STATUS OF *LEISHMANIA* PARASITE SPECIES, SANDFLY VECTOR SPECIES AND RESERVOIR HOST SPECIES IN MT. ELGON, KENYA

BY

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THESIS SUBMITTED TO THE SCHOOL OF SCIENCE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY (PARASITOLOGY), UNIVERSITY OF ELDORET, KENYA

MAY, 2024

DECLARATION

Declaration by the student

This thesis is my original work and has not been presented for a degree in any other university. No part of this thesis may be reproduced without the prior permission of the author and/or the University of Eldoret.

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DEDICATION

To my dear wife Mrs Catherine Ombula, for all the moral support and sacrifice that made me complete this work and to my beloved children Brian, Lavendar, Lydia and Albert for though they are young they were able to withstand the hectic moments even when I stayed away from them for somedays, in field, laboratory and library collecting information to piece down this thesis.

ABSTRACT

Leishmaniasis is a major public health problem experienced globally and is caused by parasite species of the genus *Leishmania*, transmitted through the bite of infected female phlebotomine sandfly. The current study determined natural infection of *Leishmania* species in sandfly vector and in reservoir hosts species inhabiting caves in Mount Elgon in Kenya. The study also determined bloodmeal sources for the sandflies. A total of 5,688 sandflies were collected using Center for Disease Control (CDC) light traps and analysed for *Leishmania* infectivity by microscopy and taxonomy by morphological and molecular characterization sequencing using Cytochrome c oxidase subunit 1 (COI). The vector species abundance was determined as quantitative counts per trap site was analysed by One Way ANOVA. Blood, liver and spleen smears were prepared from 199 bats and 25 hyraxes collected from the same caves and observed microscopically. The microscopy results showed *Leishmania* (amastigotes) in reservoir hosts' macrophages, while some of the *Leishmania* parasites were observed extra-cellularly having been released from ruptured cells during preparation of the films/smears. In this study, bats belonging to five species were morphologically identified with significant differences in their parasite abundance between the species (χ^2 = 16.7845, df = 7, P = 0.0002). The bat species had higher overall prevalence of the parasite infections compared to the hyraxes ((χ^2 = 7.342, df. =1, p = 0.0043), with *Cardioderma cor*, *Chaerephon pumilus* and *Rhinolophus clivosus* having more than 5% prevalence of the *Leishmania* infections. Analysis of 200 blood fed sandflies by ELISA showed that 120(60%) fed on humans, 55(28%) fed on bats, 16(8%) fed on hyraxes and 9(5%) fed on cats. It was concluded that transmission of *Leishmania* parasites in the study area involved three wild reservoir hosts (bats, hyraxes and cats) and two vector species *Phlebotomus pedifer* and *Phlebotomus elgonensis*. This study demonstrated for the first time, the detection of *L. major* and *L. tropica* in *P. pedifer* sandflies from Mt. Elgon. This study suggests that disruption of the life cycle of the *Leishmania* parasites should be done targeting the phlebotomine vectors and the reservoir animals in the region. However, more studies are needed to get sufficient information on other factors influencing the transmission of *Leishmania* in the region to enable effective implementation of preventive and control measures.

Key words: Mt. Elgon, caves, sandfly vectors, reservoir hosts, *Leishmania* parasites

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LIST OF ABBREVIATIONS

ATL	Adult T-cell leukemia-lymphoma
BLAST	Basic Local Alignment Search Tool
CL	Cutaneous Leishmaniasis
COI	Cytochrome c oxidase subunit 1
DAT	Direct Agglutination Test
DNA	Deoxyribonucleic Acid
DNP	Dinder National Park
ELISA	Enzyme-linked Immunosorbent Assay
HRM	High Resolution Melting
ICIPE	International Centre of Insect Physiology and Ecology
IFAT	Immunofluorescence
KEMRI	Kenya Medical Research Institute
KWS	Kenya Wild Life Service
LDU	Leishman Donovan Units
LPG	Glycoconjugate Lipophospho Glycan
ML	Muco-cutenous Leishmaniasis
NNN	Novy- Nicolle –McNeal
NTD	Neglected Tropical Diseases
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RT-PCR	Real Time Polymerase Chain Reaction
SSA	Sub Saharan Africa
SYBR	Synergy Brands
VL	Visceral Leishmaniasis
WHO	World Health Organization
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer

ACKNOWLEDGEMENT

I express my sincere thanks to my supervisors; Prof. Moses M. Ngeiywa and Dr. Judith A. Makwali for their invaluable suggestions and useful criticism of this work and without whom the present study would never have been successful. I must thank the key informant residents of Mount Elgon region for the direction and cooperation in the whole interview and initial mapping of the study area and specific caves where the subjects of the study were found before the commencement of data collection process. My special thanks go to the National Research Fund (NRF) for the financial support for this research work. Similarly, I extend my gratitude to the University of Eldoret for the Eldoret Research Fund (UoERF) grant. The patience, understanding, encouragement and support by my dear mother, E. Isangalo, loving wife, C. Ombula and my adorable children, Brian, Lavendar, Lydia and Albert cannot go unmentioned.

I also express my sincere gratitude to the village elder Mr. Kirui Philip who acted as a tour guide to enable us locate the study sites and also guard our traps. Similarly, I appreciate the technical assistance of Mr, Johnstone Ingonga who coordinated laboratory processes at both KEMRI and ICIPE laboratories. Last but not least, I acknowledge the expert scholarly contribution of the late Dr. Christopher Anjili for his encouraging advice, unlimited support and professional criticism he provided during the field work.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Leishmaniasis, categorized as a neglected tropical disease (NTD), poses a substantial challenge to public health, resulting in notable morbidity and mortality among humans (Feres, 2019; Machado *et al.*, 2019; Maurício, 2018; Nweze *et al.*, 2020). However This disease occurs across 98 countries (World Health Organization (WHO), 2020) distributed throughout the tropic and subtropic regions of Africa, Southern Europe, Middle East, Asia, as well as Central and Latin America (Pigott *et al.*, 2014; Pisarski, 2019; Sunyoto *et al.*, 2019; Wamai *et al.*, 2020). This geographic distribution of Leishmaniasis is separated into two groups: Old World and New World Leishmaniasis (Inceboz, 2019a). "Old World" Leishmaniasis occurs in the Mediterranean Basin, Middle East, and Africa (Cortes *et al.*, 2020). "New World" Leishmaniasis is found in Mexico, South and Central America (Saberi *et al.*, 2019).
A total of 1.2 billion people worldwide are currently under the threat of the disease (WHO, 2020) with approximately 70,000 deaths occurring annually (Alvar *et al.*, 2012; Bhunia and Shit, 2020). High prevalence of the disease occurs in the Indian Subcontinent and Sub-Saharan Africa (SSA) (Abongomera *et al.*, 2020; Ngere *et al.*, 2020). Leishmaniasis presents itself in three clinical forms: cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (ML) and visceral leishmaniasis (VL) (Alemayehu and Alemayehu, 2017; Rojas-Jaimes *et al.*, 2019).

The sandflies inject the infective promastigotes, during blood meals, the promastigotes that reach the puncture wound are phagocytized by macrophages and transform into amastigotes. The amastigotes multiply in infected cells and affect

different tissues, depending on the type of *Leishmania* species (Alemayehu & Alemayehu, 2017)

Across the world, cutaneous leishmaniasis (CL) is the most prevalent form of the disease and is responsible for between 600,000 to 1 million new cases annually, with 90% occurring in the Middle East and North Africa (Aronson and Joya, 2019; WHO, 2020). Infection with CL causes skin lesions, mainly ulcers on exposed parts of the body, leaving life-long scars and serious disability (Bettaieb *et al.*, 2020)

Mucocutaneous leishmaniasis (ML) has restricted distribution in Ethiopia in the old world while in the new world it occurs in Central and Latin America only (Giovanny *et al.*, 2020). The disease presents in the form of lesion development on the skin which destroy the mucous membranes of the nose, mouth and throat cavities (Garrido-Jareño *et al.*, 2020). Visceral leishmaniasis (VL) is mostly distributed in Sub Saharan Africa, South Asia, as well as South and Central America (Bi *et al.*, 2018). High-burden countries such as India, Bangladesh, Sudan, Ethiopia, and Brazil, account for 90% of VL patients while lower rates of VL occur in Southern Europe, Central Asia, China, Kenya, Uganda and the Middle East including Iran (Alemayehu and Alemayehu, 2017; Kanyina, 2020; Namulen *et al.*, 2021). The disease is characterized by swelling of the human spleen and liver, and anaemia and is the most severe form of leishmaniasis, often fatal if not treated (Chakravarty *et al.*, 2019; van Griensven and Diro, 2019; Ibarra-Meneses *et al.*, 2020; Tekalign *et al.*, 2020).

The leishmaniasis causing organisms are called *Leishmania*. There are around 20 subgenera of flagellate parasites in the genus *Leishmania*. (Mahdy *et al.*, 2016;Cotton, 2017; Salah *et al.*, 2020).

The Eurocentric perspective categorizes *Leishmania* parasites into "Old World" species, such as *L. infantum, L. donovan, L. major, L. aethiopica, and L. tropica*, and "New World" species, including *L. amazonensis, L. chagasi, L. mexicana, L. naiffi, L. braziliensis,* and *L. guyanensis* (Mitropoulos *et al*., 2010; Kevric *et al*., 2015). Visceral *Leishmania*, more prevalent in the East African region, is primarily caused by *L. donovani* and *L. infantum* (Dulacha *et al*., 2019; Kühne *et al*., 2019; Bhunia and Shit, 2020). Phlebotomine sandflies (Diptera: Psychodidae: Phlebotominae) serve as natural vectors for *Leishmania* parasites and are crucial in the disease's transmission (Trájer and Sebestyén, 2019; Waitz *et al*., 2019). Out of approximately 900 species of sandflies, about 10 percent, mainly from the genera *Phlebotomus* and Lutzomyia, have been implicated as vectors of *Leishmania* (Cotton, 2017; Salah *et al*., 2020; Sousa-Paula *et al*., 2021).

Reservoir hosts play a pivotal role in maintaining the life cycle of *Leishmania* parasites and are central to the transmission of zoonotic and sylvatic *Leishmania* infections (Fatemi *et al*., 2018; Maia *et al*., 2018). Accurate identification of reservoir hosts is crucial. There are two main sources of human leishmaniases: commensal or domestic animals, zoonotic leishmaniases in which the wild animals are the reservoir hosts, and anthroponotic leishmaniases, where infected humans serve as the reservoir host (Roque and Jansen, 2014; Gholamrezaei *et al*., 2016). Various species of domestic, wild, and synanthropic mammals, including cats, dogs, foxes, jackals, wolves, rock hyraxes, rodents, mongoose, porcupines, primates, armadillos, and reptiles, among others, have been identified as reservoir hosts of *Leishmania* parasites in different parts of the world (Jambulingam *et al*., 2017; Alcover *et al*., 2020). Some sandflies in Kenya have been observed feeding on lizards and snakes (Mutinga *et al*., 1984), and it has been reported that lizards also harbor *Leishmania* parasites,

including those affecting humans, as well as malarial parasites (Dipeolu and Mutinga, 1989; Mutinga and Dipeolu, 1989).

Determining whether suspected animals act as reservoirs in a particular environment requires local studies involving ecological and parasitological analyses. This is essential because reservoir hosts for *Leishmania* parasites are often complex and exhibit regional and temporal variations (Campino and Maia, 2018; Cotton, 2017; Machado *et al*., 2019; Rezaei *et al*., 2020). Compounding this complexity is the fact that in many regions, the role of animals as reservoirs for human *Leishmania* remains unknown (Martn-Sánchez *et al*., 2020a; Molina *et al*., 2020).

Successful transmission of *Leishmania* depends on the presence of the pathogen, the vector, and the host species (de Oliveira *et al*., 2017; Martín-Sánchez *et al*., 2020b). Given the intricate life cycle of the parasites and the involvement of reservoir animals alongside human hosts, understanding *Leishmania* transmission and epidemiology presents challenges (Al-Bajalan *et al*., 2018). The transmission of *Leishmania* parasites begins when a sandfly feeds on the blood of an infected person or animal reservoir, acquiring parasites in the process (Conceiço-Silva and Morgado, 2019). Consequently, investigations into reservoir hosts that serve as blood meals for sandfly vectors must be conducted within these environments.

While most studies have concentrated on visceral leishmaniasis (VL) due to its life-threatening nature, leishmaniasis research in Kenya is expanding, with an increasing background prevalence of cutaneous leishmaniasis (CL) (Ngere *et al*., 2020). However, the lack of comprehensive data on CL prevalence nationwide exacerbates the situation, with much of the existing data being based on studies conducted over 20 years ago (Tonui, 2006). Despite CL lesions often healing without treatment, the

process can be lengthy and leave disfiguring scars, particularly on the face, leading to persistent psychological morbidity. Addressing CL and minimizing its psychological impact requires more concerted efforts in epidemiological research and control strategy development (Owino *et al*., 2019).

In Kenya, CL is caused by three distinct species of *Leishmania* parasites: *L. major*, *L. tropica*, and *L. aethiopica* (Odiwuor *et al*., 2012; Njau *et al*., 2017). The sandflies *P. guggisbergi, P. duboscqi,* and *P. pedifer* have been identified as vectors for *L. tropica*, *L. major*, and *L. aethiopica,* respectively (Anjili *et al*., 2011; Owino *et al*., 2019). *P. pedifer* is predominantly found in caves in Kenya, notably in Mount Elgon, according to research by Anjili *et al*. (2011) and Owino *et al*. (2019).

Sandflies have a big impact on epidemiology of leishmaniasis (Courtenay *et al*., 2017). When a sandfly is infected, it regurgitates *Leishmania* parasites into the victim or consumes them together with the bloodmeal (Tapathi and Nailwal 2021). Understanding the species of sandflies and the sources of their bloodmeal is essential for discovering possible ecological reservoirs of the disease, identifying prospective vectors, and understanding the processes of disease transmission (Gitari *et al.,* 2018). This is because it's possible that sandflies feed on a variety of vertebrate host blood, each of which may harbour a distinct form of *Leishmania* parasite. It is crucial to have access to this information in order to create effective disease prevention and response activities (Ngere *et al*., 2020).

As early as 1921, 1930, and 1932 new reports of phlebotomine sandflies in Kenya were made (Minter, 1964). Research has only so far allowed the identification of two genera: *Phlebotomus* and *Sergentomyia*. The number of sandflies in Kenya has increased to 48 species and subspecies as of 2011 (Ngure *et al*., 2009; Anjili *et al*.,

2011). Despite not being known to spread any illnesses, the sandflies in the genus *Sergentomyia* can nonetheless be a nuisance because of their biting behaviour (Killick-Kendrick *et al*., 1986). Significant *Leishmania* carriers are *Phlebotomus* sandflies.

The genus *Phlebotomus* in Kenya is represented in five subgenera, namely *Phlebotomus, Larroussius, Synphlebotomus, Paraphlebotomus* and *Anaphlebotomus* (Anjili*et al.*, 2011). The genus *Sergentomyia* has the largest number of sandflies, and is represented by four subgenera, namely: *Sergentomyia, Sintonius, Grassomyia* and *Parvidens* (Sang *et al.*, 1993a). Further evidence indicate the spread of leshmaniasis beyond the traditional areas of the Rift Valley mainly Keiyo-Marakwet, Naivasha, Laikipia, Baringo, Turkana, Pokot and North Eastern counties of Isiolo, Wajir and Mandera (Tonui, 2006; Lomurukai, 2020). There is more evidence of emergence and spread of leishmaniasis in Western Kenya and Uganda (Ngure *et al.*, 2009; Bengtson *et al.*, 2020). However, published information on the occurrence and abundance of the sandfly vectors are few in the Western Kenya areas.

1.2 Statement of the Problem

Leishmaniasis is still a dangerous condition, particularly the CL and VL types, which can be lethal if untreated for an extended length of time (Inceboz, 2019a). According to Tonui (2006) and Ngere *et al*. (2020), the illness causes around 600 cases per year in Kenya, with a case fatality rate of up to 7%.*Leishmania* parasite life cycle involves residence time in reservoir hosts, which are passed to the vectors from where the vector transmits it to the human hosts (Melby *et al.*, 2019) during vector blood meals. Yet in many regions of Kenya, studies on the identities and status of *Leishmania* parasite infection of reservoir hosts and the vectors remain largely unknown. Some

information on leishmaniasis vectors in Kenya is available but the studies were conducted over decades ago and most of them were based on the sandfly vector alone (Heisch *et al.*, 1962; Wijers and Minter, 1962; Mutinga and Ngoka, 1983; Ngumbi *et al.*, 1992; Sang *et al.*, 1993b; Killick-Kendrick *et al.*, 1994; Anjili *et al.*, 2011).

The Rift Valley and Central Kenya both have a number of cutaneous leishmaniasis outbreaks. One of these was the Utut centre of *Leishmania tropica*, where widespread transmission was seen between Gilgil and Elementaita on the floor of the Rift Valley (Sang *et al*., 1994; Owino *et al*., 2019). In Utut centre there is an abandoned forest reserve on top of a lava flow with many caves and rock crevices home to sandflies and other species, including hyraxes, high infection and scar rates were reported among illicit charcoal burners (Gitari *et al.,* 2018). Multiple lesions occurred often, notably in the head area (Owino *et al.,* 2019).

Researchers Mutinga and Odhiambo (1986b) tested the vectorial potential of wild *Phlebotomus pedifer* by feeding it to volunteer patients within one of Mt. Elgon's caves. In order to identify the route of infection, sandfly infection rates, and subsequently their vectorial capacity, female *P. pedifer* were allowed to feed on patients who had lesions of cutaneous leishmaniasis. The fed sandflies were held for intervals of 3–8, and 9–13 days, respectively, before being dissected for parasite analysis. According to the findings, a larger infection rate was produced by the extended time frame (9–13 days) (Mutinga and Odhiambo, 1986b). In a different investigation, hamsters were subcutaneously injected with all sandfly parasite isolates, resulting in cutaneous lesions on the experimental hamsters' noses. All infections were concentrated in the anterior stations of the gut of the experimental *P. pedifer* sandfly species (Mutinga and Odhiambo, 1986a).

On the slopes of Mount Elgon, Kenya, Sang *et al*. (1993a) conducted a survey of cutaneous leishmaniasis, which included a thorough analysis of two communities where cases were known to occur. The sickness seemed to be confined to an escarpment region between 1750 and 1900 metres in elevation. Houses were not inhabited by vector sandflies, and the region's caves seemed to be the main source of transmission. *Leishmania aethopica* was isolated from the sandflies (Sang *et al*., 1993a). Reservoir and vector species' distribution is constantly changing as a result of climate change and unpredictable weather patterns (Ready, 2008; Fischer *et al*., 2011). Moreover, there has been emergence of vector species in areas where none was recorded in the past (Ready, 2008).

Infectivity of the vectors with *Leishmania* parasite species during blood meal is what determines *Leishmania* transmission (Dillon and Lane, 1993; Bennai *et al*., 2018). However, there is little information work on the *Leishmania* species infection status of the vectors in Kenya, and how it spreads. Studies have shown that *Leishmania* emerged and expanded in Western Kenya (Ngure *et al*., 2009; Bengtson *et al*., 2020). However, not much work has been carried out on the prevalence of sandfly vectors in these parts of Western Kenya (Anjili, 2011; Mukhwana *et al*., 2018).

According to Mutinga *et al*. (1994) and Sang *et al*. (1994), CL is usually diagnosed in Kenya by examining the disease's characteristic acute lesions and then locating the amastigotes using microscopy or tissue cultures (Appendix IX). Since all *Leishmania* amastigotes are physically identical, this method has low sensitivity and is only partially effective in differentiating the causal *Leishmania* species (Mebrahtu *et al*., 1992). Due to its better sensitivity than traditional microscopy, serological methods appear to have been used for decades to detect *Leishmania* antibodies (Dias *et al*.,

2018). In particular, the direct agglutination test (DAT) was shown to be the best suitable for field application in Kenya (Mbati *et al*., 1999). However, DAT has limited accuracy in immunosuppressed people and is prone to inaccurate interpretations due to cross reactivity, particularly with more phylogenetically related species. In diagnostic and epidemiological studies, the use of Polymerase Chain Reaction (PCR)-based molecular methods with a high degree of sensitivity and specificity has increased in order to identify and categorize *Leishmania* parasites in reservoir hosts and vectors to the species level (Belli *et al.,* 1998; Lombardi *et al.,* 2014).

In the current study, sandfly species that transmit CL parasite species in Mt. Elgon, Kenya, were identified using a combination of morphological processes employing taxonomic keys and current molecular tools (COI-PCR). ELISA was used for blood meal analysis to identify the origins of blood in the vector.

1.3 Objectives of the Study

1.3.1 General Objective

The general objective of this study was to investigate the sandfly and wild mammal species inhabiting caves in Mt Elgon that are responsible for the transmission of leishmaniasis disease in the area

1.3.2 Specific Objectives

i. To determine the distribution of sandfly species in selected Mt. Elgon cave habitats

ii. To determine the prevalence of *Leishmania* parasite infections in sandfly vector species in selected Mt. Elgon cave habitats

iii. To determine the sources of bloodmeal in sandfly vectors collected from selected Mt. Elgon caves using ELISA technique

iv. To identify the species of *Leishmania* parasites and sandfly vectors of Mt. Elgon cave habitats in Kenya using molecular techniques

1.3.3 Null Hypotheses

H_{O1}: There is no significant difference in the distribution of sandfly species in Mt. Elgon caves

H_{O2}: There are no significant differences in intensity and prevalence of *Leishmania* parasite infection in sandfly vector species in Mt. Elgon cave habitats

H_{O3}: There are no significant differences in sources of bloodmeal in sandfly vectors collected from Mt. Elgon cave habitats

H_{O4}: There is no significant difference in taxonomic identification of *Leishmania* parasites and sandflies between morphological and molecular techniques

1.4 Significance and Justification of the study

Leishmania parasites which have been repeatedly isolated from wild-caught sandflies have been shown to carry the same parasites found in patients suffering from leishmaniasis (Killick- Kendrick, 1990). Thus, it is important to isolate parasites from sandflies identified in any given area and compare them with the parasites isolated from mammalian hosts in the same area. However, some *Leishmania* parasites have been isolated from wild-caught sandflies including parasites in the guts and malphigian tubules of *Sergentomyia garnhami* Heisch, Guggisberg and Teesdale, *S. antennatus* Newstead (Kaddu *et al.*, 1988) and *Leishmania aethopica* in the oesophagus of *P. pedifer* (Pareyn *et al.,*2020)

With the growth of ecotourism, including tourist visits to caves, the threat of leishmaniasis to the health of the visitors is a matter of concern. This prompted the study of cave inhabiting phlebotomine sandflies to determine their vector potential and to identify potential health risks to the people living in close proximity to the caves and to the visitors. This information would lead to further public health recommendations to protect the local residents and the visitors from being infected by *Leishmania*. The focus of this study was to contribute to the knowledge of the roles of insect and mammalian cave fauna (Phlebotomidae and some mammalian vertebrates) in the transmission of *Leishmania* in Mt. Elgon cave habitats in Kenya.

Identifying the reservoirs of *Leishmania* parasites is useful because it will enable proper planning and implementation of complete intervention strategies against *Leishmania* in the region. The intervention strategies will also target the reservoirs of the disease. The local communities will be sensitized to become more aware of the risk factors responsible for the leishmaniasis disease and hence will take precautions to eliminate or minimize the risks.

Because seasonality, abundances, and infection rates all give strong evidence on the relative vectoral importance of each sandfly species, studies on the population dynamics of vector species are essential and must take these factors into account. Studies on the biology of sandfly vectors are also crucial because they shed light on the complicated *Leishmania* parasite's intricate dynamics of transmission. Despite reports of human leishmaniases in the region (Sang *et al.*, 1993b), patterns of abundance and natural infection rates of sandfly species with *Leishmania* parasites were examined in the current study in sampled cave habitats in the Mt. Elgon region.

In the new world, where leishmaniasis is common, natural *Leishmania* infections in bats have been recorded in recent years. However, there has been little study published in these new world countries. In countries like Kenya and Ethiopia, studies using ELISA on blood-meal from sandflies produced positive findings for bats, hyrax, humans, goats, and cats (Kassahun *et al.,* 2015). Studies carried out in Mt. Elgon reported the presence of parasite in both the bats and sandflies pointing to the possibility of bats maintaining transmission of the *Leishmania* parasites in Mt. Elgon (Makwali, 2021). More studies are is required to determine the role of bats in the spread of *Leishmania* parasites in the old-world nations where leishmaniasis is endemic.

To produce epidemiological data that is more accurate, emerging technologies must be combined with traditional methodologies. Extremely sensitive and specific diagnostic procedures must be employed to accurately identify and characterise the various CL parasites, phlebotomine vectors, and reservoir hosts whose geographic distribution may overlap (Rodrguez-Cortés *et al*., 2010).

CHAPTER TWO

LITERATURE REVIEW

2.1 Scale of the problem caused by leishmaniasis

According to Torres-Guerrero *et al*. (2017), Cunze *et al*. (2019); Ghatee *et al*. (2020), obligatory intramacrophage protozoa of the genus *Leishmania* are the cause of leishmaniasis, a significant vector-borne illness. According to Hailu *et al*. (2016), leishmaniasis is endemic in parts of the tropics, Southern Europe, subtropics, and Central America. Leishmaniasis may be found in a variety of environments, including deserts in West Asia and the Middle East and rainforests in Central and Southern America (WHO, 2020). Over 90% of VL cases worldwide are found in nations including India, Bangladesh, Nepal, Sudan, and Brazil (Ghatee *et al*., 2020). Leishmaniasis has been seen to spread globally and regionally throughout the past several decades (Wamai *et al*., 2020). More than 100 million individuals are thought to be affected with visceral leishmaniasis (VL), which has killed thousands of people over the past few years (Bi *et al*., 2018).

According to statistics, human activity is largely responsible for the increase in the number of risk factors for leishmaniasis. Numerous urbanisations, deforestation, which fuels climate change, extensive migration, immunosuppression, starvation, and treatment failure, are among these risk factors (Chapman *et al.,* 2020). Human exposure to infected sandflies may rise as a result of population migrations and changes to the environment that affects the range and density of the vectors and animal reservoirs (Courtenay *et al*., 2017; Inceboz, 2019a).

2.2 *Leishmania* vectors

According to reports, the leishmaniasis illness has spread geographically over the past several decades following an increase in vector populations due to climate change (Cotton, 2017). The little dipteran insect known as a sandfly is currently the sole recognised vector of *Leishmania*. The popular name, however, can be confusing since, in certain places, it is also used to refer to other non-vectors (Bates *et al*., 2015). There are a number of sandfly species that can spread the parasite *Leishmania* (Anjili *et al*., 2011).

According to Shimabukuro *et al*. (2017) and Galati (2018), the blood-sucking sandfly vectors of leishmaniasis and other illnesses including bartonellosis (Carrion's sickness), *Phlebotomus* fever (sandfly fever), and vesicular stomatitis are members of the subfamily *Phlebotominae*. Sandflies go through a full metamorphosis, much like other true flies (Order: Diptera), and have four distinct life stages: the egg, larva, pupa, and adult (Durán-Luz *et al*., 2019). The embryonic stages of sandflies need rather warm, wet settings to grow, but unlike mosquitoes, they do not need standing water (Svobodova *et al.,* 2009). Sandflies are regularly observed close to rodent habitats because these needs are typically met by animal burrows and excrement (Wagner and Stuckenberg, 2016).

Phlebotomus, *Sergentomyia*, and *Lutzomyia* are three genera that make up the bulk of the more than 800 species of sandflies that have been discovered (Maroli *et al*., 2013; Nzelu *et al*., 2014). *Phlebotomus* and *Sergentomyia* are found throughout the Old World. Out of an estimated 800 species of *phlebotomine* sandflies, only 98 species of the genera *Phlebotomus* and *Lutzomyia* are now known to transmit human *Leishmaniases* (Bates *et al*., 2015). The primary vector source in the Old World is the

genus *Phlebotomus,* whereas the only vector source in the New World is *Lutzomyia* (Depaquit, 2014; Sousa-Paula *et al*., 2021). Other species and other genera have been proposed as possible vectors; however there are currently stringent and challenging conditions for implicating vectors (Lainson, 2010). These requirements are as follows: To be able to transfer the parasite to a host while digesting a blood meal, sandflies must be present in the region where the disease is being reported, be able to sustain the parasite's full development, and remain there. Additionally, the disease pathogen must be regularly separated from natural sandflies captured (Ready, 2011).

Table 2.1 lists species of sandflies that might spread leishmaniasis in different countries. Researchers have occasionally relied significantly on epidemiological evidence to infer the presence of the species because the aforementioned prerequisites haven't always been satisfied (Ready, 2011).

Table: 2.1: Several sandfly species have been implicated as actual or prospective carriers of distinct *Leishmania* disease strains in different geographical areas and nations (Ready, 2011)

Region/country	Vector spp.	Disease type
Argentina/Mexico	*Lutzomyia longipalpis*	Cutaneous/visceral
Belize/Mexico	*L. olmeca*	Cutaneous
Panama	*L. panamensis*	Cutaneous
Brazil	*L. whitmani/intermedia*	Cutaneous
Columbia	*L. evansi, gomezi*	Urban
Venezuela	*L. vallesi, gomezi*	Cutaneous
Sudan	*Phlebotomus langeroniorientalis*	Visceral
Kenya/Ethiopia	*P. martini*	Visceral
	P. aculeatus	Visceral
	P. elgonensis	cutaneous
	P. guggisbergi	cutaneous
	P. longipes	Visceral
	P. orientalis	Visceral
	P. pedifer	cutaneous
Palestinian	*P. papatasi*	Cutaneous
	P. sergentii	Cutaneous
	P. syriacus	Visceral
N.W. Africa	*P. dubosqi*	Cutaneous
Greece	*P. neglectus*	Visceral
India	*P. papatasi*	Cutaneous
India	*P. argentipes*	Visceral
Saudi Arabia	*P. papatasi*	Cutaneous
Monaco	*P. perniciosus*	Visceral
	P. ariasi	Visceral
Egypt	*P. langeroni*	Visceral
China	*P. alexandri*	Visceral
	P. chinensis	Visceral
	P. longiductus	Visceral

According to the groundbreaking classification by Abonnence *et al*. (1965), *P. rondani* and *Sergentomyia Franca* were suggested as the two genera for Old World species, while *Lutzomyia Franca, Brumptomyia Franca*, Parrot, and Warileya, Hertig were proposed as the three genera for New World species (Theodor, 1948). Leng and Lewis (1987) also noted that the genus *Chinius* encompasses certain Chinese sandfly species with ancient characteristics. Among modern Old World taxonomists, the three genera *Sergentomyia*, *Phlebotomus*, and *Chinius* are predominantly recognized.

The genus *Phlebotomus* is well-documented, comprising 96 species, 11 subgenera, and 17 subspecies (Depaquit *et al*., 2013). Similarly, the three genera *Brumptomyia, Lutzomyia*, and Warileya, which house 11 species groups and 15 subgenera of Neotropical sandflies, are familiar to entomologists (Lewis *et al*., 1977). Although there have been proposed alterations, none have achieved widespread agreement. In the most recent comprehensive research, 464 species of Neotropical phlebotomine sandflies were categorized into 23 genera, 20 subgenera, three species groupings, and 28 series (Brazil & Brazil, 2018).

As per Killick-Kendrick *et al*. (1997), phlebotomine sandflies are predominantly found in warm climates across Africa, Asia, Australia, southern Europe, and the Americas. Their distribution extends to Southwest Canada just above latitude 50°N in the north and Northern France and Mongolia in the south (Saghafipour *et al*., 2017), with their southernmost limit terminating at the Dead Sea. Despite this broad distribution, they are notably absent from the Pacific islands and New Zealand (Marcondes, 2007).

The life cycle of phlebotomine sandflies is well-documented (Thies *et al*., 2018; Munstermann, 2019; Jamal *et al*., 2020). According to Costa *et al*. (2019),

phlebotomine sandflies undergo four developmental stages: egg, larva (four instars), pupa, and adult. Unlike mosquitoes, their juvenile stages do not rely on standing water for development; instead, they thrive in warm and somewhat humid environments (Killick-Kendrick, 1990). After adult females lay their eggs in areas rich in organic material like animal dung and soil, the newly hatched larvae find refuge, food, and water (Desjeux, 2004).

Killick-Kendrick *et al.* (1986) reported that phlebotomines fly at a speed of approximately 1 m/s, which is notably slower than mosquitoes. This limited flying speed restricts their dispersion range, as they cannot fly at wind speeds higher than this rate. Consequently, adult activities are often confined to locations near larval nesting sites, with their typical flying range being around 300 meters (Nassif *et al.*, 2017). Studies using mark-release-recapture techniques have shown that forest species tend to have more localized dispersion compared to peridomestic species. For example, *Phlebotomus* ariasi can spread over a distance of 2 km (Killick-Kendrick *et al.*, 1994), while species from Neotropical forests typically do not spread over distances exceeding 1 km (WHO, 2016).

Males of phlebotomine sandflies gather in leks on or near hosts, where they produce sex pheromones to attract females. According to Schur and Jacobson (2004), the vibrations of males' wings are crucial in persuading females to mate. Mosquito wings, for comparison, vibrate at 130 beats per second. The microhabitats at resting sites are typically cool, humid, and dark (Killick-Kendrick, 1999), often in close proximity to larval breeding sites. Temperature and rainfall are the primary factors influencing the seasonal activity of adult sandflies (Pareyn *et al.*, 2020).

Understanding how *Leishmania* spp. are transmitted across mammalian hosts by sandflies of the genus *Sergentomyia* requires clarification. Most of the knowledge on phlebotomine sandflies comes from research conducted by Killick-Kendrick (1999) and the WHO (2007), supplemented by information from recent literature. WHO records include only nations with confirmed endemic human leishmaniases (Rojas-Jaimes *et al*., 2019).

A total of fourty two (42) species are known to be or are proven or probable vectors in the Old World, with specific associations: 20 species are linked to the transmission of *Leishmania infantum*, six with *Leishmania donovani*, seven with *Leishmania major,* seven with *Leishmania tropica,* and three with *Leishmania aethiopica* (Dantas-Torres *et al*., 2014). Notably, *Phlebotomus sergenti* has been implicated in the transmission of both *L. tropica* and *L. aethiopica* in certain areas of Ethiopia (Maroli *et al*., 2013), while the role of *Phlebotomus alexandri* in the transmission of both recognized species of the *L. donovani* complex (*L. donovanis.*s and *L. infantum*) in some regions of China, and potentially in other countries remains unclear.

In contrast to *Phlebotomus* species, some *Lutzomyia* species are likely capable of spreading several *Leishmania* species; for example, *Lu. Migonei* has been shown to harbor four distinct parasite species. To be considered a disease vector, a species must meet several generally recognized standards (Killick-Kendrick, 1990; WHO, 2007): (a) The vector must feed on human blood; (b) In zoonotic forms of leishmaniasis, the vector must also bite and imbibe the reservoir host(s) for blood; (c) The vector must be naturally infected with the same *Leishmania* species that infects humans, which must be proven by comparison; and (e) the vector must be able to transmit the parasite by bite to a susceptible host while consuming a blood meal.

It's crucial to highlight that even when using established standards to assess the "degree" of incrimination, determining whether a species is a "proven,""strongly suspected," or "suspected" vector can be subject to interpretation or extremely challenging to ascertain. This complexity is extensively detailed by Dantas-Torres *et al*. (2017) in their explanation of permissive vectors. For instance, in a study where 80% of Neotropical *Lutzomyia longipalpis* intentionally fed on blood containing Old World wild-type *L. major*, significant promastigote infections were discovered following blood digestion. This underscores the importance of considering the epidemiological context alongside established criteria.

Moreover, satisfying the fifth requirement, which involves demonstrating the transmissibility of *Leishmania* spp. to vulnerable hosts, poses significant challenges. Sandflies must first be 'naturally' infected by ingesting the appropriate stage and number of parasites. Then, those that survive in laboratory conditions after blood digestion must be induced to feed again on susceptible hosts (Dantas-Torres *et al*., 2010).

Oliveira *et al*. (2005) evaluated the following requirements for reliable vectorial incrimination in light of these challenges: First, there should be epidemiological evidence indicating that the geographic ranges of the vector and the human illness coincide. Second, there should be evidence demonstrating that the vector promotes naturally occurring gut infections with promastigotes of the same *Leishmania* species that infect humans. These factors collectively suggest that the vector feeds on human blood. Additionally, when confirmed vectors are purportedly absent from endemic habitats or when the only phlebotomine sandflies that bite humans are species meeting

the outlined criteria, the evidence for species incrimination is strengthened (Dantas-Torres *et al*., 2010).

Phlebotomus argentipes in the Old World is now considered a possible vector of cutaneous leishmaniasis (CL) caused by the *L. donovani* agent in Sri Lanka, as suggested by Maroli *et al*. (2013). Additionally, the vectorial role of *Phlebotomus orientalis* for *L. donovani* in Kenya has been confirmed (Mutinga *et al*., 1989), and *Phlebotomus salehi* has been implicated in the transmission of *L. major*, while members of the *Phlebotomus major* complex are associated with *L. infantum* in Iran (Azizi *et al*., 2006). *P. sergenti* has also been identified as the vector of CL caused by *L. tropica* in Algeria (Khezzani and Bouchemal, 2017) and Tunisia (Bousslimi *et al*., 2010). However, it's worth noting that the *Leishmania*l agent *Leishmania killicki,* mentioned in the latter two records, belongs to the largely polymorphic *L. tropica* taxon.

In China, *Phlebotomus sichuanensis* and *Phlebotomus chinensis* are two distinct species that play a role in the transmission of *L. infantum* in endemic regions, as reported by Leng and Lewis (1987) (Assimina *et al*., 2008). Recent data implicates *Lutzomyia forattinii* and *Lu. migoneia* as new potential vectors of visceral leishmaniaisis (VL) in the New World. Shiambukuro *et al*. (2017) discovered *Lu. forattinii* alongside the isomorphic *Lutzomyia cruzi* in the Brazilian state of Mato Grosso. Naturally occurring parasites identified as *L. infantum* affected both species, suggesting their potential role in disease transmission. *Lutzomyia migonei,* traditionally considered the VL vector in La Banda, Argentina (Salomón *et al*., 2011), has also been proposed as a possible vector in Brazil (Pernabuco state), where *L. infantum* DNA has been found in wild-caught specimens. This finding suggests that in

areas where the normal VL vector, *Lu. Longipalpis*, is absent, this species may be responsible for disease transmission.

Regarding cutaneous leishmaniasis (CL) vectors, the following details are now included: (a) *Lutzomyia nuneztovari anglesi* has been identified as a vector of *L. amazonensis* in Bolivia, based on anthropophilic, biochemical identification of wild isolates and effective experimental infection (Martinez *et al*., 1999); (b) *Lutzomyia ayacuchensis* was recently found in Peru naturally infected by promastigotes typed as *L. guyanensis* (Kato *et al*., 2015); (c) In Venezuela, *Lu. migonei* has been reported as a putative vector of *L. mexicana* and *L. guyanensis* (Feliciangeli *et al*., 2004); (d) In Brazil, repeated observations of natural promastigote infections identified as *L. braziliensis*, associated with anthropophilic behavior and a spatial distribution related to human CL; and (e) Previous reports have implicated *Lutzomyia gomezi* as a proven vector of *L. bruzi* (Shiambukuro *et al*., 2017).

Estimates indicate that there are currently 98 recognized phlebotomine species and subspecies across all continents except Antarctica, along with 29 fossil species. The Americas host 512 living and 17 prehistoric taxa. Throughout the 20th century, the genus *Phlebotomus rondani* Berté has been divided and expanded. Most experts now acknowledge at least six genera, including *Brumptomyia* and *Lutzomyia* in the Americas, and *Phlebotomus* and *Sergentomyia* in the Eastern Hemisphere. Phylogenetic studies have cast doubt on the monophyly of the genera *Phlebotomus*, *Sergentomyia*, and *Lutzomyia*, complicating practical classification and revisions despite the presence of most mammalophilic species and vectors (Zahraei-Ramazani *et al*., 2018).

Phylogenetic studies employing a non-numerical cladistic approach have identified the tribes *Phlebotominae* and *Hertigiinae*. Phlebotomini comprises 931 extant species, categorized into 30 genera and six sub-tribes, including *Sergentomyinae, Australophlebotominae, Brumptomyiina, Lutzomyiina,* and *Psychodopygina.* The majority of Latin American experts concur with the classification of American genera, and some generic recommendations have been supported by numerical phylogenetic studies of faunas from China and the Oriental region. Several species of *Phlebotomus* are known to transmit the more prevalent visceral leishmaniasis (VL) caused by *Leishmania donovani* and *Leishmania infantum* in Eastern Africa (Dulacha *et al*., 2019; Kühne *et al*., 2019; Bhunia and Shit, 2020).

Forty eight species of sandflies occur in Kenya (Anjili *et al*., 2011). Three species, namely *Phlebotomus pedifer*, *P. martini* Parrot and *P. duboscqi* have been confirmed as vectors for *L. aethiopica, L. donovani* and *L. major* respectively (Owino *et al.,* 2019). Some species, namely *Phlebotomus rodhaini Parrot, Sergentomyia garnhami, S. squamipleuris Newstead, S. africanus Newstead, S. kirki Parrot, S. ingrami Newstead, S. antennatus Newstead, S. graingeri Heisch, S. guggisbergi* and Teesdale and *S. clydei* Sinton have been shown to carry various flagellates whose characterization have not been carried out (Minter, 1964; Mutinga and Kamau, 1986; Mutinga *et al*., 1986b).

Despite being tiny, sandflies may bite deeply and create extremely itchy papules, some of which can turn into blisters and become infected if scratched (Kirk and Lewis, 1951). According to Kirk and Lewis (1951), the human victims are typically bitten on their faces, hands, arms, ankles, and legs, the body parts that are most exposed when sitting outside on the verandas of tropical homes or close to termite

hills. Therefore, even if the anthropophilic sandfly species do not harbour the disease-transmitting parasites, their overall geographic distribution should be identified and the public should be made aware of the potential harm the sandflies may do.

Several caves were discovered to be home to phlebotomine sandflies during earlier investigations of the Kenyan side of Mt. Elgon (Mutinga and Odhiambo, 1986b; Sang *et al*., 1993a; Mukhwana *et al*., 2018). *Phlebotomus pedifer,* the presumed parasite's vector, was mostly discovered in a strip of escarpments between 1750 and 1900 metres in height, accounting for more than half of the identified male sandflies found on Mount Elgon's slopes (Makwali, 2021;Svobodova *et al*., 2003). These male sandflies were linked to females with *Leishmania* parasite infections and those that attack people.The caves, which were mostly home to *P. elgonensis*, were located at intermediate and higher altitudes and were connected to significant promontories and river basins (Sang *et al*., 1993a). In a previous research (Svobodova *et al*., 2003), specifics on the distribution of caves on the slopes of Mount Elgon and their sandfly ecology were reported.

Investigation by Sang and Chance (1993) demonstrated that the sandflies found in the caves in Mount Elgon can be categorised into three groups based on the species and altitude of distribution; the low altitude escarpment area (1750-1900m) where *P. pedifer* are the majority, the middle region (1900-2300m) where both *P. pedifer* and *P. elgonensis* occurred, and the higher altitude areas (2300-2600m) where *P. elgonesis* were dominant. Additionally, Sang *et al*. (1993a) showed that cutaneous leishmaniasis (CL) was restricted to Mount Elgon's lower southern slopes, where *P. pedifer* was the predominant sandfly species and a known CL vector.

The study by Sang and Chance (1993) established that on the Kenyan side of Mount Elgon, there were 237 caves and out of these he sampled 229 caves and 202 of them had sandflies. *Phlebotomus elongonesis* were the main sandflies inhabiting these caves. Sang and Chance (1993) also observed that there was a possibility that not all *P. pedifer* at elevated altitudes of 1900 meters and *P.elgonesis* in lower altitudes were prone to *Leishmania aethiopca* infection

Sang and Chance (1993) found members of the genus *Segentomyia* in smaller caves that were just 2 or 3 metres long. Western Kenya's Bungoma and Trans Nzoia Counties were discovered to have an endemic case of cutaneous leishmaniasis caused by *Leishmania aethiopica* Bray (Mutinga, 1975a; Mukhwana *et al*., 2018). The rock hyrax, tree hyrax, and giant rat were implicated as the parasite's reservoir hosts at these sites (Mutinga, 1975b), while sandflies of two species, *Phlebotomus pedifer* and *P. elgonensis*, were implicated as the parasite's vectors (Ngoka *et al*., 1975). On the agricultural slopes of Mount Elgon, these sandflies are dispersed differentially, with *P. elgonensis* inhabiting the higher altitudes and *P. pedifer* the lower levels (Sang and Chance, 1993). The likelihood of contracting an illness from a sandfly bite has also been linked to proximity to caves (Sang and Chance, 1993). The majority of the animals in the caves are bats, which have never been examined for infection with *Leishmania* parasites. It was necessary to look into their function as reservoir hosts and maintenance of the parasite's illness spread. These investigations were a major emphasis of the current study.

In addition to the leishmaniases and other parasites that infect sandflies, sandflies can also transmit other illnesses. Sandflies in California were discovered to be infected by the gregarines of the species *Lankestria mackieii* (Ayala, 1973). A few types of

sandflies can spread *Bartonella bacilliformis*. According to Mutinga (1975), *Bartonella* is the cause of bartonellosis, also known as Carrion's sickness.

Sandfly fever is brought about by certain *Phlebotomus* species, especially *Phlebotomus papatasi* Gabbi and Visentini (Salomon 2011). According to Chaniotis *et al*. (1968), sandflies have the ability to spread arboviruses like the vesicular stomatitis virus in Panama. According to Ayala (1971), *Phlebotomus vexator* occidentalis is the carrier of a trypanosome species that infects the toad *Bufo boreas halophilus.*

Nzelu *et al*. (2014) conducted research in Ghana and found no convincing evidence supporting the transmission of human leishmaniasis by sandflies in the genus *Sergentomyia*. However, other studies, such as that by Mutinga *et al*. (1994), suggest that this may indeed be the case. Senghor *et al*. (2016) presented potential evidence regarding the transmission of *L. infantum*, the causative agent of VL in Senegal, via *Sergentomyia* sandflies (*S. dubia and S. schwetzi*), utilizing ecological, parasitological, and molecular data. Furthermore, the discovery of human blood meals and *Leishmania* DNA and parasites in various species of *Sergentomyia* sandflies (Tateng *et al*., 2018; Maia and Depaquit, 2016) provides additional evidence for the potential involvement of these insects in *Leishmania* transmission. Consequently, *Sergentomyia* sandflies are still considered to be vectors of *Leishmania*.

2.3 *Leishmania* parasites

The macrophages of vertebrate hosts are where *Leishmania* parasites like to live. Here, they may be found as spherical, non-motile amastigotes that range in size from 3 to 7 μm (Claborn, 2017). Infected macrophages are consumed by the sandfly during bloodmeal bites, and amastigotes are then discharged into the insect's stomach

(Killick-Kendrick *et al*., 1994). The amastigotes quickly change into the motile, extended, flagellated promastigote form, which is 10–20μm in length (Gradoni, 2018). The promastigotes subsequently go to the sandfly's digestive system, where they reproduce via binary fission while living extracellularly as metacyclic promastigotes (Mozaffari *et al*., 2020). According to Rodriguez *et al*. (2018), macrophage killing of parasites is selectively inhibited.

The main surface glycoconjugate, lipophosphoglycan (LPG), creates a thick glycocalyx that covers the whole promastigote surface, including the flagellum (Mule *et al*., 2020). Procylic organisms, also known as procylics, express shorter LPG molecules than mature metacyclics, which contain the capping at the terminal -galactose residues with -arabinose and elongate by doubling the number of repeating disaccharides units by two to three. The host does not lyse the metacyclic organisms as they migrate to the proboscis and triggers the conventional complement pathway in the host (Valente *et al*., 2019). Instead, the organism is expelled from the midgut in its whole metacyclic form. While feeding on the blood of a mammalian host, the sandfly delivers the metacyclic *Leishmania* promastigotes into the host (Alhajri, 2019).

Once within the mammalian host, the promastigotes are absorbed by the macrophages, which swiftly transform them into amastigotes, survive, and multiply there before lysing the macrophages (Horta *et al*., 2020). The subsequent cycle is initiated when new macrophages take up the discharged amastigotes. All the phagocyte- and macrophage-containing organs eventually get infected, particularly the spleen, liver, and bone marrow (Requena and Soto, 2017).

According to Abonnenc (1972) and Lainson & Shaw (1978), there are three clinical kinds of human *Leishmaniases*: visceral leishmaniasis (VL), cutaneous leishmaniasis

(CL), and muco-cutaneous leishmaniasis (MCL). Visceral leishmaniasis, often known as kala-azar, is one of the most serious parasite diseases, especially in India, Nepal, and Sudan (Bi *et al*., 2018). It is caused by parasites from the *Leishmania donovani* group. More recent genetic study has revealed that *L. donovani* is the only *Leishmania* species that causes VL in East Africa, contrary to earlier studies that suggested many *Leishmania* species were to blame (Kanyina, 2020).

In the majority of VL endemic regions, *Phlebotomus orientalis,* which is associated with *Acaciaseyal/Balanities aegyptiaca* woodland (Depquit *et al*., 2013) and flourishes on black cotton soils (Lewis and Ward, 1987), appears to be the only vector of *L. donovani*. The exception to this norm is that *P. martini* is believed to be the main VL vector in a small region of Tuscany and causes illness (Adamczick *et al*., 2018).

According to Assimina *et al*. (2008), children between the ages of 7 and 14 are most commonly affected by leishmaniasis illnesses like Kala-azar. The signs and symptoms of kala-azar include fever, anaemia, a low white blood cell count, wasting, splenomegaly, and a severe protein imbalance in the serum (Zuckerman and Lainson, 1977). Unlike Kala-azar, that can be deadly if untreated, oriental sore, also known as cutaneous leishmaniasis can leave a person with severely disfiguring scars.

However, in some hosts, including humans, there are violent host-cell reactions to the parasites that result in skin lesions or severe pathological changes in the internal organs, such as in Kala-azar (visceral leishmaniasis) (Lainson and Shaw, 1978). Natural vertebrate hosts exhibit few or no pathological effects that are produced by the parasites. According to the species, the parasites inside the vertebrate host have an oval shape called amastigotes that range in size from 1.5 to 3.0 by 3.0 to 6.5

micrometres (Cheng, 1986). *Leishmania* infects the hosts' macrophages since they are reticuloendothelial system tissue parasites. When a female sandfly feeds on the infected host, amastigotes are consumed in blood from the epidermis or peripheral blood arteries (Figure 2.1). They develop into the promastigote form, an extended form with a flagellum, in three days, and eventually move to the front of the sandfly gut (Adler, 1964).

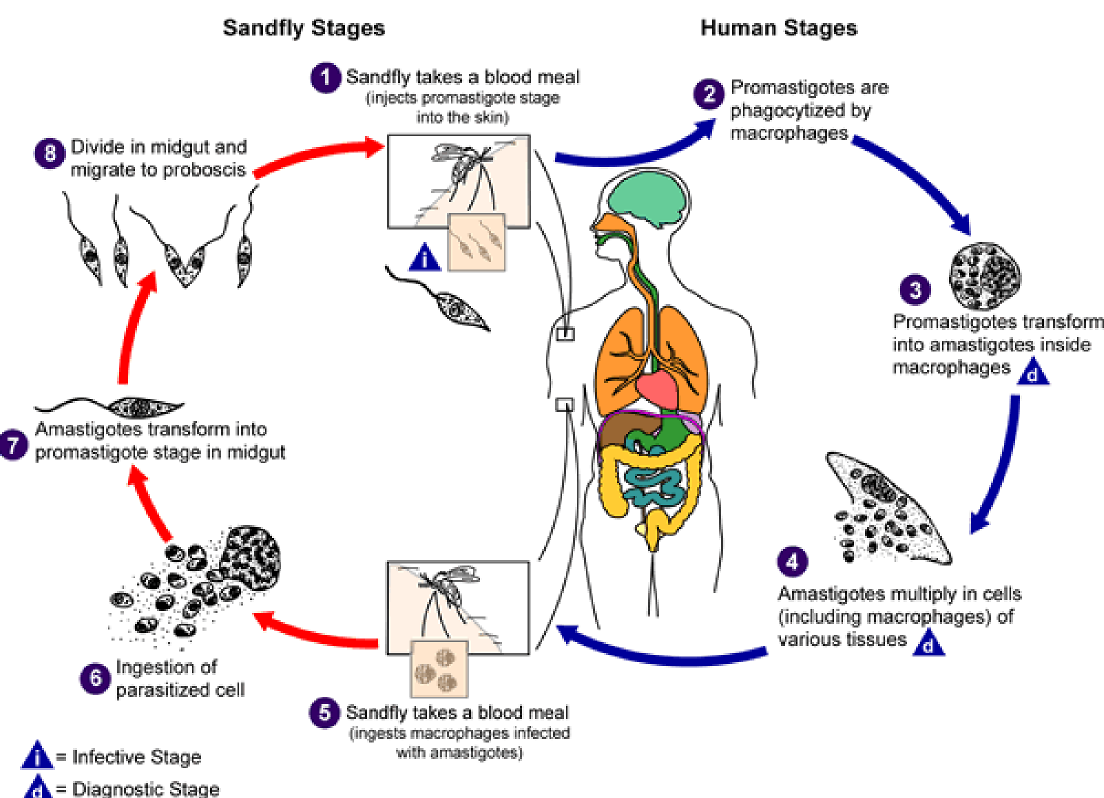

Figure 2.1: Life cycle of human cutaneous *Leishmania* (Source CDC, 2013)

Sandflies of the genus *Phlebotomus* transmit *L.donovani*, which causes mucosal and visceral leishmaniasis (Abdalla, 1982). Visceral leishmaniasis is spread to people in

East Africa by *P. martini*, a species of the *Synphlebotomus* Theodor group that may also infect other animals, including as wild canidae and dogs (Lewis and Ward, 1987). In certain parts of Kenya, *Sergentomyia garnhami* has been identified as a potential Kala-azar vector (Mutinga and Odhiambo, 1982). The most notable landscape feature linked to the outbreak of visceral leishmaniasis in Kenya is the presence of deteriorated termite mounds made by *Macrotermes bellicosus* Smeathman (Kirk and Lewis 1946). Many sandfly species, including *P. martini*, *p. vansomerenae*, and the *Synphlebotomus* complex, breed and rest in termite hills (Molyneux and Ashford, 1983; Mutinga and Kamau, 1986; Mutinga *et al*., 1989). This complex is thought to be the main vector of *L. donovani* in the Eastern Province of Kenya.

Essentially, cutaneous leishmaniaisis caused by the parasites *L. tropica* and *L. major*, which are spread by members of the genus *Phlebotomus, L. tropica* can harm both humans and canines (Ashford 1996). Chronic oriental sores caused by *L. tropica* in males which can take up to a year to appear, are frequently solitary, dry lesions that predominantly affect the face, and only become ulcerated after many months (Njau, 2010).

According to Zuckermann and Lainson (1977), *L. major* causes cutaneous leishmaniasis in humans that develops quickly and lasts only a few weeks. Lesions whichgenerally appear on the lower extremities are frequently exposed, moist, and they ulcerate within one to three weeks (Ashford and Bettini, 1987). Human highland cutaneous leishmaniasis is caused by *L. aethiopica*, which is spread by *P. pedifer* in Ethiopia and some regions of Kenya, particularly in Mount Elgon (Mutinga, 1975). Protracted lasting cutaneous sores with a protracted incubation time are brought on by *L.aethiopica.*

Phlebotomus duboscqi has been shown to be the primary zoonotic vector of *L. major* in the Baringo district, with *S. ingrami* and *P. martini* serving as alternate zoonotic vectors, according to Mutinga (1986 a) and Mutinga *et al*., (1986b).In Sudan, the dermal lesions in areas with endemic visceral leishmaniasis are caused by *L. donovani* and not *L. tropica* (WHO 2010), demonstrating the need for parasite characterization to go hand in hand with clinical manifestation and pathology of the disease.

Morphological identification of *Leishmania* parasites using microscopy in field collected sandflies can be time-consuming due to low parasite load and the need for consistent observation under a microscope. Large sample sizes and potential contamination risks make it challenging to use the samples again (Brewster *et al*., 1998). PCR-based techniques that target a variety of conserved genes, including ITS1 have been used (Azpurua *et al*., 2010; Svobodova *et al*., 2009). Previous studies have used encoding areas like Cytochrome b gene, minicircle kDNA, and 18S rRNA to identify *Leishmania* parasite in sandfly vectors (Jara *et al*., 2003, Rausay *et al.,* 2000, Azimi *et al*., 2010)

A very sensitive DNA primer is required because, as reported in the earlier investigations, the parasite burden in normally infected sandflies is lower. Because a large sample size can be processed concurrently and PCR is more sensitive to identifying infection, it is preferred over microscopy. Additionally, PCR saves time and has been utilised for investigations of a comparable kind in different nations, enabling comparison of results (Kato *et al*., 2005).

Sandflies are often collected and processed using one of two approaches in research looking at the frequency of *Leishmania* infection in sandflies: either pooling the insects or testing each one separately (Bhattarai *et al*., 2009)

Sandflies are collected and processed for research on *Leishmania* infection frequency using pooling or individual testing.According to several studies (Bhattarai *et al*., 2009; Dinesh *et al*., 2000; Tiwary *et al*., 2012), the pooling approach is chosen due to its simplicity, speed, and effectiveness, but determining infected sandflies in a positive pool is challenging and data on specific sandflies may be lost.Individual sandflies can be assessed in order to get around these restrictions. *Leishmania* infection rates ranged from 0.7% to 2.0% in wild sandfly populations, according to earlier investigationsby Rocha *et al*., 2010; Paiva *et al*., 2010; Sanchez-Garcia *et al*., 2010; Svobodova *et al*., 2009)

In a research by Tiwary (2013), the number of *Leishmania* parasites in spontaneously infected sandflies was quantified by real-time PCR using the standard curve absolute quantification technique. Gravid sandflies had the largest parasitic loads of the infected sandflies, followed by theunfed and blood-fed ones.

In a research by Kobets (2011), three distinct methods light and fluorescence microscopy, modified limiting dilution test, and PCR together with ELISA were employed to identify and measure *Leishmania* parasites in the infected mouse tissues.

Giemsa-stained tissue smears were quantified for the number of parasites as the average number of amastigotes from 100 microscopy fields. Kobets *et al*. (2010) used histological analysis to demonstrate the presence of parasites in the inguinal lymph nodes of *L. major,* while Kurey *et al*. (2009) demonstrated that the parasites might enter the spleen of the *L. tropica* (Kobets *et al*,2010). As a result, the enhanced PCR

together with ELISA was validated using a traditional method of parasite count in tissue smears (Kobets *et al*. 2010).

2.4 Reservoir hosts of *Leishmania*

Leishmania life cycle requires reservoir host, a complex pathogen-vector-reservoir relationship with temporal and geographical variations (Maia *et al*., 2018).

The concept of a reservoir has evolved dramatically during the past century, necessitating a deeper examination of zoonosis. According to Dostalova *et al*. (2012), an animal is regarded a reservoir host of *Leishmaniases* if an infectious agent can stay there long enough for the animal to serve as a source of parasites for the vector. It must be shown that the parasite population is dependent on a specific animal for its long-term maintenance in order for a reservoir host to be recognized and implicated (WHO, 2010). A "good" reservoir should be easily vulnerable to the parasites, dwell in close proximity to people, and be able to provide the vectors with parasites.

Although the occurrence might vary substantially depending on the season, a sizeable proportion of people should contract the parasites over their lifespan. Because the maturity of the parasites in the female phlebotomine sandfly depends on blood, a suitable reservoir host should offer a considerable blood source for sandflies, and both should interact, rest, and reproduce in the same environment. According to Tripathi and Nailwal (2021) and WHO (2010), **i**nfections with chronic evolution require animal survival until transmission season, and *Leishmania* parasites in reservoir hosts must share biochemistry and genetics with humans.

From small to large, wild, domestic, and synanthropic mammals, a number of them, including rock hyraxes, rodents, foxes, dogs, cats, and other domestic animals, have been reported to act as reservoir hosts for *Leishmania* species particularly in the

Indian subcontinent ; India, Nepal, and Bangladesh, and East Africa; Ethiopia and Kenya (Bhattarai *et al*., 2009) .*L. tropica* and *L. donovani*, are thought to be anthroponotic, spreading from person to person via the sandfly vector (Bhattarai *et al*., 2009). However, there is some evidence suggesting that zoonotic transmission may also be involved. The presence or absence of reservoir hosts affects the dispersion of *Leishmania* parasites, yet reservoir hosts are frequently left out of analyses. They have a significantly wider range of dispersion, which is one of the causes high spread of the *Leishmania* parasites (Alcover *et al.,* 2020).

It appears that the interactions between the parasites and their host(s) are frequently very complicated. This interaction, which is seen as a self-sustaining and autonomous system in zoonotic cutaneous leishmaniaisis (ZCL), involves the parasite, the sandfly, and the mammalian host(s). The reservoir host in this system, according to Ashford (2000), has the following traits: (1) is abundant or plenty in numbers; (2) constitutes a significant portion of the mammalian biomass; (3) is frequently a gregarious species; and (4) lives for a long enough period of time to carry the disease into the following transmission season. The WHO (2000) has established a number of standards for proving that animals are reservoir hosts for the *Leishmania* parasites.

Epidemiological classifications for potential infection of host species include major, secondary, minor reservoir hosts, and accidental hosts. Major reservoir hosts permanently sustain parasites in the environment (Bourdeau *et al.,* 2020). Secondary reservoir hosts transmit infection but cannot maintain parasite transmission without primary hosts, while accidental hosts do not contribute to the transmission cycle and infections typically go undetected in these hosts. (de Avila *et al.,* 2018; WHO, 2010).

Leishmania infection patterns in mammalian host species are influenced by host factors, parasite traits, exposure, and local environmental conditions. A specific mammalian host species may not have the same role in the transmission cycle at all locations and times (Desjeux, 2004).Transmission in infected mammals follows the 20/80 rule (80% of transmission is accounted for by 20% of individuals which are the super-spreaders) with transmission associated with a small percentage, similar to vector competence (Bourdeau *et al.,* 2020). Dynamic parasite transmission nets can cause parasites to be eradicated from host populations before being reintroduced later (Schur and Jacobson, 2004).

The role of an infected animal in the transmission cycle depends on the parasite's mode of dispersal and the host-parasite relationship. These factors influence the parasite's ability to transmit and maintain itself from the intermediate host or external environment. The host species' ability as a reservoir host is a crucial factor affecting their transmissibility. A reservoir system is a unique system of mammals responsible for maintaining the parasite, with each species participating in transmission at a specific time and place. Transmissibility competence changes during infection duration, allowing different roles for animals or individuals throughout a parasite's life cycle (Schur and Jacobson, 2004, Bourdeau *et al.,* 2020)

Amplifier hosts are mammals with a trait that facilitates transmission of infections by maintaining infection and storing more parasites in blood and skin (WHO 2010). Maintenance hosts are mammals that can infect and maintain infections, transforming into amplifier hosts based on host health conditions and immune suppression (Schur and Jacobson, 2004, Bourdeau *et al.,* 2020)

Asymptomatic infection is often linked to long-standing host-parasite partnerships, but not all prehistoric relationships evolved harmoniously (Cunzee *et al*., 2019). The parasite's ability to spread depends on its reproductive strategy, and virulence and pathogenicity may be considered fitness features due to their potential to increase transmission and positive selection (WHO, 1990).

Since asymptomatic infection is frequently linked to long-standing host-parasite interactions, a reservoir host is regarded as asymptomatic (WHO, 1990). Although some prehistoric host-parasite interactions may have facilitated the parasite's propensity to spread, it is presently believed that not all of them did as reported by (Cunzee *et al.,* 2019), who asserts that the parasite's ability to spread depends on its reproductive strategy. Given their capacity to increase parasite transmission and the likelihood of positive selection, virulence and pathogenicity may occasionally be seen as fitness features. Since *Leishmania* reservoirs show geographical and temporal variety, only a local inquiry including ecological and parasitological studies can determine if these "potential reservoirs" may operate as reservoir in a specific environment.

As the sole known reservoir for the parasite, health officials have advised the elimination of seropositive dogs in cases of human visceral leishmaniaisis caused by *L. infantum* (Killick-Kendrick *et al*., 1997). In fact, multiple research (Lainson and Rangel, 2005; Dantas-Torres, 2009) have shown that dogs are epidemiologically significant as reservoirs in various locales. However, it has already been suggested that cats and opossums may play a role in the transmission cycle of *L. infantum* in urban settings rather than dogs (Ashford *et al.,* 1998; Baneth, *et al*., 2008).

It has been disregarded that wild animals can act as a source of infection for vectors in peridomiciliary regions; even though some of these places are extremely near to sylvatic habitats.The potential involvement of these animals is a significant additional issue that must be taken into account when proposing management methods for this zoonosis. A peridomestic transmission system may be continuously reinfected by species from other orders of mammals that persist and act as a source of infection for phlebotomine sandfly vectors.

Brazil's dog culling effectiveness is low due to lack of structured surveillance, high intervals, rapid replacement, and resistance from owners to euthanizing infected dogs (Bourdeau *et al*., 2020). Dantas-Torres *et al.* (2019) suggest that species can be kept alive in the wild using a reservoir system, consisting of varying degrees of transmissibility competence among mammals. This theory aligns with Ashford's reservoir concept over 30 years ago. Although there are many sick individuals, each can only transfer the virus for a little time while holding onto the infection for a long time. These parasite species' evolutionary success may be explained by a tradeoff in this system. The accumulation of multiple short periods of infection in several infected animals ensures the survival and spread of these *Leishmania* species (Dantas-Torres *et al.,* 2010).

Several canine species, particularly the domestic dog and the two fox species *Lycalopex vetulus* and *Cerdocyon thous,* are affected by the zoonotic illness visceral leishmaniaisis in the New World (Agrawal *et al*., 2013). The three fox species; *Vulpes vulpes*, *V. corsac*, and *V. zerda* as well as domestic dogs serve as the primary reservoir hosts in the Mediterranean areas of North Africa and South Europe (Singh and Sundar, 2014). It is interesting that in all VL zoonotic foci when the illness is

caused by *Leishmania infantum* or *L. chagasi*, the dog is regarded as the major reservoir host (Millán *et al*., 2014).

Although zoonotic foci were found in several of these outbreaks, the transmission of *L. donovani* in East Africa is likely to be anthroponotic, particularly during epidemic circumstances (Bhattacharyya *et al*., 2013). According to observations, the outbreaks of VL among campers in remote parts of Southern and Eastern Sudan were the original cause of the zoonotic spread of the disease in the area (Zijlstra and El-Hassan, 2001; Mohammed *et al*., 2018). Dogs infected in a village environment were found in the Atbara River region of eastern Sudan (Meheus *et al*., 2013; Baleela *et al*., 2014).The Egyptian mongoose (*Herpestes ichneumon*), which may serve as the parasite's principal reservoir host in this ecosystem, was also shown to have significant infection rates of *L. donovani* in unoccupied forest regions of Dinder National Park (DNP) (Pareyn *et al*., 2019). Domestic dogs may be important reservoir hosts for *Leishmania donovani* in eastern Sudan and other parts of East Africa, according to research (Baleela *et al.,* 2014). The likelihood of interactions between the vector and other wild animals is typically overlooked in these experiments, though.

The most often examined animals are dogs and rats, which have long been believed to serve as reservoirs for natural *Leishmania* infections in domestic, peridomestic, and wild animals (Baneth *et al.,* 2008). Research on *Leishmania* parasites in animals like hares and marsupials has shifted attention away from sylvatic reservoir hosts. Old World bat species have previously tested positive for *Leishmania,* and *Rhinolophus lender* has been suspected to be a reservoir host on Mt. Elgon, Kenya (Makwali, 2021).

2.5 Pattern of *Leishmania* species infection in reservoir hosts

Limited research on *Leishmania spp.* transmission through mammalian host species, often used interchangeably as "hosts" and "reservoirs."(Ashford, 1996). There are not many studies that looked at these parasites infected wild hosts for an extended period of time (de Avila *et al.,* 2018). Conducting an epidemiological investigation involves infection follow-up and sampling potential reservoir host species among mammalian populations to understand the role of mammalian species in *Leishmania spp.* transmission.

It is similarly important to have a thorough methodological strategy, which should include the diagnosis of infection using both direct and indirect parasitological tests to gauge transmissibility competence (Bourdeau *et al*., 2020). To support the interpretation of the data gathered from field study, it is required, wherever possible, to undertake experimental investigations on potential natural reservoirs (Roque *et al*., 2010). Direct inspection and blood-culture methods struggle to find *Leishmania spp.* in wild animals, with sensitivity varying across studies and depending on parasite burden, tissue, and technician skill (Campino *et al*., 2013). Gold-standard techniques involve cultures of puncture wounds or hematopoietic tissues. However, good outcomes do not always indicate host transmission, as vector-parasite accessibility is determined by the source. Positive skin or blood cultures indicate transmissibility (Cheesbrough, 2005 b).

The sensitivity of direct observation of parasites in skin fragments is reduced, but when combined with the identification of the etiologic agent, this approach also validates the vitality and, hence, the transmissibility of the parasite. Positive results from internal organs (such as the liver, spleen, bone marrow, and lymph nodes) may

not always signify infectivity to the vector, but positive cultures invariably show the presence of living parasites (Cheesbrough, 2005 b). The immunofluorescence assay test method (IFAT) and the Enzyme-Linked Immunno Asay (ELISA) assays are two of the most often used serological tests that show infection (Schoone *et al.,* 2001).

For IFAT (Bhaattacharyya *et al.,* 2013), the test's sensitivity and specificity vary from 90% to 100% and 80% to 100%, respectively, while for ELISA (Marcondes *et al*., 2011), they range from 80% to 99.5% and 81% to 100%, respectively. Serologically positive but parasitologically negative hosts have been exposed to *Leishmania* infection and are thus anticipated to still be infected, but they are not always necessary for the persistence of the parasite in nature and are not always parasite reservoirs.

Since the polymerase chain reaction (PCR) identifies DNA fragments that are constitutive to the parasites, it may be seen as a parasitological test. Depending on the tissue being studied, this method may achieve sensitivity and specificity levels close to 100% (Aransay *et al*., 2000; Baker *et al.,* 1986). PCR undoubtedly shows the parasite's existence, but it does not show that the parasite is intact (Fryauff *et al*., 2004). Both ELISA and PCR were utilised in the current investigation because of their excellent sensitivity and specificity.

Recent research suggests parasite burden in skin may be linked to infectiousness during natural infection due to transmissibility (Courtenay *et al*., 2017). The use of PCR as the gold standard for diagnosis to guide treatment, although cure of human leishmaniaisis is still up for controversy, despite the fact that it is thought to be exceedingly sensitive (Kato *et al.,*2005; Jara *et al.,* 2003). Due to the absence of

standardised procedures and species-specific molecular targets, PCR diagnosis of wild and synanthropic animals remains difficult.

Despite the fact that *Leishmania* parasites have been known for many years, little is known about their epidemiology, in part because most research has focused on human health (Alemayehau & Alemayehau, 2017). Leishmaniaisis epidemiology has been assessed from a "one health" perspective in the past ten years (Pareyn *et al.,* 2019).

Mt. Elgon Sub-County cave areas in Bungoma County, Western Kenya, are endemic for cutaneous leishmaniaisis, which is brought on by *Leishmania tropica* and *Leishmania aethopica* Bray (Mutinga, 1975a). The rock hyrax, *Procavia capensis* Pallas, the tree hyrax, *Dendrohyrax arboreus* Grey (*Hyracoidea: Procaviidae*), and the giant rat, *Cricetomys gambianus* Waterhouse (*Rodentia: Cricetomynae*), have all been identified as the parasite's reservoir hosts in these locations (Mutinga, 1975b). *Phlebotomus* (*Larroussius*) *pedifer* and *P. (L.) elgonensis* are the two species of phlebotomine sandflies that have been caught in cave settings (Lewis *et al*., 1974; Ngoka *et al*., 1975). On the slopes of Mount Elgon, these sandflies are dispersed individually; with *P. elgonensis* inhabiting the higher altitudes and *P. pedifer* the lower levels (Sang and Chance, 1993). According to Killick-Kendrick (1987), being close to caves has traditionally been thought to increase the risk of contracting an illness from a female sandfly bite.

Sandflies are attracted to specific animals for bloodmeals and this has been the basis of performing bloodmeal analyses to determine upon which hosts a particular sandfly species feed (Sales KGda *et al.,* 2015). Specific sandfly vectors are different in different endemic areas. They however can be recognized by one or more of the following attributes: (i) their distribution agrees with that of *Leishmania*, (ii) they are

comparatively easy to infect with the local *Leishmania*; (iii) the infection in the sandflies extends forwards into the head (pharynx and pre pharynx) (iv) the infection persists for the remainder of the life of the sandfly; and (v) the sandflies are sufficiently prevalent to maintain the cycle of infection of the parasites" (Giantsis *et al.,* 2021)

Contrary to Baringo County, two caves; Chepkutuny and Chemai can be discovered in a semi-urban setting in Bungoma County. The sandfly fauna in this region has not been well explored, and the caves have not been checked for reservoir hosts and for particular sandflies carrying *Leishmania* parasites. Hyraxes, rats, porcupines, wild cats, and bats can be found in or close to the caves, according to information from locals and observations made on the ground.

Incidential hosts, not relevant for long-term persistence of parasites, may be considered secondary reservoir hosts, occasionally responsible for transmission and maintenance of parasite life cycles (Mutinga and Odhiambo, 1986a). According to Ashford (1996), reservoir hosts fulfill the following criteria: (1) abundance and gregariousness of the host (2) long lived or survival at least during a non-transmission season of the parasite (3) remain infected for a long time without causing acute disease and (4) sandfly bites to display the parasite in their skins or blood circulation. Given that the huge rats are already known reservoirs for the illness and that there are not enough of them in the caves to support ongoing disease transmission, the hyraxes meet these requirements (Kassahun *et al*., 2015). It is crucial to look at how bats and hyraxes contribute to the parasite's maintenance of its life cycle and transmission. In this work, *Leishmania* parasite infection was established using microscopy, serology,

and culture methods on tissues smears of bats and hyraxes that had been captured and put to sleep.

According to the majority of publications accessed, VL in East Africa is thought to be anthroponotic, and similar to VL in the Indian subcontinent (WHO, 2015; Al-Salem *et al*., 2016; Jones and Welburn, 2021). Nevertheless, there is evidence suggesting that zoonotic transmission may be occurring in unreliable reservoir hosts (Baneth, 2008). Natural L. *donovani* complex infections in canines, domestic animals, and rodents have all been documented in Ethiopia (Bashaye *et al*., 2009; Lemma *et al*., 2014).

Despite an upsurge in leishmaniaisi*s* research in Kenya, many of these studies have focused on VL since it poses a greater risk to life (Hailu *et al*., 2016), which favours CL's ongoing spread in the shadows. The absence of information on CL prevalence across the nation (Odiwuor *et al*., 2012; Gitari *et al*., 2018) makes this situation worse. Although it is known that some parasite species may create CL lesions that heal on their own (Bailey *et al*., 2017), this process can take months or years, leaving behind deep, disfiguring scars that are mostly seen on the face (Hailu *et al*., 2016; Bailey *et al*., 2017). As a result, this signal highlights the necessity for intensified measures to combat CL and to connect the ongoing psychological morbidity caused by its scars.

Kenya's rare sporadic CL reports reveal that *Leishmania* parasite species *L. tropica, L. major,* and *L. aethiopica* are the predominant *Leishmania* parasite species responsible for the disease (Njau, 2010; Odiwuor *et al*., 2012). *L. major* is found in lowland regions of Baringo and Kitui counties, while *L. tropica* was first discovered in central and Rift Valley regions (Tonui, 2006; Odiwuor *et al*., 2012). *L. aethiopica* is found in steep areas like Mount Elgon and the Rift Valley escarpments. The vectors of these

parasites are sandflies *P. duboscqi, P. guggisbergi, and P. pedifer. P. guggisbergi* and *P. pedifer* are mostly found in caves in Kenya, while *P. duboscqi* has a restricted distribution in Baringo County (Maroli *et al*., 2013; Mukhwana *et al*., 2018). Small rodents and rock hyraxes (*Procavia capensis*) have been shown to be the primary reservoir hosts of *L. major* and *L. tropica,* respectively (Anjili *et al*., 2011 & Odiwuor *et al*., 2012). Additionally, it has been suggested that the giant rat (*Cricetomys gambianus*), tree hyrax (*Dendrohyrax arboreus*), and rock hyrax (*Procavia capensis*) are the reservoirs of *L. aethiopica* in Kenya (Anjili *et al*., 2011).

Leishmania parasites are inoculated into hosts during blood feeding by infected sandflies (Lestinova *et al.,* 2017; de Avila *et al,* 2018) identifying sandfly species and their bloodmeal sources is crucial for incriminating vectors, understanding disease epidemiology, transmission dynamics, and identifying ecological reservoirs. Sandflies feed on various vertebrate hosts, potentially harboring different *Leishmania* parasite species.

Vector incrimination involves detecting *Leishmania* parasites in vectors. Microscopy sensitivity decreases with low parastemia, and most sandflies with parasite-positive *Leishmania* are often overlooked (Bennai *et al*., 2018). PCR-based molecular approaches have been used to identify and describe *Leishmania* parasites to the species level in reservoir hosts and vectors. Accurate identification and characterization of numerous *Leishmania* parasites are crucial for developing disease intervention and management plans (de Avila *et al.,* 2018).

CL-causing *L. aethiopica* is endemic to the slopes of Mt. Elgon in Western Kenya (Mukhwana *et al*., 2018). The rock hyrax *Procavia capensis* Thomas, the tree hyrax *Dendrohyrax arboreus* Smith, and the gigantic rat *Cricetomys gambianus* Waterhouse

have all been shown to have *Leishmania* parasites in this region (Mutinga *et al*., 1988; Anjili *et al*., 1994). The parasite, which is zoonotic in this region and readily feeds on these murines, is spread by *Phlebotomus (Larroussius) pedifer* Lewis, Mutinga, and Ashford. Up until recently, no other animal reservoir hosts have been discovered (Makwali, 2021). One isolate from a goat in Kenya that underwent isoenzyme characterisation revealed that *L. aethiopica* served as the infection's parasite (Mutinga, 1975).

According to Fourie (1972), the average mature male and female hyrax weighs 4.0 kg and 3.6 kg, respectively, and has a head-to-body length of 45–60 cm; these are some of the features used for morphological identification.

It might be challenging to differentiate the bush or yellow-spotted hyrax from the rock hyrax because of their similar appearances (Appendix IX). With a head-to-body length of 46.5 to 56 cm and a weight range of 2.3 to 3.6 kg, the bush hyrax is a little smaller species. A slender body, a thinner cranium, and lighter-colored pelage are among its primary morphological distinctions (Barry and Shoshani, 2000).

2.6 *Leishmania* parasites transmission from reservoirs to vectors

Most descriptions of *Leishmania spp*. reservoirs rely on natural infection findings, and lacking information on the host's epidemiological importance for parasite maintenance. Despite significant changes in concepts and methods, these descriptions remain unreliable (Ashford, 1997; Giantsis *et al*., 2021). The concept of reservoirs requires rigorous evaluation of field and laboratory investigations to understand the function of mammalian host species in *Leishmania* transmission. Despite advances in understanding, there is still a lack of information on *Leishmania's* spread between hosts and vectors.

The female sandflies need blood in order to provide the protein needed for the development of their eggs (Bourdeau *et al*., 2020). *Leishmania* is spread when a sandfly consumes blood from an infected host during one blood meal and then passes the parasite to another host during a later blood meal. The blood sources of arthropods that feed on hosts' blood have been identified using a variety of serological techniques (Schoone *et al.,* 2001). Precipitin test and hemagglutination inhibition tests, which were first used in the bloodmeal study of blood feeding sandflies (Svobodova *et al.,* 2009), were subsequently superseded by enzyme linked immunosorbent assay (ELISA) as described by Svobodova *et al*., (2003).

Precipitin ring test and counter-current immune-electrophoresis were used in Maharashtra, India, to identify *P. argentipes* bloodmeals (Dhanda and Gill, 1982). Bloodmeal preference was determined in North Bihar using a modified ouchterlony gel diffusion approach (Schoone *et al.,* 2001), while bloodmeals of wild captured flies were found in Pondicherry using the agarose gel diffusion method (Srinivasan and Panicker, 1992). By analysing their bloodmeals using the gel diffusion technique in West Bengal, *P. argentipes* host preferences were examined (Kushwaha *et al.,* 2018). Multiple blood meals were more frequently discovered in *P. argentipes* than in *P. papatasi* Scopoli, according to Palit *et al*. (2005) research. The requirement for relatively fresh blood and the absence of species-specific antibodies against each prospective animal host are limitations of serological techniques (Kato *et al*., 2005). The invention of polymerase chain reaction (PCR) mainly addressed these flaws. Bloodmeal identification has been made simpler and the test's sensitivity has been markedly increased using PCR-based molecular techniques (Mukabana *et al*., 2002). In order to identify the source of sandfly bloodmeal, the current investigation employed ELISA.

Evidently, sandfly vectors exhibit host preferences during feeding and are crucial players in the dynamics of vector-borne diseases. In a study including people, donkeys, cows, sheep, and goats in north Ethiopia, more *P. orientalis* females were drawn to and went into engorgement on the donkey and cow than on the other hosts (Gebresilassie *et al*., 2015). *P. orientalis* is primarily fed by bovine blood, with 91.6% of engorged females positive for bovine blood in northwest Ethiopia. Only 8% fed on cattle, but 28% were human and 36% mixed. Dog-baited traps in eastern Sudan attract the highest number of female sandflies, followed by Egyptian mongoose and common genet.The study highlights the importance of these animals as bloodmeal sources for *P. orientalis* (Gebresilassie *et al*. 2015)

According to Gebresilassie *et al*. (2015), *P. orientalis* females were more drawn to ground squirrels (*Xerus rutilus*), hares (*Lepus sp*.), gerbils (*Tatera robusta*), and spiny rats (*Acomys cahirinus*) than other smaller wild animals. Strong evidence that the species is largely zoophilic with opportunistic feeding preferences dependent on the accessibility of bloodmeal host was provided by the tendency of female *P. orientalis* to engorge in large numbers on specific kinds of domestic as well as wild animals. The VL epidemiology considers dogs to be the most significant species among domesticated animals. In Latin America and the Mediterranean area, they are essential reservoir hosts in the cycle of transmission of the closely related *L. infantum* (Ready, 2014).

Dogs in VL foci in eastern Sudan and Ethiopia have positive *Leishmania* DNA or seropositivity. A longitudinal study reported 72.5%, 74.3%, and 42.9% prevalence rates in 1998, 1999, and 2000, respectively. However, only 6.9% of dogs had anti-*Leishmania* antibodies in a cross-sectional study in eastern Sudan in 2002.

Hassan *et al.* (2009) found differences in canine seropositivity between their study and that of Elinaiem *et al.* (1998), possibly due to sampling timing, low dog density in eastern Sudan, and differences in dog ownership among tribes. In 2005, 5% of dog cases caused by the *L. donovani* complex were recorded in the highland region of northwest Ethiopia. In 2006, 2.8% and 3.8% of dogs tested positive, while 39.1% of canines in 2008 and 2009 studies tested positive for *Leishmania.*

The temporal lapse between human and canine outbreaks may be the cause of the disparity in human and canine VL risk. Dog ownership significantly increased the risk of human VL in eastern Sudan, lowlands, highlands, and northwest Ethiopia. In eastern Sudan, 68.7% of seropositive donkeys, 21.4% of cows, and 8.5% of goats were found. In northwest Ethiopia, high seropositivity for *L. donovani* was found in cattle (41.9%) and donkeys (33.3%), while low seropositivity was observed in goats (10%) and sheep (4.8%) (Asford 2000; Bashaye *et al*., 2009)

In northwest Ethiopia, having sheep or goats as pets was linked to a higher risk of human VL (Quinnell and Courtenay, 2009). On the other hand, domestic animals present in the garden at night were deemed protective in eastern Sudan (Hassan *et al*., 2009). According to Lemma *et al*. (2009), using pesticide to cattle enhanced the incidence of human VL in eastern Uganda and western Kenya.Consequently, the risk of contracting an infection is lower when sleeping in an animal-populated area. The primary VL vector in these areas, *P. martini*, exhibits zoophilic behaviours, which may help to explain this (Baneth *et al*., 2008). Application of insecticides to animals reveals a change in sandfly host seeking behaviour (Lemma *et al*., 2009).

In the 1960s, researchers discovered *Leishmania* infection in southern Sudan among three rodent species and two carnivore species. The infection was caused by *L.*

donovani, making it less likely to contract when sleeping in animal-populated areas. The primary vector, *P. martini,* exhibits zoophilic behaviors (Mala *et al.,* 2018; Baneth *et al*., 2008). Application of insecticides to animals reveals a change in sandfly host seeking behaviour (WHO 2010; Quinnell and Courtenay, 2009).

Three rodent species (rat, *Rattus rattus,* spiny mouse, A*comys cahirinus*, Nile grass rat; *Arvicanthis niloticus luctuosus*) and two carnivore species (Senegal geneta, *Genetta genetta senegalensis* and Sudanese serval, and *Felis serval phillipsi*) were found to have *Leishmania* infection in southern Sudan in the 1960s. The infection, according to the scientists, was brought on by *L. donovani*.

Dogs may serve as *L. donovani* reservoir due to high seroprevalence (Bashaye *et al*., 2009; Hassan *et al*., 2009; Quinnell and Courtenay, 2009), long-lasting and seemingly asymptomatic infection course. Dogs are a major local reservoir for *L. donovani*, as shown by the genetic similarity between canine and human strains (Baleela *et al*., 2014). The role of dogs as VL reservoir hosts is still debatable, however, as evidenced by the direct agglutination test's (DAT) inability to isolate *L. donovani* from the positive dog's skin, lymph nodes, liver, spleen, or bone marrow (Gramicia and Gradoni 2005). Due to the paucity of research tracking the progression of the illness in affected animals, it is unclear what role other domestic animals have in VL transmission. Apart from pyrexia, sheep experimentally infected with *L. donovani* promastigotes exhibited no symptoms of VL. Throughout the 8-month sample period, no parasites were found in the visceral organs (Anjili *et al*., 1998). Donkeys recovered from experimental *L. chagasi* infection, no *L. longipalpis* parasite infection was observed after a blood meal on affected animals (Mukhapadhyay and Chakravarty

1981). According to the findings from Nepal, *L. donovani* infection in goats might last for at least a few months (Bhattarai *et al*., 2009).

Blood meal analysis indicates domestic animals likely transmit VL through female sandflies' blood, indicating their involvement in the transmission (Gebre-Michael *et al*., 2010; Gebresilassie *et al*., 2015a). The Egyptian mongoose was suggested as a potential reservoir host of *L. donovani* in Eastern Sudan among wild animals (Elnaiem *et al.*, 1998). According to Elnaiem *et al***.** (1998), Egyptian Mangoose is a species that is widespread and gregarious, and according to Hassan *et al*. (2009), *P. orientalis* females find it appealing. However, according to Elnaiem *et al*. (1998), no skin samples produced a *Leishmania*-specific PCR results. There is little information available on the role that domestic and wild animals play in CL transmission. To identify the vector(s), determine the animal reservoir(s), or determine the existence or absence of zoonosis in research regions, in-depth investigations of the vector(s), reservoir host(s), and transmission dynamics are extremely important. To develop effective control measures and intervention techniques targeting the vectors and reservoir hosts, additional information is essential.

2.7 Morphological and molecular identification of vectors and parasites

The morphology of male and female sandflies is very different from one another. External characteristics can be used to identify the sandfly. The terminal section of the male sandfly's abdomen often has clasping structures made up of gonocoxites, parameres, cerci, and aedeagus. The females, on the other hand, lack these clasping structures; their abdomen end is blunt, and the only appendage present is the cercus. Both teeth and a pigment patch are absent in the *Phlebotomus spp.* (Lewis, 1978). However, the cibarium of some *Phlebotomus* species may only consist of a few

sparsely spaced tiny spicules (Lewis, 1978; Dantas-Torres 2014). Therefore, it is crucial to determine the genus based on the combination of all the aforementioned physical characteristics. As a result, the position of the hairs and the shape of the hair sockets in the posterior of abdominal segments 2 to 6 are mentioned as diagnostic features for identification to the genus level differentiation (Lewis, 1978; Dantas-Torres *et al*., 2014). However, given that these specimens required a cleansing procedure before mounting, their observations revealed that they were not the typical traits that, in actuality, distinguish the two taxa. The wing and cibarium characteristics, on the other hand, which were discovered to contain hair sockets comparable in form to those in *Phlebotomus spp*., verified that the specimens belonged to the genus *Sergentomyia*. The arrangement of the spines on the gonostyle served as the primary basis for dividing male sandflies into genera (Dantas-Torres *et al*., 2014)

When the spines are subterminal in *Sergentomyia*, they are often found in pairs. The gonostyle of *Phlebotomus spp.,* on the other hand, has spines that are not grouped in pairs (Dantas-Torres *et al*., 2014). The identification of sandflies is still accomplished using morphology-based characteristics of the pharynx, spermathecae, cibarium, and in the case of adult males, terminalia (Dantas-Torres *et al*., 2014), of the adult males.

A Kenyan study by Owino *et al.* (2021) identified mounted sandfly species morphologically using male external genitalia and female pharynx, antennae, and spermatheca. Kumar *et al.* (2012) amplified the sandfly c oxidase subunit I (CO1) cytochrome from mitochondrial gene to confirm morphological species identifications. Hanafi (2005) asserts that physical characteristics, particularly internal aspects like the spermatheca, cibarium, and throat in females and genitalia in males,

may be used to distinguish between different sandfly species. For identification under a microscope, the head and terminal segments of the sandfly can be removed, put in a drop of clearing medium, and then mounted on a slide with a drop of Puris media (Lewis, 1982).

Examining numerous samples for information, such as the low infection rate of sandflies with *Leishmania* (0.01-1%) in endemic areas, is crucial (Hashiguchi and Gomez, 1991; Kato *et al*., 2007), it appears that this morphological technique is laborious and time-consuming.

The use of many genetic markers, such as the ITS2 and cytb-nd1 regions, for the molecular identification of phlebotomine sandflies has been demonstrated in studies by Dantas-Torres *et al*., (2014), Dantas-Torres *et al*., (2010), Latrofa *et al*., (2012). Similarly, protein profiling using MALDI-TOF (MS, a matrix-assisted laser desorption/ionization time of flight mass spectrometry) has been suggested as a viable method for identifying phlebotomine sandflies (Dvorak *et al*., 2014). However, microscopic inspection and the important morphological characteristics, such as the pharynx, spermathecae, and cibarium of female insects as well as male terminalia, are still the main methods for identifying these insects (Abonnenc, 1972; Rahola *et al*., 2013). Therefore, morphological keys for phlebotomine sandfly identification are essential for research on these insects.

CHAPTER THREE

METHODOLOGY

3.1 Study Area

The research study took place within the Chemai and Chepkutuny cave sites, situated in Mt. Elgon Sub-County, Bungoma County, and Western Kenya. These sites specifically encompassed Chepkutuny A cave (N 00049 881/E0340 42 994/ elevation 1950 M), Chepkutuny B cave (N 00049 877/E 034043 881/ elevation 1920M), Chepkutuny C cave (N 00045 884/E034044 998/ elevation 1900M), Chemai A (N00049’ 881N/034043 233’E elevation 1827 M), and Chemai B (N00°50 546’E 034043 233/ elevation 1801M). The selection of this area was influenced by a rise in suspected cases of cutaneous leishmaniaisis (CL) reported by local residents seeking medical attention at Mt. Elgon Sub-County Hospital in Kapsokwony and neighboring medical facilities. Within these varied environments, including fault escarpment ranges, rock crevices, and caves, potential habitats for sandflies, large rats, and rock hyraxes (*Procavia capensis*) were identified (Mukhwana *et al*., 2018; Anjili *et al*., 2011). Moreover, evidence of human activities in and around the caves was observed, such as children playing inside the caves, footprints of humans and animals, including livestock, social indicators like used condoms and discarded bottles of water and alcohol, as well as hyrax defecation. These observations collectively indicated a close interaction among humans, livestock, wild animals, and sandflies within the cave environments.

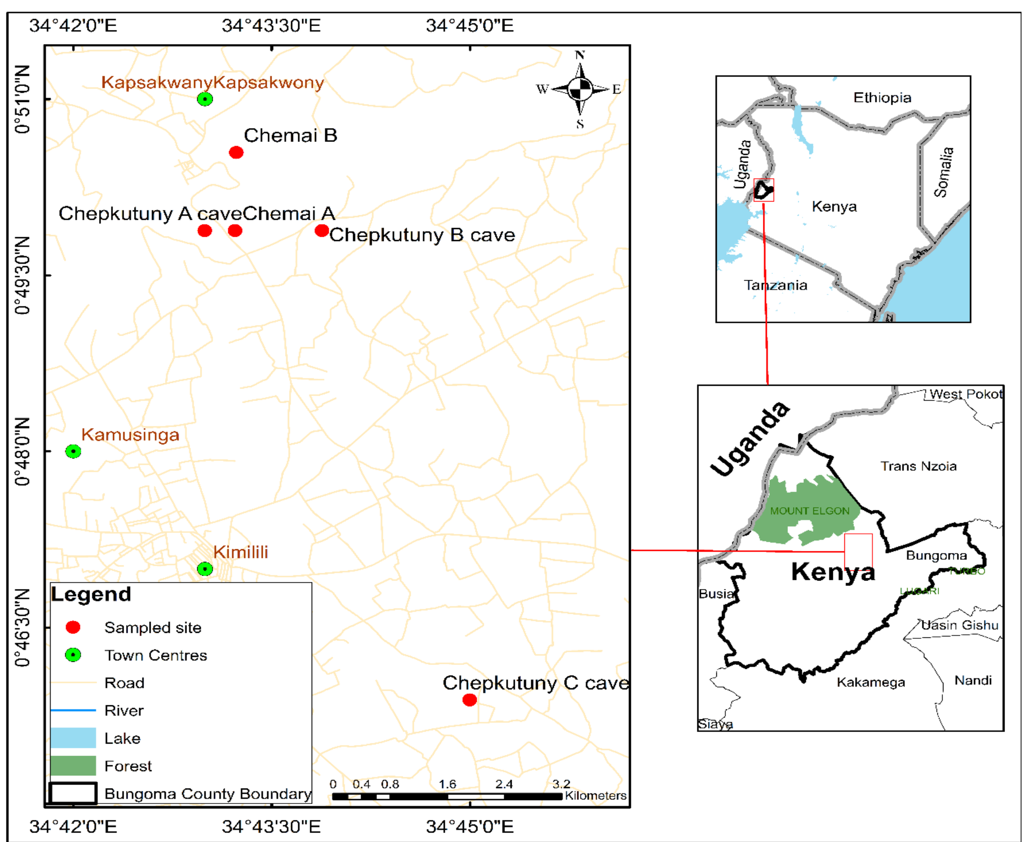

Figure 3.1: Map of Mount Elgon subcounty in Bungoma County showing the study area

3.2 Study design

An observational study design was adopted to investigate natural infections of *Leishmania* in bats, other suspected reservoir hosts, and in sandflies. Bats and sandflies were collected from the Chemai and Chepkutuny cave sites located in Mount Elgon, Bungoma County, from February 2017 to January 2019. The study was both cross-sectional and longitudinal, with bats, hyraxes, and sandflies of both sexes being sampled for one week each month. For sandfly collection, three CDC traps were used per site over a 24-month period. For bats, a single mist net was set at the entrance of each cave, while one hyrax trap cage was set up per site.

The selected caves, which were of approximately the same size, were situated at different sites along the escarpments, allowing for the collection of high-quality data and research materials due to their accessibility. The selection of these caves was also based on information obtained from interviews with residents of nearby homesteads, which indicated the presence of many bats, rodents, and hyraxes in the caves. Permission for the collection and use of research animals in Mount Elgon was granted by KWS under permit number WRTI-0089-08-21 (Appendix XIII).

3.3 Sample size determinations for suspected reservoir hosts

Sample size for each suspected reservoir host species was obtained using Fisher's Formula (Rosner. 1995).

For example, the sample size for the bats was determined as follows;

$$n=\frac{Z^2P(1.0-P)}{d^2}$$

Z = 1.96

n= sample size (where population >10000)

Z= normal deviation at the desired confidence .In this case it will be taken at 95%, Z value at 95% is 1.96

P= Proportion of the population with the desired characteristic

d^2 = Degree of precission; will be taken to be 10% (where the proportion of the population with the characteristic is not known, then 50% rule will be is used). For the current study;

d = was taken from any value between 3 and 10

P = Value was based on prevalence rate of a study in Ethiopia (Kassahun *et al.,* 2015) that was 4.9% *Leishmania* kDNA detection in bats

The expected sample size for bats that was collected during the study:

Was therefore $= \frac{1.96^2 \times 0.049 \times 0.951}{0.03^2}$

$= 198.905 \approx 199$

The expected sample size for bats was achieved since there were high bats populations in the caves of Mt. Elgon. The same formula was used to calculate the sample size for the other reservoir hosts.

3.4 Trapping of sandflies

Sandflies were collected in the investigated caves using ten micro light traps (Model 512; John W. Hock Company, Gainesville, FL) from the Centres for Disease Control and Prevention (CDC). Each cave had two traps, one placed at the entry and the other one placed inside. Sandflies are known to be low-flying, largely hopping insects, hence the traps were placed between 0.5 and 1.5 metres above the ground (at each site) (Appendix IV). To cover the rainy and dry season times, the traps were laid on eight consecutive nights every month for 24 months. The collected sand flies were numbered, labeled, and dissected for examination on the head and the last three abdominal segments for detection and identification of *Leishmania* parasite infection as well as for morphological taxonomic identification of the sandflies.

3.5 Identification of sandfly species

According to existing taxonomic keys/guides (Abonnence *et al*., 1965; Lewis, 1982; Berdjane-Brouk, 2012; Gebresilassie *et al*., 2015), species identification of sandflies by morphological observations was based on the external genitalia of males and characteristics of the throat, antennae, male aedeagus, and number of coite hairs and

spermatheca of females. Morphological characters were measured using the Quick PHOTO MICRO 3.0 software (Promicra, Prague, Czech Republic).The females have usually been considered difficult or impossible to distinguish. A comparison of the base of the spermathecal ducts is made and it is shown that all four can be identified by this character alone.

The study involved dividing sandflies into male and female groups using an Olympus SZ60 stereomicroscope. A random sample of 200 female sandflies was used to identify *Leishmania* parasites in the research area. 100 male sandflies were dissected for taxonomy, and each dissected sandfly was processed in PBS. The thorax was removed and the head and thorax junction were placed on a glass slide. The number of *Leishmania* promastigotes observed in the midguts of dissected sandflies was recorded. The head and hind segment of the abdomen were mounted for morphological identification of the sandfly using taxonomic keys from Lewis (Lewis, 1978; Lewis, 1982), Abonnec (Abonnence *et al*., 1965), Berdjane-Brouk (2012), and Gebresilassie *et al*. (2015).

3.6 Identification of suspected reservoir hosts for *Leishmania* parasites

3.6.1 Capture of bats and their species identification

The potential bat reservoir hosts were collected;using one mist net for each cave, bats were caught at the cave entrance between the hours of 18:00 and 6:00 the following morning to make up one trapping night. This was done for eight consecutive nights each month for a total of 24 months. Just before dusk, a mist net was erected; the nets were unwound, elevated on the two poles, and positioned at the cave entrance. Bats have trouble hearing the nets through their ultrasonic cries and become entangled and caught in the net as a result. Early in the morning, bats were taken out of the net,

numbered, and given intraperitoneal injections of ketamine and xylazine to put them to sleep. All captured bats were photographed, weighed, and the external morphological characteristics, such as sex, size, hair colour, length of the forearm, ear shape, and kind of membrane pertaining to tail form, were noted (Appendix XI). The keys provided by Patterson and Webala (2012) and experts from the National Museum of Kenya were used to identify each specific bat.

Some blood was drawn from the bat for slide film/smear preparation before the bats were sacrificed, dissected and their liver and spleens removed for their impression smears that were prepared and preserved in ethanol for subsequent culture, microscopy and analysis for *Leishmania* amastigotes (Appendix V)

Male and female bat counts, as well as a variety of morphometric measures, were noted in the field when specimens were collected from Mt. Elgon. Based on the wear and tear on their small teeth, adult bats' morphological traits were measured, and these included forearm length, overall length, tail length, right hind foot length, ear-length, Tragus length, and bat weight.

In order to document the species that were taken to facilitate subsequent identification at the National Museums of Kenya, a small number of individuals from each category of species that could not be adequately identified in the field were collected and kept as museum voucher specimens.

3.6.2 Capture of rock hyraxes and their species identification

Five cages were built as described by Sale, 1970; Millar, 1971 & Fourie, 1972 to successfully catch hyraxes. Each cage measured 30× 30× 75 cm and was made from with wire mesh walls and a single trapdoor controlled by a treadle. Oranges, maize ear tips, potatoes, and bananas were used as bait in the cages. Since ambient

temperature influences the body temperature of the rock hyrax and hence impacts its mobility (Fourie 1972), cages were placed in areas that had enough shade protection throughout the day. The trapped rock hyraxes were held by a restraint rope before being removed from the traps and each rock hyrax was immediately covered with a blanket once it was taken out of its cage to reduce stress during handling and further handling of individuals when being investigated was done as fast as possible (Barry and Shoshani 2000). The hyraxes were euthanized using barbiturates (Amobarbital, 65-200 mg). After administering barbiturates to kill the hyraxes, 2 ml of peripheral blood was drawn and put into a specimen container. The liver and spleen samples were then taken, labeled packaged and preserved using 40% formalin until use. A total of 25 hyraxes were captured in the five caves and identified to species level. All the collected hyraxes and bats were categorized based on morphological features then representative samples of each category taken to the National Museum of Kenya (NMK) for assistance in their detailed taxonomical identification.

3.7 Relative abundances of the sandflies in the study sites

All collected sandflies were classified as either male or female, and the resulting numbers of each gender were identified and documented per species, categorized according to their morphological characteristics. Abundance was calculated as the average number of sandflies collected per trapping night per cave.

3.8 Bat, hyraxes and sandfly infection status with *Leishmania* parasites

3.8.1 Microscopic analysis

Liver and spleen tissues of bats and hyraxes were crushed to prepare impression smears. These smears were fixed on slides using methanol, stained with Giemsa, and examined under a microscope for *Leishmania* (amastigote form). Typically,

Leishmania amastigotes are located within macrophages, but some may be seen extracellularly, having been released from ruptured macrophage cells during smear preparation (Cheesbrough, 2005b).

From the 199 collected bats, twenty randomly selected slides of liver and spleen impression smears from each bat species in the study area were examined microscopically for the presence of *Leishmania* amastigotes (Appendix VI). The mean number of amastigotes from 100 microscopy fields was used to calculate the quantity of *Leishmania* parasites in Giemsa-stained tissue smears (Kobet, 2011; Kobet *et al*., 2010).

3.8.2 Parasite Culturing

The *Leishmania* parasites were cultured in order to provide larger amounts of parasites materials for analysis. To determine the circulating *Leishmania* parasites in Mt. Elgon, a random sample of two hundred blood fed sandflies were pooled into 18 pools (12 sandflies: n=5; 11 sandflies: n=10 and 10 sandflies n=3) these were analyzed together with DNA from individual parasites.

The two hundred engorged sandflies whose guts were suspected to have promastigotes were triturated into a drop of saline and the mixture aspirated into a syringe and inoculated into Novy, Mac'Neal and Nicolle's (NNN) medium as described by Taylor and Baker (1978). The culture media were kept for five days at room temperature and then smears were made to check for any *Leishmania* parasites. The slides were stained with Giemsa, and then observed under the microscope at a magnification of $\times 100$ using oil immersion.

The parasites were isolated using blood-enriched media. A cellular deposit was formed after centrifuging the aseptically collected blood (1 to 2 ml), which was then

diluted with 10 ml of citrated saline and added to the culture medium. The use of good sterile techniques, the addition of antibodies penicillin (200 IU/ml), streptomycin (200 g/ml) and gentamycin (100 g/ml) to the medium and 5-fluorocytosine (500 g/ml) as an antimycotic agent (Schur and Jacobson, 2004), helped to avoid contamination of the culture media by bacteria, yeast species, or other fungi.

3.8.3 Establishing the source of bloodmeal in engorged female sandflies

Out of four hundred blood-fed female sandflies that were collected during the study period, two hundred (200) were randomly selected from the study population for bloodmeal source determination. The samples were analyzed by ELISA method as described by Ngumbi, (1992). Test for sandfly bloodmeal sources was done for bat, hyrax, cat, and human because they were identified at the proximity of the caves.(Appendix I, II &III)

3.9 Molecular identification of sandfly species

3.9.1 DNA extraction from sandflies

In order to avoid cross-contamination among the specimen homogenizations, the remaining components of each dissected sandfly specimen were homogenised in 180 ul of buffer (ATLQIAGEN, Hannover, Germany) taking precaution not to avoid contamination between the specimens. Following the manufacturer's instructions, genomic DNA was extracted from each homogenate using the DNeasy Blood and Tissue Kit (QIAGEN, Hannover, Germany). For subsequent analysis, the isolated DNA was stored in a freezer at -20 degrees Celsius and utilized as molecular assay templates.

3.9.2 Sandfly Identification using mitochondrial Cytochrome c Oxidase Subunit I (COI)

To confirm the morphological identifications of the sandfly species, amplification of sandfly mitochondrial cytochrome c oxidase subunit I (COI) was performed following the procedure outlined by Kumar *et al*. (2012). Polymerase Chain Reaction (PCR) purification kit was used based on the manufacturer's recommendations and the samples were submitted to International Centre of Insect Physiology and Ecology (ICIPE) for that was carried out using the appropriate forward and reverse primers: forward LCO-1490 (5'-GTTCAACAAATCATAAAGTATTGG-3') and reverse, HCO 2198 (5'-TTAACTTCAGGGTGACCAAAAAATCA-3') The primers were acquired from BioSource in the United Kingdom. Following the manufacturer's instructions, the resulting 700 bp COI amplicons were purified using a QIAGEN PCR purification kit. Subsequently, they were sent to ICIPE for further submission to the University of Cape Town in South Africa for sequencing, utilizing the forward primer. Consensus sequences for each sample were extracted by editing the returning COI chromatograms in Unipro UGENE (v. 1.3). A BLAST search for sequence similarity and multiple sequence alignment in MEGA 7 using the Clustal W tool was then performed, following the methods described by Mount (2008) and Lo (2013).

3.10 Molecular Detection and Identification of *Leishmania* Parasites

3.10.1 ITS1-PCR-RFLP Assay for *Leishmania* Detection and Identification

Initially, all DNA pools underwent concentration measurement and validation with reference primers. Subsequently, successful parasite cultures that were established were tested by PCR amplification of approximately 320 bp of the *Leishmania* internal transcribed spacer 1 (*ITS1*) using LITSR (5'-CTTGATCATTTTCCGATG-3') and L5.8S (5'-TGTTACCACTTATCGCACTT-3') primers, following the protocol

outlined by Aransay *et al*. (2000). The 20 µl reaction mixture contained 1x Dream *Taq* buffer with 2 mM $MgCl_2$ (Thermo Scientific,USA), 0.25 mM dNTPs mix, 500 nM for the two primers, 0.125 U of Dream *Taq* DNA polymerase (Thermo Scientific, USA), 3.5-8.2 ng of DNA template and nuclease free water (Sigma, St. Louis, USA). A SimpliAmpThermal Cycler (Applied Biosystems, Loughborough, UK) was used to conduct each PCR experiment. Denaturation at 98 degrees Celsius for two minutes was followed by 35 cycles of denaturation at 95 degrees Celsius for twenty seconds, annealing at 53 degrees Celsius for thirty seconds, and lastly extension at 72 degrees Celsius for thirty seconds. A last extension at 72 degrees Celsius for five minutes came next.

The PCR results were visualized using a Gel Doc TM EZ imager. The infecting *Leishmania* species was identified using HaeIII-RFLP analysis on positive pool and parasite cultures with anticipated band size (Schonian *et al*., 2003). The HaeIII digestions were conducted in a reaction volume of 20µI comprising 10 units of HaeIII enzyme, 1x Fast Digest buffer, and 17µ l of PCR products from Thermo Scientific in the United States. The reaction conditions included an initial incubation at 37°C for two hours, followed by an end-of-reaction incubation at 80°C for 20 minutes. Subsequently, the ITS1-PCR products of the positive samples were further purified using the QIAquick PCR purification kit (Qiagen, CA, USA). These purified products were then submitted for sequencing with both the forward and reverse primers to determine the *Leishmania* species.

3.10.2 Nested kDNA - PCR Assay for *Leishmania* Parasite Detection and Identification

To enhance the sensitivity of *Leishmania* parasite screening, the methods outlined by Eroglu *et al*. (2011) and Rass *et al*. (2012) were employed. Two sets of primers targeting the DNA of the *Leishmania* kinetoplast were used to amplify the pooled sandfly DNA. This region was selected as a target due to the high copy numbers present there (Kocher *et al*., 2018). For the first PCR reaction, the primers CSB2XF and CSB1XR (5'-CCAGTAGCAGAAACTCCGTTCA-3') and 13Z (5'-ATTGGGTTGGTAAAATAG-3') and LiR (5'-TGGCAGAACGCCCCT-3') were used; the second reaction, as previously described by Noyes *et al*., (1998) and Aflatoonia *et al*., (2014).

3.10.3 q PCR-HRM Assay for *Leishmania* parasite Detection and Identification

All samples that tested negative for *Leishmania* parasites by ITS1-PCR and nested kDNA-PCR were subsequently screened using ITS1-qPCR-HRM. This technique, known for its high sensitivity and specificity in detecting *Leishmania* species, was chosen based on recommendations by Talmi-Frank *et al*. (2010) and Almeida *et al*. (2014). To enhance the resolution of identifying the *Leishmania* species, ITS1 primers designed by BioSource UK were used to generate amplification products of variable sizes between the species.

The primers were ITSIF (5’-CTCGTTATGTGAGCCGTTATCC-3’) and ITS1R (5’GTCTTTCCCACATACACAGC-3’)

The reactions were carried out using the Agilent Technologies Stratagene Mx3005P real-time PCR thermocycler with High Resolution Melting (HRM). The final reaction volume contained 1x Luna universal qPCR Sybr Green-based master mix, 500nM

primers, 1 μl template, and nuclease-free water. Denaturation was performed at 95°C for one minute, followed by 40 cycles of annealing, denaturation, and extension at 60°C for 20 seconds each. HRM was conducted by denaturing the qPCR products at 95°C for one minute, cooling them at 50°C for 30 seconds and then gradually increasing the temperature by 0.1°C increments every 5 seconds while measuring the fluorescence change.

3.10.4 Sandfly COI sequences and phylogenetic analysis

To obtain consensus sequences for each sample, the chromatograms of all the sandfly COI sequences were edited using Unipro UGENE (v1.3) after trimming. The resulting consensus sequences were aligned using the MUSCLE program (Edgar, 2004) with homologous sequences identified through sequence similarity searches in GenBank using the Basic Local Alignment Search Tool (BLAST), as described previously by Azimi *et al*. (2010). Following the methodology outlined by Edgar *et al*. (2004), the General-Time-Reversible (GTR) sequence evolution model was applied to construct the maximum-likelihood phylogeny of the sandfly COI sequence alignments. Tree topologies were computed with over 1000 bootstrap replicates.

3.11 Data Management and Analyses

The Statistical Package for Social Sciences (SPSS) version 23.0 was utilized to assess the consistency of all collected sandfly information, including trapping locations, GPS coordinates, collection dates, and trap numbers. Descriptive statistics were employed to calculate and present the frequency and percentage distribution for each sandfly species per location. Quantitative counts of each species per trap, per trapping night, and per location were used to determine species abundance. Species diversity was calculated using the Shannon Weiner diversity index (H'). The sex ratio for each

species was calculated as (number of males / number of females) x 100. Differences in species infection prevalence were analyzed using the Chi-square (χ^2) test. Sex ratio differences were also assessed using the chi-square test. Differences in sandfly distribution within the caves were analyzed using One-Way ANOVA. Significance was considered at $P < 0.05$.

CHAPTER FOUR

RESULTS

4.1 Determination of distribution of sandfly vector species in Mt. Elgon cave habitats

4.1.1 Status of sandfly vector species in sampled Mt. Elgon cave sites

A total of 5688 sandflies comprising of 4403 (77.4%) female and 1285 (22.6%) males were collected during the twenty-four (24) months sampling period from the five study caves of Chemai and Chepkutuny. The collected sandflies that were identified belonged to four species using morphological keys. Morphological investigation identified four sandfly species that were *P. pedifer, P. longipes, and P.elgonensis* and *S. schwetzi*. (Appendix VII) Males were identified by the presence of prominent genital terminalia called claspers, while females had a pair of anal recti. The main feature that differentiated the sundlfy species was he number of inner surface coite hairs; 39(30—47), n=42 for *P. pedifer*, 31(30-37), n=14 for *p. longipes,* 43(39-48, n=20 for *P. elgonensis* and 45(44-49) for *S. schwetzi* (Appendix VIII)

The sandfly species identified namely; *Phlebotomus pedifer, P. elgonensis, P. longipes* and *Sergentomyia schwetzi. P. pedifer* was more predominated in all the five sites (Figure 4.1). For Chemai A site, *Sergentomyia schwetzi* was significantly ($F_{0.05\ (3,\ 8)} = 102.00$, $p<0.0001$) more abundant than *P. elgonensis* and *P. pedifer*. For Chemai B site, *P. pedifer* and *Sergentomyia schwetzi* were significantly ($F_{0.05\ (3,\ 8)} = 7.23$, $p=0.0115$) more abundant than *P. elgonensis* and *P. longipes*. In Chepkutuny A site, *P. elgonensis* and *Sergentomyia schwetzi* were significantly ($F_{0.05\ (3,\ 8)} = 602.75$, $p<0.0001$) more abundant than *P. elgonensis* and *P. longipes*. In Chepkutuny B site, *P. pedifer* and *P. longipes* were more abundant while *P.*

elgonensis less abundant with a significant difference ($F_{0.05\ (3,\ 8)}$ =19.00, p=0.0005). In Chepkutuny C, *P. longipes* and *Sergentomyia schwetzi* were abundant than *P. elgonensis* and *P. longipes* with a significant difference ($F_{0.05\ (3,\ 8)}$ =242.75, p<0.0001) as portrayed in Figure 4.1.

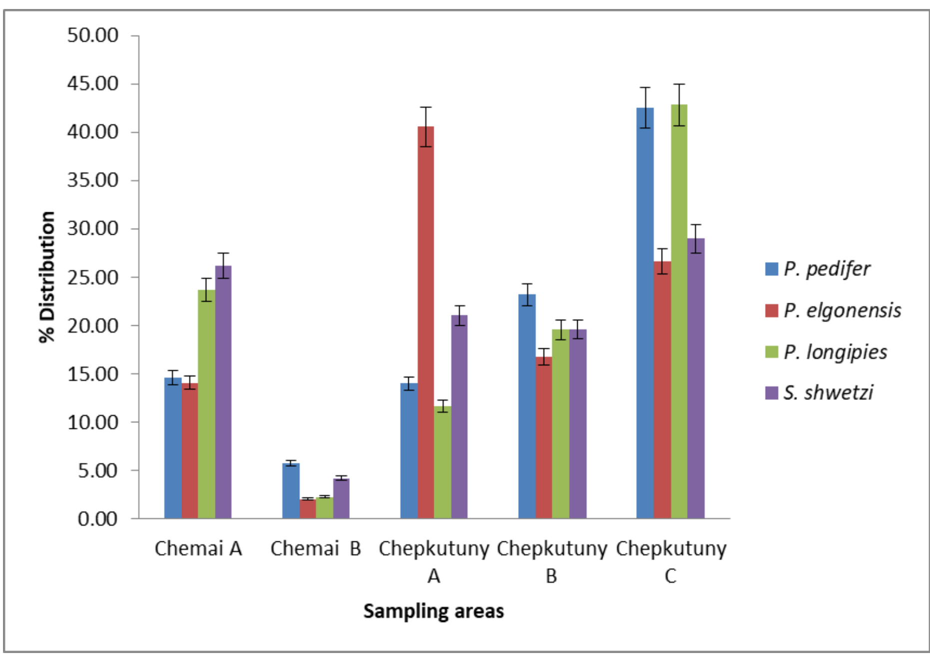

Figure 4.1: Distribution (%±5% error) of sandfly vector species in sampled in five Mt. Elgon caves

4.1.2: Seasonal distribution of sandfly vector species in Mt. Elgon cave sites

The number of sandflies captured during the rainy season (48.03%), was higher than those (51.97%) captured during the dry season. However, there was no statistically significant difference between the collections in rainy and dry seasons (χ^2 = 0.16, d.f. =1, p= 0.6892)

The difference between the collected numbers of male (χ^2 = 3.24, d.f. =1, p= 0.0719) and female (χ^2 = 1.44, d.f. =1, p= 0.2301) sandfly populations was not influenced by season as illustrated in Table 4.1

Table 4.1: Seasonal distribution of sandfly vector species in Mt. Elgon cave sites by sex category

	Dry season	Wet season	Chi square (χ^2)
Male	771(60.0)	514(40.0)	χ^2 = 3.24, d.f. =1, p= 0.0719
Female	2553(57.9)	1850(42.1)	χ^2 = 1.44, d.f. =1, p= 0.2301

Numbers in parenthesis are in percentages (%)

4.1.3 Sex ratios (Male: Female) of sandfly species collected from five caves in Mt. Elgon

In all sampled sites, *P. pedifer* females constituted the largest mean proportion (78.25%) while male constituted only 22.75% with a significant difference (χ^2 = 31.36, d.f.=1, p<0.0001) between the female and male collections. Proportion of male to female *P. pedifer* differed significantly between sites as follows; Chemai A (χ^2 = 14.44, d.f.=1, p= 0.0001), Chemai B, (χ^2 = 16.0 with 1 d.f. P = 0.0001), Chepkutuny A, (χ^2 = 57.76, d.f. =1, p<0.0001) and Chepkutuny B (χ^2 = 51.84, d.f. =1, p<0.0001) as shown in Table 4.2.

For the *P. elgonensis,* the proportion of male to female per site differed significantly in Chemai B (χ^2 = 36.0, d.f.=1, p<0.0001) with male taking the larger proportion of 80.00%, Chepkutuny A (χ^2 = 51.84, d.f.=1, p<0.0001) with female taking the larger proportion of 86.00%, Chepkutuny B (χ^2 = 43.56, d.f.=1, p<0.0001) with female taking the larger proportion of 83.00% and Chepkutuny C (χ^2 = 16.0, d.f.=1, p= 0.0001) with male taking the larger proportion of 70.00% as illustrated in Table 4.2.

For *P. longpies* the proportion of male to female per site differed significantly in Chemai B (χ^2 = 57.76, d.f.=1, p<0.0001) with female taking the larger proportion of 88.00%, Chepkutuny A (χ^2 = 49.0, d.f.=1, p<0.0001) with female taking the larger

proportion of 85.00%, Chepkutuny B (χ^2 = 10.24, d.f.=1, p= 0.0014) with female taking the larger proportion of 66.00% and Chepkutuny C (χ^2 = 5.76, d.f.=1, p= 0.0164) with male taking the larger proportion of 62.00% as illustrated in Table 4.2.

For *S. schwetzi* the proportion of male to female per site differed significantly in Chemai A (χ^2 = 5.76, d.f.=1, p= 0.0164) with female taking the larger proportion, Chemai B (χ^2 = 4.0, d.f.=1, p= 0.0455) with females taking the largest proportion of 86.00%, Chepkutuny A (χ^2 = 88.36, d.f.=1, p=<0.0001) with female taking the larger proportion of 97.00%, Chepkutuny B (χ^2 = 4.0, d.f.=1, p= 0.0455) with female taking the larger proportion of 60.00% and Chepkutuny C (χ^2 = 11.56, d.f.=1, p= 0.0007) with males taking the smaller proportion of 33.00% as shown in Table 4.2.

Table 4.2: Sex ratios (Male: Female) of sandfly species collected from five caves in Mt. Elgon

Vector Species	Site	Male (%)	Female (%)	Chi square (χ^2), df, p value
P. pedifer	Chemai A	31.00	69.00	χ^2= 14.44, d.f.=1, p= 0.0001
	Chemai B	30.00	70.00	χ^2= 16.00, d.f.=1, p= 0.0001
	Chepkutuny A	12.00	88.00	χ^2 = 57.76, d.f.=1, p<0.0001
	Chepkutuny B	14.00	86.00	χ^2 = 51.84, d.f.=1, p<0.0001
P.elgonensis	Chemai A	41.00	59.00	χ^2 = 3.24, d.f.=1, p= 0.0719
	Chemai B	80.00	20.00	χ^2 = 36.00, d.f.=1, p<0.0001
	Chepkutuny A	14.00	86.00	χ^2 = 51.84, d.f.=1, p<0.0001
	Chepkutuny B	17.00	83.00	χ^2 = 43.56, d.f.=1, p<0.0001
	Chepkutuny C	70.00	30.00	χ^2 = 16.00, d.f.=1, p= 0.0001
P. longipes	Chemai A	50.00	50.00	χ^2 = 0.00, d.f.=1, p= 1.0000
	Chemai B	12.00	88.00	χ^2=57.76, d.f.=1, p<0.0001
	Chepkutuny A	15.00	85.00	χ^2 = 49.0, d.f.=1, p<0.0001
	Chepkutuny B	34.00	66.00	χ^2 = 10.24, d.f.=1, p= 0.0014
	Chepkutuny C	38.00	62.00	χ^2 = 5.76, d.f.=1, p= 0.0164
S. schwetzi	Chemai A	38.00	62.00	χ^2 = 5.76, d.f.=1, p= 0.0164
	Chemai B	40.00	60.00	χ^2 = 4.0, d.f.=1, p= 0.0455
	Chepkutuny A	3.00	97.00	χ^2 = 88.36, d.f.=1, p<0.0001
	Chepkutuny B	40.00	60.00	χ^2 = 4.0, d.f.=1, p= 0.0455
	Chepkutuny C	67.00	33.00	χ^2 = 11.56, d.f.=1, p= 0.0007

4.2 Abundance of *Leishmania* parasite infections in sandfly vector species in Mt. Elgon cave habitats

4.2.1 Abundance of *Leishmania* parasite infections

The mean abundance of *Leishmania* parasite infections in *P. pedifer* was high in Chepkutuny C (1974.67±6.43) followed by Chepkutuny B (1075.67±5.03) while the lowest was recorded in Chemai B (265.67±20.60) with significant difference between the sites of collections ($F_{0.05\ (4,\ 10)}$ =3359.16, $p<0.0001$). Significant differences were recorded between the sandflies collected from all the sites as shown in Table 4.3.

For *P. elgonensis,* mean abundance was high in Chepkutuny A (295.00±2.00) followed by Chepkutuny C (194.00±2.65) while the lowest was recorded in Chemai B (14.67±2.52) with a significant difference ($F_{0.05\ (4,\ 10)}$ =1481.63, $p<0.0001$). Significant differences were recorded between all the sites as illustrated in Table 4.3.

P. longipes mean abundance was high in Chepkutuny C (106.67±4.93) and lowest in Chemai B (5.67±2.08) with a significant difference ($F_{0.05\ (4,\ 10)}$ =240.17, $p<0.0001$). Significant difference was recorded between all the sites as illustrated in Table 4.3.

S. schwetzi mean abundance was high in Chepkutuny C (20.67±2.08) and Chemai A (18.67±2.08) and lowest in Chemai B (3.00±2.00) with a significant difference ($F_{0.05\ (4,\ 10)}$ =27.45, $p<0.0001$). Significant difference was recorded between Chemai B and all other sites, Chepkutuny B and Chepkutuny C as shown in Table 4.3.

Table 4.3: *Leishmania* promastigotes infections (mean parasite load/density ± SD) in sandfly vector species collected from five Mt. Elgon cave sites

Site	*P. pedifer*	*P. elgonensis*	*P. longipes*	*S. schwetzi*
Chemai A	676.67±37.17^{a}	102.33±2.08^{a}	59.00±1.00^{a}	18.67±2.08^{cd}
Chemai B	265.67±20.60^{b}	14.67±2.52^{b}	5.67±2.08^{b}	3.00±2.00^{a}
Chepkutuny A	648.67±4.73^{c}	295.00±2.00^{c}	29.00±1.00^{c}	15.00±3.00^{bc}
Chepkutuny B	1075.67±5.03^{d}	121.67±9.50^{d}	48.67±7.64^{d}	14.00±2.00^{b}
Chepkutuny C	1974.67±6.43^{e}	194.00±2.65^{e}	106.67±4.93^{e}	20.67±2.08^{d}
F ratio	3359.16	1481.63	240.17	27.45
p-value	0.0000	0.0000	0.0000	0.0000

Means±SD followed by different letter superscripts in the same column are significantly different at $p<0.05$

4.2.2 Diversity of *Leishmania* parasites species infecting sandfly vector species in sampled Mt. Elgon caves

The overall Shannon Weiner diversity index (*H*') for the *Leishmania* parasites species infecting sandfly vector species in sampled Mt. Elgon caves was 0.621*H'* with eveness of 0.4652e^H/S. For the sites, Chepkutuny A had the highest Shannon Weiner diversity index of 0.4784 H' and evenness of 0.5587 e^H/S followed by Chemai A site (*H'* =0.3366) while Chemai B had the lowest diversity index (0.1519*H'*) as illustrated in Table 4.4. Significant difference was recorded between all sites ($p<0.05$).

Table 4.4: Diversity of *Leishmania* parasites species infecting sandfly vector species in sampled Mt. Elgon caves

Sampling Sites	Shannon Weiner diversity index (*H'*)	Evenness (e^H/S)
Chemai A	0.3566^{a}	0.5074
Chemai B	0.1519^{b}	0.1519
Chepkutuny A	0.4784^{c}	0.5587
Chepkutuny B	0.2603^{d}	0.4275
Chepkutuny C	0.2509^{e}	0.422
Overall	0.6210	0.4652

Indices with different superscript letters in the same column are significantly ($P<0.05$) different from each other

4.2.3 The prevalence of *Leishmania*infection in the vector during the survey

The overall percentage of sandflies infected with *Leishmania* was 7.13%. When the prevalence of *Leishmania* infection for each species of sandfly vector was computed, it was found that a large proportion of *P. pedifer* was infected with *Leishmania* with a prevalence of 9.21%. Similarly; a large proportion of *P. elgonensis* was infected with *Leishmania* with a prevalence of 8.01%. *P. longipes* infection prevalence with *Leishmania* was 4.51% whiles that of *S. Schwetzi* was 6.78%. This is a significant observation since it is usually suggested that S. *Schwetzi* and others in the same genus are not competent to transmit *Leishmania* parasites. *Leishmania* prevalence rates differed significantly between species of vectors (χ^2 = 18.0693, d.f. =3, p= 0.0004) as illustrated in Figure 4.2.

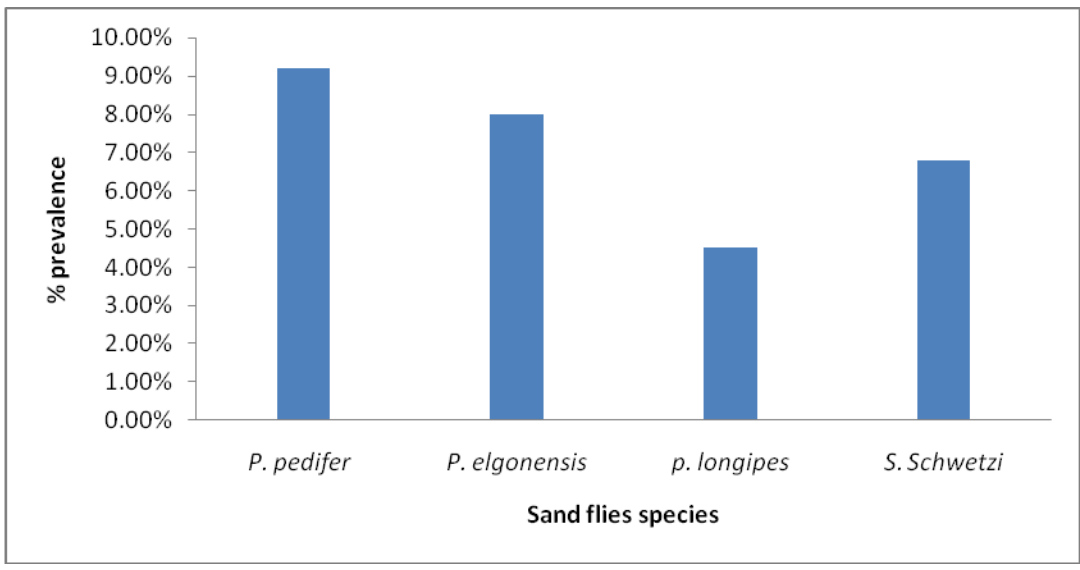

Figure 4.2: The prevalence of *Leishmania* infection in the sandfly vector species during the study in Mt Elgon.

4.3 Sources of bloodmeal in sandfly vectors collected from Mt. Elgon caves.

4.3.1 Distribution of collected bats and hyraxes species sampled in Mt. Elgon caves.

A total of one hundred and nighty nine (199) bats belonging to five species including; *Cardioderma cor, Chaerephon pumilus, Rhinolophus clivosus, Nycteris hispida* and *Nycteris nana,* were captured (Appendix XII). Rock hyrax (*Procavia capensis*) and bush hyrax (*Procavia habessinica)* were the two species of hyraxes that were taken from the research locations and identified as described in previous articles.(Barry and Shoshani, 2000; Chase-Grey, 2011a; Munyai and Foord, 2012; Olds and Shoshani, 1982) (Appendix XI).Twenty-five (25) hyraxes that were captured belonged to two species namely *Procavia habessinica* and *Procavia capensis johnstoni*. The total number of bats and hyraxes sampled were two hundred and twenty-four (224) animals. The distribution of the bats species was 49.75% *Cardioderma cor*; 21.11% *Chaerephon pumilus*, 16.58% *Rhinolophus clivosus*, 7.54% *Nycteris hispida* while *Nycteris nana* (5.03%) constituted the lowest proportion with a significant difference (χ^2 = 63.9434, d.f.=4, p<0.0001) between the species proportions. The collected hyraxes belonged to two species: *Procavia habessinica* (40.00%) and *Procavia capensis johnstoni* (60.00%) as illustrated in Figure 4.4 which varied with a significant difference (χ^2 = 4.0, d.f. =1, p= 0.0455).

In terms of bats species distribution across the sites, Chepkutuny C had a significantly (χ^2 = 55.55, d.f.=4, p<0.0001) higher population of bats (49.74% of the total collected) compared to the other sites which had nearly the same proportions of bats species (p>0.05) as shown in Table 4.5.

Table 4.5: Distribution of collected bats and hyraxes species populations in sampled Mt. Elgon caves

		Total	Chepkutuny A	Chepkutuny B	Chepkutuny C	Chemai A	Chemai B
Host	Species	N(%)	n(%)	n(%)	n(%)	n(%)	n(%)
Bats	*Cardioderm acor*	99(49.75)	12(49.9)	12(49.91)	13(49.88)	13(49.85)	49(49.7)
	Chaerephon pumilus	42(21.11)	5(21.17)	5(21.18)	6(21.16)	5(21)	21(21.08)
	Rhinolophus clivosus	33(16.58)	4(16.63)	4(16.64)	4(16.63)	4(16.5)	16(16.57)
	Nycteris hispida	15(7.54)	2(7.56)	2(7.56)	2(7.56)	2(7.5)	7(7.53)
	Nycteris nana	10(5.03)	1(5.04)	1(5.04)	1(5.04)	1(5)	5(5.02)
	Chi square (χ^2)	χ^2= 63.9434, d.f.=4, p<0.0001	χ^2= 63.7536, d.f.=4, p<0.0001	χ^2= 63.7342, d.f.=4, p<0.0001	χ^2= 63.7731, d.f.=4, p<0.0001	χ^2= 64.2839, d.f.=4, p<0.0001	χ^2= 64.2839, d.f.=4, p<0.0001
Hyraxes	*Procavia habessinica*	10(40)	1(42)	1(40.33)	1(43.67)	1(41.67)	5(41.42)
	Procavia capensisjohnstoni	15(60)	2(63)	2(60.5)	2(65.5)	2(62.5)	7 (62.13)
	Chi square (χ^2)	χ^2= 4.0, d.f.=1, p= 0.0455	χ^2= 4.2, d.f.=1, p= 0.0404	χ^2= 4.3739, d.f.=1, p= 0.0365	χ^2= 4.43976, d.f.=1, p= 0.0351	χ^2= 4.24008, d.f.=1, p= 0.0395	χ^2= 4.26173, d.f.=1, p= 0.0390

The highest average number of bats was recorded in Chemai B (44.84±17.68 SD) while the other site recorded almost the same number with a significant difference ($F_{0.05\ (4,\ 20)}$ =2.76, p=0.0461) across the sites.

For the hyraxes, the total collected number for all sites was 25. Chemai B recorded the highest significant number of the hyraxes (F ($_{0.05\ (4,\ 20)}$ =10.12, p=0.0129) the significant difference was between Chepkutuny A, Chepkutuny B, Chepkutuny C, Chemai A with Chemai B as portrayed in Figure 4.3.

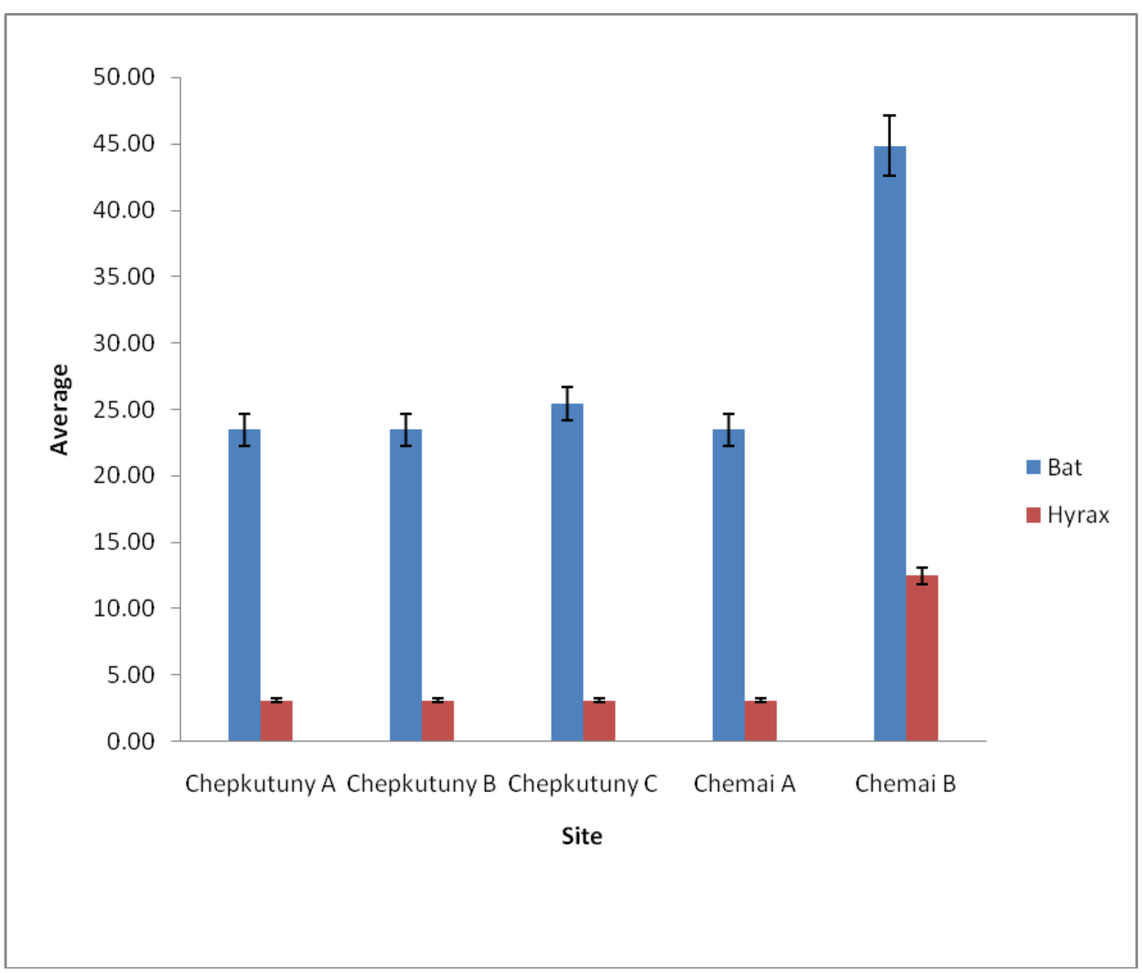

Figure 4.3: Bats and hyraxes mean numbers per collection site

4.3.2 Diversity of bats and hyraxes species sampled in selected Mt. Elgon caves

Total Shannon Weiner diversity index of combined bats and hyraxes' species was 1.59 *H'*. Shannon Weiner diversity index of bats species in Mt. Elgon caves was 1.32 *H'* while that of hyraxes was 0.67*H'*as illustrated in Figure 4.4.

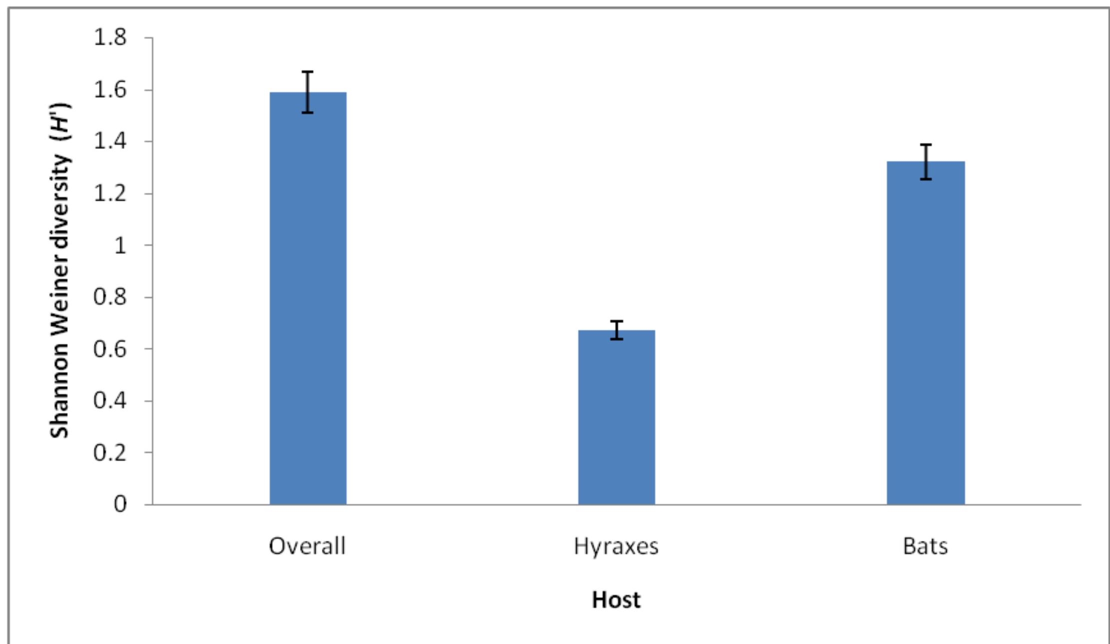

Figure 4.4: Shannon Weiner diversity of bats and hyraxes sampled in Mt. Elgon caves

4.3.3 Prevalence of bats and hyraxes infected with *Leishmania* parasites in Mt. Elgon caves

The overall prevalence of bats infected with *Leishmania* parasites was 47%. Chepkutuny C cave had the highest recorded prevalence of amastigotes in the bat samples at 80%, followed by Chepkutuny A at 60%, Chepkutuny B at 50%, Chemai A at 30% and the least was Chemai B at 25% with a significant difference (χ^2= 41.2245, d.f. =4, $p<0.0001$) between the infection rates of bats collected from the different caves.

Bat species had significantly higher prevalence of *Leishmania* parasite infections compared to hyraxes (χ^2 = 7.342, df. =1, p = 0.0043). Among the species of bats, *Cardioderma cor* had the highest prevalence of *Leishmania* infections (70.10%) followed by *Chaerephon pumilus* (55.00%) and *Rhinolophus clivosus*(53.30%) while *Nycteris nana* had the least (25.00%) with a significant difference (χ^2= 27.6489, d.f.=4, p = 0.0000). Meanwhile, the two species of hyraxes combined had less than 30% *Leishmania* parasite infections and did not differ significantly in prevalence of *Leishmania* infection (χ^2= 0.9653, d.f.=1, p= 0.3258) as shown in Table 4.6.

Table 4.6: Wild reservoir host species infection prevalence with *Leishmania* parasites in sampled Mt. Elgon caves

Host	Species	Prevalence (%)	Chi square (χ^2)
Bats	*Cardioderma cor*	70.1	χ^2= 27.6489,
	Chaerephon pumilus	55.0	d.f.=4,
	Rhinolophus clivosus	53.3	p = 0.0000
	Nycteris hispida	33.3	
	Nycteris nana	25.0	
Hyraxes	*Procavia habessinica*	28.6	χ^2= 0.9653,
	Procavia capensis johnstoni	22.2	d.f.=1,
			p= 0.3258

4.3.4 Distribution of Sources of bloodmeal in vectors collected from sampled Mt. Elgon caves

Serologic analysis by ELISA on 200 engorged *P. pedifer* sandflies showed that the flies had fed on human, bat, hyrax and cat blood. Percentage distribution of blood types showed that 60.00% sandflies fed on human, 28.00% on bat, 9.00% on hyrax and 3.00% fed on cat with a significant (χ^2= 82.8, d.f.=3, p< 0.0001) difference between the sources of blood.

In terms of sites, a large proportion of blood meal sources for *P. pedifer*came from human (Chemai A (75.00%), Chepkutuny C (62.50%) Chepkutuny A (50.00%),

Chepkutuny B (50.00%) and Chemai B (6.67%)) with a significant difference from other sources (p<0.0001). The second most preferred source of blood meal for *P. pedifer* was bats (Chemai B (33.33%), Chepkutuny B (30.00%), Chepkutuny A, (25.00%), Chepkutuny C (25.00%) and Chemai A (25.00%),). However, *P. pedifer* did not source blood meal from hyrax and cats in Chemai A and Chemai B as shown in Table 4.7.

Table 4.7: Sandfly (*P. pedifer)* blood meal sources in sampled caves in Mt. Elgon, Kenya

Capture site	No of human Human blood meal (n and %)	No of Bat Bat blood meal (n and %)	No of Hyrax blood meal (n and %)	No of cat Cat blood meal (n and %)	Chi square (χ^2) p value
Chepkutuny A	10(50.00%)	5(25.00%)	3(15.00%)	2(10.00%)	χ^2= 38.0, d.f.=3, $p< 0.0001$
Chepkutuny B	25(50.00%)	15(30.00%)	6(12.00%)	4(8.00%)	χ^2= 44.32, d.f.=3, $p< 0.0001$
Chepkutuny C	50(63.50%)	20(25.00%)	7(8.75%)	3(3.75%)	χ^2= 87.81, d.f.=3, $p< 0.0001$
Chemai A	15(75.00%)	5(25.00%)	0(0.00%)	0(0.00%)	χ^2= 25.0, d.f.=1, $p< 0.0001$
Chemai B	20(67.67%)	10(33.33%)	0(0.00%)	0(0.00%)	χ^2= 12.12, d.f.=1, $p=0.0005$
Total	120(60.00%)	54(28.00%)	17(9.00%)	9(3.00%)	χ^2= 78.96, d.f.=3, $p< 0.0001$

4.3.5 The prevalence of *Leishmania* amastigotes in reservoir hosts species in sampled Mt. Elgon caves based on liver analysis

The prevalence of *Leishmania* amastigotes in reservoir hosts species collected during the study and based on liver analysis are shown in Figure 4.5. Prevalence of *Leishmania* amastigotes in bats species specifically *Cardioderma cor* was (55.97%) followed by *Chaerphon pumilus* (31.09%) while the lower rate was recorded in *Rhinolophus clivosus* (6.59%), *Nyectris hispida* (4.10%) and *Nycteris nana* (2.23%) with a significant difference (χ^2= 108.3, d.f. =4, p<0.0001) in bat species infection with *Leishmani a*amastigotes.

For the hyraxes, the prevalence of *Leishmania* amastigotes was highest in *Procavia capensis johnstoni* species (55.17%) and non-significantly (χ^2= 1.0, d.f.=1, p= 0.3173) lower in *Procavia habessinica* (44.82%) as illustrated in Figure 4.5.

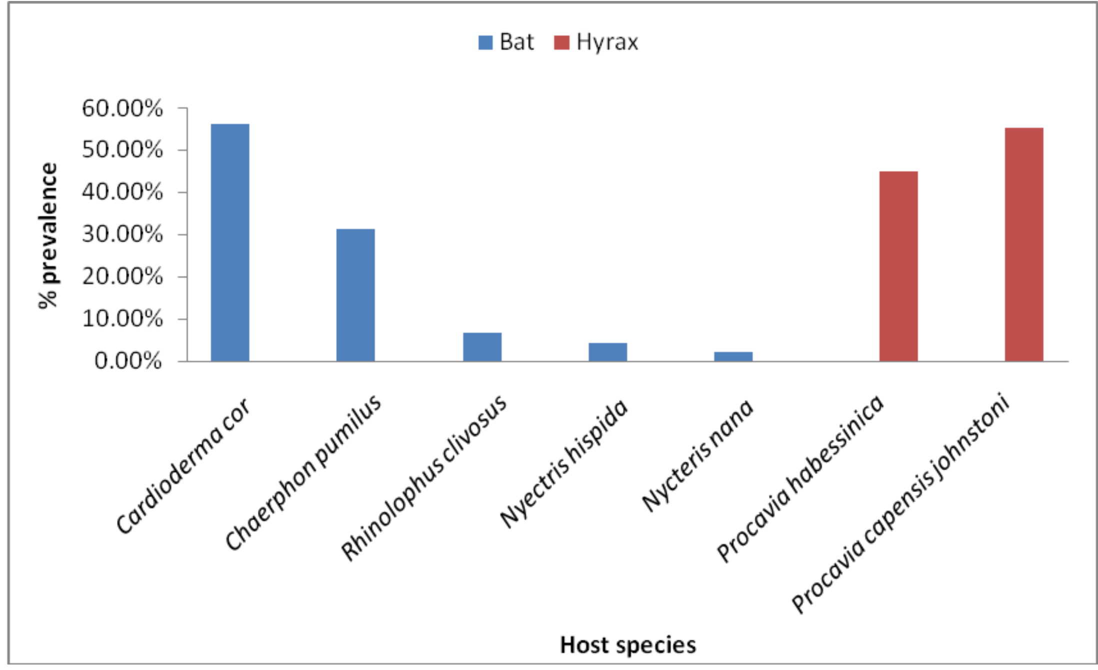

Figure 4.5: Prevalence of *Leishmania* amastigotes in bat and hyraxes host species

4.4 Sandfly species taxonomy by molecular analysis

The morphological identities of the sampled sandfly specimens were verified by PCR and sequencing analysis of the sandfly gene. The analyses revealed an expected amplicon size of 700-bp. Further analyses of the 700-bp fragment using molecular tools confirmed the morphological identifications**.** Based on their molecular properties, three separate *Phlebotomus* species with varied morphological identifications were represented by two randomly chosen COI sequences (n=2). The gene sequence from the *P. pedifer* identified morphologically was aligned with the *P. pedifer* (KP473774.1) sequence obtained from the GenBank, which had a high degree of similarity (90-100%) and demonstrated that the two sequences were closely related. The morphological identifications were confirmed when they were subsequently placed together by phylogenetic analysis into the *Paraphlebotomus* clade. Based on their molecular features, randomly chosen COI sequences (n = 2) from several morphologically recognised female *Phlebotomus* species were validated. Alignments of the gene sequence for *P. elgonensis*, which was identified morphologically, revealed significant levels of similarity (90–95%) (GenBank accession numbers KB473775.1 and KB473776.1, respectively). These were all placed in the same clade by phylogenetic analysis, indicating a tight link between them.

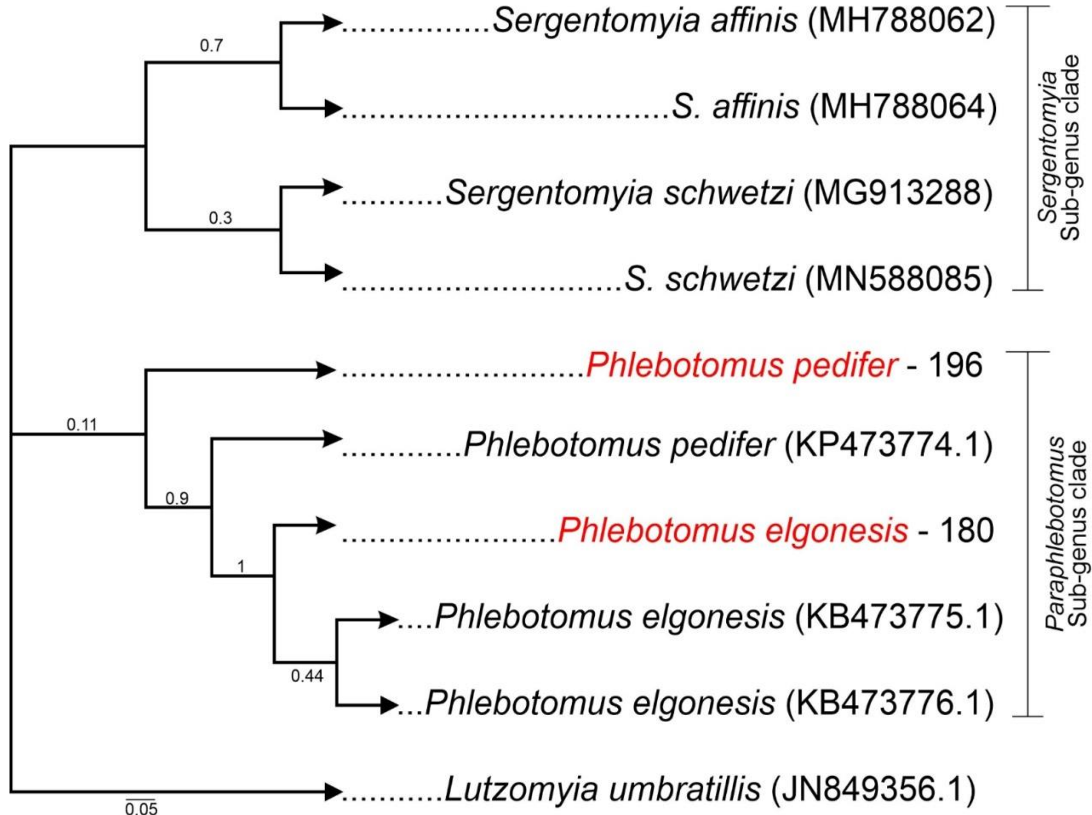

Figure 4.6: Phylogenetic analysis of the sandfly mitochondrial COI sequence by maximum likelihood method

The nodes display the proportions into which the related taxa grouped themselves. The accession numbers (in brackets) of the GenBank homologs are also mentioned, along with the specified sample identities (in red with their respective codes, 180 and 196 for *P. elgonensis* and *P. pedifer,* respectively).

Lutzomyia umbratilis, a New World sandfly species (GenBank accession: JN849356.1), was used as the out group for anchoring the tree constructed from 700-bp segments.

Due to failure to get primers for a BLAST search with *Sergentomyia schwetzi* (GenBank accessions: MG 913288 - MN588085), we were unable to do the gene sequencing for *S. schwetzi.*

Based on real-time PCR-HRM and sequencing of the PCR-HRM amplicons, twelve samples of *Phlebotomus pedifer* sandflies were shown to be positive for *Leishmania* parasites. 7.0% (n = 14/200) of female sandflies in the research region had *Leishmania* infection overall. 8.0% (16/200) of *P. pedifer* had an infection.

The ITS1 sequence (GenBank accession:MT648857) from one of the positive *P. pedifer* samples was used in a BLAST search of the National Centre for Biotechnology Information (NCBI) database, which indicated a high percentage of identity (99%) with *L. major* sequences in GenBank. The sequences from two of the positive *P. pedifer* samples (GenBank accession numbers MT648855-MT648856) also shared 97–100% of their similarities with GenBank sequences for *L. tropica*. The study's ITS1 sequences were separated into *Leishmania major* and *Leishmania tropica* clusters in the phylogenetic tree, with bootstrap support values of 90% and 99%, respectively (Figure 4.7). *Leishmania* species that have been isolated from hosts in various places and whose sequences are available in GenBank were found in these clusters.

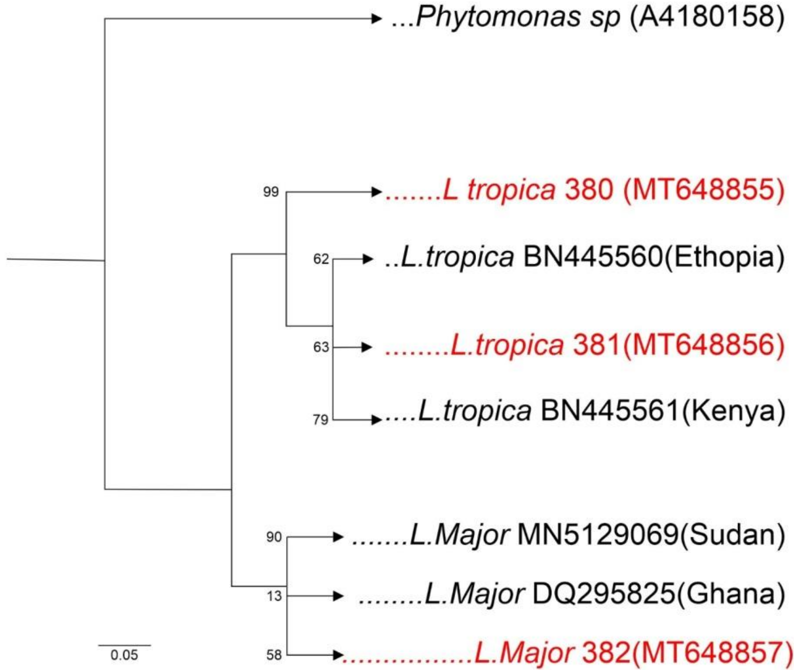

Figure 4.7: Phylogenetic tree of the *Leishmania* ITS1 gene sequence by maximum likelihood method

The 350 bp and 320 bp segments for *L. major* and *L. tropica*, respectively, were used to build the phylogeny, with *Phytomonas spp.* (AY180158) serving as the out group for rooting. The sequences from *L. major* and *L. tropica* that were found in this investigation are bolded. The primary nodes' bootstrap percentages are consistent throughout 1000 replications. The scale bar for the branch length shows how many nucleotide changes there are at each location.

Using ITS1-PCR-RFLP, it was determined that none of the pooled sandfly samples contained *Leishmania* parasites. However, the ITS1-PCR results of two parasites isolated from sandflies and successfully grown generated bands of around 320 bp. For

the isolates, HaeIII digestion of the products produced an RFLP pattern resembling that of *Leishmania tropica* (Figure 4.8).

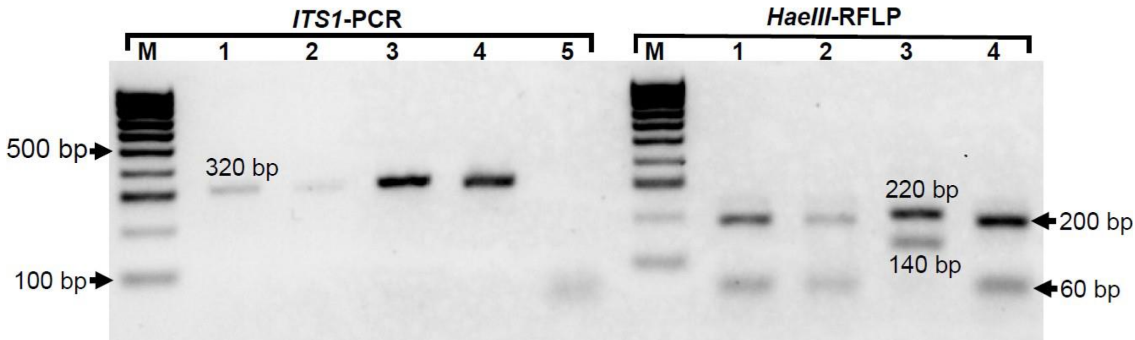

Figure 4.8: *Leishmania* parasite ITSI DNA fragments.

Left: ITS1-PCR products and Right: Restriction fragment length polymorphisms of ITS1 region by HaellIM: 100 bp ladder; 1 and 2: The faint bands may be due to inadequate amplication of the DNA. *Leishmania* spp. Isolated from sandflies; 3 and 4: *L. major* (Friedlin strain) and *L. tropica* (Lv357 strain) positive controls; 5: negative control.

Molecular sizes of each species' RFLP product are displayed, including *L. major's* (220 and 140 bp) and *L. tropica's* (200 and 60 bp) ladders kDNA-PCR nested

The results of the ITS1-PCR-RFLP were confirmed by the cultured Leishmania isolates, which generated a 750 bp amplicon, the P. pedifer-specific band (Appendix X). However, none of the pooled DNA was discovered to be positive for Leishmania species. Two sandfly sample pools were then tested for Leishmania DNA using the ITS1- real-time PCR and HRM qPCR-HRM Assays. Three samples of the P. pedifer species tested positive for Leishmania species, and L. tropica and L. major were found. Individual sandflies from these pools also tested positive for L. major. The naturally infected sandfly's HRM profile of L. major was different from the controls.

CHAPTER FIVE

DISCUSSION

5.1 Determination of the distribution of sandfly vector species in Mt. Elgon cave habitats

An entomological survey was carried out in five caves (namely, Chemai A, Chemai B, Chepkutuny A, Chepkutuny B and Chepkutuny C) in Mount Elgon Sub-County, Bungoma County, in Western Kenya. One trapping method (CDC light traps) was used to collect sandflies during the study period. All the sites (Chemai A, Chemai B, Chepkutuny A, Chepkutuny B and Chepkutuny C) were found to be positive for sandflies. Over the course of the twenty-four (24) month sampling period, 5,688 sandflies were gathered from the five research caves. The mean abundance of *P. pedifer* was highest among the 5688 collected flies in Chepkutuny C, followed by Chepkutuny B, and lowest in Chemai B, with a statistically significant difference between the two.

The higher population of sandflies collected in Chepkutuny C may be explained by the narrow entrance nature of Chepkutuny C cave, which enables it to retain more moisture and warmth as suggested in a previous study (Adamczick*et al*., 2018). Lack of disturbance by human and livestock activities in Chepkutuny C due to its small entrance could have allowed un-interfered developments of the fly's immature stages on the floor of the cave. The larger population of bats in the same cave is another possible factor; bat's excreta provide organic nutrients to developing sandfly larvae (Claborn, 2017).

Only two genera of sandfly were identified in the surveyed sites, belonging to the *Sergentomyia* and *Phlebotomus*; however, *Phlebotomus* genus was found to have the

higher prevalence of *Leishmania* parasites and thus can be considered as the major vector of *Leishmania* in the study area.

The *Phlebotomus (Larroussius)* species *P. pedifer, P. elogonensis, P. longipes,* and *S. schwetzi* species were encountered in the present research sandflies in the five caves. However, based on the morphological identification of the males, *P. pedifer* was found in all studied sites, but primarily at lower elevations (1772-1900 m), whereas *P. elgonensis* was the predominant species at higher elevations, as previously noted (Mutinga and Ngoka, 1983). *P. longipes* and *S. schwetzi* were found in all the sites that were sampled during the current study.

Studies on sandfly morphology by Killick-Kendrick *et al.* (1994) confirmed that males of *P. pedifer* and *P. longipes* can be distinguished based on the slight difference in the aedeagus shape and additionally also the number of inner surface coxite hairs, being 50–60 for *P. pedifer* and 35–50 for *P. longipes*. In contrast, females of the two species are considered indistinguishable as reported by Lewis *et al.* (1974) and Lewis & Mutinga, (1972), except for a slight difference in the base of the spermathecal ducts, which often still results in inconclusive identifications as observed by Killick-Kendrick *et al.* (1994).

Results of the present study show that the tips of the aedeagi of the Kenya's CL vectors varied among all collected specimens, being partially dependent on the orientation of the specimen on the slide. Also, the number of coxite hairs overlapped significantly between *P. pedifer* and *P. longipes* and seems more geographically dependent than species-specific as observed in the study sites. It may be concluded that these morphological characters are not appropriate for species identification Even though *P.elogonensis is* morphologically closely related to *P. pedifer* and *P.*

longipes as reported by Killick-Kendrick, *et al.*, (1994), our results show that this species can easily be distinguished based on molecular techniques.

Leishmania promastigotes are frequently more prevalent in the female sandfly guts at lower elevations (below 1900 m), which is also the region where *P. pedifer* is most prevalent and where there are more occurrences of cutaneous *Leishmania* (Mutinga and Ngoka, 1983; Mutinga and Odhiambo, 1986b). This was in agreement with Makwali's (2021) hypothesis that *P. pedifer* can be thevector of cutaneous *Leishmania* in the Mount Elgon area; though more research should be done to identify alternative vector species.

Higher abundance of the sandfly species occurred during November to April which coincided with the dry period while the lower abundance occurred during May to October which coincided with the long rainy period. As a result, the rainy season recorded 48.03% of the total sandflies that were collected, while the dry season recorded the majority (51.97%), with no statistical significant difference between the seasons ($p>0.05$). The seasons had little impact on the distribution of either the male or female sandflies. This could be explained by the relatively stable weather patterns in the protected cave habitats where sandflies rest and breed.

Given that sandfly larvae go through a process called diapause, which allows them to survive the cold environment and emerge as adults in the ensuing warm weather, the monthly (temporal) distribution of sandflies was found to be similar for all detected species as previously reported by (Cissé *et al*., (2020), with a smaller sandfly population by the start of the wet cold season in May and a population peak in January that is the onset of dry season. However, in a related research conducted in Agool, Egypt, sandfly populations peaked not only in June but also in September,

October and December that were dry months. Abdel-Bedai *et al*. (2012) in El Nekheil and other studies in Asir (Alten*et al*., 2015) in Saudi Arabia and Egypt (Hanafi *et al*., 2007) also noted this pattern of seasonal abundance in AbyarAlmashy, Almaliliah, and Mondasa. Saudi Arabia and Egypt have been researched, and their climatic patterns are likely different from the pattern in Western Kenya, where the current study was conducted.

In all sampled sites, the largest proportion was that of *P. pedifer* female male represented a smaller percentage which was significantly different. Proportion of male to female *P. pedifer* differed significantly across all the sites studied as was observed in a previous study (Claborn, 2017).The most likely explanation is because sticky traps were employed in Claborn's (2017) investigation instead of the CDC trapping technique, which has been shown to be effective for host-seeking females (Alten *et al*., 2015). For the *P. elgonensis,* the proportion of male to female per site differed significantly in Chemai B with males taking the larger proportion of 80.00%, this may be due to the presence of only two sources of blood meal, humans and the bats, since female sandflies require host blood for the development of immature stages of sandflies. Chepkutuny A had female taking the larger proportion of 88.00%, in Chepkutuny B,females took the larger proportion of 83.00%.This was probably because there were more sources of blood for the development of the immature stages. In Chepkutuny C the males took the larger proportion of 70.00%. However, the fewer female sandflies present showed a preference for human blood meal.

For *P. longipes* the proportion of male to female per site differed significantly.In Chemai B the females took the larger proportion of 88.00%.Humans and bats were the only sources of blood meal for the sandflies. In Chepkutuny A, females took the

larger proportion of 85.00%.The female sandflies fed on all the four hosts, human, bats, hyraxes and cats. In Chepkutuny B females took the larger proportion of 66.00% with all the four hosts being fed on while Chepkutuny C had males taking the larger proportion and high male collections occurred near the breeding sites of the sandflies, high female numbers were also identified in other study carried out in Morocco (Ouanaimi *et al.*, 2015).However the few females recorded showed high preference for human blood.

For *S. schwetzi* the proportion of male to female per site differed significantly.In Chemai A females took the larger proportion, Chemai B where females took the larger proportion of 88.00%, there were only two sources of blood meal for the sandflies, the human and bats. In Chepkutuny A with female took the larger proportion of 85.00%, there were humans, bats, hyraxes and cats providing the blood meal for the female sandflies. In Chepkutuny B females took the larger proportion of 66.00% and in Chepkutuny C males took the larger proportion of 62.00%. Female sandflies require a blood meal from the host for the development of the immature stages. Most of the study sites except Chepkutuny A were located below 1900m and it has been demonstrated that more *P. pedifer* occupy this habitat (Makwali, (2021).

L. aethopica was first reported in Endebess in Mt. Elgon by Mutinga (1975) and subsequently by Dawit *et al,* (2013) in *P. elgonensis* and *P. pedifer*. Similarly Mukwana *et al.,* (2018) reported presence of *L. aethopica* in *P. pedifer* at Cheptobot and Kimikung sites. However in the current study, *L. aethopica* was not observed in the sandfly vectors collected from the different sites sampled.This may be because the sampled sites were located at alititudes below 2000m above sea level compared to the other study (Mukhwana *et al*., 2018)

Seasonal differences in the male-to-female (sex) ratio of several species found in the same region as well as within the same species in other regions suggest the need for more research on the geographic and ecological systems as well as the variables affecting the fecundity of Mt. Elgon sandflies. This is probably going to provide information on the Mt. Elgon region's sand flies' mating season and behaviours, information that might be utilised to develop fresh management methods. At this focus, the proportions of gravid flies may be a reliable predictor of their physiological age and of epidemiologic significance. As previously noted (Bi *et al*., 2018), the *Leishmania* infection rate by culture of sandfly intestinal contents ranges from 0.3% to 1.5% in June. In the current study, culturing yielded significant quantities of parasites for subsequent analysis. This observation provides insight into the sandfly's infectiousness and can help plan control strategies for cutaneous *Leishmania*.

The male/female ratio of 1:1.24 proved that there were more female sandflies than males. This is consistent with prior investigations where sandflies were captured using CDC light traps. For instance, Mukhwana *et al*. (2018) showed that in all their research sites, there were more female than male sandflies caught using CDC light traps (Mukhwana *et al*., 2018). However, it could be that the CDC trapping technique picks female sandflies that are looking for hosts to feed (Alten *et al*., 2015). For the growth and maturation of their eggs, female sandflies need a blood meal (Sales KG da *et al*., 2015). Male flies often have less dispersal capacity than female flies due to the fact that their mouthparts are not designed for sucking blood (Ryan *et al.,* 2006). This could also explain why, despite a significant population of sandflies being collected , fewer male than female flies were found in the Mt. Elgon caves, and this suggests that *Leishmania* is therefore transimitted primarily by the blood sucking female sandflies.

Leishmania parasites must be dispersed by sandflies in order to proliferate (Makwali, 2021). All of the research sites had high numbers of sandflies of the genus *Phlebotomus* which suggests a high risk of CL transmission in the study area.. Unquestionably, the frequency of sandfly infection with *Leishmania* parasites is a sign of the vector's ability to transmit illness (Bennai *et al*., 2018).

5.2 Prevalence of *Leishmania* parasite infections in sandfly vector species in Mt. Elgon cave habitats

In terms ofdiversity of *Leishmania* parasite infecting sandfly vector species in Mt. Elgon, the overall Shannon Weiner diversity index (*H*') for the *Leishmania* parasite infections of sandfly vector species in Mt. Elgon was high at 0.621 *H*' with high evenness at 0.4652 e^H/S . For the sites, Chepkutuny A had the highest Shannon Weiner diversity index and evenness followed by Chemai A site while Chemai B had the lowest diversity index.

The most predominant sandfly species in the sampled Mt.Elgon caves were those of the *Phelobotmus* group. This is in agreement with Mutinga & Odhiambo's findings (1986 a) that both *P. pedifer* and *P. Elgonensis* are common and widespread in Mt. Egon, Kenya. *Sergentomyia schwetzi* was previously reported (Heisch *et al.* 1956) to have a high incidence in Kitui and was caught throughout the year. Similar results were reported by Mutinga *et al.* (1982) in West Pokot for the *Phelobotomus* members.

The *Leishmania* parasites can be maintained within the lizard host as evidenced in the findings of the research performed on the *Leishmania* parasite byForawi (1986) who isolated *L. major* from a lizard in West Pokot. The current study has shown parasites of *Leishmania* in bat and hyrax hosts. However, lizard was not investigated in the current study. The current results shows that phlebotomine sandflies in Mt. Elgon

harbour *Leishmania* parasite Ombaka *et al.,(*2023) and the presence of amastigotes in bats suggests that they could be among the wild reservoir hosts for the parasites in the area.

The Mt Elgon region has several caves with a lot of movementof local people and tourists.Nearby farming activities could easily transport and introduce new parasites and vectors of the disease. Some of the fly species caught, for example, *S. schwetzi* and *P. pedifer* have been found to be alternate zoonotic vectors of *Leishmania* in Baringo District (Mutinga *et al.,* 1986a). The same may be said of Mt. Elgon as shown by cutaneous lesions among the local residents. Experimental evidence on the vectorial capability of most of the various species of phlebotomine sandflies in Kenya is an important and urgent aspect of the epidemiology of leishmaniases (Kaddu, 1986). This would enhance the knowledge and facilitate the forecasting of potential disease (leishmaniaisis) outbreak in any area, where potential vectors are found. People would then not have to be taken "unawares" as it happened in the mid fifties (Mutinga and Kamau, 1986) when the study of sandflies "received a sudden impetus after a serious epidemic of kala-azar occurred in Kitui District" (Minter, 1964a).

The position of bats as a *Phlebotomus pedifer* natural blood supply has significant ramifications for the management of leishmaniases. Now that the current study has established that bats are reservoirs for *Leishmania* parasites (Makwali 2021), their great capacity for dispersion and lifespan may have significant implications on the persistence and spread of these parasites. Some bats, such as young ones, choose to spend the entire night within caves even though adult bats often leave them while sandflies are feeding at night. In environments where bats and *Phlebotomus pedifer* cohabit, blood meals from the sand flies were examined in order to ascertain whether

they naturally feed on bats blood. This study successfully demonstrated that the bats do, in fact, offer a consistent source of blood to the resident female sandflies.

The overall prevalence of bats infection status with *Leishmania* parasites in the current study was 49% (49/100). In terms of infection per cave, Chepkutuny C cave had the highest recorded prevalence of amastigotes in the bat's samples at 80% (16/20), followed by Chepkutuny A 60 % (12/20), Chepkutuny B 50 % (10/20), Chemai A 30% (6/20) and the least was Chemai B 25% (5/20). Ethiopian research found four positive bats in endemic *Leishmania* foci, while four were found in non-endemic regions. Human *L. tropica* infections are linked to the Awash-Methara foci (Hailu *et al*., 2006a), as well as phlebotomine sandfly infections (Gebre-Michael *et al*., 2004) and rodent infections (Kassahun *et al*., 2015). Our findings, however, indicate that bats captured in Mount Elgon's caves had a greater frequency of infection. In a different study conducted in Ethiopia, sandflies that were caught in the research region had *L. tropica* infections (Gebresilassie *et al*., 2015). Despite the fact that *L. tropica* is thought to be anthroponotic, infections in dogs (Baneth *et al*., 2014), red foxes, golden jackals, and rodents (Svobodova *et al*., 2003, and Talmi-Frank *et al*., 2010) have been well recorded typically in zoonotic foci (Sang *et al*., 1994). The discovery of this parasite in both bats and hyraxes raises the prospect of zoonotic transmission in the specific research region and confirms that bats in the current study serve as the natural reservoir hosts for *Leishmania* parasites in the Mt. Elgon *Leishmania* focus.

The fact that the geographical spread of the parasite on Mt. Elgon is significantly greater than anticipated may help to explain the discovery of the highest 49.7% *Leishmania* positive bats in Chemai B among the five caves analysed. We can barely

rule out the chance that bats from *Leishmania* endemic regions may transfer to non-endemic areas since bats have the capacity to migrate from place to place and across a large distance.

In general, a host's function in a reservoir system can be determined by whether or not it meets certain criteria, including the geographical overlap of the distribution of the vectors and hosts, the formation of a large biomass, gregariousness, and longevity, as well as the presence of a naturally occurring infection and the ability to transmit parasites (Ashford, 1996). Some of these processes include bats, and given their propensity to colonise new areas and travel great distances, they would be good bridge hosts for *Leishmania*. Bat colonies are primarily found in caves and crevices, providing suitable conditions for sandfly breeding and nocturnal resting in ambient temperatures and humidity (Feliciangeli, 2004). Bats may be an important potential natural blood supply for sandflies, according to laboratory feeding trials on *Lutzomyia longipalpis*, the most widely disseminated vector of New World VL (Lampo *et al*., 2000).

5.3 Sources of bloodmeal in vectors collected from Mt. Elgon caves

During the current study, it was established that wild reservoir hosts of *Leishmania* in the study area included bats and hyraxes vertebrate species. Between the two vertebrates, majority of the reservoir hosts were bats belonging to five species including *Cardioderma cor, Chaerephon pumilus, Rhinolophus clivosus*, *Nyectris hispida* and *Nycteris nana*. Several research conducted during the past 10 years in Mexico, Brazil, Ethiopia, and Kenya have examined the role of bats as possible hosts for certain *Leishmania* species (Berzunza-Cruz *et al*., 2015; Kassahun *et al*., 2015; Riva *et al*., 2021; Makwali,2021). The fact that bats were infected with *Leishmania*

parasites in all of these investigations emphasises the importance of this nocturnal animal as a potential host of *Leishmania* species in many regions of the world.

The current study supports the work done by Makwali, (2021) who also reported presence of *Leishmania* parasites in bats in Mt. Elgon in Kenya; hence bats are potential reservoir hosts in the transmission of *Leishmania* in the region. Furthermore, bat blood was detected in blood-fed female sandflies and some of the sandflies were infected with *Leishmania* parasites.

The overall prevalence of bats infected with *Leishmania* parasites was 49%**.** In terms of infection per cave, Chepkutuny C cave had the highest recorded prevalence of amastigotes in the bats samples at 80%, followed by Chepkutuny A at 60%, Chepkutuny B 50%, Chemai A at 30% and the least was Chemai B at 25% which were significantly different. In comparison to hyraxes, bat species exhibited a much greater rate of *Leishmania* parasite infections. This could be because there are many bats in the caves where sandflies are also present. Among the species of bats, *Cardioderma cor* exhibited the highest prevalence of *Leishmania* infections, followed by *Chaerephon pumilus* and *Rhinolophus clivosus,* while *Nycteris nana* displayed the lowest rate. In contrast, the combined prevalence of *Leishmania* infections in the liver of the two hyrax species was less than 50% compared to the bat species. Additionally, no amastigotes were observed in their blood specimens. The two hyrax species identified in the collection were *Procavia habessinica* and *Procavia capensis johnstoni,* respectively. *Procacavia capensis johnstoni* and *Procavia habessinica* each had a prevalence of amastigotes of 55.1% and 44.82%, respectively.In a similar study in Israel by Dalit Talro *et al*. (2010) that hyraxes in Maale Adumin in Central Israel had a high prevalence of *L. tropica* infection. A rock hyrax was discovered to be

infected with *Leishmania* parasites in the Utut *Leishmania* area, raising the possibility that it might serve as a reservoir for the illness and cause zoonosis (Sang *et al*., 1994). The parasites were not biochemically identified. So, there is no evidence that these were *L. tropica.* The current study is in agreement with work done in Utut.

According to other research conducted in northern and central Israel, *Leishmania* parasites were present in 10–13% of hyraxes sampled (Jacobson *et al*., 2003; Svobodova *et al*., 2006). 80% of the tested hyraxes in the same study, using Western blot serology assay proved positive for *L. tropica.* In another study, samples of the spleen, abdominal hair, and blood clots from the heart were aseptically taken from naturally deceased bats at the wildlife recovery centre in an effort to find *L. infantum* DNA in the common pipstrelle bat (*Pipistrellus pipistrullus*) from the area of Madrid. 16 of the 27 (59.2%) pipstrelle bats tested positive for *L. infantum*. The scientists found that samples from the spleen had the best sensitivity for detecting the parasite (87%), as well as the maximum number of positive results (14). They also demonstrated that the PCR approach has 100% specificity for finding *L. infantum*.

Three of the 199 bats captured in Mt. Elgon for the current study tested positive for *Leishmania major*, and blood meal analysis from a female *Phlebotomus pedifer* caught in these caves confirmed that this sandfly species sucked blood from bats. Bats' ecology and behavior indicate their crucial role as reservoirs for infectious diseases like Ebola virus (Schur and Jacobson 2004) and different kinetoplastids spread by vectors (Quinnell and Coutenay 2009). Bats may serve as natural blood supply for sandflies after laboratory feeding operation (Lampo *et al*., 2000). Additionally, it is known that bats are hosts to various trypanosomes that are transmitted by sandflies (Williams, 1976). Importantly, because both species live in

caves, bats and sandflies regularly coexist in environments where there is considerable potential for sandflies to prey on bats (Feliciangeli, 2004). Despite efforts elsewhere (Millan *et al*., 2014; Rotureau *et al*., 2006; Rajendran *et al*., 1985; Mutinga, 1975; and Morsy *et al*., 1987), the extent of *Leishmania* natural infection in the Old World bats remains unknown, and cases of Chiropteran *Leishmania* infections had not previously been reported in Kenya (Makwali, 2021); however, this study's confirmation of these cases suggests they do exist.

According to the blood-meal study, phlebotomine sandflies on Mt. Elgon sucked blood from bats, hyraxes, humans, and cats. Additional creatures, including rodents, snakes, monkeys, elephants, and many others that frequent the caves on Mount Elgon may be potential reservoir for the parasites. It appears that the investigated sandfly species indeed have preferred blood-feeding hosts, namely humans and bats. This sandfly species fed on bats, and 120 out of 200 engorged female *Phlebotomus pedifer* *c*apture*d* in the caves of Mt. Elgon, Kenya, had human blood in their systems.Similarly 54 out of 200 engorged sandflies fed on bats blood. These figures, however, were only tallied in the caves of Chepkutuny A, Chepkutuny B, and Chepkutunyi C. This could be explained by the three Chepkutuny caves' proximity to structures used for human living. Similar to this, 9 out of 200 *P. pedifer* samples tested positive for Cat. This was seen in Chepkutuny A, Chepkutunyi B, and Chepkutuny C; this may be because cats commonly visit or live nearby in human settlement in those three caves. Domestic cats undoubtedly spend a lot of time in caves searching for food (rats and mice), where they risk getting sandfly bites.

There is no record of any studies in Kenya in which bats have been specifically screened for *Leishmania* parasites apart from a recent study by Makwali, (2021) and

the current study that have revealed presence of the parasites in the bats. The current study analysed bloodmeal in engorged *P. pedifer* using ELISA test which showed that indeed the shadflies had fed on bat blood (Ombaka *et al.,* 2022)

Understanding disease transmission cycles, possible vectors, and parasite epidemiology are necessary for effective CL management. An important stage in the identification of disease transmission patterns in endemic regions and in the vector incrimination of sandflies is the determination of their natural illnesses (Dehghani *et al*., 2021). The most reliable method for proving natural *Leishmania* infections in sandflies has been microscopy for many years (Quiroga *et al*., 2017). However, this method's sensitivity declines when parasite loads are reduced, and the majority of infections in the vectors are frequently overlooked. The present work used more sensitive and specific serological and molecular approaches in addition to microscopy to identify the source of blood meals and discover parasites in vectors, hosts, and their identifications. Thus, this study offers more up-to-date information on the diversity of sandfly species and the frequency of *Leishmania* infections in vectors from Mt. Elgon caves, in Kenya.

The current study was conducted using descriptive research methods, which entail describing, documenting, analysing, and interpreting existing situations (Sang and Chance 1993). It includes some sort of comparison and contrast and looks for connections between already-existing, unaltered variables. The findings showed that Mt. Elgon has four major sandfly species that are important in epidemiology of *Leishmania*. Three of the four species were of the genus *Phlebotomus,* while one (*Schwetzi*) was from the genus *Sergentomyia*.

Relative humidity may have also influenced the abundance of sandflies in the caves. These flies have previously been found to be perennial in relatively dry climates in Kitui by Minter (1964b) and in Marigat by Basimike (1988). It seems that other factors apart from rainfall influence the occurrence of some of the sandfly species. Sandfly adults were suppressed by heavy rains, such as in June, and their population gradually increased as the rains subsided. But the breeding sites have earlier on been reported by Mutinga *et al*. (1989) to increase during the rainy season in drier areas. This means that as the rains recede the adult population emerges and finally reaches the peak.

5.4 Sandfly species taxonomy by molecular analysis

Real-time PCR-based amplification of the *Leishmania* ITS1 and High-Resolution Melting (HRM) analysis has demonstrated superior sensitivity compared to nested kDNA-PCR and ITS1-PCR for detecting *Leishmania* infections in sandflies. This highly precise approach can also differentiate between *L. major*, *L. tropica*, and mixed infections based on the melting temperatures of the infections (Talmi-Frank *et al*., 2010). Real-time PCR is particularly suitable for cutaneous *Leishmania* (CL) parasite screening and diagnosis due to its high specificity and sensitivity, especially in endemic areas where multiple *Leishmania* species coexist. For epidemiological research on *Leishmania* parasites in naturally captured sandflies, a combination of several molecular approaches is beneficial for precise identification and characterization of the infecting parasites. Further analysis of infection in samples of field-collected sandflies may yield unexpected outcomes, such as co-infections.

Initially, *Leishmania* parasite infection status was determined using microscopy, which remains an invaluable and useful method for demonstrating the presence of

parasites in tissues (Kobets *et al*., 2010). Moreover, given that Mount Elgon is recognized for having CL cases, further research is warranted to ascertain whether *P. pedifer* and *P. elgonensis* serve as possible permissive vectors of *L. major*, based on genetic identification of *L. major* and *L. tropica* infections.

The increasing cases of cutaneous leishmaniasis (CL) in Mount Elgon may be attributed to the high prevalence of sandfly infections as well as the diversity and abundance of vectors. As indicated in current and previous publications (Njau, 2010; Anjili *et al*., 2011), the identification of *L. tropica* in *P. pedifer* supports the hypothesis that this sandfly is the vector of *L. tropica* and consequently, CL in Mount Elgon, Kenya. The detection of *L. major* in *P. pedifer* also suggests that the sandfly may be a potential permissive vector for both cutaneous and visceral *Leishmania* parasites.

In order to implicate sandflies as vectors, it must first be shown that sandflies carry the *Leishmania* parasite. Epidemiological scepticism is raised about a sandfly species' potential involvement as a vector if it occurs often in an area with high *Leishmania* prevalence and behaves anthropophilically (Berdjane-Brouket *et al*., 2012). If the same species is discovered to have live transmissible promastigotes similar to those that are isolated from the vertebrate reservoirs, this theory is supported. Apparently, sandflies are known to be infected with *Leishmania* like parasites. In this work, we used microscopy to show that *Leishmania* promastigotes were present in the mid-guts of all the dissected female sandflies. Although microscopy has long been the gold standard for identifying *Leishmania* infections in sandflies (Quiroga *et al*., 2017), its sensitivity declines over time as the sandfly parasite burden rises. This limited sensitivity may be the reason why the infected sandflies in the current investigation

showed few viable infections. Due to its constrained scope and practical difficulties, this study did not collect sandflies from human residences.

We discovered *Leishmania* spp. by the use of ITS1-PCR and sequencing, which is consistent with a research conducted in Eastern Kenya (Nzeu *et al*., 2014).

The likelihood of disease transmission in the study area of Mt. Elgon can be estimated by comparing *Leishmania* species isolated from humans with those found in sandflies.

Leishmania spp. ITS1 sequences established in the current study were subjected to phylogenetic analysis, which indicated that the sequences were closely related to other *Leishmania* species isolated from humans (Odiwuor *et al*., 2012). With bootstrap support values of 99%, the *L. major* sequence clustered and was closely linked to the *L. major* sequence that was recovered from a CL patient in Ghana (Fryuff *et al*., 2006). The sequence of *L. tropica* that we discovered was closely linked to other *L. tropica* sequences isolated from people. The division of the two sequences into monophyletic groups, however, would indicate that the lineage sorting was not complete.

The presence of *Leishmania major* and *L. tropica* in *P. pedifer* sandflies from Mt. Elgon is consistent with studies conducted elsewhere and in other parts of Kenya (Mutinga *et al*., 1994; Jaouadi *et al*., 2015; Campino *et al*., 2013; Nzelu *et al*., 2014) that revealed *Leishmania* spp., human pathogens, to be naturally present in these sandflies

Sergentomyia sandflies are known to harbor *Leishmania* like parasites (Senghor *et al*., 2016).We discovered *Leishmania* parasite infections in *S. schwetzi,* notwithstanding Sadlova *et al*. 2013's claim that *Leishmania* spp. that were harmful to humans cannot

undergo growth to maturity in *S. schwetzi*. More laboratory and field research is required to determine *S. schwetzi's* suitability as a possible vector for the spread of *L. major* and *L. tropica.*

The findings of this study show that *P. pedifer* may have a role in the spread of *Leishmania* species in Kenya, and they also offer helpful information in understanding the epidemiology of the illness and developing intervention measures.

The presence of *L. tropica* and *L. Major* in *P. pedifer,* which preys mostly on humans, provides additional evidence that this sandfly species may be a potential vector for the CL and VL *Leishmania* species in the study area, Mt. Elgon region in Kenya.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

A total of 5688 sandflies 4403 female and 1285 male belonging to two different subgenera: subgenus *paraphlebotomus* and subgenus *Sergentomyia* species were collected from the sampledcaves in Mt Elgon Sub-County

- Four sandfly species were collected from study sites namely *Phlebotomus pedifer, P. elgonensis, P. longipies* and *Sergentomyia schwetzi* with *P.pedifer* being the most abundant species in all he study sites. These four sandfly species showed a significant difference in their distribution in the different caves

  Light traps are effective in capturing live sandflies, which are necessary for dissection in order to find *Leishmania* parasites.

  In terms of seasonal distribution a larger proportion of sandflies was collected during the dry season than in wet season

  In all the sampled sites, *P. pedifer* female constituted significantly higher prevalence (78.25%) than male (22.75%) of the collections.

- The study found high *Leishmania* parasite infections in sandfly vector species, with a large proportion of *P. pedifer* being the most infected. There was a significant difference in the prevalence of *Leishmania* parasite infection in sandfly vector species in the cave habitats

  Abundance of *Leishmania* parasite infections in sandfly vector species in sampled Mt. Elgon cave habitats showed that infections in *P. pedifer* was

significantly high in Chepkutuny C (1974.67±6.43) followed by Chepkutuny B (1075.67±5.03) while the lowest was recorded in Chemai B (265.67±20.60).

The prevalence of *Leishmania* parasites infections in sandflies *P. pedifer*, *P. elgonensis, p. longipes* and *S.schwetzi* showed a decreasing trend of 9.21%, 8.01%, 4.51% and 6.78% respectively in the sampled caves.

The overall percentage of sandflies infected with *Leishmania* was 7.13%. When the prevalence of *Leishmania* infection for each species of sandfly vector was computed, it was found that a large proportion of *P. pedifer* was infected with *Leishmania* with a prevalence of 9.21%. Therefore, in this study, it was also demonstrated that *P. pedifer*is the most abundant sandfly species distributed across the assessed caves in Mt. Elgon.

- It was established that there are two main wild reservoir hosts; bats and hyraxes that were the main source of blood meal for the sandflies in the study area. There was a significant difference in the source of blood meal in sandfly vectors collected from Mt. Elgon caves

The reservoir hosts identified in Mt Elgon during this study were two, the bats and hyraxes. The five species of bats were; *Cardioderma cor, Chaerephon pumilus, Rhinolophus clivosus, Nycteris hispida* and *Nycteris nana* while the hyraxes that were captured belonged to two species namely *Procavia habessinica* and *Procavia capensis johnstoni*. In terms of bats species distribution across the sites, Chepkutuny C had a higher proportion of bats (49.74%) compared to the other sites

Given that the evaluated vectors do not only feed on people, the preference of sandflies for blood meals as hosts raised the prospect of zoonotic transmission of *Leishmania* parasites in the tested Mt. Elgon caves.

The following findings were validated when linking the sandfly vector and wild reservoir hosts to the spread of cutaneous leishmaniasis (CL) in the Mt. Elgon region: In the cave environments, two wild reservoir hosts (bats and hyraxes) and one vector species (*P. pedifer* sandfly) were implicated in the transmission of *Lieshmania* parasites. The current study further verified that in Mt Elgon CL foci, there is ongoing transmission of the *Leishmania* parasite between people, wild reservoir hosts, and phlebotomine sandflies. These findings imply that both zoonotic and anthroponotic transmission of CL occurs in the studied region. Therefore, efforts should be made to interrupt the *Leishmania* parasites' life cycle by focusing on their human hosts, sandfly vectors, and reservoir animals.

- It was demonstrated through molecular techniques that two sandfly species; *P. pedifer* and *P. elogonensis* inhabited the caves. Molecular techniques also showed that *L. tropica* and *L. major* were the parasites found in the caves. Four sandfly species were identified morphological and out of these, only two; *P. pedifer* and *P. elgonensis* were further identified by molecular techniques due to limited finance.

This study demonstrated, for the first time, the detection of *L. major* and *L. tropica* in *P. pedifer* sandflies from Mt.Elgon, Kenya.

6.2 Recommendations

In attempting to incriminate the sandfly vector and reservoir hosts transmitting cutaneous leishmaniasis in Mt. Elgon region, the following should be undertaken;

- Implement focused vector control programs to reduce sandfly populations, especially in high-risk areas like caves, to mitigate *Leishmania* transmission.
- Establish comprehensive surveillance measures to monitor the prevalence and distribution of *Leishmania* parasites, sandfly vector species, and reservoir hosts in Mt. Elgon, Kenya.
- Implement measures to manage and control reservoir hosts, such as bats and hyraxes, to curb the spread of *Leishmania* parasites and minimize the risk of infection.
- Increase public awareness about *Leishmania* transmission and prevention methods, particularly targeting residents and tourists visiting caves in Mt. Elgon, to promote proactive measures.
- It is necessary to explore the host's susceptibility and establish their infectivity by the vectors in order to corroborate the involvement of reservoir hosts in the *Leishmania* life cycle; these tasks were outside the purview of the current investigation and therefore more research needed on the same
- *P. pedifer* further's demonstration of both *L. major* and *L. tropica* shows that it may be a potential vector of the parasites, therefore they shoud be culured for futher characterization in order to be sure.

- The findings of this study will be useful to health officials (the department of public health) in the Mt. Elgon region for improved planning of sandfly management measures aimed at preventing cutaneous leishmaniasis there.

- Additional research is required to ascertain the role of *P. pedifer* in the transmission cycle of the parasites *Leishmania tropica* and *Leishmania major*. This can be done by examining the parasites' developmental stages in particular sandflies that were collected in the field and by conducting transmission experiments.

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APPPENDICES

Appendix I: Preparation of Clearing and Mounting Medium

Clearing Medium

Preparation of chloral hydrate and phenol

- Saturate solution of chloral hydrate (using light heat)
- Dissolve the phenol using a water bath
- Mix equal volumes of chloral hydrate and phenol together in a dark bottle.

Mounting Medium

Puri's Medium

Distilled water…………………………… 10 ml

Gum Arabic ……………………………… 8 gm

Chloral hydrate …………………………… 70 gm

Glycerin…………………………………… 5 ml

Acetic acid ……………………………….. 3 ml

Using a water bath (80˚C), dissolve gum in water. Add chloral hydrate while stirring. Add glycerin and acetic acid. Filter the solution into a dark bottle.

Appendix II: Blood Meal ELISA for Determination of Host Preference

PREPARATION OF SOLUTIONS

Phosphate buffered saline (PBS), pH 7.4

Use stock laboratory PBS or add 1 bottle Dulbeccols PBS to 1litre distilled water. Mix and adjust pH if necessary. Store all of the following solutions at 4□C.

Boiling Casein, 0.5% (BC)	500ml	1litre
Casein (Baker no. E397-07)	2.50gs	5.0gs
0.1N NaoH	50.00ml	100ml
PBS, pH 7.4	450.00ml	900.00ml
Thermesol 1g/10ml dH_2O or powder 0.50ml 1.00ml		
	0.05gms	0.10gms
phenol red 1gm/10ml dH_2O or powder	0.10ml 0.20ml	
	0.01gm	0.02gm

Suspend casein in 0.1N NaOH and bring to a boil.

After casein is dissolved, slowly add the PBS. Allow cooling and adjusting the pH to acidic with HCl.

Add the thermersol and phenol red.

Shelf-life one week.

Wash solution (PBS-Tween)

PBS plus Tween. Add 0.5ml of Tween - 20 to 1 litre of PBS. Mix well. Do not store, make every day.

Enzyme Diluent (BC-Tween)

100ml BC +25µl Tween 20. Do not store; make every day.

TESTING FOR HUMAN, BAT, HYRAX AND CAT BLOOD SOURCE HOSTS

Sample preparation

Negative control - grind individual male sandfly in 500 µl PBS

Positive control - for each host serum: To 500 µl PBS, add 5□l host serum control.

To PVC flex plate (Dynatech) add:

-Column 1:50 µl / well of eight negative controls

-Column 2: 50 µl /well of eight positive controls i.e. Chicken, Human, Pig, Cat, Horse, Cow, Goat, bat, hyrax and Dog)

One well should be designated as a blank control and receive 50 µl of PBS alone. The remaining wells of the plate should receive 50 µl / well of sandfly blood meal sample. Incubate overnight. Wash the plate with PBS-Tween (wash solution 3) two times.

Enzyme-conjugate preparation

(Stock solutions are 0.5mg/ml)

1. To 5ml of BC-Tween (enzyme dilute, 4), add:

-HRP phosphate anti- Human: 2.5 µl (1:2000 dilutions)

-Bat, hyrax and cat hosphate conjugate: 20.0 µl (1:250 dilution)

-Sera of each host except the one being tested for: 10 µl (1:500 dilution).

N/B Add serum from all hosts except those for which enzyme conjugate was added

2. All conjugates should be diluted 1:2000. 2.5 µl of peroxidase conjugate.

Cow which is phosphate conjugate should be diluted 1: 250 = 20 µl of conjugate (bovine).

3. Add 50 µl /well of prepared enzyme conjugate solution.

4. Incubate for 1hour.

5. Wash the plate with PBS-Tween three times.

Peroxidase substrate preparation

Mix solution A and B together in 1:1 i.e. 5ml = 5ml per plate.

6. Add 100 µl of peroxidase substrate in each well.

7. Incubate for 30 minutes. Read absorbance at 414nm.

To observe readings

8. Wash plate with PBS-Tween three times.

Add two tablets + 8ml of dH_2O+ 2ml diethanolamine buffer. This is the phosphate substrate

9. Add 100 µl of the phosphate substrate to each well.

10. Incubate for 5 hours and read absorbance at 414 nm.

Appendix III: Plates for Lab and Field Work

Plate:

Type of ELISA: bloodmeal template record sheet

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	-ve	Cat										
B	**-ve**	Goat										
C	-ve	Human										
D	-ve	Bat										
E	-ve	Hyrax										
F	-ve	**Cow**										
G	-ve	**Pig**										
H	-ve	**Chicken**										

Positives

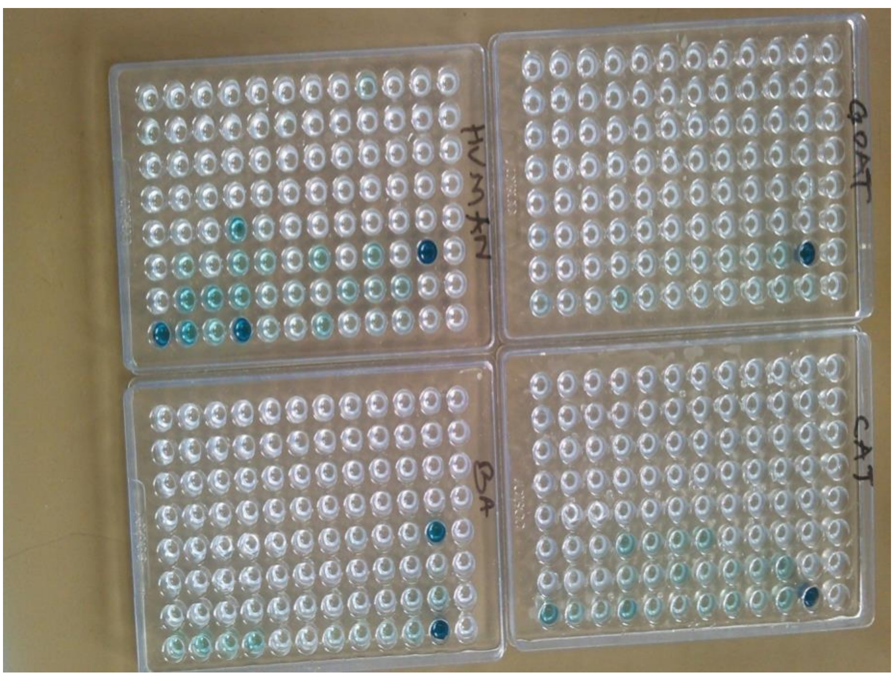
Human: 4A, 7A, 9A, 11A, 12A

Cat: 3B, 7B, 3A, 5A, 6A, 9A, 12A

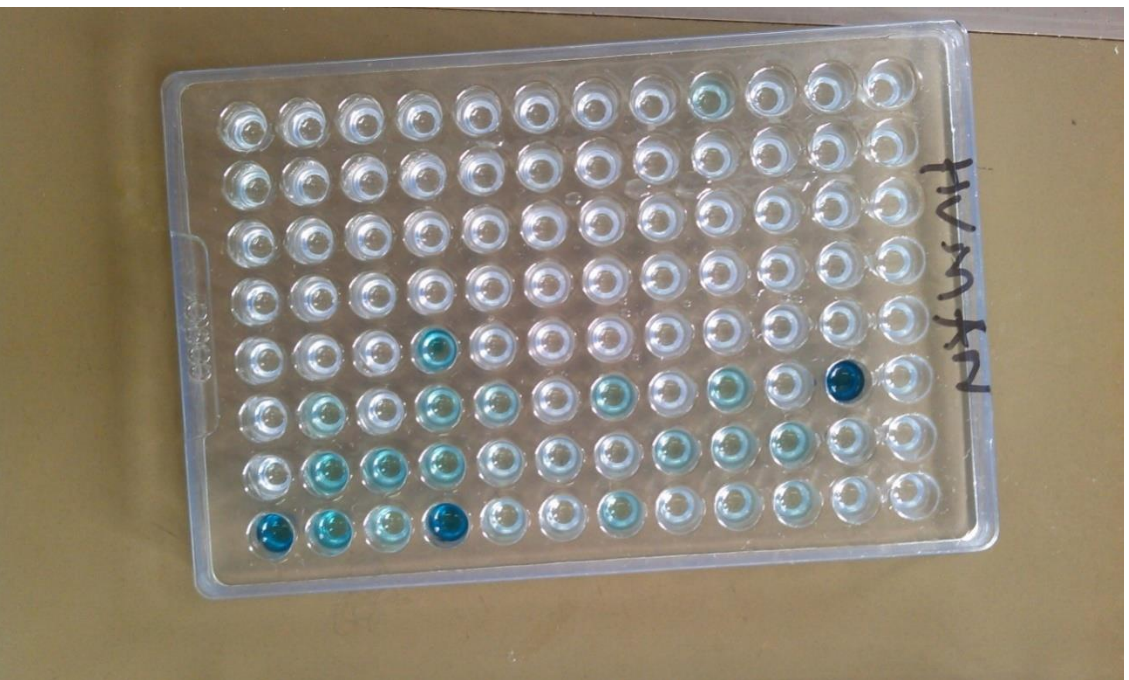
Goat: 3B, 7A, 12A

Bat: 7A, 8A, 12A

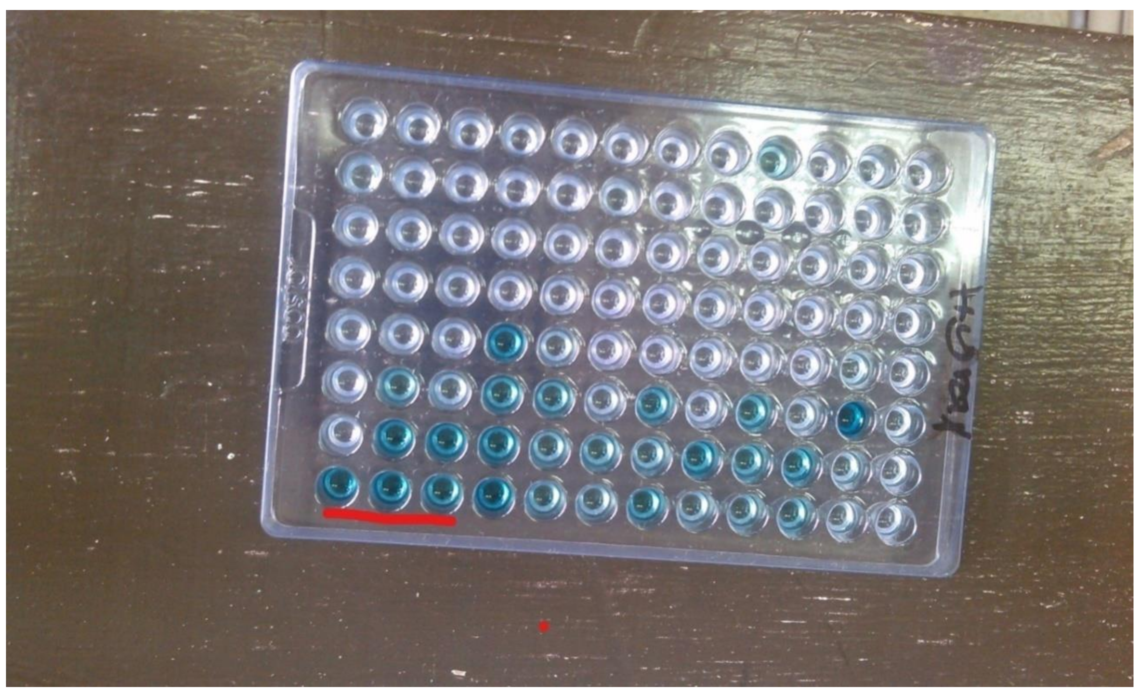
Hyrax: 13B, 14B



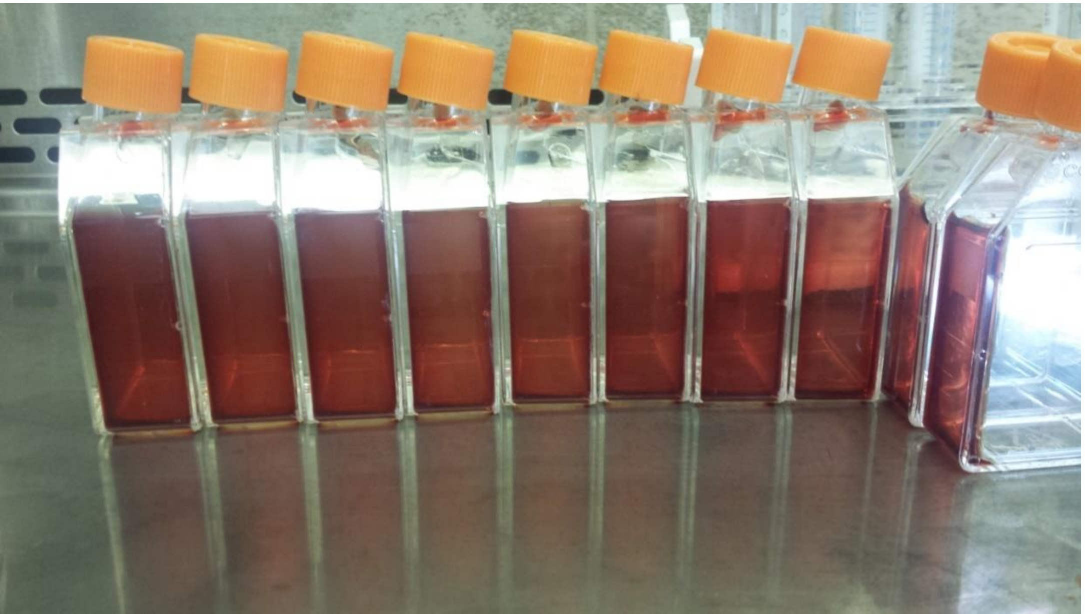
This shows positive blood meals from the above mentioned animals bat, human, goat, and cat.



The dark blue is positive controls and the light blue are sand flies that were found to have fed on human blood



The three marked red are positive control from hyrax blood. The rest shows that sandflies had positive blood meal from hyrax.

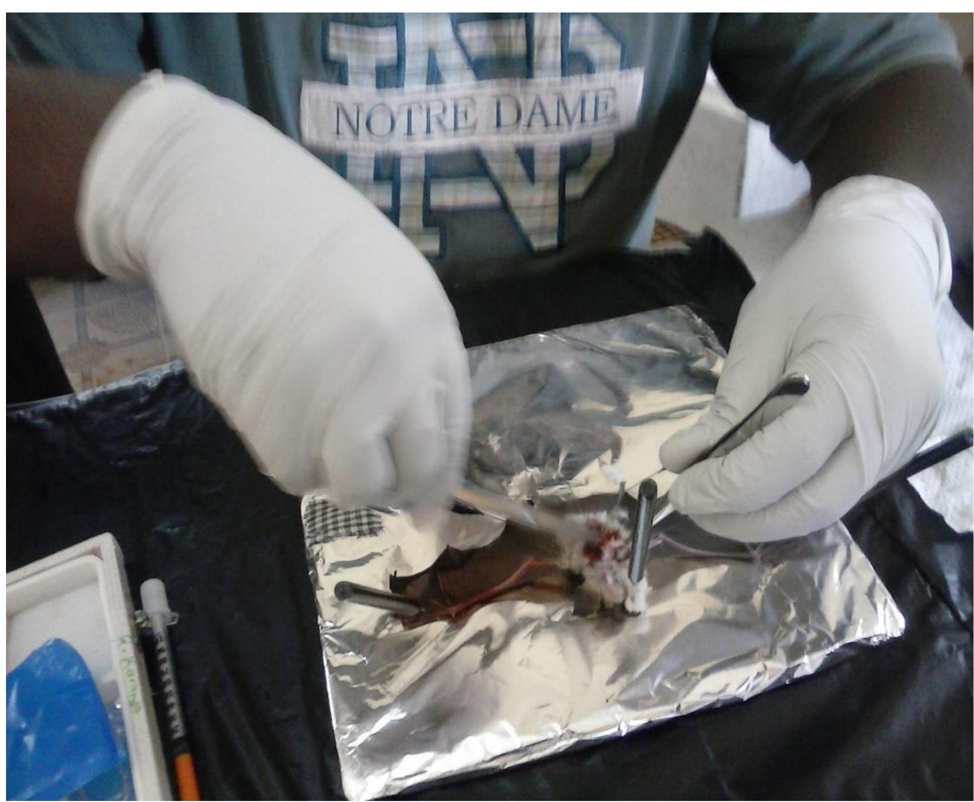


NNN MEDIA

Appendix IV: CDC Trap Lying

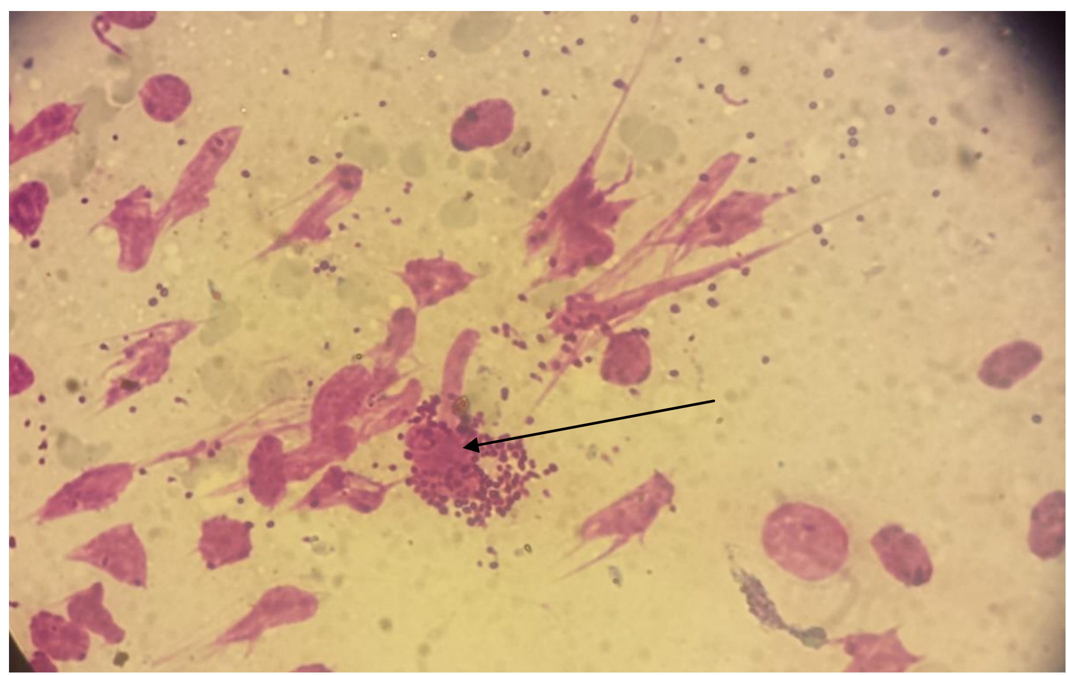
CDC SANDFLY TRAP SET IN ONE OF THE CAVES (Source: Author, 2017)

Appendix V: Preparation and dissection of bat

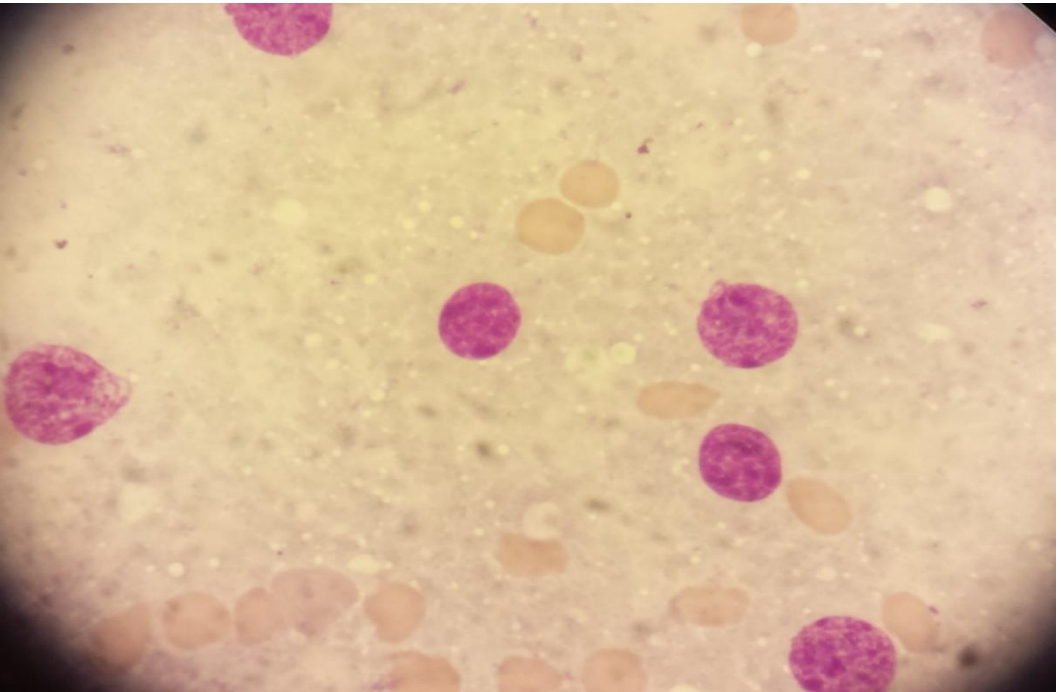


Disection of bat (*Cardioderma cor*) in the lab (Source: Author, 2017)

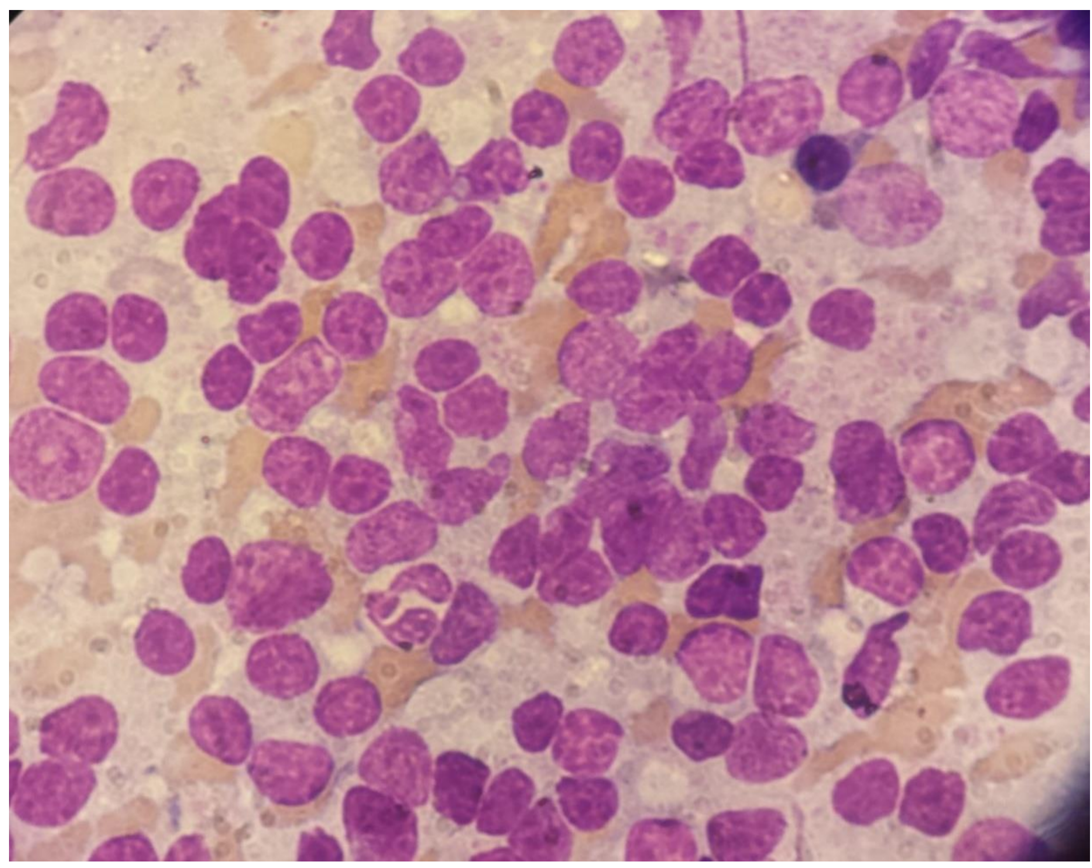
Appendix VI: Slides presentation



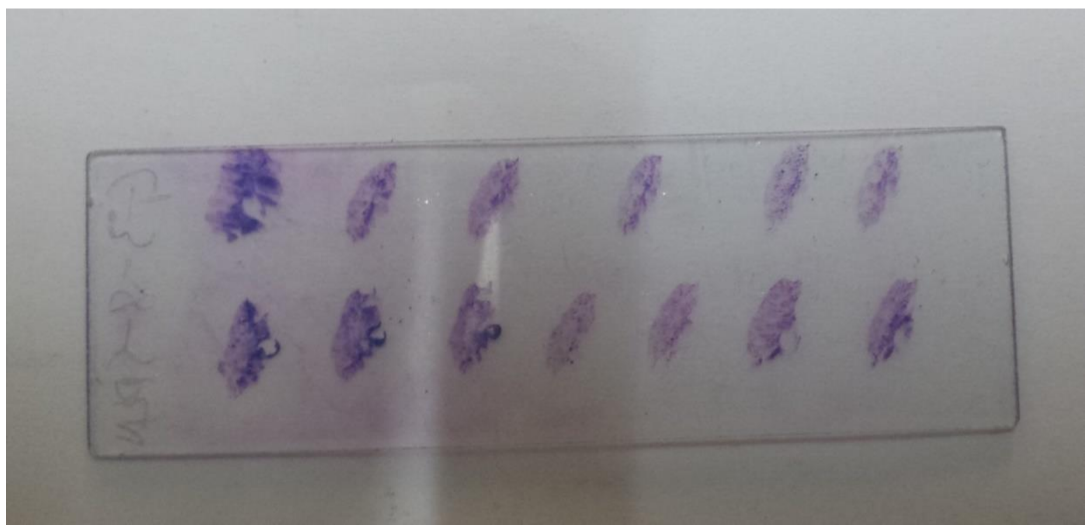
Positive slide with amastigotes in bat macrophages and some outside the cells



Liver impression of a negative smear

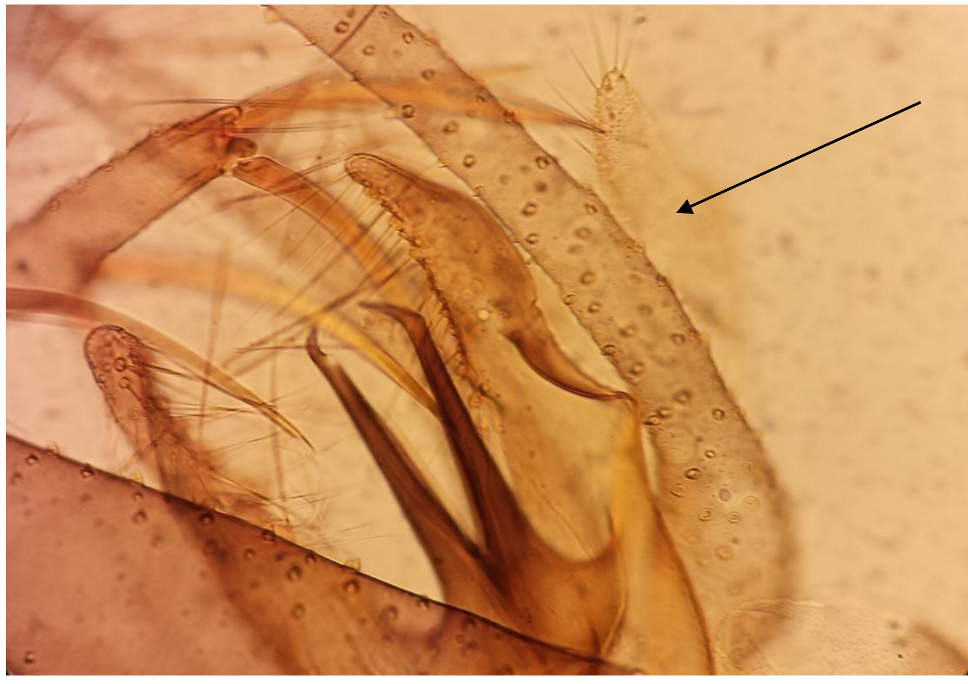


Splenic impression of a negative smear

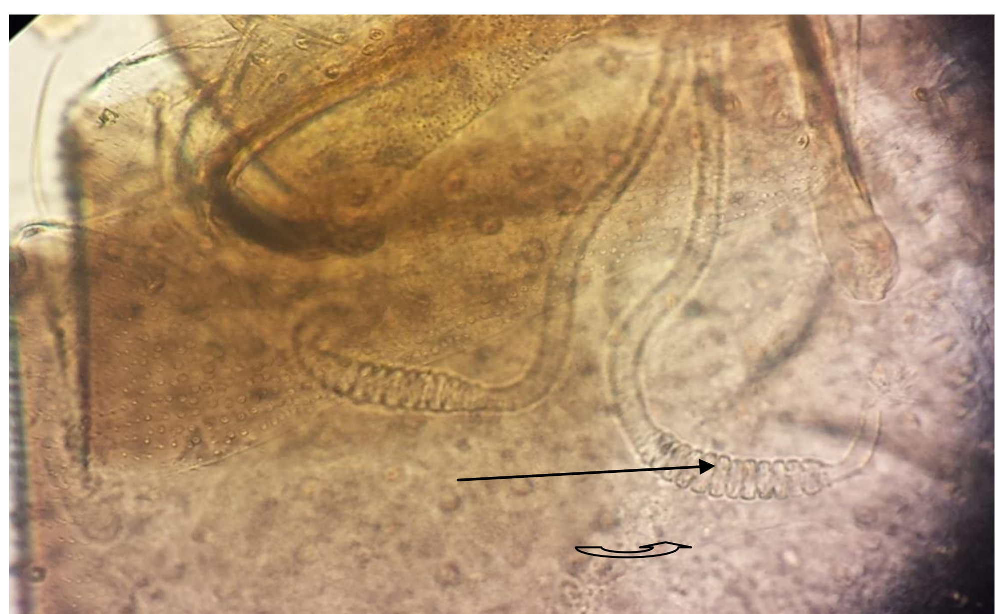


Splenic impressions on a slide

AppendixVII: Male and female *P. pedifer* Genitalia

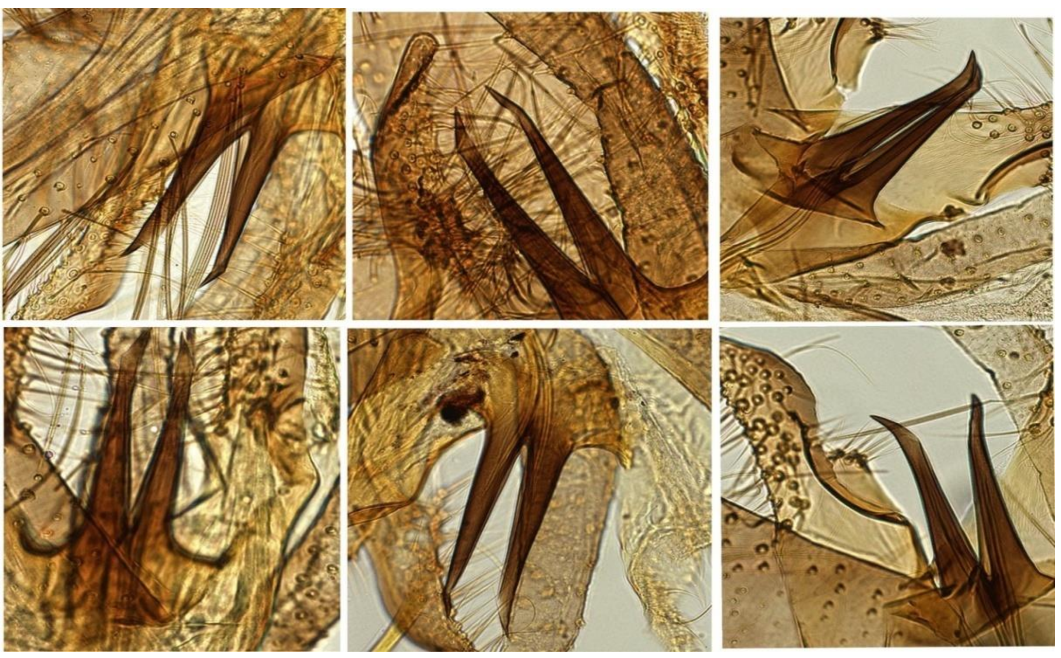


Male *P pedifer* genitalia: Genital filaments



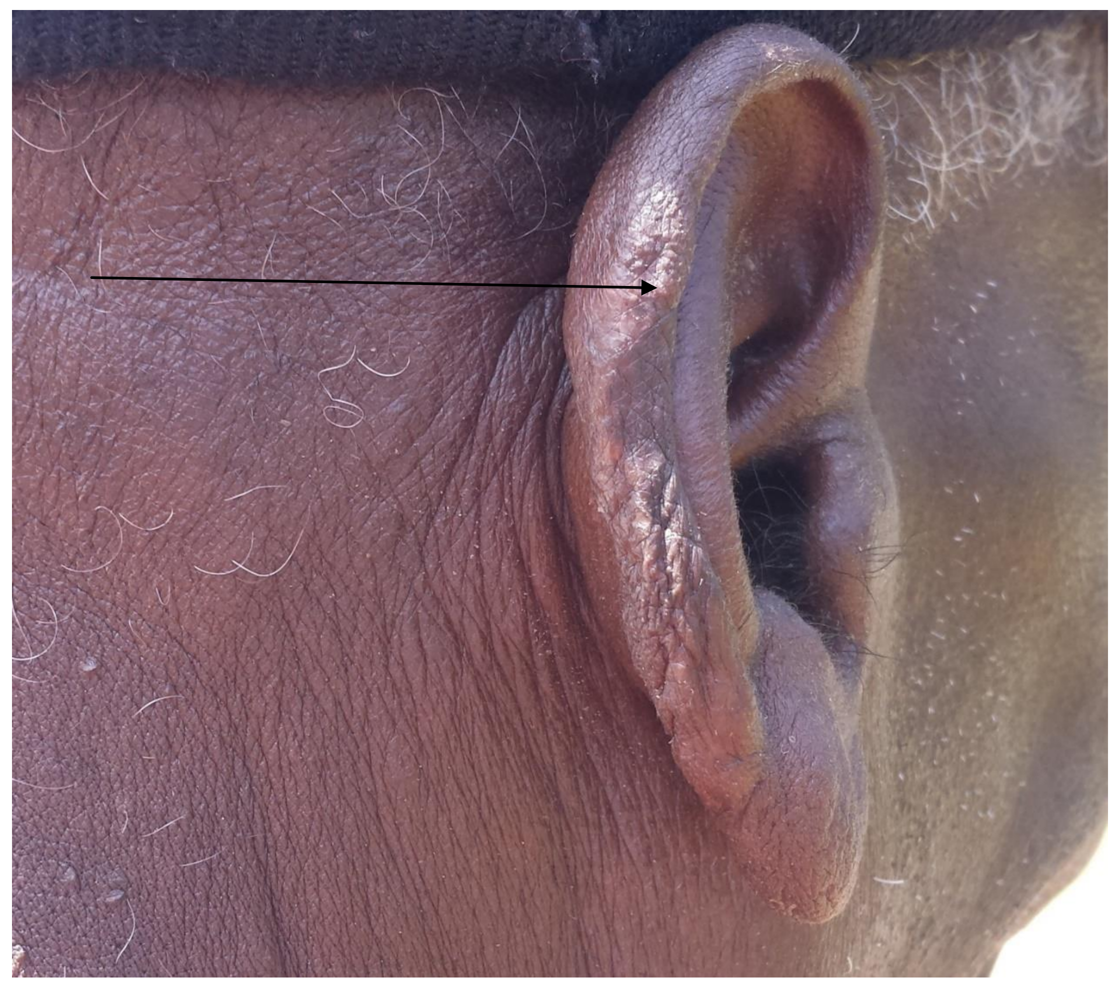
Spermatheca of female *P. pedifer*

AppendixVIII: Aedeagi of *P. Phlebotomus* and *P. longipes*



Aedeagi of male *Phlebotomus longipes* (left two columns 1 and 2) and *P. pedifer* (right column 3). *Phlebotomus longipes* was acquired from Mt. Elgon caves.

Appendix IX: Cutaneous lession on one of the residence of the study area

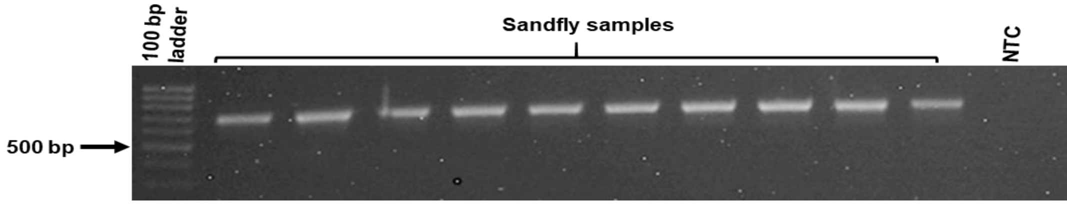


Cutaneous lession on the ear of one of the residents of the study area

(Source: Author, 2017)

Appendix X: PCR analysis

Supplementary figure



S1; Fig: Molecular identification of sandfly species based on PCR analysis of a 700-bp COI gene. NTC: non-template control.

Appendix XI: Rock hyraxes

Rock hyrax (*Procavia capensis*) photographed using a ReconyxHyperfire H500 camera trap. (Source: Author, 2017)

Bush hyrax (*Procavia habessinica*) photographed using a ReconyxHyperfire H500/H600 camera trap. (Source: Author, 2017)

Appendix XII   Bats of Mt Elgon

Plate 4.1: *Cardioderma cor* (Heart-nosed Bat). Source: Author, 2017

Plate 4.2: *Nycteris hispida* (Hairy Slit-faced Bat). Source: Author, 2017

Plate 4.3: *Chaerephon pumilus* (Little free-tailed bat). Source: Author, 2017

Plate 4.4: *Nyecteris nana* (Dark-winged lesser House Bat). Source: Author, 2017

Appendix XIII: Wildlife permits

WRTI/RP/118.6

9th September 2021

Kennedy Ombula Ombaka
University of Eldoret
P.O. Box 1125-30100
Eldoret.
Email: ombulaken@gmail.com

Dear Kennedy,

Research Permit

We acknowledge your application for a permit to regularize your PhD research, which you undertook without a permit.

We note that your research was under the University of Eldoret and titled ***'Molecular identification and serological detection of Leishmania infection in naturally infected sand fly and bat species in Mount Elgon, Kenya'.***

Having considered your request on the grounds of unawareness of the requirements, you are granted a permit ref No. **WRTI-0089-08-21 for reference to your research outputs**, hence the permit is not valid for field-work beyond the date of Issue. You are to pay WRTI research fee of **Ksh 12,000**.

We hope your study will enhance the understanding of host-vector interactions and transmission of cutaneous Leishmaniasis in Kenya.

You will submit final reports of your research findings to the undersigned.

Yours Sincerely,

DR. PATRICK OMONDI, OGW
AG. DIRECTOR/ CEO

Copy to: WRTI Head Research Permitting & Compliance

WILDLIFE RESEARCH & TRAINING INSTITUTE

P.O. Box 842 – 20117, Naivasha, Kenya – Tel: +254-050-2020577 / 2020267 / 0700 000 321 / 0731 919 465 E-mail: wrti.kenya@gmail.co

Appendix XIV: Similarity Report

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Paper ID	1988126
Submission Date	2024-06-12 12:46:56

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