Epidemiological study on tick-borne parasitic diseases in livestock from Tanzania and Kenya

2023

RINGO Aaron Edmond

Graduate School of Animal and Veterinary Sciences and Agriculture

Obihiro University of Agriculture and Veterinary Medicine

タンザニアとケニアの家畜におけるマダニ媒介性寄生虫症の疫学的研究

令和５年

(2023)

獣医科学専攻博士課程

RINGO Aaron Edmond

Table of Contents

Abbreviations

18S rRNA	-	component of the small subunit ribosomal ribonucleic acid
AoMSP-4	-	*Anaplasma ovis* major surface protein-4
BLASTn	-	basic local alignment search tool nucleotide
CI	-	Confidence interval
DDW	-	double distilled water
DNA	-	deoxyribonucleic Acid
dNTPs	-	deoxyribonucleoside triphosphote
ECF	-	east coast fever
EDTA	-	ethylenediaminetetraacetic acid
Fig.	-	figure
groEL	-	heat shock operon protein
ID	-	identification
JSPS	-	Japan society for the promotion of science
Km	-	kilometer
MANRLF	-	ministry of agriculture, natural resources, livestock and fisheries
MEGA	-	molecular evolutionary genetic analysis
MPSP	-	major piroplasm surface protein
MSP-5	-	major surface protein-5
NCBI	-	national center for biotechnology information
nPCR	-	nested polymerase chain reaction
NRCPD	-	national research center for protozoan diseases

OR	-	odds ratio
P104	-	*Theileria parva* microneme rhoptry protein 104 kDa gene
PCR	-	polymerase chain reaction
RAP-1a	-	roptry-associated protein-1a
RNA	-	ribonucleic acid
SBP-2	-	spherical body protein-2
SNPs	-	single nucleotide polymorphisms
spp.	-	species
TBDs	-	tick-borne diseases
TBPs	-	tick-borne pathogens
TSHZ	-	Tanzania short horn zebu
UV	-	ultra violet light

Unit abbreviations

%	-	percentage
℃	-	degree Celsius
bp	-	base pair
E	-	east
ml	-	milliliter
mm	-	millimeter
S	-	south
µl	-	microliter
µM	-	micromole

General introduction

Tanzania and Kenya are neighboring countries located in the eastern coast of Africa, just south of the horn of Africa. Tanzania, has a square kilometer of 945100 in size, and is bordered with Uganda and Kenya in the north, Rwanda, Burundi and the Democratic Republic of Congo in the west, in the south it is Zambia, Malawi and Mozambique while the eastern side is Indian ocean. Kenya has a size of 582,600 km^2 and is bordered in the south by Tanzania, in the west is Uganda. Indian ocean and Somalia are in the east while in the north it is Ethiopia. Both Tanzania and Kenya have lowlands and highlands terrains including the highest peak Africa (5895m) which is located in Tanzania. Both countries are located in the tropical climatic zones, and in both countries the climate involve the hot and humid coasts, semi-arid plateaus which are hot and dry. However, the highlands are temperate with sub-tropical weather. In both countries, agriculture and livestock sectors are the main activities and involves the largest part of their population.

The main tick-borne diseases (TBDs) impacting livestock industry in both countries include theileriosis, babesiosis, anaplasmosis and ehrlichiosis. Theileriosis in cattle is caused by several protozoan pathogens including, *Theileria parva*, *T. mutans*, *T. taurotragi*, *T. buffeli* and *T. velifera*. East coast fever (ECF) caused by *T. parva* is transmitted trans-stadially by *Rhipicephalus appendiculatus* tick and is considered as a leading cause of deaths in cattle in the east, central and southern Africa (Muraguri et al., 2005; Swai et al., 2007). The general clinical symptoms of ECF involves fever, lymphadenopathy, labored breathing, corneal opacity, anorexia, nasal discharge and anemia.

Bovine babesiosis is caused by *Babesia* spp., *B. bigemina* and *B. bovis* and are transmitted by *Rhipicephalus microplus* and *R. decoloratus* (Lynen et al., 2007). The disease is manifested by the following symptoms hemoglobinuria, fever, jaundice, anemia, abortion in pregnant animals,

reduced milk production and neurological signs such as convulsion, posterial paralysis and loss of coordination, death usually follows if not treated.

Bovine anaplasmosis is another important disease of cattle caused by *Anaplasma marginale* and transmitted by *R. microplus,* which is regarded as the principal vector of the pathogen in cattle in Tanzania (Swai et al., 2007). The main clinical symptoms of bovine anaplasmosis include fever, jaundice, anorexia, progressive anemia, weakness, inappetence, loss of coordination, loss of milk production, brown urine and sudden death. The disease is rarely severe in calves or young stocks but is generally fatal in adult cattle, and on recovery the animal remains carrier of the disease agent and hence, the source of infection (Kocan et al., 2010). Generally, in Tanzania the disease is considered the second most important tick-borne disease of cattle after ECF.

Bovine ehrlichiosis is a disease of domestic and wild ruminants like cattle, sheep, goats, buffalo and yaks, and is caused by *Ehrlichia ruminantium* (Makala et al., 2003; Allsopp, 2010). In Tanzania, a reported tick vector transmitting the disease is *Amblyomma. variegatum* (Kerario et al., 2017). Generally, the indigenous breeds of cattle are more resistant to ehrlichiosis than the exotic breeds. The main clinical symptoms of ehrlichiosis are depression, respiratory distress, neurological signs and sudden death.

Small ruminants (goats and sheep) are important livestock in Tanzania and Kenya. Similar to cattle the large population of small ruminant in the two eastern countries are owned by the pastoralists and are grazed together with cattle. In most cases the small ruminants are kept for meat. As for cattle, the most important tick-borne diseases of small ruminants include theileriosis, anaplasmosis, babesiosis and ehrlichiosis (Bilgic et al., 2017).

Caprine and ovine theileriosis are mainly caused by *Theileria lestoquardi*, *T. ovis, T. recondita* and *T. separata.* Among these species, *T. lestoquardi* is the most pathogenic *Theileria* spp. in small

ruminants causing malignant ovine theileriosis which is characterized by generalized lymphadenopathy, fever, listness, anorexia, diarrhea and constipation. The other *Theileria* species causes benign theileriosis in sheep and goats (Altay et al., 2007).

Several species of *Babesia* have been reported to cause ovine and caprine babesiosis including *Babesia ovis, B. motasi* and *B. crassa* (Schnittger et al., 2003). Among the three species, *B. ovis* is the most virulent species especially in sheep and causes severe anemia, fever, hemoglobinuria, icterus and occasionally death with the case fatality ranging between 30℃ – 50% (Aktas et al., 2007).

Ovine anaplasmosis is caused by Gram-negative bacterial organisms, namely, *Anaplasma ovis* and *A. phagocytophilum* (Yousefi et al., 2017). The *A. ovis* infection is transmitted biologically and mechanically by ticks, blood-sucking insects and contaminated fomites. Ovine anaplasmosis is often sub-clinical in health animals, but under stressful condition such as poor health condition, climatic change, pregnancy, mixed infections, heavy tick burden, worm infestation, vaccination and transportation in long distances may trigger the development of clinical signs (Stuen, 2016). The clinical symptoms of ovine anaplasmosis include anemia, bloody diarrhea, pyrexia, jaundice, nasal discharge and nervous signs.

In Tanzania and Kenya, most of the tick-borne diseases have effective therapies provided the animals are treated early and accurately. Tick-borne diseases usually develop severe clinical signs the longer the animal remain untreated. The most challenging part in the management of TBDs in most sub-Saharan countries including Tanzania and Kenya is the presence of accurate and fast diagnosis of TBDs. The most commonly used diagnostic methods include the following, The use of clinical signs has been the main and most common method in the region. The veterinarians use their skills and experience to diagnose the diseases based on the clinical signs

shown by the animals. However, this method is unreliable due to the human errors, but most importantly, the overlapping of the clinical symptoms and the presence of co-infections in infected animals that changes the patterns of the clinical symptoms.

The other popular method used for the diagnosis of TBDs in Tanzania and Kenya is microscopy. Microscopy remains the basic and common tool for the diagnosis of tick-borne protozoan and rickettsial diseases in the sub-Saharan countries including Tanzania and Kenya. However, human error and incompetence has often produced wrong diagnosis leading to unsuccessful treatment. The other challenge of microscopy the personnel experience is the presence of different states of the diseases, for acute stage of the disease results can be produced correctly, however, in the chronic stages of the disease microscope cannot detect the pathogens. In addition, some pathogens like *Babesia bovis* always show low level of parasitemia in their hosts, therefore through microscopy it is likely to miss the pathogen and produce wrong results.

Serological tests have by far produced reliable results in the diagnosis of TBDs in the sub-Saharan Africa. It has fewer disputes compared to microscopy and the use of clinical signs. However, availability of reagents has always caused havoc in the use of serological tests in Tanzania and most sub-Saharan Africa.

Lately, molecular technology has apparently been used in the diagnosis of TBDs. This tool has high sensitivity and specificity and produces the most accurate reliable results. However, the main challenge of this technology is the scarcity of qualified human resource in the region and more importantly it is expensive to run especially in the developing countries like Tanzania and Kenya. Therefore, the use of molecular technology has remained in major towns and referral laboratories of the countries. Therefore, based on the aforementioned background status on the

diagnosis of TBDs in Tanzania, Kenya and other sub-Saharan countries, TBDs will remain a big challenge to the livestock industry.

In Tanzania and Kenya, the use of acaricides has predominately remain the main control method of TBDs, most farmers rely heavily on the routine use of acaricides to control vector borne diseases. However, with the insufficient extension services, the misuse of acaricides has become a big factor in failure to control ticks. Remarkably, the major concern recently is the growing alert of ticks becoming acaricide resistant. The acaricide resistant is growingly becoming another disconcertment of TBDs in the region. The other TBD's control method practiced mainly by the pastoralists is the exposure of the animals at the young age (less than 1 year) to TBDs so as they develop immunity against any further reinfection. The exposed animals will remain carriers of the diseases and will not show clinical symptoms when re-infected by the parasites in the field. Therefore, the animals remain apparently health regardless of the presence of ticks and the pathogens. A condition is termed endemic stability.

Remarkably, despite all the challenges in the TBDs management in Tanzania and Kenya, little information is available on the detailed epidemiological status of TBDs. Therefore, the overall aim of these studies was to analyze the occurrence, distribution and genetic diversity of protozoan and rickettsial pathogens using sensitive molecular technology in blood samples collected from cattle and small ruminants of Tanzania and Kenya.

Chapter 1

Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania

1.1 Introduction

Tick-borne pathogens (TBPs) pose major threat to livestock in tropical and sub-tropical regions (Jensenius et al., 2006; Gomes et al., 2013). In Africa, infection of cattle and other ruminants with TBPs cause significant economic losses to livestock farmers (Tembue et al., 2011). The main tick-borne diseases impacting cattle include east coast fever, babesiosis, anaplasmosis and ehrlichiosis. East coast fever is caused by *Theileria parva* while *Babesia bovis* and *B. bigemina* are the major causes of bovine babesiosis. Infections of cattle with *Anaplasma marginale* and *Ehrlichia ruminantium* cause anaplasmosis and ehrlichiosis, respectively (Simuunza et al., 2011). Bovine cerebral theileriosis (turning sickness) is another important tick-borne disease affecting cattle. *Theileria taurotragi,* previously considered a pathogen of Eland (Martin and Brocklesby, 1960), *T. parva* and occasionally *Theileria annulata* are considered main causes of this disease (Devos & Roos, 1981).

Tanzania has approximately 25 million herds of cattle, representing the third largest cattle population in Africa (Engida et al., 2015). However, cattle productivity (milk and meat) is ranked low among African countries (Engida et al., 2015). One of the key obstacles contributing to low productivity is prevailing infectious diseases, notably, tick-borne diseases (Kivaria, 2006).

Pemba Island like in many other parts of Tanzania, most of the household depends on livestock farming. The management system practiced in Pemba Island is intensive management system and semi-intensive management system. The breeds of cattle kept in Pemba are the exotic dairy breeds for milk production and the indigenous short horn zebu for beef. However, the dairy and beef

industry in the island is not growing as expected. The main culprit for stagnant growth of livestock in Pemba is reported to be tick-borne diseases (Woodford et al., 1990).

Despite the significance of the tick-borne pathogens in cattle, little information is available in terms of their occurrence, distribution and the degree of enzootic stability in Pemba Island, Tanzania. Therefore, the aim of this study was to analyze the presence and genetic diversity of tick-borne protozoan and rickettsial pathogens infecting cattle on Pemba Island, Tanzania.

1.2 Materials and methods

1.2.1 Study area

Pemba Island is located in the Indian Ocean, 50 km from the mainland of Tanzania (Fig. 1.1). The island is located between latitude 39.606019°S and 39.86269°S and longitude -4.907837°E and -5.419286°E. The size of the island is 988 square km, with four districts: Mkoani, Chake, Wete and Micheweni. The topography is characterized by numerous small valleys and hills. Pemba experiences bimodal rainfall pattern. The average rainfall per annum is 1860 mm, which falls mostly between March and May (long rains) and October to December (short rains). The climate is tropical sub-humid with average temperatures of 26ºC annually. The vegetation can be classified into four types, which are tropical moist forest, coastal rag shrubs, clove plantations and farmlands (Vreysen and Khamis, 1999).

1.2.2 Sample collection and DNA extraction

Blood samples were collected from clinically healthy cattle in April and May 2017, using sterile needles and vacutainer tubes coated with EDTA. Sampled cattle were mostly indigenous breeds and their crosses with Friesian, Jersey and Brown Swiss. Approximately 3–5 ml of blood was drawn from the jugular vein of the animal, transported to the laboratory in cool boxes and then

kept at 4ºC in the laboratory for no more than a few days until DNA extraction was conducted. A total of 245 blood samples were collected randomly from different farms in all four districts of Mkoani, Micheweni, Chake and Wete, whereby the number of samples collected were 65, 60, 60 and 60 respectively. Samples were collected from animals of around one and a half years of age and above. DNA was extracted from 200 µl of blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and stored at -30ºC until used.

1.2.3 Ethical statement

The owners of the selected farms were informed of the study and provided their approval for sampling of their cattle. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Approval ID: 29 – 68).

1.2.4 Molecular detection of *Theileria, Anaplasma, Babesia* and *Ehrlichia* species

Nested PCR was used to screen all the samples with species-specific primers (Table 1.1) for *A. marginale, B. bovis, B. bigemina* and *E. ruminantium* detection. *Theileria* species were screened using the genus specific 18S rRNA primers and positive samples were sequenced to confirm the species. After species confirmation, *T. parva*-positive samples were screened again and sequenced using species-specific primers for p104 gene. The thermo-cycling conditions were set as described previously (Table 1.1). The reaction mixture had a final volume of 10 µl, containing 0.5 mM of each primer, 1 µl of dNTP mix and 0.1 µl of Ex *Taq* polymerase (Takara Bio, Japan), 1 µl of 10×standard *Taq* buffer, 1 µl of DNA template and 5.9 µl of double distilled water. The positive controls were positive samples from previous studies (Adjou Moumouni et al., 2015); (Jirapattharasate et al., 2016), while double-distilled water (DDW) was used as negative control.

PCRs were run in a thermal cycler (Bio rad, USA). The PCR products were electrophoresed on a 2% agarose gel and stained by ethidium bromide and then viewed under UV transilluminater.

1.2.5 Cloning and sequencing

For sequencing, 1 - 5 positive samples were randomly selected per detected pathogens. Amplicons were extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen). The concentration of the extracts was measured using a NanoDrop 2000 spectrophotometer (Thermofisher, USA). The extracted PCR product (6 µl) was ligated into a pGEM-T Easy vector (2 µl) (Promega, USA) with a T4 DNA ligase (1µl) in a restriction buffer (1 µl). Thereafter, the ligation mixture was incubated at 16ºC for 3 h and then kept at 4ºC overnight. The plasmid construct was transformed into *Escherichia coli* DH5α competent cells and then extracted using NucleoSpin® Plasmid QuickPure (Machery-Nagel, Germany) kit". The PCR product, ligated to the plasmid, was sequenced with Big-dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) using a 3100 Genetic Analyzer (Applied Biosystems, USA). Four clones were sequenced for each amplicon. The sequencing primers used were M13/pUC. Nucleotide sequences were produced using 3100 Genetic Analyzers (Applied Biosystems, USA). The nucleotide sequence identities were determined by performing GenBank BLASTn analysis with genes on the NCBI database.

1.2.6 Phylogenetic analysis

A phylogenetic tree was constructed using MEGA version 7.0 program (Kumar et al., 2018) with DNA sequences obtained from this study and those from the same pathogens already available in the GenBank. Neighbor joining method was used for phylogenetic tree analysis of *A. marginale* MSP-5 gene, *T. parva* p104 genes, *B. bovis* SBP-2 gene, *B. bigemina* RAP-1a gene and

E. ruminantium pCS20 gene. Bootstrap analysis with 1050 replications was used to estimate the confidence of the nodes and branches of the trees.

1.2.7 Nucleotide sequences accession numbers

Gene sequences obtained in this study were deposited in GenBank database of the National Center for Biotechnology Information using Bankit. The GenBank accession numbers were assigned to the sequenced genes as shown in Table 1.2.

1.2.8 Statistical analysis

Pearson's chi-square (χ^2) and Fisher's Exact test were used to statistically analyze the prevalence of the pathogens detected. The significance of co-infections was assessed by Odds Ratio calculation using MedCalc software. A p-value < 0.05 was considered statistically significant.

1.3 Results

1.3.1 Overall prevalence

The overall infection rates of cattle by tick-borne pathogens detected by PCR are presented in Table 1.3. The PCR revealed that 175/245 (71.4%) of the cattle were infected by one to six of the detected pathogens. The most frequently observed pathogens among the 245 samples were *Theileria* spp. occurring in 62.5% of the samples, *B. bigemina* (17.6%), *A. marginale* (15.9%), *E. ruminantium* (7.4%) and *B. bovis* (4.5%) (Table 1.3).

1.3.2 Identification of *Theileria* species

The 18S rRNA sequences of *Theileria* spp.-positive samples revealed four distinct *Theileria* species, namely, *T. parva*, *T. mutans*, *T. taurotragi*, and *T. ovis* (Table 1.4). Of the 153 examined *Theileria*-positive samples, *Theileria mutans* was the most frequent (occurring in 68.6% of the

samples) followed by *T. taurotragi* (48.4%), *T. parva* (41.2%), and *T. ovis* (1.9%) (Table 1.5). All *T. parva*-positive samples were also positive for *T. parva*-specific p104 gene.

1.3.3 Prevalence of co-infections

A total of 115 cattle (46.9%) were simultaneously co-infected with two or more pathogens. Overall, 99 different species combinations were observed. Pathogen co-infections ranged from double to sextuple (Table 1.6). The majority (63.5%) of the 115 co-infections occurred as double infections. The most frequent co-occurrences included *T. mutans* and *T. taurotragi*, *T. mutans* and *A. marginale* and a triple infection of *T. parva*, *T. mutans* and *T. taurotragi*. However, the prevalence of co-infections was not statistically significant.

1.3.4 Comparative sequence analysis of p104, RAP-1a, SBP-2, MSP-5 and pCS20 genes

All *T. parva, B. bigemina, B. bovis, A. marginale* and *E. ruminantium* sequences in this study were of the expected sizes of 277 bp, 412 bp, 580 bp, 195 bp and 278 bp, respectively. The percentage nucleotide identity among the three *T. parva* p104 gene sequences (MG210825 – MG210827) ranged from 99.3% to 100%. These sequences shared 99% nucleotide identity with sequences from Kenya (KP347566 and KP347565). Furthermore, the three *B. bigemina* RAP-1a gene sequences (MG210822 – MG210824) shared nucleotide identity ranging from 99.5% to 100% among themselves. They showed 99% nucleotide identity with sequences from Kenya (KP347559) and Egypt (KF192811). However, *B. bovis* SBP-2 gene sequences (MG344151 – MG344153) shared sequence identity of 66% to 100%. The three SBP-2 gene sequences from this study showed 97% sequence identity with a sequence from Thailand (KT460090). Meanwhile, the *A. marginale* MSP-5 gene sequences (MG700524 – MG700527) shared 100% nucleotide identity, and had 100% identity with sequences from Egypt (KU042081 and KU042083) and 99% with KP347554 from Kenya. On the other hand, *E. ruminantium* pCS20 gene sequences (MG334253 –

MG334255) nucleotide identity ranged from 99.6% to 100%. They shared 100% nucleotide identity with sequences from Mozambique (KX373601) and Sudan (AB218277).

1.3.5 Phylogenetic analysis

Phylogenetic trees of *T. parva, B. bigemina, B. bovis, E. ruminantium* and *A. marginale* in this study were constructed based on p104, RAP-1a, SBP-2, pCS20 and MSP-5 genes respectively using sequences available in GenBank. All the *T. parva* sequences from this study clustered together in the same clade with sequences from Kenya (KP437566 and KP437565) (Fig. 1.2). The two sequences of *B. bigemina* (MG210822 and MG210823) clustered with sequences from Asian countries including Philippines (JX648554), Indonesia (KY484520, KY484521 and KY562848) and Syria (AB617644). The other sequence (MG210824) was in a separate clade with sequences from Turkey (KC515388 and KT220513) as shown in Fig. 1.3. Two *B. bovis* sequences (MG344151 and MG344152) clustered in the same clade whereas the third sequence (MG344153) formed a separate branch (Fig. 1.4). Meanwhile, *E. ruminantium* sequences were in the same clade with other sequences from Mozambique (KX373601) and Sudan (AB218277) (Fig. 1.5). Furthermore, phylogenetic analysis revealed that *A. marginale* sequences isolated in this study were in the same clade with sequences from Mongolia (JQ735905 and AB703240), Egypt (KU042081), Cuba (JF270381) and Colombia (KX365052) (Fig. 1.6).

1.4 Discussion

Tick-borne protozoan and rickettsial pathogens cause economically important infectious diseases affecting livestock worldwide (Altay et al., 2008; Dantas-Torres et al., 2012; Aktas et al., 2012). However, information on the occurrence and genetic diversity of these pathogens is lacking in several regions of the world including Pemba Island, Tanzania. In the present study, I performed

a molecular survey of tick-transmitted pathogens on blood samples of cattle collected from Pemba Island, Tanzania. My findings revealed that *T. mutans, T. parva, T. taurotragi, B. bigemina, B. bovis, E. ruminantium* and *A. marginale* are present in cattle from the study area.

The higher prevalence of *T. mutans* (68.6%) as compared to other pathogens is in agreement with previous studies in Kenya ((Njiiri et al., 2015), Uganda ((Byaruhanga et al., 2016) and Zambia ((Simuunza et al., 2011). The higher prevalence of this pathogen in the horn of Africa towards southern Africa may be explained by the ability of hosts to carry this pathogen for a longer time at high level after infection without showing clinical signs (Asiimwe et al., 2013). Consequently, *T. mutans* might be acquired by calves early in life, after which they remain life-long carriers and a source of infection to new hosts (Hailemariam et al., 2017). This may explain the high prevalence of *T. mutans* in this study. Alternatively, but less likely, cattle might be more susceptible to *T. mutans* infection than other TBD pathogens (Simuunza et al., 2011). Importantly, *T. mutans* have been reported to have some strains which are pathogenic (Young et al., 1978; Paling et al., 1981; Moll et al., 1986). The pathogenic strains reported in eastern Africa cause severe anaemia, icterus, enlarged lymph nodes, weight loss and sometimes death (Pfitzer, 2009; Byaruhanga et al., 2016) reported that *T. mutans* causes ECF-like clinical signs in cattle. Therefore, this pathogen can be considered of economic important to farmers in the region as they can develop serious disease to exotic breeds or newly introduced cattle.

East Coast Fever (ECF) caused by *T. parva* is the most economically important TBD in Tanzania causing high morbidity and mortality in cattle (Swai et al., 2009). The prevalence of *T. parva* (41.2%) is in agreement with similar studies in the region (Swai et al., 2005), Kenya ((Njiiri et al., 2015), western Uganda (Tayebwa et al., 2018) and North eastern Uganda (Byaruhanga et al., 2016) similar results were reported. The relatively higher prevalence of *T. parva* in this study

could be due to higher number of indigenous cattle which are freely grazing in pastures and are continuously exposed to ticks. Additionally, indigenous cattle are relatively resistant to challenges of ECF pathogens. Therefore, they carry the parasite for long periods without showing any signs of illness due to endemic stability (Kariuki et al., 1995; Simuunza et al., 2011). Consequently, they continuously infect ticks which subsequently cause new infections to cattle including the crosses and newly introduced cattle in the area which are highly susceptible and develop serious disease. In the phylogenetic analyses, the p104 gene sequences of *T. parva* from this study were found in a single clade (Fig. 1.2), which suggests that a single genotype of *T. parva* is circulating in the cattle population of the study area.

Theileria taurotragi causes theileriosis in cattle and elands. Recent studies have associated this pathogen with Bovine Cerebral Theileriosis (Turning sickness) in indigenous East African Zebu cattle (Binta et al., 1998; Catalano et al., 2015). In this study the prevalence of *T. taurotragi* was 48.4%. It is higher compared to previous studies in Kenya (Njiiri et al., 2015), Uganda (Byaruhanga et al., 2016) and Zambia (Simuunza et al., 2011). The prevalence of *T. taurotragi* coincides with that of *T. parva* in the study area, this could be attributed to the fact that they share the same tick vector, *R. appendiculatus*.

Babesiosis caused by *B. bigemina* and *B. bovis* is an important infectious disease of cattle in Tanzania (Swai et al., 2005). The prevalence of *B. bigemina* (17.6%) was lower compared to the previous studies in Tanzania (Swai et al., 2005) and Kenya (Adjou Moumouni et al., 2015). The low prevalence of *B. bigemina* in this study could be due to the high temperatures experienced on Pemba Island, as high temperature has been reported to inhibit the development or eliminate *B. bigemina* infection in ticks (Kocan, 1995). This hypothesis is further supported by the low prevalence of this pathogen in drier areas of northern Uganda (Byaruhanga et al., 2016) and in dry

season in Zambia (Simuunza et al., 2011). Phylogenetic analyses of RAP-1a suggested the presence of different genotypes of *B. bigemina* strain populations in Pemba island (Fig. 1.3). A similar trend was observed in *B. bovis* which recorded the lowest prevalence (4.5%). The low prevalence of this parasite in the region could be associated with the low key distribution of the pathogen rather than the presence and distribution of the vector and hosts. The SBP-2 phylogenetic analysis suggests the presence of different genotypes of *B. bovis* strain in the study area (Fig. 1.4).

Ehrlichiosis caused by *E. ruminantium* had a prevalence of 7.4%. The low prevalence is in agreement with previous studies in southwestern Ethiopia (Hailemariam et al., 2017) and North-eastern Uganda (Byaruhanga et al., 2016). This might be attributed by the biological nature of the pathogen. It has been reported the pathogen resides mostly in endothelial cells of blood vessels and is only periodically found in the blood stream (Andrew & Norval, 1989; Lorusso et al., 2016). The presence of this pathogen together with its vector (*Amblyomma* ticks) reported by (Mamiro et al., 2016) in Tanzania should be taken into account when considering to improve breeds, by introducing exotic breeds of cattle (*Bos taurus*). Phylogenetic analysis of the pCS20 sequences of *E. ruminantium* of this study showed high identity to strains detected in Sudan and Mozambique which falls in the Southern-Central Africa and Caribbean clade as shown in Fig. 1.5.

Anaplasmosis caused by *A. marginale* is known to be pathogenic to domestic ruminants, high producing dairy cattle are highly susceptible (Hailemariam et al., 2017). In this study we report a prevalence of 15.9%. However, higher prevalence were reported in Southern highland and Tanga region of Tanzania (Swai et al., 2005), Kenya (Gachohi et al., 2010) and in Uganda (Kabi, 2008). The lower prevalence of *A. marginale* in this study, could be attributed to high proportion of indigenous zebu cattle sampled. The indigenous zebu cattle have been reported by (Swai et al., 2007) to have inherent resistance to ticks, which could lead to lower *A. marginale* infection. Tick

resistant traits most likely contributed to the lower prevalence of *A. marginale*. In phylogenetic analyses, the *A. marginale* MSP-5 sequences of this study were detected in a single clade of the tree (Fig. 1.6), which suggest that MSP-5 gene is conserved among field isolates from different geographical location.

A complex pattern of co-infections was observed in this study. *Theileria* spp. were the most common pathogens in co-infections. The interaction between different TBPs can be much more complex and involve ecological, epidemiological and also clinical aspects (Baneth, 2014; Diuk-Wasser et al., 2016). Results of a recent study suggested that heterologous protection effect of less pathogenic *Theileria* co-infections against *T. parva* infection may be occurring (Byaruhanga et al., 2016). In western Kenya, a study showed that infections with a less pathogenic *Theileria* spp. (such as *T. mutans*) were associated with reduction of ECF fatality and mortality in a population of East African short horn zebu cattle (Woolhouse et al., 2015). Therefore, the frequent co-infections with *T. mutans* and *T. parva* observed in this study can be of ecological importance in the reduction of ECF fatalities and mortalities in cattle in the study area. However, pathogenic effect of *T. mutans* which is considered less pathogenic may still occur. Notably, co-infected hosts usually develop clinical symptoms that considerably deviate from the typical patterns observed in animals infected with single pathogen, which may prevent accurate diagnosis and lead to treatment failure.

In conclusion, this study revealed high prevalence and diversity of tick-borne pathogens in cattle in the study area, *Theileria* spp. (*T. parva*, *T. mutans* and *T. taurotragi*) were the most prevalent pathogens. Co-infections were more common than single infections with *Theileria* species mostly involved in co-infection as compared to other detected pathogens. This might have implications for potential interaction of pathogens and the patterns of clinical symptoms. These

findings contribute to our understanding of the occurrence and genetic diversity of tick-borne pathogens in Tanzania and will be useful in the formulation of control strategies.

1.5 Summary

In this study, the occurrence of *Theileria* spp., *Babesia* spp., *Anaplasma* spp. and *Ehrlichia* spp. in 245 blood samples collected from cattle on Pemba Island, Tanzania was investigated. Polymerase chain reaction (PCR) and gene sequencing was used to detect and identify pathogens. PCR screening revealed overall infection rates of 62.4% for *Theileria* spp., 17.6% for *Babesia bigemina*, 15.9% for *Anaplasma marginale*, 7.4% for *Ehrlichia ruminantium* and 4.5% for *Babesia bovis*. Further analysis using sequences of *Theileria* spp. 18S rRNA revealed infection of cattle with *Theileria mutans* (68.6%), *Theileria taurotragi* (48.4%), *Theileria parva* (41.2%), and *Theileria ovis* (1.9%). Co-infections of cattle, with up to six tick-borne pathogens, were revealed in 46.9% of the samples. Sequence analysis indicated that *T. parva* p104, *E. ruminantium* pCS20 and *A. marginale* MSP-5 genes are conserved among cattle blood samples in Pemba, with 99.3% - 100%, 99.6% - 100% and 100% sequence identity values, respectively. In contrast, the *B. bigemina* RAP-1a and *B. bovis* SBP-2 gene sequences were relatively diverse with 99.5% - 99.9% and 66.4% - 98.7% sequence identity values respectively. The phylogenetic analyses revealed that *T. parva* p104, *E. ruminantium* pCS20 and *A. marginale* MSP-5 gene sequences clustered in the same clade with other isolates from other countries. In contrast, the *B. bigemina* RAP-1 and *B. bovis* SBP-2 gene sequences showed significant differences in the genotypes, as they appeared in separate clades. This study provides important data for understanding the epidemiology of tick-borne diseases, and is expected to improve the approach for diagnosis and control of tick-borne diseases in Tanzania.

Table 1.1 List of primers used in the assays.

Target gene	Assay	Primer sequences (5'⟶3')		Fragment	Annealing temp.	References
		Forward	Reverse	(bp)	(°C)	
Theileria spp. (18S rRNA)	PCR	GAAACGGCTACCACATCT	AGTTTCCCCGTGTTGAGT	778	55	(Cao et al., 2013)
	nPCR	TTAAACCTCTTCCAGAGT	TCAGCCTTGCGACCATAC	581	55	
B. bigemina (Bbig.RAP-1)	PCR	GAGTCTGCCAAATCCTTAC	TCCTCTACAGCTGCTTCG	879	55	(Terkawi et al., 2011)
	nPCR	AGCTTGCTTTCACAACTCGCC	TTGGTGCTTTGACCGACGACAT	412	55	
B. bovis (BbSBP-2)	PCR	CTGGAAGTGGATCTCATGCAACC	TCACGAGCACTCTACGGCTTTGCAG	1236	64	(AbouLaila et al., 2010)
	nPCR	GAATCTAGGCATATAAGGCAT	ATCCCCTCCTAAGGTTGGCTAC	580	58	
T. parva (p104)	PCR	ATTTAAGGAACCTGACGTGACTGC	TAAGATGCCGACTATTAATGACACC	496	65	(Ota et al., 2009)
	nPCR	GGCCAAGGT CTCCTTCAGATTACG	TGGGTGTGTTTCCTCGTCATCTGC	277	60	
T. orientalis (MPSP)	PCR	CTTTGCCTAGGATACTTCCT	ACGGCAAGTGGTGAGAACT	777	58	(Kaba et al., 2009)
T. annulata (Tams)	PCR	GTAACCTTTAAAAACGT	GTTACGAACATGGGTTT	721	55	(d'Oliveira et al., 1995)
	nPCR	CACCTCAACATACCCC	TGACCCACTTATCGTCC	453	60	
A. marginale (MSP5)	PCR	GTGTTCCTGGGGTACTCCTATGTGAACAAG	AAGCATGTGACCGCTGACAAACTTAAACAG	547	78 -69	(Ybañez et al., 2012)
	nPCR	AAGCACATGTTGGTAATATTCGGCTTCTCA	AATTCTCGCATCAAAAGACTTGTGGTACTC	195	78-69	
E. ruminantium (pCS20)	PCR	ACTAGTAGAAATTGCACAATCYAT	RCTDGCWGCTTTYTGTTCAGCTAK	400	61	(Farougou et al., 2012)
	nPCR	ACTAGTAGAAATTGCACAATCYAT	AACTTGGWGCRRGDARTCCTT	278	61	

Table 1.2 Accession numbers assigned to the isolates.

Pathogen	Target gene	Accession number	Sequence size (bp)
Anaplasma marginale	MSP5	MG700524	195
		MG700525	195
		MG700526	195
		MG700527	195
Babesia bovis	SBP-2	MG725962	582
		MG725963	540
Babesia bigemina	RAP-1	MG210822	412
		MG210823	412
		MG210824	412
Ehrlichia ruminantium	pCS20	MG334253	279
		MG334254	279
		MG334255	279
Theileria spp.	18S rRNA	MG725959	521
		MG725960	520
		MG725961	520

		MG755213	243
		MG755214	243
		MG755215	243
		MG755216	260
		MG755217	260
Theileria parva	p104	MG210825	278
		MG210826	278
		MG210827	278
		MG700531	278
		MG700532	278

Table 1.3 Identification of *Theileria* species by BLASTn analysis of 18S rRNA sequences of the isolates from Tanzania.

Accession numbers	Highest BLASTn match	Accession number of match	% identity
MG700532	*T. parva*	KP347566	100
MG700531	*T. parva*	KP347566	100
MG755216	*T. mutans*	KU206320, FJ869899	100
MG755217	*T. mutans*	KU206309, FJ869899	100
MG755213	*T. taurotragi*	L19082	100
	T. taurotragi	KY352037	98
MG755214	*T. taurotragi*	L19082	100
MG725959	*T. ovis*	KT851427 – 38	100
MG725960	*T. ovis*	KT851427 – 38, KX273858	100
MG725961	*T. ovis*	KT851427 – 38, KX273858	100

Table 1.4 Tick borne pathogens detected in cattle from Pemba, Tanzania.

Pathogen detected	number of positive (%)
Single infection	
T. parva	11 (4.49)
T. mutans	18 (7.35)
T. taurotragi	13 (5.31)
B. bigemina	8 (3.27)
B. bovis	2 (0.82)
E. ruminantium	3 (1.22)
A. marginale	5 (2.04)
Double infection	
T. parva + *T. mutans*	9 (3.67)
T. parva + *T. taurotragi*	6 (2.45)
T. parva + *B bigemina*	5 (2.04)
T. parva + *A. marginale*	1 (0.41)
T. parva + *B. bovis*	1 (0.41)
T. mutans + *T. taurotragi*	14 (5.71)
T. mutans + *B. bigemina*	8 (3.27)
T. mutans + *E. ruminantium*	3 (1.22)

T. mutans + *B. bovis*	1 (0.41)
T. mutans + *A. marginale*	13 (5.31)
T. taurotragi + *B. bigemina*	1 (0.41)
T. taurotragi + *A. marginale*	4 (1.63)
B. bigemina + *E. ruminantium*	3 (1.22)
B. bigemina + *A. marginale*	2 (0.82)
E. ruminantium + *A. marginale*	2 (0.82)
Triple infection	
T. parva + *T. mutans* + *T. taurotragi*	13 (5.31)
T. parva + *T. mutans* + *B. bigemina*	2 (0.82)
T. parva + *T. mutans* + *E. ruminantium*	2 (0.82)
T. parva + *T. mutans* + *B. bovis*	2 (0.82)
T. parva + *T. taurotragi* + *A. marginale*	2 (0.82)
T. mutans + *T. taurotragi* + *B. bigemina*	3 (1.22)
T. taurotragi + *B. bigemina* + *A. marginale*	1 (0.41)
Quadruple infection	
T. parva + *T. mutans* + *T. taurotragi* + *B. bigemina*	2 (0.82)
T. parva + *T. mutans* + *T. taurotragi* + *E. ruminantium*	2 (0.82)
T. parva + *T. mutans* + *T. taurotragi* + *B. bovis*	1 (0.41)
T. parva + *T. mutans* + *T. taurotragi* + *A. marginale*	2 (0.82)

T. mutans + *T. taurotragi* + *B. bigemina* + *A. marginale*	1 (0.41)
T. mutans + *T. taurotragi* + *B. bigemina* + *B. bovis*	2 (0.82)
T. mutans + *T. taurotragi* + *B. bigemina* + *A. marginale*	3 (1.22)
T. mutans + *T. taurotragi* + *B. bovis* + *A. marginale*	1 (0.41)
Quintuple infection	
T. parva + *T. mutans* + *T. taurotragi* +*B. bigemina* + *E. ruminantium*	1 (0.41)
T. mutans + *T. taurotragi* + *B. bigemina* + *E. ruminantium* + *A. marginale*	1 (0.41)
Sextuple infection	
T. parva + *T. mutans* + *T. taurotragi* + *E. ruminantium* + *B. bovis* + *A. marginale*	1 (0.41)
Total	175 (71.43)

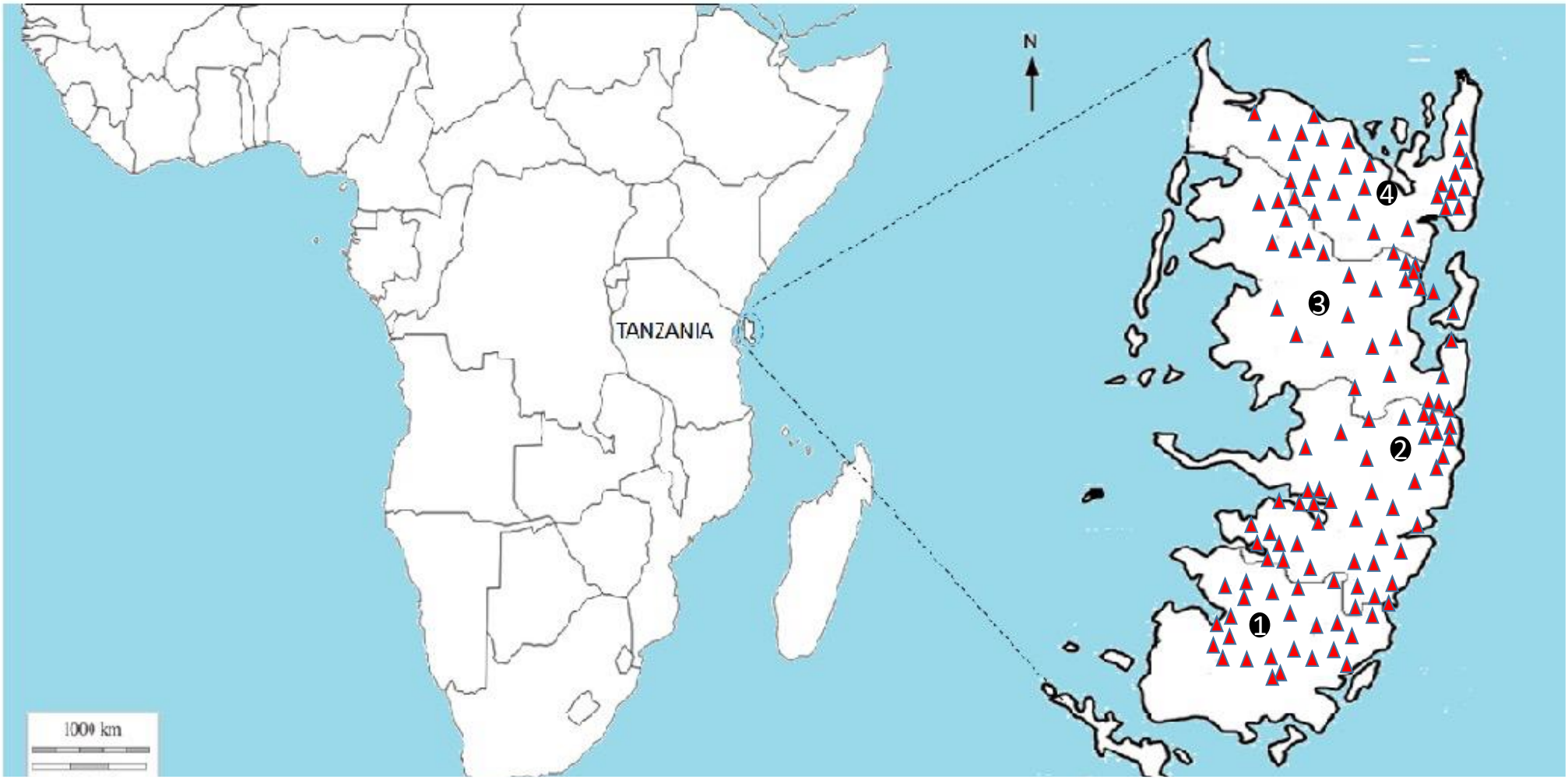

Figure 1.1 Map of Pemba Island, Tanzania showing sample collection sites.

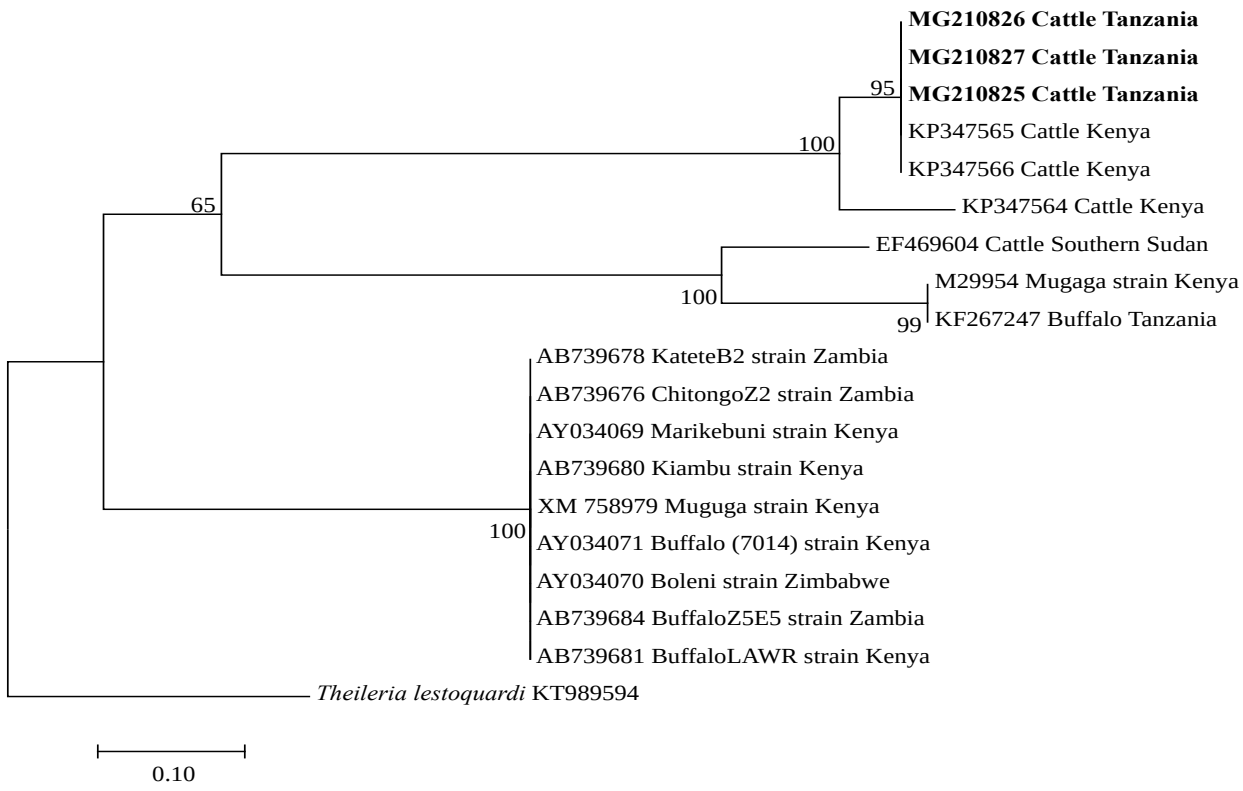

Figure 1.2 Phylogenetic analysis by neighbor joining method based on *Theileria parva* p104 gene sequences. Numbers at nodes represent percentage occurrence of clade in 1050 bootstrap replication of data. Sequences from this study are shown in bold font. The p104 gene of *T. lestoquardi* (KT989594) was used as an outgroup.

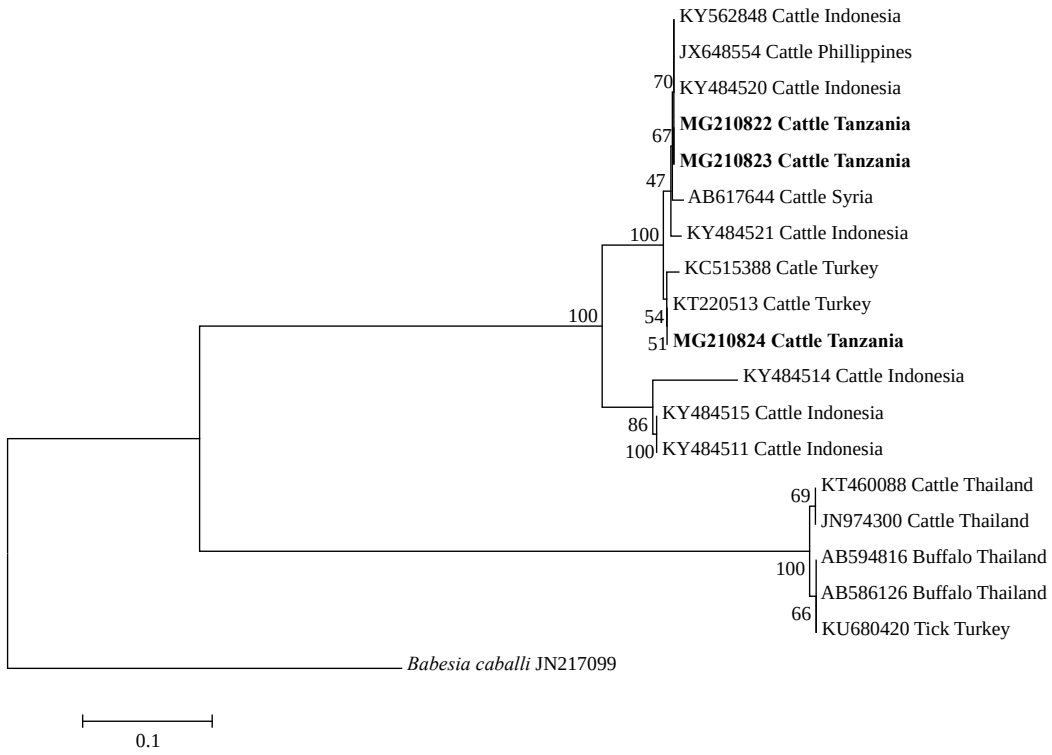

Figure 1.3 Phylogenetic analysis by neighbor joining method based on *Babesia bigemina* RAP-1a gene sequences. Number at the nodes represent percentage occurrence of clade in 1050 bootstrap replication of data. Sequences from this study are shown in bold font. The RAP-1a gene of *Babesia caballi* (JN217099) was used as outgroup.

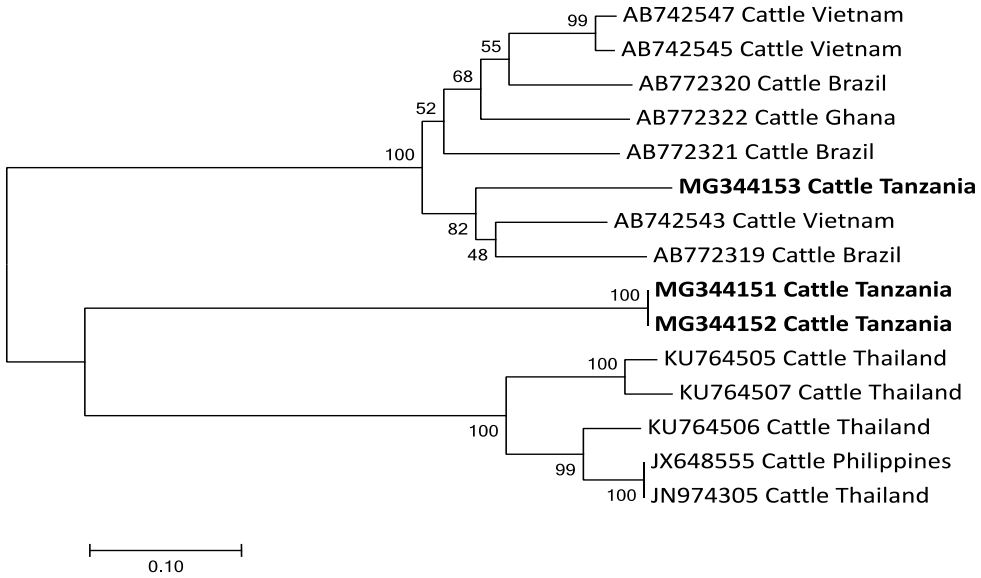

Figure 1.4 Phylogenetic analysis by neighbor joining method based on *Babesia bovis* SBP-2 gene sequences. Numbers at nodes represent percentage occurrence of clade in 1050 bootstrap replication of data. Sequences from this study are shown in bold font.

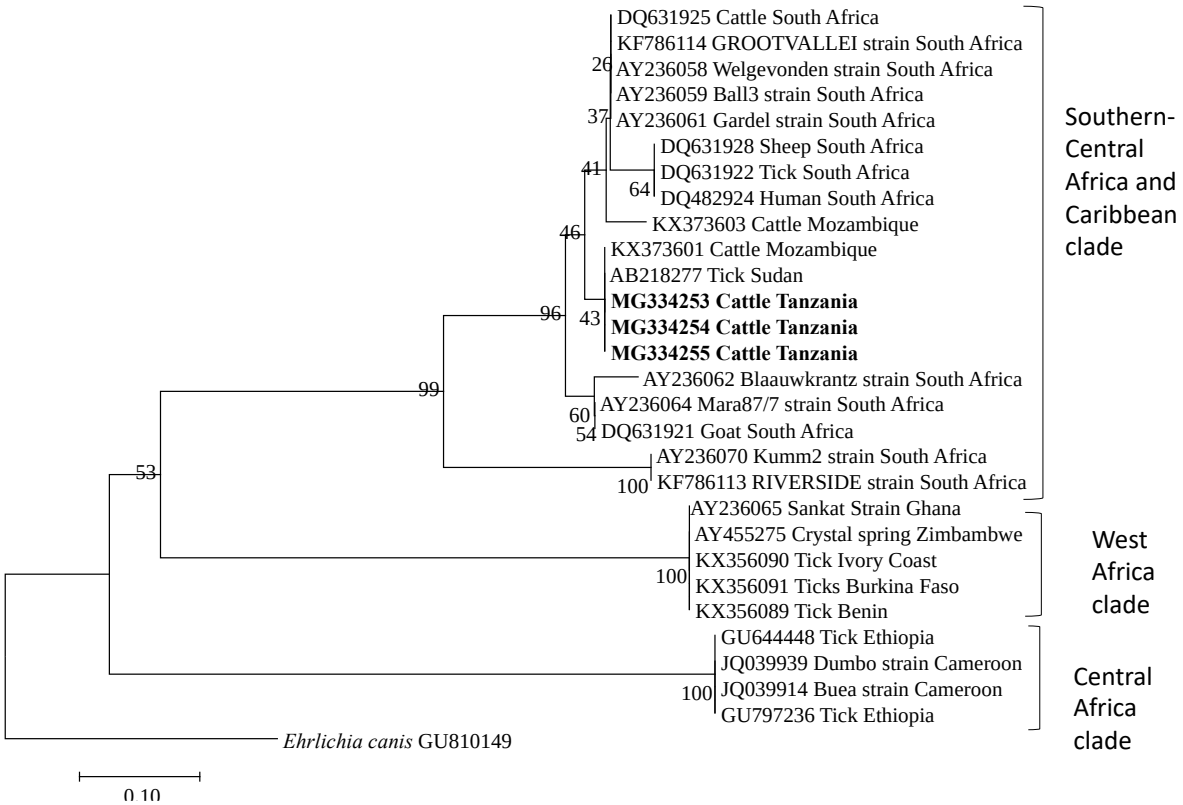

Figure 1.5 Phylogenetic analysis by neighbor joining method based on *Ehrlichia ruminantium* pCS20 gene sequences. Numbers at the nodes represent percentage occurrence of clade in 1050 bootstrap replication of data. Sequences from this study are shown in bold font. The pCS20 gene of *E. canis* (GU810149) was used as an outgroup.

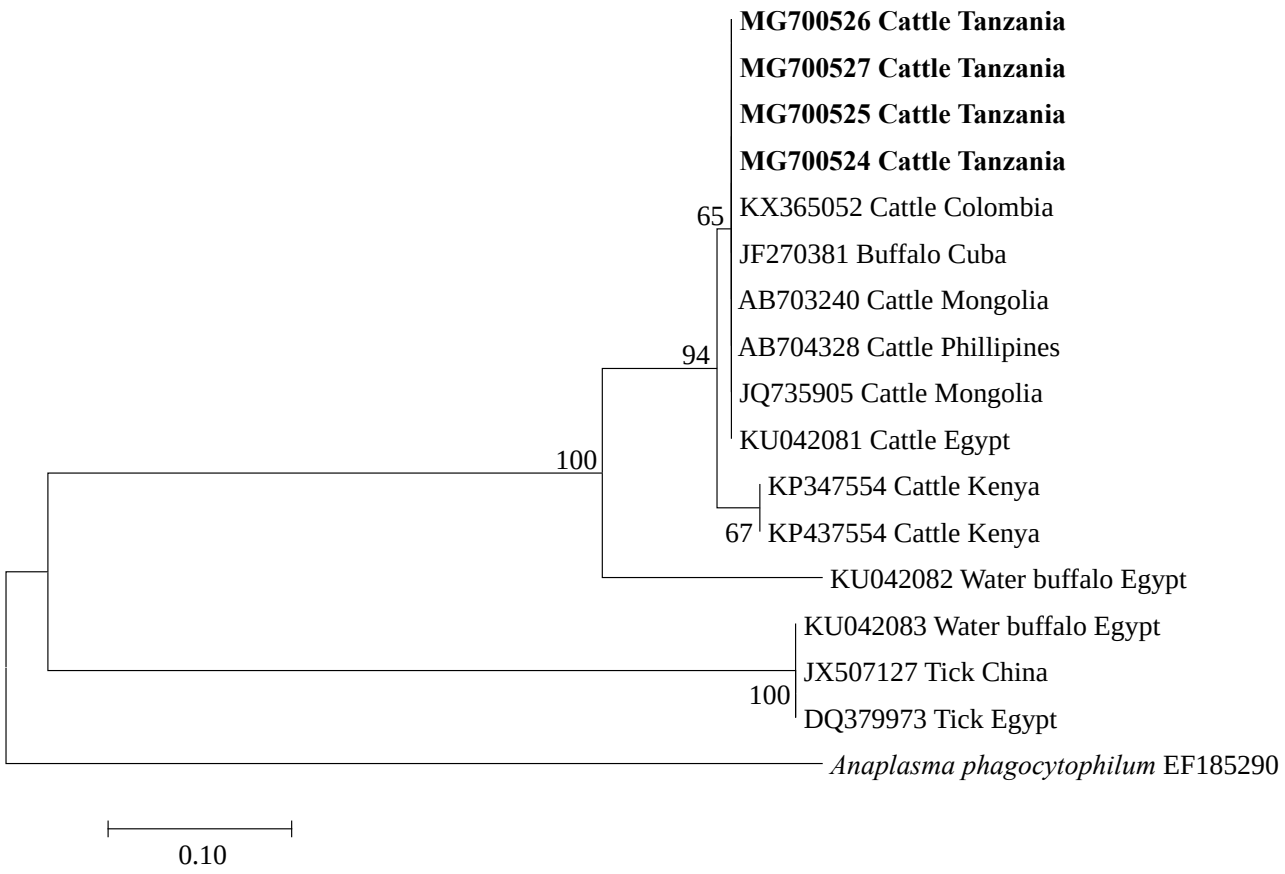

Figure 1.6 Phylogenetic analysis by neighbor joining method based on *Anaplasma marginale* MSP-5 gene sequences. Numbers at the nodes represent percentage occurrence of clade in 1050 bootstrap replication of data. Sequences from this study are shown in bold font. The MSP-5 gene of *A. phagocytophilum* (EF185290) was used as an outgroup.

Chapter 2

Molecular detection and characterization of tick-borne haemoparasites among cattle on Zanzibar Island, Tanzania

2.1 Introduction

In Tanzania, a country considered to be with the third largest population of cattle in Africa (Kivaria, 2006; Engida et al., 2015), ticks and tick-borne diseases pose great economic losses to the farmers. The losses are mainly due to production costs, mortalities, and the cost of veterinary diagnosis, treatment and tick control (Kivaria, 2006). Tick-transmitted diseases are mainly bovine theileriosis caused by *Theileria parva* and *T. taurotragi*, bovine anaplasmosis caused by *Anaplasma marginale*, bovine babesiosis caused by *Babesia bovis* and *B. bigemina* and bovine ehrlichiosis caused by *Ehrlichia ruminantium* (Hailemariam et al., 2017; Tembo et al., 2018).

In Zanzibar, cattle husbandry is of great importance, about 35% of agricultural households are engaged in livestock keeping especially cattle. Despite the significant number of households involved in livestock in Zanzibar, the sector contributes 5.9% of the country's gross domestic products (Engida et al., 2015). The low contribution of livestock sector to the country's economy has been associated among other factors with the diseases. Reports from the livestock sector in Zanzibar unveil that tick-borne diseases are estimated to contribute more than 70% of the total cattle deaths on the island. Despite the damage caused by tick-borne pathogens (TBPs) to the livestock sector in the country, studies on the distribution and genetic diversity of these pathogens are remarkably scarce. Therefore, the present study aimed to fill in the information gap by determining the occurrence, distribution and genetic diversity of the TBPs in Zanzibar, Tanzania. The results of this study are expected to provide important information that will serve as a scientific basis for the control strategies of tick-borne diseases in Tanzania.

2.2 Materials and methods

2.2.1 Study area

Zanzibar Island famously known as Unguja is located in the Indian ocean just about 35 km off the coast of Tanzania mainland (Fig. 2.1). The island is located at latitude -6º 09'50.18" S and longitude 39º 11'52.55" E. The size of the island is 1,666 km^2 with the highest peak of 123 meters above sea level. Zanzibar experiences a total annual rainfall of 1,600 mm, with long rainfall season starting from end of March to June and short rains in October to December. The climate is tropical sub-humid with annual average temperatures of 26℃.

2.2.2 Sample collection and genomic DNA isolation

A total of 236 blood samples were collected randomly from clinically healthy cattle in different farms. The samples were collected from the four districts of Zanzibar Island, Central (n = 77), North (n = 66), West (n = 52) and South (n = 41). The blood samples were collected in June and July 2019. The sampled animals were aged a year old and above. Different cattle breeds were sampled in different locations including the indigenous Tanzanian short horn zebu cattle and their cross breeds with Friesian, Ayrshire, Jersey and Brown Swiss. Approximately 3 - 5 ml of whole blood was taken from each selected animal by jugular venipuncture using sterile vacutainer needles and collected in the sterile vacutainer tubes coated with ethylenediaminetetraacetic acid (EDTA). The samples were transported to the lab in cool boxes to be refrigerated at 4°C for few days until DNA isolation was performed. Genomic DNA was extracted from 200 µl of blood sample using Qiagen blood DNA extraction kit (Qiagen, German), following the manufacturer's instructions and stored at -30°C until further analysis.

2.2.3 Ethical statement

Consent of sampling the animals was released by all cattle owners and the Ministry of Agriculture, Natural Resources, Livestock and Fisheries - Zanzibar (MANRLF) on a condition

that experienced, practicing veterinarians should draw blood from the animals and proper restraining procedures should be applied while collecting blood. All the required procedures were carried out based on ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Approval ID: 29 - 68).

2.2.4 Molecular detection of the tick-borne pathogens using species-specific primers

To detect the presence of *B. bigemina, B. bovis* and *A. marginale* nested PCR was used with species-specific assays (Table 1.1). The *Theileria* species group were screened using the genus specific 18S rRNA primers, the positive samples were sequenced to confirm the species. However, *T. parva* was screened again using species-specific primer p104. The reaction mixtures of the PCR and the thermocycling conditions were performed as described earlier in **chapter 1.**

2.2.5 Sequencing of the PCR positive samples

For genetic characterization five to eight of the PCR positive samples for *B. bigemina, B. bovis* and *A. marginale* were randomly selected and sequenced. Meanwhile, all *Theileria* positive samples were sequenced to confirm the species. The PCR amplicons were purified from the agarose gel by QIAquick Gel Extraction Kit (QIAGEN, Germany). The concentration of the PCR products was checked by NanoDrop 2000 spectrophotometer (Thermofisher, USA). All sequencing analysis assays were performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, USA). The sequences produced were analyzed by web-based software called mixed sequence reader to check for heterozygous base-calling like indels, Single Nucleotide Polymorphisms (SNPs) and short tandem repeats. The nucleotide sequence identities were obtained by performing GenBank BLASTn analysis with sequences on the GenBank database. The percentage identity between the nucleotide sequences were calculated using an online program called sequence manipulation suite (SMS).

2.2.6 Phylogenetic analysis

Genetic relatedness of *T. parva* (p104), *A. marginale (*MSP5), *B. bigemina* (RAP1a) and *B. bovis* (SBP-2) gene sequences of this study to those of the same gene deposited in the GenBank were performed by phylogenetic analysis, using MEGA version X (Kumar et al., 2018). The neighbour joining and maximum likelihood methods were used with a bootstrap analysis set at 1000 replicates.

2.2.7 Nucleotide sequences accession numbers

Gene sequences produced from this study were deposited in the GenBank database of the National Centre for Biotechnology Information (NCBI) using Bankit and the rRNA submission portal (submit.ncbi.nlm.nih.gov/subs/genbank/). The GenBank accession numbers obtained in this study are as follows; MN807306 - MN807311 for *B. bigemina* (RAP1a), MN807312 - MN807317 for *A. marginale* (MSP-5), MN807318 - MN807325 for *T. parva* (p104), MN814425 - MN814428 for *B. bovis* (SBP-2), MN726629 - MN726637 for *T. taurotragi* (18S rRNA), MN726645 - MN726650 for *T. mutans* (18S rRNA) and MN818575 - MN818578 for *T. velifera* (18S rRNA).

2.2.8 Statistical analysis

Pearson's chi-square ($\chi 2$) and Fisher's Exact test were used to statistically analyze the prevalence of the pathogens detected. The significance of co-infections was determined by Odds Ratio using MedCalc software. A p-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Overall prevalence

Out of 236 blood samples examined by PCR, 152 samples (64.5%) were positive by at least one of the seven pathogens detected (Table 2.2). The most frequently observed pathogens in this study were *Theileria mutans* 90 (38.1%), *T. parva* 81 (34.3%), *T. taurotragi* 73 (30.9%), *Anaplasma*

marginale 24 (10.2%), *Babesia bigemina* 12 (5.1%), *T. velifera* 8 (3.4%) and *B. bovis* 5 (2.1%) (Table 2.2). The North district showed higher infection rate of tick-borne pathogens in Zanzibar 47 (71%) followed by Central 52 (67%), West 34 (65%) and South 19 (46%) (Table 2.4).

2.3.2 Prevalence of mixed infections

A total of 86 (36.4%) animals were co-infected with two or more pathogens. In total 67 pathogens were involved in the co-infection. The pathogens co-infection ranged from double to quintuple co-infections. Double co-infections contributed majority of 41 (61.2%) of the total co-infections. The most frequent co-occurrences were the triple co-infection of *T. parva, T. taurotragi* and *T. mutans* followed by the double co-infections of *T. parva* and *T. taurotragi* as well as *T. parva* and *T. mutans* (Table 2.3).

2.3.3 Comparative sequence analyses of p104, RAP1a, MSP5 and SBP2 genes

The size of sequences of *T. parva, B. bigemina, A. marginale* and *B. bovis* from this study were 277bp, 412bp, 195bp and 580bp, respectively. The percentage nucleotide identity of all 8 *T. parva* p104 gene sequences (MN807318-MN807325) ranged from 99.64% to 100% among themselves. These sequences showed 100% nucleotide sequence identity with sequences from previous studies conducted in Tanzania (MG700531, MG700532 and MG210825) and Kenya (KP347566). Meanwhile, the percentage identity of all 6 *A. marginale* MSP-5 gene sequences (MN807312-MN807317) was 100% within themselves. These sequences showed 100% nucleotide sequence identity with sequences from Sri Lanka (LC467711) and India (MK834272). On the other hand, the percentage nucleotide identity of all six *B. bigemina* (RAP1a) gene sequences (MN807306-MN807311) ranged from 99.51% to 100%. These sequences showed 100% nucleotide identity with sequences from previous studies conducted in Tanzania (MG210822 and MG210823) and (MK481015) sequence from South Africa. Furthermore, the percentage nucleotide identity of all 4 *B. bovis* (SBP-2) gene sequences (MN814425-MN814428) ranged from 99.66% to 100%. However,

these sequences shared 99.66% nucleotide sequence identity with (MG725962) from previous study in Tanzania and 96.91% with (AB742545) sequence from Vietnam.

2.3.4 Phylogenetic analysis

In this study, phylogenetic trees of *T. parva*, *B. bovis*, *B. bigemina* and *A. marginale* were constructed based on p104, SBP-2, RAP-1a and MSP-5 genes respectively against sequences extracted from the NCBI GenBank. The two sequences MN807319 and MN807325 of *T. parva* clustered together on one clade on the phylogenetic tree, while the other sequence MN807321 appeared in a separate clade with sequences MG210826 and MG700532 from Tanzania and KP347566 Kenya (Fig. 2.2). On the other hand, all the four sequences MN814425 - MN814428 of *B. bovis* used in the phylogenetic tree of this study appeared in the same clade, which also involved sequence MG725962 from Tanzania (Fig. 2.4). Meanwhile, sequences MN807307 and MN807309 of *B. bigemina* generated in this study clustered together in the same clades with sequences MG210822 and MG210823 from Tanzania, KY484512 and KY484518 Indonesia and MH265105 isolated from water buffalo in Philippines, while the other two sequences of *B. bigemina* from this study appeared in a separate clade formed with other sequences from Tanzania (MG210824), Uganda (MG426198), Kenya (KP893330), Benin (KX685384) and Turkey (KC515387 and KC515388) (Fig. 2.3). Furthermore, phylogenetic analysis revealed that the four *A. marginale* sequences generated in this study were in the same clade with other sequences MG700524 and MG700527 from Tanzania, MF159030 Benin, MK481012 South Africa, MK164572 Thailand and LC467710 isolated from water buffalo in Sri Lanka (Fig. 2.5).

2.4 Discussion

In this study, I used PCR assays to perform molecular survey of tick-transmitted pathogens on blood samples of cattle collected from Zanzibar Island, Tanzania. The findings revealed that *T.*

parva, *T. mutans*, *T. taurotragi*, *T. velifera*, *B. bigemina*, *B. bovis* and *A. marginale* occur in the island. Remarkably, high rate of co-infections was observed among the detected pathogens.

In the present study, theileriosis caused by haemoprotozoan parasites, namely, *T. parva* (34.3%), *T. taurotragi* (30.9%), *T. mutans* (38.1%) and *T. velifera* (3.4%) showed relatively higher prevalence than the other detected pathogens.

East Coast Fever (ECF) caused by *T. parva* is the most economically important tick-borne disease (TBD) in Tanzania causing high morbidity and mortality in cattle (Swai et al., 2009). The prevalence of *T. parva* (34.3%) in this study, supports the previous studies conducted elsewhere in the region. Studies in Tanzania (Swai et al., 2009), Kenya (Njiiri et al., 2015) and Uganda (Tayebwa et al., 2018) reported similar results. The relatively high prevalence of *T. parva* could be due to the ability of Tanzanian Short Horn Zebu (TSHZ) cattle to tolerate the infection caused by this pathogen in the study area. The TSHZ cattle are in higher proportion in the sampled animals can carry the pathogen for longer periods of time and will continuously infect ticks and subsequently other cattle (Laisser et al., 2017). Moreover, the indigenous breeds (TSHZ) are fairly resistant to ECF due to endemic stability and can carry the pathogen for prolonged periods of time without developing clinical signs of the disease (Kariuki et al., 1995). In phylogenetic analyses, the p104 gene sequences of *T. parva* from this study appeared in different clades, which suggests that different genotypes of *T. parva* are circulating in the cattle population of the Zanzibar Island.

Cattle infected by *T. taurotragi* develop signs which are relatively similar to ECF, and can easily be confused by ECF clinically. The higher prevalence (30.9%) of *T. taurotragi* is consistent with the results I reported in a neighbouring island of Pemba suggesting the two islands have favourable environment for the multiplication of the pathogen and its vector *Rhipicephalus appendiculatus.*

T. mutans is known to be less pathogenic in cattle although there are some reported strains in Eastern Africa which cause severe anaemia, icterus, enlarged lymph nodes, weight loss and sometimes death (Paling et al., 1981; Moll et al., 1986). In the present study, *T. mutans* had the highest infection rate (38.1%), which is similar to results of previous studies conducted in east and central Africa regions including Uganda (Byaruhanga et al., 2016), Kenya (Njiiri et al., 2015) and Zambia (Simuunza et al., 2011). The higher prevalence of *T. mutans* in the present study, could be due to the ability of host to carry the pathogen for long periods of time without showing clinical signs as reported by (Asiimwe et al., 2013). Presumably, the infection is acquired by calves early in life after which the calves remain life-long carriers (Hailemariam et al., 2017).

The *T. velifera* is a non-pathogenic parasite of cattle. In this study, it recorded low prevalence (3.4%) compared to the previous studies in the region reported in Kenya (Njiiri et al., 2015), Zambia (Tembo et al., 2018), Ethiopia (Gebrekidan et al., 2014) and Uganda (Oura et al., 2004). The lower prevalence of this pathogen in this study suggests that there is low key distribution of this pathogen in Zanzibar. However, to confirm this hypothesis further investigation needs to be done in the study area. The vector of this pathogen, *A. variegatum* is well distributed in Tanzania (Mamiro et al., 2016; Kerario et al., 2017).

In this study, the prevalence of *B. bigemina* was 5.1%, this is lower compared to the previous studies conducted in the region. In Tanzania (Swai et al., 2007) and Kenya (Adjou Moumouni et al., 2015) studies reported relatively higher prevalence. The lower prevalence of this pathogen in this study could be due to higher temperatures experienced in Zanzibar Island in the long periods of the year. The higher temperatures have been reported by (Kocan, 1995) to inhibit and eventually eliminate *B. bigemina* in ticks. Previous studies conducted in the drier areas of north-eastern Uganda (Byaruhanga et al., 2016) and in Zambia during the dry season by (Simuunza et al., 2011) supports this hypothesis by reporting low prevalence of this pathogen. The

phylogenetic analyses of the RAP-1a gene sequences of *B. bigemina* showed that isolates from this study were found in different clades, which suggests the presence of different genotypes of *B. bigemina* strains in Zanzibar Island. On the other hand, *B. bovis* recorded the lowest prevalence (2.1%) in this study, the results are consistent with the previous studies in the region reported in Ngong-Kenya (Adjou Moumouni et al., 2015) and Zambia (Simuunza et al., 2011). These findings suggest that there is low key distribution of the pathogen in the region rather than the presence and distribution of the vector. However, *R. microplus,* a vector of this pathogen is well distributed in Tanzania (Mamiro et al., 2016). In the phylogenetic analyses, the SBP-2 gene sequences of *B. bovis* from this study appeared in a single clade, which suggest that the gene is conserved in the study area.

Bovine anaplasmosis caused by a bacterium *Anaplasma marginale* is an important tick-borne disease of cattle, the parasite is more pathogenic to naïve exotic breeds of cattle (Hailemariam et al., 2017). This study reports a prevalence of 10.2% which is lower compared to similar studies in Tanzania (Swai et al., 2007), Kenya (Gachohi et al., 2010) and Uganda (Kabi, 2008). This could presumably be due to the inherent resistant nature of the indigenous zebu cattle to the vector ticks (Swai et al., 2007). Therefore, the pathogen is poorly transmitted from ticks to the indigenous zebu cattle, considering that, in this study zebu cattle were in higher proportion. The phylogenetic tree of MSP-5 gene sequences of *A. marginale* revealed that all 5 isolates of this study appear in a single clade. This finding suggests that MSP-5 gene of *A. marginale* is conserved within Tanzania.

Remarkably, in the present study, co-infections were the common finding. Mixed infections were detected in 86 (36.4%) animals and overall, 67 different pathogen combinations were involved in co-infection. The higher rate of mixed infection in this study is in correlation with the previous study I conducted in a neighbouring island of Pemba, in Tanzania. The most frequent co-

occurrence in the present study was recorded for *T. parva*, *T. taurotragi* and *T. mutans*. Coincidentally, the same pathogens had higher co-infection rates in the study conducted in Pemba. These pathogens were the most frequent encountered in both studies. The higher prevalence of these pathogens and their co-infections in these two studies could be due to a favourable environmental climate and vegetation in both islands for the multiplication of *R. appendiculatus* a vector for *T. parva* and *T. taurotragi*, and *A. variegatum* for *T. mutans*. Additionally, the mild and non-pathogenic *Theileria* species like *T. mutans* and *T. taurotragi* can be carried by the indigenous cattle (TSHZ) for prolonged period of time in the study areas. Consequently, the presence of these pathogens in TSHZ will potentially trigger their immune system resulting in development of immunity against *Theileria* spp., which can protect them against a more pathogenic *Theileria* spp. like *T. parva* reducing the severity of the disease. This phenomenon is termed heterologous protection (Woolhouse et al., 2015). However, mixed infections between different tick-borne pathogens can be much more complex and involve ecological, epidemiological and clinical aspect of the host animals (Baneth, 2014; Diuk-Wasser et al., 2016; Hailemariam et al., 2017). Generally, mixed infections in a host may increase or decrease the pathogenicity of existing infections. Notably, the animals that are co-infected can develop different patterns of clinical symptoms that deviates from a typical pattern of a disease in an animal infected by single pathogen hence leading to incorrect diagnosis and eventually treatment failure.

In conclusion, this study revealed a relatively higher prevalence of *Theileria* species (*T. parva*, *T. taurotragi* and *T. mutans*) compared to the other detected pathogens (*B. bigemina*, *B. bovis* and *A. marginale*). There was low genetic diversity of the detected tick-borne pathogens. Mixed infections were common, with *Theileria* species highly involved in co-infections. The epidemiological findings revealed in this study will provide important information on tick-borne diseases in Tanzania and will be used as scientific basis for planning future control strategies.

2.5 Summary

This study was performed to investigate the occurrence and species composition of bovine TBPs in cattle kept on Zanzibar Island. A total of 236 blood samples were randomly collected in cattle population in June and July, 2019. I used Polymerase Chain Reaction (PCR) and gene sequencing to detect and identify pathogens. PCR screening of all 236 samples revealed that 64.5% of animals were infected by TBPs, including *T. mutans* (38.1%), *Theileria parva* (34.3%), *T. taurotragi* (30.9%), *Anaplasma marginale* (10.2%), *Babesia bigemina* (5.1%), *T. velifera* (3.4%) and *B. bovis* (2.1%). Overall, a total of 86 animals (36.4%) were co-infected with two up to five pathogens. The pathogens mostly involved in the co-infection were *T. parva*, *T. taurotragi* and *T. mutans*. Sequence analysis indicated that *T. parva* p104 and *B. bigemina* RAP1a genes are diverse among the sampled animals in Zanzibar Island, with 99.64%–100% and 99.51%–100% nucleotide sequence identity value respectively. In contrast, the *A. marginale* MSP-5 and *B. bovis* SBP-2 genes are conserved, with 100% and 99.66%-100% nucleotide sequence identity values respectively. The phylogenetic analyses revealed that *T. parva* p104 and *B. bigemina* RAP-1a gene sequences showed significant differences of genotypes, as they appear in different clades. Meanwhile, *A. marginale* MSP-5 and *B. bovis* SBP-2 gene sequences appear in the same clade with other sequences extracted from the NCBI GenBank. The epidemiological findings revealed in this study will provide important information on tick-borne diseases in Tanzania and will be used as scientific basis for planning future control strategies.

Table 2.2 Prevalence of detected pathogens.

Pathogen	Central district	South district	North district	West district	Overall
T. parva	25 (32.5%)	10 (24.4%)	25 (37.9%)	21 (40.4%)	81 (34.3%)
T. taurotragi	38 (49.4)	9 (21.9%)	18 (27.3%)	8 (15.4%)	73 (30.9%)
T. mutans	33 (42.9%)	11 (26.8%)	40 (60.6%)	16 (30.8%)	90 (38.1%)
T. velifera	0 (0)	2 (4.9%)	5 (7.8%)	1 (1.9%)	8 (3.4%)
B. bovis	0 (0)	0 (0%)	3 (4.6%)	2 (3.9%)	5 (2.1%)
B. bigemina	8 (10.4%)	0 (0%)	2 (3.1%)	2 (3.9%)	12 (5.1%)
A. marginale	15 (19.5%)	5 (12.2%)	3 (4.6%)	1 (1.9%)	24 (10.2%)

Table 2.3 Observed level, frequency and number of species combination and involved in co-infections.

Level of co-infections	Frequency	%	No. of species combination	No. of pathogens involved
Double	41	47.7	11	22
Triple	36	41.9	8	24
Quadruple	8	9.3	4	16
Quintuple	1	1.2	1	5
Overall	86	100	24	67

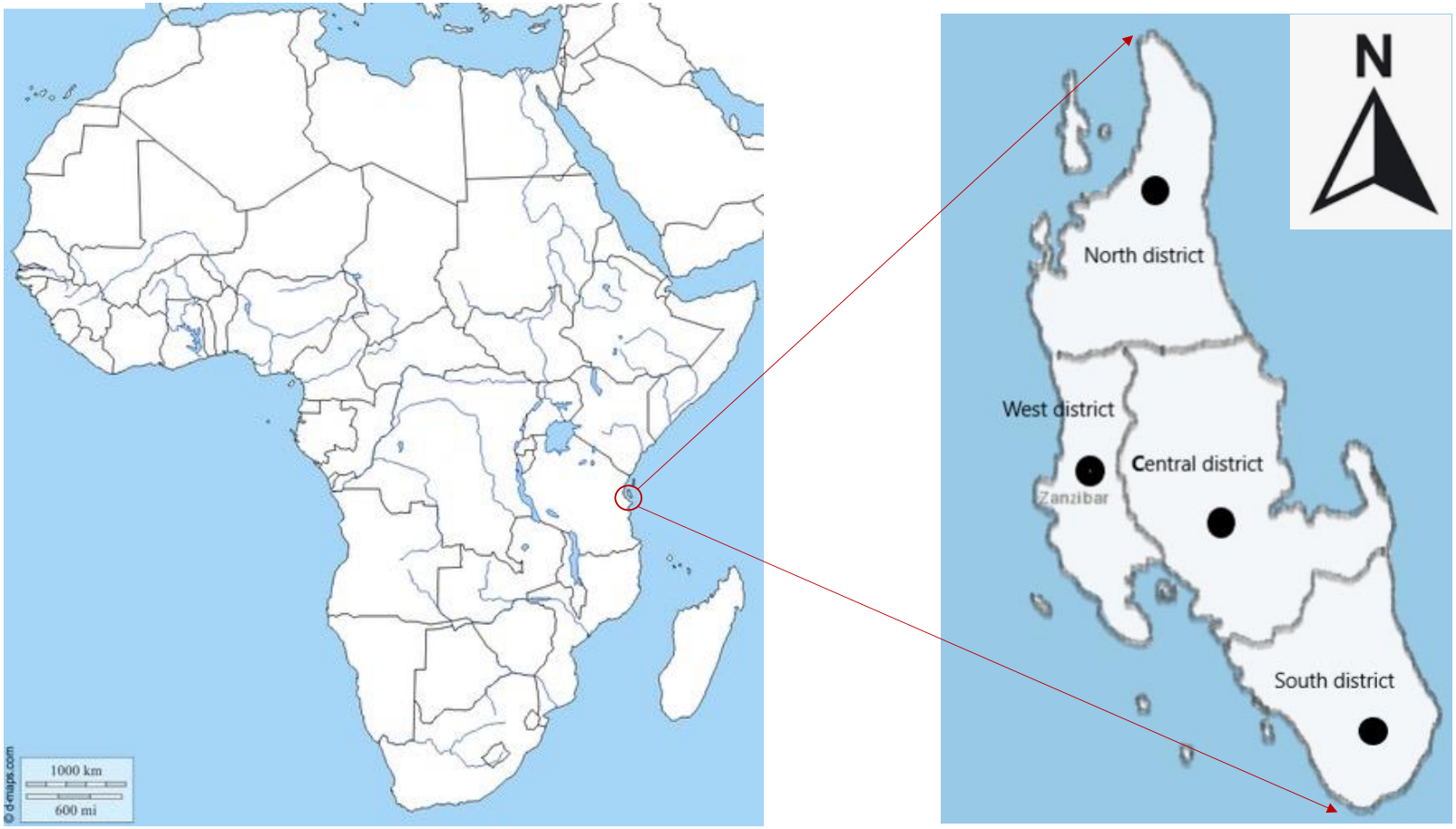

Figure 2.1 Map of Zanzibar Island, Tanzania showing the four districts (North, West, Central and South) where samples were collected.

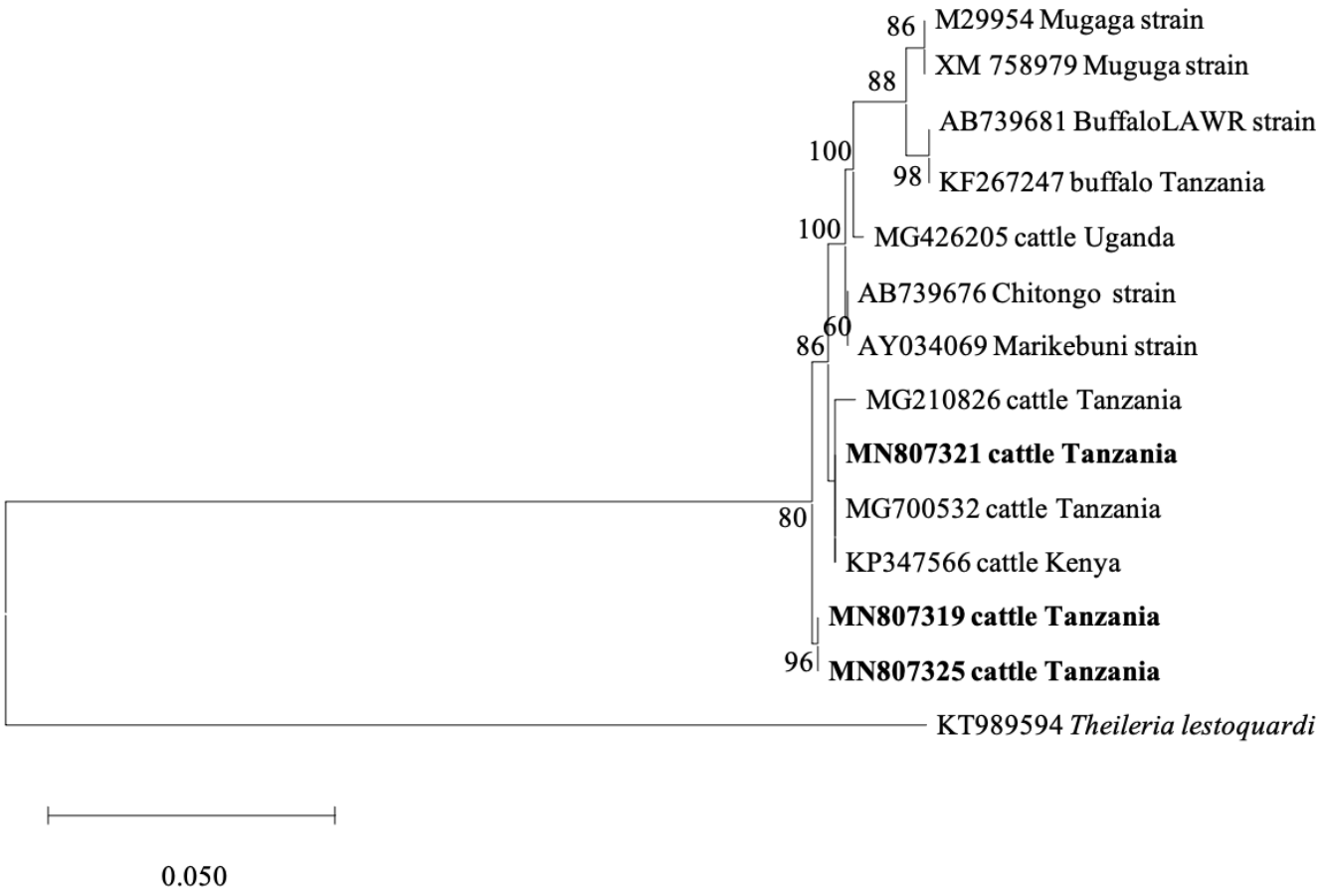

Figure 2.2 Phylogenetic analysis of *Theileria parva* identified in this study based on p104 gene sequences using neighbour joining method. The number at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in bold font. The p104 gene of *Theileria lestoquardi* (KT989594) was used as an outgroup.

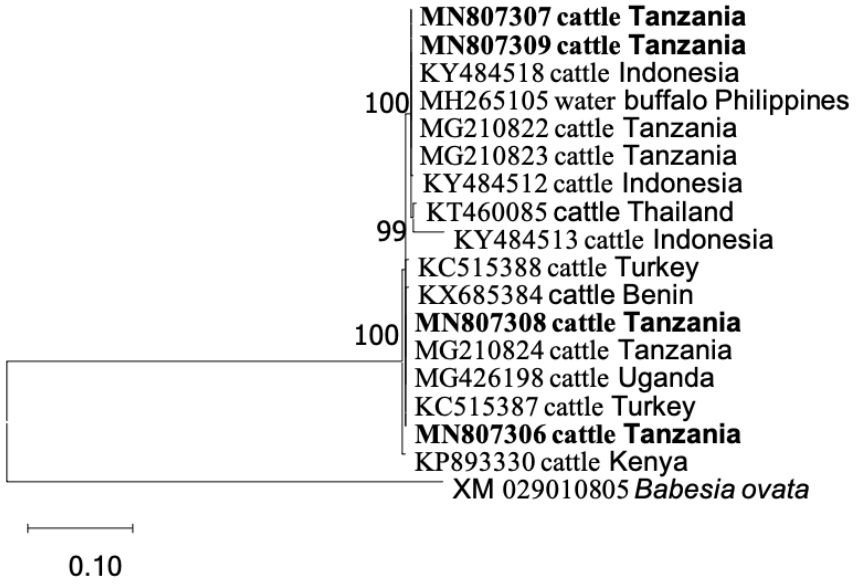

Figure 2.3 Phylogenetic analysis of *Babesia bigemina* identified in this study based on RAP-1a gene sequences using neighbour joining method. The number at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in bold font. The RAP-1a gene of *Babesia ovata* (XM029010805) was used as an outgroup.

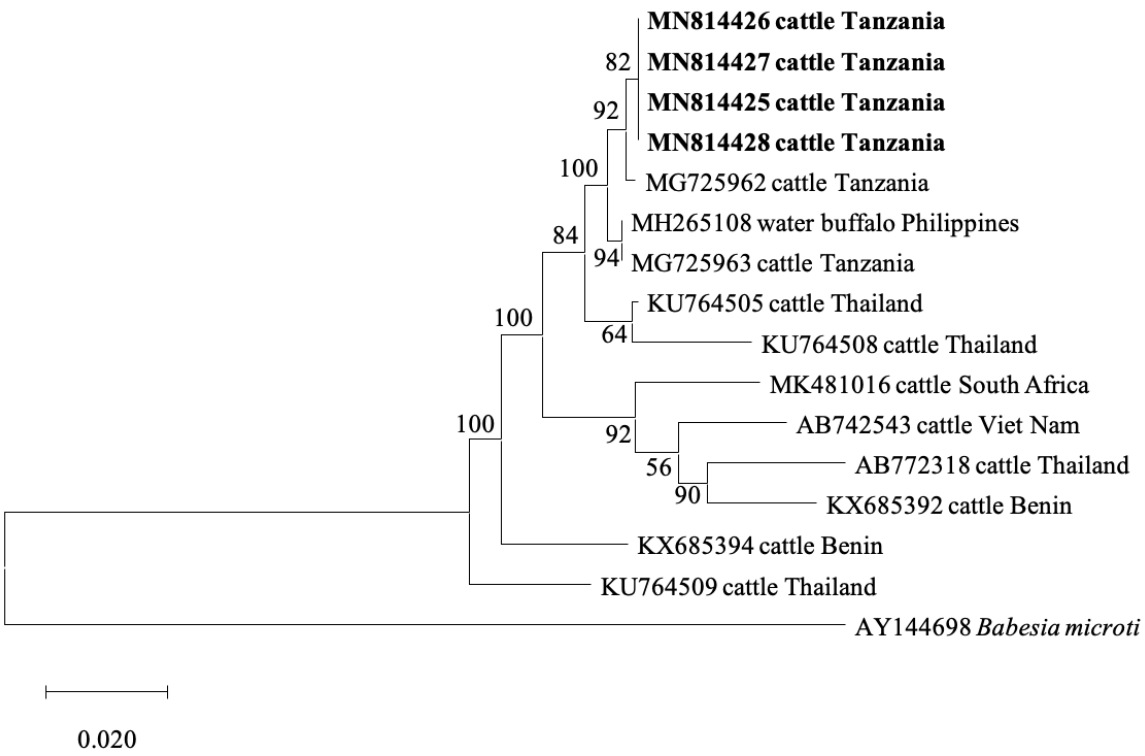

Figure 2.4 Phylogenetic analysis of *Babesia bovis* identified in this study based on SBP-2 gene sequences using maximum likelihood method. The number at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in bold font. The SBP-2 gene of *Babesia microti* (AY144698) was used as an outgroup.

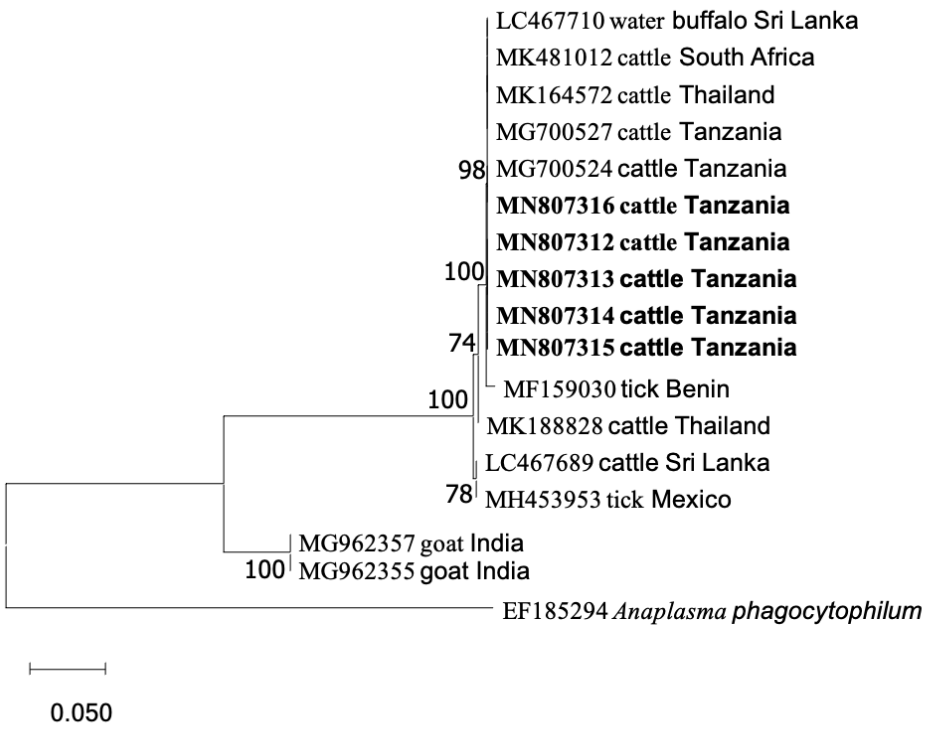

Figure 2.5 Phylogenetic analysis of *Anaplasma marginale* identified in this study based on MSP-5 gene sequences using maximum likelihood method. The number at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in bold font. The MSP-5 gene of *Anaplasma phagocytophilum* (EF185294) was used as an outgroup.

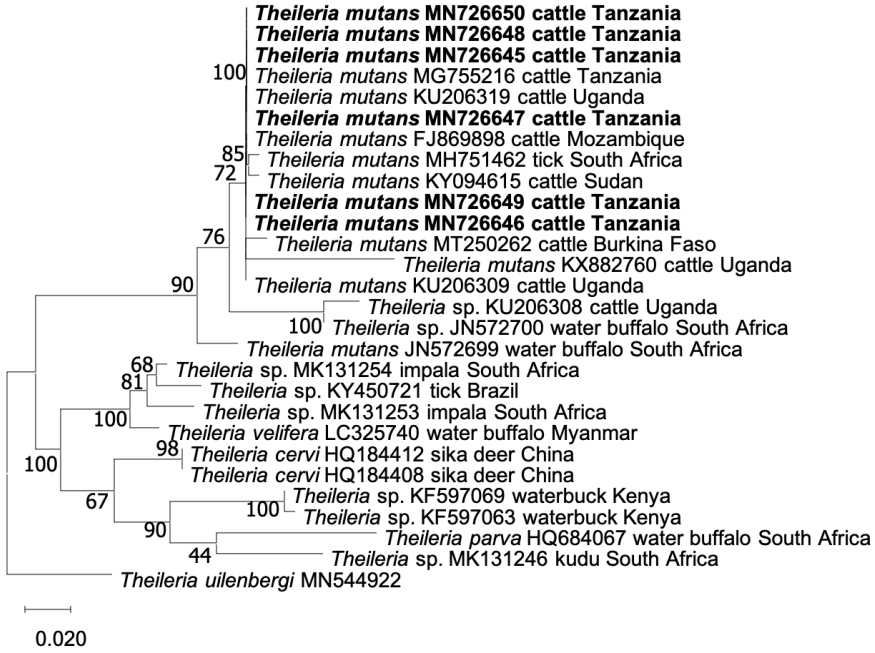

Figure 2.6 Phylogenetic analysis of *Theileria mutans* identified in this study based on 18S rRNA gene sequences using maximum likelihood method. The number at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in bold font. The 18S rRNA gene of *Theileria uilenbergi* (MN544922) was used as an outgroup.

Chapter 3

Molecular investigation of tick-borne haemoparasites isolated from indigenous zebu cattle in Tanga region, Tanzania

3.1 Introduction

The pastoral communities in sub-Saharan Africa practice a livestock production system that exposes cattle and other livestock to ticks and tick-borne diseases (TBDs) (Kasozi et al., 2014; Byaruhanga et al., 2016). In Tanzania, the indigenous zebu cattle (*Bos indicus*), accounted for more than 96% of the total cattle population in the country. Most of these are owned by pastoralists (Kurwijila et al., 2012). These animals are continuously moved from one point to another in search of pastures and water. Consequently, these animals are continuously exposed to ticks and tick-transmitted pathogens. Under these circumstances, farmers are subjected to losses attributed to TBDs, which include production losses, mortality, and the veterinary cost for diagnosis, treatment, and control of TBDs (Homewood et al., 2006; Kivaria, 2006; Ocaido et al., 2009). Given the importance of cattle husbandry in Tanzania, coupled with more than 70% of the human population engaged in the livestock sector (Engida et al., 2015) with a large proportion of cattle (close to 96%) owned by pastoralists and agro-pastoralists (Kurwijila et al., 2012). A community which practices a free-range system, moving their animals from one location to the other in search of pasture and water. The pastoral communities mostly keep the indigenous zebu cattle and these animals are continuously exposed to different species of ticks in the pastures (Ndagala et al., 1990). The main challenge of the pastoralists apart from the shortage of pastures and water is diseases. In Tanzania, 56% of the annually reported mortalities of cattle are caused by tick-borne diseases (Kivaria, 2006; Swai et al., 2009).

Tanga region is among a few regions in Tanzania that has a large open land which is semi-arid and is practically used by the pastoralists as pastures of their livestock. Large groups of indigenous zebu cattle are grazed in the semi-arid land of Handeni, Kilindi and Korogwe districts.

Despite the importance of livestock TBDs in the country, information on the epidemiology of these diseases and the genetic composition of the causative agents in Tanzania is limited. Therefore, this study aimed to investigate the occurrence and genetic diversity of bovine tick-borne

pathogens of veterinary significance in the indigenous zebu cattle of northeastern coast of Tanzania.

3.2 Materials and methods

3.2.1 Study area

This study was conducted in the Tanga region, which is located on the northeast coast of Tanzania. The region covers 26,680 km^2, about 2% of the size of Tanzania. Tanga region lies between latitude 4.965088° S and 5.5743° S and longitude 38.2744° E and 38.7787° E (Figure 3.1). The sample collection sites are located in the Handeni district, where the topography is characterized by low lands and rises to the average elevation of 696 m. The district is characterized by short vegetation towards the inlands which also becomes semi-arid and dry. Handeni has an annual precipitation of 600 mm which begins from April to May, followed by a dry season to December. From January to March, the area is cool with some occasional precipitation. The average temperature is between 26℃ to 32℃. The district is composed of 21 wards.

3.2.2 Sample collection and DNA extraction

A total of 250 blood samples were collected randomly from apparently healthy indigenous zebu cattle in five herds located in different wards of Handeni district: Kwamsisi (n=51), Komkonga (n=49), Kabuku (n=50), Kwamatuku (n=50), and Kwachaga (n=50) (Figure 1). Of the sampled animals, 184 were female cattle and 66 were males. Samples were collected between May and June 2019. The five herds were managed under extensive grazing on communal land by pastoralists. Each herd consisted of approximately about two hundred cattle. For each animal, approximately 4 ml of blood was collected from the jugular vein using a sterile vacutainer needle and collected in vacutainer tubes (BD Biosciences, USA) coated with ethylenediaminetetraacetic acid (EDTA). We targeted cattle of one and a half years old and above. Animal age was obtained through farmer's records and by using the horns of the sampled animals. Samples were temporarily stored in cool boxes in the field before being shifted to the laboratory for refrigeration at 4℃. DNA was extracted from 200 µl of whole blood using QIAamp DNA Blood Mini Kit (Qiagen, German) following the manufacturer's protocol, and stored at -80℃ until the time of laboratory screening of pathogens.

3.2.3 Ethical statement

The permission of sampling the animals was granted by the Ministry of Livestock and Fisheries-Tanzania, and all the farmers were informed about the importance of the study and gave their consent, under the condition that blood should be drawn by experienced veterinarians and proper restraint procedures should be applied to avoid any injuries to their animals. All the required procedures for sample collection were carried out based on the ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (Animal experiment permit no. 20 - 128).

3.2.4 Molecular detection of tick-borne pathogens

DNA samples were screened for *B. bigemina*, *B. bovis*, *A. marginale*, and *E. ruminantium* by nested PCR with species-specific primer assays (Table 1.1). *Theileria* spp. were screened using the genus-specific 18S rRNA primers. Thereafter, the *Theileria* genus positive samples were screened using the *Theileria* species-specific primer assays. PCR composition and volume were performed as described in **chapter 1.**

3.2.5 Sequencing of the PCR-positive samples

We randomly selected 5 – 10 positive samples (approximately 10%) from each detected pathogen for sequencing. The amplicons were extracted from the agarose gel by QIAquick Gel Extraction Kit (Qiagen, German). The concentration of each extracted PCR product was checked by NanoDrop 2000 spectrophotometer (Thermofisher, USA). The Big Dye terminator cycle sequencing kit (Applied Biosystems, USA) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA) were used to perform all sequencing assays. The produced sequence reads were checked and analyzed by the web-based software named Mixed Sequence Reader (MSR) (http://MSR.cs.nthu.edu.tw/), to clean them for heterozygous base calling such as indels, tandem repeats, and single nucleotide polymorphism (SNIP). The sequence reads for each sample were trimmed and assembled by SeqMan Pro software from DNASTAR Lasergene to get the consensus sequence for each selected positive sample. GenBank BLASTn analysis was used to check the sequence identities with sequences previously deposited in the GenBank database. The sequences of the same pathogens were checked for percentage identity by using an online software tool called Sequence Identity and Similarity (SIAS).

3.2.6 Phylogenetic analysis

Gene sequences of *T. parva* (p104), *B. bigemina* (RAP-1a), *A. marginale* (groEL), and *T. mutans* and *T. taurotragi* (V4 region of the 18S rRNA) obtained in this study and those deposited

in the GenBank database reported from previous studies were used for phylogenetic analysis, using MEGA version XI (Tamura et al., 2021). Maximum likelihood and neighbor-joining methods were used with the bootstrap set at 1,000 replicates.

3.2.7 Nucleotide sequence accession numbers

Gene sequences generated from the present study were deposited in the Genbank database of the National Center for Biotechnology Information (NCBI) using BankIt for genomic DNA sequences and the ribosomal RNA submission portal (submit.ncbi.nlm.nih.gov/subs/genbank/) for the ribosomal RNA sequences. The GenBank accession numbers assigned to the sequences of this study are as follows: OP390271 – OP390278 for *T. parva* p104, OP390279 – OP390284 for *B. bigemina* RAP-1a, OP414689 – OP414693 for *A. marginale* groEL, OP379365 – OP379368 for *T. mutans* V4 region of the 18S rRNA, OP380376 – OP380382 for *T. taurotragi* V4 region of the 18S rRNA.

3.2.8 Statistical analysis

The prevalence of the detected pathogens was statistically analyzed by Pearson's chi-square (X^2) and Fisher's exact test and the significance of the co-infections was determined by odds-ratio calculation using MedCalc software. A *p*-value <0.05 was considered statistically significant.

3.3 Results

3.3.1 Overall infection rate

Out of the 250 cattle screened by PCR, 216 cattle (86.4%) were positive for at least one of the five detected pathogens, while 34 cattle (13.6%) were not infected by any of the screened pathogens (Table 3.3). The detected pathogens in this study were *T. mutans* (48%; 120/250), *A. marginale* (32.4%), *T. parva* (25.6%), *T. taurotragi* (20.8%), and *B. bigemina* (13.2%) (Table 3.2). Based on animal sex, 58 males (87.9%) and 153 females (83.2%) were infected by one or more of the screened pathogens (Table 3.4). There were no significant differences observed based on sex (Table 3.4). The overall prevalence based on location is indicated in Table 3.2. There were no significant differences in infection rates based on the five locations (Table 3.2). *B. bovis* and *E. ruminantium* were not detected in this study.

3.3.2 Co-infection analysis

The proportion of cattle in which two or more pathogen species were detected is referred to as co-infections. Out of 250 cattle in this study, 112 cattle (44.8%) were found to be simultaneously

infected with two or more pathogens. Overall, 48 concurrent infections were observed in this study (Table 3.3). The pathogen co-infections ranged from double to quadruple (Table 3.3). The double co-infection (79.5%) contributed the majority of the pathogens involved in the co-infections, followed by the triple co-infection (18.8%) and there were two cases (1.8%) of quadruple co-infections (Table 3.5). The most frequent combinations of co-infection were *T. mutans* + *A. marginale* (18), followed by *T. mutans* + *T. parva* (17), and *T. mutans* + *T. taurotragi* (14) (Table 3). The co-infections based on location showed that Kwachaga had the highest prevalence (54%), followed by Kwamsisi (50.9%), Komkonga (42.9%) while Kabuku and Kwamatuku both had 19 (38%) (Table 3.3). There were no significant differences in co-infection rates based on location.

3.3.3 Comparative gene sequence analyses

The sequences of each detected pathogen were compared for nucleotide identity among themselves and with the corresponding sequences isolated previously in Tanzania or neighboring countries. The percent nucleotide identities of eight *T. parva* p104 gene sequences (OP390271 – OP390278) ranged from 98.92% – 100% with each other. In addition, these sequences showed percent identity values of 98.20% – 99.28% with sequences MG700532, MG700532, MG210825, and MN807320 obtained previously in Tanzania. Meanwhile, the shared percent nucleotide identity values of six *B. bigemina* RAP-1a gene sequences (OP390279 – OP390284) obtained in this study ranged from 99.27% – 100%. These sequences also showed an identity value of 99.51% with sequences MG210822 – MG210824 obtained from previous studies conducted in Tanzania. Interestingly, these sequences were more identical (99.76%) with sequences MG426199 and KP347559 from Uganda and Kenya, respectively. For *A. marginale*, the nucleotide identity values of five groEL gene sequences (OP414689 – OP414693) showed high identity values of 99.88% to 100%. However, these sequences showed a 99.10% identity value with sequences KY523020 – KY523026 from Uganda. Furthermore, sequences (OP379365 – OP379368) of the 18S rRNA gene of *T. mutans* showed low percentage identity values ranging from 22.45% to 60.77% among them, however, they showed higher identity values of up to 98.85% with sequences MN726645, MN726648, MN726650 and MG755217 isolated previously in Tanzania. Finally, the 18S rRNA gene sequences (OP380376 – OP380382) of *T. taurotragi* showed high diversity between them by identity values of 21.81% to 94.65%. Surprisingly, these sequences showed a high identity value (100%) with sequences MN726630, MN726631, MN726635, and MG755215 isolated in previous studies in Tanzania.

3.3.4 Phylogenetic analysis

In this study, phylogenetic trees of *T. parva*, *B. bigemina*, *A. marginale,* and *T. mutans* were constructed based on their respective genes (p104, RAP-1a, groEL, and V4 region of 18S rRNA) together with sequences extracted from the NCBI GenBank database. All sequences of the p104 gene of *T. parva* were clustered together on a phylogenetic tree (Figure 3.2). Notably, sequence MN810050 from Uganda and KP347564 from Kenya showed a close relationship with the clade that contains sequences of this study (Figure 3.2). For *B. bigemina*, the RAP-1a gene sequences OP390281 and OP390279 of this study appeared in the same clade together with sequences KY484520 from Indonesia, MK481015 from South Africa, and MG210822 – MG210823 from Tanzania. The other three sequences OP390280, OP390283, and OP390282 of this study appeared in a different clade with sequences MN870655 from Egypt, MN807306 from Tanzania, and MG426198 from Uganda (Figure 3.3). Moreover, all *A. marginale* sequences OP414690 – OP414693 of this study appeared in a single clade on a phylogenetic tree plus a sequence KC113455 from the Philippines (Figure 3.4). For *T. mutans*, the V4 region of 18S rRNA gene sequences OP379365 and OP379366 of this study formed a clade of their own, while the other OP379367 appeared isolated from the other sequences in the tree. Moreover, sequence OP379368.

3.4 Discussion

This study demonstrates a high prevalence of tick-borne pathogen infections among indigenous zebu cattle in the pastoral community of the Tanga region, Tanzania. However, some of the detected pathogens showed some degrees of diversity. Our findings revealed that *T. mutans, T. parva*, *T. taurotragi*, *B. bigemina,* and *A. marginale* were present in the sampled animals. Remarkably, high rates of co-infections were observed in the sampled cattle.

The proportion of cattle infected with *T. mutans* in this study was higher compared to other detected pathogens. The prevalence of *T. mutans*, a pathogen transmitted by the *Am. variegatum* tick is in agreement with previous studies conducted in Tanzania (Swai et al., 2005) and neighboring countries of Malawi (Chatanga et al., 2022), Zambia (Simuunza et al., 2011; Tembo et al., 2018), Uganda (Byaruhanga et al., 2016), and Kenya (Njiiri et al., 2015). The higher prevalence of this pathogen in this study can be explained by the fact that in endemic areas, cattle can acquire these pathogens and carry them for long periods without manifesting clinical symptoms (Asiimwe et al., 2013). Presumably, Hailemariam et al., (2017) reported that calves

acquire the infection when young (5 to 6 months) in endemic areas and they remain lifelong carriers of the disease whereby they continuously infect ticks and subsequently cause new infections in cattle. The other possibility could be that the *Am. variegatum* ticks reported in Tanzania (Lynen et al., 2007; Mamiro et al., 2016; Kerario et al., 2017) could be widely distributed and efficiently transmit the pathogen.

Importantly, some strains of *T. mutans* have been reported to be pathogenic and cause severe anemia, icterus, enlarged lymph nodes, weight loss, and sometimes death (Paling et al., 1981; Moll et al., 1986). Therefore, this pathogen is of economic importance to the farmers and requires proper intervention as it can cause serious diseases in exotic breeds or newly introduced naive cattle. Equally important, is that the symptoms manifested by *T. mutans* infections are somehow similar to *T. parva* infections, therefore, clinically these two infections can be confused in the field. The phylogenetic tree analysis for the V4 region of the 18S rRNA gene sequences of *T. mutans* shows that the sequences were clustered in different clades, which implies that different genotypes of *T. mutans* are circulating in a population of cattle in the study area.

About a quarter of the sampled cattle were positive for *T. parva* in this study. This pathogen is the causative agent of ECF in cattle and is the most economically important protozoan tick-borne pathogen in Tanzania (Laisser et al., 2014). Notably, the prevalence of *T. parva* in this study is relatively lower compared to studies I conducted in the nearby islands of Pemba and Zanzibar, in Tanzania. However, these findings are consistent with the other study conducted in the regions of Mara, Singida, and Mbeya in Tanzania (Kerario et al., 2017). Furthermore, similar results were reported in the neighboring countries of Uganda (Muhanguzi et al., 2014; Byaruhanga et al., 2016) and Zambia (Tembo et al., 2018). The lower infection rates of *T. parva* in this study could be attributed to the fact that the indigenous zebu cattle (kept under an extensive management system) are fairly resistant to *T. parva* infections (Gachohi et al., 2014). This is due to the continuous exposure to this pathogen from the early stages of their lives; therefore, cattle develop immunity against the *T. parva* infections. These animals, which are mostly under the traditional management system, tend to develop a state of endemic stability by developing antibodies against *T. parva* infections which protect them from any further reinfection (Kazungu et al., 2015). Moreover, the other possibility could be the reduced abundance and poor distribution of its vector *R. appendiculatus* in the study area, which is dry and semi-arid with very low precipitations. Tanzania, like any other tropical country, has been affected by climate change in various agroecological

zones, culminating to change in rainfall patterns, reduced rainfall, and the emergence of drought in different zones (Kimaro & Chibinga, 2013). Drought has been hypothesized to reduce the abundance and distribution of tick vectors like *R. appendiculatus* (Yeoman et al., 1967; Gachohi et al., 2014). The phylogenetic tree analysis shows that the *T. parva* p104 gene sequences of this study were clustered in a single clade, suggesting that the p104 gene is conserved in the sampled cattle.

Theileria taurotragi is a piroplasm parasite that causes benign theileriosis in cattle. This study reported a low infection rate of *T. taurotragi*. However, *T. taurotragi* and *T. parva* are transmitted by the same vector *R. appendiculatus*. Coincidentally, the two pathogens in this study had a relatively lower infection rate, which can be presumably related to the low abundance and distribution of their vector. The comparison of *T. taurotragi* prevalence with other studies conducted in Tanzania shows that it was lower compared to previous studies I conducted in the nearby islands of Pemba and Zanzibar, which may suggest that the sampling sites of the present study could not provide the same favorable environment for the multiplication of the pathogen and its vector. More importantly, *T. taurotragi* has been associated with bovine cerebral theileriosis (Turning sickness) in indigenous zebu cattle in east, central and southern Africa (Binta et al., 1998; Catalano et al., 2015). This implies that the pathogen is of economic importance in this region.

Babesiosis is an important disease in cattle and other ruminants. In Tanzania, bovine babesiosis is caused by *B. bigemina* and *B. bovis* (Lynen et al., 2008). *Rhipicephalus microplus* is currently the main vector and efficiently transmits both *Babesia* spp., while *R. (Boophilus) decoloratus* was previously the reported vector of *Babesia* spp. in the country (Lynen et al., 2008). Notably, the prevalence of *B. bigemina* in this study is consistent with the previous studies conducted in Tanzania (Swai et al., 2007). Moreover, similar results were reported in the neighboring countries by Tayebwa et al. (2018) in Uganda, Adjou Moumouni et al. (2015) in Kenya, and Chatanga et al. (2022) in Malawi. The consistency in prevalence in the region could be due to the emergence of the *R. microplus* tick, which is vastly distributed due to its high ability to reproduce and its ability to adapt to different climates (Sungirai et al., 2018). The phylogenetic tree analyses show that sequences of the RAP-1a gene of this study appeared in different clades which suggests that different isolates of *B. bigemina* are circulating in a population of cattle in the study area.

Bovine anaplasmosis is caused by the rickettsial parasite *A. marginale* and transmitted by *R. microplus* in Tanzania (Lynen et al., 2008). The bacterium is an important tick-borne pathogen of cattle and is more devastating in adult exotic breeds (Hailemariam et al., 2017). In this study, we report a slightly higher prevalence than the previous reports in Tanzania (Swai et al., 2005). Similar findings have been reported in the neighboring countries of Burundi (Nyabongo et al., 2021), Malawi (Chatanga et al., 2022), and Zambia (Tembo et al., 2018). The higher prevalence of *A. marginale* in this study could presumably be due to the wide distribution of *R. microplus* in the coastal areas of Tanzania (Lynen et al., 2008). The other possibility for a higher prevalence of this pathogen can be due to the ability of the indigenous zebu cattle to develop resistance against TBDs. This has been a protective mechanism used by the indigenous zebu cattle to survive the challenge of tick-borne infections. Therefore, when infected with *A. marginale*, they have a fast mechanism of seroconversion and developing immunity against the pathogen, hence, they can continuously be infected without showing clinical symptoms (Magona et al., 2011). The phylogenetic tree analysis of the groEL gene sequences revealed that sequences of this study were clustered in the same clade, suggesting that similar genotypes of *A. marginale* are present and circulating in the population of cattle in the study area.

Remarkably, this study reports a high prevalence of co-infections, implying that a significant number of cattle were concurrently infected with multiple parasite infections at the time of sampling. Concisely, the co-infections scenario in endemic areas has been documented to increase or decrease the pathogenicity of the infections, depending on the pattern of the parasites involved in the co-infections (Woolhouse et al., 2015). This heterologous reactivity plays role in the patterns of morbidity and mortality of the diseases. Therefore, in parasitic diseases, the outcome of the co-infection can either be protective to the host or may lead to severe infections to the host depending on the pathogens involved in the co-infections. Altogether, the interaction of tick-borne parasites during co-infections in the indigenous zebu cattle in endemic areas suggests that there can be ecological and epidemiological mechanisms to survive the challenges of the TBDs (Baneth et al., 2014; Diuk-Wasser et al., 2016). However, the higher prevalence of co-infections reported in this study suggests that a poor management system is imposed by the pastoralists on their animals in this study.

In conclusion, this study revealed a high prevalence of tick-borne pathogens in indigenous zebu cattle of the Tanga region in Tanzania. Additionally, some degree of genetic diversity in the

detected pathogens was observed. The study also showed that co-infections were more common than single infections, which implies that the interaction of the pathogens involved might have an effect on the pattern of clinical symptoms manifestation and therefore, complicate the diagnosis of the diseases. The epidemiological data produced in this study provide significant information on tick-borne diseases in the area and will serve as a scientific basis for planning future control strategies.

3.5 Summary

Tick-borne diseases (TBDs) are a major hindrance to livestock production in pastoral communities of Africa. Although information on tick-borne infections is necessary for setting up control measures, this information is limited in pastoral communities of Tanzania. Therefore, this study aimed to provide an overview of the tick-borne infections in indigenous cattle of Tanzania. A total of 250 blood samples were collected from the indigenous zebu cattle in the Tanga region, Tanzania. Then, we conducted a molecular survey using Polymerase Chain Reaction (PCR) and gene sequencing to detect and identify selected tick-borne pathogens. PCR assays were conducted using assays based on *Theileria* spp. 18S rRNA, *Theileria parva* p104, *Theileria mutans* and *T. taurotragi* V4 region of the 18S rRNA, *Babesia bigemina* RAP-1a, *B. bovis* SBP-2, *Anaplasma marginale* heat shock protein groEL, and *Ehrlichia ruminantium* pCS20.

PCR screening revealed an overall infection rate of (48%) for *T. mutans*, (32.4%) for *A. marginale,* (25.6%) for *T. parva*, (20.8%) for *T. taurotragi*, (13.2%) for *B. bigemina*. Co-infections of up to four pathogens were revealed in 44.8% of the cattle samples.

Sequence analysis indicated that *T. parva* p104 and *A. marginale* groEL genes were conserved among the sampled animals with sequence identity values of 98.92 – 100% and 99.88% – 100%, respectively. On the other hand, *the B. bigemina* RAP-1a gene and the V4 region of the 18S rRNA of *T. mutans* genes were diverse among the sampled cattle, indicating the sequence identity values of 99.27% - 100% and 22.45% - 60.77%, respectively. The phylogenetic analyses revealed that *T. parva* p104 and *A. marginale* groEL gene sequences of this study were clustered in a clade. In contrast, the *B. bigemina* RAP-1a and the *T. mutans* V4 region of the 18S rRNA gene sequences appeared in the different clades. This study provides important basement data for understanding the epidemiology of tick-borne diseases and will serve as a scientific basis for planning future control strategies.

Table 3.2 Overall prevalence of the detected pathogens.

Pathogen	Kwamsisi (n=51)	Kabuku (n=50)	Kwamatuku (n=50)	Kwachaga (n=50)	Komkonga (n=49)	Overall (n=250)
Theileria parva	14 (27.5%)	16 (32%)	5 (10%)	18 (36%)	11 (22.5%)	64 (25.6%)
Theileria mutans	30 (58.8%)	28 (56%)	20 (40%)	22 (44%)	20 (40.8%)	120 (48%)
Theileria Taurotragi	11 (21.6%)	8 (16%)	14 (28%)	13 (26%)	6 (12.2%)	52 (20.8%)
Babesia bigemina	8 (15.7%)	7 (14%)	1 (2%)	7 (14%)	10 (20.4%)	33 (13.2%)
Anaplasma marginal	15 (29.4%)	13 (26%)	20 (40%)	26 (52%)	7 (14.3%)	81 (32.4%)

Table 3.3 Tick-borne pathogens detected from Handeni, Tanga.

Single infection	Kwamsisi (n=51)	Kabuku (n=50)	Kwamatuku (n=50)	Kwachaga (n=50)	Komkonga (n=49)	Total no. positive (%)
T. parva	2 (4%)	3 (6%)	2 (4%)	3 (6%)	5 (10%)	15 (6)
T. mutans	14 (28%)	11 (22%)	7 (14%)	5 (10%)	9 (18%)	46 (18)
T. taurotragi	1 (2%)	2 (4%)	4 (8%)	0 (0%)	2 (4%)	9 (4)
B. bigemina	1 (2%)	3 (6%)	0 (0%)	3 (6%)	6 (12%)	11 (4)
A. marginale	3 (6%)	1 (2%)	9 (18%)	9 (18%)	1 (2%)	23 (9)
Sub total	19 (37%)	20 (40%)	22 (44%)	20 (40%)	23 (47%)	104 (42)
Double infections						
T. parva + T. mutans	3 (6%)	5 (10%)	2 (4%)	4 (8%)	3 (6%)	17 (7)
T. parva + T. taurotragi	0 (0%)	1 (2%)	0 (0%)	1 (2%)	2 (4%)	4 (2)
T. parva + B. bigemina	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	1 (0)
T. parva + A. marginale	4 (8%)	2 (4%)	0 (0%)	2 (4%)	4 (8%)	12 (5)
T. mutans + T. taurotragi	5 (10%)	1 (2%)	5 (10%)	1 (2%)	2 (4%)	14 (6)
T mutans + B. bigemina	2 (4%)	2 (4%)	0 (0%)	2 (4%)	2 (4%)	8 (3)

T. mutans + *A. marginale*	2 (4%)	4 (8%)	5 (10%)	4 (8%)	3 (6%)	18 (7)
T. taurotragi + *B. bigemina*	2 (4%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	3 (1)
T. taurotragi + *A. marginale*	0 (0%)	2 (4%)	4 (8%)	5 (10%)	1 (2%)	12 (5)
B. bigemina + *A. marginale*	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0)
Sub total	18 (35%)	17 (34%)	16 (32%)	21 (42%)	17 (35%)	89 (36)
Triple infections						
T. parva + *T. mutans* + *T. taurotragi*	0 (0%)	0 (0%)	1 (1%)	2 (4%)	1 (2%)	4 (2)
T. parva + *B. bigemina* + *A. marginale*	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0)
T. parva + *T. mutans* + *B. bigemina*	1 (2%)	1 (2%)	0 (0%)	0 (0%)	1 (2%)	3 (1)
T. parva + *T. taurotragi* + *A. marginale*	1 (2%)	0 (0%)	1 (2%)	1 (2%)	1 (1%)	4 (2)
T. mutans + *B. bigemina* + *A. marginale*	1 (2%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)	3 (1)
T. taurotragi + *B. bigemina* + A. *marginale*	1 (2%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	2 (1)
T. mutans + *T. taurotragi* + *B. bigemina*	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0)
T. mutans + *T. taurotragi* + *A. marginale*	1 (2%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)	3 (1)
Sub total	7 (14%)	1 (2%)	3 (6%)	6 (12%)	4 (8%)	21 (8)
Quadruple infections						
T. parva + *T. mutans* + *T. taurotragi* + *A. marginale*	1 (2%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	2 (1)
Total positive samples	45 (88%)	39 (78%)	41 (82%)	47 (94%)	44 (90%)	216 (86)

Table 3.4 The prevalence of tick-borne pathogens detected based on the animal sex.

Sex	*T. parva*	*T. mutans*	*T. taurotragi*	*B. bigemina*	*A. marginale*
Male (n=66)	16 (24.2%)	37 (56.1%)	9 (13.6%)	11 (16.7%)	20 (30.3%)
Female (n=184)	48 (26.1%)	83 (45.1%)	43 (23.4%)	22 (11.9%)	61 (33.2%)

Table 3.5 Observed co-infections, frequencies, number of species combination and pathogens involved.

Co-infections	Frequence	%	Spp combination	Pathogens involved
Double	89	79.4	10	20
Triple	21	18.8	8	24
Quadruple	2	1.8	1	4
Overall	112	100	19	48

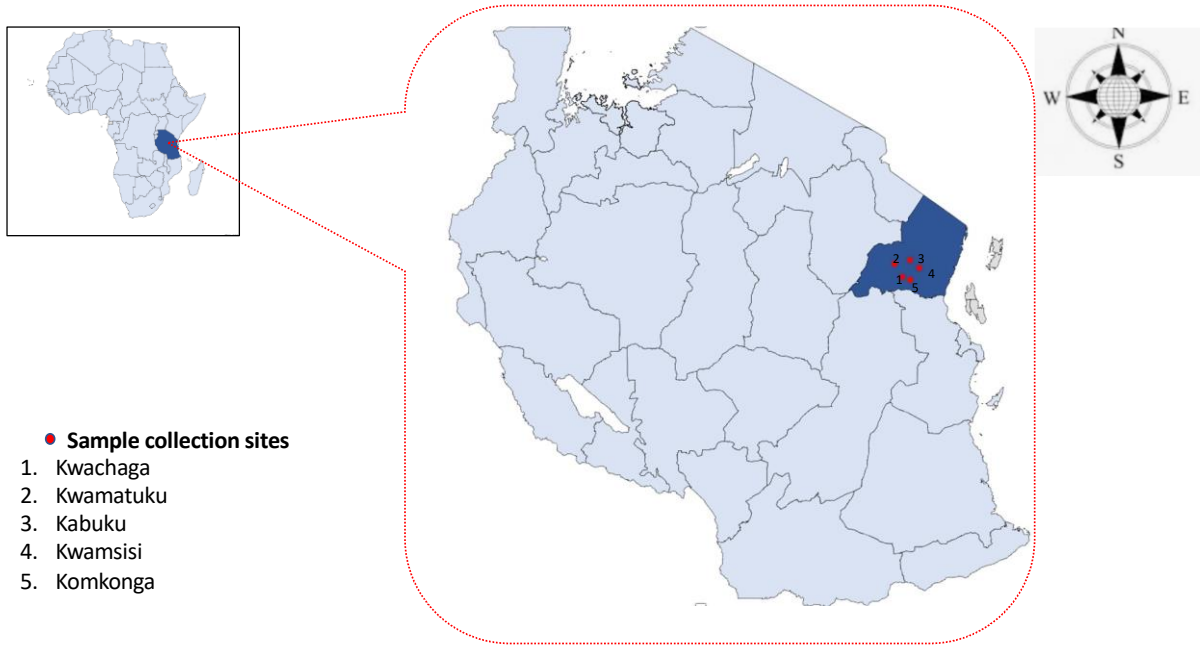

Figure 3.1 Map of Tanzania showing sample collection sites in Tanga region, Tanzania.

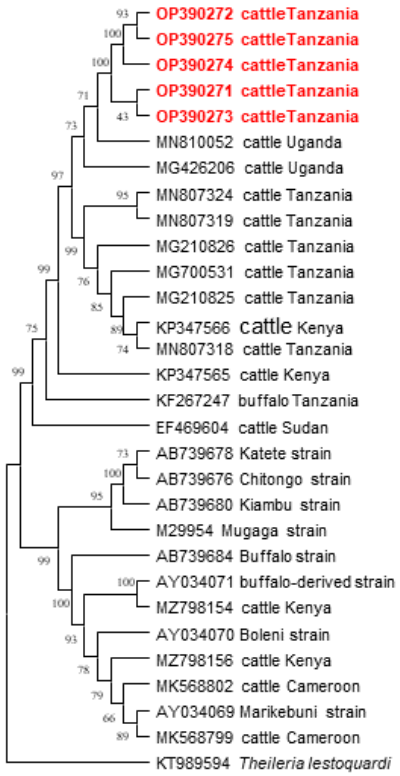

Figure 3.2 The phylogenetic analysis of *Theileria parva* identified in this study based on (p104) gene sequences. The tree was constructed by MEGA version 11 using the Maximum Likelihood method. The numbers at nodes represent percentage occurrence of clade in 1000 bootstrap replication of data. Sequences from this study are shown in red color. *Theileria lestoquardi* (KT989594) was used as an outgroup.

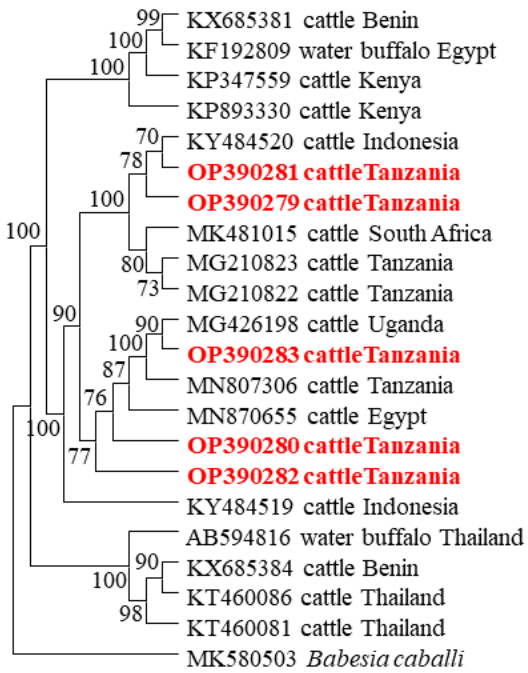

Figure 3.3 The phylogenetic analysis of *Babesia bigemina* identified in this study based on (RAP-1a) gene. The tree was constructed by MEGA version 11 using Maximum Likelihood method, the confidence of occurrence of the nodes was assessed by bootstrap in 1000 replication. The sequences of this study are shown by red color. *Babesia caballi* (MK580503) was used as an outgroup.

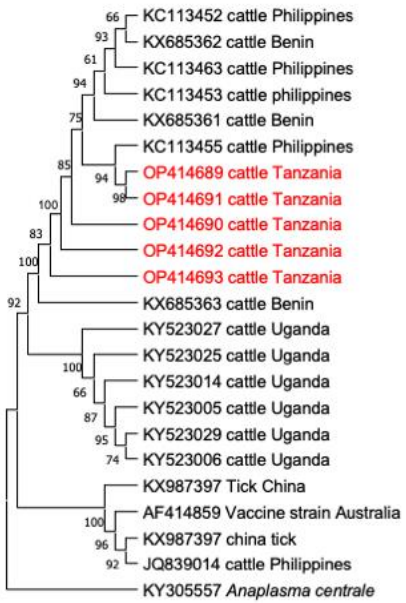

Figure 3.4 The phylogenetic analysis of *Anaplasma marginale* identified in this study based on (groEL) gene. The tree was constructed by MEGA version 11 using Maximum Likelihood method, the confidence of occurrence of the nodes was assessed by bootstrap in 1000 replication. The sequences of this study are shown by red color.

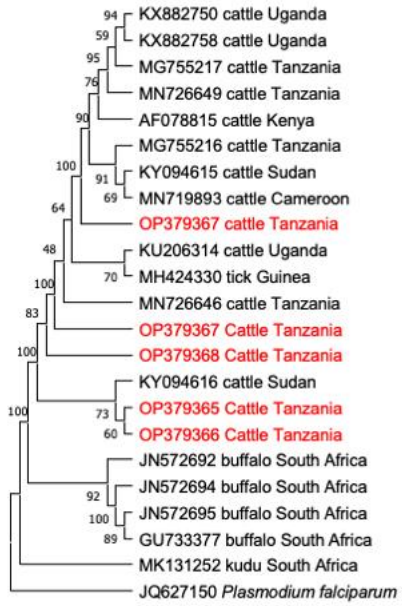

Figure 3.5 The phylogenetic analysis of *Theileria mutans* identified in this study based on (18S rRNA) gene. The tree was constructed by MEGA version 11 using Maximum Likelihood method, the confidence of occurrence of the nodes was assessed by bootstrap in 1000 replication. The sequences of this study are shown by red color.

Chapter 4

Molecular detection and genetic characterization of pathogenic *Theileria, Anaplasma* and *Ehrlichia* species amongst apparently healthy sheep in eastern and western Kenya

4.1 Introduction

Tick-borne diseases (TBDs), caused by *Theileria, Babesia, Anaplasma* and *Ehrlichia*, are common in tropical and sub-tropical regions of the world, where the distribution of hosts, pathogens and vectors overlap in higher magnitude (Bilgic et al., 2017). Over the years, more focus of TBDs studies have been directed to bovine pathogens while small ruminants received limited attention (Yin et al., 2007). Due to the growing socio-economic importance of small ruminants, more attention is now being directed towards pathogens of sheep and goats (Bilgic et al., 2017).

Sheep are among the most important livestock in sub-Saharan Africa as far as socio-economic importance is concerned (Yin et al., 2007). They are kept for meat (lamb, hogget or mutton), milk, fleece, manure and cultural rituals. Large number of sheep are kept by the pastoralists, therefore exposure to ticks is high. The most important tick-borne pathogens of small ruminants include *Theileria ovis, T. separata, T. lestoquardi* and *T. recondita* which cause theileriosis. *Babesia ovis* and *B. motasi* cause babesiosis. *Anaplasma ovis* causes anaplasmosis, whereas *A. phagocytophilum* causes tick-borne fever and *Ehrlichia ruminantium* causes ehrlichiosis. *T. lestoquardi, B. ovis, B. motasi, A. phagocytophilum* and *E. ruminantium* are considered to be pathogenic while the rest are less pathogenic in sheep (Uilenberg, 1995; Schnittger et al., 2000; Razmi & Yaghfoori, 2013).

Economic losses incurred from the TBDs include mortality, production losses, veterinary costs and ticks control (Jonsson et al., 2008a). In sheep, most of TBDs manifest themselves as sub-

clinical infections (Bilgic et al., 2017). Animals that survive the acute phase of infection develops a life-long carrier state, which is associated with significant production and economic losses (Gharbi et al., 2006; Uilenberg, 1995). Therefore, the pathogens regarded as less pathogenic should equally be considered important, as they continuously infect ticks when they are in carrier state resulting in new infection to uninfected animals (Razmi & Yaghfoori, 2013).

Horn of Africa is one of the three regions of sub-Saharan Africa which are considered to be highly populated by sheep and other livestock (Hanotte et al., 2000). Regardless of the large population of sheep in the region, little information is available on the presence and genetic diversity of tick-borne pathogens. This study was conducted to fill in this information gap. Blood samples were collected from sheep in Machakos and Homabay counties in Kenya and analyzed for the occurrence and genetic diversity of ovine tick-borne pathogens using PCR and sequencing.

4.2 Materials and methods

4.2.1 Study area

Blood samples were collected from sheep in Machakos and Homabay counties (Fig. 4.1). Machakos is located about 63 km south east of Nairobi Kenya. The local climate is semiarid with hilly terrain with an altitude of 1000 to 2100 meters above sea level. The climate of this area is temperate and subtropical whereby temperatures are modified by altitude. The area has summer rainfall while the vegetation is Savannah and rain starts at the end of March to May. Homabay County is located in west southern Kenya along Lake Victoria. Climate is inland equatorial with temperatures ranging from 17.1^{o}C to 34.8^{o}C.

4.2.2 Sample collection and DNA extraction

A total of 76 blood samples were collected from clinically healthy sheep, using sterile needles and EDTA coated vacutainer tubes. An average of 3 – 5 ml of blood was drawn from the jugular vein of the animal, kept in cool boxes until transported to the laboratory. Fifty-two and 24 samples were collected in Machakos and Homabay counties respectively. Samples were collected from different farms targeting animals of around two years of age and above. Genomic DNA was extracted as described in **chapter 1.**

4.2.3 Ethical statement

The owners of the selected farms were informed about the study and provided their approval for sample collection from their sheep. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Permit for animal experiment: 280080, DNA experiment 1219-2; Pathogen: 2015727).

4.2.4 Molecular detection of tick-borne pathogens

All samples were screened using PCR with primers obtained from previous studies for *Theileria* spp. 18S rRNA, *B. ovis* 18S rRNA, *A. ovis* Major surface protein 4 (AoMSP-4), *Anaplasma* spp. 16S rRNA and *E. ruminantium* pCS20 genes (Table 4.1). Thereafter, *Theileria* spp. and *Anaplasma* spp. positive samples were sequenced to confirm the species. PCR conditions are indicated in Table 4.1. The reaction mixture and composition were performed as described in **chapter 1**. The double distilled water (DDW) was used as negative control, while positive controls were positive samples from my previous study. PCRs were run in a thermal cycler (Bio Rad, USA). The PCR products were electrophoresed on a 2% agarose gel and then stained with ethidium bromide and viewed under UV transilluminater.

4.2.5 Cloning and sequencing

About 3-5 positive samples per detected pathogen were randomly selected for sequencing. Amplicons were purified using QIAquick Gel Extraction Kit (Qiagen, German) according the manufacturer's protocol. The procedure for checking the concentration of the extracts, cloning and sequencing of the amplicon were performed as described in **chapter 1.**

4.2.6 Phylogenetic analysis

The sequences obtained in this study were compared to sequences of the same pathogens from other regions of the world by phylogenetic analysis using MEGA version 7.0 software 10. Maximum likelihood method was used to construct phylogenetic trees for *T. ovis, A. ovis, E. ruminantium* and uncultured *Anaplasma* spp. Bootstrap analysis with 1000 replications was used to estimate the confidence of the nodes and branches of the trees.

4.2.7 Nucleotide sequence accession numbers

Sequences obtained in this study were deposited in Genbank database of the National Center for Biotechnology Information using BankIt, the sequences were assigned the following accession numbers: MF360021 to MF360025 for *T. ovis* 18S rRNA; MF360026 to MF360029 for *A. ovis* MSP4; MG637125 to MG637127 for uncultured *Anaplasma* spp. 16S rRNA and MG544303 to MG544305 for *E. ruminantium* pCS20.

4.3 Results

4.3.1 Overall infection rates

Theileria spp. had an overall infection rate of (51.3%), *A. ovis* (34.2%), *E. ruminantium* (7.9%) and uncultured *Anaplasma* spp. (40.8%). *Theileria ovis* was the only *Theileria* species identified following the sequencing of all PCR positive *Theileria* spp. while for *Anaplasma* spp. the

uncultured *Anaplasma* spp. and *A. ovis* were identified. Meanwhile, *B. ovis* and *T. lestoquardi* were not detected in this study.

4.3.2 Infection rates based on location

The infection rates detected in Homabay were (54.2%), (33.3%), (8.3%) and (41.7%) for *T. ovis, A. ovis, E. ruminantium* and uncultured *Anaplasma* spp., respectively. In Machakos the infection rates were (50%), (34.6%), (7.7%) and (26.9%) for *T. ovis, A. ovis, E. ruminantium* and uncultured *Anaplasma* spp., respectively.

4.3.3 Mixed infections

Several sets of co-infections were revealed in this study (Table 4.2). Multiple infection was found in (61.8%) samples. *T. ovis* was the pathogen most frequently associated with multiple infections (46.1%) followed closely with *A. ovis* (44.7%) (Table 3.2). *T. ovis* + *A. ovis* co-infection had the highest overall prevalence. A triple infection (*A. ovis* + *T. ovis* + uncultured *Anaplasma* spp.) with an overall prevalence of (7.9%) was revealed in this study (Table 4.2).

4.3.4 Comparative sequence analysis

The PCR products of all the *T. ovis, A. ovis* and *E. ruminantium* isolated in this study were of the expected sizes of 520 bp, 347 bp and 279 bp, respectively. The three uncultured *Anaplasma* spp. amplified products were of different sizes (398 bp, 367 bp and 344 bp). The percentage identity among the 5 nucleotide sequences (MF360021 – MF360025) of *T. ovis* 18S rRNA ranged from 99.61% to 100%. These sequences shared 100% identity with previous sequences deposited in GenBank from Sudan (AY260171 and MG333457) and Tanzania (AY260174). Furthermore, the four nucleotide sequences (MF360026 – MF360029) of *A. ovis* MSP4 shared 97.98% to 100% nucleotide identity, and had 98% nucleotide identity with the other sequences from Tunisia (KY659324, KY659320, KM285220 and KC432643). Meanwhile, the three nucleotide sequences

(MG544303 – MG544305) of *E. ruminantium* pCS20 shared nucleotide identity of 99.64%, and had 100% sequence identity with other GenBank sequences from Ethiopia (GU797236), Benin (KX356089), Ivory Coast (KX356090) and Cameroon (JQ039914 and JQ039939). As for uncultured *Anaplasma* spp. 16S rRNA, the three sequences MG637125 – MG637127 from this study, shared 100% nucleotide identity, and showed 100% nucleotide identity with sequences from Japan (AY969011) and USA (KJ942183).

4.3.5 Phylogenetic analysis

Phylogenetic trees of *T. ovis*, *A. ovis*, uncultured *Anaplasma* spp. and *E. ruminantium* were constructed based on 18S rRNA, MSP-4, 16S rRNA and pCS20 gene sequences, respectively which have been generated in this study and those downloaded from GenBank. The *T. ovis* sequences from this study clustered with the *T. ovis* sequences from the GenBank, making a separate clade from other *Theileria* species (Fig. 4.2). Three *A. ovis* MSP-4 isolates from this study were in the same clade while one sequence formed a separate branch (Fig. 4.3). Similarly, for *E. ruminantium* all the three isolates from this study were located in the same clade (Fig. 4.4). Meanwhile, the uncultured *Anaplasma* spp. sequences of this study appeared in the same clade separated from other *Anaplasma* sequences from the GenBank (Fig. 4.5).

4.4 Discussion

Despite the wide distribution of ovine tick-borne diseases in tropical and subtropical regions of the world (Ros-García et al., 2013), and how important they are in livestock improvement (Jongejan & Uilenberg, 1994; Jonsson et al., 2008; Bilgic et al., 2017), very little information is available regarding their presence and distribution (Ros-García et al., 2013). In the present study, I performed molecular detection and analysis of tick-transmitted protozoan and rickettsial

pathogens from blood samples of sheep collected from two counties of Homabay and Machakos in Kenya. The following pathogens were detected: *Theileria ovis, Anaplasma ovis, Ehrlichia ruminantium* and uncultured *Anaplasma* spp.

Theileriosis in sheep can be caused by several *Theileria* spp. In this study, only *T. ovis* was detected (51.3%). The study revealed *T. ovis* as the most prevalent pathogen, which is in contrast to the previous studies (Wamuyu et al., 2015; Mwamuye et al., 2017; Omondi et al., 2017) that did not detect this pathogen in Kenya. However, (Adjou Moumouni et al., 2015) reported this pathogen in cattle reared in Ngong. The prevalence of *T. ovis* was compared to other studies from neighboring countries. In Ethiopia, (Gebrekidan et al., 2014) reported a prevalence of 92%, (El Imam et al., 2016) reported a prevalence of 88.6% in Sudan. Therefore, this pathogen is widely distributed in different parts of Africa. *Rhipicephalus evertsi evertsi* which transmits *T. ovis* trans-stadially has been reported to exist in Kenya (Omondi et al., 2017), which suggests that *T. ovis* is endemic in this region of the horn of Africa. Normally, *T. ovis* is known to cause benign type of theileriosis and is less pathogenic to sheep (Uilenberg, 1995; Schnittger et al., 2000b; G. R. Razmi et al., 2003), with low economic importance in sheep (Mtshali et al., 2016). However, it cannot be neglected as it can cause disease under stressful situations.

The 18S rRNA gene sequence of *T. ovis* from this study shared high identity (100%) with isolates from neighboring countries including Sudan (AY260171 and MG333457) and Tanzania (AY260174). This could be due to vast movement of animals in this region by the pastoralists. Therefore, leading to high chances of animals from different areas to cross over and exchange ticks while searching for pastures and water. Moreover, the phylogenetic tree showed all 18S rRNA sequences (Fig. 4.2) in this study appeared in the cluster with other *T. ovis* sequences extracted from the GenBank which suggest that, the gene is highly conserved.

Anaplasmosis caused by *A. ovis* generally is considered to cause sub-clinical symptoms in sheep, although this disease has been reported to cause severe disease in Bighorn sheep (Tibbitts et al., 1992; Renneker et al., 2013). Acute disease tends to be associated with stress factors such as co-infection, hot weather, deworming, vaccination, heavy tick burden, long distance transportation and animal movement (Renneker et al., 2013). In this study, a prevalence of 34.2% was detected for this pathogen, this is higher than previously reported in Kenya by (Omondi et al., 2017), whilst, this pathogen was not detected in Maasai Mara and Shimba Hills National Reserves. Data from other studies in Africa showed high prevalence of *A. ovis* in Tunisia (Belkahia et al., 2014), Algeria (Atef Aouadi, 2017) and South Africa that I did. These observations suggest *A. ovis* has a wide distribution in different geographical areas of Africa. However, in Kenya and other parts of sub-Saharan Africa large number of sheep are owned by the pastoralists, who take long distance movements daily, in sunny days searching for pastures and water (Byaruhanga et al., 2016). This potentially leads to animal stress and eventually results into disease severity. The low production of local breeds of sheep could be associated with diseases which are endemic in sub-Saharan Africa.

Phylogenetic analysis of *A. ovis* MSP-4 sequences from this study (Fig. 4.3) and those extracted from GenBank revealed that the three genotypes from this study clustered in the same clade while the other sequence formed a separate branch (Fig. 4.3). This shows considerable genetic divergence of sequences from this study.

Ehrlichia ruminantium, transmitted by ticks of genus *Amblyomma*, causes heartwater in domestic and wild ruminants in sub-Saharan Africa, Madagascar, and some Caribbean islands (Allsopp, 2010). The disease is one of the major obstacle in improving livestock production in Africa (Cangi et al., 2017). In this study, *E. ruminantium* was detected in sheep from both

Machakos and Homabay counties, with the overall occurrence of 7.9%, this supports the previous studies in Kenya (Omondi et al., 2017) that reported similar prevalence. (Wesonga et al., 2006) reported high prevalence in Narok. The presence of several *Amblyomma* species including *A. variegatum* has been reported by (Omondi et al., 2017) in Kenya, which suggests that ovine Ehrlichiosis is endemic in Kenya. In phylogenetic analysis, all three isolates obtained in this study (Fig. 4.4) formed a supported clade with those from other parts of Africa. This can be associated with the wide distribution of the main vector *A. variegatum* in sub-Sahara Africa, and the movement of animals by pastoralist that manifest the genetic spread of this pathogen. Moreover, this pathogen is regarded as an extremely diverse organism, and it has raised concern as it is adapting to canine and human hosts (Allsopp et al., 2005). Even more crucial for diversity is the recombination that occurs naturally between different genotypes of *E. ruminantium* in the field which makes it difficult to develop a vaccine (Allsopp, 2010).

uncultured *Anaplasma* spp. is a Gram-negative bacterium detected in this study. It is a new *Anaplasma* sp. detected in Kenya. The overall prevalence is 31.6% from both sampled counties. Noteworthy, Phylogenetic analyses showed the two isolates of this bacteria clustered together, and closely related to *Anaplasma marginale* (Fig. 4.5).

Generally, the overall co-infection rates in this study were 61.8%, with *T. ovis* showing high association in co-infection. The co-infection (*T. ovis* + *A. ovis*) (27.3%) was the most common, this could be associated with high prevalence of the two pathogens in this study. Moreover, the two pathogens share the same vector, *Rhipicephalus evertsi evertsi*.

In this study, all pathogens were detected from the apparently healthy animals, which suggests that sheep in Kenya have established an enzootic stability status against these pathogens. It shows that animals were infected at the early stages of their lives and developed immunity

against the detected pathogens. These animals carry the pathogens without showing any clinical signs, but they continuously transmit the pathogens to ticks and eventually to new animals. Factors like increased infection rates especially in rainy season when tick activity is increased or decreased immunity of the hosts can break the enzootic stability and these animals will develop clinical signs.

Babesia ovis and *Theileria lestoquardi* were not detected in this study, which supports the previous study (Omondi et al., 2017), although further studies with greater coverage may be needed to ascertain their absence.

In conclusion, the results obtained in this study indicate the occurrence and diversity of *T. ovis, A. ovis, E. ruminantium* and uncultured *Anaplasma* spp. in sheep from Machakos and Homabay counties. Mixed infections are common in the study area and therefore, disease diagnosis can be complex. On that account, proper diagnostic tests are required for accurate diagnosis. Further studies covering larger sample size and wider geographical coverage are required to estimate the risk factors associated with these diseases and their economic importance.

4.5 Summary

In this study, the occurrence and genetic diversity of *Theileria* spp., *Anaplasma* spp., *Babesia ovis* and *Ehrlichia ruminantium* in sheep from Machakos and Homabay counties of Kenya was analyzed. A total of 76 blood samples from apparently healthy sheep were screened using PCR. The assays were conducted using primers based on *Theileria* spp. 18S rRNA, *Anaplasma ovis Major surface protein-4* (AoMSP-4), *B. ovis* 18S rRNA, *E. ruminantium* pCS20 and *Anaplasma* spp. 16S rRNA. Overall infection rates for *Theileria* spp., *A. ovis, E. ruminantium* and *Anaplasma* spp. were 39/76 (51.3%), 26/76 (34.2%), 6/76 (7.9%) and 31/76 (40.8%), respectively. The overall co-infection was 47/76 (61.8%). All *Theileria* spp. positive samples were confirmed

to be *Theileria ovis* on sequencing. A phylogenetic analysis of 18S rRNA gene sequences revealed that isolates of this study clustered with *T. ovis* sequences from other regions suggesting this gene is highly conserved. *E. ruminantium* pCS20 sequences were in the same clade on the phylogenetic tree. However, three AoMSP-4 sequences appeared in the same clade while one sequence formed a separate branch revealing genetic divergence to the other sequences. The 16S rRNA sequencing revealed uncultured *Anaplasma* spp. and *A. ovis*. The phylogenetic analyses of uncultured *Anaplasma* spp. revealed that the two sequences formed a divergent clade signifying genetic differences to other isolates. This study provides important information regarding tick-borne pathogens occurrence and their degree of genetic diversity among sheep in Kenya.

Table 4.1 List of primers used for PCR assays.

Target gene	Assay	Primer sequences (5' → 3')		Fragment	Annealing	References
		Forward	Reverse	(bp)	Temp (°C)	
B. ovis (SSU rRNA)	PCR	TGGGCAGGACCTTGGTTCTTCT	CCGCGTAGCGCCGGCTAAATA	549	62	(Aktas et al., 2005)
A. ovis (AoMSP4)	PCR	TGAAGGGAGCGGGGTCATGGG	GAGTAATTGCAGCCAGGCACTCT	347	62	(Torino A. et al., 2012)
Anaplasma spp.(16S rRNA)	PCR	GGTTTAATTCGATGCAACGCGA	CGTATTCACCGTGGCATG	430	78-69	(Bekker et al., 2002)
	nPCR	GGTTTAATTCGATGCAACGCGA	GCTCAGCCTTGCGACGT	335	78-69	(Simuunza et al., 2011)
E. ruminantium (pCS20)	PCR	ACTAGTAGAAATTGCACAATCYAT	RCTDGCWGCTTTYTGTTCAGCTAK	400	61	(Farougou et al., 2012)
	nPCR	ACTAGTAGAAATTGCACAATCYAT	TGATAACTTGGWGCRRGDARTCCTT	278	61	
Theileria spp. (18S rRNA)	PCR	GAAACGGCTACCACATCT	AGTTTCCCCGTGTTGAGT	778	55	(Cao et al., 2013)
	nPCR	TTAAACCTCTTCCAGAGT	TCAGCCTTGCGACCATAC	581	55	

Table 4.2 Mixed infections detected in this study.

Pathogens	County		
	Homa-bay (n=24)	Machakos (n=52)	Overall (n=76)
T. ovis + *A. ovis*	4 (16.7%)	14 (26.9%)	18 (23.7%)
A. ovis + *A. phagocytophilum*	3 (12.5%)	5 (9.6%)	8 (10.5%)
A. ovis + *E. ruminantium*	2 (8.3%)	0 (0%)	2 (2.6%)
T. ovis + *A. phagocytophilum*	5 (20.8%)	6 (11.5%)	11 (14.5%)
A. phagocytophilum+ *E. ruminantium*	2 (8.3%)	0 (0%)	2 (2.6%)
A. ovis + *T. ovis* + *A. phagocytophilum*	2 (8.3%)	4 (7.7%)	6 (7.9%)

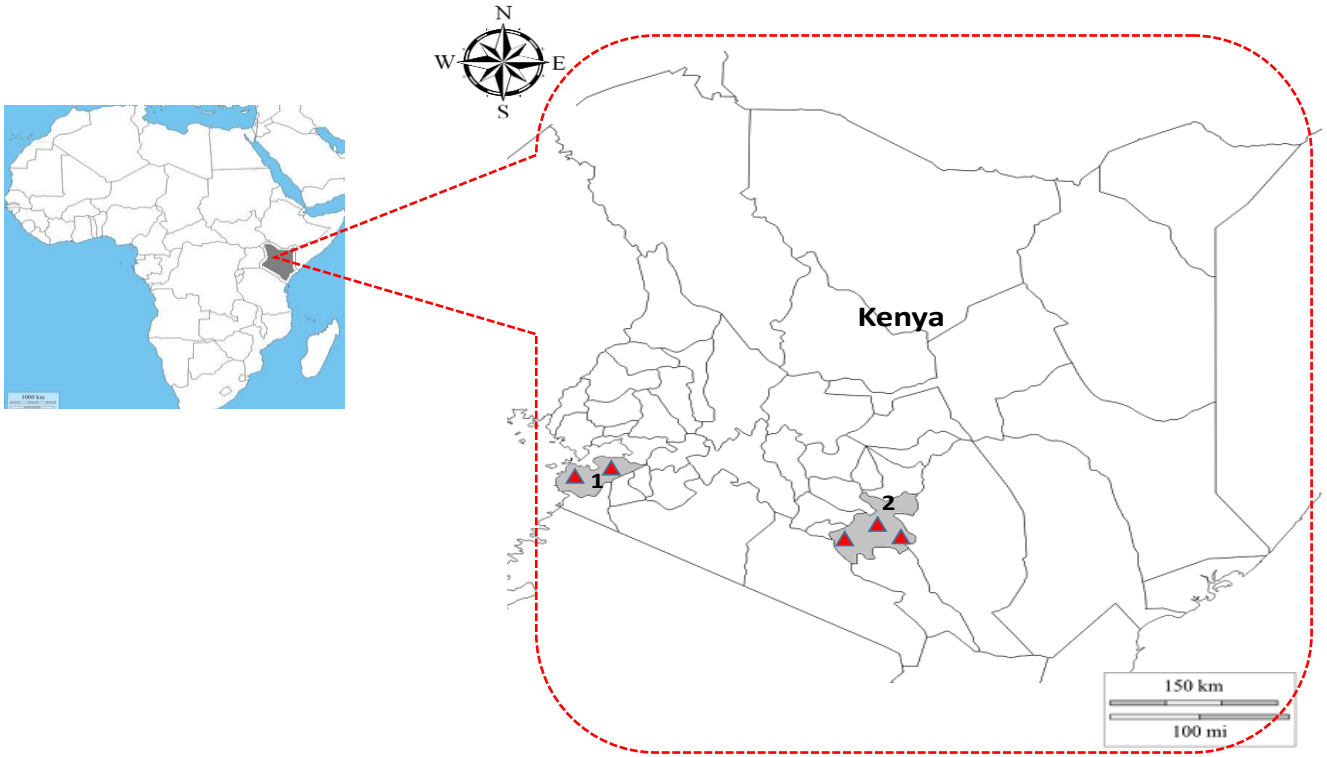

Figure 4.1 Map of Kenya showing the two counties where samples were collected, Homabay (1) in western Kenya, and Machakos (2) in central Kenya. The red triangles are sample collection farms.

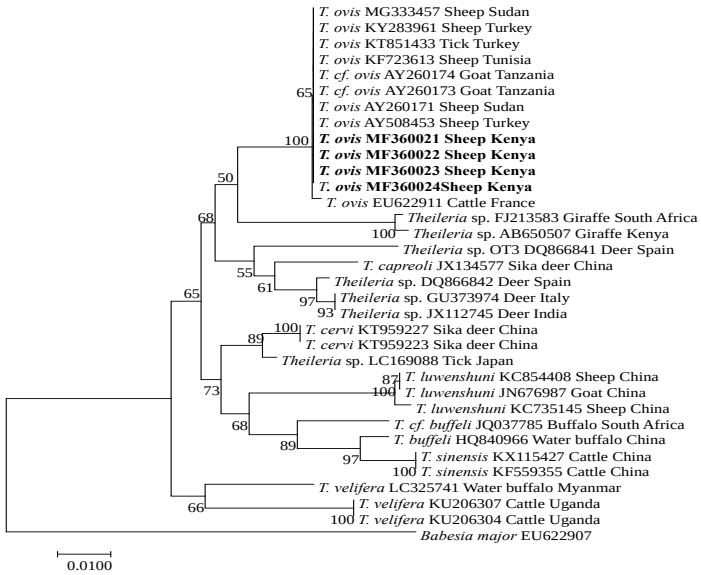

Figure 4.2 Phylogenetic analysis of *Theileria ovis* based on 18S rRNA. The tree was constructed using Maximum likelihood method, MEGA version 7. The sequences generated in this study are shown in bold font. Number at the nodes represent the percentage of occurrence of clades in 1000 bootstrap replication of the taxa. The evolutionary distances were computed using the p-distance method (Kumar et al., 2016). The 18S rRNA gene sequence of *Babesia major* (EU622907) was used as an outgroup.

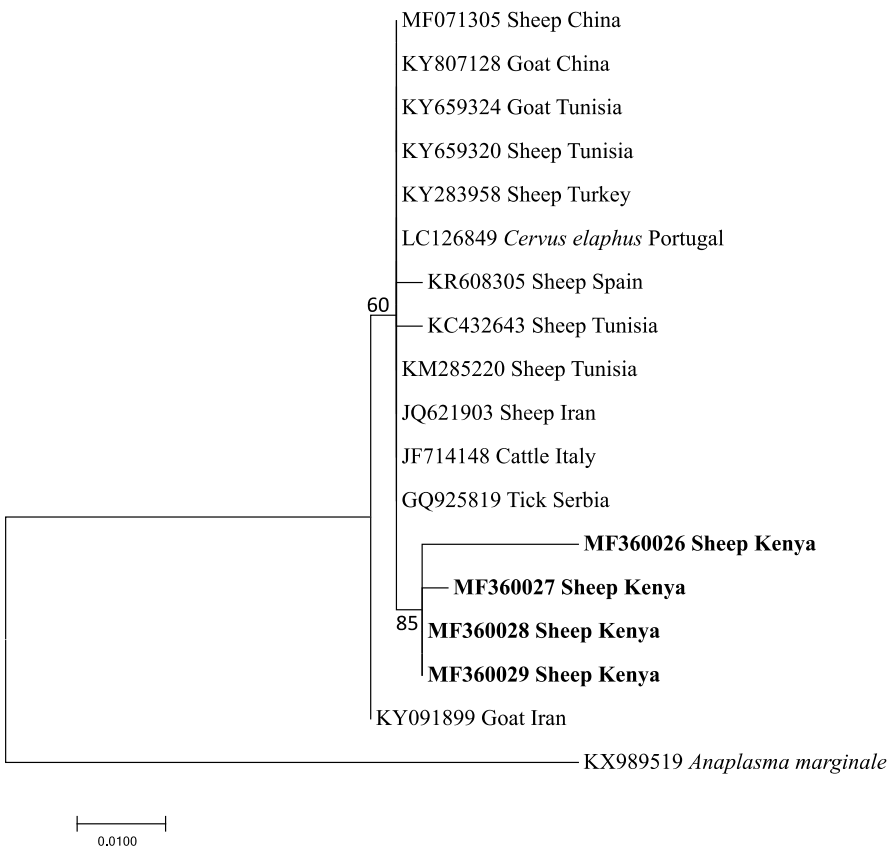

Figure 4.3 Phylogenetic analysis of *Anaplasma ovis* based on AoMSP-4. The tree was constructed using Maximum likelihood method, MEGA version 7. The sequences determined in this study are shown in bold font. Numbers at the nodes represent the percentage of occurrence of clades in 1000 bootstrap replication of the taxa. The evolutionary distances were computed using the p-distance method (Kumar et al., 2016). The MSP-4 gene sequence of *Anaplasma marginale* (KX989519) was used as an outgroup.

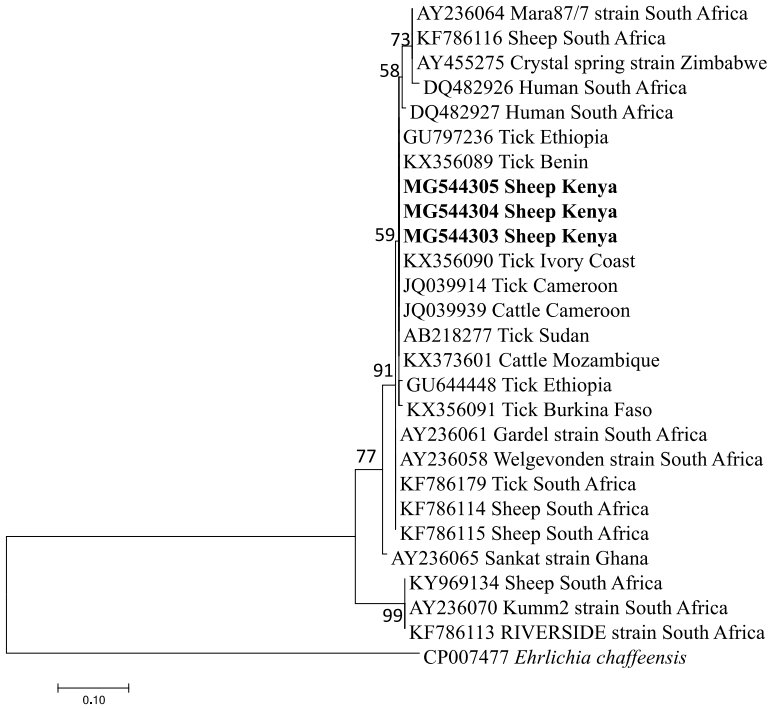

Figure 4.4 Phylogenetic analysis of *Ehrlichia ruminantium* based on pCS20. The tree was constructed using Maximum likelihood method, MEGA version 7. The sequences in bold font are from this study. The number at the nodes represent the percentage of occurrence of clades in 1000 bootstrap replication of the taxa. The evolutionary distances were computed using the p-distance method (Kumar et al., 2016). The pCS20 gene sequence of *Ehrlichia chaffeensis* (CP007477) was used as an outgroup.

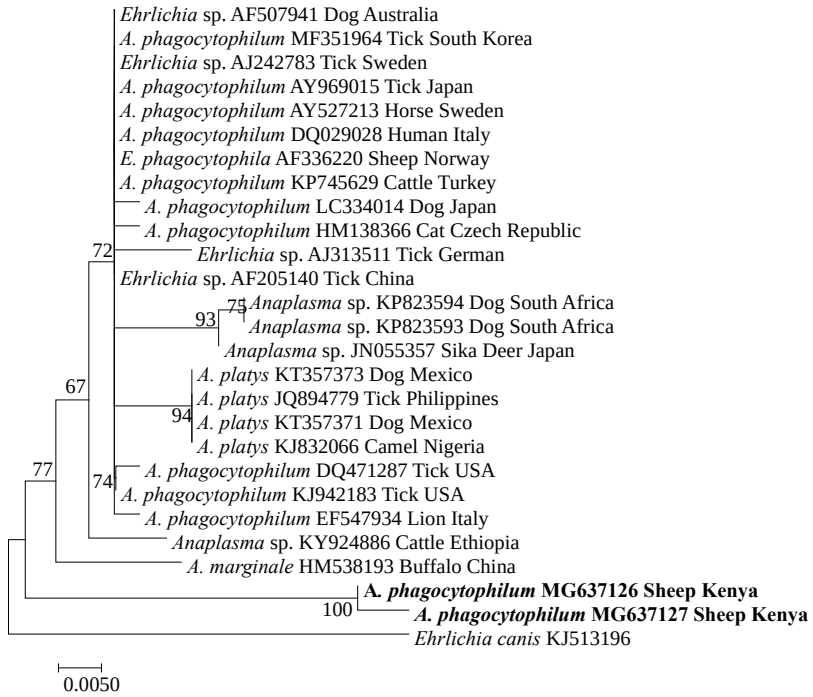

Figure 4.5 Phylogenetic analysis of uncultured *Anaplasma* spp. based on 16S rRNA. The tree was constructed using Maximum likelihood method, MEGA version 7. The sequences in bold font are from this study. The number at the nodes represent the percentage of occurrence of the clades in 1000 bootstrap replication of the taxa. The evolutionary distances were computed using t he p-distance method (Kumar et al., 2016). the 16S rRNA gene sequence of *Anaplasma ovis* (KX579073) was used as an outgroup.

General discussion

Tick-borne diseases (TBDs) are a serious constraint to livestock production in East Africa and the rest of tropical and subtropical regions and impact on the performance of livestock and the livelihoods of resource-poor farming communities in the region (Altay et al., 2008; Dantas-Torres et al., 2012; Aktas et al., 2012). In Tanzania and Kenya, detailed studies on tick-borne pathogens (TBPs) in cattle and small ruminants using sensitive molecular detection methods are limited. Therefore, the current project has conducted epidemiological studies on ticks and tick-borne diseases of cattle in Tanzania (Tanga region, Pemba and Zanzibar Island) and sheep in Kenya (Machakos and Homa-bay counties), respectively. The studies aimed to analyse the occurrence and genetic composition of tick-borne diseases in cattle and sheep in Tanzania and Kenya. The three studies conducted in Tanzania, chapter 1,2 and 3 were conducted in Pemba Island, Zanzibar Islands and Tanga region, respectively. Tanga region is located in the eastern coast of Tanzania and the two islands are located in the Indian ocean, on eastern coast of Tanzania.

The three locations were selected for these studies based on the following factors. Tanga region is known for its large population of cattle especially the indigenous breeds. The indigenous breeds are predominantly owned by the pastoralist who moves from one location to the other in search of pasture and water. On the other hand, Zanzibar and Pemba Islands receives cattle and other livestock from different locations of the Tanzania mainland. Moreover, the two islands receive different classes of livestock from the neighbouring country of Kenya. The animals shipped to the two islands are the exotic breeds for dairy purposes and the indigenous breeds for beef. Remarkably, the health status of the imported animals in the two islands is poorly assessed. Consequently, the two islands are most likely to carry the pathogens that are brought by animals from different Agro-ecosystems of the Tanzania mainland and the neighbouring country of Kenya.

The other factor is the climatic condition of the two islands, Pemba Island experiences bimodal rainfall pattern. The average rainfall per annum is 1860 mm, which falls mostly between March and May (long rains) and October to December (short rains). The climate is tropical sub-humid with average temperatures of 26ºC annually. The vegetation can be classified into four types, which are tropical moist forest, coastal rag shrubs, clove plantations and farmlands (Vreysen & Khamis, 1999). Similar climatic condition is experienced in Zanzibar Island, which has a total annual rainfall of 1,600 mm, with long rainfall season starting from end of March to June and short rains in October to December. The climate is tropical sub-humid with annual average temperatures of 26°C. Notably, each island provides favorable environment for the survival and multiplication of tick vectors. Attributable to the factors analyzed earlier the prevalence and the genetic relationship of the detected pathogens in chapter 1 and chapter 2 are more less indistinguishable.

Noteworthy, the three chapters describing results of Pemba and Zanzibar Islands and Tanga region shows that, among the detected pathogens were theileriosis causal agents, namely, *Theileria parva*, *T. taurotragi* and *T. mutans* which were detected in both islands and Tanga and the prevalence was closely similar. The prevalence of *T. parva* in Pemba was 41.2% while in Zanzibar was 34.3%, and Tanga 25.6%. *T. taurotragi* in Pemba was 48.4% and Zanzibar was 30.9% and Tanga was 20.8% while for *T. mutans* in Pemba was 68.6%, Zanzibar was 38.1% and Tanga was 20.8%. In general terms, the presence and their prevalence indicate that the two islands and the coastal region of Tanzania provide similar epidemiological state for the thriving of different species of ticks and the pathogens they are harboring.

Contrastingly, the other detected pathogens in chapter 1,2 and 3 showed that Pemba Islands had higher prevalence than Zanzibar Island and Tanga. The prevalence of *Babesia bigemina* in Pemba was 17.6% almost three times the prevalence in Zanzibar (5.1%) but in Tanga was 13.2%.

This suggests that probably Pemba Island provide a more favorable ecological environment for the *Rhipicephalus microplus* tick which is widely distributed in the coastal areas of Tanzania including Pemba and Zanzibar Islands (Lynen et al., 2007). The prevalence of *B. bovis* was 4.5% in Pemba Island more than twice that of Zanzibar Island (2.1%) and this pathogen was not detected in Tanga region. The higher prevalence observed in Pemba Island can follow similar explanation provided earlier on *B. bigemina* as the two pathogens are transmitted by the same vector tick *R. microplus*. Moreover, *Anaplasma marginale* detection was higher again in Pemba Island 15.9% compared to 10.2% in Zanzibar Island. The higher prevalence of this pathogen in Pemba than Zanzibar Island could be due to the reason that regardless of similar climatic condition, Pemba is much wetter and covered by green vegetation than Zanzibar, which are conditions adding more favorable ecological environment for the thriving of the vector ticks. However, the prevalence of this pathogen in Tanga was the highest (32.4%). This could be attributed by the fact that indigenous cattle are relatively resistant to *A. marginale* and they can harbour the pathogens without showing the clinical symptoms. Furthermore, the presence of *Ehrlichia ruminantium* (7.4%) on Pemba Island compared to Zanzibar Island and Tanga where the pathogen was not detected, can be explained by the ecology of Pemba which is wetter than the other two locations which should be an added favorable condition for the thriving of *Amblyomma* tick.

Alarmingly, both Islands and Tanga region provided relatively higher rate of mixed infections. Pemba Island again had a higher rate of mixed infections (46.9%), followed by Tanga region 44.8% and Zanzibar Island had 36.4%. This suggests that the detected pathogens are endemic in the three study sites and farmers are not adequately using acaricides for the control of ticks. Therefore, animals are potentially living with the infections without showing the clinical signs as they have developed some degree of immunity due to endemicity of the pathogens. This

however, makes them to serve as potential reservoirs for offspring and younger animals which will then suffer from the infections.

A study conducted in Kenya in chapter 4 on sheep, was designed purposely on the two locations of Machakos and Homabay counties. Machakos is located in a semi-arid tropical climate with the Savannah vegetation, while Homa-bay county is again semi-arid and temperatures ranging from 26℃ to 34℃. Additionally, Homa-bay is located near the Ruma National Park. In Kenya, Tanzania and most other sub-Saharan countries large proportion of their total livestock population are owned by the pastoralists. Pastoralists keeps their livestock in free range system and they move their animals from one location to the other in search of pasture and water. Due to the increased challenges of land use and growth of the human population in the regions, pastoralists have become marginalized and left with the arid and semi-arid land for pastures. In that sense, the large proportion of small ruminants and other livestock are reared in the climate similar to the climate provided by the two locations of the study in chapter 4.

In this study, the pathogens detected were *Anaplasma ovis* (34.2%), *Theileria ovis* (51.3%), *E. ruminantium* (7.9%) and uncharacterized *Anaplasma* species (40%). The results shows that these pathogens are common among small ruminants owned by the pastoralists. Extraordinarily, the sampled animals as shown in chapter 4 were apparently healthy and yet they were carrying these pathogens. Inevitably, this shows that the detected pathogens are endemic in the study areas and most likely in the East African region. Interestingly, in the two locations of this study *Babesia ovis* and *Theileria lestoquardi* were not detected, this outcome is in agreement with the few other similar studies conducted in Ghana and the other I did in South Africa, which did not detect the two pathogens. The scenario suggests that, the two pathogens might not be present in the region

or are in low key in this particular region. However, more extensive molecular and large scale sero-epidemiological studies with larger sample size to confirm this hypothesis are recommended.

In conclusion, tick-borne diseases are present in Tanzania and Kenya, these diseases are well distributed in the region. Animals have shown they can carry the pathogens without showing the clinical symptoms which suggests that the tick-borne diseases are endemic in the two East African countries. The distribution of tick-borne diseases in the region shows that despite the geographical, ecological and epidemiological differences tick-borne diseases are widely distributed in the two countries.

General summary

Tick-borne diseases (TBDs) pose a major challenge to the livestock industry in many tropical and sub-tropical countries. In East Africa, Tanzania and Kenya are among the countries located just south of the horn of Africa. This region is popularly known for a large population of livestock. However, TBDs in the region present a serious threat to the livestock sector particularly cattle and small ruminants. The most important TBDs in the region are theileriosis, anaplasmosis, babesiosis and ehrlichiosis. Despite the damage incurred by these diseases in Tanzania and Kenya, limited epidemiological data on the occurrence and distribution of TBDs in the two countries is available. Therefore, several molecular studies were carried out in different locations of the two countries to address the problem.

In chapter 1, a total of 245 blood samples from different breeds of cattle were randomly collected on Pemba Island, in Tanzania. Polymerase chain reaction (PCR) and sequencing was used to detect and identify the tick-borne pathogens (TBPs). The assays were performed using primers based on *Theileria* spp. (18S rRNA), *Babesia bovis* (SBP-2), *B. bigemina* (RAP-1a), *Anaplasma marginale* (MSP-5 and groEL), *Ehrlichia ruminantium* (pCS20), *T. parva* (p104), *T. mutans* (18S rRNA), and *T. taurotragi* (18S rRNA). PCR screening of cattle samples collected on Pemba Island revealed overall infection rates for *Theileria* spp. (62.4%), *B. bigemina* (17.6%), *A. marginale* (15.9%), *E. ruminantium* (7.4%) and *B. bovis* (4.5%). Further analysis using sequences of *Theileria* spp. (18S rRNA) revealed infection of cattle with *T. mutans* (68.6%), *T. taurotragi* (48.4%), *T. parva* (41.2%), and *T. ovis* (1.9%). Co-infections of cattle, with up to six TBPs, were revealed in 46.9% of the samples. Sequence analysis indicated that *T. parva* (p104), *E. ruminantium* (pCS20) and *A. marginale* (MSP-5) genes are conserved among cattle blood samples in Pemba, with 99.3% - 100%, 99.6% - 100% and 100% sequence identity values, respectively. In

contrast, the *B. bigemina* (RAP-1a) and *B. bovis* (SBP-2) gene sequences were relatively diverse with 99.5% - 99.9% and 66.4% - 98.7% sequence identity values, respectively. The phylogenetic analyses revealed that *T. parva* (p104), *E. ruminantium* (pCS20) and *A. marginale* (MSP-5) gene sequences clustered in the same clade with other isolates from other countries. In contrast, the *B. bigemina* (RAP-1a) and *B. bovis* (SBP-2) gene sequences showed significant differences in the genotypes, as they appeared in separate clades. The data provided should improve the understanding of the epidemiology of tick-borne diseases, and is expected to improve the approach for diagnosis and control of tick-borne diseases in Tanzania.

In chapter 2, blood samples were collected randomly in 236 cattle of different breeds from Zanzibar Island, Tanzania. The PCR and sequencing were used to screen the samples for detection of TBPs. The assays were performed using primers described in chapter 1. The PCR screening revealed that 64.5% of animals were infected by TBPs, including *T. mutans* (38.1%), *T. parva* (34.3%), *T. taurotragi* (30.9%), *A. marginale* (10.2%), *B. bigemina* (5.1%), *T. velifera* (3.4%) and *B. bovis* (2.1%). Overall, a total of 86 animals (36.4%) were co-infected with up to five pathogens concomitantly. The pathogens mostly involved in the co-infection were *T. parva*, *T. taurotragi* and *T. mutans*. Sequence analysis indicated that *T. parva* (p104) and *B. bigemina* (RAP-1a) genes are diverse among the sampled animals on Zanzibar Island, with 99.64%–100% and 99.51%–100% nucleotide sequence identity value, respectively. In contrast, the *A. marginale* (MSP-5) and *B. bovis* (SBP-2) genes are conserved, with 100% and 99.66%-100% nucleotide sequence identity values respectively. The phylogenetic analyses revealed that *T. parva* (p104) and *B. bigemina* (RAP-1a) gene sequences showed significant differences of genotypes, as they appear in different clades. Meanwhile, *A. marginale* (MSP-5) and *B. bovis* (SBP-2) gene sequences appear in the same clade with other sequences extracted from the NCBI GenBank. The epidemiological findings

revealed in this study will provide important information on tick-borne diseases in Tanzania and will be used as scientific basis for planning future control strategies.

In chapter 3, a total of 250 blood samples were randomly collected from indigenous cattle of Tanga region, Tanzania. The assays performed were based on primers described in chapter 1. The results show an overall infection rate for *T. mutans* (48%), *A. marginale* (32.4%), *T. parva* (25.6%), *T. taurotragi* (20.8%) and *B. bigemina* (13.2%). Co-infections of up to four pathogens were revealed in 44.8% of the cattle samples. Sequence analysis indicated that *T. parva* (p104) and *A. marginale* (groEL) genes were conserved among the sampled animals with sequence identity values of 98.92 – 100% and 99.88% – 100%, respectively. On the other hand, the *B. bigemina* (RAP-1a) gene and the (V4 region of the 18S rRNA of *T. mutans* genes were diverse among the sampled cattle, indicating the sequence identity values of 99.27% - 100% and 22.45% - 60.77%, respectively. The phylogenetic analyses revealed that *T. parva* (p104) and *A. marginale* (groEL) gene sequences of this study were clustered in a clade. In contrast, the *B. bigemina* (RAP-1a) and the *T. mutans* (V4 region of the 18S rRNA) gene sequences appeared in the different clades. The findings revealed in this work shows that indigenous breed of cattle reared in free range system in Tanzania can be the source of tick-borne infections in other naïve breeds of cattle in the country. Therefore, this information is useful in the control of tick-borne diseases.

In chapter 4, blood samples were randomly collected from 76 apparently healthy sheep in Machakos and Homa-bay counties in Kenya. The assays were performed using primers based on *Theileria* spp. (18S rRNA), *Anaplasma* spp. (16S rRNA), *Babesia ovis* (18S rRNA), *A. ovis* (AoMSP-4) and *E. ruminantium* (pCS20). The overall infection rates of sheep samples collected in Machakos and Homabay counties in Kenya for *Theileria* spp. (51.3%), *Anaplasma* spp. (40.8%), *A. ovis* (34.2%) and *E. ruminantium* (7.9%). The overall co-infection was (61.8%). All *Theileria*

spp. positive samples were confirmed to be *T. ovis* on sequencing. A phylogenetic analysis of (18S rRNA) gene sequences revealed that isolates of this study clustered with *T. ovis* sequences from other regions suggesting this gene is highly conserved. The *E. ruminantium* (pCS20) sequences were in the same clade on the phylogenetic tree. However, three (AoMSP-4) sequences appeared in the same clade while one sequence formed a separate branch revealing genetic divergence to the other sequences. The 16S rRNA sequencing revealed uncultured *Anaplasma* spp. and *A. ovis*. The phylogenetic analyses of uncultured *Anaplasma* spp. revealed that the two sequences formed a divergent clade signifying genetic differences to other isolates. This study provides important information regarding tick-borne pathogens occurrence and their degree of genetic diversity among sheep in Kenya.

In conclusion, the studies reveal that TBDs are well distributed in Tanzania and Kenya. *Theileria* spp. were the most prevalent TBPs in the region led by *Theileria mutans*, *T. parva* and *T. taurotragi*. Moreover, mixed infection in cattle and sheep were the prominent findings in the study areas. These data provide basement epidemiological background which will contribute to the future control strategies of tick-borne diseases in Tanzania and Kenya.

和文要約

マダニ媒介性感染症は、多くの熱帯および亜熱帯地域の畜産業に対し大きな脅威となっている。タンザニアとケニアは、共に東アフリカに位置しており、畜産業が盛んな地域として知られているが、家畜におけるマダニ媒介性寄生虫症の被害は甚大なものとされている。この地域で最も重要な家畜のマダニ媒介性寄生虫症は、一般的にタイレリア症、アナプラズマ症、バベシア症およびエーリキア症とされている。しかしながら、当該地域の家畜におけるマダニ媒介性寄生虫症の発生と分布に関する詳細な疫学的データは限られている。そこで、本研究ではタンザニアとケニヤの主要家畜である牛と羊におけるマダニ媒介性寄生虫症の流行実態の一端を解明するために分子疫学的調査を実施した。

第 1 章では、タンザニアのペンバ島で採集した 245 頭の牛の血液サンプル中のマダニ媒介性病原体について PCR 法によるスクリーニングとシークエンス解析を行った。その結果、*Theileria* spp. (62.4%) 、*B. bigemina* (17.6%) 、*A. marginale* (15.9%) 、*E. ruminantium* (7.4%) および *B. bovis* (4.5%)が検出された。*Theileria* 属に属する種の特定を行ったところ、 *T. mutans* (68.6%)、*T. taurotragi* (48.4%)、*T. parva* (41.2%)、および *T. ovis* (1.9%) が検出された。PCR 産物の塩基配列に基づいて系統解析を行ったところ、*T. parva* (p104)、 *E. ruminantium* (pCS20)、および *A. marginale* (MSP-5) の標的遺伝子塩基配列が、他のアフリカの国と地域の分離株と近縁のクラスターに位置していることが判明した。対照的に、*B. bigemina* (RAP-1a) と *B. bovis* (SBP-2) の標的遺伝子配列は、他のアフリカの国と地域で分離された株とは独自のクラスターを形成した。これらのデータは、タンザニアにおけるマダニ媒介性寄生虫症の流行実態の一端を解明し、さらにコントロール対策を構築する上で一助となると考えられる。

第 2 章では、タンザニアのザンジバル島で採集した 236 頭の牛の血液サンプル中のマダニ媒介性病原体について PCR 法によるスクリーニングとシークエンス解析を行った。その結果、*T. mutans* (38.1%)、*T. parva* (34.3%)、*T. taurotragi* (30.9%)、*A. marginale* (10.2%)、*B. bigemina* (5.1%)、*T. velifera* (3.4%)および *B. bovis* (2.1%)が検出された。系統解析では、*T. parva* (p104) 、*B. bigemina* (RAP-1a) および *B. bovis* (SBP-2)の標的遺伝子配列は、タンザニア独自のクラスターを形成した。一方、*A. marginale* (MSP-5)の標的遺伝子配列は、地理的に離れた国及び地域の分離株と近縁のクラスタを形成した。これらの結果は、タンザニアにおけるマダニ媒介性寄生虫症に関する重要な情報を提供し、将来の制御戦略を構築する上で有用な科学的根拠として活用できると考えられる。

第3章では、タンザニアの陸地であるタンガ地域で採集した250頭の牛の血液サンプル中のマダニ媒介性病原体についてPCR法によるスクリーニングとシークエンス解析を行った。その結果、*T. mutans*（48%）、*T. parva*（25.6%）、*T. taurotragi*（20.8%）、*B. bigemina*（13.2%）および*A. marginale*（32.4%）が検出された。系統解析では、*T. parva*（p104）および*A. marginale*（groEL）の標的遺伝子配列がタンザニア独自のクラスターを形成した。対照的に、*B. bigemina*（RAP-1a）および*T. mutans*（18S rRNAのV4領域）の標的遺伝子配列は、アフリカと世界中で分離された株と近縁関係にあることが判明した。これらの結果は、タンザニアの放し飼いシステムで飼育された土着の牛の品種が、他のナイーブ品種への伝播の感染源になり得ることが示唆された。したがって、これらの情報はタンザニアの内陸地域におけるマダニ媒介性寄生虫症の制御戦略を構築する上有効に活用できると考えられる。

第4章では、ケニアのマチャコスとホマベイ地域の羊76頭から採集した血液サンプル中のマダニ媒介性病原体についてPCR法によるスクリーニングとシークエンス解析を行った。その結果、*T. ovis*（51.3%）、*A. ovis*（34.2%）、および*E. ruminantium*（7.9%）が検出された。系統解析では、*T. ovis*（18S rRNA）の標的遺伝子配列は、ケニア分離株において高度に保存され、アフリカの他の地域やトルコで分離された株と近縁関係にあった。また、*E. ruminantium*（pCS20）塩基配列は東アフリカで分離された株で独自のクラスターを形成した。*A. ovis*（AoMSP-4）の標的塩基配列では、３つのケニア分離株が同一クラスターに属し、また、一つの分離株は他の３株と分岐を形成した。これらの結果から、ケニアの羊の間でマダニ媒介性病原体の発生とその遺伝的多様性の関する有用な情報を提供し、小型反芻動物におけるマダニ媒介性寄生虫症の診断と制御戦略の構築に有用であると考えられる。

以上のように、この研究ではタンザニアとケニアの主要家畜である牛と羊におけるマダニ媒介性寄生虫症の流行実態の一端を明らかにした。そのなかでも*T. mutans*、 *T. parva*および*T. taurotragi* 最も広く分布しているマダニ媒介性病原体であることが明らかとなった。さらに、多くの牛と羊が複数のマダニ媒介病原体に同時感染していることが示唆された。これらのデータは、タンザニアとケニアにおける家畜のマダニ媒介性寄生虫症の制御戦略構築に役立つ基礎データを提供するものと考えられる。

Acknowledgements

This research work was carried out at the National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine. Firstly, I would like to appreciate the opportunity I got from the Japan Society for the Promotion of Science, RONPAKU Program (JSPS RONPAKU) to pursue my PhD degree study.

Foremost, I would like to express my sincere gratitude to my supervisor Prof. XUAN Xuenan for yet giving me another opportunity to work in his laboratory. I am grateful to Prof. XUAN for the continuous support, patience, motivation, enthusiasm, and immense knowledge. His guidance helped me through the period of conducting research experiments and writing of the thesis. I could not have imagined having a better supervisor and mentor for my PhD study.

Besides my supervisor, I thank the rest of my thesis committee members: Prof. YOKOYAMA Naoaki, Prof. NISHIKAWA Yoshifumi, and Prof. NOBORU Inoue for their encouragement, insightful comments and hard questions.

I am grateful to Dr. Paul Franck Adjou Moumouni and Dr. Charoonluk Jirapattharasate for enlightening me the first glance of research when I first came to the Host and Defense Laboratory. I will forever cherish your support and tireless guidance.

I appreciate the expertise guidance in writing manuscripts to Associate Prof. Oriel Thekisoe, you made a lot of genuine comments that built me and made me a better researcher.

My sincere thanks also go to all the Professors of NRCPD, led by Prof. KAWAZU Shin-ichiro, Prof. SUZUKI Hiroshi, Prof. IGARASHI Makoto, Associate Prof. UMEMIYA-SHIRAFUJI Rika, Associate Prof. FUKUMOTO Shinya, Associate Prof. ASADA Masahito and Assistant Prof. SUGANOMA Keisuke for their professional interactions in the presentations and within the NRCPD vicinities.

I would like to thank my fellow members in Host and Defense laboratory: Shengwei Ji, Eloiza May Galon, Shimaa Abd El-Salam El-Sayed, Uday Kumar Mohanta, Zhuowei Ma, Thanh Thom DO, Boniface Chikufenji, Iqra Zafar, Moaz Amer and Hang Li for the stimulating interactions, and support we shared together.

I thank all the African community in and around Obihiro University for the valuable time we had together.

Last but not the least, I would like to thank my family: My parents Edmond Ringo and Victoria Ringo for raising me up and making me a better person, my wife Daniella and kids Adrian and Charlotte for their precious time I had with them after hard work in lab.

I dedicate this work and achievement to my late father Mr. Edmond Ringo.

"NAMSHUKURU MWENYEZI MUNGU KWA MAFANIKIO HAYA"

References

AbouLaila, M., Yokoyama, N., & Igarashi, I. (2010). Development and evaluation of two nested PCR assays for the detection of *Babesia bovi*s from cattle blood. *Veterinary Parasitology*, 172, 65-70.

Adjou Moumouni, P. F., Aboge, G. O., Terkawi, M. A., Masatani, T., Cao, S., Kamyingkird, K., Jirapattharasate, C., Zhou, M., Wang, G., Liu, M., Iguchi, A., Vudriko, P., Ybanez, A. P., Inokuma, H., Shirafuji-Umemiya, R., Suzuki, H., & Xuan, X. (2015). Molecular detection and characterization of *Babesia bovis, Babesia bigemina, Theileria* species and *Anaplasma marginale* isolated from cattle in Kenya. *Parasites and Vectors*, *8*(1).

Aktas, M., Altay, K., & Dumanli, N. (n.d.-a). Determination of prevalence and risk factors for infection with *Babesia ovis* in small ruminants from Turkey by polymerase chain reaction.

Aktas, M., Altay, K., & Dumanli, N. (2005). PCR-based detection of *Theileria ovis* in *Rhipicephalus bursa* adult ticks. *Veterinary parasitology*, 140, 259-263

Aktas, Munir, Altay, K., Ozubek, S., & Dumanli, N. (2012). A survey of ixodid ticks feeding on cattle and prevalence of tick-borne pathogens in the Black Sea region of Turkey. *Veterinary Parasitology, 187*, 567–571.

Allsopp, B. A. (2010). Natural history of *Ehrlichia ruminantium*. *Veterinary Parasitology*, 167, 123–135.

Allsopp, M. T., Louw, M., & Meyer, E. C. (2005). *Ehrlichia ruminantium*: an emerging human pathogen? *Annals of the New York Academy of Sciences*, 1063, 358–360.

Altay, K., Aydin, M. F., Dumanli, N., & Aktas, M. (2008). Molecular detection of *Theileria* and *Babesia* infections in cattle. *Veterinary Parasitology*, 158, 295–301.

Altay, K., Dumanli, N., & Aktas, M. (2005). Molecular identification, genetic diversity and distribution of *Theileria* and *Babesia* species infecting small ruminants. *Veterinary parasitology*, 147, 161-165.

Andrew, H. R., & Norval, R. A. I. (1989). The carrier status of sheep, cattle and African buffalo recovered from Heartwater. *Veterinary Parasitology*, 34, 261–266.

António A.M. Tembue, Fábio J.M. Silva, Jenevaldo B. Silva, Tiago M. Santos, Huarrisson A. Santos, , Cleber O. Soares, and Adivaldo H. Fonseca, (2011). Risk factors associated with the frequency of antibodies against *Babesia bovis* and *Babesia bigemina* in cattle in southern Mozambique. *Pesiquisa Veterinaria Brasileira*, 31, 663–666.

Asiimwe, B. B., Weir, W., Tait, A., Lubega, G. W., & Oura, C. A. L. (2013). Haemoparasite infection kinetics and the population structure of *Theileria parva* on a single farm in Uganda. *Veterinary Parasitology*, 193, 8–14.

Atef Aouadi, H. L. (2017). Molecular evidence of tick-borne hemoprotozoan-parasites (*Theileria ovis* and *Babesia ovis*) and bacteria in ticks and blood from small ruminants in Northern Algeria. *Comparative Immunology, Microbiology and Infectious Diseases*, 50, 34–39.

Baneth, G. (2014). Tick-borne infections of animals and humans: a common ground. *International Journal for Parasitology*, 44, 591–596.

Bekker, C. P. J., de Vos, S., Taoufik, A., Sparagano, O. A. E., & Jongejan, F. (2002). Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Veterinary Microbiology*, 89, 223–238.

Belkahia, H., Said, M. Ben, Hamdi, S. El, Yahiaoui, M., Gharbi, M., Daaloul-Jedidi, M., Mhadhbi, M., Jedidi, M., Darghouth, M. A., Klabi, I., Zribi, L., & Messadi, L. (2014). First molecular

identification and genetic characterization of *Anaplasma ovis* in sheep from Tunisia. *Small Ruminant Research,* 121, 404–410.

Berggoetz, M., Schmid, M., Ston, D., Wyss, V., Chevillon, C., Pretorius, A. M., & Gern, L. (2014). Tick-borne pathogens in the blood of wild and domestic ungulates in South Africa: Interplay of game and livestock. *Ticks and Tick-Borne Diseases*, 5, 166–175.

Bilgic, H. B., Bakırcı, S., Kose, O., Unlu, A. H., Hacılarlıoglu, S., Eren, H., Weir, W., & Karagenc, T. (2017). Prevalence of tick-borne haemoparasites in small ruminants in Turkey and diagnostic sensitivity of single-PCR and RLB. *Parasites and Vectors,* 10, 211.

Binta, M. G., Losho, T., Allsopp, B. A., & Mushi, E. Z. (1998). Isolation of *Theileria taurotragi* and *Theileria mutans* from cattle in Botswana. *Veterinary Parasitology,* 77, 83–91.

Byamukama, B., Vudriko, P., Agnes Tumwebaze, M., Stuart Tayebwa, D., Byaruhanga, J., Kamilo Angwe, M., Li, J., May Galon, E., Ringo, A., Liu, M., Li, Y., Ji, S., Abdo Rizk, M., Frank Adjou Moumouni, P., Lee, S.-H., Sevinc, F., & Xuan, X. (2021). Molecular detection of selected tick-borne pathogens infecting cattle at the wildlife-livestock interface of Queen Elizabeth National Park in Kasese District, Uganda. *Ticks and tick-borne diseases,* 12, 101772

Byaruhanga, C., Collins, N. E., Knobel, D., Chaisi, M. E., Vorster, I., Steyn, H. C., & Oosthuizen, M. C. (2016). Molecular investigation of tick-borne haemoparasite infections among transhumant zebu cattle in Karamoja Region, Uganda. *Veterinary Parasitology: Regional Studies and Reports*, 3, 27–35.

Cangi, N., Pinarello, V., Bournez, L., Lefrançois, T., Albina, E., Neves, L., & Vachiéry, N. (2017). Efficient high-throughput molecular method to detect *Ehrlichia ruminantium* in ticks. *Parasites & Vectors*, 10.

Cao, S., Zhang, S., Jia, L., Xue, S., Yu, L., Kamyingkird, K., Moumouni, P. F. A., Moussa, A. A. E. M., Zhou, M., Zhang, Y., Terkawi, M. A., Masatani, T., Nishikawa, Y., & Xuan, X. (2013). Molecular Detection of *Theileria* species in sheep from Northern China. *Journal of Veterinary Medical Science*, 75, 1227–1230.

Catalano, D., Biasibetti, E., Lynen, G., Di Giulio, G., De Meneghi, D., Tomassone, L., Valenza, F., & Capucchio, M. T. (2015). "Ormilo disease" a disorder of zebu cattle in Tanzania: Bovine cerebral theileriosis or new protozoan disease? *Tropical Animal Health and Production*, 47, 895–901.

D., Muhanguzi, E., Mat, ovu, an, d C., Wai, & swa. (2010). Prevalence and characterization of *Theileria* and *Babesia* species in cattle under different husbandry systems in Western Uganda. *International Journal of Animal and Veterinary Advances*, 2, 51–58.

Dantas-Torres, F., Chomel, B. B., & Otranto, D. (2012). Ticks and tick-borne diseases: a One Health perspective. *Trends in Parasitology*, 28, 437–446.

Devos, A. J., & Roos, J. A. (1981). The isolation of *Theileria taurotragi* in south africa. *Onderstepoort Journal of Veterinary Research*, 48, 149–153.

Diuk-Wasser, M. A., Vannier, E., & Krause, P. J. (2016). Coinfection by Ixodes tick-borne pathogens: Ecological, epidemiological, and clinical consequences. *Trends in Parasitology*, 32, 30–42.

d'Oliveira, C., van der Weide, M., Habela, M. A., Jacquiet, P., & Jongejan, F. (1995). Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *Journal of Clinical Microbiology*, 33, 2665–2669.

E S Swai, A N Mbise, V Kessy, E Kaaya, P. S. and P. M. L. (2005). Farm constraints, cattle disease perception and tick management practices in pastoral Maasai community-Ngorongoro,

Tanzania. Livestock Research for Rural Development. *Livestock Research for Rural Development,* 17, 2

El Imam, A. H., Hassan, S. M., Gameel, A. A., El Hussein, A. M., Taha, K. M., & Oosthuizen, M. C. (2016). Molecular identification of different *Theileria* and *Babesia* species infecting sheep in Sudan. *Annals of Parasitology*, 62, 47–54.

Engida, E., Guthiga, P., & Karugia, J. (2015). The role of livestock in the Tanzanian economy: policy analysis using a dynamic computable general equilibrium model for Tanzania. *International Association of Agricultural Economists*, 15.

Farougou, S., Adakal, H., & Boko, K. C. (2012). Prevalence of *Amblyomma variegatum* infection by *Ehrlichia ruminantium* in cattle extensive herds in Benin poster and others view project Modélisation des niches écologiques de Rhipicephalus View project. *Revue Méd. Vét*, 163, 261–266.

Franck, P., Moumouni, A., Luc Aplogan, G., Katahira, H., Gao, Y., Guo, H., Efstratiou, A., Jirapattharasate, C., Wang, G., Liu, M., Ringo, A. E., Umemiya-Shirafuji, R., Suzuki, H., & Xuan, X. (2017). Prevalence, risk factors, and genetic diversity of veterinary important tick-borne pathogens in cattle from Rhipicephalus microplus-invaded and non-invaded areas of Benin.*Ticks and tick-borne diseases,* 9, 450-464

Gachohi, J. M., Ngumi, P. N., Kitala, P. M., & Skilton, R. A. (2010). Estimating seroprevalence and variation to four tick-borne infections and determination of associated risk factors in cattle under traditional mixed farming system in Mbeere district, Kenya. *Preventive Veterinary Medicine*, 95, 208–223.

Gebrekidan, H., Hailu, A., Kassahun, A., Rohoušová, I., Maia, C., Talmi-Frank, D., Warburg, A., & Baneth, G. (2014). Theileria infection in domestic ruminants in northern Ethiopia. *Veterinary Parasitology*, 200, 31–38.

Gharbi, M., Sassi, L., Dorchies, P., & Darghouth, M. A. (2006). Infection of calves with *Theileria annulata* in Tunisia: Economic analysis and evaluation of the potential benefit of vaccination. *Veterinary Parasitology*, 137, 231–241.

Gomes, J., Soares, R., Santos, M., Santos-Gomes, G., Botelho, A., Amaro, A., & Inácio, J. (2013). Detection of *Theileria* and *Babesia* infections amongst asymptomatic cattle in Portugal. *Ticks and Tick-Borne Diseases*, 4, 148–151.

Hailemariam, Z., Krücken, J., Baumann, M., Ahmed, J. S., Clausen, P. H., & Nijhof, A. M. (2017). Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia. *PLoS ONE*, 12, (11).

Hanotte, O., Tawah, C. L., Bradley, D. G., Okomo, M., Verjee, Y., Ochieng, J., & Rege, J. E. O. (2000). Geographic distribution and frequency of a taurine *Bos taurus* and an indicine *Bos indicus* Y specific allele amongst sub-Saharan African cattle breeds. *Molecular Ecology*, 9, 387–396.

Jensenius, M., Parola, P., & Raoult, D. (2006). Threats to international travellers posed by tick-borne diseases. *Travel Medicine and Infectious Disease*, 4, 4–13.

Jirapattharasate, C., Franck, P., Moumouni, A., Cao, S., Iguchi, A., Liu, M., Wang, G., Zhou, M., Vudriko, P., Changbunjong, T., Sungpradit, S., Ratanakorn, P., Moonarmart, W., Sedwisai, P., Weluwanarak, T., Wongsawang, W., Suzuki, H., & Xuan, X. (2016). Molecular epidemiology of bovine *Babesia* spp. and *Theileria orientalis* parasites in beef cattle from northern and northeastern Thailand. *Parasitology International*, 65, 62–69.

Jongejan, F., & Uilenberg, G. (1994). Ticks and control methods. *Revue Scientifique et Technique (International Office of Epizootics)*, 13, 1201–1226.

Jonsson, N. N., Bock, R. E., & Jorgensen, W. K. (2008a). Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Veterinary Parasitology*, 155, 1–9.

Jonsson, N. N., Bock, R. E., & Jorgensen, W. K. (2008b). Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Veterinary Parasitology*, 155, 1–9.

Kabi, F. (2008). Sero-prevalences of Tick-borne infections among the Nkedi zebu and Ankole cattle in Soroti district, Uganda. *Journal of Protozoology Research*, 18, 61–70.

Kariuki, D. P., Young, A. S., Morzaria, S. P., Lesan, A. C., Mining, S. K., Omwoyo, P., Wafula, J. L. M., & Molyneux, D. H. (1995). *Theileria parva* carrier state in naturally infected and artificially immunised cattle. *Health and Production journal*, 27, 15–25.

Kerario, I. I., Muleya, W., Chenyambuga, S., Koski, M., & Simuunza, M. (2017). Abundance and distribution of Ixodid tick species infesting cattle reared under traditional farming systems in Tanzania. *African Journal of Agricultural Research*, 12, 286–299.

Kivaria, F. M. (2006). Estimated direct economic costs associated with tick-borne diseases on cattle in Tanzania. *Tropical Animal Health Production journal*, 38, 291–299.

Kocan, K. M. (1995). Targeting ticks for control of selected hemoparasitic diseases of cattle. *Veterinary Parasitology*, 57, 7–121.

Kocan, K. M., De La Fuente, J., Blouin, E. F., Coetzee, J. F., Ewing, S. A., & Kocan, K. M. (2010). The natural history of *Anaplasma marginale*. *Veterinary Parasitology*, 167, 95–107.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33, 1870–1874.

Laisser, E. L. K., Chenyambuga, S. W., Karimuribo, E. D., Msalya, G., Kipanyula, M. J., Mwilawa, A. J., Mdegela, R. H., & Kusiluka, L. J. M. (2017). A review on prevalence, control measure, and tolerance of Tanzania shorthorn zebu cattle to east coast fever in Tanzania. *Tropical Animal Health and Production*, 49, 813–822.

Levi Hakwale Makala, Peter Mangani, Kozo Fujisaki, H. Nagasawa. (2003). The current status of major tick borne diseases in Zambia. *Veterinary Research*, 35, 467–483.

Lorusso, V., Wijnveld, M., Majekodunmi, A. O., Dongkum, C., Fajinmi, A., Dogo, A. G., Thrusfield, M., Mugenyi, A., Vaumourin, E., Igweh, A. C., Jongejan, F., Welburn, S. C., & Picozzi, K. (2016). Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. *Parasites & Vectors*, 9.

Lynen, G., Zeman, P., Bakuname, C., Giulio, G. di, Mtui, P., Sanka, P., Jongejan, F., Lynen, G., Bakuname, · C, Mtui, · P, Sanka, · P, Zeman, P., & Jongejan, F. (2007). Cattle ticks of the genera *Rhipicephalus* and *Amblyomma* of economic importance in Tanzania: distribution assessed with GIS based on an extensive Weld survey. *Experiment Applied Acarology*, 43, 303–319.

Mamiro, K. A., Magwisha, H. B., Rukambile, E. J., Ruheta, M. R., Kimboka, E. J., Malulu, D. J., & Malele, I. I. (2016). Occurrence of ticks in cattle in the new pastoral farming areas in Rufiji district, Tanzania. *Journal of Veterinary Medicine*, 2016, 1–5.

Martin and Brocklesby, 1960. (1960). The development of Theileria = *Cytauxzoon taurotragi* from eland in its tick vector *Rhipicephalus appendiculatus*. *Parasitology*, 81, 129.

Moll, G., Lohding, A., Young, A. S., & Leitch, B. L. (1986a). Epidemiology of theileriosis in calves in an endemic area of Kenya. *Veterinary Parasitology*, 19, 255–273.

Mtshali, K., Khumalo, Z., Nakao, R., Grab, D. J., Sugimoto, C., & Thekisoe, O. (2016). Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *The Journal of Veterinary Medical Science*, 77, 1573–1579.

Muraguri, G. R., McLeod, A., McDermott, J. J., & Taylor, N. (2005). The incidence of calf morbidity and mortality due to vector-borne infections in smallholder dairy farms in Kwale district, Kenya. *Veterinary Parasitology*, 130, 305–315.

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

Njiiri, N. E., Mark, B., Bronsvoort, D., Collins, N. E., Steyn, H. C., Troskie, M., Vorster, I., Thumbi, S. M., Sibeko, K. P., Jennings, A., Conradie Van Wyk, I., Mbole-Kariuki, M., Kiara, H., Poole, E. J., Hanotte, O., Coetzer, K., Oosthuizen, M. C., Woolhouse, M., & Toye, P. (2015). The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. *Veterinary Parasitology*, 210, 69–76.

Omondi, D., Masiga, D. K., Fielding, B. C., Kariuki, E., Ajamma, Y. U., Mwamuye, M. M., Ouso, D. O., & Villinger, J. (2017). Molecular detection of tick-borne pathogen diversities in ticks

from livestock and reptiles along the shores and adjacent islands of lake Victoria and Lake Baringo, Kenya. *Frontiers in Veterinary Science*, 4, 1–15.

Ota, N., Mizuno, D., Kuboki, N., Igarashi, I., Nakamura, Y., Yamashina, H., Hanzaike, T., Fujii, K., Onoe, S., Hata, H., Kondo, S., Matsui, S., Koga, M., Matsumoto, K., Inokuma, H., & Yokoyama, N. (2009). Epidemiological survey of *Theileria orientalis* infection in grazing cattle in the eastern part of Hokkaido, Japan. *Journal Veterinary Medical Science*, 71, 937–944.

Oura, C. A. L., Bishop, R. P., Wampande, E. M., Lubega, G. W., & Tait, A. (2004). Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. *International Journal for Parasitology*, 34, 603–613.

Paling, R. W., Grootenhuis, J. G., & Young, A. S. (1981). Isolation of *Theileria mutans* from Kenyan buffalo, and transmission by *Amblyomma gemma*. *Veterinary Parasitology*, 8, 31–37.

Patalinghug Ybañez, A., Sivakumar, T., Haidee, R., Ybañez, D., Ratilla, J. C., Perez, Z. O., Gabotero, S. R., Hakimi, H., Kawazu, S.-I., Matsumoto, K., Yokoyama, N., & Inokuma, H. (2012). First Molecular Characterization of *Anaplasma marginale* in cattle and *Rhipicephalus (Boophilus) microplus* Ticks in Cebu, Philippines. *Journal of Veterinary Medical Science,*75, 27-36

Pfitzer, S. (2009). Occurrence of tick-borne haemoparasites in nyala (*Tragelaphus angasii*) in KwaZulu-Natal and Eastern Cape Province , South Africa. *UPSpace Institutional Repository*, *June*, spi.

Razmi, G. R., Hosseini, M., & Aslani, M. R. (2003). Identification of tick vectors of ovine theileriosis in an endemic region of Iran. *Veterinary Parasitology*, 116, 1–6.

Razmi, G., & Yaghfoori, S. (2013). Molecular surveillance of *Theileria ovis, Theileria lestoquardi* and *Theileria annulata* infection in sheep and ixodid ticks in Iran. *Onderstepoort Journal of Veterinary Research*, 80(1).

Renneker, S., Abdo, J., Salih, D. E. A., Karagenç, T., Bilgiç, H., Torina, A., Oliva, A. G., Campos, J., Kullmann, B., Ahmed, J., & Seitzer, U. (2013). Can *Anaplasma ovis* in Small Ruminants be Neglected any Longer? *Transboundary and Emerging Diseases*, 60, 105–112.

Ros-García, A., Barandika, J. F., García-Pérez, A. L., Juste, R. A., & Hurtado, A. (2013). Assessment of exposure to piroplasms in sheep grazing in communal mountain pastures by using a multiplex DNA bead-based suspension array. *Parasites and Vectors*, 6, 1–11.

Schnittger, L., Yin, H., Jianxun, L., Ludwig, W., Shayan, P., Rahbari, S., Voss-Holtmann, A., & Ahmed, J. S. (2000a). Ribosomal small-subunit RNA gene-sequence analysis of *Theileria lestoquardi* and a *Theileria* species highly pathogenic for small ruminants in China. *Parasitology Research*, 86, 352–358

Simuunza, M., Weir, W., Courcier, E., Tait, A., & Shiels, B. (2011). Epidemiological analysis of tick-borne diseases in Zambia. *Veterinary Parasitology*, 175, 331–342.

Stuen, S. (2016). Haemoparasites in small ruminants in European countries: Challenges and clinical relevance. *Small Ruminant Research*, 142, 22–27.

Swai, E. S., Karimuribo, E. D., Kambarage, D. M., & Moshy, W. E. (2009). A longitudinal study on morbidity and mortality in youngstock smallholder dairy cattle with special reference to tick borne infections in Tanga region, Tanzania. *Veterinary Parasitology*, 160, 34–42.

Swai, E. S., Karimuribo, E. D., Kambarage, D. M., Moshy, W. E., & Mbise, A. N. (2007). A comparison of seroprevalence and risk factors for *Theileria parva* and *T. mutans* in

smallholder dairy cattle in the Tanga and Iringa regions of Tanzania. *Veterinary Journal*, 174, 390–396.

Tayebwa, D. S., Vudriko, P., Tuvshintulga, B., Guswanto, A., Nugraha, A. B., Gantuya, S., Batiha, G. E. S., Musinguzi, S. P., Komugisha, M., Bbira, J. S., Okwee-Acai, J., Tweyongyere, R., Wampande, E. M., Byaruhanga, J., Adjou Moumouni, P. F., Sivakumar, T., Yokoyama, N., & Igarashi, I. (2018a). Molecular epidemiology of *Babesia* species, *Theileria parva*, and *Anaplasma marginale* infecting cattle and the tick control malpractices in Central and Eastern Uganda. *Ticks and Tick-Borne Diseases*, 9, 1475–1483.

Tembo, S., Collins, N. E., Sibeko-Matjila, K. P., Troskie, M., Vorster, I., Byaruhanga, C., & Oosthuizen, M. C. (2018). Occurrence of tick-borne haemoparasites in cattle in the Mungwi District, Northern Province, Zambia. *Ticks and Tick-Borne Diseases*, 9, 707–717.

Terkawi, M. A., Huyen, N. X., Shinuo, C., Inpankaew, T., Maklon, K., Aboulaila, M., Ueno, A., Goo, Y. K., Yokoyama, N., Jittapalapong, S., Xuan, X., & Igarashi, I. (2011). Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the northeast region of Thailand. *Veterinary Parasitology*, 178, 201–207.

Tibbitts, T., Goff, W., Foreyt, W., & Stiller, D. (1992). Susceptibility of two rocky mountain bighorn sheep to experimental infection with *Anaplasma ovis. Journal of Wildlife Diseases*, 28, 125–129.

Torino A., Agnone, A., Blanda, V., Alongi, A., D'Agostino, R., Caracappa, S., Marino, A. M. F., di Marco, V., de La Fuente, J., Renneker, S., Abdo, J., Salih, D. E. A. A., Karagenç, T., Bilgiç, H., Torina, A., Oliva, A. G., Campos, J., Kullmann, B., Ahmed, J. S., Wilson, M. (2017). Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *Veterinary Parasitology*, 8, 1573–1579.

Uilenberg, G. (1995). International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Veterinary Parasitology*, 57, 19–41.

Vreysen, M. J. B., & Khamis, I. S. (1999). Notes on the Ecology of a Natural Glossina austeni (Diptera: Glossinidae) Population in the Jozani Forest, Unguja Island of Zanzibar. *International Journal of Tropical Insect Science*, 19, 99–108.

Wamuyu, L., Obanda, V., Kariuki, D., Gakuya, F., Makanda, M., Otiende, M., & Ommeh, S. (2015). Molecular Detection and Characterization of Theileria Infecting Wildebeest (*Connochaetes taurinus*) in the Maasai Mara National Reserve, Kenya. *Pathogens*, 4, 626–638.

Wesonga, F. D., Orinda, G. O., Ngae, G. N., & Grootenhuis, J. (2006). Comparative tick counts on game, cattle and sheep on a working game ranch in Kenya. *Tropical Animal Health and Production*, 38, 35–42.

Woodford, J. D., Jones, T. W., Rae, P. F., Boid, R., & Bell-Sakyi, L. (1990). Seroepidemiological studies of bovine babesiosis on Pemba Island, Tanzania. *Veterinary Parasitology*, 37, 175–184.

Woolhouse, M. E. J., Thumbi, S. M., Jennings, A., Chase-Topping, M., Callaby, R., Kiara, H., Oosthuizen, M. C., Mbole-Kariuki, M. N., Conradie, I., Handel, I. G., Poole, E. J., Njiiri, E., Collins, N. E., Murray, G., Tapio, M., Auguet, O. T., Weir, W., Morrison, W. I., Kruuk, L. E. B., Toye, P. G. (2015). Co-infections determine patterns of mortality in a population exposed to parasite infection. *Science Advances*, 1, 1400026–1400026.

Yin, H., Schnittger, L., Luo, J., Seitzer, U., & Ahmed, J. S. (2007). Ovine theileriosis in China: A new look at an old story. *Parasitology Research*, 101, 191–195.

Young, A. S., Purnell, R. E., Payne, R. C., Brown, C. G. D., & Kanhai, G. K. (1978). Studies on the transmission and course of infection of a Kenyan strain of *Theileria mutans*. *Parasitology*, 76, 99

Yousefi, A., Rahbari, S., Shayan, P., Sadeghi-dehkordi, Z., & Bahonar, A. (2017). Molecular detection of *Anaplasma marginale* and *Anaplasma ovis* in sheep and goat in west highland pasture of Iran. *Asian Pacific Journal of Tropical Biomedicine*, 7, 455–459.