニ媒介性動物感染症の原因である、家畜の生産に悪影響を及ぼしている。ダニ媒介性感染症の疫学情報は、自然界での感染動態や伝播経路を監視するのに役立ち、病原体制御法を構築するために重要である。マラウイ共和国では現在、家畜のダニ媒介性病原体に関する分子疫学データは限られている。本論文は、マラウイの牛、羊、山羊、犬の原虫およびリケッチア病原体を、分子技術を用いて検出し、その特徴を明らかにすることを目的としている。

第一章では、マラウイの牛に流行するダニ媒介性病原体の検出を分子診断技術により実施した。PCR により検査した 191 頭の牛の血液由来 DNA サンプルのうち、合計 177 頭（92.7%）が少なくとも 1 つのダニ媒介性病原体に陽性であった。塩基配列解析の結果、*Anaplasma bovis*、*Anaplasma marginale*、*Ananplasma platys*-like、*Anaplasma* sp. 、*Babesia bigemina*、*Ehrlichia* sp.、*Theileria mutans*、*Theileria parva*、*Theileria taurotragi*、*Theileria velifera* が検出された。また、152 頭（79.6%）では２つ以上の病原体の共感染が確認された。本研究により、マラウイの牛におけるダニ媒介性病原体の感染率は非常に高いことが明らかとなった。さらに、病原体の種類によって異なる治療法が必要となるため、高い共感染率はダニ媒介性感染症の診断と治療を困難とする一因であることが示された。

第二章では、牛の CD8 陽性 T 細胞により認識される *Theileria parva* 抗原遺伝子 *Tp1* と *Tp2* をマーカーに、マラウィに流行する *Theileria parva* の遺伝的多様性を調査した。検査した 446 頭の牛のうち、254 頭（54.5%）が *Theileria parva* 種特異的 PCR が陽性であった。塩基配列解析の結果、*Tp1* 遺伝子に比べ *Tp2* 遺伝子ではより高い遺伝的多様性が認められた。すなわち、*Tp1* では 14 箇所（3.65%）、*Tp2* では 156 箇所（33.12%）に一塩基多型が認められ、さらに *Tp1* では塩基欠失があった。その結果、*Tp1* と *Tp2* の遺伝子にはそれぞれ 6 個と 10 個のアミノ酸バリアントが存在した。ほとんどの配列は、*Theileria parva* 弱毒ワウチンを構成する Muguga 株および Kiambu 5 株と同一または類似していた。このことは、ワクチン株がワクチン未接種の牛群に感染拡大したこと、あるいはワクチン株と類似の遺伝子型を持った原虫がマラウイに存在していることを示唆している。本研究は、マラウイにおける *Theileria parva* の防除に Muguga カクテル弱毒ワクチンを使用することが有効であることを支持するものである。

第三章では、マラウイの中央部と南部にある 6 つの地域から採集した牛血液サンプルを材料に、サテライトマーカーによる *Theileria parva* の集団遺伝構造を調査した。解析の結果、マラウイの牛には少なくとも 2 つの *Theileria parva* 遺伝集団が感染していることが明らかとなった。一方の

遺伝集団は、全ての採集地点から検出され、弱毒ワクチン株の一つである Muguga 株を含む優占集団であった。もう一方の集団は、そのほとんどが中央部の農場からのサンプルで構成されていた。本研究によりマラウイにおける *Theileria parva* の流行株の大半が、Muguga 株と近縁であることが明らかとなった。

第四章では、マラウイの羊と山羊のダニ媒介性病原体の検出を分子診断技術により実施した。マラウイ中央部と南部の 2 つの農場から採取した 8 頭の羊および 99 頭のヤギの血液 DNA サンプルを収集した。PCR の結果、79 頭（73.8%）が少なくとも 1 つのダニ媒介性病原体に感染しており、そのうち 47 頭（43.9%）で２つ以上の病原体の共感染がみられた。塩基配列解析の結果、*Anaplasma ovis*、*Babesia gibsoni*-like、*Ehrlichia ruminantium*、*Ehrlichia canis*、*Theileria mutans*、*Theileria ovis*、*Theileria separata*、*Theileria* sp. MSD-like が検出された。本研究は、小型反芻家畜に感染するダニ媒介性病原体 の実態を分子技術を用いて確認した、初めての試みである。

第五章では、マラウイにおける犬のダニ媒介性病原体の検出を分子診断技術により実施した。マラウイの主要都市である Blantyre、Lilongwe、Mzuzu で採取した 209 頭の犬の血液 DNA サンプルを用いた。調査された犬のうち、93 頭（44.5%）が少なくとも 1 つのダニ媒介性病原体に感染していた。そのうち 16 頭（7.8%）では、少なくとも２つ以上の病原体の共感染がみられた。配列解析の結果、マラウイでは初めて *Anaplasma platys*、*Babesia rossi*、*Babesia vogeli*，*Ehrlichia canis*，*Hepatozoon canis* に犬が感染していることを分子レベルで明らかにした。

これら一連の研究は、マラウイの家畜におけるダニ媒介性病原体の高い感染状況を示している。調査したすべての動物種で複数の病原体による混合感染率が高かったことから、マラウイにおけるダニ媒介性感染症の適切な管理のためには、マダニ媒介性病原体の診断法を改善する必要がある。また、殺ダニ剤によるマダニ防除がマラウイで主に実践されている疾病管理法であるが、殺ダニ剤に対するマダニの抵抗性獲得が世界的に懸念されており、新たなダニ媒介性感染症の防除法を導入する必要がある。