# MOLECULAR DETECTION OF SELECTED HAEMOPATHOGENS IN DOMESTIC ANIMALS AND THEIR ASSOCIATED ECTOPARASITIC BITING KEDS (GENUS *Hippobosca*) COLLECTED FROM LAISAMIS, NORTHERN KENYA

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# Molecular Detection of Selected Haemopathogens in Domestic Animals and Their Associated Ectoparasitic Biting Keds (Genus *Hippobosca*) Collected from Laisamis, northern Kenya

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with our approval as university supervisors.

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## DEDICATION

This thesis is dedicated to my parents; Mr. Tarcisio Mwaki and Mrs. Caroline Kithira, my siblings; Pamela Kathure, Amos Kimathi, and Denis Mutugi, and my family for supporting me and giving me an easy moment during my studies.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **DNA** | Deoxyribonucleic acid |
| **icipe** | International Center of Insect Physiology and Ecology |
| **HRMA** | High-resolution melt analysis |
| **HRM** | High-resolution melting |
| **WHO** | World health organization |
| **COI** | Cytochrome C oxidase subunit I |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **IACUC** | Institutional Animal Care and Use Committee |
| **GDP** | Gross domestic product |
| **NCBI** | National Centre for Biotechnology Information |
| **qPCR** | Quantitative polymerase chain reaction |
| **BLAST** | Basic Local Alignment Search Tool |
| **MAFFT** | Multiple Alignment using Fast Fourier Transform |
| **ITS1** | Internal Transcribed Spacer 1 |
| **SDGs** | Sustainable Development Goals |

## ABSTRACT

Production of domestic animals plays a central role to the livelihood of pastoralists inhabiting marginalized arid and semi-arid regions of northern Kenya. Pastoralists rely on their domestic animals such as goat, cattle and sheep for nutrition, through milk and meat production and income through sale of animal products. However, production of domestic animals is limited by pests and diseases. Hippoboscids (keds) and ticks are the predominant blood-feeding ectoparasites of domestic animals in northern Kenya. Therefore, the aim of this study was to identify hippoboscids infesting various domestic animals (goat, sheep, donkey, and dog) in Laisamis, Marsabit County, northern Kenya, and detect haemopathogens (*Trypanosoma* spp., *Anaplasma* spp., *Ehrlichia* spp., *Theileria* spp., *Babesia* spp., *Clostridium perfringens*, and *Brucella* spp.) in both the domestic animals and their associated ectoparasitic biting keds. The field study research design was cross-sectional, and domestic animals including goats, sheep, donkeys, and dogs, and their ectoparasitic hippoboscids were sampled for collection. Fresh blood (n = 389) from the animals were stored in EDTA vacutainers tubes and preserved immediately in liquid nitrogen while hippoboscids (n = 235) were preserved in absolute ethanol and transported to *icipe* laboratories in Kasarani for screening of pathogens. Genomic DNA was extracted from whole hippoboscids and blood samples, and PCR-HRMA (High Resolution Melt Analysis) performed for detection of pathogens by comparison of HRMA profiles of the test samples with the known positive controls. DNA sequencing of samples with unique HRM profiles then followed to confirm the specific species of the pathogens. Geneious Prime software was used in analysis of the sequences. Morphological examination, confirmed through gene sequencing, revealed two hippoboscid species: *Hippobosca variegata* and *Hippobosca longipennis*). Among the pathogens detected in goats included: *Anaplasma ovis* (84.5%), a novel *Anaplasma* sp. (11.8%), *Trypanosoma vivax* (7.3%), *Ehrlichia canis* (66.1%), and *Theileria ovis* (0.8%). Similarly, *A. ovis* (93.5%), *E. canis* (22.2%), and *T. ovis* (38.9%) were detected in sheep. Donkeys, were positive for '*Candidatus* Anaplasma camelii' (11.1%), *T. vivax* (22.2%), *E. canis* (25%), and *Theileria equi* (13.9%). In addition, keds carried the following pathogens; goat/sheep keds - *T. vivax* (29.3%), *Trypanosoma evansi* (0.86%), *Trypanosoma godfreyi* (0.86%), and *E. canis* (51.7%); donkey keds - *T. vivax* (18.2%) and *E. canis* (63.6%); and; dog keds - *T. vivax* (15.7%), *T. evansi* (0.9%), *Trypanosoma simiae* (0.9%), *E. canis* (76%), *Clostridium perfringens* (46.3%), *Bartonella schoenbuchensis* (76%), and *Brucella abortus* (5.6%). Dog keds harboured the most pathogens, suggesting dogs as the key reservoirs of pathogens in the study area. Pathogens such as *Trypanosoma vivax* and *Ehrlichia canis* were detected in both the blood and hippoboscids collected from the domesticated animals, suggesting the potential utility of hippoboscids in xenosurveillance of pathogens in their associated hosts. The presence of zoonotic pathogens such as *Brucella abortus*, in domestic animals of the study area calls for an urgent need for further surveillance of pathogens circulating in Kenyan domestic animals, and establish the possible role of keds in disease transmission. Information from this study will guide the policy makers and livestock farmers in disease control and management.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

Hippoboscids are obligate ectoparasites of vertebrates belonging to the family hippoboscidae. They are also known as keds or louse flies (Petersen *et al*., 2007; Rahola *et al*., 2011). Insects in the family hippoboscidae are one of the most important blood-suckers of birds and ruminants. They are specialized larviparous ectoparasitic flies that spend most of their adult life within the fur and feathers of their mammal and avian hosts respectively (Rani *et al*., 2011). Their bodies are distinctly dorsoventrally flattened (from back to belly), allowing them to slide between the feathers and scuttle around in the fur of their hosts. Across the world, Hippoboscidae retains three subfamilies (Ornithomyinae, Hippoboscinae, and Lipopteninae), 21 genera, and 213 species (Dick *et al*., 2007). Hippoboscoidea is the superfamily of this Diptera family and it also contains the Glossinidae or tsetse flies and two families of bat flies, the Streblidae and the Nycteribiidae (Rahola *et al*., 2011).

In northern Kenya, various hippoboscid species have been reported including *Hippobosca camelina* **(Figure 1.1)**, a predominant ectoparasite of camels (Kidambasi *et al*., 2020). Moreover, hippoboscids act as vectors of many infectious agents including bacteria, helminths, protozoan and viruses (Rahola *et al*., 2011) and have been shown to be mechanical transmitters of camel associated bacteria, '*Candidatus* Anaplasma camelii' (Bargul *et al*., 2021). Unfortunately, these hippoboscids (keds), other pests such as ticks that are endemic in northern Kenya, and diseases, hinder livestock production.

**Figure 1.1:** ***Hippobosca camelina*** (Bargul *et al*., 2021)

Keds cause economic losses in various ways, including annoyance and psychological disturbances produced during the act of biting and feeding, the diseases they transmit (Bargul *et al*., 2021), and expenditure incurred by farmers in controlling them (Narladkar, 2018). Livestock production plays a key economic role to the livelihood of pastoralists living in the marginalized arid and semi-arid regions of northern Kenya (Mburu *et al*., 2017). These pastoralists largely depend on livestock as a source of meat and milk, income from selling livestock, and as a mode of transport by mainly donkeys and camels.

About 75% of newly emerging diseases currently affecting humans originated from animals (Jones *et al*., 2008). Hemopathogens of livestock, particularly those of zoonotic importance, are responsible for some of the most serious emerging infectious diseases facing sub-Saharan Africa and the rest of the world (Rosenberg *et al*., 2018). In Laisamis, northern Kenya,, hemoparasites that cause babesiosis, theileriosis, rickettsiosis, anaplasmosis, and ehrlichiosis are a major impediment to livestock productivity and public health (Bargul *et al.*, 2021).

Currently, there is little surveillance of pathogens harbored by the livestock and the role of keds in spreading various diseases. Therefore, this calls for an urgent need for research studies to profile infectious and zoonotic pathogens circulating in livestock for better understanding of disease prevalence, mode of transmission, and for control. In this

study, we aimed to identify *Hippobosca* spp. in Laisamis, the study area, screen for selected hemopathogens (*Anaplasma, Trypanosoma, Clostridium, Ehrlichia, Brucella, Theileria,* and *Babesia* spp.) in goats, sheep, and donkeys, and in keds collected from goats, sheep, dogs, and donkeys, and determine the potential role of the hippoboscids in xenodiagnosis of disease pathogens to their corresponding host species.

### 1.2 Statement of the problem

Domestic animals are often the sole source of nutrition and livelihoods among nomadic and pastoral communities in arid zones. Unfortunately, their productivity is usually constrained by several factors, chiefly pests and diseases causing huge agricultural losses leading to increased poverty. Biting flies such as tabanids, *Stomoxys*, hippoboscids, and ticks are the common haematophagous ectoparasites of livestock in Marsabit County, northern Kenya (Bargul *et al*., 2021; Getahun *et al*., 2020; Getange *et al*., 2021; Kidambasi *et al*., 2020). Keds infest and blood-feed on all livestock species, domestic and wild animals. In addition, keds also feed on humans and in the process, could transmit zoonotic pathogens.

Haemopathogens of domestic animals, particularly those of zoonotic importance are responsible for some of the most serious emerging infectious diseases facing Sub-Saharan Africa and the rest of the world today (Rosenberg *et al*., 2018). In Kenya, haemoparasites that cause babesiosis, theileriosis, rickettsiosis and related diseases like anaplasmosis and ehrlichiosis, are a major impediment to productivity of the domestic animals and public health, thus leading to huge economic loss and health concerns (Kiara *et al.,* 2014).

In Laisamis, northern Kenya, extensive research has not been done to catalogue pathogens harbored by domestic animals and their common hippoboscids which are among the major pests of domestic animals reared by pastoralists. Moreover, the interaction of domestic animals and humans has the potential to facilitate the spread of zoonotic pathogens (Omondi *et al*., 2017), thus, studies on pathogens present in the

domestic animals and their ectoparasitic hippoboscids is critical for mitigation and design of disease control strategies as well as further studies on possible transmission between the hippoboscids and the animals.

### 1.3 Justification

The main economic activity of pastoralists living in the arid and semi-arid areas of northern Kenya is production from their domestic animals, for example, meat and milk. Pastoralists depend on their domestic animals as source of food, income, and as a mode of transport. Dogs are mostly used as source of security.

About 75% of newly emerging diseases currently affecting humans originated from animals (Jones *et al*., 2008). Previous studies have shown the presence of various pathogens such as *Ehrlichia chaffeenis*, *Coxiella burnetti*, *Brucella* spp. *Rickettsia aeschlimannii* and trypanosomes in camels and their associated vectors among the pastoralist communities in Marsabit county, northern Kenya (Getahun *et al*., 2020; Getange *et al*., 2021; Kidambasi *et al*., 2020).

In Laisamis, Marsabit county in northern Kenya, little surveillance studies have been done to profile pathogens circulating in the domestic animals and their associated keds. Additionally, major pests of domestic animals including ticks and other biting flies like hippoboscids cause economic losses by their annoyance and psychological disturbances produced during the act of biting and feeding, and the diseases they transmit as a lot of money is used to buy drugs to treat the animals and to control the pests (Bargul *et al*., 2021; Narladkar, 2018).

Pastoralism supports about 20 million people, produces about 90% of the meat consumed in East Africa, and contributes about 13% of the GDP in Kenya (Nyariki & Amwata, 2019). Unfortunately, diseases that affect cattle, sheep, and goats, are endemic in northern Kenya and are a key constraint to production of domestic animals. Thus, early screening of these haemopathogens in domestic animals and their ectoparasitic

hippoboscids will provide information that will aid in disease management and improve on production of the domestic animals, hence, saving on the economy and providing food security. This will further contribute to the realisation of vision 2030 and sustainable development goal II (SDG), zero hunger, which heavily relies on resilient agricultural practices.

### 1.4 Research questions

i) What are the *Hippobosca* species found on domesticated animals in Laisamis, northern Kenya?

ii) What are the pathogens circulating in blood and hippoboscids of domesticated animals in Laisamis, northern Kenya?

iii) Can hippoboscids be used in xenodiagnosis of disease pathogens to their associated hosts?

### 1.5 Hypothesis

There is no presence of pathogens in blood of domestic animals and their associated ectoparasitic hippoboscids in Laisamis, northern Kenya.

### 1.6 Objectives

#### 1.6.1 General objective

To investigate the haemopathogens in domestic animals and their associated ectoparasitic biting keds (Genus *Hippobosca*) collected from Laisamis, northern Kenya.

#### 1.6.2 Specific objectives

i) To identify *Hippobosca* spp. infesting various domestic animals (goat, sheep, donkey, and dog) in Laisamis, northern Kenya.

ii) To screen and characterize the haemopathogens (*Trypanosoma* spp., *Anaplasma spp.*, *Ehrlichia* spp., *Theileria* spp., *Babesia* spp., *Clostridium perfringens*, and *Brucella* spp.) in domestic animals (goat, sheep, donkey, and dog) and their ectoparasitic hippoboscids collected in Laisamis, northern Kenya.

iii) To determine the potential of hippoboscids in xenodiagnosis of disease pathogens to their corresponding host species.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 Hippoboscids as disease vectors

The general classification of hippoboscids is as follows;

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Diptera
Superfamily: Hippoboscoidea
Family: Hippoboscidae
Genus: *Hippobosca*

Members of the hippoboscidae are known to act as vectors of many infectious agents: protozoan, bacteria, helminthes, and viruses (Rahola *et al*., 2011). They certainly transmit mammal Trypanosomatidae of the genus *Megatrypanum* (Oyieke & Reid, 2003). They are the only known vectors of *Haemoproteus*, an apicomplexan parasite of birds (Rahola *et al*., 2011). A study on the presence of various *Rickettsia* spp. in *Melophagus ovinus*, sheep hippoboscid, reports DNA of two Rickettsia species, *Rickettsia raoultii* and *Rickettsia slovaca* (Liu *et al.*, 2016). Additionally, *Melophagus ovinus* play a role in the transmission of *Bartonella* among ruminants (Halos *et al*., 2004).

In northern Kenya, a camel-specific ked, *Hippobosca camelina*, has been shown to mechanically transmit '*Candidatus* Anaplasma camelii' to mice and rabbits (Bargul *et al.*, 2021). Further, the fly *Hippobosca longipennis* is thought to transmit the larva of the filarial nematoda *Acanthocheilonema dracunculoides* to hyenas and domestic dogs in Kenya *(*Nelson *et al*., 1963; Rahola *et al*., 2011). *Hippobosca longipennis*, also known as the dog louse fly, is a blood sucking ectoparasite found on domesticated and feral dogs and wild carnivores such as lions and cheetahs, and is distributed in the Middle East and

Asia, including China and India and, the arid and semi-arid regions of Africa (Rani *et al*., 2011). *H. longipennis* has been reported to infest Indian dogs in places like Uttar Pradesh, Himachal Pradesh, Punjab and the eastern zone of Maharashtra state (Sharma *et al*., 2009).

*H. variegata*, an ectoparasite of cattle and domestic equines such as donkeys (Oboňa *et al*., 2019), is mostly distributed in Asia and sub-Saharan Africa. In addition, not much data is published about the vectorial capacity of these hippoboscids, thus, calls for more research to understand the vectorial competence of these flies. These keds also descend and occasionally feed on humans and in the process, could transmit zoonotic pathogens (Getahun *et al*., 2020).

### 2.2 Selected haemopathogens of domestic animals and their impacts

Haemopathogens in animals, particularly of zoonotic importance, attract attention in both livestock and public health sectors since their impacts are felt at the household, regional, national and global levels. Some of their impacts are on food safety and security, socio-economics and human health (McElwain & Thumbi, 2017). Some of the common pathogens that mostly infect animals in Kenya include *Trypanosoma* spp., *Anaplasma* spp., *Ehrlichia* spp., *Theileria* spp., *Babesia* spp. and *Brucella* spp. (Kidambasi *et al*., 2020; Mwamuye *et al*., 2017; Omondi *et al*., 2017).

#### 2.2.1 Trypanosoma *species*

Trypanosomes are protozoan parasites in the family trypanosomatidae that are mostly transmitted by tsetse flies. *Glossina* spp. are the biological vectors of African trypanosomes, the causative agents of human and animal trypanosomiasis that cause debilitating diseases in Africa (Petersen *et al.*, 2007). The three most important species of trypanosomes are *Trypanosoma congolense, Trypanosoma vivax* and *Trypanosoma brucei*. Trypanosomes lead to African animal trypanosomiasis, a disease that causes

serious economic losses in livestock due to mortalities, morbidities, quarantine, and costs of prevention and treatment of this disease (Muhanguzi *et al*., 2017).

In northern Kenya, trypanosome species such as *T. vivax*, *T. congolense* and *T. brucei* have been reported in camels but their pathological effects are not well known (Getahun *et al*., 2020). Additionally, animal trypanosomiasis is also reported in tsetse-free belts, where transmission is usually promoted by other biting flies of *Tabanus* and *Stomoxys* spp., and the parasites species mostly associated with the disease include *T. evansi* and *T. vivax* (Desquesnes *et al*., 2013). *T evansi* causes trypanosomiasis in domestic animals such cattle, camels, and pigs as well as in wild animals like water buffaloes (Dargantes *et al*., 2009). Generally, the infected animals exhibit anaemia, abortion, lethargy, weight loss, infertility, reduced milk and meat production, and death when the animal is not treated (Desquesnes *et al*., 2013).

### 2.2.2 *Anaplasma species*

These pathogens are obligate intracellular bacteria transmitted by arthropods, mainly ixodid ticks, from one vertebrate host to another. They allow their vertebrate hosts to be reservoirs by causing a persistent infection in them (Rar & Golovljova, 2011; Rikihisa *et al*., 2010). The persistent infection caused by these pathogens can lead to death in animals due to co-infection by *Staphylococcus aureus*, *Mannheimia/Bibersteinia* and other opportunistic diseases (Dugat *et al*., 2015).

Various *Anaplasma* spp. cause anaplasmosis including *A. marginale, A. centrale, A. ovis*, and *A. mesaeterum* (Aktas *et al*., 2011; Iweriebor *et al*., 2017) whereby *A. marginale*, *A. ovis* and *A. bovis* causes anaplasmosis affecting domestic animals while *Anaplasma phagocytophilum* causes human anaplasmosis (Silveira *et al*., 2012; Stuen *et al*., 2014). Infection by these pathogens lead to serious economic losses, for example, bovine anaplasmosis caused by *Anaplasma marginale* is responsible for economic losses reaching 57 and 22 million USD in India and Australia respectively (Aubry & Geale, 2011; Nair *et al*., 2013). *Anaplasma marginale* is the most virulent of the known

*Anaplasma* spp. and is characterized by a progressive hemolytic anaemia (Aubry & Geale, 2011). Further, it is the most commonly documented *Anaplasma* spp. in Kenya (Peter *et al*., 2020).

On the other hand, *Anaplasma phagocytophilum* is the major zoonotic pathogen (Stuen *et al*., 2014). *Anaplasma centrale* is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare (Aubry & Geale, 2011) In northern Kenya, anaplasmosis disease presents major challenges to productivity of domestic animals (Bargul *et al*., 2021). Recent studies conducted in northern Kenya have shown subclinical infection of "*Candidatus* Anaplama camelii" in Camels (Getange *et al*., 2021; Kidambasi *et al*., 2020). Biological and mechanical pathway are the two common routes through which *Anaplasma* spp. is transmitted, and a recent study in Kenya has shown that Camel keds (*Hippobosca camelina*) can transmit this "*Ca*. A. camelii" to small laboratory animals (Bargul *et al*., 2021).

### 2.2.3 *Ehrlichia* species

The genus *Ehrlichia* comprises of six recognized species: *Ehrlichia canis, E. ewingii, E. chaffeensis, E. ruminantium, E. minasensis, and E. muris* (Bastos *et al*., 2015; Mnisi *et al*., 2022; Paddock & Childs, 2003). *Ehrlichia* spp. are causative agents of ehrlichiosis, a disease that is among the most important public health issue across the world, especially in tropical and subtropical regions (Zhang *et al*., 2017). It's a rickettsial disease that affects various mammals such as goats, sheep, dogs and humans. Studies have shown evidence of *Ehrlichia* infection in goats in Wuhan, China on the basis of clinical signs, histopathological, gross lesions, serum-biochemical and PCR (Zhang *et al*., 2017). Infected goats exhibit mild pyrexia, lethargy, lack of appetite, serious or mild nasal discharge and elevated rectal temperature (Loftis *et al.*, 2008).

*Ehrlichia ruminantium* is the main *Ehrlichia* species infecting domestic animals such as cattle in Africa and the Caribbean Islands, and is transmitted by infected ticks in the genus *Amblyomma* (B. A. Allsopp, 2015). *E. ruminantium* infections cause severe

economic losses in Africa, where approximately 150 million animals are at risk of infection (Basil A. Allsopp, 2010), thereby negatively affecting livelihoods that depend on domestic animals. *Ehrlichia canis* is a canine pathogen that causes canine ehrlichiosis in dogs (Harrus & Waner, 2011). Cases of Ehrlichiosis have been reported in dogs, mouse and humans in China (Dong *et al*., 2013; Xu *et al.*, 2015). The canine ehrlichiosisis is characterized by a high fever, lethargy, anorexia, hemorrhagic tendencies, lymphadenomegaly, depression and splenomegaly (Harrus & Waner, 2011). *Ehrlichia muris* is a pathogen of wild rodents that is related to *E. canis*, and it has been detected in patients exposed to hard ticks in the United States of America (Pritt *et al*., 2017), however, its zoonotic potential is not clear.

Recent study in northern Kenya, particularly Marsabit county, has shown infection of camels by a novel *Ehrlichia* sp., '*Candidatus* Ehrlichia regneryi' that is closely related to *Ehrlichia canis* (Getange *et al*., 2021). Further, in the same study, *E. chaffeensis* and *E. ruminantium* were detected in camel ticks as well as in sheep blood and their associated ticks. On the other hand, ticks collected from camel herds in Moyale constituency, were found to harbor an *Ehrlichia* sp. related to *E. ruminantium* which is known to cause heartwater disease in dromedary camels (Younan *et al*., 2021).

### 2.2.4 Piroplasms

Piroplasms of the genus *Theileria* and *Babesia* are tick-borne haemoprotozoans. The diseases they cause are theileriosis and babesiosis respectively, with babesiosis being one of the most important tick-borne infections of domestic animals (Beck *et al*., 2009). Theileriosis and babesiosis in domestic animals lead to severe economic losses by impairing the development and productivity of the livestock industry (Zhang *et al.*, 2014). These diseases may also lead to clinical infections in domestic animals, humans and wild animals as well (Aydin *et al.*, 2015). Prior investigation during the prolonged drought in 2009 in Wamba, Samburu District, presence of *Babesia* parasites were found on blood smears of several donkeys and one Grevy's zebra (Hawkins *et al.*, 2015). Further, during the 2016 acute camel death syndrome (ACDS) in Marsabit and Wajir, one

case of *Babesia* sp. was reported in blood samples collected from ACDS affected camels (Younan *et al*., 2021). Piroplasmosis in donkeys, caused by *Theileria and Babesia* spp. causes huge economic losses because infected animals ultimately have a reduced capacity to work (Kumar *et al*., 2009; Mekibib *et al.*, 2010).

Moreover, *Theileria* spp. infect many domestic animals worldwide and cause diseases with the greatest economic impact in ruminants such as East Coast fever caused by *Theileria parva* and tropical theileriosis caused by *Theileria annulate* (Boes & Durham, 2017). In Kenya, theileriosis caused by *T. parva* (East Coast fever) has been associated with high mortalities in cattle (Gachohi *et al*., 2012). Studies have also reported presence of *Theileria mutans, Theileria annulate, Theileria equi,* and *Theileria ovis* in domestic animals such as camels worldwide (Qablan *et al*., 2012; Sazmand & Joachim, 2017).

*Babesia* spp. contribute to huge economic losses reaching to millions of USD mostly in Australia and India (Aubry & Geale, 2011; Nair *et al*., 2013). *Babesia bigemina*, which is transmitted by *Rhipicephalus decoloratus*, is the main species of *Babesia* found to infect cattle in Kenya (Wesonga *et al*., 2010). Recent studies have also detected *Babesia bovis* in camels in Egypt (El-Sayed *et al*., 2021) and *Babesia caballi* in camels in Iran (Mirahmadi *et al*., 2022). Impacts such as treatment costs of acute infections, abortions, decreased performance and deaths caused by these haemo-parasites are responsible for significant losses to the equine industry (Rothschild, 2013).

### 2.2.5 *Brucella* species

Bacteria that belong to this genus are nonmotile, facultative anaerobic, intracellular gram-negative coccobacilli (Baek *et al*., 2003; Kakoma *et al*., 2003). There are five known species that cause disease in domesticated animals and they include *B. melitensis* in goats, *B. ovis* in sheep, *B. canis* in dogs, *B. abortus* in cattle, and *B. suis* in pigs. Only four of these have zoonotic potential, that is, *B. melitensis, B. abortus, B. suis,* and *B. canis* in rare cases (Corbel, 2006). These bacteria cause a disease known as brucellosis. This disease is endemic in many developing countries of Africa, Latin America and Asia

(Islam *et al*., 2018) and is one of the most important zoonotic diseases infecting humans and domesticated animals. It's classified as one of the top neglected zoonosis by World Health organization (World Health Organization (WHO), 2007). Brucellosis leads to abortions, infertility and reduced milk yield resulting in tremendous economic loss in livestock production (McDermott *et al*., 2013).

*Brucella* species have been shown to be of high public health and socio-economic importance in northern Kenya (Kairu-Wanyoike *et al*., 2019). Moreover, brucellosis is among the top priority zoonosis in Kenya, due to the socio-economic burden and amenability to control with a challenge of establishing its true morbidity and socio-economic impact due to misdiagnosis and underreporting (Munyua *et al*., 2016). Studies indicate high prevalence in livestock and humans; however, this varies with the livestock production system and geographical region (Njeru *et al.*, 2016).

Transmission of brucellosis to humans occurs through direct contact with infected animals either through mucous membranes or skin abrasions and ingestion of unpasteurized dairy products (Havas *et al.*, 2013). Additionally, transmission of *Brucella* pathogens can also occur mechanically via bites of contaminated stable flies (Baldacchino *et al*., 2013). There is limited data on the prevalence of brucellosis in domestic animals of northern Kenya, however, recent studies have shown presence of *Brucella* in camels of Marsabit County (Akoko *et al*., 2021). Since brucellosis is a zoonotic disease, pastoralists or individuals who are in contact with domestic animals such as farmers are at high risk of infection. Hence, there is need for urgent studies to catalogue infectious pathogens harbored by domestic animals in northern Kenya, that will lead to disease control, management, and reduction in transmission.

### 2.3 Molecular techniques used in detection of pathogens

The detection of pathogenic bacteria is key to the prevention and identification of problems related to health and safety in both humans and animals. Polymerase chain reaction (PCR), culture and colony counting methods as well as immunology-based

methods are the most common tools used for pathogen detection, and they involve DNA analysis, counting of bacteria, and antigen–antibody interactions, respectively (Lazcka *et al*., 2007). With increasing concerns about public health and the development of molecular techniques, new detection tools and the combination of existing approaches have increased the abilities to monitor pathogenic bacteria by exploring new biomarkers, analyzing various genes such as functional genes, quantification, and increasing the sensitivity and accuracy of detection (Zhang *et al*., 2021).

Culture-based methods, a traditional detection method of pathogenic bacteria, are low-cost, easy to operate, and highly standardized, and thus are broadly used for the regulatory purposes of pathogenic bacteria monitoring such as enumerating FIB in bathing water (Tiwari *et al*., 2021). However, the main disadvantages of these methods are that they are time and labor-consuming, can have false negative/positive results, can lack ability to differentiate between the target and other non-target endogenous microorganisms of the same samples, and have the inability to detect viable but nonculturable (VBNC) cells (Sohier *et al*., 2014). Therefore, molecular methods, a rapid analyzing tool with high accuracy and specificity, are gradually emerging as the most popular detection approach for pathogens (Zhang *et al*., 2021).

Molecular methods can be divided into two major groups according to the biological markers being used, that is, the nucleic acid targeting method and protein/antigen targeting method (Deshmukh *et al*., 2016). The nucleic acid targeting method includes fluorescence amplification-based methods, such as the polymerase chain reaction (PCR), digital PCR (dPCR), quantitative or real-time PCR (qPCR), deoxyribonucleic acid (DNA) microarray, fluorescence in situ hybridization (FISH), and molecular beacon, and sequencing-based methods such as Illumina sequencing, pyrosequencing, and nanopore sequencing. The protein and antigen targeting method includes a traditional antibody–antigen interaction method similar to immunological methods (lateral flow tests (LFTs)) and enzyme-linked immunosorbent assays (ELISA) (Ramírez-Castillo *et al*., 2015).

PCR is a technique to amplify segments of DNA whereby primers designed based on the knowledge of specific gene sequences of a target microorganism are used, and a series of repeated temperature changes is required (Zhang *et al*., 2018). Through this method, a small sample of a DNA sequence could be rapidly amplified into a large amount, thus enabling the detection and quantification of a low amount of the target DNA sequence (Zhang *et al*., 2021).

High-resolution melting (HRM) analysis is a post PCR method that measures the fluorescence reduction of intercalating dye in the process of dissociation of double stranded DNA with gradual increase in temperature after the region of interest within the DNA sequence is first amplified using PCR (Michael Chatzidimopoulos *et al*., 2019). HRM analysis generates sequence-related melting profiles and can reveal sequence variations at the level of a single nucleotide (Chatzidimopoulos *et al*., 2014). This method has become increasingly popular due to its simplicity, flexibility, non-destructive nature, superb sensitivity and specificity (Vossen *et al*., 2009). In Kenya, PCR-HRM has been used by researchers to detect infectious pathogens in camels and their biting ectoparasites such as ticks and camel keds (*Hippobosca camelina*) (Getange *et al*., 2021; Kidambasi *et al*., 2020).

### 2.4 Role of xenodiagnosis in detection of pathogens in infected hosts

The emergence of infectious diseases can have devastating public health and economic consequences. Being able to identify such events at early stages could significantly mitigate some of these outcomes. In many developing regions of the world, current surveillance methods are hindered by the costs associated with collecting, storing and shipping clinical specimens. Xenodiagnosis is a diagnostic procedure that entails detection of a parasite or pathogen through screening of an arthropod vector that has fed on an infected host (Enriquez *et al*., 2014). It can be performed in two ways: In direct xenodiagnosis, whereby live insects are used to detect viable disease organisms in individuals with presumptive infections, or Indirect xenodiagnosis, whereby insect feeds on heparinized blood through a feeder membrane (i.e., chicken skin) (Singh *et al*., 2020).

Xenodiagnosis was introduced in the early 20th century as a way of detecting trypanosomes in mammalian hosts by feeding laboratory-bred reduviid bugs on the animals, and its utilization has been shown in diagnosis of arboviral infections by use of mosquitoes (Mourya *et al*., 2007). It is a sensitive parasitological tool that acts as the most direct way for testing the infectiousness of hosts to the vectors, and has been used previously to investigate the dynamics and epidemiology of *Leishmania donovani* transmission in india (Singh *et al*., 2020).

Previous studies have also shown the implementation of xenodiagnoses to better characterize the parasitological status of seropositive animals (cattle and pigs), through detection of trypanosomes in infected hosts characterized by low parasitaemia levels (Wombou Toukam *et al*., 2011). Further, xenodiagnosis on dogs with visceral leishmaniasis using sand flies (*Lutzomyia longipalpis*), the vector, has also been reported previously (Magalhães-Junior *et al*., 2016).

In Kenya, a recent report showed the potential utility of camel keds (*Hippobosca camelina*) in xenodiagnoses of disease pathogens in their camel host, whereby there was occurrence in keds of pathogens that were similarly present in their camel host from which they were collected (Kidambasi *et al*., 2020). Recently, sand flies have also been utilized in evaluating the infectiousness of humans in an area endemic for visceral leishmaniasis in India (Singh et al., 2021). Data from previous studies, demonstrate the feasibility of xenosurveillance and suggest that it could be used to detect and predict pathogen outbreaks at earlier, more actionable stages of emergence (Grubaugh *et al*., 2015). Therefore, xenosurveillance could be of aid in disease control and management at an early stage.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study site

This study was conducted in Laisamis sub-County (1° 36' 0" N, 37° 48' 0" E), Marsabit County **(Figure 3.1)**. Laisamis is located in the northern part of Kenya bordering Ethiopia to the North, Turkana County to the West, Samburu and Isiolo Counties to the South, and Wajir County to the East. It occupies an area of 20,290 km$^2$ that comprises of five County Assembly Wards, with Laisamis ward covering an area of 3,885 km$^2$, falling within arid and semi-arid climatic conditions (Marsabit CIDP 2018-2022). The main economic activity in this region is livestock rearing with limited crop production. The main livestock kept includes camels, goats, sheep, cattle, and donkeys, and to a smaller extent poultry (Wanyoike *et al*., 2018).

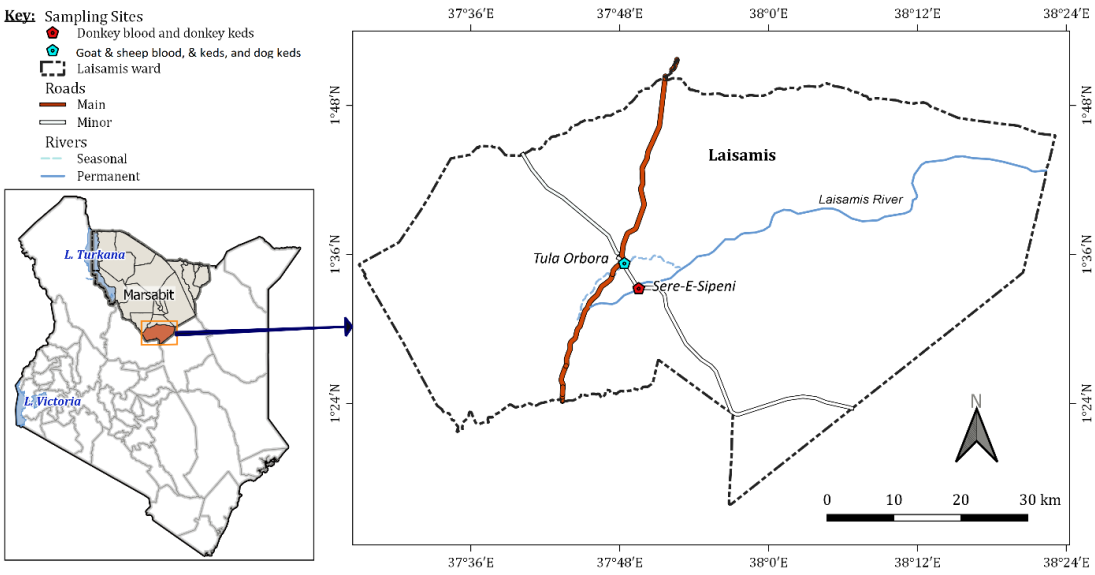


**Figure 3.1: A map of Kenya showing the study sites in Laisamis, Marsabit County.**

The samples collected from each site are shown on the map key.

### 3.2 Study design

A cross-sectional study design was employed in this study.

### 3.3 Sample size determination

The sample size (N) of the livestock was estimated using the formula based on the normal approximation to the binomial distribution (Cochran, 1977);

$$N = \frac{Z^2(p(1-p))}{d^2}$$

Where ***N*** is the maximum sample size required, ***Z*** is the z-score for a given confidence level, ***p*** is the estimated prevalence, and ***d*** is a statistical error. Since the prevalence of infections by *Anaplasma* spp. in co-herded sheep in the study area is estimated to be 88% (Getange *et al*., 2021):

$$N = \frac{1.96^2(0.88(1-0.88))}{0.05^2} = 162$$

However, this target was not achieved for all the samples since only 108 co-herded sheep, and 36 donkeys were available for sampling. The target was achieved for goats since 245 of them were available for sampling and all the samples were used in the study.

### 3.4 Ethical approval

This study strictly adhered to the experimental guidelines and procedures approved by the International Centre of Insect Physiology and Ecology (*icipe)* Institutional Animal Care and Use Committee (REF: IACUC/ICIPE/003/2018) and the Pwani University Ethics Review (approval number: ERC/EXT/002/2020). All the animals (goats, sheep, and donkeys) were handled carefully to minimize pain and discomfort during sampling.

Verbal consent was obtained from livestock owners prior to collection of samples since written consent was not possible as the livestock keepers could neither read nor write.

### 3.5 Sample collection

Samples were collected in two field-sampling trips and each sampling site was geo-referenced with a Global Positioning System (GPS). Goat and sheep blood, keds on co-herded goats and sheep, and dog keds were collected along Laisamis River at Tula Orbora which is one of the livestock watering points (1° 35' 16.4" N, 37° 48' 22.5" E) in the first sampling conducted in July 2019. Donkey blood and donkey keds were collected at Sere-e-Sipeni (1°33'14.9"N, 37°49'32.1"E) in the second field sampling conducted in February 2020.

#### 3.5.1 Collection of blood samples

About 5 mL of blood from the jugular vein of 245 goats, 108 sheep and 36 donkeys of both sexes were collected into 5 mL EDTA vacutainers (Plymouth PLG, UK), then immediately preserved in liquid nitrogen for transportation to the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi for analysis.

#### 3.5.2 Collection of hippoboscids

Keds associated with the goats, sheep, donkeys, and dogs were collected from their hosts by handpicking at night as previously reported (Kidambasi *et al*., 2020). Keds were preserved in absolute ethanol ready for transportation to *icipe* for molecular screening of pathogens.

### 3.6 Morphological identification of hippoboscids

Keds for use in molecular and morphological identification were sorted in the Molecular Biology and Bioinformatics Unit (MBBU) laboratories at *icipe*, Nairobi. Species

identification based on morphology was done through comparison with known hippoboscid collections at *icipe*, with emphasis on the color pattern of the scutellum.

### 3.7 DNA extraction

Individual keds were surface-sterilized with 70% ethanol and left to air dry for 10 min on a paper towel in a clean hood. Each fly was then placed into a clean 1.5 mL Eppendorf tube containing sterile 250 mg of zirconia beads with 2 mm diameter (Stratech, UK). The flies were ground in liquid nitrogen using a Mini-Beadbeater-16 for 3 min (BioSpec, Bartlesville, OK, USA) until they were completely crushed into powder.

Genomic DNA was then extracted from the keds and blood samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

### 3.8 Molecular identification of hippoboscids

Representative samples from each morphologically identified hippoboscid species were selected for molecular identification. DNA extracted from the leg of the representative flies was amplified by PCR using COI primers to further confirm the species identity by sequencing. The PCRs were carried out in 20 µL reaction volumes, containing 4.0 µL of 5× HOT FIREPol Blend mix (Solis BioDyne, Estonia), 1 µL of 10 pmol of each primer (LCO1490F & HCO2198R), 12.0 µL PCR water and 2.0 µL of template DNA. PCR amplification was preceded by an initial enzyme activation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 56°C for 30 sec, and primer extension step at 72°C for 30 sec; then a final elongation at 72°C for 7 min. 5.0 µL of the resulting amplicon was resolved through gel electrophoresis in ethidium bromide stained 1.5% agarose gel, and DNA fragments visualized under ultraviolet light using Kodak Gel Logic 200 Imaging System (SPW Industrial, Laguna Hills, CA, USA), for confirmation of a successful amplification. About 10.0 µL of the remaining PCR amplicons were purified to remove

excess primers and unused dNTPs using ExoSAP-IT™ kit, according to manufacturer's instruction. The purified amplicons were then sent for sequencing by Macrogen, Inc. (Amsterdam, Netherlands).

### 3.9 Screening of pathogens using PCR - HRM

Hemopathogens including *Anaplasma*, *Ehrlichia*, piroplasms (*Theileria* and *Babesia* spp.), animal African trypanosomes, *Clostridium perfringens* and *Brucella* spp. were targeted for amplification using PCR followed by DNA fragment analysis based on high-resolution melting (HRM) analysis (Šimenc & Potočnik, 2011). Rotor-Gene Q (Qiagen, Hannover, Germany), Quant Studio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Mic qPCR (Bio Molecular Systems, Upper Coomera, Queensland, Australia) thermocyclers were used for PCR-HRM analysis for pathogen detection.

Genus-specific *Anaplasmataceae* primers were used for amplification of the 16S rRNA gene target of *Ehrlichia* and *Anaplasma* spp. (Mwamuye *et al*., 2017), while *Theileria* and *Babesia* spp. were screened simultaneously using primers which target the hypervariable V4 region of 18S rRNA genes (Gubbels *et al*., 1999). *Clostridium perfringens* bacteria was screened using a specific primer targeting the 16S rRNA gene (Wu *et al*., 2009). A set of genus-specific primers described by (Probert et al., 2004) that targets the bcsp31 gene was used for the identification of *Brucella* spp. A universal set of primer that targets the trypanosomal internal transcribed spacer region was used for screening of animal African trypanosomes (Njiru *et al*., 2005). PCR primers used in screening of pathogens are listed in **Table 3.1**.

**Table 3.1: PCR primers for pathogen detection.**

| Primer name | 5' to 3' sequence | Target organism | Target gene | Amplicon size (bp) | Primer reference |
|---|---|---|---|---|---|
| *Anaplasma*JV_F | CGGTGGAGCATGTGGTTTAATTC | *Anaplasma* spp. | Partial 16S rRNA | 300 | (Mwamuye *et al*., 2017) |
| *Anaplasma*JV_R | CGRCGTTGCAACCTATTGTAGTC | | | | |
| *Ehrlichia*JV_F | GCAACCCTCATCCTTAGTTACCA | *Ehrlichia* spp. | 16S rRNA | 300 | (Mwamuye *et al*., 2017) |
| *Ehrlichia*JV_R | TGTTACGACTTCACCCTAGTCAC | | | | |
| *Ehrlichia 16S* F | CGTAAAGGGCACGTAGGTGGACTA | *Ehrlichia* spp. | 16S rRNA | 200 | (Tokarz *et al*., 2009) |
| *Ehrlichia 16S* R | CACCTCAGTGTCAGTATCGAACCA | | | | |
| EHR 16SD | GGTACCYACAGAAGAAGTCC | *Ehrlichia* & *Anaplasma* spp. | Longer 16S rRNA | 1000 | (Parola *et al*., 2000) |
| 1492R | GGTTACCTTGTTACGACTT | | | | |
| ITS1_CF | CCGGAAGTTCACCGATATTG | *Trypanosoma* spp. | ITS1 | 250-720 | (Njiru *et al*., 2005) |
| ITS1_BR | TTGCTGCGTTCTTCAAC-GAA | | | | |
| RLB_F | GAGGTAGTGACAAGAAATAACAATA | *Theileria* & *Babesia* spp. | 18S rRNA | 450 | (Gubbels *et al*., 1999) |
| RLB_R | TCTTCGATCCCCTAACTTTC | | | | |
| Br_F | GCTCGGTTGCCAATATCAATGC | *Brucella* spp. | bcsp31 | 223 | (Probert *et al*., 2004) |
| Br_R | GGGTAAAGCGTCGCCAGAAG | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Cp_F | AAAGATGGCATCATCATTCAAC | *Clostridium perfringens* | 16S rRNA | 279 | (Wu *et al*., 2009) |
| Cp_R | TACCGTCATTATCTTCCCCAAA | | | | |
| LCO1490F | GGTCAACAAATCATAAAGATATTGG | *Hippobosca* spp. | Cytochrome c oxidase | 710 | (Folmer *et al*., 1994) |
| HCO2198R | TAAACTTCAGGGTGACCAAAAAATCA | | subunit I | | |

PCR-HRM assays were performed in runs of 10 µL reaction volumes, containing 6 µL nuclease-free water, 2 µL of 5× HOT FIREPol EvaGreen HRM mix (no ROX) (Solis BioDyne, Estonia), 0.5 µL of 10 pmol of each primer and 1 µL of template DNA. PCR conditions in the Rotor-Gene, Quant Studio and Mic qPCR for detection of *Ehrlichia*, *Anaplasma* and piroplasms (*Theileria* and *Babesia*) were preceded by an initial enzyme activation step at 95°C for 15 min followed by 10 cycles of denaturation at 94°C for 20 sec, touch-down annealing from 64°C with a decrease of 1°C after each cycle for 25 sec, and primer extension step at 72°C for 30 sec. Then, another 30 cycles each of: denaturation at 94°C for 20 sec, touch-down annealing from 55°C with a decrease of 1°C after every 5 cycles for 50 sec, and extension at 72°C for 30 sec, with a final elongation at 72°C for 3 min.

Specific annealing temperatures of 55°C, 53.9°C, and 63.2°C were used for detection of *Trypanosome* spp., *Clostridium perfringens*, and *Brucella* spp., respectively. The PCR conditions were initial enzyme activation at 95°C for 15 min, 40 cycles of; denaturation at 95°C for 30 sec, annealing for 30 sec, and extension at 72°C for 30 sec, with a final elongation at 72°C for 7 min.

HRM analysis proceeded immediately after the PCR with a gradual increase in temperature from 75°C to 95°C with 2 sec increases of 0.1°C between successive fluorescence acquisitions. The melting curves were visualized based on the fluorescence signals and the change in fluorescence with time (dF/dT) plotted against change in temperature (°C).

Rotor-Gene Q Series Software 2.1.0 (Build 9), Quant Studio™ Design and Analysis Software version 1.5.1 (Mwamuye *et al*., 2017), and micPCR Software v2.8.1 were used to assess melt profiles of the test samples in comparison with that of the known positive controls to confirm detection of pathogens. DNA Sequencing of representative samples with distinct melting curves proceeded to confirm presence of the specific genus and species of the pathogens detected.

### 3.10 PCR amplicons purification and Sanger sequencing

Selected representative samples with expected and distinct melting curves relative to the known positive controls were amplified in larger PCR reaction volumes of 15 µL. 5 µL of the PCR amplicons were then resolved through gel electrophoresis in 2% (*w/v*) agarose gel stained with ethidium bromide, and DNA fragments visualized under ultraviolet light using Kodak Gel Logic 200 Imaging System (SPW Industrial, Laguna Hills, CA, USA), for confirmation of a successful amplification. The remaining volume of PCR amplicons (about 10 µL of each sample) were purified using ExoSAP-IT PCR Product Cleanup kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. Purified samples were then incubated at 37ºC for 15 mins and 85ºC for 15 mins in a Proflex thermocycler (Applied Biosystems) prior to Sanger sequencing by Macrogen, Inc. (Amsterdam, Netherlands).

### 3.11 Data analysis

Data acquired after screening of the pathogens in the blood samples and their ectoparasitic hippoboscids were recorded in a Microsoft Excel Spreadsheet Program version 18.2110.13110.0 (Microsoft Corp.). The Rotor-Gene Q Series 2.1.0 (Build 9), Quant Studio™ Design and Analysis Software v1.5.1, and micPCR Software v2.8.1 were used for HRM analysis.

All nucleotide sequences were edited and aligned with closely related sequences available in the NCBI GenBank nr database using the MAFFT plugin in Geneious Prime

software version 2020.2.1 (created by Biomatters, Auckland, New Zealand) (Kearse *et al*., 2012). The Basic Local Alignment Search Tool (BLAST) was used to query related sequences available in the GenBank nr database (www.ncbi.nlm.nih.gov/BLAST/). Maps were generated by feeding the coordinates of the sampling locations into a GIS software, QGIS v3.16.

### 3.12 Phylogenetic analysis

Maximum likelihood phylogenetic trees of the sequence alignments were constructed using PhyML v3.0 (Guindon *et al*., 2010). Topologies of the tree were estimated using nearest neighbor interchange (NNI) improvements over 1,000 bootstrap replicates and the Akaike information criterion for automatic model selection was employed in the phylogenies. FigTree v1.4.3 (Rambaut, 2016) was used to visualize the phylogenetic trees.

# CHAPTER FOUR

# RESULTS

## 4.1 Identification of hippoboscids

Hippoboscid flies that were collected on goats and sheep (116), donkeys (11), and dogs (108), were identified morphologically through comparison with known hippoboscid collections at *icipe* with emphasis on the color pattern of the scutellum (Bargul *et al*., under review). DNA sequencing was done after PCR amplification with COI primers to further confirm the species identity, and the sequences deposited in GenBank database and assigned accession numbers as follows: *Hippobosca variegata* (MW128366) and *Hippobosca longipennis* (MW128365). The morphological identification of the keds matched with the molecular identification of two ked species; *Hippobosca variegata* and *Hippobosca longipennis*. *Hippobosca variegata* (**Figure 4.1**) was collected from goats, sheep, and donkeys, while *Hippobosca longipennis* (**Figure 4.2**) was collected from dogs only.


**Figure 4.1: *Hippobosca variegata.***

The scutellum color pattern is yellow-brown-yellow-brown-yellow

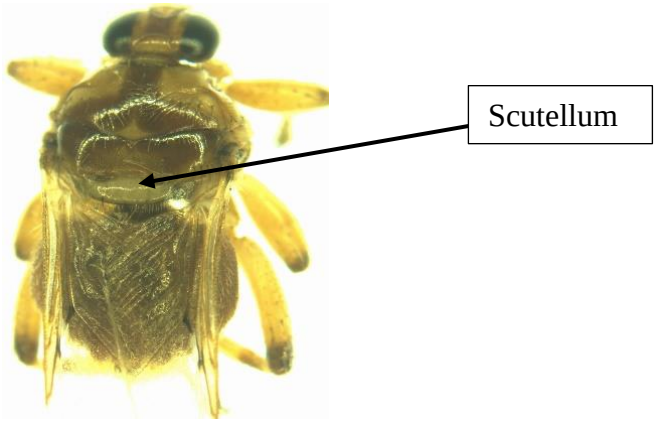


**Figure 4.2: *Hippobosca longipennis.***

The scutellum color pattern is yellow only.

### 4.2 Pathogen detection

A total of 389 blood samples from goats (245), sheep (108) and donkeys (36), as well as 235 keds from goats and sheep (116), dogs (108) and donkeys (11) were randomly collected in Laisamis Sub-County, Marsabit County of northern Kenya. Out of the 389 blood samples screened, 87.7% (341/389) were positive for *Anaplasma* spp., 50.1% (195/389) were positive for *Ehrlichia canis*, 12.6% (49/389) were positive for *Theileria* spp., and 6.7% (26/389) were positive for *Trypanosoma* spp.

Out of the 235 ked samples screened, 63.4% (149/235) tested positive for *Ehrlichia canis*, 34.9% (82/235) tested positive for *Bartonella schoenbuchensis*, 24.3% (57/235) were positive for *Trypanosoma* spp., 21.3% (50/235) tested positive for *Clostridium perfringens*, and only 2.6% (6/235) tested positive for *Brucella abortus.* All pathogens detected in this study are listed in **Table 4.1**.

**Table 4.1: Summary of pathogens detected in domestic animals and their keds.**

| Pathogens | Prevalence of hemopathogens in blood and ked samples | | | | | |
|---|---|---|---|---|---|---|
| | **Goat blood (n=245)** | **Sheep blood (n=108)** | **Goat/sheep keds (n=116)** | **Dog keds (n=108)** | **Donkey blood (n=36)** | **Donkey keds (n=11)** |
| *Trypanosoma* spp. | *T. vivax* = 18 (7.3%) | — | *T. vivax* = 34 (29.3%)<br>*T. evansi* = 1 (0.9%)<br>*T. godfreyi* = 1 (0.9%) | *T. vivax* = 17 (15.7%)<br>*T. simiae* = 1 (0.9%)<br>*T. evansi* = 1 (0.9%) | *T. vivax* = 8 (22.2%) | *T. vivax* = 2 (18.2%) |
| *Anaplasma* spp. | *Anaplasma ovis* = 207 (84.5%)<br>Novel *Anaplasma* sp. = 29 (11.8%) | *Anaplasma ovis* = 101 (93.5%) | — | — | '*Candidatus* Anaplasma camelii' = 4 (11.1%) | — |
| *Ehrlichia canis* | 162 (66.1%) | 24 (22.2%) | 60 (51.7%) | 82 (76%) | 9 (25%) | 7 (63.6%) |
| *Theileria/Babesia* spp. | *Theileria ovis* = 2 (0.8%) | *Theileria ovis* = 42 (38.9%) | — | — | *Theileria equi* = 5 (13.9%) | — |
| **Brucella abortus* | — | — | — | 6 (5.6%) | — | — |
| **Clostridium perfringens* | — | — | — | 50 (46.3%) | — | — |
| **Bartonella schoenbuchensis* | — | — | — | 82 (76%) | — | — |

*Zoonotic pathogens; dash (—) means the pathogen was not detected.

NCBI BLAST results of study sequences of pathogens against the GenBank reference sequences are shown in **Table 4.2**.

**Table 4.2: NCBI BLAST results of study sequences of pathogens against the GenBank reference sequences.**

| Pathogens | Host | GenBank accession numbers | | Percentage similarity |
|---|---|---|---|---|
| | | Study sequences | GenBank reference sequences | |
| *Anaplasma ovis* | Goat | MZ203400 | MG869525 | 100% |
| | Sheep | OM282854 | MG869525 | |
| novel *Anaplasma* sp. | Goat | MZ203399 | MG869525 | 96.8% |
| *Theileria ovis* | Sheep | OM282856 | MN712508 | 100% |
| *Theileria equi* | Donkey | OM282857 | MK063829 | 100% |
| *Candidatus* Anaplasma camelii | Donkey | MZ203398 | MT510533 | 100% |
| *T. evansi* | Goat/sheep keds | MZ221830 | FJ712715 | 99.7% |
| *T. simiae* | Dog keds | MZ221829 | JN673387 | 99% |
| *E. canis* | Dog keds | OM282855 | MT499360 | 100% |
| *Bartonella schoenbuchensis* | Dog keds | MZ203403 | KJ639882 | 100% |
| *Clostridium perfringens* | Dog keds | MZ203401 | MT613499 | 100% |

### 4.2.1 Pathogen detection in blood samples

*Goat blood samples:*

Goat blood (245) tested positive for *Anaplasma ovis* 84.5% (207), novel *Anaplasma* sp. 11.8% (29), *Ehrlichia canis* 66.1% (162), *Trypanosoma vivax* 7.3% (18), and *Theileria ovis* 0.8% (2) (**Table 4.1**). After performing PCR-HRMA, the melting profiles of *Anaplasma* spp. in goat appeared to be distinctly different for both the *Anaplasma ovis* and the novel *Anaplasma* sp. The melting point of *Anaplasma ovis* was at about $89^0$C, while that of the novel *Anaplasma* sp. was at about $88^0$C (**Figure 4.3**).

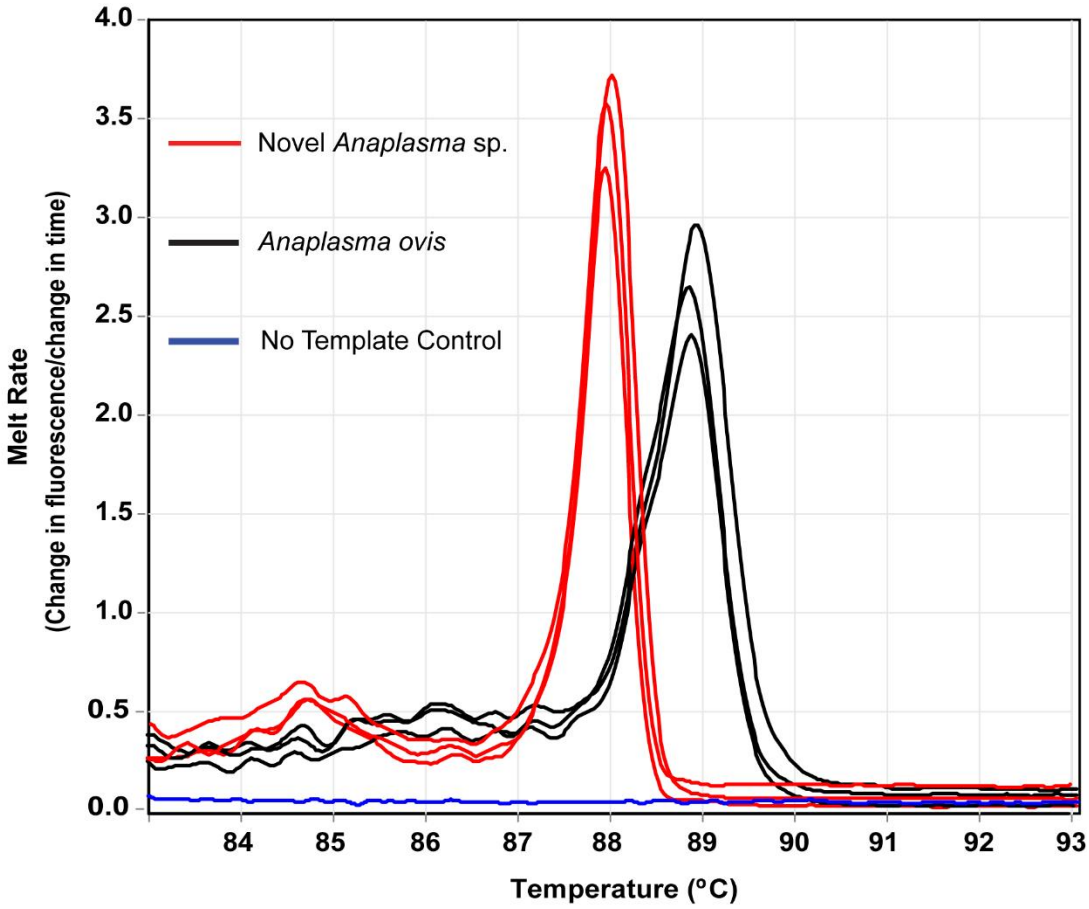


**Figure 4.3: HRM melt curves of amplification of 16S rRNA of *Anaplasma* spp. in goats.**

On alignment of edited *Anaplasma* 16S rRNA sequences with closely related sequences retrieved from NCBI GenBank nr database, most samples showed a 100% similarity to *A. ovis* (GenBank accession MG869525). However, some sequences were distinctly different in bases from the GenBank-retrieved *Anaplasma ovis* among other sequences with an identity of 96.8% and below (**Figure 4.4**).

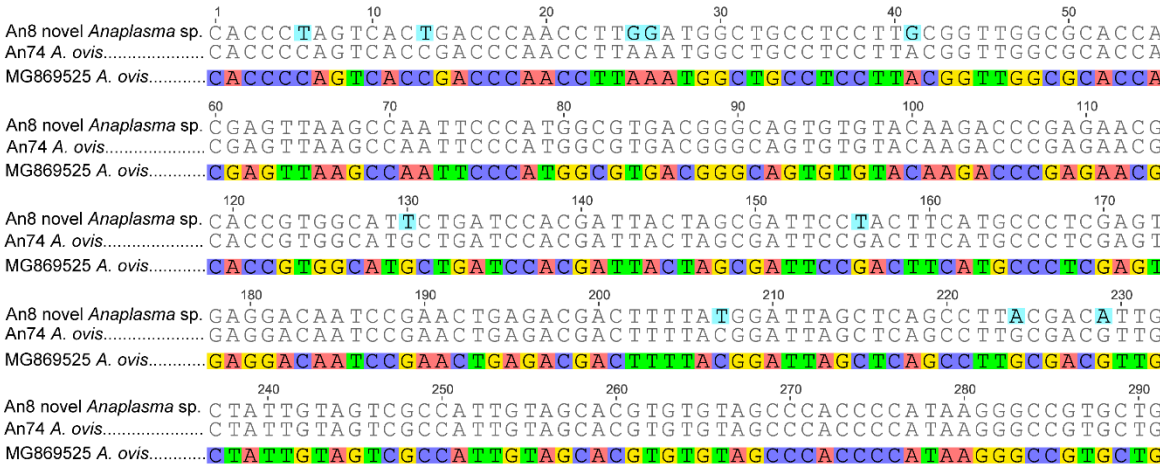


**Figure 4.4: Multiple sequence alignment of 16S rRNA sequences of *Anaplasma* spp.**

This figure shows the multiple sequence alignment of 16S rRNA sequences of (i) novel *Anaplasma* sp. (study sample An8) and (ii) *A. ovis* (An74) amplified from goat blood, and (iii) the GenBank-retrieved sequence of *A. ovis*, accession MG869525. Nucleotide changes were identified between MG869525 and An8 sequences. For example, at position 25 and 26 in the above alignment, 'AA' in the GenBank sequence MG869525 is replaced by 'GG' in the query sequence An8 (study sample). On the other hand, An74 sequence (from this study) was 100% identical to the *A. ovis* sequence MG869525 from GenBank.

*Sheep blood samples:*

*Anaplasma ovis* 93.5% (101), *Ehrlichia canis* 22.2% (24), and *Theileria ovis* 38.9% (42) were present in sheep blood (108) (**Table 4.1**). Further, pairwise alignment of the edited *Theileria* 18S rRNA sequences with closely related GenBank retrieved sequence of *Theileria ovis* (GenBank accession MN712508), showed 100% similarity (**Appendix I**).

*Donkey blood samples:*

Donkey blood samples (36) were found to be infected with *Ehrlichia canis* 25% (9), *'Candidatus* Anaplasma camelii' 11.1% (4), *Trypanosoma vivax* 22.2% (8) and *Theileria equi* 13.9% (5) (**Table 4.1**). Pairwise alignment of the 200-bp *Ehrlichia* 16S

rRNA sequence with GenBank retrieved sequence of *Ehrlichia canis* (GenBank accession MT499360), showed 100% similarity (**Appendix II**). Further, BLAST analysis of the edited *Theileria* 18S rRNA sequences with closely related sequences retrieved from NCBI GenBank nr database, showed 100% identity with *Theileria equi* (**Table 4.2**). Similarly, BLAST analysis of the *Anaplasma* 16S rRNA sequences, showed 100% similarity to *'Candidatus* Anaplasma camelii' (**Table 4.2**).

### 4.2.2 Pathogen detection in keds

*Goat and sheep keds:*

In keds collected from co-herded goats and sheep, we detected *Ehrlichia canis* 51.7% (60/116) and three trypanosome species, namely *Trypanosoma vivax* 29.3% (34/116), *Trypanosoma evansi* 0.9% (1/116) and *Trypanosoma godfreyi* 0.9% (1/116) (**Table 4.1**).

*Donkey keds:*

*Ehrlichia canis* 63.6% (7/11) and *Trypanosoma vivax* 18.2% (2/11) were detected in keds collected from donkeys (**Table 4.1**).

*Dog keds:*

The keds collected from dogs were found to harbor *Ehrlichia canis* 76% (82/108), *Trypanosoma vivax* 15.7% (17/108), *Trypanosoma simiae* 0.9% (1/108), and *Trypanosoma evansi* 0.9% (1/108) in dog keds (**Table 4.1**). Also, the dog keds harbored *Clostridium perfringens* 46.3% (50/108), *Brucella abortus* 5.6% (6/108) and *Bartonella schoenbuchensis* 76% (82/108). Similarly, BLAST analysis of the 200-bp *Ehrlichia* 16S rRNA sequences showed an identity of 100% with *Ehrlichia canis* sequenced from ticks and fleas collected from companion dogs and cats in East and Southeast Asia (GenBank accession MT499360) (**Table 4.2**). All the ked samples were negative for *Anaplasma* spp. and piroplasms (*Theileria* and *Babesia* spp.). Moreover, only keds obtained from

dogs were found to be positive for *Brucella abortus*, *Clostridium perfringens* and *Bartonella schoenbuchensis.*

Sequences of pathogens detected in this study were deposited in GenBank database and assigned accession numbers as follows: *Anaplasma ovis* (MZ203400) and novel *Anaplasma* sp. (MZ203399) in goat, *Anaplasma ovis* (OM282854) and *Theileria ovis* (OM282856) in sheep, *Candidatus* Anaplasma camelii (MZ203398) and *Theileria equi* (OM282857) in donkey, *Bartonella schoenbuchensis* (MZ203403), *Clostridium perfringens* (MZ203401), *T. simiae* (MZ221829), and *E. canis* (OM282855) in dog keds, and *T. evansi* (MZ221830) in goat/sheep keds (**Table 4.2**).

### 4.3 Potential role of hippoboscids in xenodiagnosis

We detected *Trypanosoma vivax* and *Ehrlichia canis* in both the hippoboscids and their associated hosts. Infection with *Ehrlichia canis* was present in goat blood 66.1% (162/245), sheep blood 22.2% (24/108), and keds collected from the co-herded goats and sheep 51.7% (60/116). Similarly, *Ehrlichia canis* was also present in donkey blood 25% (9/36) and their associated keds 63.6% (7/11). On the other hand, infection with *T. vivax* was detected in goat blood 7.3% (18/245) and keds collected from the co-herded goats and sheep 29.3% (34/116), and, in donkey blood 22.2% (8/36) and their associated keds 18.2% (2/11), but was not detected in blood collected from sheep (**Table 4.1**).

### 4.4 Phylogenetic analysis of *Anaplasma* spp. 16S rRNA sequences

The phylogenetic tree comparing sequences of 16S rRNA gene fragments of *Anaplasma* (900-1000 bp) from this study to other sequences of the same gene available in GenBank is presented in **Figure 4.5**. Phylogenetic relationships and molecular evolution were inferred using maximum likelihood method. The tree was drawn to scale representing a 2% evolutionary change in nucleotides per site with the topologies estimated using nearest neighbor interchange improvements over 1,000 bootstrap replicates. Homology analysis of *Anaplasma ovis* (OM282854 and MZ203400) sequences from this study

showed that they are identical to each other and the published *Anaplasma ovis* from China (MG869525) and Kenya (MW467549), while, '*Ca.* Anaplasma camelii' sequence from this study (MZ203398) also showed that it's related to the published '*Ca.* Anaplasma camelii' from Kenya (MT510533). Further, the phylogenetic analysis clearly shows the genetic diversity between the novel *Anaplasma* sp. with the published *A. ovis* from China (MG869525) and Kenya (MW467549) in **Figure 4.5**.

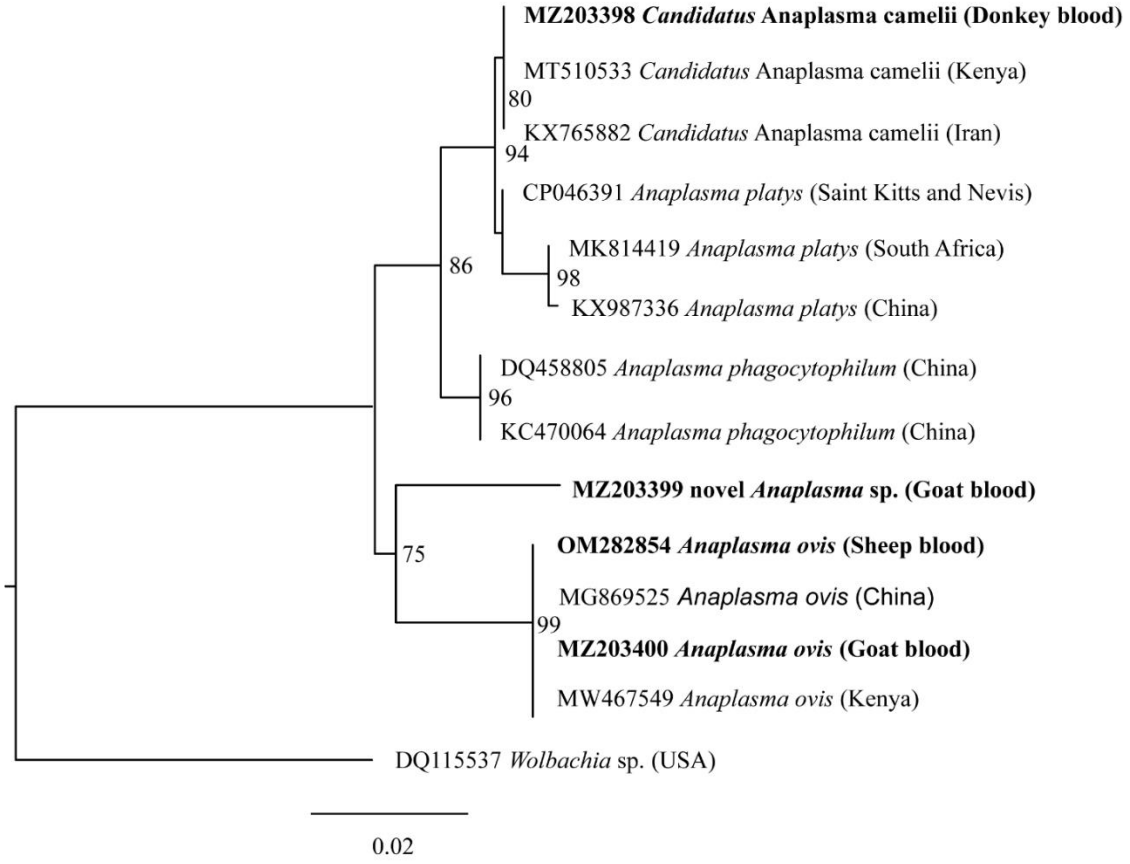


**Figure 4.5: Maximum likelihood phylogenetic tree of 16S rRNA gene of *Anaplasma* spp.**

The tree shows the close relation and genetic divergence of *Anaplasma* spp. sequences from this study and that retrieved from GenBank. Bootstrap values at the major nodes are of percentage agreement among 1000 bootstrap replicates. The GenBank accession numbers, species identification and country of origin are indicated for each sequence.

Sequences from this study are indicated in bold, and the associated study sample indicated at the end. *Wolbachia* endosymbiont (GenBank accession DQ115537) 16S rRNA was used as the outgroup.

# CHAPTER FIVE
# DISCUSSION

In northern Kenya, there is little information about the different species of hippoboscids infesting domestic animals, and infectious pathogens circulating in domestic animals due to lack of proper disease surveillance. In this study, we aimed to identify the different hippoboscid species infesting domestic animals, and also determine the occurrence and prevalence of selected haemopathogens in domestic animals (goats, sheep and donkeys) and their predominant ectoparasitic keds (collected from goats, sheep, donkeys and dogs) in the area of study in Laisamis, Marsabit County of northern Kenya.

We report the identification of two hippoboscid species, *Hippobosca variegata* (GenBank accession MW128366) and *Hippobosca longipennis* (GenBank accession MW128365), infesting goats, sheep, donkeys, and dogs respectively, in Laisamis. These two species can be identified easily by observing the color pattern of the scutellum, with *H. variegata* having a scutellum color pattern of yellow-brown-yellow-brown-yellow, and *H. longipennis* having a scutellum color pattern of yellow only (Bargul *et al*., under review). We observed that *H. longipennis* were only found on dogs, while *H. variegata* had a wide host range as they were found on goats, sheep, and donkeys. *H. longipennis* flies have been reported previously to heavily infest on dogs in Turkana, Kenya (Nelson, 1963).

In addition to the two hippoboscid species identified, a camel-specific *Hippobosca camelina* has also been reported previously to infest on camels in the area of study (Kidambasi *et al*., 2020). These hippoboscids are blood-feeding obligate ectoparasites of vertebrates belonging to the family hippoboscidae (Rahola *et al*., 2011). They mainly infest the underbelly of their host, but can also be found on other parts of the body such as the ears, girth, neck and hump (Kidambasi *et al*., 2020). Additionally, these flies have claspers for firm attachment to the skin hairs of the host during feeding or resting.

The occurrence and prevalence of selected hemopathogens in the domestic animals and their predominant ectoparasitic keds was also investigated in Laisamis, the area of study, and our findings revealed *Trypanosoma vivax* as the predominant trypanosome species in domestic animals and keds, outside the tsetse belts, with an infection rate of 6.7% (26/389) and 22.6% (53/235), respectively. This agrees with a previous study that reported *T. vivax* as the major case of trypanosome infection outside tsetse infested areas in western Kenya (Thumbi *et al*., 2010). Similarly, *T. vivax* infection in camels and camel keds was previously reported from the same study area (Kidambasi *et al*., 2020), suggesting *T. vivax* as the major cause of trypanosomiasis in Laisamis, northern Kenya.

Moreover, many keds can acquire blood meal at the same time from one animal that is infected by trypanosomes, therefore, the possibility of their positivity rate being higher compared to that of their hosts. *T. vivax* is known to be pathogenic to goats, sheep and equids (Galiza *et al.,* 2011), and trypanosomiasis disease caused by this pathogen is among the most important diseases constraining livestock productivity and agricultural development, for instance, in Ethiopia (Bedada & Dagnachew, 2012).

Donkeys were found to harbor *Trypanosoma vivax*, *Ehrlichia canis*, '*Ca*. Anaplasma camelii', and *Theileria equi*, with *E. canis* being the most prevalent pathogen 25% (9/36). Currently, *Ehrlichia canis* is receiving increasing attention due to its high morbidity and mortality in animals (Bunroddith *et al*., 2018). Detection of this canine pathogen (*E. canis*) in donkeys is not surprising because the pastoralist farmers rear mixed livestock species together with other domestic animals including dogs, thus the donkeys could have acquired this pathogen from infected dogs through tick bites. This pathogen causes ehrlichiosis, an emerging disease of domestic animals mainly transmitted by ticks, and has previously been reported to infect dogs, cattle, humans, and goats (Zhang *et al*., 2017).

Additionally, donkeys tested positive for the camel associated bacteria, '*Ca.* Anaplasma camelii', previously reported in Kenyan camels (Kidambasi *et al*., 2020). The presence of this camel pathogen in donkeys could be due to the fact that donkeys were co-herded

with camels, thus, the donkeys could have acquired the pathogen from infected camels. Keds are the common ectoparasites infesting domestic animals in the study area and have been reported as mechanical vectors of this bacterial pathogen (Bargul *et al*., 2021). Further research is needed to determine the zoonotic potential as well as the pathogenic role of this pathogen in donkeys and other livestock species. Among the piroplasms screened, *Theileria equi* (13.9%) was detected in donkeys. Pathogens associated with this genus are among the causative agents of equine piroplasmosis and have previously been shown to infect donkeys in some parts of Kenya including Mwingi (Oduori *et al*., 2015).

Goats were found to be infected with *T. vivax*, *E. canis*, *Theileria ovis*, *A. ovis* and a novel *Anaplasma* species. Similarly, sheep were also found to harbor *E. canis*, *Theileria ovis,* and *A. ovis. Theileria ovis* was more prevalent in sheep (38.9%) than in goats (0.8%) which is consistent with a study done on livestock in Palestine (Azmi *et al*., 2019). *T. ovis* is known to cause ovine theileriosis, a disease that is among the most important infectious diseases affecting small ruminants, leading to significant economic losses to farmers (Al-Hosary *et al*., 2021). *E. canis* and *T. vivax* were detected in most of the samples analyzed, including keds, suggesting these two are the common pathogens circulating in livestock herds in the study area. More studies will be needed to understand the vectors of these pathogens and whether keds are competent vectors of the pathogens.

Further, a high prevalence of *Anaplasma ovis* was detected in goats (84.5%) and sheep (93.5%). This finding is consistent with a study done in Tunisia, North Africa, in goats and sheep (Said *et al*., 2015). This high prevalence of *Anaplasma ovis* could also be attributed to the presence of ticks in most of the domestic animals. *Anaplasma ovis* is distributed worldwide and considered a major cause of anaplasmosis in small ruminants in tropical and subtropical regions of the world with general clinical effects ranging from fever, fatigue, low milk production and abortion but with a low mortality rate (Stuen *et al*., 2011). Moreover, *A. ovis* has also been reported in dairy goats in a previous study in

Corsica, France, after an extensive survey due to health and production problems encountered in the goat flocks (Cabezas-Cruz, Gallois, *et al*., 2019).

Among the goat blood samples analysed, we discovered 11.8% (29/245) of the samples were infected with an unidentified *Anaplasma*-like species. The *Anaplasma* sp. shared 96.77% sequence identity with *A. ovis* sequenced from goat blood in China (GenBank accession MG869525). The GC content of this novel *Anaplasma* sp. was 52.9%, and had clear and distinct differences in the bases on alignment with the retrieved *Anaplasma ovis* from NCBI GenBank nr database. The pathogenic role of this *Anaplasma*-like species in goats is not understood. However, it is related to *A. ovis*, which is known to be pathogenic in goats, sheep, and some wild ruminants (Said *et al*., 2015).

We detected more hemopathogens in dog keds than in other keds including, *T. vivax, T. evansi, T. simiae, E. canis, Clostridium perfringens, Bartonella schoenbuchensis,* and *Brucella abortus.* Keds collected from dogs acquired these pathogens from infected dogs during their bloodmeal feeding. Most dogs had a free-roaming lifestyle in the study region, hence, it is possible that dogs were at a higher risk of being infected with a wide range of pathogens. We were unable to collect blood sample from dogs during sampling because we lacked proper protective gear against dog bites and also the dogs were not vaccinated from rabies and thus, collecting blood from dogs was considered very risky.

The highest prevalence rates among all the pathogens detected in keds obtained from dogs were observed in *E. canis* (76%) and *B. schoenbuchensis* (76%). This high prevalence rate of these pathogens in dog keds, could be due to the high competition of pathogens circulating in the same host population, considering the possible interactions between the pathogens and, host immune system, and host life cycle as well (Poletto *et al.*, 2015). Previous studies have also reported detection of *B. schoenbuchensis* in deer ked by PCR test with a prevalence rate of more than 60% (Szewczyk *et al*., 2017).

*Bartonella schoenbuchensis* is one of the most important species that causes bartonellosis and it has been reported to cause infections in humans, cattle and wild

animals such as the cervids in Asia, North America and Europe (Rolain *et al*., 2003; Vayssier-Taussat *et al*., 2016). In addition, *Bartonella* infection often manifests as various cardiovascular, neurological and rheumatologic conditions, making it a public health concern since pastoralists, farmers and veterinarians who interact with domestic animals are at a high risk of infection (Maggi *et al*., 2012). There is little information on presence of *E. canis* in keds, but this pathogen has been detected previously in *Rhipicephalus sanguineus* (the brown dog tick), the biological vector of the pathogen (Cabezas-Cruz, Allain, *et al*., 2019).

We also report the first occurrence of *Clostridium perfringens* in dog keds. This pathogen is an important cause of enteric diseases in humans and domestic animals and is responsible for several forms of enterotoxaemia, which differs in clinical manifestation and severity according to the toxigenic type involved and specific toxins produced (Singh et al., 2018). Majorly, it affects small ruminants worldwide causing heavy mortality and significant economic impact (Sumithra *et al*., 2013). *Clostridium perfringens* has been shown to cause death in dogs due to hemorrhagic gastroenteritis of the gastrointestinal tract, thus, more research is needed to better understand the role of this bacterium in enteric disease of dogs (Schlegel *et al*., 2012).

This study also reports detection of *Brucella sp.* (5.6%) in dog keds that was closely related to *Brucella abortus* sequenced from cattle milk in India, showing a sequence identity of 98.68% (GenBank accession MK881176). Brucellosis in dogs is mainly associated with *B. canis* and not *B. abortus*, which mainly occur in cattle. However, cross-species transmission of *Brucella* spp. is possible and this is consistent with a study done in Argentina that detected *B. abortus* in farm dogs (Mortola *et al*., 2019). Further, *B. abortus* is a common source of human infection with a high zoonotic potential, and cause a disease called Bang's disease in humans (Kaden *et al*., 2018). *Brucella* species have been shown to be of high public health and socio-economic importance in northern Kenya, and mainly transmitted to humans through ingestion of unpasteurized dairy products or raw/undercooked animal products (Kairu-Wanyoike *et al*., 2019).

*T. vivax* (15.7%) detected in keds obtained from dogs was more prevalent than *T. evansi* (0.9%) and *T. simiae* (0.9%) that had a low prevalence rate. Presence of these trypanosome species in dog keds suggests that the keds took up this infection during their bloodmeal acquisition from dogs that were initially infected with trypanosomes possibly from other biting flies like *Stomoxys* and *Tabanids*. Similarly, *T. vivax* (29.3%) was also more prevalent than *T. evansi* (0.9%) and *T. godfreyi* (0.9%) in keds obtained from goats. This low infection rate could be attributed to disease stability in the area, change in climate and seasonal outbreaks (Gutierrez *et al*., 2006).

Further, both *T. vivax* and *T. evansi* have recently been detected in camel keds in northern Kenya (Kidambasi *et al*., 2020). Moreover, *T. godfreyi* and *T. simiae* have also been shown to infect a wide range of domestic animals including pigs, cattle, camels, dogs and goats with *T. simiae* being highly pathogenic to domestic pigs (Hamill *et al*., 2013; Simwango *et al*., 2017). *T. simiae* and *T. godfreyi* are among the *Trypanosome* spp. that cause African animal trypanosomiasis with tsetse flies being their main vector (Isaac *et al*., 2016). This is the first report of occurrence of these two *Trypanosome* spp. in keds.

Screening of keds collected from donkeys showed detection of *T. vivax* (18.2%) and *E. canis* (63.6%) as the common pathogens. Detection of these pathogens in donkeys and goats, as well as in their associated ectoparasitic keds shows the xenodiagnostic potential of using keds to indirectly screen for pathogens occurring in their associated hosts. This finding is consistent with a recent report that showed occurrence in keds of pathogens that were similarly present in their camel host from which they were collected, and further proposed the potential use of keds in xenodiagnosis (Kidambasi *et al*., 2020). Hippoboscid species have been implicated in transmission of pathogens (Rahola *et al*., 2011). For example, in transmission of mammalian trypanosomatidae of the genus *Megatrypanum*, and are suspected to be vectors of *T. avium* and *T. corvi* in birds (Svobodová *et al*., 2015).

It is likely that when keds bite their hosts to acquire bloodmeals, they also take up haemopathogens if the host is infected. Sheep blood in this study was not found to be infected with *T. vivax*, but goat and donkey blood, and keds collected from donkeys and co-herded goats and sheep, were found to be infected with *T. vivax*. This could possibly be attributed to the fact that goats and donkeys were initially infected with trypanosomes from other biting flies like *Stomoxys* and *Tabanids* that have previously been shown to be present in the study area (Kidambasi *et al*., 2020).

However, detection of pathogens in keds does not incriminate them as vectors, but more studies should be done to determine the vector competence of these keds. Keds can be good candidates for xenosurveillance and they can be collected easily for molecular screening to detect pathogens acquired from naturally infected animals in the process of feeding. Importantly, this xenosurveillance detection provides a less invasive approach than the currently available painful blood collection procedures. A recent report has also shown the utilization of sand flies in evaluating the infectiousness of humans in an area endemic for visceral leishmaniasis in India (Singh *et al*., 2021).

# CHAPTER SIX

# CONCLUSIONS AND RECOMMENDATIONS

## 6.1 Conclusions

This study confirms *Hippobosca variegata* and *Hippobosca longipennis* to be among the major pests infesting livestock in Laisamis, Marsabit County of northern Kenya. Our findings also show the detection of various selected infectious hemopathogens of veterinary and public health concern in the domesticated animals, and their associated ectoparasitic biting keds. Notably, we sequenced multiple hemopathogens in dog keds, including zoonotic ones (*Brucella abortus*), suggesting dogs, which closely interact with livestock and humans, as key reservoirs of diseases in Laisamis.

Our findings also suggest the potential use of hippoboscids in xenodiagnosis for detection of haemopathogens in their associated hosts, thus bypassing the need to obtain blood samples via jugular venipuncture for pathogen detection. Further, findings from this study contributes to increase in knowledge on the prevalence rate and presence of the various pathogens circulating in livestock and their ectoparasitic biting keds in Laisamis, northern Kenya. Information from this study is crucial in guiding policy makers and livestock farmers in vector and disease control in the northern Kenya community.

## 6.2 Recommendations

Based on the findings from this study, the following can be recommended:

1. Further surveillance studies to map out and increase the understanding of the epidemiology of circulating livestock diseases in different study areas in northern Kenya.
2. Further studies to establish the role of keds in disease transmission, particularly of zoonotic importance, since they also occasionally feed on humans.
3. Further research studies to catalogue additional blood-borne pathogens including viruses occurring both in hippoboscids and their hosts, to provide more

information to support usage of hippoboscids in xenomonitoring of animal diseases.

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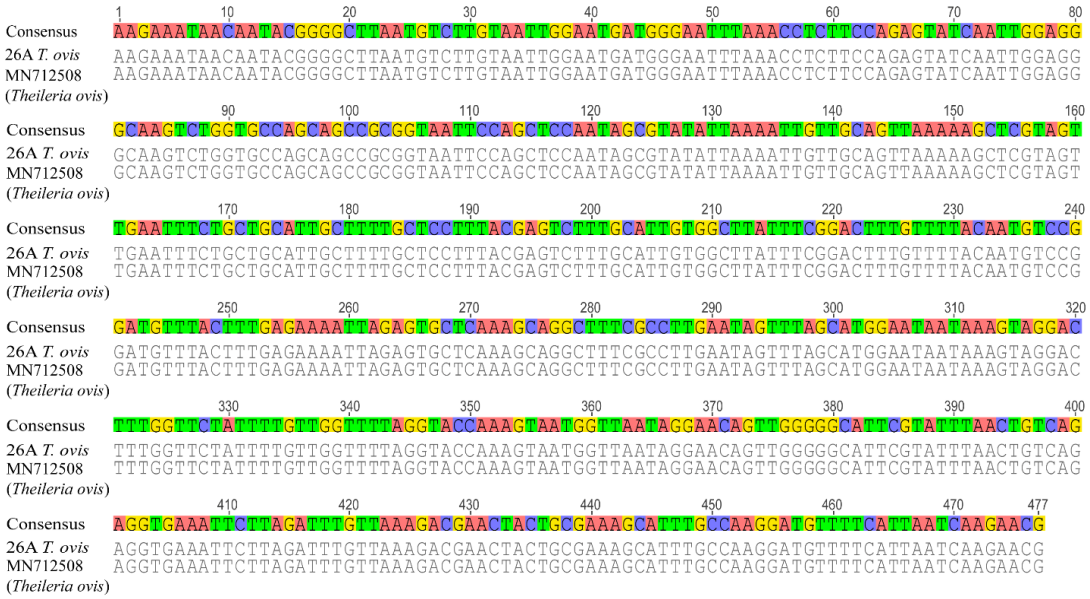
# APPENDICES

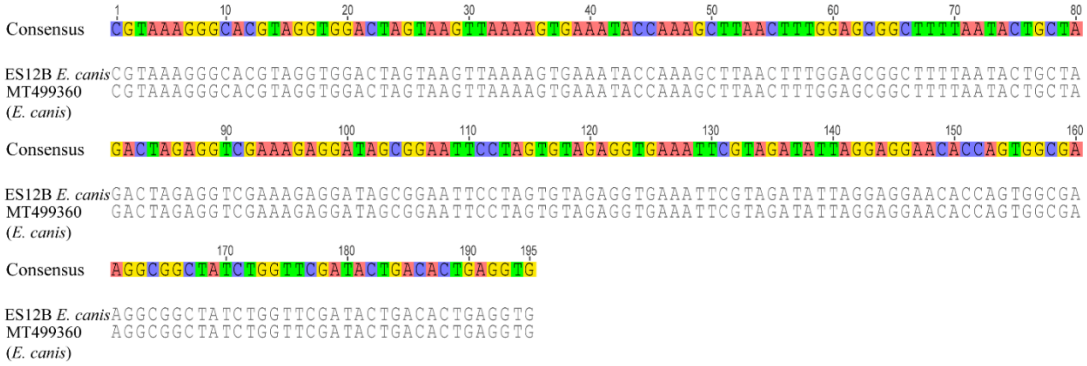
## Appendix I: Pairwise alignment of 18s rRNA sequence of *Theileria ovis*

This figure shows the pairwise alignment of 18S rRNA sequences of *Theileria ovis* in sheep (study sample 26A) and the GenBank-retrieved sequence of *Theileria ovis*, accession MN712508. The percentage similarity is 100%.



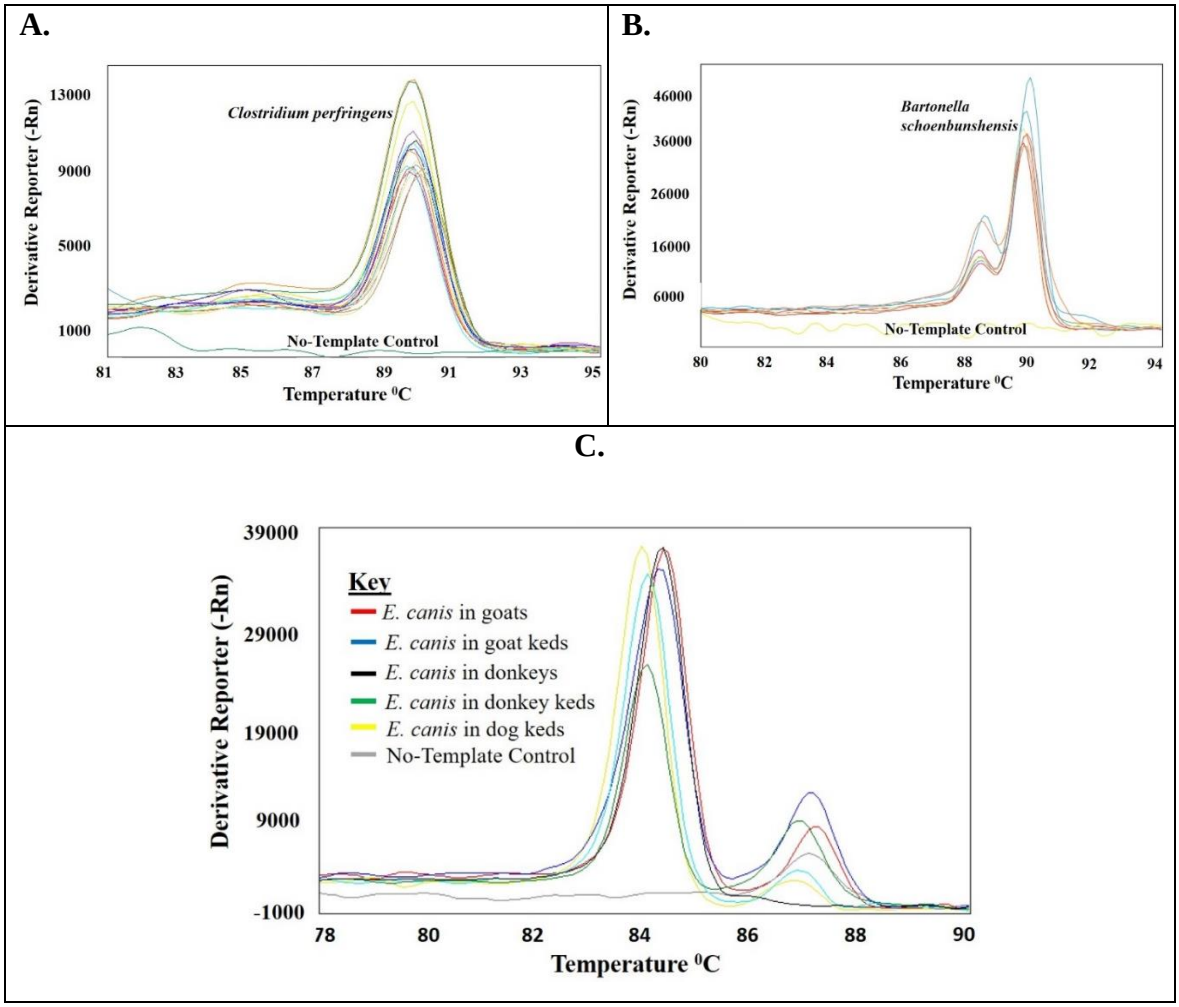
**Appendix II: Pairwise alignment of 16s rRNA sequence of *Ehrlichia canis***

This figure shows the pairwise alignment of 16S rRNA sequences of *Ehrlichia canis* in donkey (study sample ES12B) and the GenBank-retrieved sequence of *Ehrlichia canis*, accession MT499360. The percentage similarity is 100%.



**Appendix III: HRM melt curves of amplification of 16S rRNA of pathogens in domestic animals and keds.**

**A.** *Clostridium perfringens* in dog keds, **B.** *Bartonella schoenbuchensis* in dog keds, and **C.** *Ehrlichia canis* in domestic animals and their associated keds. These melt curves were generated by Quant Studio 3 Real-Time PCR System.



# Appendix IV: Ethical approval

icipe
African Insect Science for Food and Health

*IACUCAnimalUseForm2017*

## IACUC - ANIMAL USE FORM

**IACUC ref no. IcipeACUC2018-003**
**IACUC use only**

This form is to be submitted to iacuc@icipe.org after completing all sections and all endorsements (see Annex for instructions).

**POINTS TO NOTE:**

1. This form is valid, unchanged, for one year.
2. If minor changes are required then the **IACUC - ANIMAL USE MINOR AMENDMENT FORM** (see Annex for attachment – ***IACUCMinorAmendmentForm2017***) should be completed and sent to the IACUC committee for consideration.
3. The committee requires the form to be submitted to the IACUC committee at least two weeks before the start of the activity to enable sufficient time for it to be approved.
4. No activity shall be approved unless the current file format (***IACUCAnimalUseForm2017***) is used.
5. On completion of filling in the Animal Use Form, ensure that the Risk Assessment Form (see 'Usefull documents" Annex (Page 5) for attachment) is also filled, if required, and submitted to the Institute Biosafety Committee (IBC) committee.
6. The *icipe* IACUC committee follows international standards for animal care and use; the following sections are used to confirm that researchers have adequately considered the 3R's (Replacement, Reduction, Refinement) relating to the use animals in research and training.
7. Only persons named on this form (section 3 – 5) shall be allowed to handle and/or carry out sampling of the animals.
8. Upon completion of the activity, the PI shall complete the PI Report Back form (see Annex for attachment) and send it to the IACUC.
9. The animals used belong to the PI during the period of the activity (as indicated on this form) and the ownership can be transferred to the Farm three weeks after completion of the study through a written agreement with copy to IACUC.
10. Experiments that do not involve breeding, male animals should be castrated to avoid aggressive behavior.
11. Animals should be dehorned to avoid fighting and bullying.

| 1. Title of Research Activity (experiment, survey or training): | (Max. 30 words) |
|---|---|
| Do camel hippoboscids play role in transmission of pathogenic animal trypanosomes in northern Kenya? | |

| 2. Team, location & grant code under which activity falls: | e.g. BT02 NBO WEL004 |
|---|---|
| **Team:** Dr Joel Bargul (THRiVE-2 postdoctoral fellow), Dr Daniel Masiga (sponsor), and Prof Mark Carrington (Cambridge THRiVE-2 mentor)<br>**Location:** icipe's Animal Health Department<br>**Grant code:** B7120F (THRiVE-II Postdoc – Joel) | |

| 3. Principal Investigator (PI) or Team Leader:<br>(Person ultimately responsible for this activity) | Dr. Joel Ltilitan Bargul | Home Tel.<br>Mobile Tel. | +254 726862228 |
|---|---|---|---|
| 4. Collaborators: | icipe & University of Cambridge, UK.<br>The UK mentor at the Univ. of Cambridge is not involved in any way with animal experiments to be only conducted at icipe by Joel Bargul. | | |
| 5. Technicians/staff involved in animal sampling & handling: | Mr. Jeremiah Ojude | | |

| 6. Overall objectives of the activity: | *(Provide numbered list)* |
|---|---|
| (i) To study the prevalence of camel trypanosomiasis in northern Kenya<br>(ii) To characterize haematophagous biting flies of genus *Hippobosca* associated with camels in northern Kenya<br>(iii) To identify trypanosomes species associated with the fly and its camel host<br>(iv) To identify the bloodmeal sources of hippoboscids.<br>(v) To determine vectorial capacity of camel hippoboscids in transmission of trypanosomiasis. | |

| 7. Background information: | *Give information that the reviewers can use to understand the activity, include references* |
|---|---|

## Appendix V: Publication in a peer review journal

Open Research Africa

Open Research Africa 2022, 5:23 Last updated: 06 JUN 2022

Check for updates

RESEARCH ARTICLE

# Molecular detection of novel *Anaplasma* sp. and zoonotic hemopathogens in livestock and their hematophagous biting keds (genus *Hippobosca*) from Laisamis, northern Kenya

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**Abstract**

**Background:** Livestock are key sources of livelihood among pastoral communities. Livestock productivity is chiefly constrained by pests and diseases. Due to inadequate disease surveillance in northern Kenya, little is known about pathogens circulating within livestock and the role of livestock-associated biting keds (genus *Hippobosca*) in disease transmission. We aimed to identify the prevalence of selected hemopathogens in livestock and their associated blood-feeding keds.
**Methods:** We randomly collected 389 blood samples from goats (245), sheep (108), and donkeys (36), as well as 235 keds from both goats and sheep (116), donkeys (11), and dogs (108) in Laisamis, Marsabit County, northern Kenya. We screened all samples for selected hemopathogens by high-resolution melting (HRM) analysis and sequencing of PCR products amplified using primers specific to the genera: *Anaplasma, Trypanosoma, Clostridium, Ehrlichia, Brucella, Theileria,* and *Babesia.*
**Results:** In goats, we detected *Anaplasma ovis* (84.5%), a novel *Anaplasma* sp. (11.8%), *Trypanosoma vivax* (7.3%), *Ehrlichia canis* (66.1%), and *Theileria ovis* (0.8%). We also detected *A. ovis* (93.5%), *E. canis* (22.2%), and *T. ovis* (38.9%) in sheep. In donkeys, we detected '*Candidatus* Anaplasma camelii' (11.1%), *T. vivax* (22.2%), *E. canis* (25%), and *Theileria equi* (13.9%). In addition, keds carried the following pathogens; goat/sheep keds - *T. vivax* (29.3%), *Trypanosoma evansi*

(0.86%), *Trypanosoma godfreyi* (0.86%), and *E. canis* (51.7%); donkey keds - *T. vivax* (18.2%) and *E. canis* (63.6%); and dog keds - *T. vivax* (15.7%), *T. evansi* (0.9%), *Trypanosoma simiae* (0.9%), *E. canis* (76%), *Clostridium perfringens* (46.3%), *Bartonella schoenbuchensis* (76%), and *Brucella abortus* (5.6%).

**Conclusions:** We found that livestock and their associated ectoparasitic biting keds carry a number of infectious hemopathogens, including the zoonotic *B. abortus*. Dog keds harbored the most pathogens, suggesting dogs, which closely interact with livestock and humans, as key reservoirs of diseases in Laisamis. These findings can guide policy makers in disease control.

**Keywords**

Vector-borne diseases, Hippobosca, high-resolution melting analysis, hemopathogens, keds

This article is included in the Developing Excellence in Leadership, Training and Science Africa gateway.

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**Author roles: Mwaki DM**: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation; **Kidambasi KO**: Conceptualization, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Kinyua J**: Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – Review & Editing; **Ogila K**: Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – Review & Editing; **Kigen C**: Conceptualization, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Getange D**: Conceptualization, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Villinger J**: Conceptualization, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Masiga DK**: Conceptualization, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Carrington M**: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Validation, Visualization, Writing – Review & Editing; **Bargul JL**: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*



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**First published:** 06 Jun 2022, **5**:23 https://doi.org/10.12688/openresafrica.13404.1

## Introduction

Livestock in Africa are considered as one of the most valuable agricultural assets for the rural and urban poor, and accounts for about 40% of the agricultural GDP (Malabo Montpellier Panel, 2020). In 2018, Africa's total livestock population was estimated at 2 billion poultry birds, 438 million goats, 384 million sheep, about 356 million cattle, 40.5 million pigs, almost 31 million camels, and 38 million equines (including 30 million donkeys, 6.5 million horses, and 885,000 mules) (Malabo Montpellier Panel, 2020). This represents about one-third of the world's livestock population (Otte *et al.*, 2019). Moreover, livestock production plays a key economic role to the livelihood of pastoralists living in the marginalized arid and semi-arid regions of northern Kenya (Mburu *et al.*, 2017). These pastoralists largely depend on livestock as a source of meat and milk, income from selling livestock, and donkeys and camels also serve as a mode of transport. Pastoralism supports about 20 million people, produces about 90% of the meat consumed in East Africa and contributes to about 13% of the GDP in Kenya (Nyariki & Amwata, 2019).

Unfortunately, livestock production is hindered by pests and diseases, which are endemic in northern Kenya (Perry & Grace, 2009). Hemopathogens of livestock, particularly those of zoonotic importance, are responsible for some of the most serious emerging infectious diseases facing sub-Saharan Africa and the rest of the world (Rosenberg *et al.*, 2018). About 75% of newly emerging diseases currently affecting humans originated in animals (Jones *et al.*, 2008). In Kenya, hemoparasites that cause babesiosis, theileriosis, rickettsiosis, anaplasmosis, and ehrlichiosis are a major impediment to livestock productivity and public health (Kiara *et al.*, 2014).

Bacterial diseases in livestock include bartonellosis caused by *Bartonella* spp., which is mainly transmitted by biting arthropod vectors such as ticks and reported widely in both wild and domestic mammals such as dogs, cats, and cattle (Ereqat *et al.*, 2016). In addition, brucellosis, a zoonotic disease, has been reported worldwide and mainly causes infections to the genitals of animals, abortion, and fetal death (Probert *et al.*, 2004). *Brucella* species have been shown to be of high public health and socio-economic importance in northern Kenya (Kairu-Wanyoike *et al.*, 2019). Parasitic protozoal infections, for example African animal trypanosomiasis, cause debilitating diseases in livestock and serious economic losses in Africa (Petersen *et al.*, 2007). Etiological agents such as *Clostridium perfringens* cause enteric diseases such as enterotoxaemia in both humans and livestock, mostly goats and sheep (Singh *et al.*, 2018).

Livestock act as reservoirs of infectious pathogens that can be transmitted by various vectors. Ticks and biting flies such as *Stomoxys* spp. and tabanids are vectors of infectious pathogens including bacteria, viruses (*e.g.*, Rift Valley fever viruses), rickettsiae (*Coxiella, Anaplasma*), and protozoa (*T. evansi, T. vivax*, *T. simiae*) (Baldacchino *et al.*, 2013; Narladkar, 2018). Hippoboscid flies, commonly known as keds and belonging to the family Hippoboscidae within the superfamily Hippoboscoidea, are obligate ectoparasites of vertebrates, both domestic and wild animals and birds (Petersen *et al.*, 2007; Rahola *et al.*, 2011). Members of Hippoboscidae act as vectors of many infectious agents including bacteria, viruses, and protozoans (Rahola *et al.*, 2011). Keds cause economic losses in various ways, including annoyance and psychological disturbances produced during the act of biting and feeding, the diseases they transmit (Bargul *et al.*, 2021), and expenditure incurred by farmers in controlling them (Narladkar, 2018). The painful bites inflicted on the bloodmeal host by keds result in skin lesions and by feeding on blood, they contribute to anaemia (Oyieke & Reid, 2003).

In northern Kenya, keds and ticks are common external pests of livestock, found on livestock all year round (Bargul *et al.*, 2021). Keds are known to infest and blood-feed on all livestock species, domestic and wild animals. In addition, keds also feed on humans and in the process, could transmit zoonotic pathogens (Getahun *et al.*, 2020). To date, little efforts have been put into surveillance of pathogens harbored by the livestock and the role of keds in spreading various diseases. This calls for an urgent need for research studies to catalogue livestock infectious and zoonotic pathogens circulating in livestock for a better understanding of disease prevalence, transmission routes, and for control. In this study, we screened for selected hemopathogens (*Anaplasma, Trypanosoma, Clostridium, Ehrlichia, Brucella, Theileria,* and *Babesia* spp.) in goats, sheep, and donkeys, and in keds collected from goats, sheep, dogs, and donkeys.

## Methods

### Study site

The study was conducted in Laisamis sub-County (1° 36' 0" N, 37° 48' 0" E) in Marsabit County, northern Kenya (Figure 1). Marsabit County borders Ethiopia to the North, Turkana County to the West, Samburu and Isiolo Counties to the South, and Wajir County to the East. Laisamis sub-County occupies an area of 20,290 km$^2$ that comprises five County Assembly Wards, among which Laisamis Ward (3,885 km$^2$), the area of this study has arid and semi-arid climatic conditions (Marsabit CIDP, 2018). The main economic activity in this region is livestock rearing with limited crop production. The main livestock species kept in Marsabit County include approximately 217,360 camels, 2,029,490 goats, 1,851,452 sheep, 420,000 cattle, 81,900 donkeys, and 45,860 poultry (Marsabit CIDP, 2018).

### Sample collection

Samples were collected in two field-sampling trips and each sampling site was geo-referenced with a global positioning system (GPS). Goat and sheep blood, keds on goats, sheep, and dog keds were collected in July 2019 along the Laisamis River at Tula Orbora, which is one of the main livestock watering points (1° 35' 16.4" N, 37° 48' 22.5" E). Donkey blood and donkey keds were collected at Sere-e-Sipeni (1°33'14.9" N, 37°49'32.1" E) in February 2020.

### Ethical approval

This study was conducted in strict adherence to the experimental guidelines and procedures approved by the International

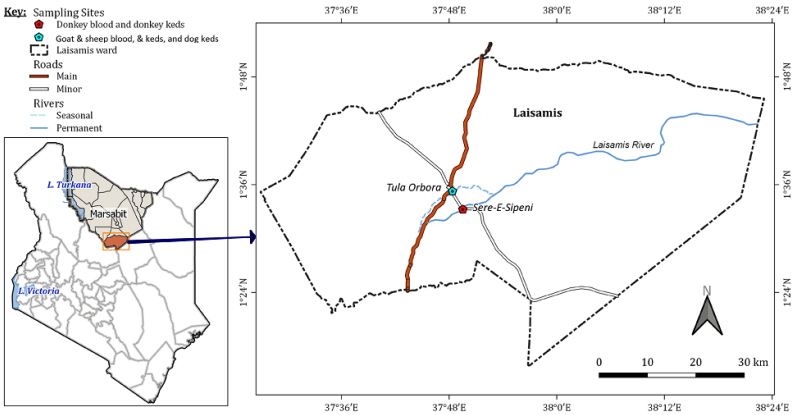


**Figure 1. A map of Kenya showing the study sites in Laisamis, Marsabit County.** The samples collected from each site are shown on the map key.

Centre of Insect Physiology and Ecology (icipe*)* Institutional Animal Care and Use Committee (REF: IACUC/ICIPE/003/2018) and the Pwani University Ethics Review (approval number: ERC/EXT/002/2020). Goats, sheep, and donkeys were handled carefully to minimize pain and discomfort. Verbal consent was obtained from livestock owners prior to collection of samples. Written consent was not possible as the livestock keepers could neither read nor write.

### Blood collection

About 5 mL of blood was obtained from the jugular vein of 245 goats (22 males and 223 females), 108 sheep (8 males and 100 females), and 36 donkeys (18 males and 18 females) of both sexes. Each sample was collected into 5 mL EDTA vacutainers (Plymouth PLG, UK), and kept under cold chain during the sampling exercise. Immediately after completion of the sampling process, all blood samples were preserved in liquid nitrogen for transportation to *icipe*, Nairobi, for molecular detection of pathogens.

### Collection and identification of livestock keds

Keds that infested goats, sheep, donkeys, and dogs were collected from their hosts by handpicking at night as previously reported (Kidambasi *et al.*, 2020). Freshly collected keds were preserved in absolute ethanol ready for transportation to *icipe* for molecular screening of pathogens. Keds for use in molecular and morphological identification were sorted at *icipe* (Nairobi). Species identification based on morphology was done through comparison with known hippoboscid collections at *icipe*.

### DNA extraction

Keds were surface-sterilized with 70% ethanol and left to air dry for 10 min on a paper towel in a clean hood. Each fly was then placed into a clean 1.5-mL Eppendorf tube containing 250 mg of sterile zirconia beads of 2-mm diameter (Stratech, UK). The flies were homogenized in liquid nitrogen using a Mini-Beadbeater-16 for 3 min (BioSpec, Bartlesville, OK, USA). Genomic DNA was extracted from individual flies and blood samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

### PCR-HRM for pathogen detection

Hemopathogens including *Anaplasma*, *Ehrlichia*, piroplasms (*Theileria* and *Babesia* spp.), animal African trypanosomes, *Clostridium perfringens* and *Brucella* spp. were amplified using pathogen-specific PCRs (Table 1) followed by DNA fragment analysis based on high-resolution melting (HRM) analysis. Rotor-Gene Q (Qiagen, Hannover, Germany), Quant Studio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Mic qPCR (Bio Molecular Systems, Upper Coomera, Queensland, Australia) thermocyclers were used for PCR-HRM analysis for pathogen detection.

Genus-specific *Anaplasmataceae* primers were used for amplification of the 16S rRNA gene of *Ehrlichia* and *Anaplasma* spp. (Mwamuye *et al.*, 2017), while *Theileria* and *Babesia* spp. were screened simultaneously using primers that target the hypervariable V4 region of the 18S rRNA gene (Gubbels *et al.*, 1999). *Clostridium perfringens* was detected using specific primers targeting the 16S rRNA gene (Wu *et al.*, 2009).

**Table 1. PCR primers for pathogen detection.**

| Primer name | 5' to 3' sequence | Target organism | Target gene | Amplicon size (bp) | Primer reference |
|---|---|---|---|---|---|
| *Anaplasma*JV_F<br>*Anaplasma*JV_R | CGGTGGAGCATGTGGTTTAATTC<br>CGRCGTTGCAACCTATTGTAGTC | *Anaplasma* spp. | Partial 16S rRNA | 300 | (Mwamuye *et al.*, 2017) |
| *Ehrlichia*JV_F<br>*Ehrlichia*JV_R | GCAACCCTCATCCTTAGTTACCA<br>TGTTACGACTTCACCCTAGTCAC | *Ehrlichia* spp. | 16S rRNA | 300 | (Mwamuye *et al.*, 2017) |
| *Ehrlichia 16S* F<br>*Ehrlichia 16S* R | CGTAAAGGGCACGTAGGTGGACTA<br>CACCTCAGTGTCAGTATCGAACCA | *Ehrlichia* spp. | 16S rRNA | 200 | (Tokarz *et al.*, 2009) |
| EHR 16SD<br>1492R | GGTACCYACAGAAGAAGTCC<br>GGTTACCTTGTTACGACTT | *Ehrlichia* and *Anaplasma* spp. | Longer 16S rRNA | 1000 | (Parola *et al.*, 2000) |
| ITS1_CF<br>ITS1_BR | CCGGAAGTTCACCGATATTG<br>TTGCTGCGTTCTTCAAC- GAA | *Trypanosoma* spp. | ITS1 | 250–720 | (Njiru *et al.*, 2005) |
| RLB_F<br>RLB_R | GAGGTAGTGACAAGAAATAACAATA<br>TCTTCGATCCCCTAACTTTC | *Theileria* and *Babesia* spp. | 18S rRNA | 450 | (Gubbels *et al.*, 1999) |
| Br_F<br>Br_R | GCTCGGTTGCCAATATCAATGC<br>GGGTAAAGCGTCGCCAGAAG | *Brucella* spp. | bcsp31 | 223 | (Probert *et al.*, 2004) |
| Cp_F<br>Cp_R | AAAGATGGCATCATCATTCAAC<br>TACCGTCATTATCTTCCCCAAA | *Clostridium perfringens* | 16S rRNA | 279 | (Wu *et al.*, 2009) |

A set of genus-specific primers described by (Probert *et al.*, 2004) that targets the bcsp31 gene was used for identification of *Brucella* spp. A universal set of primer that targets the trypanosomal internal transcribed spacer I (ITS-I) region was used for detection of animal African trypanosomes (Njiru *et al.*, 2005).

PCR-HRM assays were performed in runs of 10 µL reaction volumes, containing 6 µL nuclease-free water, 2 µL of 5×HOT FIREPol EvaGreen HRM mix (no ROX) (Solis BioDyne, Estonia), 0.5 µL of 10 pmol of each primer and 1 µL of template DNA.

PCR conditions in the Rotor-Gene, Quant Studio and Mic qPCR for detection of *Ehrlichia, Anaplasma* and piroplasms (*Theileria* and *Babesia*) were preceded by an initial enzyme activation step at 95°C for 15 min followed by 10 cycles of denaturation at 94°C for 20 sec, touch-down annealing from 64°C with a decrease of 1°C after each cycle for 25 sec, and primer extension step at 72°C for 30 sec. Then, another 30 cycles each of: denaturation at 94°C for 20 sec, touch-down annealing from 55°C with a decrease of 1°C after every 5 cycles for 50 sec, and extension at 72°C for 30 sec, with a final elongation at 72°C for 3 min.

Specific annealing temperatures of 55°C, 53.9°C, and 63.2°C were used for detection of *Trypanosome* spp., *Clostridium perfringens*, and *Brucella* spp., respectively. The PCR conditions were initial enzyme activation at 95°C for 15 min, 40 cycles of: denaturation at 95°C for 30 sec, annealing for 30 sec, and extension at 72°C for 30 sec, with a final elongation at 72°C for 7 min.

HRM analysis proceeded immediately after PCR with a gradual increase in temperature from 75°C to 95°C with 2 sec increase of 0.1°C between successive fluorescence acquisitions. The melting curves were visualized based on the fluorescence signals and the change in fluorescence with time (dF/dT) plotted against change in temperature (°C).

Rotor-Gene Q Series Software 2.1.0 (Build 9), Quant Studio Design and Analysis Software version 1.5.1 (Mwamuye *et al.*, 2017), and micPCR Software v2.8.1 were used to assess melt profiles of the test samples in comparison with that of the known positive controls to confirm detection of pathogens. DNA sequencing of representative samples showing distinct melting curves proceeded to identify the pathogens.

### Purification of PCR amplicons and gene sequencing

Representative samples with expected and distinct melting curves relative to the known positive controls were amplified in larger PCR reaction volumes of 15 µL. Five µL of the PCR amplicons were resolved through 2% ethidium bromide-stained agarose gel electrophoresis followed by visualization of the DNA under ultraviolet light using Kodak Gel Logic 200 Imaging System (SPW Industrial, Laguna Hills, CA, USA). About 10 µL of each sample with clear bands was purified using ExoSAP-IT PCR Product Cleanup kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. Purified samples were then incubated at 37°C for 15 min and 85°C for 15 min in a Proflex thermocycler (Applied Biosystems) prior to Sanger sequencing by Macrogen, Inc. (Amsterdam, Netherlands). All sequences generated by this study were deposited in the GenBank (NCBI) database and assigned accession numbers.

### Data analysis
The Rotor-Gene Q Series 2.1.0 (Build 9), Quant Studio Design and Analysis Software v1.5.1, and micPCR Software v2.8.1 were used for HRM analysis. Data on pathogens from blood and ked samples were recorded in a Microsoft Excel Spreadsheet Program version 18.2110.13110.0 (Microsoft Corp.).

All nucleotide sequences were edited and aligned with closely related sequences from the NCBI GenBank nr database using the MAFFT plugin in Geneious Prime software version 2020.2.1 (created by Biomatters, Auckland, New Zealand; open source alternatives: UGENE, BioEdit) (Kearse *et al.*, 2012). The Basic Local Alignment Search Tool (BLAST) was used to query related sequences available in the GenBank nr database. Maps showing the sampling sites were generated by feeding the coordinates of the sampling locations into a GIS software, QGIS v3.16.

### Phylogenetic analysis
Maximum likelihood phylogenetic trees of the sequence alignments were constructed using PhyML v3.0 (Guindon *et al.*, 2010). Tree topologies were estimated using nearest neighbor interchange improvements over 1,000 bootstrap replicates and the Akaike information criterion for automatic model selection was employed in the phylogenies. FigTree v1.4.3 (Rambaut, 2016) was used to visualize the phylogenetic trees.

## Results
### Detection of pathogens
A total of 389 blood samples from goats (245), sheep (108) and donkeys (36), as well as 235 keds from goats and sheep (116), dogs (108) and donkeys (11) were randomly collected in Laisamis Sub-County, Marsabit County of northern Kenya. Out of the 389 blood samples screened, 87.7% (341/389) tested positive for *Anaplasma* spp., 50.1% (195/389) tested positive for *Ehrlichia canis*, 12.6% (49/389) tested positive for *Theileria* spp., and 6.7% (26/389) tested positive for *Trypanosoma* spp.

Out of 235 ked samples screened, 63.4% (149/235) were positive for *E. canis*, 34.9% (82/235) were positive for *Bartonella schoenbuchensis,* 24.3% (57/235) were positive for *Trypanosoma* spp., 21.3% (50/235) were positive for *Clostridium perfringens*, and only 2.6% (6/235) were positive for *B. abortus.* All the pathogens detected in this study are listed in Table 2.

### Pathogen detection in blood samples
***Goat and sheep blood samples.*** In goat blood (245), we detected *Anaplasma ovis* 84.5% (207), novel *Anaplasma* sp. 11.8% (29), *E. canis* 66.1% (162), *Trypanosoma vivax* 7.3% (18), and *Theileria ovis* 0.8% (2) by PCR-HRM (Figure 2).

We also detected *A. ovis* 93.5% (101), *E. canis* 22.2% (24), and *T. ovis* 38.9% (42) in sheep blood (108).

Alignment of the edited *Anaplasma* 16S rRNA sequences with closely related sequences queried on NCBI GenBank nr database, showed that most samples were 100% identical to *A. ovis* (GenBank accession MG869525). However, some sequences were distinctly different from the queried *A. ovis* among other sequences with an identity of 96.8% and below (Figure 3). In addition, alignment of the edited *Theileria* 18S

**Table 2. Summary of hemopathogens detected in livestock and their keds.**

| Pathogens | Prevalence of hemopathogens in blood and ked samples | | | | | |
|---|---|---|---|---|---|---|
| | Goat blood (n=245) | Sheep blood (n=108) | Goat/sheep keds (n=116) | Dog keds (n=108) | Donkey blood (n=36) | Donkey keds (n=11) |
| *Trypanosoma* spp. | *Trypanosoma vivax* = 18 (7.3%) | — | *Trypanosoma vivax* = 34 (29.3%) *Trypanosoma evansi* = 1 (0.86%) *Trypanosoma godfreyi* = 1 (0.86%) | *Trypanosoma vivax* = 17 (15.7%) *Trypanosoma simiae* = 1 (0.9%) *Trypanosoma evansi* = 1 (0.9%) | *Trypanosoma vivax* = 8 (22.2%) | *Trypanosoma vivax* = 2 (18.2%) |
| *Anaplasma* spp. | *Anaplasma ovis* = 207 (84.5%) Novel *Anaplasma* sp. = 29 (11.8%) | *Anaplasma ovis* = 101 (93.5%) | — | — | *'Candidatus* Anaplasma camelii' = 4 (11.1%) | — |
| *Ehrlichia canis* | 162 (66.1%) | 24 (22.2%) | 60 (51.7%) | 82 (76%) | 9 (25%) | 7 (63.6%) |
| *Theileria/ Babesia* spp. | *Theileria ovis* = 2 (0.8%) | *Theileria ovis* = 42 (38.9%) | — | — | *Theileria equi* = 5 (13.9%) | — |
| **Brucella abortus* | — | — | — | 6 (5.6%) | — | — |
| **Clostridium perfringens* | — | — | — | 50 (46.3%) | — | — |
| **Bartonella schoenbuchensis* | — | — | — | 82 (76%) | — | — |

*Zoonotic pathogens; dash (–) means the pathogen was not detected.

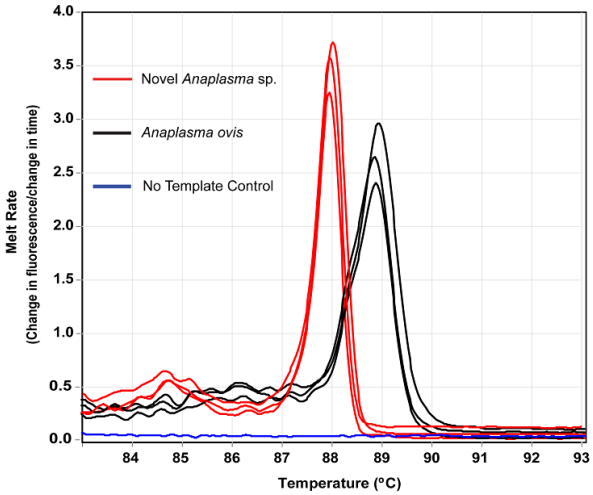


**Figure 2. Melt curves of amplification of 16S rRNA of *Anaplasma* spp. in goats.**

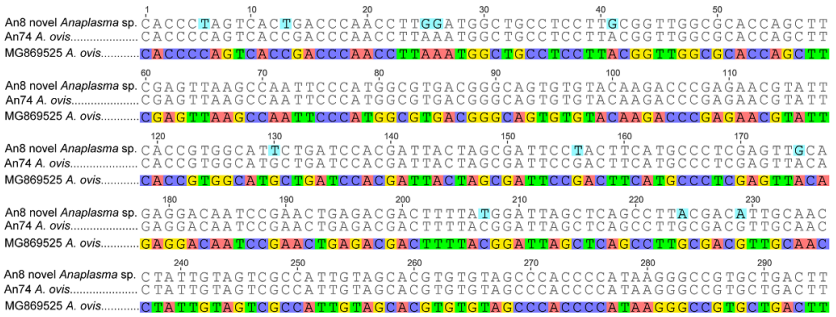


**Figure 3. Multiple sequence alignment of 16S rRNA sequences of (i) novel *Anaplasma* sp. (from study sample An8) and (ii) *A. ovis* (An74) amplified from goat blood, and (iii) the GenBank-retrieved sequence of *A. ovis*, accession MG869525.** Nucleotide changes were identified between MG869525 and An8 sequences. For instance, at position 25 and 26 in the above alignment, 'AA' in the GenBank sequence MG869525 is replaced by 'GG' in the query sequence An8 (study sample). In contrast, An74 sequence (from this study) was 100% identical to the *A. ovis* sequence MG869525 from GenBank.

rRNA sequences with closely related sequences showed 100% similarity to *T. ovis* (GenBank accession MN712508).

***Donkey blood samples.*** In donkey blood samples (36), we detected *E. canis* 25% (9), '*Candidatus* Anaplasma camelii' 11.1% (4), *Trypanosoma vivax* 22.2% (8) and *Theileria equi* 13.9% (5). Analysis of the 200-bp *Ehrlichia* 16S rRNA sequences showed 100% identity to *E. canis* sequenced from ticks and fleas collected from companion dogs and cats in East and Southeast Asia (GenBank accession MT499360). On the other

hand, alignment of the edited *Theileria* 18S rRNA sequences with closely related sequences queried on NCBI GenBank nr database, showed 100% identity with *Theileria equi* (GenBank accession MK063829). Additionally, analysis of the *Anaplasma* 16S rRNA sequences showed 100% identity to '*Candidatus* Anaplasma camelii' (GenBank accession MT510533). *Brucella* spp. and *Clostridium perfringens* were not detected in donkey and goat blood.

### Pathogen detection in keds

***Goat and sheep keds.*** The keds collected from co-herded goats and sheep were found to harbor *E. canis* 51.7% (60/116) and three trypanosome species, namely *T. vivax* 29.3% (34/116), *Trypanosoma evansi* 0.86% (1/116) and *Trypanosoma godfreyi* 0.86% (1/116).

***Donkey keds.*** The pathogens that were detected in keds collected from donkeys included; *E. canis* 63.6% (7/11) and *T. vivax* 18.2% (2/11).

***Dog keds.*** We detected *E. canis* 76% (82/108), *T. vivax* 15.7% (17/108), *Trypanosoma simiae* 0.9% (1/108), and *T. evansi* 0.9% (1/108) in dog keds. Also, the dog keds harbored *Clostridium perfringens* 46.3% (50/108), *B. abortus* 5.6% (6/108) and *Bartonella schoenbuchensis* 76% (82/108).

Similarly, analysis of the 200-bp *Ehrlichia* 16S rRNA sequences showed 100% identity with *E. canis* sequenced from ticks and fleas collected from companion dogs and cats in East and Southeast Asia (GenBank accession MT499360). All the ked samples were negative for *Anaplasma* spp. and piroplasms (*Theileria* and *Babesia* spp.). Moreover, only keds obtained from dogs were positive for *B. abortus*, *C. perfringens* and *B. schoenbuchensis.* The *Brucella* bcsp31 gene sequences showed a 98.68% identity with *B. abortus* sequenced from cattle milk DNA in India (GenBank accession MK881176).

The morphological identification of the keds matched with the molecular identification of two ked species: *Hippobosca variegata* (GenBank accession MW128366) and *Hippobosca longipennis* (GenBank accession MW128365).

### Phylogenetic analysis of Anaplasma spp. 16S rRNA sequences

The phylogenetic tree comparing sequences of 16S rRNA gene fragments of *Anaplasma* (900–1000 bp) from this study to other sequences of the same gene available in GenBank is presented in (Figure 4). Phylogenetic relationships and molecular evolution were inferred using the maximum likelihood method. Tree Topologies were estimated using nearest neighbor interchange improvements over 1,000 bootstrap

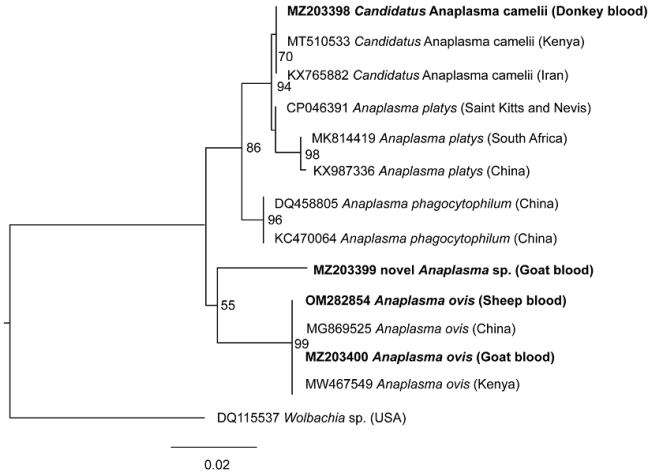


**Figure 4. Maximum likelihood phylogenetic tree of 16S rRNA gene of *Anaplasma* spp.** The tree shows the close relation as well as genetic divergence of sequences from this study and that queried from GenBank. Bootstrap values at the major nodes are of percentage agreement among 1000 bootstrap replicates. GenBank accession numbers, species identification and country of origin are indicated for each sequence. Sequences from this study are indicated in bold, and the associated sample indicated at the end. *Wolbachia* endosymbiont (GenBank accession DQ115537) 16S rRNA was used as the outgroup.

replicates. The tree was drawn to scale representing a 2% evolutionary change in nucleotides per site.

## Discussion

There is little information about infectious pathogens, particularly zoonotic, circulating in livestock of northern Kenya, due to lack of proper disease surveillance. In the area of study in Laisamis, northern Kenya, we aimed to determine the occurrence and prevalence of selected hemopathogens in livestock (goats, sheep and donkeys) and their predominant ectoparasitic keds (collected from goats, sheep, donkeys and dogs).

Our findings revealed *T. vivax* as the predominant trypanosome species in livestock and keds, outside the tsetse belts, with an infection rate of 6.7% (26/389) and 22.6% (53/235), respectively. This agrees with a previous report that showed *T. vivax* as the major case of trypanosome infection outside tsetse-infested areas in western Kenya (Thumbi *et al.*, 2010). This species is known to be pathogenic to goats, sheep and equids (Galiza *et al.*, 2011). Similarly, *T. vivax* infection in camels and camel keds was previously reported from the same study area (Kidambasi *et al.*, 2020), suggesting *T. vivax* as the major cause of trypanosomiasis in Laisamis, northern Kenya. Trypanosomiasis disease caused by this pathogen is among the most important diseases limiting livestock productivity and agricultural development, for example in Ethiopia (Bedada & Dagnachew, 2012).

Donkeys harbored *T. vivax*, *E. canis*, '*Ca.* Anaplasma camelii', and *T. equi*, with *E. canis* being the most prevalent pathogen 25% (9/36). *E. canis* is receiving increasing attention due to its high morbidity and mortality in animals (Bunroddith *et al.*, 2018). Detection of the canine pathogen, *E. canis*, in donkeys is not surprising because the pastoralist farmers rear mixed livestock species together with other domestic animals including dogs; thus, the donkeys could have acquired this pathogen from infected dogs through insect or tick bites. Ehrlichiosis is an emerging disease of domestic animals mainly transmitted by ticks and has previously been reported to infect dogs, cattle, humans, and goats (Zhang *et al.*, 2017).

Further, donkeys were found to be infected with the camel-associated bacteria, '*Ca.* Anaplasma camelii', previously reported in Kenyan camels (Kidambasi *et al.*, 2020). The presence of this camel pathogen in donkeys could be attributed to co-herding of donkeys with camels, and the donkeys could have acquired the pathogen from infected camels. Keds are common ectoparasites infesting livestock in the study area and have been reported as mechanical vectors of this bacterial pathogen (Bargul *et al.*, 2021). Further research is needed to determine the zoonotic potential as well as the pathogenic role of this pathogen in donkeys and other livestock species. *T. equi* (13.9%) was also detected in donkeys. Pathogens associated with this genus are among the causative agents of equine piroplasmosis and have previously been shown to infect donkeys in some parts of Kenya including Mwingi (Oduori *et al.*, 2015).

Goats harbored *T. vivax*, *E. canis*, *T. ovis*, *A. ovis* and a novel *Anaplasma* species. Sheep were also found to harbor *E. canis*, *T. ovis,* and *A. ovis. T. ovis* was more prevalent in sheep (38.9%) than in goats (0.8%). This finding is consistent with a study done on livestock in Palestine (Azmi *et al.*, 2019). Ovine theileriosis caused by *T. ovis* is among the most important infectious diseases affecting small ruminants, leading to significant economic losses to farmers (Al-Hosary *et al.*, 2021). *E. canis* and *T. vivax* were detected in most of the samples analyzed, including keds, suggesting these two are the common pathogens circulating in livestock herds in the study area. Further research will be needed to understand the vectors of these pathogens and whether keds are competent vectors of the pathogens.

Additionally, a high prevalence of *A. ovis* was detected in goats (84.5%) and sheep (93.5%), which is consistent with findings in a study done in Tunisia, North Africa, in goats and sheep by PCR (Said *et al.*, 2015). This high prevalence of *A. ovis* could also be attributed to ticks that were present in most of the domestic animals. *A. ovis* is distributed worldwide and considered a major cause of small ruminant anaplasmosis in tropical and subtropical regions of the world, with general clinical effects ranging from fever, fatigue, low milk production and abortion but with a low mortality rate (Stuen & Longbottom, 2011). A previous study in Corsica, France, reported the presence of *A. ovis* in dairy goats after an extensive survey due to health and production problems encountered in the goat flocks (Cabezas-Cruz *et al.*, 2019b).

We discovered an unidentified *Anaplasma*-like species in 11.8% (29/245) of goats analysed. The *Anaplasma* sp. shared 96.77% sequence identity with *A. ovis* sequenced from goat blood in China (GenBank accession MG869525). The GC content of this novel *Anaplasma* sp. was 52.9% with clear observation of differences in the bases with the queried *Anaplasma* spp. from NCBI GenBank nr database (Figure 3). The pathogenic role of this *Anaplasma*-like species in goats is not understood. However, it is related to *A. ovis*, which is known to be pathogenic in sheep, goats, and some wild ruminants (Said *et al.*, 2015).

More hemopathogens were detected in dog keds than in other keds, and they include *T. vivax, T. evansi, T. simiae, E. canis, C. perfringens, B. schoenbuchensis,* and *B. abortus.* Due to the fact that most dogs had a free-roaming lifestyle in the study region, it is possible that dogs were at a higher risk of being infected with a wide range of pathogens. Therefore, keds collected from dogs acquired these pathogens from infected dogs during their bloodmeal feeding. We were unable to collect blood from dogs during sampling because we lacked proper protective gear against dog bites, and the dogs were not vaccinated from rabies; thus, collecting blood from dogs was considered too high-risk.

Among all the pathogens detected in keds obtained from dogs, *E. canis* (76%) and *B. schoenbuchensis* (76%) had the highest prevalence rates. The high prevalence rate of these pathogens could be attributed to the high competition of pathogens circulating in the same host population, considering the

possible interactions between the pathogens and, host immune system, and host life cycle as well (Poletto *et al.*, 2015). In previous studies, *B. schoenbuchensis* was also detected in deer ked by PCR test with a prevalence rate of more than 60% (Szewczyk *et al.*, 2017). *B. schoenbuchensis* is one of the most important species that cause bartonellosis and has been reported to cause infections in humans, cattle and wild animals such as the cervids in Asia, North America and Europe (Rolain *et al.*, 2003; Vayssier-Taussat *et al.*, 2016). Additionally, *Bartonella* infection often manifests as various cardiovascular, neurological and rheumatologic conditions, making it a public health concern since pastoralists, farmers and veterinarians who interact with domestic animals are at a high risk of infection (Maggi *et al.*, 2012). There is little information on the presence of *E. canis* in keds, but this pathogen has been detected in *Rhipicephalus sanguineus* (the brown dog tick), the biological vector of the pathogen (Cabezas-Cruz *et al.*, 2019a).

This study also reports the first occurrence of *C. perfringens* in dog keds. This pathogen is an important cause of enteric diseases in humans and domestic animals and is responsible for several forms of enterotoxaemia, which differs in clinical manifestation and severity according to the toxigenic type involved and specific toxins produced (Singh *et al.*, 2018). It affects small ruminants worldwide, causing heavy mortality and significant economic impact (Sumithra *et al.*, 2013). In previous reports, this pathogen has been shown to cause death in dogs due to hemorrhagic gastroenteritis of the gastrointestinal tract; thus further research is needed to better understand the role of this bacterium in enteric diseases of dogs (Schlegel *et al.*, 2012).

The *Brucella* sp. (5.6%) detected in dog keds was closely related to *B. abortus* sequenced from cattle milk in India, showing a sequence identity of 98.68% (GenBank accession MK881176). Brucellosis in dogs is mainly associated with *B. canis* and not *B. abortus*, which mainly occur in cattle. However, cross-species transmission of *Brucella* spp. is possible and this is consistent with a study done in Argentina that detected *B. abortus* in farm dogs (Mortola *et al.*, 2019). Additionally, *B. abortus* is a common source of human infection with a high zoonotic potential, and cause a disease called Bang's disease in humans (Kaden *et al.*, 2018). *Brucella* species have been shown to be of high public health and socio-economic importance in northern Kenya, and mainly transmitted to humans through ingestion of unpasteurized dairy products or raw/undercooked animal products (Kairu-Wanyoike *et al.*, 2019).

Among trypanosomes detected in keds obtained from dogs, *T. vivax* (15.7%) was more prevalent than *T. evansi* (0.9%) and *T. simiae* (0.9%), which had low prevalence rates. The presence of these trypanosome species in dog keds suggests that the keds were infected during their bloodmeal acquisition from dogs that were initially infected with trypanosomes from ticks and possibly from other biting flies like *Stomoxys*. Additionally, the dogs had a free-roaming lifestyle and thus, it is also possible that they were infected when moving into neighboring tsetse-infested regions. Similarly, *T. vivax* (29.3%) was also more prevalent than *T. evansi* (0.86%) and *T. godfreyi* (0.86%) in keds obtained from goats. This low infection rate could be attributed to disease stability in the area, change in climate and seasonal outbreaks (Gutierrez *et al.*, 2006). Both *T. vivax* and *T. evansi* have recently been detected in camel keds in northern Kenya (Kidambasi *et al.*, 2020). Further, previous studies have shown *T. godfreyi* and *T. simiae* to infect a wide range of domestic animals including pigs, cattle, camels, dogs and goats with *T. simiae* being highly pathogenic to domestic pigs (Hamill *et al.*, 2013; Simwango *et al.*, 2017). *T. simiae* and *T. godfreyi* are among the *Trypanosome* spp. that cause African animal trypanosomiasis with tsetse flies being their main vector (Isaac *et al.*, 2016). This is the first report of occurrence of these two *Trypanosome* spp. in keds.

The molecular data from keds collected from donkeys showed detection of *T. vivax* (18.2%) and *E. canis* (63.6%) as the common pathogens. Detection of these pathogens in donkeys and goats, as well as in their associated ectoparasitic keds, shows the xenodiagnostic potential of using keds to indirectly screen for pathogens occurring in their associated hosts. Similarly, a recent report demonstrated the occurrence in keds of pathogens that were similarly present in their camel host from which they were collected, and further proposed the potential use of keds in xenodiagnosis (Kidambasi *et al.*, 2020). However, detection of pathogens in keds does not incriminate them as vectors, but studies should be carried out to determine the vector competence of these keds. Keds are known to transmit mammalian trypanosomatidae of the genus *Megatrypanum* and are suspected to be vectors of *T. avium* and *T. corvi* in birds (Svobodová *et al.*, 2015).

The impact of zoonotic pathogens is often underestimated due to limited surveillance and insufficient data of disease burden in most developing countries (Munyua *et al.*, 2016). This study reveals that the domesticated animals, as well as keds collected from them, carried infectious pathogens of veterinary and public health concern. Notably, we sequenced multiple hemopathogens in dog keds, including zoonotic ones (*B. abortus*, *B. schoenbuchensis*, and *C. perfringens*). Close association of humans with domestic animals infested by keds and other disease vectors increases chances of pathogen transmission. It is therefore crucial to conduct further studies to map out circulating livestock diseases in northern Kenya and establish the role of keds in disease transmission.

## Conclusions

We detected various selected infectious hemopathogens present in livestock and their associated ectoparasitic biting keds in northern Kenya, which calls for further surveillance studies to increase the understanding of the epidemiology of livestock diseases and the transmission of zoonotic ones by insect vectors such as keds that also occasionally feed on humans. This will guide the policy makers and livestock farmers in disease control.

## Data availability

### Underlying data

NCBI GenBank: Uncultured Anaplasma sp. clone An74 16S ribosomal RNA gene, partial sequence (16S rRNA of *Anaplasma ovis* in goat), accession number MZ203400: https://identifiers.org/ncbiprotein:MZ203400

NCBI GenBank: Uncultured Anaplasma sp. clone An8 16S ribosomal RNA gene, partial sequence (16S rRNA of novel *Anaplasma* sp. in goat), accession number MZ203399: https://identifiers.org/ncbiprotein:MZ203399

NCBI GenBank: Anaplasma ovis isolate 92B 16S ribosomal RNA gene, partial sequence (16S rRNA of *Anaplasma ovis* in sheep), accession number OM282854: https://identifiers.org/ncbiprotein:OM282854

NCBI GenBank: Candidatus Anaplasma camelii clone An4B 16S ribosomal RNA gene, partial sequence (16S rRNA of *Candidatus* Anaplasma camelii in donkey), accession number MZ203398: https://identifiers.org/ncbiprotein:MZ203398

NCBI GenBank: Uncultured Bartonella sp. clone EJ72 16S ribosomal RNA gene, partial sequence (16S rRNA of *Bartonella schoenbuchensis* in dog keds), accession number MZ203403: https://identifiers.org/ncbiprotein:MZ203403

NCBI GenBank: Uncultured Clostridium sp. clone Dg48 16S ribosomal RNA gene, partial sequence (16S rRNA of *Clostridium perfringens* in dog keds), accession number MZ203401: https://identifiers.org/ncbiprotein:MZ203401

NCBI GenBank: Trypanosoma simiae voucher T52 small subunit ribosomal RNA gene and internal transcribed spacer 1, partial sequence (*T. simiae* ITS1 in dog keds), accession number MZ221829: https://identifiers.org/ncbiprotein:MZ221829

NCBI GenBank: Trypanosoma evansi voucher T64 small subunit ribosomal RNA gene and internal transcribed spacer 1, partial sequence (*T. evansi* ITS1 in dog keds), accession number MZ221830: https://identifiers.org/ncbiprotein:MZ221830

NCBI GenBank: Ehrlichia canis isolate ES72 16S ribosomal RNA gene, partial sequence (Short 16S rRNA of *E. canis* in dog keds), accession number OM282855: https://identifiers.org/ncbiprotein:OM282855

NCBI GenBank: Theileria ovis isolate 26A small subunit ribosomal RNA gene, partial sequence (18S rRNA of *Theileria ovis* in sheep), accession number OM282856: https://identifiers.org/ncbiprotein:OM282856

NCBI GenBank: Theileria equi isolate 15D small subunit ribosomal RNA gene, partial sequence (18S rRNA of *Theileria equi* in donkey), accession number OM282857: https://identifiers.org/ncbiprotein:OM282857

Figshare: Detection of hemopathogens in goat, sheep, donkey, and their associated hematophagous biting keds, and dog keds, https://doi.org/10.6084/m9.figshare.18586028.v1 (Mwaki *et al.*, 2022)

This project contains the following underlying data:

- Raw HRM Rotor Gene, Quant Studio and micPCR HRM data files for detection of pathogens in goats, sheep, donkeys and their associated biting keds, and dog keds. The HRM data files for *Anaplasma* spp. and *Ehrlichia* sp. can be accessed using Rotor Gene Q software, Quant Studio™ Design and Analysis software, and micPCR software, while data files for the other pathogens can be accessed using Quant Studio™ Design and Analysis software.



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