EPIDEMIOLOGY OF BABESIA INFECTION IN SHEEP IN KAJIADO CENTRAL SUB-COUNTY, KENYA

BY

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DECLARATION

This thesis is my original work and has not been presented for Award of degree in any other University

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DEDICATION

I dedicated this thesis to my late father, my mother, and my beloved sisters and brothers.

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ABBREVIATIONS AND ACRONYMS

ANOVA	-	Analysis Of Variance
BUN	-	Blood Urea Nitrogen
CCPP	-	Contagious Caprine Pleuropneumonia
DIC	-	Disseminated intravascular coagulation
DNA	-	Deoxy ribonucleic Acid
ELISA	-	Enzyme-linked immunosorbent assay
FAO	-	Food and Agriculture Organization
Hb	-	Hemoglobin
HDL	-	High-density lipoproteins
ICT	-	In-Circuit Test
IFAT	-	The immunofluorescence antibody test
IMDP	-	Imidocarb Dipropionate
Km^2	-	Square kilometre
LDL	-	Low -density lipoproteins
MCHC	-	Mean corpuscular hemoglobin concentration
MCV	-	Mean corpuscular volume
MS	-	Microsoft
OIE	-	World Organization for Animal Health
PCR	-	Polymerase Chain Reaction
PPR	-	Peste des Petits Ruminants disease
PCV	-	Packed cell volume
RBC	-	Red blood cells

RLB	-	Reverse Line blot hybridization.
rRNA	-	Ribosomal RNA
UN	-	United Nations.
WBC	-	White blood cells

ABSTRACT

Ovine *Babesia* infection is a tick borne disease caused by hematotropic parasites of the genus *Babesia. Babesia* infections in sheep and goats are caused by three *Babesia* species, namely *Babesia motasi, Babesia. crassa and Babesia ovis*. Significant economic losses due to the disease have been reported in tropical and subtropical part of the world yet the status of *Babesia* infection in Kenya remains largely unclear.

The objectives of the study were to 1) To determine the *Babesia* haemoparasites infecting sheep in Kajiado central 2) To identify the possible risk factors associated with the *Babesia* infection of sheep in Kajiado central sub County 3) To compare the performance of microscopy and PCR in detecting *Babesia* infection of sheep in the study areas. This study was a cross-sectional study and was conducted in purposively selected locations of Oldonyork, Matasi, Bissil, Namanga Portland, Ngaatatock, Sajiloni, and Oloosuyian in Kajiado central sub-county. Farmers owning sheep were initially identified and random sample of 31 flocks selected to participate in the study. To determine risks factors associated with *Babesia* infection of sheep, data on flock characteristics and management practices including common diseases and treatment status of the flocks were collected using questionnaires administered to sheep owners.

A total of 395 blood samples were also collected from sheep and blood smears prepared for subsequent examination by microscopy for presence of *Babesia* parasites. Thereafter, DNA was extracted from the blood samples and the presence of *Babesia* DNA was determined by polymerase chain reaction. The collected data were entered in MS Excel® and analyzed by using Stata® statistical program. Kappa statistic was used to assess agreement between PCR and microscopy tests. A univariate analysis was done to screen for possible risk factors associated with infection of sheep with *Babesia* ($p<10\%$).

Microscopic examinations revealed presence of *Babesia* parasites in sheep erythrocytes. PCR analysis confirmed the presence of 239 bp specific band corresponding to the DNA of *Babesia* species. Of the 395 sheep sampled, 22 (5.6%) were positive for *Babesia spp*. upon microscopic examination whereas 13 (3.3%) were positive for the presence of *Babesia spp* by PCR. The level of agreement between microscopic test and PCR was 73%.Farmers managed diseases either by spraying with acaricides (96.8%), deworming or by injections (3.2%). The sex of sheep and presence of ticks on dogs were significantly associated with ovine *Babesia* infection. The findings indicate that sheep in Kajiado County are infected with *Babesia* parasites and it would be interesting to establish their pathogenicity.

This study provides useful baseline data, which when shared with relevant government authorities, could be useful in designing surveillance strategies for effective control and better management of sheep. Subsequently, it is recommended that further research on molecular epidemiology of *Babesia spp* in sheep in pastoralist areas, especially in Kajiado to build on the results obtained in this study.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Babesia infection is a tick-borne disease caused by hematotropic parasites of the genus *Babesia* (Homer *et al*., 2000) The disease affects mammals and birds and significantly impacts on the health of farm and pet animals. Therefore, *Babesia* infection is associated with huge economic losses worldwide. Several animal species are thought to be potential *Babesia* hosts. All vertebrates are potential carriers as they also serve as hosts for the parasites (Leonhard *et al.*, 2012). *Babesia* infection also occurs in domestic animals including cattle, sheep, goats, horses, dogs, cats and pigs. The disease is very common in small ruminants (Theodoropoulos *et al*., 2006). *Babesia ovis (B. ovis)* is pathogenic especially in sheep and its case-fatality in susceptible hosts range from 30 to 50 % in field infections. *Babesia* infection has also been reported in various wild carnivore and ungulate species (Hashemi-Fesharki, 1977).

Babesia infection in domesticated small ruminants is caused by three species, namely *Babesia motasi (B. motasi), Babesia crassa (B. crassa)* and *B. ovis* (Friedhoff *et al.,* 1988). The blood parasite is transmitted by ticks of the genus *Rhipicephalus*. *B. ovis* is endemic in southern Europe, the Middle East, and central Asia. The geographical distribution of *Babesia* infection in South-East Asia and in Africa is however not well known (Friedhoff *et al*., 1997).

The economic loss associated with *Babesia* infection in sheep production in tropical and subtropical areas is enormous (Bai *et al*., 2002).*Babesia* infection of sheep remains an important impediment to meat and milk production because of the resulting parasitemia and death (Caracappa *et al*., 1999). The blood picture is usually drastically affected in infected animals (Radostits *et al.*, 2000). *Babesia* infection is more common in small ruminants and a prevalence

of between 30% and 50% have been reported in Iran (Gonzales *et al*., 2007; Klockiewicz *et al*., 2007; Criado-Fornelio *et al*., 2003). In Korea, a *Babesia* parasite, which is capable of infecting the human, has been reported to be very close to the *Babesia* infecting sheep in China (Jung-Yeon *et al*., 2007). Although *Babesia* infections reported above have resulted in significant economic losses in tropical and subtropical part of the world, information on the status of the infection of sheep in Kenya and especially in Kajiado central is still largely lacking. In this regard, this thesis reports the epidemiology of *Babesia* infection in sheep reared in Kajiado central sub-county with focus on occurrence of the infection, risk factors associated with the disease as well as test agreements between microscopy and PCR when used for the detecting the infection.

1.2 Problem statement

Babesia infection is an emerging zoonotic disease that affects livestock and human with life-threatening implications, particularly in the immune compromised individuals. *Babesia* infection of sheep has been reported in a number of countries including; Iran (Dehkordi *et al*., 2010), China (Guan *et al*., 2012), Sudan (Osman *et al*., 1997) and Somalia (Ahmed *et al*., 2013). *Babesia* infection of sheep causes losses in production of milk, meat, and other livestock by-products with possibility of death in severe cases (Perez *et al*., 2010). The infection causes severe economic losses to sheep farmers in tropical and sub-tropical regions. In Kenya, *Babesia* infection is ranked among the most common causes of economic losses in animal production (Wesonga *et al*., 2010). The occurrence and epidemiology of *Babesia* infection of sheep in many African countries and especially in Kenya is still scanty and existing information is not up to date with available data generated more than 5 years ago. Moreover, the techniques used for the detection of the infection in Kenya have relied on the use of microscopy and Enzyme-linked

Immunosorbent Assay (ELISA) techniques and not molecular method such as PCR, which known to be sensitive and able to detect early infections. Therefore, there is a gap in knowledge especially with regard to the detection, general epidemiology and occurrence of *Babesia* infection in sheep. The knowledge gap is essential for the control and prevention of *Babesia* infection of sheep reared in Kajiado central sub-County in Kenya and thus preventing economic losses to sheep farmers.

1.3 Justification for the study

Sheep production is a source of important livelihood particularly in pastoralist communities of Kenya including Kajiado central sub-county. There are very limited studies on infection of sheep with *Babesia* including specific data on general epidemiology and molecular detection methods. An understanding of general epidemiology and detection of the *Babesia* infection using nucleic acid-based techniques will aid in developing molecular methods that would be key in designing sustainable strategies for the control and prevention of the disease in the country. The general epidemiological data obtained from this study provide useful baseline information that when shared with relevant government authorities, could be useful in designing appropriate surveillance strategies for effective surveillance, control and better management of the infection in the future, especially in ovine species.

1.4 Hypothesis

There exist potential risk factors for *Babesia spp* infection in sheep reared in Kajiado County and these factors may contribute to occurrence of *Babesia spp* infection in sheep.

1.5 General Objective

The general objective is to establish the occurrence and epidemiology of infection of sheep with *Babesia* in Kajiado Central

1.6 Specific objectives

1. To determine the occurrence of *Babesi*a species infections of sheep reared in Kajiado Central
2. To identify the risk factors associated with the *Babesia* infection of sheep in Kajiado Central
3. To compare the performance of microscopy and PCR for detection of *Babesia* infection in sheep in the study areas above

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aetiology and transmission

Babesia infection is as result of infection with the protozoa of the genus *Babesia,* which belongs to the family *Babesiidae,* and the order *Piroplasmida*. *Babesia spp* infection in sheep is caused by three species, namely *B. motasi, B. crassa* and *B. ovis* (Friedhoff *et al*., 1988). *Babesia* infection in sheep is transmitted by ticks through bites. The mode of transmission between the ticks and the hosts is transovarial where either nymph or the adult ticks can transmit the disease (Cruz-Flores *et al.,* 2008). The ticks become infected when they ingest babesia parasites in the blood of infected sheep (Leonhard *et al*., 2012). The parasite in sheep is transmitted by ticks of the genus *Haemaphysalis* (*H. Punctata, H. Otophila*), *Dermacentor* (*D. silvarum*), *Hyalomma spp* and *Rhipicephalus (Rhipicephalus. Bursa)* (Rehman *et al.,* 2004). The ticks are sensitive to climatic conditions and require a relative humidity of at least 80% in order to survive. Typical habitants of the ticks that transmit the infection include deciduous and coniferous woodland, heathland, moorland, rough pasture, forests and urban park (Gassner *et al*., 2011).

2.2 Pathogenesis of *Babesia* infection

The infective form of *Babesia* is known as sporozoites and is produced in the salivary gland of the tick vectors. The sporozoites are usually injected into the sheep by larvae or adult tick when feeding on the host. The parasite then attacks the host erythrocytes and then destroys them. This destruction leads to the release of hemoglobin in circulation (Alani *et al.,* 1988). The erythrocytic cycle continues until the animal dies or its immune system is overwhelmed. *Babesia* parasites may be present in the blood system in small numbers sometimes even for many years without causing the disease (Alani *et al*., 1988). There are a number of changes in hematological and

biochemical profiles linked to the destruction of erythrocytes by *Babesia* following infection of sheep. As parasitemia advances, infected animals reveal a significant decrease in erythrocytes counts, packed cell volume (PCV) level, haemoglobin (Hb)-concentration, mean corpuscular Volume (MCV), and mean corpuscular haemoglobin concentration (MCHC). The biochemical changes seen in this case include alterations of total serum protein, as well as changes in levels of albumin, urea, creatinine, triglyceride, cholesterol, high-density lipoprotein (HDL) and a decrease in low-density lipoprotein [LDL] (Bijan *et al.*, 2012). In contrast, a further increase in parasitemia level has been reported to result in a significant increase in white blood cells (WBC) count, especially neutrophils. Changes in biochemical profiles such as an increase in the concentration of blood urea nitrogen (BUN), creatinine, total protein, albumin, globulin, triglyceride, cholesterol, HDL, and LDL have also been documented (Peinado *et al*., 1995).

2.3 Clinical Signs of *Babesia* infection

After infection with the parasite, sheep develop fever and parasitemia within 2 to 4 days followed by clinical signs of the disease, which include anorexia, listlessness, anemia, moderate jaundice, and haemoglobinuria. In immunocompetent animals, hyperthermia usually returns to normal level on the fourth day after the peak pyrexia. Thereafter, parasitemia may reduce to a low level or even zero during the course of the disease (Rahbari *et al.*, 2008). Most cases of *Babesia* infection are seen in adults and animals younger than nine months, which usually remain asymptomatic. The level of parasitemia and the degree of anaemia are not usually correlated. The decrease in PCV has been reported to range from 30 to 40%. In some studies, parasitized erythrocytes have not been observed to block capillaries in the brain and it has been postulated that the failure of the cytoadherence to brain capillaries may contribute to the absence of nervous symptoms in acute babesiosis. Other studies have reported that kidneys are severely

affected exhibiting acute glomerulonephritis and it is thought that the lesions observed are due to vascular alteration and stasis, leading to anoxia of the tissues. A disseminated intravascular coagulation (DIC) syndrome has been reported in sheep infected with *Babesia* (Yeruham *et al*., 1998).

2.4 Epidemiology and Risk factors of *Babesia* infection

The occurrence of the disease is dependent on the distribution of the ticks that transmit the disease. Many studies are available on associated risk factors of the protozoan infections in different countries. These countries include Iran (Bijan *et al*., 2015), Kenya (Okuthe and Buyu, 2006), Greece (Theodoropoulos *et al.*, 2006), Rwanda (Bazarusanga *et al*., 2007), Bolivia (Gonzales *et al.*, 2007) and Uganda (Magona *et al.*, 2008). These studies have documented that age, sex, species, breed, and season, as well as feeding system and watering system are the major risk factors, which are associated with the prevalence of ovine *Babesia* infection in sheep.

2.5 Prevalence of ovine *Babesia* infection

The prevalence of *B. ovis* infection in Lohi sheep in Pakistan has been found to be 50% (Iqbal *et al*., 2011) while a separate study reported a prevalence of 34% in infected sheep on detection by PCR (Shahzad *et al*., 2013). In Nigeria, a prevalence of 7.5% has been reported for *B. ovis*-infection in sheep with prevalences of 40% for Yankasa breed, 26.7% for Ouda breed and 33.3% for the Koraji breed (Biu *et al*., 2009). A study done in Zaria Abattoir in Nigeria did not report any cases of *B. ovis* infection (Onoja *et al*., 2013). An overall prevalence of between 45% and 95% has been reported in a study done in Machakos, Kenya. This prevalence was determined by using microscopy and ELISA to detect the presence of the *Babesia* (Wesonga *et al*., 2010).

2.6 Diagnosis of ovine babesiosis

The diagnosis of ovine *Babesia* infection has been done using a range of techniques such as microscopy, serological methods, and molecular techniques. These techniques outlined in the following sections reviewed below.

2.6.1 Microscopy

In the acute phase, diagnosis of ovine *Babesia* infection is mainly based on the microscopic examination of Giemsa-stained blood smears and clinical symptoms (Aktas *et al*., 2005). Though microscopy is good for confirmatory diagnosis as revealed by the demonstration of the piroplasms in erythrocytes, the technique may not detect the parasites during subclinical infections because of low parasitemia experienced during this stage. Furthermore, examination of blood smears by microscopy may be influenced by the technical skills of the laboratory technician. In some cases, the morphological alterations of host erythrocytes and *Babesia* parasites as seen in the advanced stages of acute infections may result in false diagnoses of the infection (Uilenberg *et al.*, 2001).

2.6.2 Serological detection of ovine *Babesia* infection

Serological tests used for the detection of ovine *Babesia* infection are usually antibody-based approaches, though some may be based on the detection of circulating antigens of the parasite in the ovine blood. The samples used in these cases are mainly serum. The specific serological methods that have been used include ELISA, immune fluorescent antibody test (IFAT) and immune chromatographic test (ICT). The serological tests are useful for diagnosis of subclinical and chronic infections (Bose *et al*., 1995). For example, serological tests such as IFAT and ELISA have been used for diagnosis chronic *B. ovis* infection (Sevinc *et al.*, 2013; Ekici *et al.*, 2012; Wright 1990; Sevinc *et al*., 2007). The use of IFAT requires skilled personal and

experienced operators. Other earlier studies have also used ELISA to detect *B. ovis* antibodies (Emre *et al*., 2001, Cicek *et al*., 2004, and Duzgun *et al*., 1991).

2.6.3 Molecular detection of ovine babesiosis

Molecular tools employing the detection of nucleic acids such as DNA have been used to detect the presence of *Babesia* species in sheep blood (Shayan *et al.,* 2008). For example techniques such as PCR targeting specific genes of the parasite have been used to detect *Babesia spp* such as *B. ovis, B. crassa, and B. motasi* (Almeria *et al.*, 2001). The specific types of PCR that have been used to detect the parasites in sheep blood include conventional PCR, nested PCR, and real-time PCR.

Conventional PCR has been used in some studies to screen for *Babesia spp* including *B. ovis*. For example, Mousa*et al.* (2013) and Aktas *et al*. (2016) used 18S rRNA of the piroplasms for detection of *Babesia* species including *B. ovis*. In the study of Aktas *et al.* (2016) the PCR assay was used together with reverse line blotting assays to screen for the piroplasms in sheep blood. A conventional PCR has also been used for diagnosis of subclinical infection of sheep with *B. ovis* (Hurtado *et al.,* 2015). This method has been used together with a semi-nested PCR to improve on the sensitivity for the diagnosis of subclinical infection of sheep with *Babesia* parasites. Bijan *et al.* (2015) also determined the prevalence of *B. ovis* in sheep and goats by conventional PCR and detected the parasites in the blood of sheep and goats. This method has been used together with microscopic examinations to confirm the presence of the piroplasms in blood.

Nested PCR has also been used to detect *Babesia* species in blood of sheep just like conventional PCR and other modern PCR techniques. Niu *et al*. (2017) used nested-PCR assay targeting the rap-1a gene, to determine the prevalence of *Babesia spp* in small ruminants and ticks in China. This method detected *Babesia* haemoparasites in 3.89% of blood samples and 6.4% in tick

samples. There is also another report where this technique was used to increase the sensitivity of the PCR generating two amplified products for confirmation purposes (Guiquan *et al.*, 2008). Another study also used nested PCR to detect *Babesia* in sheep and goats (Leonhard *et al*., 2003).

In another recent study, the presence of *B. ovis* in *R bursa* has been detected by using real-time PCR. This real-time PCR was used to target surface protein D of *B. ovis* (BoSPD) and has been used to detect *B. ovis* in naturally infected sheep and ticks (Erster *et al*., 2016). In another study published in 2015, a multiplex real-time PCR assay was used for the detection of the parasite in sheep blood. This method employed a species-specific multiplex real-time PCR to detect *B. ovis* and other blood parasites including *Theileria ovis* and *Anaplasma ovis* (Hurtado *et al*., 2015).

2.7 Treatment and Control

There are many drugs that have been used for the treatment of ovine Babesiosis including Quinuronium sulphate, Acaprin, Diminazene aceturate (Berenil®) and Imidocarb dipropionate salt (Imizol®) (Hashemi-Fesharki, 1977). Treatment of *Babesia* infections is usually done to cure, control and even prevent the spread of the infections. The transmission and spread of infection of sheep with *Babesia* parasites is controlled mainly by using acaricides to control the tick-vectors and by practising good pasture management (Ahlam *et al*., 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was done in Kajiado central sub-County of Kajiado County. Kajiado is one of the counties in the former Rift Valley Province of Kenya. It has a population of 687000 and an approximate area of 21,293 km². The county borders Nairobi and extends to the Tanzania border further south. The county capital is Kajiado but the largest town is Ngong. Livestock population is high; cattle (247,000), sheep (533,000) and goats (507000). The selected study areas included oldonyorok, Matasia, Namanga, Bissil, Portland, Ngaatatock, Sajiloni, and Oloosuyian. The areas were selected based on a number of factors including the costs of sampling, the distance between the flocks and precision to be maintained in this study.

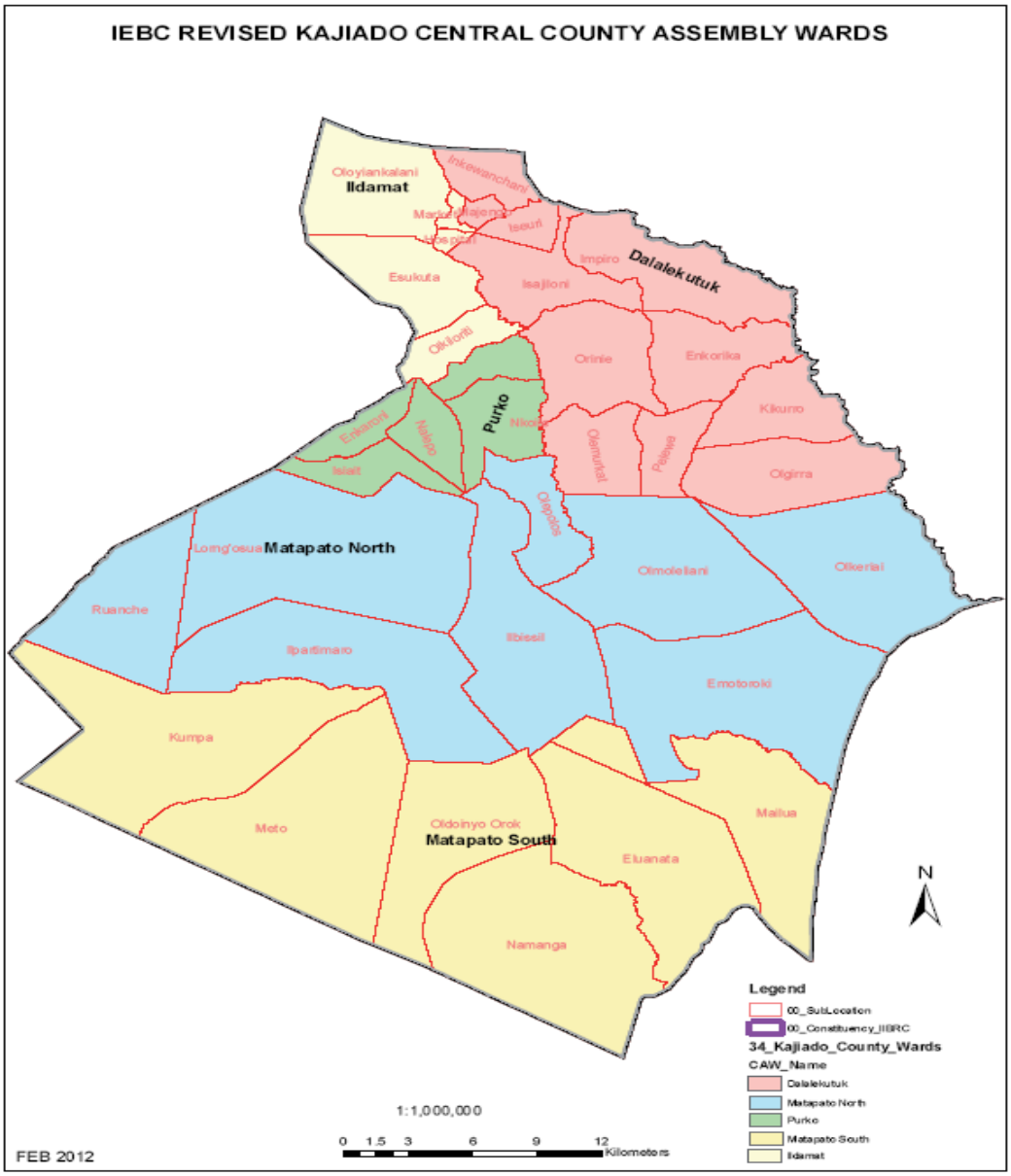

Figure 1: Map of Kajiado central sub-county showing the areas of study (Source: www.kajiado.go.ke)

3.2 Sample size determination and Sampling of flocks

This study was a cross-sectional study conducted randomly in oldonyorok, Matasi, Namanga, Bissil, Portland, Ngaatatock, Sajiloni, and Oloosuyian. The selection of the study areas was based on a number of factors including the costs of sampling, the distance between the flocks and precision to be maintained in this study.

3.2.1 Sample size determination

Sample size was calculated as described by Martin et al. (1987) using the formula

$n= (Z_{\alpha/2}^2 * p * q)/L^2$

Where n= sample size, $Z_{\alpha/2} = 1.96$ the value of Z_α required for (2-tailed test) confidence level of 95%

L = Precision of the estimate, p= a priori estimate of the prevalence of *Babesia* in sheep and

$q = 1 - p$

A prevalence estimate of 15% used based on a study done in Egypt by Hussein *et al*.(2017). A precision (L) of 0.05 was used. Thus $n= (1.96^2 * 0.15 * 0.85)/0.05^2$

n=196 heads of sheep which was adjusted to 392 to account for clustering.

3.2.2 Selection of flocks and sheep

A list of flocks in each area was obtained from local administrative leaders including village elders. The list included; the name of the farm, location (area), an estimate of the number of animals owned and if the flocks comprised of either sheep alone or both sheep-flocks and cattle herds. A total of 31 flocks were purposively selected in the study. The number of flocks selected to participate in the study was based on a number of factors including the costs of sampling, the distance between the flocks and precision to be maintained in this study. The flocks were selected using randomly generated computer numbers. A fixed number of sheep was sampled

from each flock. The sampling strategy ensured inclusion of animals from different animal age categories. A questionnaire was administered and blood samples collected during each visit to the farm.

3.3 Data collection by questionnaire administration

Data for sheep characteristics included sex, age and tick infestation. Both data on sheep and flocks characteristics were collected by administering questionnaires to the animal owners. The characteristics studied here included flocks-location, flocks-size, animal species and tick-infestation of dogs. The questionnaires were also used to capture data on challenges and opportunities on sheep rearing as well as the common diseases in the study area. Questionnaire also captured the vaccination program for sheep, last infections and tick control system.

3.4 Blood sample collection and handling

A total of 392 whole blood samples in anticoagulant were collected for microscopic examination and detection of the parasite DNA by PCR. Sheep were restrained and about 5 ml blood collected into vacutainer tubes from the jugular vein using sterilized needles. Care was taken to avoid any contamination of the samples. The samples were transported in cool boxes with ice packs to the Department of Public Health, Pharmacology and Toxicology Laboratory and stored in -20^0C pending laboratory analyses.

3.4.1 Blood smear preparation and microscopic examination

Thick and thin smears of the blood samples were made on newly labeled glass slides. The dried blood smears were fixed in absolute methyl alcohol for one minute. To detect *Babesia*, Giemsa staining was done as described by (Almeria *et al.*,2001), for about thirty minutes and the stained smears observed by a microscope under oil immersion at 100 X objective. The photos of blood

smears that revealed *Babesia*-infected erythrocytes were taken and then saved in USB flash disk for further documentation.

3.4.2 Extraction of DNA from blood

The genomic DNA of *Babesia* was extracted in a laboratory at the Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine of the University of Nairobi, Kenya, using a commercial DNA extraction kit according to the manufacturer's instructions (QIAamp DNA Blood Mini-Kit, Germany). To extract the parasite DNA, 800 µl of genomic lysis buffer was added to 200 µl of whole blood and then transferred to zymospin column in collection tubes. Thereafter, the mixture was centrifuged at 10000 revolutions per minute for 1minute and the column with bound DNA transferred into new collection tubes. The bound DNA in the column was pre-washed with 200 µl of DNA-pre-wash buffer followed by another round of washing with 500 µl of DNA wash buffer by centrifuging for 1 minute. The washed bound DNA in the column was eluted with 50 µl of DNA elution buffer and the eluted DNA was collected in 1.5 ml DNAase free-Eppendorf tubes. The DNA samples were stored at -30°C pending further PCR analysis.

3.4.3 Detection of *Babesia* DNA by PCR

A pair of specific primers targeting 16S rRNA of *Babesia spp* infecting sheep were used for the amplification by PCR using Veriti 96 well thermocycler (Applied Biosystems, Singapore).The primers that were used in this study included Bassp-forward primers (5′CAG-GAT-TGC-TTT-CGC-AAC-AAG-3′) ,and Bass- reverse (5′CCTTGACATAACCGG CGAGG3') as described by (Shayan *et al*., 2007). Another pair of primers targeting *B. ovis* secretory antigen 1 (BovSA1) was designed for confirmation of the *B. ovis*. The primer pair designed included forward primer-BovSA15'-CAG,TCT,TAG,CGG,AGA,AGC,ATA,C-3' and reverse primer BovSA1 5'-

CTT,GGG,TTA,CGA,CAG,CCT,AAA-3'. To amplify the *Babesia spp* specific DNA, a 12.5µl PCR mixture containing 2 µl DNA template, 6.25ml master mix, 0.5µl each for forward and reverse primers as well as 3.25 µl deionized distilled water was used. The amplification involved an initial cycle at 94^{o}C for 5 min followed by 35 cycles of denaturation at 94^{o}C for 45 sec, annealing at 56^{o}C for 45 seconds and extension at 72°C extension for 45 seconds. These cycles were followed by a final extension at 72°C for 10 min. The PCR -amplicons were then separated by gel electrophoresis for 45 minutes using 1.5% agarose dissolved in a total volume of 100 ml containing 0.5 x Tris-Borate EDTA buffer and 7 µl of ethidium bromide. Thereafter, the electrophoresed and stained DNA was visualized with UV transilluminator and a photograph of the gel was taken using gel viewer (Gelmax 125 Imager, UVP, Cambridge, UK). A 50bp DNA ladder was used as the molecular size marker.

3.5 Data Management and Analyses

Both field and laboratory data were entered in MS Excel® sheet and later exported to Stata for further analysis. Descriptive statistical measures including measures of central tendency (arithmetic mean, median) and measures of dispersion (standard deviation) as well as frequencies were determined. Chi-square test was used to assess the relationship between the variables which were investigated. Correlation between variables (sex, age. housing system, grazing system, tick control system), was determined using a p-value of < 0.10, which was considered as significant. Finally, Kappa statistic was used to measure the level of agreement between the microscopy test and PCR analysis, Kappa result was interpreted as follows: values ≤ 0 indicated no agreement and 0.01–0.20 indicated none to slight, 0.21–0.40 considered as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement as described by Cohen.(1960).

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of sheep owners in the study areas

All the 31(100%) farmers selected in all the study areas, which included Oldonyorok, Matasi, Namanga, Bissil, Portland, Ngaatatock, Sajiloni, and Oloosuyian participated in providing questionnaire data. The sheep owners were all married people. The breed of sheep which was mostly kept in these farms was categorized as crossbreeds (74%), but the rest were categorized as local breeds. These sheep were mostly watered from ponds (water pans) since the study area didn't have rivers and tap water, and there were no proper housing facilities for the sheep, and therefore they were mostly kept outdoors. Most of the flock sizes were over 30 sheep per flock (81%), with only 19% of the respondents reporting that they had less than 30 sheep per flock. Questionnaire data revealed that the average age in years of the respondents was 45.13 (±13.03) years. Most of them (58%; 18/31) had no formal education, and only 39 % had attained primary school education. All respondents were married and were mostly males (98%) (Table1). As shown in figure 2, 63% of the farmers in Kajiado central sub-county reared sheep mainly for income generation, but the others 41% kept them as a source of milk for consumption. Some respondents preferred keeping sheep because they had a relatively faster growth rate and were easier to handle. These flocks were mostly mixed comprising of sheep reared together with either goats or cattle. Most of these sheep flocks consisted of cross-breed sheep raised in the open housing system.

Table 1: Description of the characteristics of the sheep farmers in Kajiado central sub-county

Description of the farms characteristics (n=31)		% Descriptive statistic measure
Owner age	<45	57
	>45	43
Material status	Married	100
	Single	0
Breed	Cross	74
	Local	26
Source of water	Tap	0
	Pond	100
Housing system	Open	100
	Close	0
Flock size	<30	19
	>30	81
Flock	Mixed	74
	Sheep only	26

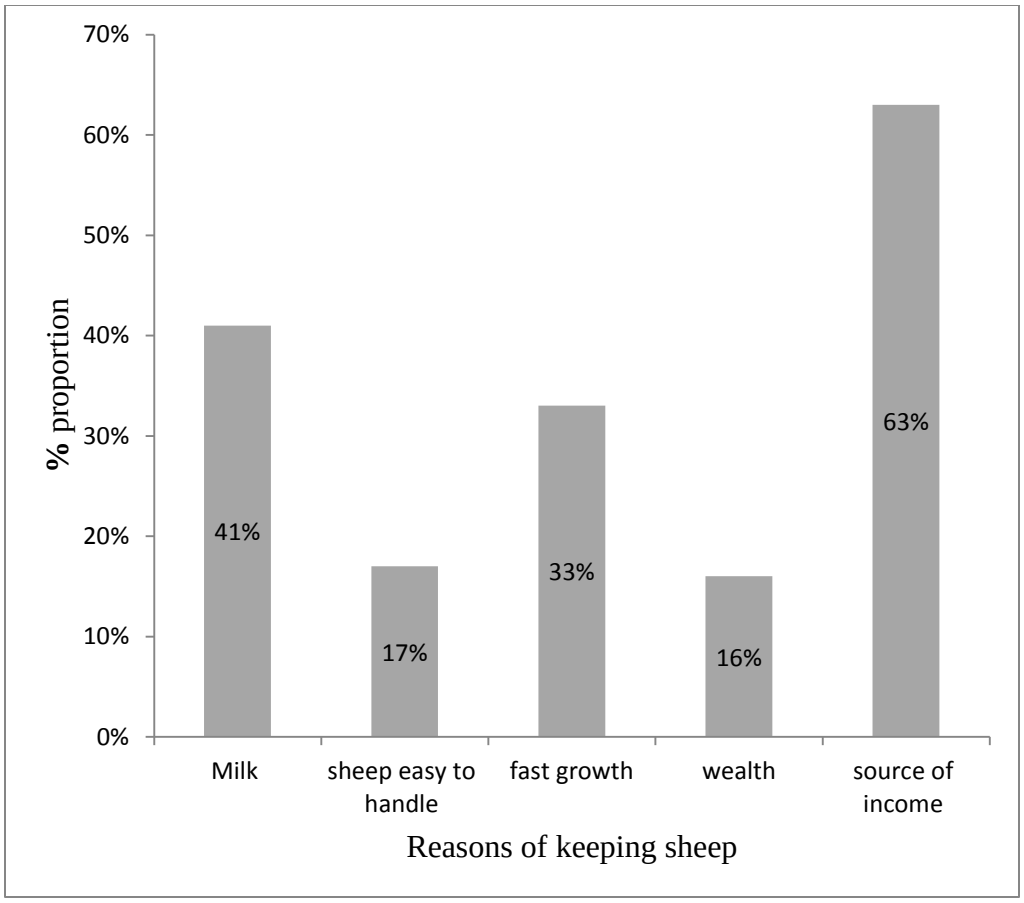

Figure 2: Reasons for keeping sheep by farmers in Kajiado central sub-county

4.2 Flock health and disease control

The common diseases reported by sheep owners included; worm infections (44%), respiratory infections (35%), Foot and Mouth disease (28%) and Heart water (27%). Other reported diseases which were reported included sheep & goat pox, diarrhea, Contagious Caprine Pleuropneumonia, enterotoxaemia, sheep pox and anaplasmosis (Figure 3). The main challenges hindering sheep production in the area included frequent disease outbreaks, wildlife conflicts especially attacks by the hyenas and, frequent human conflict especially with regard to pasture and water resources (Figure 4).

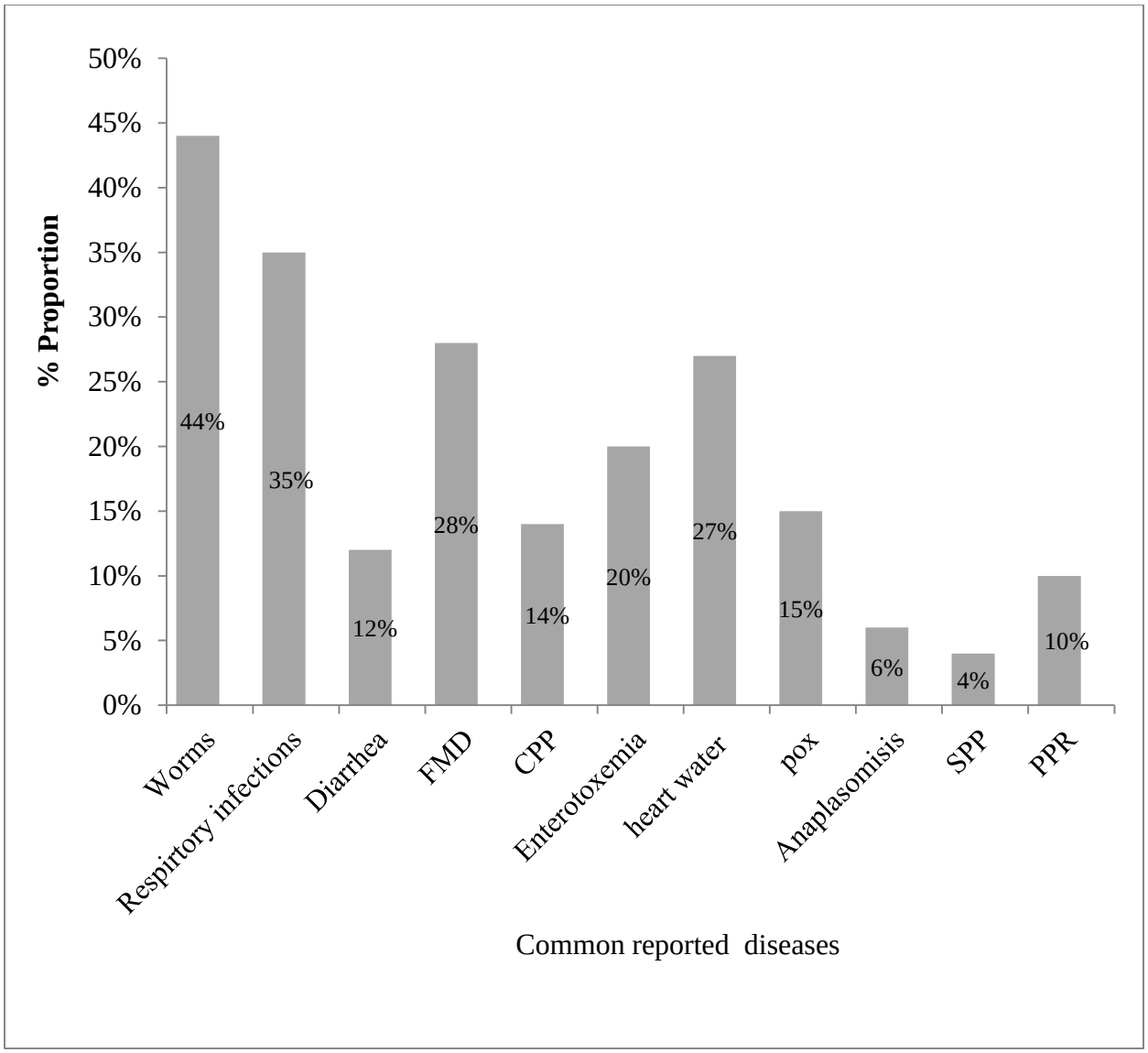

Figure 3: Common diseases reported by farmers to pose health challenges in sheep flock in Kajiado central.

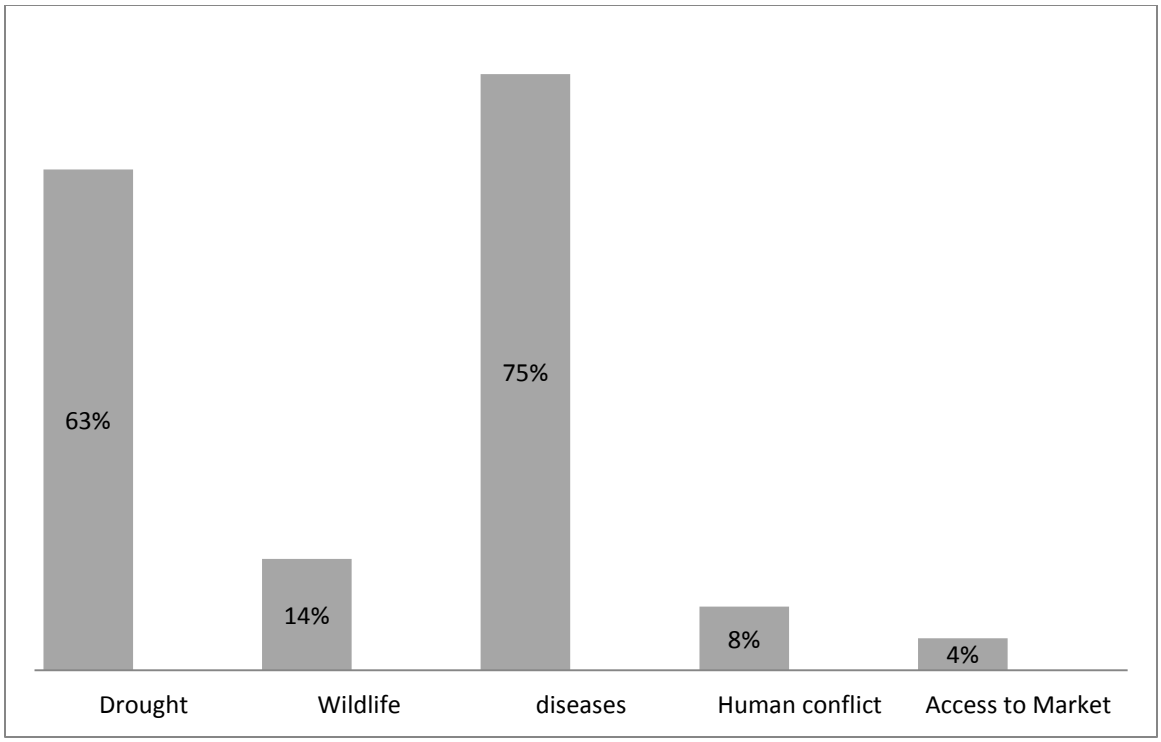

Figure 4: The challenges hindering sheep production in Kajiado central sub-county

4.3 Farming practices of animal owners

Questionnaire data also revealed that approximately 58 % of the respondents vaccinated their animals against various diseases for less than 12 months before the survey, while 16.13% had vaccinated their animals more than 12 months before the survey. These sheep owners reportedly vaccinated their animals against FMD (55%) and PPR (13%), while only six percent of the sheep farmers had vaccinated against both PPR and sheep pox. Most of the respondents (97; n=31) reportedly controlled ectoparasites by spraying of animals with acaricide, with the remainder (3%) only deworming sheep either by drenching or injection. About 42 % of these sheep farmers regularly sprayed their animals with acaricide, with about 12% of them applying acaricide twice a week and the remainder (45%) having irregular schedule for applying acaricide (Table 2).

Table 2: Acaricides-formulations, frequency and methods of their applications for tick-control by the farmers

Tick control with acaricides		% Reported
Frequency of spraying	None	45%
	Once/week	42%
	Twice/week	13%
Acaricide formulation	spray	96%
	Other	4%
Applications method	spraying	96%
	Other	4%

4.4 Sheep flock characteristics and number of sheep sampled per study area

A total of 392 sheep from the 31 flocks were sampled yielding a total of 395 blood samples. Those that were sampled were of different sex and age groups. More male sheep above 12 months than females were sampled in this study (Table 3). Twenty six percent of the samples were collected from Oloosuyian (Table 4).

Table 3: The age and sex of sheep sampled in the study areas

Age	Sex		Total
	Female	Male	
Below12 months	89	124	213
Above12 months	47	135	182
Total	136	259	395

Table 4: Distribution of number of samples collected from sheep in the selected study areas.

Location	Number sampled	Percentage (%)
Matasi	28	8%
Bissil	33	8%
Namanga	39	9%
Nagatatock	13	3%
Oloosyian	10	27%
Sajiloni	76	19%
Olonyork	67	17%
Portland	34	9%

Note: Total number of blood samples collected = 395

4.5 *Babesia* species detected by microscopy

A total of 395 blood samples were examined by microscopy to investigate for the presence of *Babesia spp. Babesia* parasites were detected in blood smears of sheep (Figure 5). Out of the samples analyzed, *Babesia* piroplasms were seen in 22 translating to an estimated prevalence of 5.57%.The highest prevalence was observed in Bissil (12.12%) followed by Sajiloni (7.89%), Ngatatock (7.69%) and Olonyork (7.46%) areas respectively. The lowest prevalence of infection of sheep with Babesia was observed in Oloosyian (1.90%). *Babesia* parasite was also detected in blood samples collected from sheep in Matasi, Portand, and Namanga (Table 5).

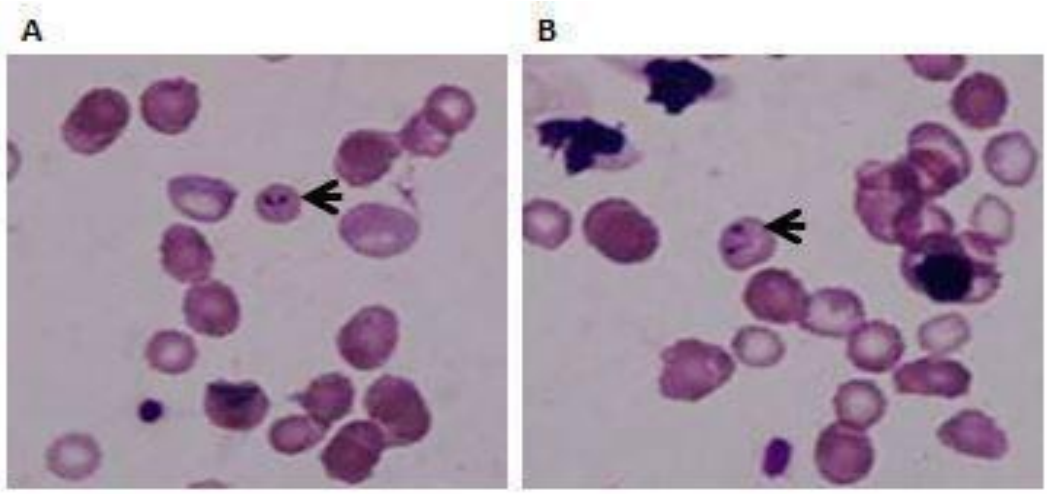

Figure 5: Blood smears showing sheep infected with Babesia spp after staining with Giemsa. The arrow heads in panels A and B show erythrocytes infected with *Babesia* species. The smears were prepared from different blood samples.

Table 5: Proportion of *Babesia spp* infection of sheep in various locations in Kajiado Central based on microscopic examination

Location	Number sampled	Positive samples (%)
Matasi	28	1 (3.57)
Bissil	33	4 (12.12)
Namanga	39	1(2.56)
Nagatatock	13	1 (7.69)
Oloosyian	105	2 (1.90)
Sajiloni	76	6 (7.89)
Olonyork	67	5 (7.46)
Portland	34	2 (5.88)

Note: Total number of blood samples examined by microscopy (395) and number positive (%): 22(5.57). The other percentages were calculated based on the total number of samples collected from each of the locations of Kajiado sub-County

4.6 *Babesia* species Detected by PCR

The 395 blood samples were examined for presence of *Babesia* parasites using PCR techniques. A specific DNA band corresponding to 239 bp was observed from some samples confirming the presence of the parasite DNA and infection of sheep with *Babesia* species (Figure 6). Out of the 395 samples analyzed, 13 samples were positive for *Babesia* DNA, translating to an overall prevalence of 3.29%. In this case the highest prevalence was reported in Ngaatatock (7.69%) followed by Bissil location (6.06%). The PCR technique did not detect *Babesia* DNA in blood samples collected from sheep reared in Portland location of Kajiado central sub-county. *Babesia* was also detected in sheep kept in Olonyork, Matasi, Sajiloni, Namanga and Oloosyian locations (Table 6.).On further PCR analysis, specific primers targeting the *B. ovis*-SA1 gene did not amplify the target gene specific for *B. ovis.*

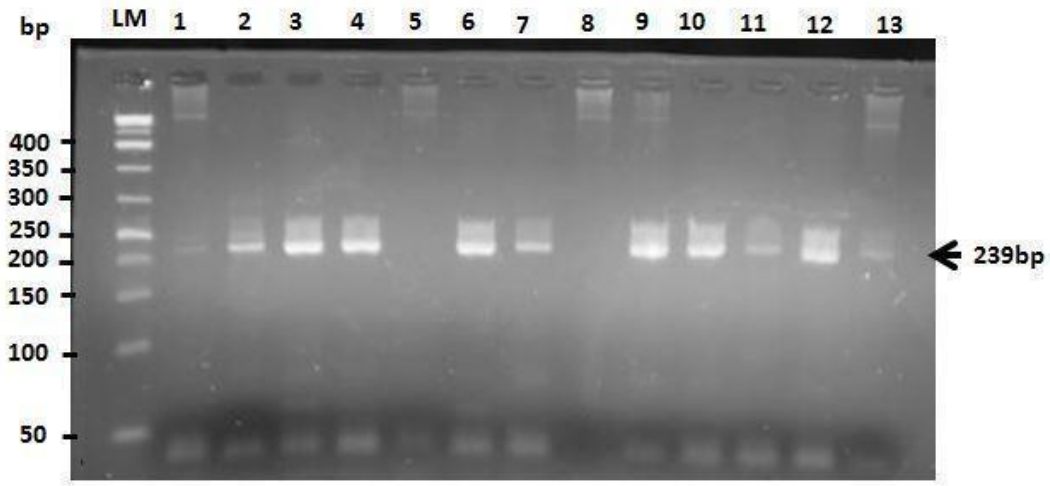

Figure 6: The PCR bands of 16S rRNA of Babesia spp isolated from sheep blood. Lanes: LM, 50 bp-Ladder Marker; 1– 4, 6 - 7, 9 - 13 are samples positive for *Babesia spp*; 5, Negative control.

Table 6: Proportion of *Babesia spp* infection of sheep in selected areas of Kajiado Central based on PCR analysis**.**

Location	Number sampled	Positive samples (%)
Matasi	28	1 (3.57)
Bissil	33	2 (6.06)
Namanga	39	1 (2.56)
Ngaatatock	13	1 (7.69)
Oloosyian	105	1 (0.95)
Sajiloni	76	3 (3.95)
Olonyork	67	4 (5.97)
Portland	34	0 (0.00)

Note: Total number of blood samples examined by microscopy (395) and number positive (%): 13 (3.29). The other percentages were calculated based on the total number of samples collected from each of the locations of Kajiado sub-County.

4.7 Test agreement between microscopy and PCR test results

A two by two tabulation of results of both microscopy and PCR was done and Kappa statistics subsequently determined. Thirteen samples were positive for both microscopy and PCR test whereas 9 samples that were positive by microscopic examination were negative by PCR test. All the samples that were negative on microscopy were also negative on PCR (Table 7). The test results of microscopic examination and PCR technique were in agreement revealing kappa value of 0.73 indicating that the test agreement was significant.

Table 7: A comparison of microscopic examination and PCR test for the detection of Babesia species detected in blood samples of sheep reared in Kajiado central.

	PCR		
Microscopy	Positive	Negative	Total
Positive	13	9	22
Negative	0	373	373
Total	13	382	395

4.8 Assessment of risk factors of *Babesia* infection in sheep

A univariate analyses was initially done to assess for association between occurrence of *Babesia* infection in sheep and each factor. Sex of the sheep and absence of ticks in dogs within the flocks were the only factors significantly associated with occurrence of *Babesia* infection ($p=0.10$) in sheep. Tick control, sex, breed, and age of the owner were not significant ($p>0.10$) (Table 8).

Table 8: Univariate analysis of potential risk factors associated with occurrence of *Babesia* infection of sheep in the study areas.

Possible risk factor	level	% Positive based on PCR	P value
Sheep age	<12months	0.05	0.756
	>12month	0.02	
Sheep sex	M	0.037	0.091
	F	0.030	
Sheep breed	Local	0.02	0.372
	Cross	0.04	
Presence of dog	No	0.9	0.062
	Yes	0.7	
Herd size	small	0.8	0.702
	Large	0.8	

Note: The risk factors, including those potentially considered as confounders, to assess for their effect on their occurrence of *Babesia* infection in sheep. The outcome of interest was based on the PCR results. The only risk factor that was significantly associated with ovine *Babesia* infection was sex of the sheep ($P \leq 0.10$).

CHAPTER FIVE

5.0 DISCUSSION

This study found that the animal owners were married men who had no formal education with an average age of 45 years although other members of the family were also involved in taking care of the animals including sheep. Similar previous studies also found that other family members including children played major role for taking care of the sheep (Boone *et al*., 2007; Frantkin, 2001; Mwanyumba, 2015). This study revealed that most of the flocks were mixed comprising of sheep, goats and cattle. In most of the cases, the indigenous and cross breed sheep were kept by the farmers. This ensured that the farmers kept animals selected for adaptive traits such as diseases resistant and drought. This explanation is supported by a previous study by Kosgey *et al* (2008) and Konig (2015). This study also found that most of the household kept different livestock species in the same place at night to maximize use of the limited space as observed in a study by Notenbaert *et al*. (2012). The use of limited space to keep different livestock species at night may be a risk a factor that may aid in the spread of ticks resulting in transmission of tick-borne diseases including babesiosis.

This study found that the biggest challenge for sheep farmers were diseases especially during the dry season when animals gather at the watering points. This disease challenge has been reported in Somalia, Ethiopia, and Sudan (Lughano and, 1996). A possible explanation for this is that when animals gather at watering sources, there is a possibility of pathogen transmission leading to increased disease outbreaks (Perry *et al.*, 2011). Farmers interviewed in this study reported that limited access to the markets was one of the major challenges that may have resulted in low animal production resulting in the lower prices of animals and their products as reported in another study done by FAO, UN and OIE (FAO-UN/OIE 2015). Human conflicts for

limited pastures and water were also a challenge for livestock farmers in Kajiado. Indeed, these challenges are not limited to the present study since other researchers have reported conflicts amongst farmers when in search of pasture and water (Boone *et al*., 2007).

This study revealed that helminthiasis, CCPP, PPR, FMD and tick-borne diseases were the common disease problems affecting animals in Kajiado central. This finding is in agreement with at study done in Kenya by Gitonga *et al*. (2016). Therefore, these data suggest that infection with Babesia in sheep may exist together with other clinically important diseases. In this regard, future studies on the epidemiology of ovine Babesia infections may be considered together with other diseases. Furthermore, it is possible that co-infections with these diseases may overburden the immune system of sheep resulting in risk of infection with ovine *Babesia.* It is also important to note that even though the farmers mainly vaccinated their animals against FMD, PPR, and sheep pox, PPR was still reported as common disease in Kajiado central. This report suggests that the vaccination strategies may not have worked or the immune system of the host animals may have been overburden due to the possible co-infections. Nevertheless, further studies are required to confirm the role of co-infection on vaccinations failure and whether this has a role in increasing the risk of infections with babesia in sheep.

Most of the sheep were infested with ticks although most farmers reported to have sprayed their animals with acaricides two times weekly. The presence of tick on sheep suggests that there is risk of the animals being infected with babesia as well as other tick-borne diseases. Additionally, the presence of ticks after spraying indicates that the ticks may have developed resistance to the acaricides. Alternately, the farmers may have used the acaricides at understrength concentration. Therefore, there is need for further studies to determine the cause of failure of the ticks to respond to the acaricides even after spraying.

The prevalence of babesia infection in Kajiado central based on microscopy was 5.57% consistent with other previous studies reported by Ferrer *et al*. (1998) and Yeruham *et al*.(1998) who reported prevalences of 6.1% and 5.56%, respectively. However, other studies reported higher prevalence of 52.1%, 23.63%, and 50.92% for ovine *Babesia* infection when IFAT was used (Papadopoulos *et al*., 1996, Karatepe *et al*., 2003 and Hosein *et al*., 2007). This disagreement may be as a result of less specificity of IFAT as compared to microscopy yielding false positive results. Another reason for the higher prevalence is because IFAT detects antibodies which tend to persist for a longer time even after the babesia parasite has been cleared from circulation.

The present study revealed a lower prevalence of 3.29% by PCR as compared to microscopic test suggesting a higher sensitivity of microscopic test although microscopic examination may in some cases present technical problems due to false morphological diagnosis. Furthermore, the technique requires special diagnostic knowledge and skills (Shayan *et al*., 2008).Specific primers designed to detect B-ovis-SA1 gene failed to amplify the target gene specific for *B. ovis* suggesting that the *Babesia* parasites observed by microscopy may not be *B. ovis*. Indeed another study done in Nigeria also failed to amplify another *B. ovis* target gene by PCR just like in this study (Onoja *et al*., 2013). One possible reason for failure to amplify the *B. ovis* specific gene in this study is because the primers designed could not anneal and amplify the secretory antigen 1 gene. Alternatively, failure to optimize the PCR assay system could not be ruled out. Therefore, further studies using other target genes for *B. ovis* or even different primers targeting the same gene are recommended.

PCR is now routinely used in many laboratories around the world for investigations of tick-borne infections including babesiosis, especially for carrier animals (Altay *et al*., 2008). The PCR used

in this study detected *Babesia* parasites in sheep up to the genus level. Therefore, even though the PCR could not detect the parasite up to the species level, it may still be possible to detect *B. ovis* with improved PCR assay system and validate the test for routine use. The low prevalence of *Babesia* infection revealed by this study suggests that the disease may not be a major threat to sheep farming in Kajiado central sub-county. Nevertheless, further large scale studies covering many areas and using many samples from different sheep are still required to confirm this claim.

A previous study found that sex of the animals is a significant risk factor associated with the occurrence of *Babesia* infection in which case male animals were reported to be prone to the infection than females (Iqbal *et al.,* 2011). This report is in agreement with the present study in which male animals were found to be more likely to be infected with *Babesia* than female animals. However, this difference appears to be marginally significant (P = 0.091) as compared to the previous study above. Although the findings above appeared to be in agreement, another study by Theodoropoulos *et al.* (2006) reported higher prevalence of *Babesia* infection in female sheep and goats in Greece and none in male sheep suggesting that sex may not be a risk factor for the infection. Nonetheless further studies using more samples and stringent significance level are needed to clarify this discrepancy.

The present study found that existence of dogs within flocks of sheep may contribute to the occurrence of *Babesia* infection in sheep (P=0.062) in agreement with previous studies done by Iqbal *et al.* (2011) and Theodoropoulos *et al*. (2006). Indeed sheep flocks raised with dogs had a higher number of infected animals suggesting that the animals may have acquired the ticks from the dogs and thus contributing to the transmission of the disease. The role of farm dogs as a risk factor for *Babesia* infection in sheep and goats is unclear, although ticks has been reported to transmit *Babesia* even after feeding on other hosts (Yeruham *et al*. 1996) and *R. sanguineus* that

usually infests dogs has been found also on sheep (Bouattour *et al.* 1999). Therefore, the role of ticks infesting dogs in the transmission of ovine *Babesia* infection needs to be studied further. On the other hand, Criado-Fornelio et al. (2003) reported the same *Babesia* species can infect a wide variety of animal hosts. This finding indicates that other animal species besides dogs may also be risk factors for infection of sheep with *Babesia.* Nevertheless, further studies may be required to confirm the role of tick infested dogs in the transmission of *Babesia* infection in flocks of sheep. Even though studies have shown that dogs do not share the *Babesia spp* with sheep, studies on possibility of the same parasite infecting dogs existing together with sheep flocks also infecting sheep may be required to clarify this claim.

This study also found that although some animals were infested with ticks, this infestation was not associated with occurrence of babesia infection in the flocks, consistent with the findings by Theodoropoulos *et al*. (2006). These findings are unexpected because under normal circumstances, infestation of animals with ticks should result in high prevalence of tick-borne diseases including babesiosis. One possible reason for this unexpected observation is that most of tick species seen were not the ones responsible for the transmission of *Babesia spp* in sheep. However, more extensive studies are required to determine the role of tick infestation of sheep and the transmission of ovine *Babesia*.in Kajiado central sub county.

Earlier studies have reported that age, flock-size and breed are risk factors associated with the occurrences of ovine babesioses in sheep (Razmi *et al*. 2003; Theodoropoulos *et al*., 2006; Iqbal *et al*., 2011). These previous findings are inconsistent with the current study, which reported a statistically non-significant association between age group, flocks size and breed, and occurrence of the infection ((P> 0.1). The inconsistent finding may be as a result of the differences in farming practices in the different geographical areas and other factors such as seasons.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The following conclusions were made from this study;

1. Sheep farming is undertaken mainly under mixed livestock enterprises, grazing and watering is done communally and may encourage disease spread.
2. Sheep reared in Kajiado central sub-county are infected with *Babesia* parasites and the prevalence of the infection appears to be low.
3. The PCR assays used in this study could detect babesia parasites up to the genus level but specific primers for *B. ovis* seemed not to be appropriate for PCR analysis.
4. Sex of sheep and tick infested-dogs kept together with sheep appeared to be the main risk factors associated with occurrence of ovine *Babesia* infection in Kajiado central.
5. The results of microscopic examination and PCR analysis appeared to be in agreement although more extensive studies is required to confirm this finding.

6.2 RECOMMENDATIONS

1. Further studies to confirm the occurrence of pathogenic *B. ovis* infection in sheep reared in Kajiado central using PCR based on other target genes and specific primers.
2. Additional studies focusing on both dry and wet seasons should be done to determine the effect of seasonal changes on the disease prevalence.
3. There is need for large scale studies to conclusively determine the test agreements between microscopic examination, ELISA and PCR assay.

CHAPTER SEVEN

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APPENDICES

Appendix 1: Questionnaire for collecting data in possible risk factors associated with occurrence of *Babesia* spp in sheep.

Household no

Date……

Introduction

My name is Dr. Khansa Ahmed, a Masters of Science student of the University of Nairobi. My research topic is about ovine *Babesia* infection, which is tick-borne disease that causes economic losses in sheep production. I hope to fill the gap on the information about the disease that can help the government to control and prevent the disease from causing economic losses.

I look forward to your assistance during the collection of field data and blood samples from sheep within your farms:

Farm details :

Owner name :

Owner age :

Education level :

Material status :

The farm location :

Animal species :

Contact :

1. Breed……..

2. Body condition

A. good b. fair c. poor

3. Season

A. rain season b. dry season

4. Feeding system

A. ground feeding b. trough feeding

5. Water system

A .tap b. pond

6. Housing system

A. open b. close

7. Floor type

……

8. Flocks size

A. large (30>) b. small (30<)

9. Common disease in the area ……

10. When is the last vaccination and for which disease? .....

11. Tick control system:

A. How many time during the

year…….

B. what do you usually use? .....

C. the type of the product

A. spray b. powder c. other

D. The method they are using ……..

12. Last infection in the farm and the treatment

…….

13. The purpose of rearing sheep……

14. The challenge they are facing to maximize the sheep production……

15. The presence of tick in the dog associated with the flocks

Thank for your help