Natural infection of ~~sand fly~~ sandfly vector and reservoir hosts species with *Leishmania* parasites in Mt. Elgon cave habitats in Kenya

Abstract

Leishmaniasis is a major public health problem caused by a parasite of the genus *Leishmania* which is transmitted through the bite of an infected female phlebotomine ~~sand fly~~ sandfly. However, the disease epidemiology on interactions of causative agents, reservoir hosts, and vectors in areas where there is a high prevalence of the disease ~~need~~ needs to be properly understood to inform effective control. The present study was conducted to determine natural infection by *Leishmania* parasites in phlebotomine ~~sand fly~~ sandfly vectors and suspected reservoir hosts found in caves in Mount Elgon in Kenya. ~~Sand flies~~ Sandflies, bats and rock hyraxes were found inhabiting the assessed caves in the study area. A total of 5688 ~~sand flies~~ sandflies collected from caves were morphologically identified and analyzed for infectivity with *Leishmania* parasites by molecular characterization sequencing using Cytochrome c oxidase subunit 1 (COI). ~~Giemsa stained~~ Giemsa-stained blood smears and spleen and liver impression smears prepared from bats, and hyraxes collected from the same caves were examined microscopically for *Leishmania* amastigotes. The ~~sand flies~~ sandflies, bats, and ~~the~~ hyraxes were identified morphologically using taxonomic keys. The results showed that randomly selected COI sequences (n = 5) of three different morphologically identified *Phlebotomus* species were positive for *Leishmania* based on their molecular characteristics. Five species of bats were identified, and they were significantly more abundant compared to hyraxes (χ^2 = 16.7845, df = 7, P = 0.0002). The bats' species had a higher prevalence of *Leishmania* parasites compared to hyraxes (F = 7.342, *P* = 0.0043). *Cardioderma cor*, *Chaerephon pumilus* and *Rhinolophus clivosus* bat species had more than 5% *Leishmania* parasite prevalence rate. *Leishmania* amastigote density was significantly higher in the liver (F = 39.232, *P* < 0.001) compared to the density in the blood (F = 75.1124, *P* < 0.001) of bats and hyraxes. This study has shown that transmission of *Leishmania* parasites in Mt. Elgon caves involves vertebrate reservoir hosts (bats and hyraxes) and the insect vector species (sandfly). Therefore, disruption of the life cycle of the *Leishmania* parasites should be done by targeting these reservoir animals and the ~~sand fly~~ sandfly vector.

Keywords: Molecular characterization, sequencing analysis, *Leishmania* parasites, ~~sand fly~~ sandfly vector, reservoir hosts, Mt. Elgon caves (Kenya),

1. Introduction

As a neglected tropical disease (NTD), leishmaniasis is a major public health problem that causes significant morbidity and mortality to humans, especially in ~~the~~ developing countries in the tropical regions of ~~Sub-Saharan~~ Sub-Saharan Africa [1, 2]. The disease is caused by the protozoa parasite of the genus *Leishmania* [3-5]. There are more than 20 flagellate parasites of the genus *Leishmania* subgenera *Leishmania* and *Viannia* which are causative agents of leishmaniasis [6, 7]. The Eurocentric worldview groups *Leishmania* parasites into Old World species: *L. major*, *L. aethiopica*, *L. infantum*, *L. donovani*, and *L. tropica* [8, 9] found in Asia, Africa, and Southern Europe and New World species such as *L. amazonensis*, *L. chagasi*, *L. mexicana*, *L. naiffi*, *L. braziliensis*, and *L. guyanensis* [10] found in South America. In Eastern Africa Region, including Kenya, the more prevalent visceral leishmaniasis (VL) is usually caused by *L. donovani* and *L. infantum* [11, 12]. while the less prevalent cutaneous leishmaniasis is caused by *L. tropica*, *L. major*, and *L. aethiopica* [6, 7]

Phlebotomine ~~sand flies~~ sandflies (Diptera: Psychodidae: Phlebotominae) are a medically important group ~~due to the fact that~~ because they are confirmed as natural vectors of *Leishmania* parasites [13-15]. There are 900 confirmed species of sandflies of which about 10 percent of these species belonging mainly to the genus *Phlebotomus* and *Lutzomyia* have been implicated as the vectors of leishmaniasis [6, 7, 16].

The reservoir hosts of the *Leishmania* parasite help in maintaining the parasite life cycle and hence are central for the transmission of zoonotic and sylvatic infections [17, 18]. Therefore, accurate identification of reservoir hosts is important for targeted control. Based on reservoir hosts, there are two main sources of human leishmaniases: zoonotic leishmaniases, in which the reservoir hosts are wild animals, commensals, or domestic animals, and: anthroponotic leishmaniases, in which the reservoir host is infected human [19, 20]. Several species of wild, domestic, and synanthropic mammals have been recorded as reservoir hosts of *Leishmania* parasites in different parts of the world. These include dogs, rock hyraxes, rodents, ~~mongoose~~ mongooses, cats, foxes, jackals, wolves, porcupines, primates, armadillos, and other domestic animals [21, 22].

The ~~reservoirs~~ reservoir hosts for *Leishmania* parasites are often so complex that they show regional and temporal variations [23], and only local studies involving ecological and parasitological analyses can determine whether the suspected animals are playing a role as reservoirs for the disease agents in a given environment [2, 7, 24]. This is made particularly worse since the role of animals as reservoirs for human leishmaniasis remains unclear in several regions of the world [25, 26]. A successful transmission requires the presence of pathogen, vector, and host species [27, 28]. The transmission and epidemiology of leishmaniasis disease ~~is~~ are complicated due to the complex life cycle of the parasites and the involvement of vectors, and reservoir animals besides human hosts [29]. The transmission of *Leishmania* parasites starts when parasites are picked up by the ~~sand fly~~ sandfly when feeding on the blood of an infected human or an infected animal reservoir [30]. Therefore, analyses of blood meals for ~~sand fly~~ sandfly vectors must be carried out in habitats where reservoir hosts (co)exist with the vector flies. In this study, the infection status of ~~sand fly~~ sandfly vector and suspected reservoirs with *Leishmania* parasites co-existing in Mt. Elgon cave habitats (Kenya) was determined using a combination of taxonomic keys with molecular tools (COI-PCR) to identify sandfly species and reservoir hosts responsible for transmitting CL parasites in Mt Elgon region, Kenya.

2. Materials and Methods

2.1 Study Area

The study was conducted in five caves in Chemai and Chepkutuny area located in Mt Elgon Sub-County in Bungoma County, Western Kenya. The study sites were Chepkutuny A cave (N $00^0$49 881/E034^0 42 994/ elevation 1829 M), Chepkutuny B cave (N $00^0$49 877/E $034^0$43 881/ elevation 1776M), Chepkutuny C cave (N $00^0$45884/ E$034^0$44 998/ elevation 1845M) Chemai A (0º49 881N/034º43 233'E elevation 1827 M), Chemai B (N0°50 546'E $034^0$43 233/ elevation 1801M). This area was selected based on the increase in suspected CL cases among the people seeking medication at health facilities in Mt. Elgon Sub-County Hospital in Kapsokowny and other health facilities in the area. All the study sites had numerous fault scarp ranges, rock crevices, and caves which are potential habitats for sandflies, giant rats, and rock hyraxes (*Procavia capensis*) [31, 32]. There was also evidence suggesting human and animal activities in and around the study sites that included: observation of children playing inside caves, ~~foot prints~~ footprints, and defecations of animals including those of livestock, social evidence such as used condoms

and hyrax nests indicated by their defecation. Consequently, all ~~these evidence suggest~~ this evidence suggests a close interaction of humans, wild animals, livestock, pets, and ~~sand flies~~ sandflies found in the caves.

2.2 Sample size determinations for reservoir hosts

Sample sizes for reservoir hosts (bats and hyraxes) were obtained using the formula:

$$n = \frac{Z^2 P(100 - P)}{d^2}$$

Z = 1.96

d = was taken from any value between 3 and 10

P = Value was based on a prevalence rate of 4.9% *Leishmania* kDNA detection in bats ~~of~~ in a study in Ethiopia [33].

The expected sample size for bats in the study was therefore

$$n = \frac{1.96^2 * 0.049(100 - 0.951)}{0.05^2} = 199$$

2.3 Determination of bat, hyrax, and ~~sand fly~~ sandfly infection status with *Leishmania* parasites

2.3.1 Direct microscopy

Direct microscopy of blood and crushed liver and spleen tissue smears of bats and hyraxes were fixed with methanol~~,~~ and stained with Giemsa for a thin film to view *Leishmania's* bodies (amastigotes of *Leishmania*). *Leishmania* bodies are usually found within the macrophages. Some of the L.D bodies can be seen extra-cellularly, released from the macrophage cells ruptured during the preparation of the film.

In stained preparations, the cytoplasm surrounded by a limiting membrane appears pale – blue. The nucleus is relatively large and stains red. The kinetoplast is situated at the right angle to the nucleus. The kinetoplast is slender, rod-shaped, and ~~is~~ stained deep red. Axoneme arises from the kinetoplast and extends to the margin of the body. The unstained vacuole space is seen alongside the Axoneme [34].

2.3.2 Morphological and molecular identification of ~~sand fly~~ sandfly species

~~Sand flies~~ Sandflies were collected from the caves using CDC light traps. The ~~sand fly~~ sandfly collections were immediately preserved in 70% ethanol. Each fly was dissected into two parts; the first part ~~consisting~~ consisted of the head and terminal which were used for morphological identification according to the morphological taxonomic keys as described by Alves *et al*., 2008) while the other body part was stored at -80˚C until it was used for the molecular identification.

Individual ethanol-fixed specimens were homogenized and lysed using a DNA extraction kit (DNeasy tissue kit, Qiagen, California, USA) following the manufacturer's instructions. The DNA extracts were subjected to the PCR gene amplification.

To verify the morphological identifications of the vectors, the sandfly mitochondrial cytochrome c oxidase subunit 1 (COI) was amplified as previously described [34, 35]. The 700 bp COI amplicons obtained were purified using the QIAGEN PCR purification kit according to the manufacturer's protocol. The COI chromatograms were edited in Unipro UGENE (v 1.3) to obtain consensus sequences for each sample, followed by sequence similarity search using BLAST and multiple sequence alignment using the Clustal W tool in MEGA 7.

Nested kDNA PCR assay

Leishmania parasite screening in the ~~vector was~~ vector was achieved by PCR amplification of the pooled sandfly DNA using primers targeting the *Leishmania* kinetoplast DNA [35, 36]**,**. Kinetoplast DNA was targeted due to its presence in high numbers [37]**.** The PCR reactions were carried out as previously described [38, 39]
The specimens which were found to be negative for *Leishmania* parasites the nested kDNA-PCR were further screened ~~by qPCR-HRM~~ by qPCR-HRM to achieve high sensitivity and specificity in detecting *Leishmania* following workflows as previously described [40, 41]. Agilent Technologies Stratagene Mx3005P ~~real-time~~ real-time PCR thermocycler was used for the assays. The 20 µl final volume contained 1x Luna universal qPCR sybr Green-based master mix (NEB), 500nM of each primer, 1 µl of template**,** and ~~nuclease-free~~ nuclease-free water. Cycling conditions included an initial denaturation at 95^{o}C for 1 minute followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and annealing and extension at 60^{o}C for 20 seconds. HRM was performed by denaturation of the qPCR products at 95^{o}C for 1 minute followed by cooling at 50^{o}C for 30 seconds for re-annealing and gradually raising the temperature by approximately 0.1^{o}C increments per 5 seconds and recording changes in ~~fluorescence~~ fluorescence.

2.3.3 Sandfly COI sequences and phylogenetic analyses

The chromatograms of all sandfly COI sequences were trimmed and edited using Geneious software (v 8.1.8) to obtain consensus sequences for each specimen. The consensus sequences were aligned using MUSCLE with homologous sequences identified by sequence similarity searches in GenBank using the Basic Local Alignment Search Tool (BLAST) as previously described [42]. Maximum-likelihood phylogeny of sandfly COI sequence alignments ~~were~~ was constructed using PhyML version 3.0 employing the general-time-reversible (GTR) sequence evolution model. Tree topologies were estimated for over 1000 bootstrap replicates following previously described workflows [43].

2.3.4 *Leishmania* parasite detection and identification in individual sandfly specimens and vertebrate host blood

To determine whether the sampled sandfly specimens were infected by *Leishmania* parasites, individual specimens were tested by PCR amplification of the *Leishmania* internal transcribed spacer 1 (ITS1) region as previously described [42]. Briefly, the amplification was carried out in a 15-µl final volume containing 1x Dream Taq buffer with 2 mM MgCl2 (Thermo 170 Scientific, USA), 0.25 mM dNTPs mix, 500 nM of each primer, 2 U of Dream Taq DNA polymerase (Thermo Scientific, USA), 5–10 ng of DNA template and nuclease-free water (Sigma, St. Louis, USA). Positive controls (DNA templates) and negative control (nuclease-free water) were included in each PCR reaction system.
Blood samples obtained from various vertebrate hosts (hyrax, human and bat) were similarly analyzed for *Leishmania* parasites to determine their infection and thus their reservoir status.

2.4 Data analyses

Details of all the collected sandflies including the trapping sites were recorded and compared for consistency using SPSS (version 23.0). Descriptive statistics were used to determine the distribution using frequency and percentage for each sandfly species per site. Species abundance was determined as the quantitative counts per trap per trapping night per site. Differences in the species prevalence were analyzed using the Chi-square (χ^2) test. Differences in abundance of parasites in host tissues and blood, sandfly and reservoir hosts species per site**,** and monthly distributions were analyzed using One-Way ANOVA. Significance was determined at $P \leq 0.05$.

3. Results

3.1 *Leishmania* parasite detection and identification

Twelve samples of *Phlebotomus* ~~sand flies~~ sandflies were found to be positive for *Leishmania* parasites based on real-time PCR-HRM and sequencing of the PCR-HRM amplicons. The overall *Leishmania* infection rate in the sampled female sandflies from the study area was 7.07% (n = 14/198). Infection rate in *P. pedifer* was 9.09% (12/132).

The pooled sandfly DNA was found to be positive for *Leishmania* parasites using ITS1-PCR-RFLP. This could be due to the high sensitivity of the ITS1 target, especially in the DNA pools where the potentially positive samples were diluted. However, ITS1-PCR products of the two successfully established parasites' DNA isolated from sandflies produced bands of approximately 320 bp. Digestion of the products with Hae-III gave an RFLP pattern characteristic of *Leishmania* for the isolates (Figure 1).

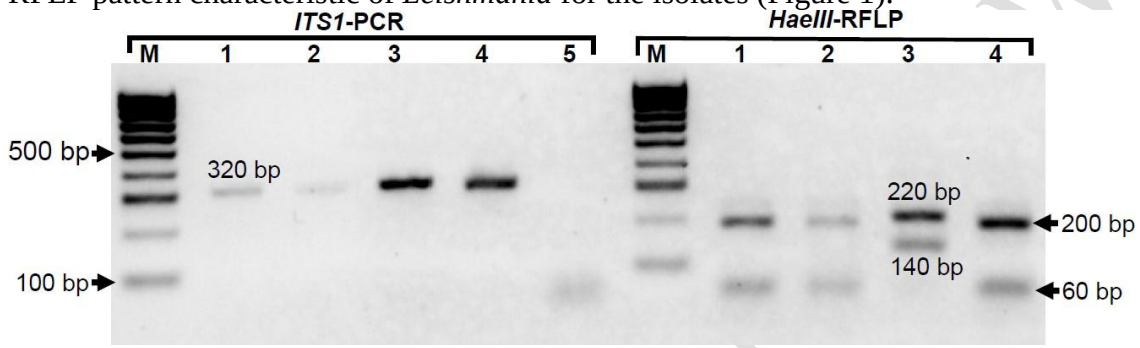

Figure 1. *Leishmania* parasite identification by ITS1-PCR-RFLP

M: 100 bp ladder; 1 and 2: *Leishmania* spp. Isolated from sandflies; 3 and 4: *L. major* (Lv357 strain) positive controls; 5: negative control. Molecular sizes of RFLP product for the species is shown at 60bp to 200 bp with a ladder at 220bp for *L. tropica*.

None of the pooled ~~sand fly~~ sandfly extracted DNA was found to be positive for *Leishmania* species using Nested kDNA-PCR and ~~were~~ was further subjected to ITS1-real-time PCR followed by HRM (qPCR-HRM) *Leishmania* DNA was detected in three sandfly DNA pools using this method. Confirmation of *Leishmania* species in individual sandflies belonging to these pools revealed *L. tropica* in three samples belonging to *P. pedifer* species. The HRM profile of *L. major* found in one sandfly varied slightly from that of the positive control.

3.2 Prevalence of bats infected with *Leishmania* parasites in Mt. Elgon caves:

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 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= 0.0000). Bat species had a 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 the prevalence of *Leishmania* infection (χ^2= 0.9653, d.f.=1, p= 0.3258) as illustrated in Table 1.

Table1: Wild reservoir host species infection prevalence with *Leishmania* parasites

Host	Species	Prevalence (%) with	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

3.3 The *Leishmania* amastigote intensity in reservoir hosts species in Mt. Elgon caves based on liver and blood analysis:

The *Leishmania* amastigote intensity in reservoir ~~hosts~~ host species collected during the survey based on liver and blood analysis are shown in Table 2. Density of *Leishmania* amastigote in bats was 16080.00±18704.2) while that in hyrax was 290.0±42.43) with no significant difference (t = 1.1281, p = 0.31048)

Among the species of bats, *Cardioderma cor* and *Chaerephon pumilus* had the highest amastigote density in the liver. Meanwhile, the two species of hyraxes had less than 50% amastigote density compared to the bat species and in the blood species there was no amastigote observed.

Table 2: Intensity of *Leishmania* amastigote parasite infection of the reservoir in the liver

Species	Species	Intensity ~~of~~*~~Leishmania~~* of *leishmania* parasites
Bats	*Cardioderma cor*	4.5×10^4
	Chaerephon pumilus	2.5×10^4
	Rhinolophus clivosus	5.3×10^3
	Nyectris hispida	3.3×10^3
	Nycteris nana	1.8×10^3
Hyraxes	*Procavia habessinica*	2.6×10^2
	Procavia capensis johnstoni	3.2×10^2

4. Discussion

Real-time PCR-based amplification of the *Leishmania* ITS1, followed by HRM, was found to be highly sensitive in identifying *Leishmania* infections in sandflies over the nested kDNA-PCR and the ITS1-PCR alone. Furthermore, this technique is highly specific in discriminating between *L. major*, *L. tropica*, and mixed infections based on their melting temperatures. The high sensitivity and specificity of real-time PCR highlight its suitability in screening and diagnosis of CL parasites, especially in endemic regions where multiple *Leishmania* species may coexist. Combining different molecular methods for the epidemiological studies of *Leishmania* in field-caught sandflies is useful for the accurate detection and characterization of the infecting parasites. Direct analysis of infection in field-collected samples may reveal unexpected results including co-infections. Moreover, molecular identification of *L. major* and *L. tropica* infections in *P. pedifer* suggested these

sandfly species may be the potential permissive vector of *L. major*, which needs to be investigated further.

The high infection prevalence in sandflies and the high vector abundance and diversity could ~~possibly~~ explain the increasing cases of CL in the Mt Elgon region. Identification of *L. tropica* in *P. pedifer* confirms this sandfly as the vector of *L. tropica* in Mt. Elgon, Kenya as observed in previous reports [32, 44]. However, *L. major* ~~was also~~ has also been identified from *P. pedifer* species, suggesting that it could be a potential permissive vector for both the CL and VL parasites.
~~Density~~ The density of *Leishmania* amastigote in bats and hyraxes ~~were~~ was significantly different. Among the species of bats, *Cardioderma cor* and *Chaerephon pumilus* had the highest amastigote density in the liver. Meanwhile, the two species of hyraxes had less than 50% amastigote density compared to the bat species and in the blood species there was no amastigote observed. This ~~is in agreement~~ agrees with studies reported elsewhere [19].

Human populations were the most preferred hosts as indicated by the high number of sandflies (55%) that ~~had~~ fed on humans [45]. This significant preference for human hosts further suggested possible transmission of *L. tropica* and possibly *L. major* in the study area. This possibility is supported by the finding of Leishmania parasites in the ~~sand flies~~ sandflies collected from caves in the study area. Bats were the second largest blood meal source constituting 30% of sandfly blood meals and the sandflies that fed on rock hyraxes constituted only 8%. ~~Presence~~ The presence of bats and rock hyraxes ~~in close proximity to~~ close to humans within caves and in abundance appears to provide an alternative source of blood meal which aids in the amplification of *Leishmania* parasites within the sandfly populations which compares to another earlier study [46].

Bats have adequate features to be naturally infected by *Leishmania* parasites and may play a role in the epidemiological cycle of leishmaniasis. The present study revealed natural *Leishmania* infection in bats. Current finding ~~indicate~~ indicates the importance of bats in the leishmaniasis disease transmission cycle in Mt. Elgon region.
Leishmaniasis continues to be an emerging disease affecting the poorest of the poor if not controlled. In incriminating the ~~sand fly~~ sandfly vector and wild reservoir hosts in the transmission of cutaneous leishmaniasis in Mt. Elgon region, the following observations were confirmed: Transmission of ~~*Lieshmania*~~ *Leishmania* parasites involved two wild reservoir hosts (bats and hyraxes) and one vector species (*P. pedifer* sandfly). Therefore, disruption of the life cycle of the *Leishmania* parasites should be undertaken by targeting the ~~sand fly~~ sandfly vector and the reservoir animals.

Ethical approval
Permission was granted by Kenya Wildlife Service (KWS) under the permit number (WRTI-0089-08-21) for the collection and use of research animals in Mt Elgon for this research work

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