Universiteit Antwerpen

Faculty Sciences
Department Biology

Transmission dynamics, distribution and diagnostics of cutaneous leishmaniasis in southwestern Ethiopia: a basis for disease management

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de wetenschappen: biologie aan de Universiteit Antwerpen
te verdedigen door

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PART I
INTRODUCTION

SUMMARY – SAMENVATTING

Summary

Cutaneous leishmaniasis (CL) is a neglected tropical disease characterized by nodular and crusty skin lesions, mainly on people's face and extremities, resulting in disfiguring scars after healing. It is caused by *Leishmania* parasites, which are transmitted by female phlebotomine sand flies during blood feeding. The disease is a major public health problem in the Ethiopian Rift Valley, where *Leishmania aethiopica* affects approximately 20,000 to 50,000 people annually. Previous reports indicate that transmission of *L. aethiopica* is zoonotic, with hyraxes serving as reservoirs. These animals share their rocky habitat with the main CL vectors, *Phlebotomus pedifer* in the south and *P. longipes* in the center and north of Ethiopia.

In this PhD thesis the three main objectives were *i)* to explore the ecology, transmission dynamics and distribution of *L. aethiopica* in southwestern Ethiopia, *ii)* to validate molecular assays for detection of *L. aethiopica* and assess which assay is most appropriate for particular sample types or research purposes, and *iii)* to determine whether the recently introduced protein profiling technique, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is a good method for unambiguous sand fly species determination in Ethiopia.

Exploration of the ecology, transmission dynamics and distribution of *L. aethiopica* in southwestern Ethiopia.

Although the baseline research on the ecology and transmission dynamics of CL has already been carried out in the 1970's in Ochollo, a CL endemic village in southwest Ethiopia, still many aspects remain to be discovered. It is important to understand how the vector population behaves, and what and where the sources of infection are in order to enable implementation of effective integrated disease control programs.

Based on field studies and experiments that we conducted in Ochollo (chapter 2 and 3), we confirm that hyraxes are the only animal reservoir of *L. aethiopica* in the area. Moreover, we show that there is most probably also human-to-human transmission, emphasizing the importance of early diagnosis and treatment of infected individuals. The sand fly population is least abundant in the wet season and most infected sand flies reside inside caves. They are mainly endophagic and are active in the evening and especially around midnight. Therefore, we recommend investing in community

knowledge (which we found to be very low in chapter 5) to prevent people's risk behavior, and providing impregnated bed nets for protection at night.

To target these interventions to affected areas, a comprehensive understanding on the spatial distribution of the vector and CL is required. We present a 250 m resolution map predicting the areas with optimal survival conditions for the vector in five administrative zones in southwest Ethiopia, which suggests a much wider distribution than reported previously (chapter 4 and 5). We also established that climate parameters are the most important predictors for the vector distribution, indicating that the distribution may alter when climate changes, leading to disease outbreaks.

Evaluation of diverse molecular approaches for *L. aethiopica* detection in different sample types for various purposes.

Because *L. aethiopica* is a unique species that only occurs in Ethiopia and some parts of Kenya, molecular assays for detection of *Leishmania* parasites have not been validated for this species and which of the many available approaches is most suitable for a certain research or clinical purpose is unclear.

We comparatively assessed the performance of different conventional and molecular diagnostic assays on two sample types of Ethiopian suspected CL patients (chapter 6). We demonstrate that in clinical practice, microscopy of skin scrapings (SS) should still be the first-line diagnostic at primary health care units. If samples are negative, SS should be sent to a referral centre where they should be tested by the recently developed, sensitive LC kDNA qPCR, with specific primers for *L. aethiopica* minicircle kinetoplast DNA.

However, this TaqMan probe assay is expensive, so if for particular research purposes an extensive sample screening is required, the almost equally sensitive, cheaper spliced-leader (SL) RNA SYBR Green qPCR assay can be applied. This assay also proved appropriate in combination with a crude extraction buffer for *Leishmania* detection in laboratory- and field-infected sand flies, making it a convenient method for high-throughput screening in eco-epidemiological research (chapter 7).

Validation of MALDI-TOF MS for unambiguous sand fly species determination in Ethiopia.

The sand fly fauna in Ethiopia is diverse and some of the species are closely related, resulting in difficulties for morphological identifications and molecular determinations are very costly, time consuming and Ethiopian species lack in reference databases.

We identified the species of field captured Ethiopian sand flies by morphology, DNA barcoding and MALDI-TOF MS protein profling and demonstrate that the latter is a rapid, suitable technique for unambiguous sand fly identification that can even distinguish very closely related species (chapter 8). Furthermore, we demonstrate that sequence and protein profiles of the previously distinct *P. pedifer* and *P. longipes* species are identical, suggesting that these two vectors are presumably a single species. Lastly, we found a sand fly species in a novel CL focus in north Ethiopia, which is probably a new species of the *Adlerius* subgenus.

Collectively, the research performed in the framework of this thesis lays groundwork for adequate CL detection and guidance of disease control and indicates the use of novel techniques for accurate, cost-effective future entomological surveys.

Samenvatting

Cutane leishmaniasis (CL) is een verwaarloosde tropische ziekte die gekenmerkt wordt door nodulaire en korstige wonden op de huid, voornamelijk op het gezicht en de ledematen van mensen, met misvormende littekens na genezing als gevolg. De infectie wordt veroorzaakt door *Leishmania* parasieten, die worden overgedragen door vrouwelijke zandvliegen van de genus *Phlebotomus* tijdens een bloedvoeding. De ziekte vormt een groot probleem voor de volksgezondheid in de middelhoge gebergtes van Ethiopië, waar de *Leishmania aethiopica* parasiet jaarlijks ongeveer 20,000 tot 50,000 mensen treft. Eerdere rapporten geven aan dat de overdracht van *L. aethiopica* zoönotisch is, waarbij klipdassen als reservoir voor de infectie dienen. Deze dieren delen hun rotsachtige habitat met de belangrijkste CL vectoren, *Phlebotomus pedifer* in het zuiden en *P. longipes* in het noorden van Ethiopië.

In dit proefschrift waren de drie belangrijkste doelstellingen: *i)* het onderzoeken van de ecologie, transmissiedynamiek en verspreiding van *L. aethiopica* in het zuidwesten van Ethiopië, *ii)* verschillende moleculaire technieken voor detectie van *L. aethiopica* valideren en bepalen welke test het meest geschikt is voor een bepaald staal-type of onderzoeksdoeleinde, en iii) bepalen of de recent geïntroduceerde techniek 'MALDI-TOF mass spectrometry', waarbij men eiwitprofielen van zandvliegen genereert, een goede methode is voor een eenduidige bepaling van zandvlieg soorten in Ethiopië.

Verkenning van de ecologie, transmissiedynamiek en verspreiding van *L. aethiopica* in het zuidwesten van Ethiopië.

Hoewel het basisonderzoek naar de ecologie en transmissiedynamiek van CL al in de jaren 70 is uitgevoerd in Ochollo, een dorp waar CL endemisch is in het zuidwesten van Ethiopië, zijn er nog veel aspecten te ontdekken. Het is belangrijk om te begrijpen hoe de vector populatie zich gedraagt en wat en waar de infectiebronnen zijn, om op basis van deze informatie strategieën voor controle van de ziekte te ontwikkelen en implementeren.

Door middel van veldstudies en experimenten die we in Ochollo hebben uitgevoerd (hoofdstuk 2 en 3) bevestigen we dat klipdassen de enige dierlijke reservoirs van *L. aethiopica* in het gebied zijn. Bovendien laten we zien dat er waarschijnlijk ook overdracht van mens op mens is (via de zandvlieg), wat het belang benadrukt van vroege diagnose en behandeling van geïnfecteerde individuen. De populatie

zandvliegen is het laagst in het natte seizoen en de meeste geïnfecteerde zandvliegen bevinden zich in grotten. Ze bijten mensen vooral binnenshuis en zijn 's avonds en specifiek rond middernacht actief. Daarom raden we aan om te investeren in het bijbrengen van kennis over de ziekte aan de gemeenschap (die we in hoofdstuk 5 erg laag bevonden) om op die manier risicogedrag van mensen te voorkomen. Bovendien adviseren we om geïmpregneerde bed netten te gebruiken om de bevolking ‘s nachts te beschermen tegen beten van zandvliegen.

Om deze interventies op getroffen gebieden te richten, moeten we weten waar de vector en CL precies voorkomen. We presenteren een kaart met een spatiale resolutie van 250 m die de gebieden met optimale overlevingscondities voor de vector voorspelt in vijf administratieve zones in het zuidwesten van Ethiopië (hoofdstuk 4 en 5). Deze kaart suggereert een veel bredere verspreiding dan eerder gerapporteerd werd voor CL. We hebben ook vastgesteld dat klimaatparameters het meeste invloed hebben op het voorkomen van de vector, wat aangeeft dat de huidige aangeduide regio’s kunnen variëren wanneer het klimaat zou veranderen, wat kan leiden tot ziekte uitbraken.

Evaluatie van diverse conventionele en moleculaire testen voor detectie van *L. aethiopica* in verschillende staal-types en voor bepaalde doeleinden.

Omdat *L. aethiopica* een soort is die enkel voorkomt in Ethiopië en sommige delen van Kenia, zijn moleculaire testen voor detectie van *Leishmania* parasieten niet gevalideerd voor deze soort. Bovendien is het moeilijk om te bepalen welk van de vele beschikbare testen het meest geschikt is voor een bepaald onderzoeks- of klinisch doel.

We hebben de prestaties van verschillende conventionele en moleculaire diagnostische *Leishmania* detectie methodes getest op twee staaltypes van vermoedelijk CL-geïnfecteerde Ethiopische individuen en vervolgens vergeleken en beoordeeld (hoofdstuk 6). We toonden aan dat microscopie van huidstalen in de klinische praktijk nog steeds de standaard methode moet zijn voor de eerstelijnsgezondheidszorg. Als de stalen negatief zijn, worden ze best getest door de recent ontwikkelde LC kDNA qPCR, met specifieke primers voor *L. aethiopica* minicirkel kinetoplast DNA.

Deze test op basis van TaqMan-probes is echter duur, dus als bepaalde onderzoeksdoeleinden het testen van een uitgebreid aantal stalen vereist, dan kan de bijna even gevoelige, goedkopere, ‘spliced-leader (SL-) RNA SYBR Green qPCR’ test worden toegepast. Deze methode bleek ook geschikt in combinatie met een ruwe

extractiebuffer voor *Leishmania* detectie in laboratorium- en veld-geïnfecteerde zandvliegen, waardoor het een handige methode is voor het testen van het grote hoeveelheid stalen in eco-epidemiologisch onderzoek (hoofdstuk 7).

Validatie van MALDI-TOF MS voor een eenduidige bepaling van zandvliegsoorten in Ethiopië.

De fauna van zandvliegen in Ethiopië is erg divers en sommige soorten zijn nauw verwant en lijken erg op mekaar, wat soms zorgt voor problemen voor morfologische identificaties. Daarnaast zijn moleculaire bepalingen gebaseerd op DNA redelijk duur, tijdrovend en Ethiopische soorten ontbreken vaak in referentiedatabases.

We identificeerden de soorten van Ethiopische zandvliegen die we in het veld vingen op basis van morfologie, twee soorten DNA-profielen en MALDI-TOF MS gegenereerde eiwitprofielen en demonstreren dat de laatste een snelle, goedkope en geschikte techniek is voor eenduidige identificatie van zandvliegen die zelfs zeer nauw verwante soorten kan onderscheiden (hoofdstuk 8). Verder laten we zien dat DNA- en eiwitprofielen van *P. pedifer* en *P. longipes* soorten identiek zijn, wat suggereert dat deze twee vectoren waarschijnlijk slechts één enkele soort zijn. Ten slotte vonden we een nieuwe zandvliegsoort in een nieuwe CL-focus in het noorden van Ethiopië die behoort tot de *Adlerius* subgenus.

Gezamenlijk legt dit onderzoek de basis voor interventies om de ziekte te controleren, een adequate detectie van *Leishmania* parasieten en wijst het op het gebruik van nieuwe technieken voor toekomstige eco-epidemiologische en entomologische onderzoeken.

CHAPTER 1

Background, rationale and objectives

Leishmania and leishmaniasis

Leishmaniasis is a neglected tropical disease (NTD) caused by parasites of the genus *Leishmania*. The flagellates are obligate intracellular and belong to the order Kinetoplastida and family Trypanosomatidae [1]. Female sand flies are the vector for *Leishmania*, mediating parasite transmission when they take blood meal from a vertebrate host [2–4]. The transmission can be anthroponotic, with humans serving as the only source of infection, or zoonotic when *Leishmania* parasites occur in animals and occasionally cause disease in humans. In this case of zoonotic transmission, a wide variety of mammals can act as reservoir hosts [5].

Leishmania life cycle

The life cycle of *Leishmania* includes two major morphological stages, namely flagellated promastigotes within the sand fly and non-flagellated amastigotes within the vertebrate host's cells. The vertebrate host can get infected when an infectious female sand fly takes a blood meal, thereby injecting metacyclic promastigotes in the host's skin. These are phagocytosed by mononuclear phagocytic cells, such as macrophages. The promastigotes transform into amastigotes intracellularly and multiply by binary fission. Eventually, the macrophages burst, releasing the amastigotes, which can infect other phagocytes of different tissues. Upon blood feeding on infected hosts, amastigotes can be taken up by a sand fly [6].

Leishmania development in the vector happens in the digestive system and can take up to two weeks. Amastigotes in the blood meal are directed to the midgut, where they transform into procyclic promastigotes, a replicative but weakly motile form. As a response to the blood meal, the midgut epithelium secretes a perithophic matrix (proteins, glycoproteins and chitin) that surrounds the blood meal, forming a mechanical barrier for the promastigotes and which may result in defecation of parasites. At the end of digestion, sand fly chitinases disintegrate the peritrophic matrix upon which promastigotes develop to long nectomonads that can attach with their flagellum between the microvilli in the midgut wall to avoid excretion with remnants of the blood meal. Once attached to the midgut, the parasites are named leptomonads which replicate and move anteriorly towards the thoracic midgut, where they accumulate. A promatigote secretory gel (PSG) is produced creating a gel-like plug which can phyisically block the gut. Then, the leptomonads evolve into non-dividing and highly motile metacyclic promastigotes (metacyclogenesis), which migrate to the

stomodeal valve of the thoracic midgut, on the edge of the PSG plug. The PSG blockage and damage caused by *Leishmania* parasites to the stomodeal valve cause that infectious sand flies probe several times and take smaller blood meals [7–9]. Moreover, the PSG blockage leads to regurgitation when the sand fly takes a blood meal, which enhances the disposition of metacyclic promastigotes and infection of the mammalian host [6,10,11].

Recent findings have shown that a second uninfected blood meal triggers dedifferentiation of metacyclic promastigotes, a process named reverse metacyclogenesis. The parasites transform into retroleptomonad promastigotes, which are highly replicative, and when transforming to metacyclic promastigotes again, the process results in enhanced sand fly infectiousness [12].

Clinical manifestations

Leishmaniasis can have a variety of clinical outcomes in humans, depending on the *Leishmania* species and the immune response of the host [3,6]. There are three major forms of leishmaniasis.

First, visceral leishmaniasis (VL) or 'kala-azar' induces fever attacks, hepatosplenomegaly and anemia, and is lethal if left untreated in 95% of the cases (Fig 1.1A). After recovery or successful treatment of VL, post-kala-azar dermal leishmaniasis (PKDL) can occur, a complication that causes a macular or nodular rash mainly on the patient's face and arms (Fig 1.1B). Second, mucocutaneous leishmaniasis (MCL) causes severe damage to mucous membranes of the nose, mouth and throat and eventually destruction of the nasal septum, lips and palate leading to extensive facial disfiguration (Fig 1.1C). Third is cutaneous leishmaniasis (CL), which can be subdivided in localized cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL). LCL is the most common form and is characterized by ulcerative skin lesions or non-ulcerative nodules that mostly appear on people's faces and extremities (Fig 1.1D). It is usually self-healing, although it leaves disfiguring permanent scars (Fig 1.1E). LCL can disseminate and form multiple non-ulcerative skin lesions involving the entire body, which is distinctive for DCL (Fig 1.1F). This is a rare form, which does not heal by itself, is difficult to treat and mainly affects people with an impaired immune system [2,3,13]. Exceptionally, CL develops into a chronic form, named leishmaniasis recidivans (LR), which can develop months or years after treatment and is characterized by confluent lesions on and around the edge of the scar [14,15]. Although CL is not fatal like VL, it is

a chronic disfiguring condition that results in severe social stigma for (post-) infected individuals.

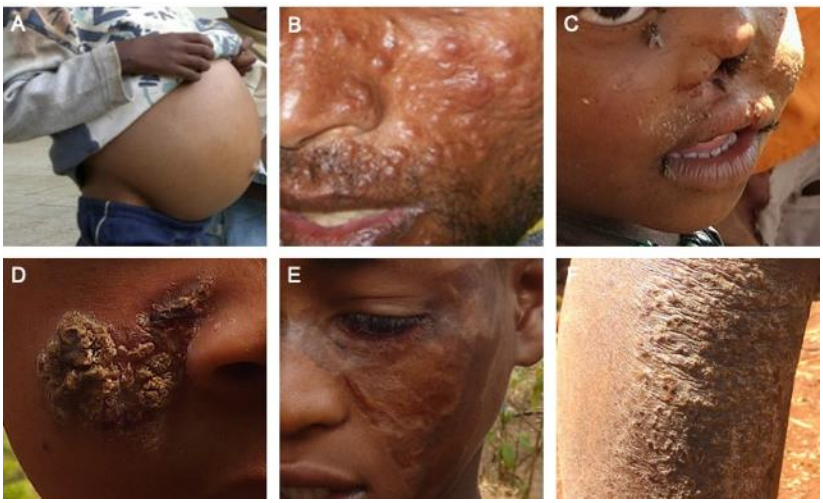


Fig 1.1 Clinical manifestations of leishmaniasis in Ethiopia. A) Hepatosplenomegaly due to a visceral leishmaniasis (VL) infection; B) post-kala-azar dermal leishmaniasis (PKDL), a complication after recovery or treatment of a VL infection; C) almost healed mucocutaneous leishmaniasis (MCL) lesion, affecting the mucosa of the nose and mouth; D) typical localized cutaneous leishmaniasis (LCL) lesion on the face; E) scar formation after LCL infection; F) diffuse cutaneous leishmaniasis (DCL) spread over a leg. *Panels A and B were derived from http://vl-ethiopia.huji.ac.il, panels C-F were made by Myrthe Pareyn*.

Epidemiology

Leishmaniasis is found in approximately 90 countries worldwide, in particular in the tropics, subtropics and southern Europe, where it is caused by 20 different *Leishmania* species. In the Old World, VL is caused by *Leishmania donovani* and *L. infantum*, while CL and MCL are due to *L. tropica*, *L. major* and *L. aethiopica*. The Centers for Disease Control and Prevention (CDC) estimates that there are annually 700,000 to 1,200,000 CL cases and 100,000 VL cases worldwide. However, the incidence of the infection is very difficult to estimate, due to difficult diagnosis and lack of adequate surveillance systems [5].

Diagnosis

Correct, timely diagnosis of the *Leishmania* species that causes the infection is of high importance for surveillance and adequate treatment. There are many diagnostic

approaches available, but which one is preferred depends on the parasite species, purpose and available resources.

Diagnosis of VL is mostly done on tissue samples or aspirates from the bone marrow and sometimes from the lymph nodes or spleen. The most common sample type for CL diagnosis is a skin scraping taken from the margin of the lesion, and sporadically lesion aspirates and dry blood spots [16,17].

The samples are commonly used for histopathological diagnosis by Giemsa staining or cultivation. This approach has shown high specificity, but is time-consuming, requires expertise and often provides false negative results. Alternatively, less invasive serological tests (e.g. immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), direct agglutination assay (DAT), Montenegro skin test (MST)) can be useful, mainly for VL diagnosis, although these tests are unable to distinguish active forms from quiescent or previous infections and suffer from cross-contamination of other infections [16,18,19]. Molecular methods, such as polymerase chain reaction (PCR) combine high sensitivity and specificity. PCRs can amplify a variety of target sequences of the *Leishmania* genome (nuclear or extra-chromosomal minicircle kinetoplast DNA (kDNA) for parasite detection, speciation and quantification. Unfortunately, this method is costly, requires technical resources and experience, making it often unavailable in resource-constrained countries [18,20,21].

New promising tests for CL diagnosis are the loop-mediated isothermal amplification (LAMP) assay and CL detect rapid test, which both need field evaluations before they can be considered for standard diagnosis [17,18].

Treatment

There are several treatment options depending on the clinical form of leishmaniasis, the *Leishmania* species causing the infection, severity of the case and patient's underlying health [2].
Currently, parenteral administration of pentavalent antimonial compounds (sodium stiboglocunate and meglumine antimoniate) are the first line treatment for CL, MCL and VL. However, the disadvantage is that this drug can have serious, yet reversible, side effects such as kidney problems and liver toxicity. Additionally, there are increasing reports of drug resistance worldwide [2,6,24,25]. Liposomal amphotericin B, on the other hand, is less toxic and can be used as a topical or intravenous treatment for CL,

MCL and VL, but is expensive and often reserved for severe immunocompromised cases [2,3,25,26]. Oral drugs for CL, MCL and VL treatment are azoles (ketonazole, itraconazole and fluconazole), which show fluctuating responsiveness depending on the *Leishmania* species, and miltefosine, for which a rise in resistance has been observed due to the lack of treatment compliance [2,3,25,27]. The effectiveness of paromomycin sulphate by parenteral injection for VL or local treatment for CL seems promising, but requires further clinical trials. For non-complicated CL, cryotherapy with liquid nitrogen or thermotherapy by radiofrequency heat could also be good treatment options, but needs evaluation on different *Leishmania* species [12,16, 17].

Although quite some treatments exist, the adverse effects result in low treatment compliance, by which drug resistance can develop. Moreover, the drugs are difficult to obtain by the patients because they are not available in many countries or the distance to treatment centres is too far. Rather than seeking help from healthcare facilities or hospitals, the symptoms are treated in local communities with traditional methods by local healers. They apply methods like herbs, holy water, cauterization with hot iron or applying battery acid. Most people that eventually show up at health facilities have a long history of illness and very severe lesions, which are very difficult to treat [26,28].

Reservoirs

Leishmaniasis can be zoonotic or anthroponotic, depending on the parasite and vector species and the ecological setting. Identification of all reservoir hosts that contribute to transmission is a prerequisite for disease control. However, reservoir identification is quite a complex process that requires a lot of knowledge [31].

Reservoir criteria

In order to determine the role of a given hosts in a reservoir system, it is not sufficient to simply detect parasites in the host. Ashford and colleagues proposed a number of parameters that should be met to include a reservoir. In particular, the species should be abundant and have a relatively long life span. A large proportion of the individuals should become infected and remain infected for a long time without acute disease development, sufficient to sustain parasites during the non-transmission season. Additionally, the species has to be attractive and accessible to sand flies for their blood meal [31,32]. However, empirical characterization of reservoirs remains challenging, so additional criteria should be taken into account. Parasites should be available in the

skin or blood in sufficient numbers to be taken up by the sand fly. Moreover, parasites need to be viable and numerous during and after transmission to ensure a feasible transmission route [33].

Old World *Leishmania* reservoirs

In the Old World, humans are the reservoir hosts for transmission of *L. tropica* and *L. donovani* and in some cases for *L. infantum* [34]. It is known that VL can also be asymptomatic in humans, but whether asymptomatically infected humans can contribute to transmission currently remains unknown, although it is an important factor to take into account [77].

Zoonotic transmission can occur in domestic or sylvatic cycles. Dogs are the principal domestic reservoirs for *L. infantum* and even dogs that are asymptomatically infected are infectious to sand flies [36]. *L. major* has a sylvatic zoonotic cycle with gerbils as the main reservoirs: in particular great gerbils (*Rhombomys opimus*) in Central Asia, fat sand rats (*Psammomys obesus*) in the Middle East and a variety of jird species (*Meriones libycus, M. shawi, M. hurrianae*) and fat sand rats in North Africa [37–40]. Moreover, the multimammate mouse (*Mastomys erythroleucus*), black rat (*Rattus rattus*) and Indian gerbil (*Tatera gambiana*) are also suspected reservoirs for *L. major* [41,42]. *L. tropica* is zoonotic in some foci, where the main suspected reservoirs are some rodent species and hyraxes (*Procavia spp*. and *Heterohyrax brucei*) [43–46]. These hyraxes are also the presumed main reservoirs for *L. aethiopica* [47,48].

Reservoir control

When *Leishmania* transmission is anthroponotic the major methods for control are adequate surveillance, active case detection and successful treatment and monitoring. This should be done in combination with measures to prevent reinfection, for which community knowledge is often lacking, although it is a very important factor [34,49].

When dogs are the main reservoir, often surveillance with serological diagnosis and subsequent antileishmanial treatment are applied, although they easily get reinfected, hence culling of infected animals is preferred in some areas. Canine leishmaniasis vaccines are under development, which reduce the symptoms in dogs, but the effect on transmission is unknown yet. Repellents on their skin or in a collar are also proven effective in some cases to reduce sand fly bites [50].

In case rodents are the source of infection, different measures can be applied, including burrow destruction, poisoning with rodenticides like zinc phosphide or anticoagulants, avoiding that their food is growing near to human settlements etc. [51,52]. For hyrax control, destruction of their habitats near human settlements, biological control (predators) or shooting are suggested interventions. However, in some countries they are protected, reducing the methods that can be used against this reservoir [31,53].

Sand flies

Phlebotomine sand flies are the insect vectors for *Leishmania* parasites. Understanding their ecology, activity and feeding behavior will provide insights in disease transmission, which is crucial for efficient implementation of control measures.

Taxonomy and morphology

Phlebotomine sand flies (Diptera, Psychodidae, Phlebotominae) are subdivided in six genera; three in the Old World, *Phlebotomus, Sergentomyia* and *Chinius*, and three in the New World, *Lutzomyia*, *Brumptomyia* and *Warileya*. Although the other genera also include human-biting sand flies, only *Phlebotomus* and *Lutzomyia* sand fly species contribute to *Leishmania* transmission [54,55].

Sand flies undergo complete metamorphosis through four developmental stages: egg, four instar larvae, pupae and adult and the complete cycle takes about one to three months (Fig 1.2A). Adult phlebotomine sand flies are small with a maximum body length of 3.5 mm and most body parts and wings are densely covered with hair. When at rest, they hold their wings in a characteristic 'V' shape (Fig 1.2B) [54,56].

Species identification of sand flies is mostly done by morphological taxonomy, by mounting the specimen's head and last segments of the abdomen, which include distinctive features (cibarium, pharyngeal armature and genitalia, Fig 1.2C and D). This approach is very tedious and demands high expertise (especially for females). Moreover, keys are often outdated, resulting in inadequate identifications [58].

Alternative techniques, like molecular identification based on different mitochondrial (*e.g.* cytochrome oxidase subunit I, cytochrome B) and ribosomal (*e.g.* ITS-2, 18S rRNA) gene targets are available and robust for phylogenetic analyses, but are costly and labor intensive in case of high-throughput screenings [58]. Sophisticated techniques are required to decide whether morphologically similar sand flies warrant recognition as

different species and to support entomological field research [34].

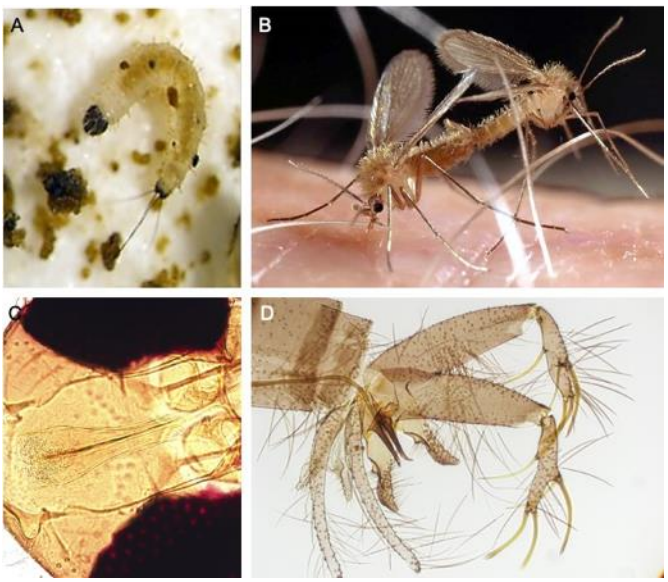


Fig 1.2 Morphology of phlebotomine sand flies. A) Third instar larva; B) female (left) and male (right) *Phlebotomus (P.) duboscqi* mating pair; C) pharynx armature and cibarium in head of *P. orientalis*; D) male genitalia of *P. pedifer. The two top panels (A and B) are derived from Lawyer et al. (2017)* [57]*, the lower panels (C and D) were made by Myrthe Pareyn.*

Biology

Breeding and resting sites

Sand fly breeding and resting sites can be very diverse depending on the species and ecological setting they inhabit.

The immature stages do not require an aquatic environment for their development, but are generally associated with a rather warm and humid environment, rich in decomposing organic content, *e.g.* animal excreta and plant materials [54,59]. Immature stages have been recovered from several habitats, but these collections are scarce and often the specimens are few in numbers. The true breeding sites of many species are therefore still to be elucidated [59,60].

Adult sand flies are found in a wide range of dark, cool and humid ecotopes, like for example caves, fissures in walls of human dwellings and stables, tree holes, animal

burrows, soil cracks and termitaries [48,56,59,61,62]. Sand flies tend to stay close to their breeding and resting sites, although males disperse even less than females and are therefore more abundant near breeding sites. In resting sites, the opposite gender occurrence is observed [63,64].

Flight and feeding behaviour

The activity of sand flies consists of a number of discrete components, including foraging, mating and breeding. The biting behavior as well as the activity pattern differs from species to species and even for one sand fly species between different ecological settings [54,56,65].

The flight speed of sand flies is about 1 m/s, making it hard for them to fly when it is windy. Overall, sand flies fly close to the ground, presumably to avoid being swept away by wind flows, because they are tiny and fragile and because the wind speed increases with altitude [34]. When there is a vertical obstacle (e.g. a wall or bed net), they move upwards close to it with intermittent stops [66]. Reports on the dispersal of sand flies range from 300 m to almost 2 km, probably depending on the species, wind and availability of sugar and blood meals [67–70].

Both male and female sand flies feed on natural sugary secretions from plants or honeydew produced by homopterous aphids. Only females feed on blood, which provides proteins necessary for egg maturation. They are active between dusk and dawn and attack their host silently. Female sand flies start to take their first blood meal around the fourth day of adulthood [54]. Some species are autogenous, meaning that they develop their first batch of eggs without a blood meal. For the following egg batches, a blood meal is required again [34]. Oviposition generally takes place about six to nine days post blood meal [57]. Few species feed only once between successive ovipositions, whereas others may take multiple blood meals in a single oviposition cycle [12,54]. Upon feeding on an infectious host, it takes eight to 12 days to develop metacyclic promastigotes, although this depends a lot on the species and air conditions [11]. Sand flies are generally opportunistic and feed on a wide variety of vertebrate hosts, although some species feed almost exclusively on a few specific vertebrate species. Their preference largely depends on the accessibility of the vertebrates in their environment [56].

Vector incrimination and competence

There are two types of vectors, specific vectors, transmitting only a single *Leishmania* species, and permissive vectors, that can maintain the growth of more than one species.

Of approximately 800 sand fly species, only 93 are proven or potential vectors. The fact that so many do not play a role in transmission depends on many aspects. Criteria for inclusion of a sand fly as a *Leishmania* vector are that they bite the reservoir host(s), have natural infections with the same parasite strain that causes human leishmaniasis and must be able to support the growth of parasites and transmit them when they take a blood meal [71]. For the latter, several parasite-vector interactions are required to make a sand fly competent for *Leishmania* transmission. Firstly, the *Leishmania* parasites should be resistant for the digestive enzymes produced in the midgut of that sand fly species. Secondly, the promastigotes should be able to bind to the inner surface of the sand fly gut and lastly, the sand flies should be able to complete metacyclogenesis of the promastigotes, resulting in metacyclic stages that can survive in the vertebrate host [34,72].

Vector control

There is a variety of vector control measures that can be used to reduce the infectious sand fly population and consequently the burden of the disease. As sand flies are terrestrial breeders and the immature stages are hard to locate, control methods are entirely directed to the adult stages. Which, where and when control methods should be applied for an integrated vector control program must be decided based on the ecology and behaviour of the vector(s). Even though some of the available methods can individually reduce the vector abundance, it is highly recommended to apply more than one method at the same time [34,73].

Measures targeting foraging sand flies

Personal protective measures are often key in preventing leishmaniasis transmission. One of the preferred methods is the use of long-lasting insecticide treated bed nets (LLITN), especially when the vector is endophagic [34]. The LLITN are usually impregnated with synthetic pyrethroids, integrated in the fibres, resulting in effectiveness for up to three years [74]. Therefore, it is considered a relatively cheap

and sustainable method compared to other interventions and if high coverage is accomplished, it can result in community protection [34,75]. There is controversy with regard to the mesh size of the nets. Some studies state that the mesh size should be smaller to prevent that sand flies can pass through the net, while others describe that larger mesh sizes are almost evenly effective, because sand flies die when they come in contact with the insecticide. This is important, as a smaller mesh size may result in a lower compliance of the LLITN use, especially in hot environments. On the contrary, the smaller the mesh size, the better the net will protect the humans against nuisance of other insects [76–78]. Overall, LLITN resulted in great reductions in vector populations and the incidence of leishmaniasis [74,79–82]. Other personal protection measures are the use of insect repellents or protective clothing impregnated with insecticides. However, these methods are expensive, impractical and potentially harmful to use [73]. Studies show variable results and it is therefore only recommended for people that are at risk for infection temporarily [83–85].

To tackle foraging sand flies that are mainly breeding in sylvatic habitats quite distant from inhabited areas, two control measures can be applied. Firstly, insecticide treated vertical barriers can be set up around sand fly breeding sites. The principle is based on the assumption that sand flies fly close to the ground when they are foraging. Upon encountering a vertical obstacle, they make short intermittent vertical flights, by which they are exposed to the insecticides impregnated in the barrier and die [66,78,86]. Secondly, attractive toxic sugar can be applied on vegetation or bait stations. Sand flies are attracted to these sites and die when they take a sugar meal from it [87,88]. Both methods have been proven successful in several studies to reduce the sand fly population [78,86–88]. However, both interventions are labour intensive and achievement of adequate, persistent coverage is difficult. Furthermore, it should be taken into account that the insecticides can be dangerous for non-targeted insects, although some studies claim that this effect is negligible if the toxic sugar solution is not applied on flowering vegetation [88,89]. Due to these reasons, these measures are in many situations impractical and undesirable for sand fly control.

Multiple studies have shown that improved housing structure is associated with a lower number of sand flies and leishmaniasis cases, because there is less vector movement through holes [29,90]. Therefore, filling up wall cracks, smoothening walls or sealing openings with meshes could have an impact on endophagic sand flies [91].

If the animal reservoir is well understood, control of foraging sand flies can also be conducted through the reservoir host. Studies show that treatment of the wild reservoir, mostly rodents, with a bait containing a systemic insecticide (i.e. fipronil, ivermectin) significantly reduced the female sand fly population. Moreover, sometimes it is combined with a rodenticide, so that the reservoir population can be reduced at the same time. On the other hand, this control measure requires frequent and careful distribution of these toxic baits inside the reservoir habitats, which is quite strenuous [92,93].

Measures targeting resting sand flies

Spraying of insecticides on resting sites of sand flies can cause a reasonable reduction in the sand fly population, but the impact on the disease varies from place to place [34,73]. Generally, indoor residual spraying (IRS) to reduce endophagic vectors is considered an effective control measure [94]. To decide which class of insecticides to use for IRS, the susceptibility of the sand flies to the insecticide should be tested, since there is resistance in some areas to certain insecticides due to its use for malaria control [95]. The insecticide needs reapplication about every five to six months, which makes it quite labour demanding. Moreover, the effectiveness of IRS depends on many other factors, including the surface type, method of application, total coverage, adequate supplies of the insecticide and spraying equipment and trained personnel. Overall, management and monitoring of IRS are crucial to ensure successful control and avoid insecticide resistance [34,81,96].

Furthermore, impregnated curtains can reduce the number of resting vectors indoors, by knocking them down before oviposition. The disadvantage of this method is that it is largely dependent on the house structure and it requires routine impregnation, about every three months [97,98].

Measures against sand fly breeding sites

If the breeding sites of the sand flies are identified, these can be used for vector control measures as well. One of the reasons for leishmaniasis outbreaks is new human settlement close to sand fly breeding sites, suggesting that relocation of these settlements could reduce the risk of transmission. Alternatively, the breeding sites can be treated by insecticides. However, it is hard to reach sufficient coverage and exposure of the insecticides to weather conditions reduces its effectiveness, so it is not

considered cost-effective and potentially harmful for the environment [94]. Breeding sites can also be reduced by for example, sanitation programs, cutting forests or demolition of rodent burrows [56,59,73]. However, environmental control has fluctuating success and is no long-term control measure. Also, the potential ecological conflict should always be considered [56,73].

Cutaneous leishmaniasis in Ethiopia

All three clinical forms are common in Ethiopia, although they occur in different areas. It is generally assumed that VL occurs in the lowlands and CL in the highlands of the country. In the course of this thesis we solely focus CL, since it is the most prevailing form in the southern Rift Valley, the target area of the VLIR-UOS project under which this PhD was carried out.

Epidemiology

Most of the CL cases are caused by *L. aethiopica,* with the exception of a single *L. tropica* infection [47,48,99–104]. However, *Leishmania* species identification has not been done extensively from CL patients to state that other species do not play a role in transmission. The World Health Organisation (WHO), the Armauer Hansen Research Institute (AHRI) and Ethiopian Federal Ministry of Health estimated that the yearly incidence of CL counts 20,000 to 50,000 cases, while only 878 cases were reported in 2018 to the WHO [105,106]. These numbers show that there is severe underreporting of CL.

CL infections occur mostly on the mountain ridges that are separated by the Ethiopian Rift Valley, ranging from North to Southwest and South to Northeast, where it presents in a patchy distribution with discrete transmission foci (Fig 1.3). Well described endemic foci are situated in four out of nine regions: Southern Nations, Nationalities, and Peoples' region (SNNPR), Amhara region, Tigray region, Oromia region, and Addis Ababa City Administration. These foci are all situated at altitudes ranging between 1,750 m and 3,000 m [47,48,100–104].

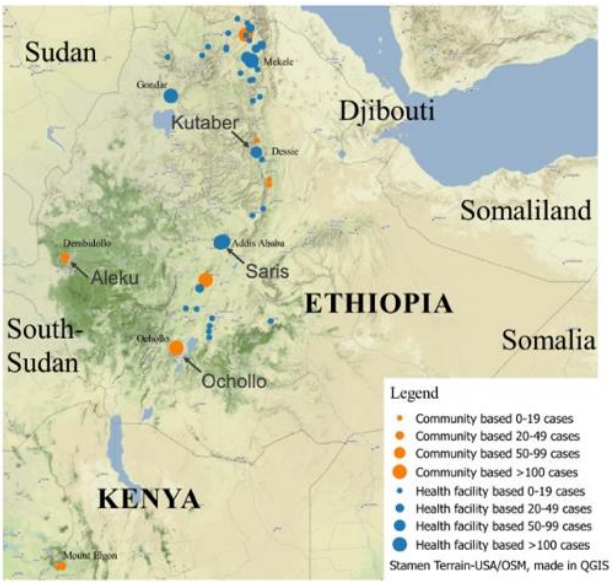


Fig 1.3 Map of published cutaneous leishmaniasis (CL) reports in Ethiopia. CL infections occur mostly in foci on the mountain ridges that are separated by the Ethiopian Rift Valley, ranging from North to Southwest and South to Northeast. Arrows in grey indicate the areas where hyraxes were found *Leishmania* positive (section 4.3.1). *Figure adapted from Van Henten et al. (2018)* [17].

Risk factors

The occurrence and spread of leishmaniasis are encouraged by a variety of socio-economic, demographic, behavioural and environmental factors.

Population movement for family visits or labour plays a big role in disease transmission. When infected individuals migrate to areas where there is no leishmaniasis, but the vector does occur, the movement may result in an outbreak. Vice versa, people that move from non-endemic to endemic areas have no immunity against the parasite and can get severely infected [107–109]. Moreover, the latest years, humans have altered the land use considerably for agricultural activities and settlements. This brought people closer to sand fly habitats, increasing their risk of infection [28,47]. Other important risk factors are the immunosuppressive state of people [28,110], the presence of domestic animals inside and around households, and the lack of sanitation in the remote villages leading to decomposing organic waste that may provide a suitable habitat for larval breeding nearby human dwellings [102]. The poor condition of these human dwellings allows sand flies to easily enter the houses for blood meal acquisition, leading to a higher parasite exposure [28].

The environmental factors that are accompanied with CL are not so well understood. A study recently indicated that there is a strong correlation with altitude, rainfall and slope, although these are rather vague parameters given the fact that the transmission occurs in particular foci only [111]. The slope indirectly points to the higher risk of infection in proximity to rocky cliffs, gorges and hyrax burrows, where the CL vectors are breeding [29,47,102]. Generally, leishmaniasis is moving towards new areas, which could be correlated to climate change, as sand flies and reservoirs have to search for new suitable living conditions, thereby exposing a susceptible population to the parasite [112].

Reservoir hosts

The transmission cycle of CL in Ethiopia is considered zoonotic (Fig 1.4). A variety of hosts have been investigated for their role in transmission, but hyraxes are assumed to be the main animal reservoirs for *L. aethiopica*. Humans are indicated as incidental hosts.

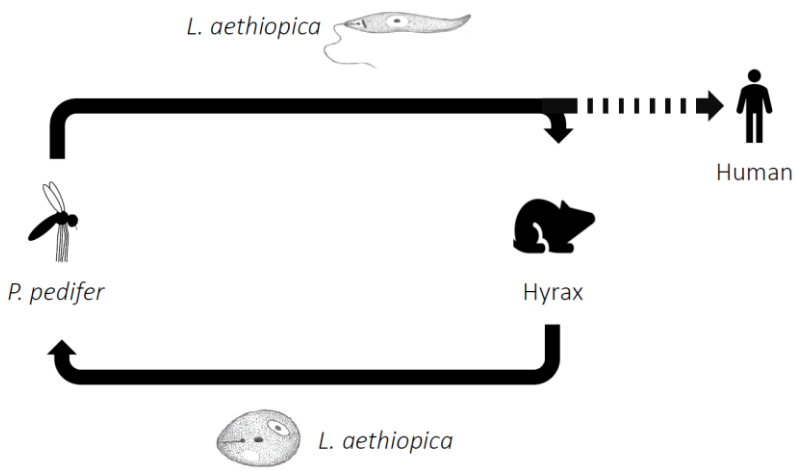


Fig 1.4 Assumed transmission cycle of *Leishmania aethiopica* in southern Ethiopia. *Phlebotomus pedifer* is the only vector and hyraxes are the reservoirs of *Leishmania aethiopica* in southern Ethiopia. Therefore, transmission is considered zoonotic with humans acting as incidental hosts. *By Myrthe Pareyn*

Hyraxes

Hyraxes (Fig 1.5) belong to the order Hyracoidea, have an average lifespan of 12 years, can weigh one to five kilograms and have a maximum length of 60 cm. There are three

genera of hyraxes, which all occur in Ethiopia, namely *Procavia* (rock hyrax), *Heterohyrax* (bush hyrax or yellow-spotted hyrax) and *Dendrohyrax* (tree hyrax). The animals can be found at a wide range of altitudes, from sea level up to 3,600 m and mainly inhabit rocky outcrops, trees and riverbanks in colonies. They are diurnal, herbivorous and show seasonality in their reproduction activities, as most births are seen during the rainy season, when food is plentiful [113]. Furthermore, they typically defecate and urinate in communal latrines that can be centuries old, which leads to accumulation of their excretions over time, eventually providing an ideal breeding site for sand flies [113,114].

In natural conditions, *Leishmania* infected hyraxes do not develop any lesions and the infection prevalence is generally high. *Heterohyrax brucei* was found naturally infected with *Leishmania* parasites in Kutaber (20.0%), Aleku (27.3%), Saris (6.3%) and Ochollo (21.1%)(arrows in Fig 1.3), all CL endemic sites in Ethiopia [47,48,115]. Moreover, 7.8% of *Procavia habessinica* specimens were found *Leishmania* positive in Kutaber [48]. Ashford and colleagues suggested that the infection rates were high enough to suppose that the parasite can sustain itself in the hyrax population [47,48,115]. However, no further studies have been carried out to evaluate their contribution to human CL. Yet, some researchers recommend to destroy their habitat and shoot or biologically control the hyrax populations to reduce disease transmission [31,47].

Fig 1.5 A bush hyrax (*Heterohyrax brucei*) captured in Ochollo. *Pictures by Myrthe Pareyn*

Other potential reservoir hosts

To unravel the complexity of CL transmission and know where to intervene in the cycle, it is important to know all players of transmission. In southern Ethiopia, grass rats (*Arvicanthis sp.*), spiny mice (*Acomys sp.*) and dwarf gerbils (*Gerbillus nanus*) have been found positive for *L. tropica* DNA [116]. A variety of bat species (*Cardioderma cor, Glauconycteris variegata, Miniopterus arenarius, Neoromicia somalica, Nycteris hispida* and *Scotophilus colias*) also tested positive for *L. tropica* and *L. major* DNA in various endemic and non-endemic areas in Ethiopia [117]. However, so far there is only a single documented human CL case due to *L. tropica* and none due to *L. major*, so the contribution of these animals to CL transmission remains unclear [99]. A giant rat (*Cricetomys sp.*) and a ground squirrel (*Xerus rutilus*) were found naturally infected with *L. aethiopica* but the latter was found in a VL endemic area in the lowlands, where human CL cases have never been reported [118,119].

Larger mammals, like dogs or livestock animals have only been investigated in VL endemic settings in Ethiopia, where they were found positive for *L. donovani* DNA [120,121]. However, one study in Kenya found a single goat infected with *L. aethiopica* [122].

Vectors

Phlebotomus (Larroussius) longipes was first described in Kenya and Ethiopia by Parrot and Martin (1939) [123]. A few decades later the species was identified as the vector for *L. aethiopica* [97]. In 1972, researchers found that there was another species occurring in the two countries that was morphologically very similar to *P. longipes*, which they named *P. (La.) pedifer* [124]. Females of the two species are indistinguishable, but males have a different appearance of the aedeagus [124,125]. Both species are incriminated vectors for *L. aethiopica* [48,103,126].

While *P. longipes* mainly occurs in northern and central Ethiopia, *P. pedifer* has only been described in Ochollo, a village in southern Ethiopia, and in some places in Kenya [47,48,68,104,127]. Both species occur in similar habitats, in particular caves, rock fissures, hollow tree boles and inside houses. In general, they have a preference for cool, dark places with a high humidity, without smoke and protected from the wind [128]. *P. longipes* is predominantly active during the night and can fly up to 240 m per night for a blood meal [48,128]. Studies show that when *P. pedifer* and *P. longipes* feed

on human CL lesions caused by *L. aethiopica*, the parasites are transmitted efficiently and differentiate into metacyclic promastigotes in the sand fly [48,129].

Besides, a large entomological study in the Awash Valley in northeastern Ethiopia at an altitude of about 1000 m, could isolate *L. aethiopica* from a single *P. sergenti* specimen and *L. tropica* infections were found in *P. sergenti* and *P. saevus*, but their vectorial competence has not yet been established [130].

Ochollo focus

Ochollo is a village in Gamo zone in the highlands of southwestern Ethiopia that has quite extensively been investigated throughout the past 50 decades. In Gamo language, CL is named “bolbo” and has been known in the area for a very long time.

Epidemiology

Ochollo village was first investigated in 1972 by Ashford and colleagues, who found among 895 study participants that 10.7% presented active lesions and 30.4% had scars. An unequal age distribution was noticed, as 41.2% of the infections appeared under the age of 6 and adults with scars stated that their first lesion appeared during childhood [48]. The epidemiology in Ochollo was studied once more over a decade later, when 3022 residents were interviewed and examined. Active lesions were present in 3.6% of the sampled population in 1987 and 3.9% in 1989, hence substantially lower than in the earlier report. Once more, 80% of the cases were within the age group of 0 to 10 years [101]. The most recent study was carried out in 2014, examining the disease among 523 primary school children. In this population, 4.0% presented with active lesions, 1.5% with active lesions and scars and 59.8% with scars, which were all predominantly located on the face [100]. Overall, CL is a very important health problem in this area and is mainly infecting young children.

Ecology

Ochollo has a very rough topography, with many boulders and cliffs nearby human dwellings and quite a dense population of hyraxes (Fig 1.6). Ashford *et al.* found four out of 19 (21.1%) of the hyraxes (*Heterohyrax brucei*) naturally infected with *Leishmania,* suggesting that also in Ochollo they are the main reservoir of the infection [48].

Earlier studies on the sand fly species composition in Ochollo revealed the presence of five species, namely *P. pedifer*, *P. aculeatus*, *Sergentomyia bedfordi, S. affinis vorax* and *S. africana magna*, of which the former is the predominant species [48,61]. However, Gebre-Michael and colleagues claimed that *P. aculeatus* was misidentified, and was actually *P. ashfordi* [131].

So far, *P. pedifer* is the only incriminated vector of *Leishmania* in the area. The species shows anthropophilic characteristics, bites mainly hyraxes and humans and the infection prevalence was estimated at 1.7% and 5.4% in two studies based on low sample sizes [48,61]. *P. pedifer* occurs inside houses and hollow tree boles, but mainly resides in cracks and fissures in basalt cliffs and rocks, where they live in close association with hyraxes [48].

Fig 1.6 Rough topography of Ochollo, with many rocky cliffs and boulders. *Pictures by Myrthe Pareyn.*

Problem statement

Leishmaniasis is a growing public health issue in Ethiopia and is spreading to areas previously unburdened by the disease. It mostly affects the poor community, among whom the knowledge on disease transmission and prevention is very limited [28,49,132]. Additionally, some health care workers have difficulties to recognize the infection. Therefore, it remains often mis- or under-diagnosed and even when clinical diagnosis is possible, the patients' remoteness to tertiary referral centers impedes a confirmed diagnosis (by microscopy or molecular techniques) and proper treatment [26,133]. The lack of a timely diagnosis and treatment for CL leads to disfiguring scars on people's faces and extremities, which has a severe psychological impact on (post-) infected individuals [134]. The epidemiological surveillance is inadequate and there is

currently no active case detection going on. Altogether, these limitations result in underreporting of CL in Ethiopia, hampering disease control.

Furthermore, the disease is assumed to be zoonotic, making the transmission dynamics very complex. Yet, there is a lack of understanding on the true contributors to transmission, which is essential to be able to deal with the sources of the infection. CL caused by *P. pedifer* is limited to a relatively small area, so there is very limited knowledge on disease transmission and the behavior and ecology of the vector [31,47]. However, a thorough understanding is pivotal to decide which, when and where interventions should be implemented to effectively reduce the sand fly population and subsequently the incidence of CL.

Thesis objectives and outline

This PhD thesis was carried out in the framework Phase 1 of the VLIR-UOS Institutional University Cooperation between Arba Minch University (AMU) and the Katholieke Universiteit Leuven, Universiteit Gent, Vrije Universiteit Brussel and Universiteit Antwerpen. The project addresses the needs of the communities in the southern Rift Valley in Ethiopia. It includes capacity building in teaching, laboratory skills and research to empower AMU and research to design sustainable solutions that answer the region's considerable challenges. The IUC program includes 6 linked projects that are in line with the national priorities of Ethiopia and the VLIR-UOS country strategy.

Project 3 'Health' encompasses research on CL, as this disease affects the community already for a very long time, thus there is an urge for research on this topic. My PhD project on the ecology of CL was carried out in synergy with the project of Behailu Merdekios, a PhD student and member of Project 3, whose focus lies on the public health issue of the disease. Together, our studies provide a good overview of the community knowledge, transmission dynamics, risk factors and distribution of CL in southwestern Ethiopia. This to lay groundwork for adequate detection of CL and implementation of prevention and control measures in southwestern Ethiopia.

Objectives

The transmission dynamics of *L. aethiopica*, transmitted by *P. pedifer* is unique and restricted to a relatively small area in southwestern Ethiopia and some parts of Kenya. Although the baseline research has been carried out in the 1970's by Ashford and

colleagues, many aspects of disease transmission remain unclear. It is important to understand which reservoirs maintain the spread of the infection and who are the key players of transmission to be able to eliminate all sources of *Leishmania* parasites. Moreover, a thorough understanding of the seasonality of the population density and habitat of the vector and its biting behavior (at which time point, where and from which hosts they prefer to take a blood meal) is pivotal to decide which and when control methods should be implemented to reduce disease transmission. Ochollo is the only reported endemic village in the whole of southern Ethiopia. It was therefore used as a model village to investigate how transmission takes place. However, in the surrounding areas that are potentially also burdened by the disease should be identified as well, in order to estimate the true magnitude of CL in southwestern Ethiopia and to be able to decide in which areas to apply control measures. Hence, the first objective of this thesis was to **explore the transmission dynamics of CL and map its distribution in southwestern Ethiopia,** which we describe in "Part II: Transmission"**.**

Furthermore, many approaches are available for detection of *Leishmania* parasites in a variety of samples, all coming with their advantages and drawbacks. For clinical samples, some assays lack sensitivity, while others are expensive or require a lot of expertise. In entomological and ecological research, a high number of specimens needs to be tested for the presence of *Leishmania*. Hence, there is need for sensitive, inexpensive, high-throughput screening methods to identify potential vectors and reservoirs and understand their ecology. For each setting and purpose, one should outweigh which method is most appropriate to use. Therefore, the second objective of this dissertation was to **evaluate the performance of different diagnostic assays for detection of *L. aethiopica* in a variety of clinical samples, field- and laboratory-infected sand flies and reservoir hosts,** which we present in "Part III: Diagnostics".

The sand fly fauna in Ethiopia is diverse and some of the species are closely related, resulting in difficulties for morphological identifications and molecular determinations are very costly, time consuming and Ethiopian species lack in reference databases. Recently, a novel protein profiling technique was introduced for sand fly species determination, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The third objective was to **assess whether MALDI-TOF MS protein profiling is a good alternative method for unambiguous sand fly species determination in Ethiopia.** This research is shown in "Part IV: Methods in sand fly research".

Project outline

Part II: Transmission

Chapter 2 describes the infection prevalence among the vector population and the habitat where most of the (infected) sand flies are present in Ochollo. Moreover, the seasonality of the vector population is presented and the role of rodents and hyraxes in transmission is discussed.

Chapter 3 focuses on the feeding behavior of the vector under natural circumstances and when hosts are equally available. Moreover, it shows the activity pattern of the vector and whether it exhibits endo- or exophagic biting behavior. Also the role of livestock in disease transmission is discussed.

Chapter 4 presents a species distribution model of *Phlebotomus pedifer*, the vector of cutaneous leishmaniasis in southwestern Ethiopia, which was carried out with a maximum entropy algorithm using microclimate, topographic and land use data.

Chapter 5 briefly reports places in southwestern Ethiopia that were previously not reported endemic, but where CL was found to occur and describes the appearance of CL lesions and the tendency of infected people to seek treatment.

Part III: Diagnostics

Chapter 6 documents the results of a comparison of five PCR methods with microscopy on two sample types collected from patients with a suspected CL lesion to advise on optimal diagnosis of *Leishmania aethiopica*.

Chapter 7 demonstrates the suitability of a crude nucleic acid extraction procedure in combination with a real-time qPCR assay targeting spliced-leader RNA for detection and quantification of *Leishmania* parasites in field- and laboratory-collected (infected) sand flies and hyrax tissue samples.

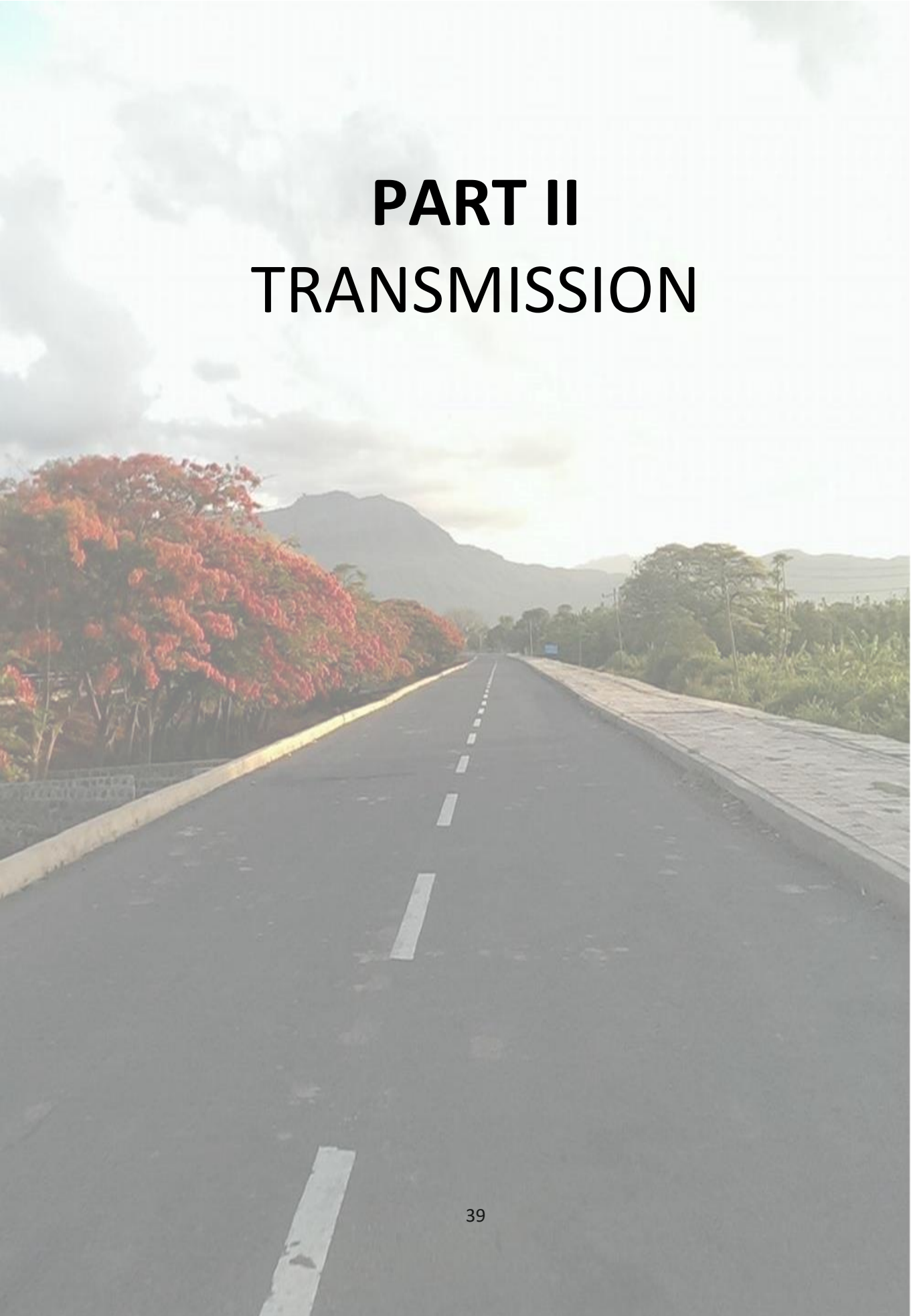
Part IV: Methods in sand fly research

Chapter 8 describes the applicability of MALDI-TOF MS protein profiling as a tool for unambiguous determination of Ethiopian sand flies in comparison with morphological and DNA-based taxonomic techniques.

Part V: Discussion

Chapter 9 provides an overall discussion and conclusions about the main results of this thesis and indicates the future perspectives for CL research and management in Ethiopia.

Capacity building briefly demonstrates the development of a molecular laboratory at Arba Minch University.



PART II
TRANSMISSION

CHAPTER 2

Ecology and seasonality of sand flies and potential reservoirs of cutaneous leishmaniasis in Ochollo, a hotspot in southern Ethiopia

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Research article

Published in PLoS Neglected Tropical Diseases, 2019, 13(8)

DOI 10.1371/journal.pntd.0007667

Abstract

Ochollo is a village in southern Ethiopia burdened with cutaneous leishmaniasis (CL), where *Phlebotomus pedif*er is the only vector for *Leishmania aethiopica* and hyraxes are confirmed reservoir hosts. A detailed description of the different players of transmission, and the ecology and seasonality of the vector needs to be established in order to accomplish efficient control programs.

Between March 2017 and February 2018, a monthly sand fly collection was carried out in different habitats and records of temperature and humidity were taken. Rodents and hyraxes were trapped in the dry and wet season. All samples were screened for *Leishmania* kinetoplast DNA (kDNA). Positive samples were further processed for determination of the *Leishmania* species and the species of the sand fly/small mammal that was found infected. Additionally, the species of 400 sand fly specimens from different habitats and seasons was identified.

17,190 *Sergentomyia* and *Phlebotomus* sand flies were caught and showed an overall kDNA prevalence of 2.6%, all were *L. aethiopica* infections only found in *P. pedifer.* The overall sand fly and *P. pedifer* abundance peaked in the dry season and was negatively correlated with the %RH. The kDNA prevalence varied over the months and was negatively correlated with the temperature. Total sand fly abundance did not differ between the sampled habitats, but *P. pedifer* was the distinct predominant species only in caves. Moreover, significantly more infected sand flies were found in caves. Only 1/192 rodents were kDNA positive, while 20.0% (5/25) of *Heterohyrax brucei* were found infected.

This study suggests that caves may be a source of multiplication of the infection. If an outdoor control program would be considered, it would be useful to focus on caves in the wet season, when the sand fly abundance is lowest. The captured rodent species appear not important for transmission and the contribution of hyraxes in transmission should be further investigated.

Background

Cutaneous leishmaniasis (CL) is a vector-borne disease caused by protozoa of the genus *Leishmania* and is listed as one of the neglected tropical diseases (NTD) [13]. It is characterized by nodules or ulcerative skin lesions, which can lead to secondary infections and disfiguring scars. In the Old World, female sand flies of some species of the genus *Phlebotomus* are the vector for *Leishmania,* as they can transmit the parasites when they take a blood meal [6,28]. Since the parasite's host range includes other mammals aside from humans in Ethiopia, CL is a zoonosis [31].

The annual CL incidence in Ethiopia is estimated around 20 000 to 50 000 cases, which is probably an underestimation [106]. Endemic foci of CL are located in the (mid-)highlands [135], widespread in the country [47,100]. The main causative agent for CL is *L. aethiopica* [136] and occasionally *L. tropica* [99]. The most common vectors are *P. pedifer and P. longipes* [47,48], although one study also obtained *L. aethiopica* promastigotes from *P. sergenti* [130].
A steep slope (>2.15 degrees), an altitude between 1700m and 3500m and an average yearly rainfall between 1300 and 1700 mm were found positively correlated with the occurrence of CL [111]. In general, areas with CL are characterized by a temperate climate and rugged environments with cliffs, serving as a favorable habitat for sand flies and hyraxes [115].

Rock hyrax (*Procavia* spp.) and bush hyrax (*Heterohyrax* spp.) are thought to be the reservoir hosts for *L. tropica* and *L. aethiopica* in Ethiopia [47,48,115], Kenya [118,126,138] and other African countries [44–46]. In northern Africa, rodents are known to be potential reservoirs for *L. major* [37,40,139] and *L. tropica* [140]. Likewise, Ethiopian rodents and bats have recently been found positive for *L. tropica* DNA [116,117]. This suggests that rodents and other small mammals, whose burrows can be used by sand flies as resting and breeding sites, might be alternative animal hosts for *L. aethiopica*. Resting sites for phlebotomine sand flies can be almost anything, as long as it is relatively cool and humid [56]. The places identified so far are cracks in basalt cliffs, fissures and holes in walls, barns, caves used by hyraxes, rodent burrows, tree trunks, etc. [47,56,62].

Ochollo is a village in the mid-highlands of southern Ethiopia where CL is mainly seen among children [100,101]. A study on primary school children in 2014 showed that 4% of the study population had active lesions, 59.8% of them had scars and 1.5% had both,

making it a considerable public health problem. Scars and lesions were predominantly localized above the neck with the highest occurrence on the cheeks [100]. One study performed a species typing on skin scrapings of 35 CL patients, confirming the hypothesis that *L. aethiopica* causes CL [141].

Former studies described two *Phlebotomus* species in Ochollo: *P. pedifer* and *P. ashfordi. P. pedifer* is the most abundant species, shows anthropophilic behavior and is so far the only incriminated vector, though, this information was based so far on relatively small sample sizes [61,130,131]. The parasite species from five naturally infected sand flies was determined and turned out to be *L. aethiopica* [61]. During a study in 1973 in Ochollo, *P. pedifer* was found to live in close association with hyraxes in cracks in rocks and sand fly blood meals were found to come from humans and hyraxes [136].

This suggested that hyraxes, which are numerous in Ochollo, might play an important role in transmission of *Leishmania* parasites. Ashford *et al.* found four out of 19 *Heterohyrax brucei* (21%) naturally infected with *Leishmania* parasites, and thereby hypothesized that hyraxes might even be able to sustain the parasite in the hyrax population, without a human factor [48].

In this study, we evaluate whether the situation of sand flies and hyraxes is still similar to 45 years ago, we investigate whether also rodents are likely as natural hosts for *Leishmania aethiopica* in the region and we describe spatial and temporal variation in the abundance of (infected) sand flies. Since CL is a zoonosis and the ecology and transmission dynamics are unique to each area, such knowledge must be obtained to lay the groundwork for adequate disease control.

Methods

Ethics Statement

Animal trapping and sample collection were conducted with authorization of the appropriate institutional and household authorities. Handling of the animals was carried out according to the 2016 Guidelines of the American Society of Mammalogists for use of small mammals in research and education. Permission from EWCA (Ethiopian Wildlife Conservation Authority) was not required according to the Ethical Clearance

Committee of Arba Minch University, since our study site was a village which is not part of a protected area and the captured species are not endangered.

Description of the study area

Ochollo is a village situated approximately 20 km north of Arba Minch at 6°11'N, 37°41'E. It lies on the western side of the Ethiopian Rift Valley, at an altitude of approximately 2100 m. It has a temperate climate, with rainy seasons in February and March (average rainfall 400 mm), and between June and September (average rainfall 600 mm) [101]. The population consists of approximately 5000 people, living on top of hills and across steep slopes. The hills give rise to caves and crevices and the landscape is overall rocky and relatively densely vegetated.

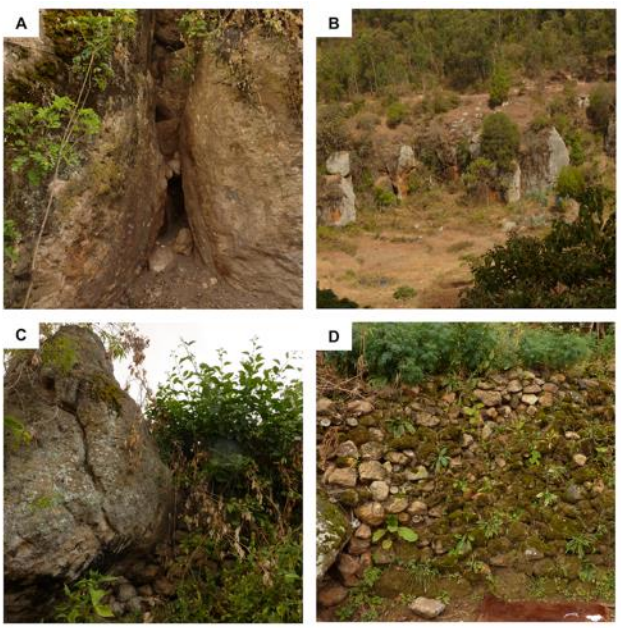


Fig 2.1 The different sample sites in Ochollo. Three habitats were used for sampling sand flies: (A-B) Caves, which are located on cliffs; (C) Rocky areas, constructed by big boulders; (D) Stone fences, manmade walls by stones covered with moss.

Sampling was carried out in three different habitats: caves, rocky areas and stone fences. Caves (Fig 2.1A) are highly abundant in Ochollo and are situated in the proximity of houses. They are located on cliffs (Fig 2.1B), where hyraxes are abundant. Rocky areas (Fig 2.1C) are a cluster of large boulders, with dark and humid crevices in

between, where hyraxes are seen at daytime. Stone fences (Fig 2.1D) are manmade walls around household compounds and footpaths, constructed by stones from the surrounding area. The stones are covered with moss, indicating a high humidity. There are many small openings between the rocks, leaving space for potential resting sites of rodents and sand flies.

Sample collection

Sand flies were collected from March 2017 until February 2018, for three consecutive nights between dusk and dawn in the three habitats every month (Additional file 2.1 Table S1). Sand flies were collected using CDC miniature light traps (John W. Hock Company, USA) and two types of sticky traps covered with sesame oil; sticky traps made from an A4 format cardboard plate covered with white papers and plastic were placed straight at the entrance of crevices, while white laminated A4 format papers were used to fold into cavities (Additional file 2.2 Fig S1). The traps were equally distributed over the sample sites belonging to the three habitats: caves, rocky areas and stone fences. Female sand flies were sorted out under the microscope. The head, wings and legs were disposed, and the thorax and abdomen were stored in 97% ethanol at -20°C until further analysis.

Rodents were trapped with Sherman live traps (76 x 89 x 229 mm, Sherman Live Trap Co., Tallahassee, FL, USA); in March, April, May, August and September 2017, 400 traps were distributed in three different habitats during three consecutive days, baited with peanut butter and flour and checked daily at sunrise and just before sunset. Hyraxes were collected by local hunters using traditional snare traps. Mammals were initially identified based on taxonomic features, weighed and measured [142]. Venous blood was collected on filter paper (dry blood spots) and stored at -20°C. Ear samples were collected (4mm x 4mm) and preserved in 97% ethanol at -20°C.

I-buttons (HQMatics, the Netherlands), small climate loggers, were placed at the sampling places to record the temperature and % relative humidity (%RH) on an hourly basis during the whole year. A short description of the weather conditions was recorded on the sampling days.

Molecular analyses

DNA extraction

DNA from individual sand flies was extracted individually by overnight incubation of the thorax and abdomen at 37°C in 50µl extraction buffer (10 mM TrisHCl pH 8, 10 mM EDTA, 0.1% SDS, 150 mM NaCl) and 0.5 µl proteinase K (200µg/ml). Afterwards, 25 µl distilled water was added and the sample was heated for 5 minutes at 95°C [143]. Negative and positive extraction controls were included, respectively being an uninfected and *L. major* (MHOM/SA/85/JISH118) infected *Lutzomyia longipalpis* acquired from the 'Laboratory of Microbiology, Parasitology and Hygiene' (LMPH, Antwerp, Belgium).

The individual sand fly extracts were cross-pooled in duplicate with an in-house pooling technique per six samples (Additional file 2.3: Fig S2). For purification of the pools, 1/10th volume 3M NaOAc (pH 5.6) and 2 volumes -20°C 100% ethanol were added to each pool and incubated overnight at -20°C. The samples were centrifuged 15 minutes at 15000 RCF at 4°C and the supernatant was removed. 500 µl of 70% ethanol (chilled at -20°C) was added for washing and the mixture was centrifuged again. After discarding the supernatant, the pellet was left to dry in a heating block at 50°C. Finally, the DNA was resuspended in 10 µl distilled water and left to rehydrate prior to using it for PCR.

DNA from ear and blood samples from rodents and hyraxes was extracted using the NucleoSpin Tissue kit (Macherey Nagel, Germany) according to the manufacturer protocol. Elution was done in 50 µl RNA/DNA free water.

Detection of Leishmania DNA

To screen the samples for *Leishmania* parasites, a real-time PCR approach based on Nicolas et al. (2002), targeting 120 bp of the kinetoplast DNA (kDNA) minicircles, was used with minor adjustments [144]. For each reaction, the positive and negative extraction controls described above, as well as PCR controls (RNA/DNA free water and *L. major* sand fly extracts respectively), acquired from LMPH, were included. The mastermix was made in a volume of 10 µl, containing 0.8 mg/ml BSA (GE Healthcare Lifescience, Belgium), 1X QUANTIMIX HotSplit Easy kit (Biotools, Spain), 0.1 µM of both primers, JW11 5'-CCT ATT TTA CAC CAA CCC CCA GT-3' and JW12 5'-GGG TAG GGG CGT

TCT GCG AAA-3' (Invitrogen, Life Technologies, Belgium), and 1 µl template. The reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium). After the initial denaturation for 15 minutes at 95°C, amplification was performed in 40 cycles with ten seconds denaturation at 95°C, ten seconds primer annealing at 58°C and 30 seconds extension at 72°C. For the melt curve, the following program was used: 15 seconds at 95°C, one minute at 60°C followed by a gradual increase of 0.3°C/s to 95°C. Validity of the PCR was determined by the positive control in duplicate that should give the same Ct value (+/- 0.5). The cut-off for positivity was determined by a Ct value of at least 5 Ct values below the lowest Ct value of all negative controls and a melting temperature of approximately 83.8°C (+/- 0.5°C).

Leishmania species determination

To identify the *Leishmania* species of the kDNA positive samples, a conventional PCR approach targeting the 350 bp ITS-1 gene based on El Tai et al. (2000) was used [145]. Positive and negative controls were included as described above. The mastermix was prepared in a volume of 15 µl, containing 0.5 µM of both primers, LITSR 5'-CTG GAT CAT TTT CCG ATG-3' and L5.8S 5'-TGA TAC CAC TTA TCG CAC TT-3' (Invitrogen, Life Technologies, Belgium), 0.2 mM dNTP (GE Healthcare Lifescience, Belgium), 1X QIAGEN PCR Buffer (Qiagen, Belgium), 0.04 U/µl HotStarTaq DNA polymerase (Qiagen, Belgium) and 1.5 µl template. The PCR was performed on Biometra T professional gradient Thermocycler (Biometra, the Netherlands). After 15 minutes initial denaturation at 95°C, the amplification was performed in 40 cycles of 30 seconds at 95°C, 30 seconds at 53°C and one minute at 72°C, followed by a final extension step of five minutes at 72°C. The results were visualized on a 1.5% agarose gel. Positive samples were sent to 'Vlaams Instituut voor Biotechnologie' (VIB, Antwerp, Belgium) for sequencing. Obtained sequences were aligned in GenBank using Basic Local Alignment Search Tool (BLAST) to determine the *Leishmania* species. Sequences showing a query coverage and identity of more than 97% were considered as a successful identification.

Sand fly species determination

Given the huge amount of sand flies collected (see further), it was not possible to identify them all morphologically to species level (also because of the effect of the oil on the sticky traps on the body condition), nor to apply molecular identification for all of them. Instead, 200 sand flies were selected from the wettest month (July 2017) and

again 200 from the driest month (January 2018) for molecular species determination. Those 400 samples were randomly selected from the trap sites, taking into account the proportion that sand flies from each trap site and habitat contributed to the total amount of sand flies in that month (Additional file 2.4: Table S2). From the sand flies captured in July 2017, the 200 samples selected for molecular screening included 118 samples from caves, 30 from rocky areas and 52 from stone fences. For January 2018, 124 sand flies were selected from caves, 26 from rocky areas and 50 from stone fences. Furthermore, we also used molecular species identification for all specimens in which kDNA was detected.

For this molecular identification, a conventional PCR targeting the 700 bp cytochrome c oxidase subunit I (COI) based on Kumar et al. 2012 was used [146]. The mastermix was made in a volume of 15 µl, consisting of 0.4 µM of each primer LCO 1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and 0.4 µM HCO 2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Invitrogen, Life Technologies, Belgium), 0.2 mM dNTP (GE Healthcare Lifescience, Belgium), 1X QIAGEN PCR Buffer (Qiagen, Belgium) and HotStarTaq DNA polymerase (Qiagen, Belgium). The PCR was performed on a Biometra T professional gradient Thermocycler (Biometra, the Netherlands). After an initial denaturation step of 15 minutes at 95°C, 35 amplification cycles were set at 40 seconds at 94°C, one minute at 45°C and one minute at 72°C and finally seven minutes at 72°C. Since no sequences for *P. pedifer* could be found in GenBank, this sand fly species was first morphologically identified and the according COI sequence was obtained with the protocol described above.

Small mammal species determination

The species of all captured small mammals was confirmed with a PCR targeting cytochrome B (350bp). The 15µl mastermix contained 1.5 µl template, 2.5 mM $MgCl_2$ (Promega, The Netherlands), 0.2 mM dNTP, 0.2 µM of each primer H15915 5'-TCT CCA TTT CTG GTT TAC AAG AC-3' and L14723 5'-ACC AAT GAC ATG AAA AAT CAT CGT T-3', 1X GoTaq flexi PCR buffer (Promega, The Netherlands) and 0.03 U/µl GoTaq G2 flexi DNA polymerase (Promega, The Netherlands). The following program was used for amplification: initial denaturation for 5 minutes at 94°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 52°C, 1.5 minute at 72°C, and a final extension step of 10 minutes at 72°C.

Amplicons of both PCRs were loaded on 1.5% gel. Successful samples were sent for

sequencing at VIB and sequences were aligned in BLAST for species identification with the same conditions for interpretation as mentioned for ITS-1 sequences.

Statistical analysis

The statistical analysis was performed with R version 3.4.3. Statistical tests were considered significant when the p-value < 0.05.

Differences in sand fly abundance

We tested whether the species distribution, over the different habitats, varied in the wettest and driest month by a generalized linear model (GLM, Poisson) using the glm function in R. The response variable was the number of sand flies of a particular species, in a specific habitat, within one month. The effects were habitat type, month, and species, and the interaction effects between the three factors. The histogram and Shapiro Wilk test on the residuals of the model indicated a normal distribution. Significance testing was done using an ANOVA Chi-square test on the model.

We tested whether the total sand fly abundance varied between habitats and seasons. We fitted general linear mixed models (GLMM) using the R package lmerTest [147]. The response variables for this model were the logarithmically transformed mean numbers of females or males, within one of the three habitat types, for a specific sample site, within one month. The fixed effects were sex, month and habitat type and interaction effects between the three fixed effects were included. Additionally, the random effect sample site nested within habitat type (different sample sites were used for each habitat type, all assigned with a unique code) was added to the model. Both, the histogram and Shapiro Wilk test on the residuals of this model indicated a normal distribution. Tests of fixed effects were based on F-tests with degrees of freedom approximated using Satterthwaite's method.

The correlation between the mean monthly temperature and %RH and the total sand fly abundance was determined. Two separate mixed models were constructed with either mean temperature or % RH as explanatory variable, because the variables were strongly correlated. The models were constructed with the fixed effects mean monthly temperature or %RH, habitat and their interaction. The random effect, response variables and the procedure of significance testing were the same as described above.

Differences in infection rate

Prevalence was always calculated based on the number of tested female sand flies. A general linear model (GLM) was coded with R package lme4 [148] to evaluate whether the kDNA positive sand flies are more abundant in certain habitats and show seasonal fluctuations. The response variables for this model were the proportions of kDNA positive sand flies per sample site per month. We added a very small number (0.005) to each of the proportions to avoid numerical problems in categories with overall zero proportions. The fixed effects were month, habitat type and their interaction. The optional vector 'weights' was included in the model, to indicate that the proportion was calculated based on the tested sand flies. The ANOVA Chi-square test on this model indicated which parameters had a significant effect. Additionally, a general linear hypothesis test (packages multcomp and zoo [149,150]) was used as a multiple comparison function, specified as a Tukey test, for the habitat type.

Besides, a GLM was generated to assess whether the temperature and %RH were correlated with the proportion of kDNA positive sand flies. The same steps were undertaken as described before for two separate models, with the mean monthly temperature or %RH, habitat type and the interaction as parameters, and the proportion of kDNA positive sand flies per sample site per month as response variable. The Chi-square test indicated which parameters had a significant effect.

Results

Small mammals, parasites and vectors

Small mammals were captured and subsequently screened with different PCR approaches (Table 2.1). We caught 192 rodents, of which one ear sample, derived from *Mus mahomet*, was positive for kDNA. The sample was not positive for ITS-1, thus the *Leishmania* species could not be determined. Of the three shrews (*Crocidura olivieri*) and four bats (*Nycteris spp.*) that were captured, none were found infected. From 25 captured hyraxes, which were all *H. brucei*, five (20.0%) ear samples gave a positive result for kDNA. Of these five samples, four were positive for ITS-1 and when aligned in GenBank, all were *L. aethiopica*. Lesions were never seen on the animals' nose or ears. None of the dry blood spot samples was positive.

Table 2.1 Small mammals captured in Ochollo and the amount (proportion %) of kDNA and ITS-1 positives.

Small mammal species	# captured	kDNA+ (%)	ITS-	BLAST
Rodents				
Arvicanthis spp.	24	0		
Dendromus spp.	3	0		
Lophuromys cf. chrysopus	24	0		
Mus cf. proconodon	21	0		
Mus mahomet	3	1 (33.3%)	0	
Mus minutoides	1	0		
Rattus rattus	41	0		
Stenocephalemys albipes	75	0		
Total rodents	192	1 (0.5%)		
Other small mammals				
Crocidura olivieri	7	0		
Nycteris spp.	4	0		
Heterohyrax brucei	25	5 (20.0%)	4	*Leishmania*

An overview of the sand fly data is presented in Additional file 2.1: Table S1. In total, 17190 (*Sergentomyia* and *Phlebotomus*) sand flies, 8410 females (48.9%) and 8780 males (51.1%), were caught between March 2017 and February 2018. Of the selection of 400 specimens from different habitats collected in January and July, 293 (73.3%) were *P. pedifer* (Table 2.2), the others were mostly *Sergentomyia spp*. and a very small number of *Phlebotomus* that matched with < 95% identity in GenBank with different *Phlebotomus* species. The overall species composition did not differ between months (χ^2_6= 1.03, p = 0.598).

Of 8410 females, 1065 were excluded for parasite screening, because of contamination or inhibition prior to or during DNA extraction (Additional file 2.1: Table S1). The reactions were determined as invalid, because positive and negative extraction controls did not give correct results. The overall percentage of sand flies positive for kDNA was 2.6% (187/7345). Assuming an overall proportion of 73.3% *P. pedifer* in the total sand fly population (Table 2.2), the proportion of the kDNA positive *P. pedifer* is 3.5% (187/5384). Of the 187 kDNA positive samples, 162 were positive for ITS-1 (86.6%,

Additional file 2.5: Fig S3), of which 58 samples were successfully sequenced (35.8%). The established species was *L. aethiopica* in all cases. The COI sequence of some morphologically identified *P. pedifer* sand flies was used as a reference sequence to align the obtained sequences with. Of the 187 kDNA positive samples, 148 were positive for COI (79.1%, Additional file 2.6: Fig S4), of which 147 samples were successfully sequenced (99.3%). All of the kDNA positive sand flies were identified as *P. pedifer*.

Table 2.2: Proportions of sand fly species in different habitats during the wettest (July 2017) and driest (January 2018) months in Ochollo.

Month	Species	Cave	Rocky area	Stone fence	All habitats
July 2017	*P. pedifer*	93 (78.8%)	16 (53.3%)	32 (61.5%)	141 (70.5%)
	Sergentomyia spp.	22 (18.6%)	13 (43.3%)	19 (36.5%)	54 (27.0%)
	Other *Phlebotomus*	3 (2.5%)	1 (3.3%)	1 (1.9%)	5 (2.5%)
January 2018	*P. pedifer*	117 (94.4%)	19 (76.0%)	16 (31.4%)	152 (76.0%)
	Sergentomyia spp.	7 (5.6%)	5 (20.0%)	35 (68.6%)	47 (23.5%)
	Other *Phlebotomus*	0 (0.0%)	1 (4.0%)	0 (0.0%)	1 (0.5%)
Overall seasons	*P. pedifer*	210 (86.8%)	35 (63.6%)	48 (46.6%)	293 (73.3%)
	Sergentomyia spp.	29 (12.0%)	18 (32.7%)	54 (52.4%)	101 (25.3%)
	Other *Phlebotomus*	3 (1.2%)	2 (3.7%)	1 (1.0%)	6 (1.5%)

Seasonal changes of the sand fly abundance and infection rate

An overview was made for the overall monthly number of captured sand flies and proportion of kDNA positive females (Fig 2.2 A and B). Fluctuations in the abundance of sand flies ranged between 779 and 2498 sand flies per month and was significantly different over the months ($F_{11,160} = 6.99$, $p < 0.001$).

The sand fly abundance was higher in the recorded dry season, with peaks in January, February and April. The population was lower from June to October, with the lowest point in August, which corresponds with the documented rainy season. Since we found no difference in the proportion of *P. pedifer* between the extreme months (Table 2.2), we assume that the seasonal patterns for the total sand fly population can be taken as a proxy for those in *P. pedifer* alone.

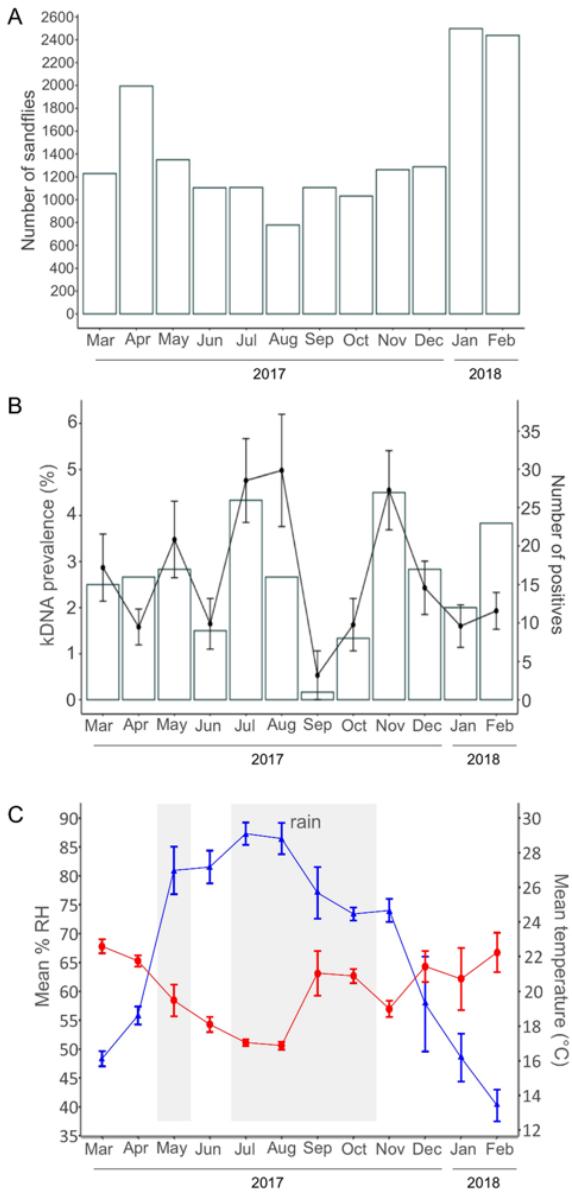


Fig 2.2 Monthly fluctuations of the (infected) sand flies and seasonal parameters (March 2017 – February 2018). (A) total number of sand flies caught per month. (B) the mean kDNA prevalence (%) among tested female sand flies per month with standard errors on the left axis (line plot) and the number of positive sand flies per month on the right axis (bar plot). (C) The mean monthly % RH (left axis) and temperature (°C, right axis) and according standard errors are respectively shown by a blue triangle and red circle line. The months when there was rainfall during the time of sand fly collection are depicted with grey background.

The kDNA prevalence among female sand flies varied significantly over the months (χ^2_{11}= = 29.73, p = 0.002), fluctuating from 0.5% to 5.0%, however the Tukey test could not show pairwise differences between months. There were peaks in kDNA prevalence rates in July and August and November. Similarly, the number of infected sand flies was highest in July and November, but also in February a relatively high number of infected sand flies was obtained, while the prevalence rate was rather low. A steep decline was observed in September, in both absolute numbers and prevalence. However, only 34% of the specimens from this month were successfully tested.

Correlation between seasonal parameters and sand fly abundance/ infection rate

The monthly temperature and % RH were recorded in Ochollo from March 2017 to February 2018 (Fig 2.2 C and Additional file 1: Table S1). Average values were used, since the recordings of different habitats were indistinguishable. The mean monthly temperature ranged between 17.0°C and 22.6°C over the year, while the % RH stretched between 40.2% and 87.3%. Mean temperature and % RH had an inverse pattern. The mean % RH rose from March to August and dropped from August to February. It showed a steep decline from November to February and had its lowest value in February, while the sand fly abundance peaked in January and February (2498 and 2439 sand flies respectively). The temperature fell from March to August and from then on increased again. It dropped to its lowest recordings in July and August (17.1°C and 17.0°C), while the prevalence then peaked highest (4.8% and 5.0%). In September and October, the temperature increased (21.0°C and 21.1°C) and simultaneously the prevalence decreased (0.5% and 1.6%). In November, a slight drop in temperature (about 2°C) was recorded and the number of positive sand flies increased from eight in October to 27 in November. The months that were recorded to have had rainfall during the sand fly collection were May, July, August, September and October.

The correlations between the mean temperature and % RH, and the average monthly number of sand flies and kDNA prevalence were obtained by GLMMs and GLMs respectively. Temperature did not have a correlation with the sand fly abundance ($F_{1,217}$ = 0.37, p = 0.955), but the % RH showed a significant negative correlation ($F_{1,217}$ = 5.95, p = 0.015). There was no interaction effect, meaning that the type of habitat (cave, stone fence or rocky area) did not influence the association between temperature and % RH and the population.

Temperature was negatively correlated with kDNA prevalence (χ_1^2 = 3.91, p = 0.048), while the % RH showed a borderline non-significant positive correlation with the kDNA prevalence (χ_1^2 = 3.79 , p = 0.051). The type of habitat did not influence the association between temperature and the kDNA prevalence, but there was an interaction effect between the type of habitat and the mean % RH (χ_2^2 = 6.87, p = 0.032).

Spatial (monthly) abundance of the (infected) sand flies

The average monthly sand fly abundance at each habitat type is presented on a logarithmic scale (Fig 2.3, left panel). Sand flies were present in each habitat during the whole year. The monthly variation of the sand fly abundance was significantly different at each habitat type ($F_{22,160}$ = 2.40, p < 0.001), as shown by the crossing lines and different peaks and drops for each habitat type, indicating that the sand fly population was not predominantly present at one of the three habitats. From a total number of 400 sand flies, 200 from July 2017 and 200 from January 2018, the number and proportion of each sand fly species in the different habitats are presented in Table 2.2.

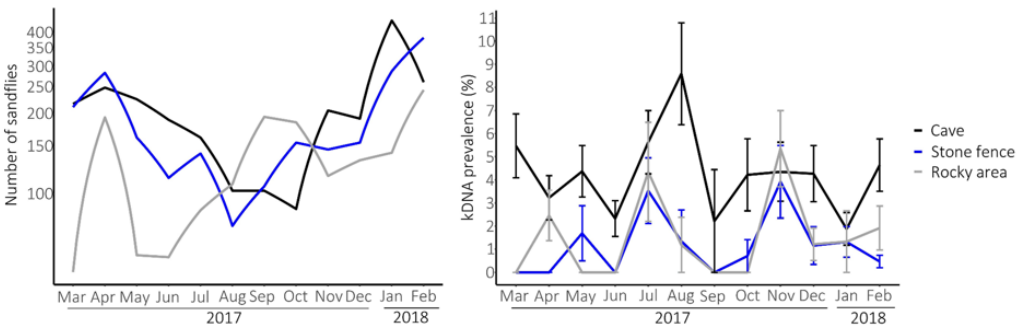


Fig 2.3 The average monthly sand fly abundance and kDNA prevalence (%) at the three sampled habitats (cave, stone fence, rocky area) in Ochollo (March 2017 – February 2018). Left panel: the average number of sand flies caught per month at each habitat presented on a logarithmic scale. Right panel: monthly kDNA prevalence (%) among tested females for each habitat. Error bars were calculated based on the standard error and mean, but were too small to visualize on the left graph. Black, blue and grey lines respectively represent the sand flies and kDNA prevalence at caves, stone fences and rocky areas.

The three-way interaction of the model on the selection of 400 sand flies of which the species was determined, indicated that the abundance of sand flies in the different habitats was significantly different in July 2017 and January 2018 (χ_0^2 = 23.62, p < 0.001). Only in caves, the population mainly consisted of *P. pedifer* in July (93/118,

78.8%) and January (117/124, 94.4%), while raw numbers and proportions of *P. pedifer* were lower at rocky areas and stone fences.

The monthly kDNA prevalence among tested females (Fig 2.3, right panel) followed a similar pattern at each habitat type (χ^2_{22}= 32.04, p = 0.077) and the habitat had a significant correlation with the prevalence (χ^2_2 = 55.01, p < 0.001). Throughout the whole year, infected sand flies were present at caves, in contrast with the stone fences and rocky areas, where there were several months without infected sand flies. At all three habitats, a peak in prevalence occurred in July or August. At stone fences and rocky areas, the maximum prevalence was reached in November.

Fig 2.4 presents which habitat was preferred by (infected) sand flies. According to the Tukey test, caves (4.27%) had a significantly higher prevalence than stone fences (0.97%, p < 0.001) and rocky areas (1.53%, p-value <0.001), which is also reflected by the black curve (Fig 2.3 left panel) that is, except for November, consistently higher than the other two. Raw numbers are presented at the bottom of Additional file 1: Table S1.

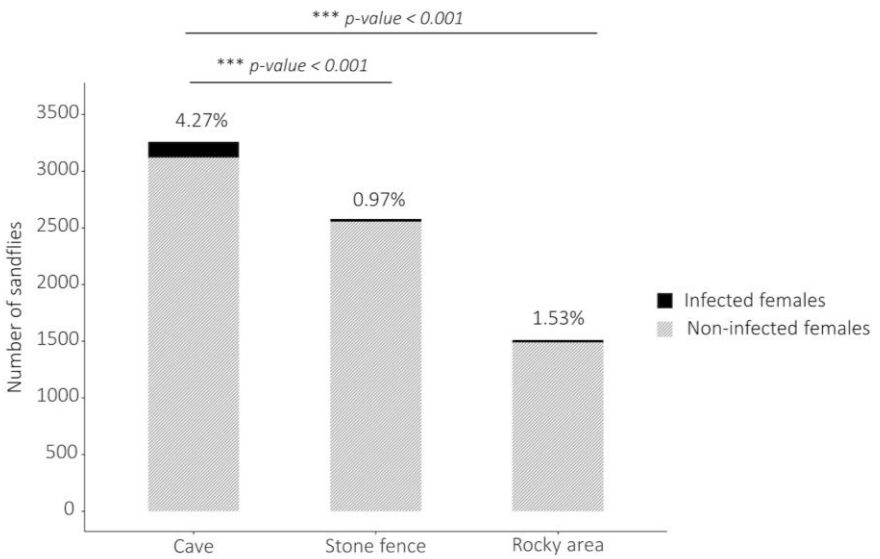


Fig 2.4 The distribution of (infected) sand flies in different habitats. The total number of non-infected and infected female sand flies in each of the three sampling habitats (cave, stone fence, rocky area) are respectively presented by the striated grey and solid black parts of the bars. P-values were obtained by a Tukey test.

Discussion

This work presents results on the parasites, small mammals and sand flies that contribute to CL transmission in southern Ethiopia, as well as the seasonal dynamics and habitat distribution of (infected) sand flies.

Small mammals, parasites and vectors

We demonstrated that a high proportion of *H. brucei*, which are abundant in Ochollo, were asymptomatically infected with *L. aethiopica*, confirming the results of Ashford et al. and Lemma et al. [47,48]. Similarly, *Procavia spp.* are described to carry *L. tropica* parasites in Ethiopia [48,115], Kenya [118] and other African countries [44,46,151], with prevalence ranging between 3.5% and 27% with seasonal variations. However, since the nomenclature *L. aethiopica* was only named as such in 1973, the aetiology of CL in Ethiopia was believed to be *L. tropica*, implying that the infections found in Ethiopia were in fact most probably due to *L. aethiopica* [136]. The high abundance and infectivity rate of hyraxes in Ochollo, in combination with the fact that they can become up to 13 years old, suggests that hyraxes might play a considerable role in the transmission of zoonotic CL, as suggested already 45 years ago. Yet, it remains to be evaluated how long the parasite can be sustained in a hyrax and how efficient it can be transmitted to *P. pedifer* [33].

Our study also assessed whether other small mammals could contribute to the transmission of CL. Only a single *Mus mahomet* was found kDNA positive, indicating that the captured rodent species do not play a major role as a source of infection in southern Ethiopia. Abebe et al. found one ground squirrel (*Xerus rutilus*) naturally infected with *L. aethiopica* in Aba Roba (1200 m), a VL endemic area, where there has never been a human case of CL [119]. Except for this report, to our knowledge *L. aethiopica* has not been found in rodents [47,48,103,152]. In contrast, studies carried out in different areas in Ethiopia found *L. tropica* in *Arvicanthis sp.*, *Gerbillus nanus* and *Acomys spp.* [116,130] and *L. major* in *Arvicanthis niloticus* [153]. We did not obtain enough samples from bats and shrews to draw any meaningful conclusions from this study. It would be interesting to acquire knowledge about the blood meal sources of *P. pedifer* in the area. If particular rodent species or other mammals appear to be a dominant blood source, new work could target these species. All dry blood spots samples were negative for kDNA, suggesting that these samples should not be used for molecular detection of *L. aethiopica*.

Based on a selected subsample of 400 sand flies, it can be concluded that *P. pedifer* (73.3%) is the predominant sand fly species in Ochollo. The rest of the sand flies mainly belong to species in the subgenus *Sergentomyia.* Only 1.5% of the sand flies in our traps were other *Phlebotomus* species. The exact species could not be determined, because sequences were not available in GenBank, but most probably it was *P. ashfordi* [61,131]. Overall, 2.6% of the sand flies (or an estimated 3.5% of *P. pedifer*) captured between March 2017 and February 2018 in Ochollo were positive for kDNA.

CL has been described as a disease of childhood in Ochollo, as about 65% of primary school children had scars or active lesions [100]. The infection at young age might be due to the children's or sand flies' behavior, but it could also be explained by the high prevalence and sand fly abundance, which indicate that there is a high intensity of *Leishmania* transmission, increasing the risk of early exposure to an infectious sand fly. Studies on the behavior of children and indoor/outdoor biting behavior of sand flies should be carried out to find out where and when transmission takes place. Two prior studies carried out in Ochollo described that promastigotes were found in 5.4% (2/37) [48] and 1.67% (5/359) [61] of the dissected sand flies. They only dissected a small number of *P. pedifer* females, in contrast with the current research, where all sand flies (*Phlebotomus* and *Sergentomyia*) were tested. The former two studies proved that *P. pedifer* is a vector for *Leishmania* by dissecting the midgut of the sand flies, while this study intended to evaluate whether other sand fly species could also carry *Leishmania* parasites. Therefore, we opted to screen a large sample size with a real-time PCR targeting kDNA. *Leishmania* parasites have a concatenated network of kDNA minicircles, which are highly abundant, making it a very sensitive fragment to target [154]. *P. pedifer* was the only infected sand fly species in Ochollo carrying exclusively *L. aethiopica* parasites. There is very limited knowledge about this vector, although this is a prerequisite for efficient vector control strategies. To know when and where to deal with the vector population, the seasonality and habitat preference were determined.

Seasonality

The relative humidity seemed to be a good indicator for rainfall, as rainfall on the days of sampling was recorded in most months with a high relative humidity. The seasons did not appear like previously described for this area [101], confirming that seasons are changing, as mentioned by inhabitants.

Our data illustrate that the sand fly abundance and *Leishmania* infection in Ochollo

were present throughout the whole year and exhibit a distinct seasonality. Since sand flies were not collected in the same number of caves, stone fences and rocky areas every month, this might give a slightly biased representation of the monthly abundance of sand flies. The % RH was found negatively correlated with the sand fly abundance, and since the % RH is a good proxy for rainfall, there is a decline in the sand fly abundance when the rainfall increases. This was reflected by the sand fly abundance, which peaked during the dry months and dropped in the rainy period. With *P. pedifer* accounting for nearly 75% of all sand flies, in the wet as well as in the dry season, it can be stated that the species' population decreases with increasing rainfall.

A study in the Mt. Elgon region in Kenya by Mukhwana et al. observed the same fluctuation in the abundance of *P. pedifer*, showing a peak in the two dry seasons and a drop in the two wet seasons [155]. Although that study was only based on 657 sand flies, it supports the current findings. No other studies were carried out on seasonality in areas where *P. pedifer* is responsible for CL transmission. *P. longipes* is the other main vector for *L. aethiopica* in Ethiopia and shows morphological and ecological similarities with *P. pedifer* [136,156]. However, contradictory to the obtained results for *P. pedifer*, an increase in rainfall was accompanied with a rise in the *P. longipes* population in a similar study conducted in Kutaber [48].

Increasing temperatures appeared to be accompanied with a drop in the number and proportion of kDNA positive sand flies, but not as distinct as the correlation between temperature and sand fly abundance. The low prevalence rate in September should be interpreted with caution. Due to inhibition, only 34% of the female sand flies caught in September were successfully tested, which might slightly lower the strength of the prevalence estimation within this month.

Studies have been done to investigate the effect of temperature on the development of *Leishmania* parasites in sand flies [157], as well as the metabolism of the sand flies [158]. Metabolic processes are slower at lower temperatures, so there is a delay in defecation when temperatures are lower, providing more time for *Leishmania* to establish an infection in the midgut [157,158].

Contradictory to our results, the seasonality of *P. pedifer* infected with *L. aethiopica* in the Mt. Elgon region in Kenya showed two drops, concurrent with the rainy seasons [155]. However, based on the tables and figures provided in the paper, it is unclear how the presented results were calculated: only 21 females were infected with

promastigotes, yet four trapping sites over a period of 12 months were described, resulting in 48 separate prevalence values. None of them were zero, raising doubts about the presented results. In Kutaber, the infection of promastigotes in *P. longipes* varied considerably over 14 months, but seemed to be independent of seasonal conditions [48].

Spatial abundance

Insight in the spatial abundance of the vector is important for potential future outdoor vector control strategies. The overall sand fly population in Ochollo was not predominantly abundant at a particular habitat, though, hyrax feces suggested to serve as larval food, were only present at caves and rocky areas. It was remarkable that in caves, on average almost 87% of the sand flies were *P. pedifer*, while at stone fences and rocky areas, which are situated closer to the people's houses, only half or even less than half of the population was *P. pedifer*. Other papers describe without statistical analysis that the preferred habitat for *P. pedifer and P. longipes* are cracks in basalt cliffs (here referred to as caves) [48,61,68,159].

The infected sand fly population was evidently more present at caves, where they were found throughout the whole year, while stone fences and rocky areas had several months without kDNA positive sand flies, implying that caves could be the source of multiplication of the infection. A study in Mt Elgon Region in Kenya observed that CL cases as well as *P. pedifer* were mainly found near caves and concluded from this that human infection with *L. aethiopica* by *P. pedifer* is happening near caves. It must be mentioned though, that no other possible habitats of *P. pedifer* were considered in that study [159]. At first sight, there seemed to be more CL cases close to the caves in Ochollo, but more research is required to investigate where the transmission particularly happens.

Public health relevance

Data on vectors and reservoirs of CL in southern Ethiopia are very limited. Until now, there are no efficient intervention programs, partially due to a potentially important zoonotic component and ecological factors associated with the disease that are left aside. Moreover, CL is moving towards new areas with susceptible people, because people are building settlements and cultivate closer to habitats of hyraxes and sand flies [47].

Our results can provide guidance for disease management programs in areas in southern Ethiopia and Kenya with a similar ecological and climatological context. Although there is still need to investigate potential indoor transmission, if outdoor vector control would be considered, it would be a good idea to focus on caves in the beginning of the wet season, when the population is at its minimum.

The role of hyraxes in CL transmission should be further investigated to assess whether they should be included in control programs. Destroying hyrax habitats close to human settlements is almost impossible in Ochollo, since the majority of the houses are surrounded by basalt cliffs in a range of 200 m. Shooting hyraxes or biological control of the population is believed not to be effective and might result in an increase in human-vector contact [31,48]. Rather than focusing on the hyraxes, attractive toxic sugar treated barrier fences around caves could be used to prevent sand flies from going towards human dwellings to acquire a blood meal [160].

Also, people should be made aware of the risk they face when building settlements or residing in the proximity of caves [132]. Additional information is required about the biting behavior of *P. pedifer* to decide whether indoor residual spraying and insecticide impregnated bed nets could be beneficial.

Acknowledgements

We are grateful to prof. Dr. Guy Caljon, Dr. Sarah Hendrickx and Rik Hendrickx from the Laboratory of Microbiology, Parasitology and Hygiene (University of Antwerp, Belgium) for their advice and positive controls. Our special thanks goes out to the village head and field workers of Ochollo, who made the sample collection possible. Also, we want to thank Glauco Camenish, from the University of Zurich (Switzerland), for guidance in the identification of the hyrax specimens.

Funding

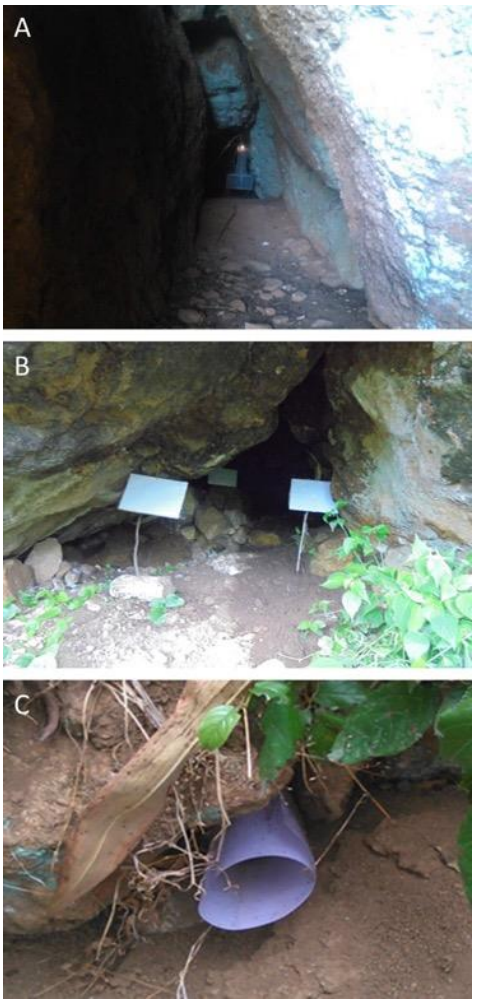
This work was supported by the University of Antwerp. MP is a fellow of the Flemish Interuniversity Council (VLIR-UOS, NDOC2016PR003).

Additional files

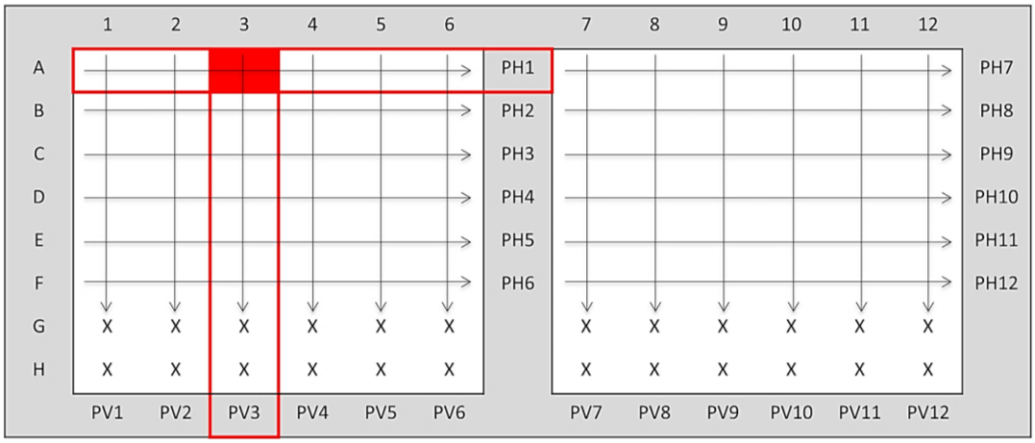
Additional file 2.1: Table S1 Overview of the monthly sample collection in Ochollo (March 2017—February 2018). The total number of sandflies, males and females caught per month. The number of not tested and tested females per month. The percentage of tested females, the number of kinetoplast DNA (kDNA) positive females and the kDNA prevalence (%) per month. *Abbreviations:* T = temperature; %RH = % relative humidity; # = number

Time	Location type	# sites	# total sand flies	# femal sand flies	# tested sand flies (%)	# tested kDNA+ sand flies (%)	Mean T (°C)	Mean %RH
Mar 2017	Cave	3	653	279	274 (98%)	15 (5.47%)	21.94	49.22
	Rocky	3	152	67	67 (100%)	0 (0%)	23.17	47.72
	Stone fence	2	424	181	181 (100%)	0 (0%)	22.72	47.86
	Overall	8	1229	527	522 (99%)	15 (2.87%)	22.61	48.32
Apr 2017	Cave	3	752	341	341 (100%)	11 (3.23%)	21.48	55.43
	Rocky	2	388	202	200 (99%)	5 (2.50%)	21.72	57.94
	Stone fence	3	855	469	468 (100%)	0 (0%)	22.24	53.10
	Overall	8	1995	1012	1009 (100%)	16 (1.59%)	21.81	55.79
May 2017	Cave	4	907	348	343 (99%)	15 (4.37%)	18.14	84.31
	Rocky	2	117	28	28 (100%)	0 (0%)	18.93	83.49
	Stone fence	2	326	121	118 (98%)	2 (1.69%)	23.74	66.56
	Overall	8	1350	497	489 (98%)	17 (3.48%)	20.27	80.94
Jun 2017	Cave	4	760	386	386 (100%)	9 (2.33%)	17.42	83.51
	Rocky	2	115	41	41 (100%)	0 (0%)	17.88	83.94
	Stone fence	2	229	119	119 (100%)	0 (0%)	20.06	70.37
	Overall	8	1104	546	546 (100%)	9 (1.65%)	18.45	81.53
Jul 2017	Cave	4	650	319	284 (89%)	16 (5.53%)	16.70	87.33
	Rocky	2	173	92	92 (100%)	4 (3.53%)	17.12	89.37
	Stone fence	2	284	177	170 (96%)	6 (4.35%)	17.46	81.11
	Overall	8	1107	588	546 (93%)	26 (4.76%)	17.09	87.31
Aug 2017	Cave	4	410	168	163 (97%)	14 (8.59%)	16.55	85.57
	Rocky	2	218	85	84 (99%)	1 (1.19%)	16.84	89.90
	Stone fence	2	151	74	74 (100%)	1 (1.35%)	17.61	77.90
	Overall	8	779	327	321 (98%)	16 (4.98%)	17.00	86.46
Sep 2017	Cave	3	308	144	45 (31%)	1 (2.22%)	21.83	73.40
	Rocky	3	585	276	101 (37%)	0 (0%)	20.42	81.62
	Stone fence	2	213	143	43 (30%)	0 (0%)	20.60	74.35
	Overall	8	1106	563	189 (34%)	1 (0.53%)	20.95	77.06
Oct 2017	Cave	4	350	173	167 (97%)	8 (4.79%)	20.39	74.56
	Rocky	2	371	187	184 (98%)	0 (0%)	21.43	72.47
	Stone fence	2	311	141	139 (99%)	0 (0%)	21.33	71.88
	Overall	8	1032	501	490 (98%)	8 (1.63%)	21.05	73.41
Nov 2017	Cave	3	619	273	254 (93%)	12 (4.72%)	18.19	76.79
	Rocky	3	351	192	184 (96%)	7 (3.80%)	19.83	71.39
	Stone fence	2	293	160	155 (99%)	8 (5.16%)	19.72	70.93
	Overall	8	1263	625	593 (95%)	27 (4.55%)	19.25	74.01

Dec 2017	Cave	3	575	310	284 (62%)	15 (5.28%)	21.79	56.28
	Rocky	3	401	255	244 (60%)	1 (0.41%)	21.32	64.04
	Stone fence	2	312	173	171 (73%)	1 (0.58%)	20.61	49.89
	Overall	8	1288	738	699 (65%)	17 (2.43%)	21.24	57.80
Jan 2018	Cave	3	1344	606	373 (62%)	7 (1.88%)	20.59	44.22
	Rocky	2	286	126	75 (60%)	1 (1.33%)	24.30	52.60
	Stone fence	3	868	417	303 (73%)	4 (1.32%)	17.34	53.00
	Overall	8	2498	1149	751 (65%)	12 (1.60%)	20.74	48.51
Feb 2018	Cave	3	790	397	345 (87%)	16 (4.64%)	22.32	37.53
	Rocky	2	492	226	208 (92%)	4 (1.92%)	24.17	43.85
	Stone fence	3	1157	714	637 (89%)	3 (0.47%)	20.19	42.06
	Overall	8	2439	1337	1190 (89%)	23 (1.93%)	22.23	40.24
Year	Cave		8118	3744	3259 (87%)	139 (4.27%)	19.92	67.35
	Rocky		3649	1777	1508 (85%)	25 (1.53%)	20.58	69.86
	Stone fence		5423	2889	2578 (89%)	23 (0.97%)	20.54	63.25
	Overall		17190	8410	7345 (87%)	187 (2.55%)	20.35	67.62



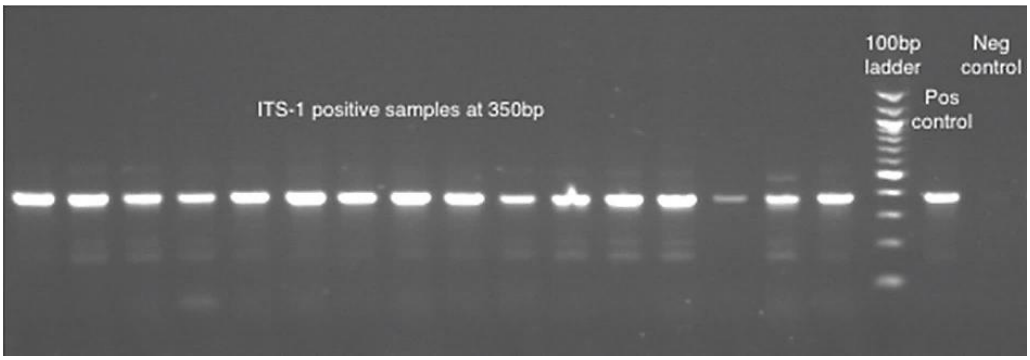
Additional file 2.2: Fig S1 Traps used for sandfly collection. A) CDC miniature light trap placed inside a cave; B) sticky traps within a cave; C) laminated papers rolled up into crevices of a stone fence.



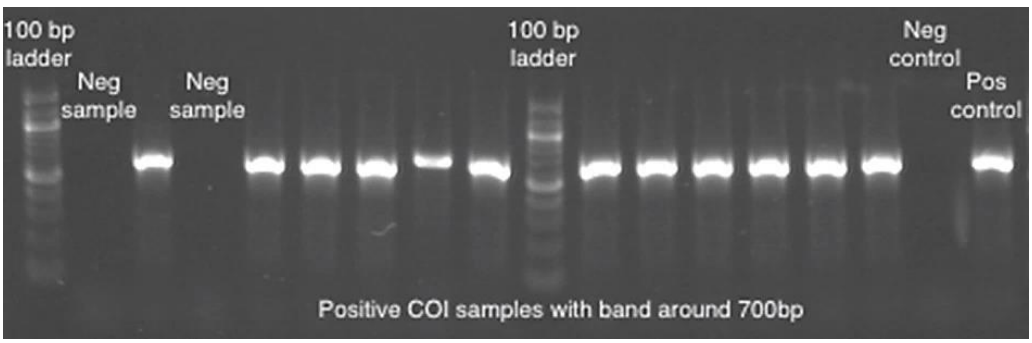
Additional file 2.3: Fig S2 Pooling of sandfly extracts before purification. Pooling was carried out vertically (PV = pool vertical) and horizontally (PH = pool horizontal) per 6 samples. Row G and H do not contain any samples. When two pools appear positive (in this case i.e. PH1 and PV3) after screening, the cross-over represents a kDNA positive sandfly.

Additional file 2.4: Table S2 Overview of the total population of sandflies and the sample that was used from each habitat and specific sample site for species determination. For species identification, 200 sandflies were collection from July 2017 and again 200 from January 2018. Sample sites are the different places where sandfly traps were placed, which contribute together to the total collection in a particular habitat. Collection describes the number of sandflies from a particular habitat or trap site that contributed to the total amount of sandflies collected during that month. This same proportion (percentages) was used for the sandflies that were brought to species level, here referred to as selection.

July 2017												
Habitat	**Cave**				**Rocky area**			**Stone fence**				**Total**
Collection / selection (% Total)	650/118 (59%)				173/30 (16%)			284/52 (26%)				1107/200
Sample site	**C2**	**C3**	**C5**	**C9**	**R8**	**R16**	**R17**	**S9**	**S12**	**S16**	**S17**	
Collection / selection	243/43	219/40	66/12	122/23	68/11	-	105/19	133/28	151/24	-	-	
% (sample site/habitat)	37%	34%	10%	19%	40%	-	60%	47%	53%	-	-	
January 2018												
Habitat	**Cave**				**Rocky area**			**Stone fence**				**Total**
Collection / selection	1344/124 (62%)				286/26 (13%)			540/50 (25%)				2170/200
Sample site	**C2**	**C3**	**C5**	**C9**	**R8**	**R16**	**R17**	**S9**	**S12**	**S16**	**S17**	
Collection / selection	550/51	273/25	521/48	-	-	204/18	82/8	-	296/27	27/3	217/20	
% (sample site/habitat)	41%	20%	39%	-	-	71%	29%	-	55%	5%	40%	



Additional file 2.5: Fig S3 ITS-1 amplicons on a PCR gel. ITS-1 amplicons show a band around 350bp. A 100bp ladder was used and, on the right, a positive control (band at 350bp) and negative control (no band) are depicted.



Additional file 2.6: Fig S4 COI amplicons on a PCR gel. COI amplicons show a band around 700bp. A 100bp ladder was used and the positive (700bp band) and negative control (no band) are shown.

CHAPTER 3

Feeding behavior and activity of *Phlebotomus pedifer* and potential reservoir hosts of *Leishmania aethiopica* in southwestern Ethiopia

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Research article

Published in PLoS Neglected Tropical Diseases, 2020, 14(3)

DOI 10.1371/journal.pntd.000794

Abstract

Cutaneous leishmaniasis (CL) is a major public health concern in Ethiopia. However, knowledge about the complex zoonotic transmission cycle is limited, hampering implementation of control strategies. We explored the feeding behavior and activity of the vector (*Phlebotomus pedifer*) and studied the role of livestock in CL transmission in southwestern Ethiopia.

Blood meal origins of engorged sand flies were determined by sequencing host DNA. A host choice experiment was performed to assess the feeding preference of *P. pedifer* when humans and hyraxes are equally accessible. Ear and nose biopsies from livestock were screened for the presence of *Leishmania* parasites. Sand flies were captured indoor and outdoor with human landing catches and CDC light traps to determine at which time and where *P. pedifer* is mostly active.

A total of 180 *P. pedifer* sand flies were found to bite hosts of 12 genera. Humans were the predominant blood meal source indoors (65.9%, $p < 0.001$), while no significant differences were determined outdoors and in caves. In caves, hyraxes were represented in blood meals equally as humans (45.5% and 42.4%, respectively), but the host choice experiment revealed that sand flies have a significant preference for feeding on hyraxes ($p = 0.009$). Only a single goat nose biopsy from 412 animal samples was found with *Leishmania* RNA. We found that *P. pedifer* is predominantly endophagic ($p = 0.003$), but occurs both indoors and outdoors. A substantial number of sand flies was active in the early evening, which increased over time reaching its maximum around midnight.

In contrast to earlier suggestions of exclusive zoonotic *Leishmania* transmission, we propose that there is also human-to-human transmission of CL in southwestern Ethiopia. Livestock does not play a role in CL transmission and combined indoor and outdoor vector control measures at night are required for efficient vector control.

Background

Cutaneous leishmaniasis (CL) is a vector born disease, caused by *Leishmania* protozoa and transmitted by female phlebotomine sand flies. It is characterized by nodules or ulcerative skin lesions on people's faces and extremities, which result in disfiguring scars after healing [6,13].
CL is a major public health concern in Ethiopia, affecting approximately 20,000 to 50,000 people annually [106], in which *Leishmania aethiopica* is responsible for the majority of the infections [47,114,136]. Ochollo, our study site, is a village in the mid-highlands of southwestern Ethiopia, where CL is endemic and is mainly affecting young children [100,161]. A recent study identified 4% of the primary school children with active lesions, 1.5% with lesions and scars and 59.8% with scars [100]. Adults are very seldom found with active lesions, because they already recovered from a childhood *Leishmania* infection, thereby becoming resistant to the development of clinical infection [162]. There are currently no control programs for CL in southern Ethiopia, mainly because of the complexity of the zoonotic transmission cycle and the limited understanding of the vector's behavior.

Previous researchers described *Phlebotomus pedifer* as the only vector in Ochollo [48,61,131,163], showing a 3.5% infection rate [163]. A study in Kenya found a high susceptibility of *P. pedifer* to *L. aethiopica* when feeding on active human CL lesions, implying that transmission is very efficient [129]. The species has been found indoors, around household compounds, in tree holes, rocky areas and inside caves [48,163]. A study in Ochollo in 1973 showed that 11 *P. pedifer* sand flies from indoors and five from caves were solely feeding on humans and hyraxes respectively [48]. However, until now relatively little is known about its biting behavior. Sand flies are generally known to be active between dusk and dawn and females feed on a wide variety of vertebrate hosts. However, the peak activity and host preference differs among sand fly species, so species specific entomological data are crucial to obtain a clear image of the transmission cycle [63,164,165].

Besides the vector, the reservoirs of the infection should be well documented. Hyraxes (*Heterohyrax bucei* and *Procavia capensis*) have been described as the reservoir of the zoonotic transmission of CL in Ethiopia [47,114,136,163]. *H. brucei* is abundant in Ochollo and a large proportion has been found infected with *L. aethiopica.* They live near human settlements, in caves and rock crevices, where sand flies and other potential hosts are abundant [48,163]. Rodents were found most probably not to play

a role in transmission in Ochollo [163], but other animals have so far not been investigated yet as carriers of *L. aethiopica*. Given that bovines are commonly bitten by the main CL vector in Ethiopia, *P. longipes*, their role in transmission needs further investigation [17,48,127].

Successful disease control requires profound understanding of the transmission cycle. Knowledge about the blood meal preference of sand flies is crucial to demonstrate which vertebrates might contribute to disease transmission and should be included in control programs. Moreover, information on where and at what time sand flies are biting is a prerequisite to decide which vector control methods should be applied.

In this study, we aimed to gather knowledge on *(i)* the blood meal sources of *P. pedifer* in different habitats and its feeding preference when hosts are equally available, *(ii)* the role of domestic animals in CL transmission, and *(iii)* the indoor and outdoor activity pattern of *P. pedifer.* This information will shed light on the natural transmission cycle of CL in southwestern Ethiopia and help in instructing control efforts in the area.

Methods

Ethics Statement

This study was reviewed, approved and monitored by the Institutional Ethics Review Board (IRB) of Arba Minch University (cmhs/1203482/111 and cmhs/120017/111). Healthy adults (> 18 years) with obvious scar formation, who have been living in Ochollo their whole life, were selected as subjects for the human landing collections and host choice experiment. Written informed consent was obtained from all human volunteers who participated. All animal handlings were carried out according to the 2016 Guidelines of the American Society of Mammologists for use of mammals in research and education and in agreement with the appropriate institutional authorities.

Study area

Ochollo is located in southwestern Ethiopia (6°11'N, 37° 41' E), about 20 km North of Arba Minch (Fig 3.1). It is a rocky area with steep slopes and basalt cliffs with caves, situated at an altitude ranging between 1600 m and 2200 m. The area has a modest climate with an average yearly temperature around 20°C and a high humidity from May until October [163]. The village covers approximately 1100 hectares and is divided into

eight sub-villages. Ochollo is densely inhabited by approximately 5000 people, which are mainly clustered on the tops of hills and steep slopes. People ranch cattle and goats, and some households have dogs. Hyraxes are abundant and live in caves and rocky areas near human residences, while rodents mainly occupy stone fences and human and animal dwellings. Houses are mainly made of mud, wood and grass, leaving many openings for sand fly entry and resting places.

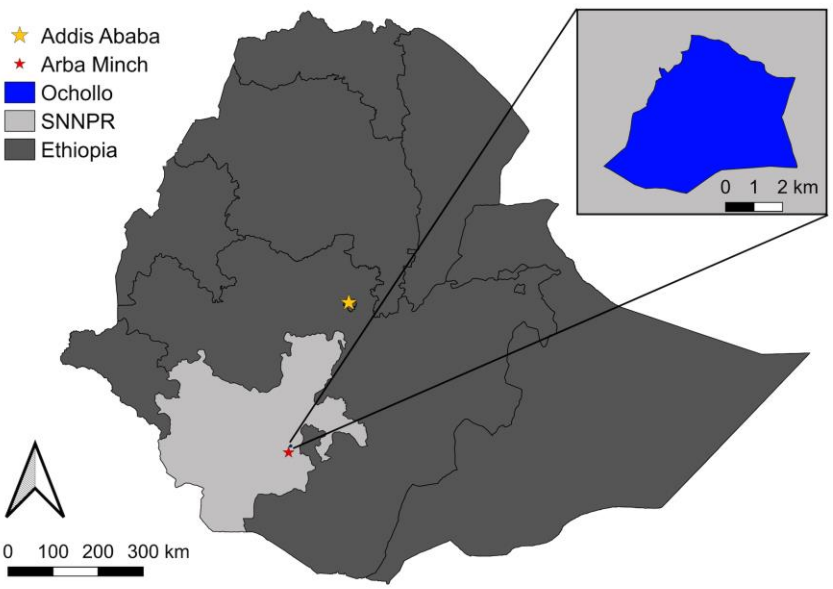

Fig 3.1 Map of the location of the study site, Ochollo, in Ethiopia [166,167]**.** SNNPR: Southern Nations, Nationalities and People's Region.

Host identification

Sand fly collection

Sand flies were collected indoors and outdoors from 72 households (nine households in each of the eight sub-villages) between February and May 2018. Additionally, ten caves were selected in the village for monthly sand fly collections from March to June 2018. Caves were located within the village, in a range of maximum 300 meters from human dwellings. Trapping was performed once per month at each sampling site with a particular entomological approach. Indoors and in caves, one CDC miniature light trap

(John W. Hock Company, Florida, USA) and five sticky traps (ST, A4 format white papers attached to card board, covered with plastic impregnated with sesame oil on both sides) were placed at the bed end and wall cracks indoors, and inside caves. Collection with the two methods was performed on separate days. Only ST were utilized outdoors (N = 5 per collection site), which were placed on wall cracks of the houses and surrounding potential sand fly breeding or resting sites. Traps were set at 18h and collected again the next morning at sunrise. Blood fed female sand flies were sorted out, and the thorax and abdomen were dissected and stored in 97% ethanol at -20°C until further analysis. No distinction was made among different stages of blood digestion in sand flies.

Blood meal analysis

DNA isolation from the blood fed specimens was performed with a NucleoSpin Tissue kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Finally, the DNA was eluted in 50µl nuclease free water. Unfed sand flies and sand flies fed on laboratory mice (*Lutzomyia longipalpis*, acquired from the Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium) were respectively used as negative and positive extraction controls.

DNA extracts were subjected to a PCR targeting a fragment of the Cytochrome B gene (*Cyt B*, 359 bp) as described by Steuber *et al*. (2005) and Carvalho *et al*. (2017) [168,169]. In short, the 15µl reaction mixture consisted of 1X Green GoTaq Flexi buffer (Promega, Leiden, Netherlands), 1.5mM $MgCl_2$ (Promega, Leiden, Netherlands), 0.5µM of both primers Cyt1 (5'-CCA TTC AAC ATC TCA GCA TGA TGA AA-3') and Cyt2 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3')(Life Technologies, Merelbeke, Belgium), 0.2 mM dNTPs (GE Healthcare Lifescience, Diegem, Belgium), 1U GoTaq G2 Flexi DNA Polymerase (Promega, Leiden, Netherlands) and 1.5µl DNA template. Amplification was carried out with an initial activation step of two minutes at 95°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 52°C and one minute at 72°C, and a final extension step of five minutes at 72°C. The PCR was performed on Biometra T professional gradient Thermocycler (Biometra, Westburg, Netherlands).

Positive and negative PCR controls and the above-mentioned extraction controls were included in each of the PCR reactions. PCR results were visualized on a 1.5% gel. After the PCR analyses were carried out at Arba Minch University in Ethiopia, the amplicons were sent to the Vlaams Instituut voor Biotechnologie (VIB) at the University of

Antwerp in Belgium for sequencing. The obtained *Cyt B* sequences were aligned in GenBank using BLAST to determine the host species that served as a blood source. Results were only included when both query coverage and identity exceeded 95%.

Sand fly species identification

If the blood meal of a specimen was successfully identified, the sand fly species was determined with a PCR targeting a 700 bp fragment of the *cytochrome c oxidase subunit I* (*COI*) gene, as described by Kumar *et al.* (2012) and Pareyn *et al.* (2019) [146,163].

Leishmania screening

Sand flies of which the blood meal was successfully determined were subjected to a real-time PCR assay targeting kinetoplast DNA (kDNA) for *Leishmania* detection. Furthermore, the *Leishmania* species of the kDNA positive specimens was determined with a PCR targeting a 350 bp fragment of the internal transcribed spacer 1 (ITS-1) gene followed by sequencing. Both assays were carried out as described in our previous study [163].

Livestock sample collection

Ear and nose biopsy samples from livestock (bovines and goats) were collected between January and April 2019. Samples originated either from animals that were slaughtered for human consumption or live animals. For the latter collection method, Xylocaine 2% gel (Astra Zeneca, Dilbeek, Belgium) was applied on the nose and ear for local anesthesia. Samples were collected using a 3 mm Biopsy puncher (Henry Schein, Vilvoorde, Belgium) and stored in 97% ethanol at -20°C until further analysis. To stop the bleeding, the incised skin wound was ligated with skin glue. Between ear and nose biopsy collections of each animal, the puncher was cleansed with 1% bleach and rinsed in distilled water, and a new puncher was used for each animal.

Leishmania detection in livestock

Nose and ear biopsies were screened for the presence of *Leishmania* nucleic acids at Arba Minch University (Ethiopia). Samples of each animal were subjected to a reverse transcriptase real-time PCR (RT-qPCR) targeting Spliced-Leader (SL-)RNA [170]. Additionally, a selection of the samples (216/412) was also screened for the presence of kDNA to confirm the results of the first assay [163]. Nose and ear biopsy samples of

each animal were pooled before extraction. Both RNA and DNA were isolated from the selection of samples that were tested by the two assays using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and NucleoSpin RNA/DNA buffer set (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. For the remaining samples, only RNA isolation was carried out using solely the first method. A naive and *L. major* infected mouse ear from an experimental infection (Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium; ethical approval UA ECD 2017-80) were respectively used as negative and positive extraction controls.

The SL-RNA and kDNA qPCR assays were carried out as described previously by Eberhardt *et al.* and Pareyn *et al.* respectively [163,170]. Extracts were 1:10 diluted before addition to the reaction mixture to avoid inhibition. Each PCR run included a no-template (negative) control and an infected (positive) control (a *L. aethiopica* infected hyrax nose biopsy DNA/RNA extract). For positive animals, ear and nose tissue samples were subjected to a separate extraction, followed by a PCR to determine the parasite RNA/DNA presence in the different tissues. Finally, the SL-RNA and kDNA positive samples were subjected to a PCR targeting ITS-1 for *Leishmania* species identification.

Sand fly activity pattern

Human landing catches

To assess the indoor and outdoor human biting rhythms of *P. pedifer*, human landing catches (HLC) were conducted in and around four different household compounds between March and August 2018. HLC were done once per month at each sampling site, indoors and outdoors on the same day. Four collectors were used for each collection night, each working for six hours. In the first part of the night (between 18h and 24h), one collector performed the HLC indoors and one outdoors. In the second part of the night (24h until 6h), two other collectors carried out the same activities. The person sitting outdoors was positioned at least 10 m from the house. The collectors sat on chairs with only their legs exposed and sand flies that landed on their legs were collected using a mouth aspirator. For each iteration of the experiment, there was an exchange in pre- and post-midnight and indoor-outdoor shifts to compensate for individual differences in attractiveness and collection skills. The collected specimens were cleared in Nesbitt's solution, washed with 70% ethanol and mounted in Hoyer's

medium. Sand flies were determined up to species level with morphological identification keys [131,156].

CDC light trap captures

Sand fly activity was also studied with CDC miniature light traps (John W. Hock Company, Florida, USA) from January to March 2019. Sand flies were captured during eight trapping sessions in a cave, which was previously determined as a hotspot of *P. pedifer* [163]. Traps were placed between 18h and 18h30 and collections were performed with an interval of 75 minutes until about 1h, eventually resulting in five collections per night. Sand flies were collected with a mouth aspirator and the number of male and female sand flies was recorded to establish the hourly activity of the sand flies. Captured sand flies were later used for the host choice experiment.

Host choice experiment

A host choice experiment was carried out using the sand flies that were captured with CDC light traps for the sand fly activity assessment [171]. Additional sand flies were trapped in other surrounding caves in Ochollo with CDC miniature light traps (John W. Hock Company, Florida, USA) in order to increase the sample size. The experiment was performed at 2h, immediately after the hourly CDC light trap captures.

The experimental set-up consisted of three connected cages (Fig 3.2) [171]. Female non-fed sand flies were placed in the middle cage. In each of the lateral cages, a particular host served as a blood meal source: a human volunteer's hand and forearm and a hyrax. Hyraxes were trapped by local people using traditional trapping methods. They were sedated during the experiment with ketamine (10mg/kg intramuscularly; Verve Human Care Laboratories, Uttarakhand, India) and placed inside the cage exposing the nose, ears and forepaws to the sand flies. Their eyes were protected from sand fly bites with a napkin. Human volunteers were lying down in a horizontal position with their head nearby the cage for CO_2 attractiveness towards the sand flies. The distance between the two hosts was approximately three meters and the experimental set-up was covered by a plastic canvas to avoid interference of wind and other potential feeding sources in the surroundings.

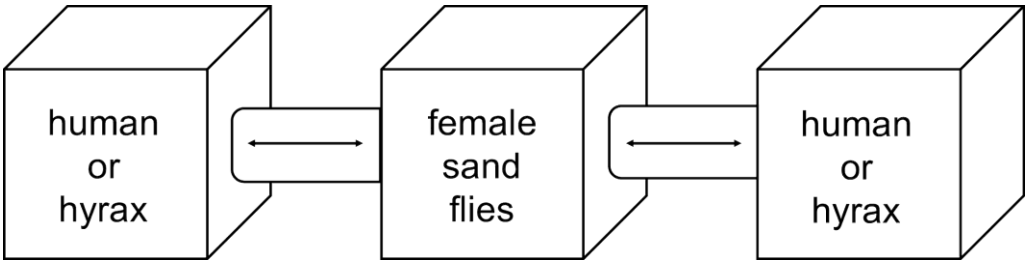

Fig 3.2 Host choice experiment set-up. Female non-fed sand flies captured from caves were transferred to the middle cage, where they were left for 30 minutes to adapt. Then, the connecting tubes to the lateral cages, where a hyrax and human hand were exposed, were opened to allow sand flies to obtain their preferred blood meal during four hours. The hosts themselves and their places were changed for each iteration of the experiment.

After 30 minutes adaptation in the middle cage, the connecting tubes to the two lateral cages were opened for four hours to allow sand flies to bite their preferred host. Blood fed sand flies were collected using a mouth aspirator and stored in 97% ethanol at -20°C until further analysis. The experiment was conducted eight times and for each iteration, the position of the hosts was changed and new subjects were used. Hyraxes were released at their trapping site after the experiment. The blood meal sources and sand fly species were determined by sequencing a fragment of the *Cyt B* and *COI* gene respectively, according to the methods described above (blood meal analysis and sand fly identification).

Data analysis

All statistical analyses were carried out in R version 3.5.0, using packages “lme4” and “lmerTest” [147,148]. P-values < 0.05 were assumed statistically significant.

Sand fly blood meal sources

To assess which host group served as an important blood meal source for sand flies, a generalized linear mixed model (GLMM) with Poisson error distribution was used. The number of sand flies that fed on a particular host, within a specific habitat, during a certain month was included as the response variable. The habitat where sand flies were captured (indoors, outdoors, cave), the host group they acquired their blood meal from and the interaction between habitat and host type were included as fixed effects. In order to correct for monthly variation in sand fly presence, we incorporated the

collection as a random effect in the model. A post hoc test, specified as Tukey test, was applied to compare the hosts groups with each other [172].
After the previous general model, GLMMs were made similarly for each habitat separately to determine the important blood meal sources in each habitat. The model was constructed as described above, but only the host group was included as a fixed variable.

Sand fly activity

The sand fly human biting rhythms indoors and outdoors were measured with HLC. We had to transform our data to a binomial distribution (0 = no sand flies were caught within an hourly time interval, 1 = one or more sand flies were captured within a time interval), because the hourly counts were low. A GLMM was used with the HLC at each time interval as the dependent variable with a binomial error distribution. The location where sand flies were trapped (indoor/outdoor) was included as a fixed effect in order to assess if sand flies bite significantly more indoors compared to outdoors. Time interval was included as a fixed effect to compare at which moment sand flies were mostly active. We used the time interval as a categorical variable instead of continuous, because our preliminary analyses showed that there was a non-linear correlation. The trapping month and sampling site were incorporated as random effects in the model.

Sand fly activity was based on the number of captured sand flies with a CDC light trap. A GLMM with Poisson error distribution was used to estimate the sand fly activity. Due to technical difficulties, we were not able to include the fifth trapping night in the final dataset. The number of male and female sand flies at a certain time interval was used as the dependent variable. Sex was implemented as a fixed effect in the model to assess whether there were more males or females. Time interval was included as a categorical fixed effect to determine during which period most individuals were present. The experiment day was incorporated as a random effect to correct for potential differences between the sampling days.

Host choice experiment

A GLMM with binomial distribution was used to determine the preferred blood meal source of sand flies when both humans and hyraxes are available. The proportion of sand flies that either fed on a hyrax or human during a single experiment was used as the response variable. The proportions were weighed by the total amount of sand flies that took a blood meal within a single experiment, since this varied between the

different experiments. The type of host and its position in the experiment were included as fixed effects to establish which host was preferred for a blood meal while correcting for potential personal and environmental bias. The experiment day was included as a random effect to correct for variation between days.

Results

Sand fly blood meal sources

A total of 11,488 sand flies were collected, of which 368 were blood fed female sand flies, which underwent procedures for blood meal origin identification. 92 (25.0%) of these samples were excluded from the analysis, as negative extraction controls tested positive, indicating contamination during the DNA isolation procedure. The *Cyt B* gene could not be amplified for 11 samples (3.0%) and the sequence identity of 83 samples (22.6%) could not be determined using the previously set cut-off requirements for the BLAST analysis. The overall analysis resulted in successful blood meal identification for 182 (49.5%) specimens. All of these specimens, except for two, turned out to be *P. pedifer*. The other two matched with several sand fly species of the subgenus *Laroussius* in GenBank with low query coverage and identity, but could not be identified up to species level. One sand fly acquired its blood meal from a human and the other one from a bush hyrax.

A total of 180 *P. pedifer* sand flies fed on 12 different hosts, presented in Table 3.1. Overall, humans were the most important blood meal source ($p < 0.001$), accounting for 59.4% of the identified origins, followed by bovines (13.9%), bush hyraxes (10.6%), goats (7.2%) and rodents (5.0%). Residual blood meals were acquired from a wide variety of vertebrates, together covering 4.0% of the determined sources. From the sand flies that fed on humans, five out of 107 (two collected from caves and three from indoors) were positive for *Leishmania* kDNA, which were all *L. aethiopica* infections.

Table 3.1 Blood meal analysis of *Phlebotomus pedifer* in Ochollo between February and May 2018. Scientific and common name of blood meal sources grouped into categories for further analysis, and the number and percentage of sand flies that fed on each host. Out of 180 sand flies, 129 were collected indoors, 18 outdoors and 33 inside caves.

Host category	Scientific name	Common name	Number	Percentage
Human	*Homo sapiens*	Human	107	59.4%
Livestock	*Bos taurus*	Bovine	25	13.9%
	Capra hircus	Goat	13	7.2%
Hyrax	*Heterohyrax brucei*	Bush hyrax	19	10.6%
Rodent	*Acomys spp.*	Spiny mouse	6	3.3%
	Grammomys sp.	Thicket rat	2	1.1%
	Arvicanthis sp.	Grass rat	1	0.6%
Other	*Myonycteris angolensis*	Bat	2	1.1%
	Canis lupus familiaris	Dog	2	1.1%
	Gallus gallus	Chicken	1	0.6%
	Felis catus	Cat	1	0.6%
	Tragelaphus sp.	Bushbuck	1	0.6%
Total identified			180	

Indoors, 129 blood fed sand flies were collected (Fig 3.3A). Significantly more sand flies (65.9%, $p < 0.001$) had fed on humans (Additional file 3.1: Table S1) compared to 23.3% that fed on livestock (16.3% on bovines and 7.0% on goats) and 5.4% on rodents. Three of the sand flies that were captured indoors had acquired their blood meal from hyraxes. No significant difference in blood meal sources could be determined from the 18 sand flies that were captured outdoors (Fig 3.3B, Additional file 3.1: Table S1), but the most important origins were again humans (38.9%) and livestock (33.3%). In caves, 33 sand flies were blood fed, but no significant differences in blood meal sources were determined (Fig 3.3C, Additional file 3.1: Table S1). Hyraxes were represented in blood meals equally as humans (45.5% and 42.4% respectively).

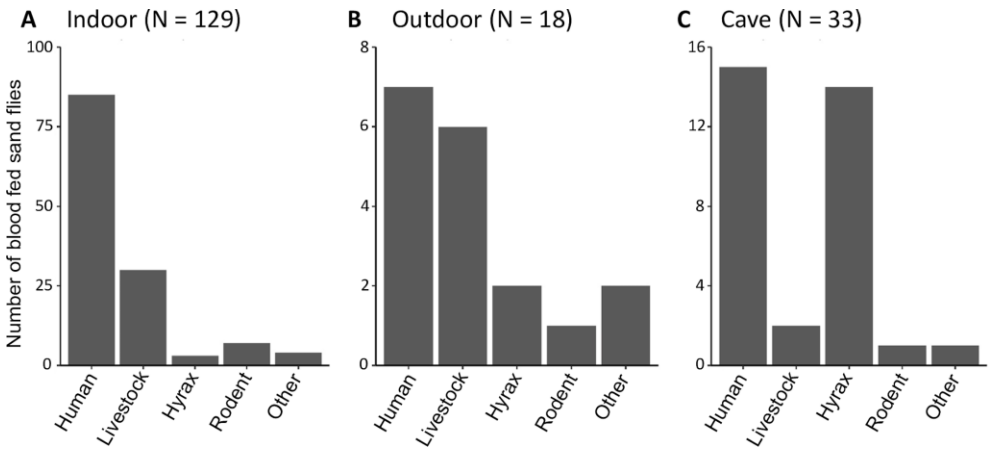

Fig 3.3 Blood meal sources of *Phlebotomus pedifer* captured in different habitats in Ochollo 2018. Blood meal sources of sand flies captured (A) indoors (B) outdoors and (C) in caves. The category livestock includes bovines and goats and the category rodents consists of *Acomys spp*., *Grammomys sp*. and *Arvicanthis sp*.. The 'other' host group includes all other vertebrates that sand flies fed on (Table 3.1).

Leishmania in livestock

A total of 412 ear and nose samples, of which 209 from bovines and 203 from goats, were collected. Of the 412 samples, 17 were collected from slaughtered bovines and 395 from live animals. The selection of the samples that were subjected to both kDNA and SL-RNA assays were all negative. The pooled sample of one live goat was positive for SL-RNA. After separate tissue extractions of this goat, the nose sample appeared positive for kDNA and SL-RNA, with a Ct value of approximately 28 in both assays. The ITS-1 gene could not be amplified for *Leishmania* species identification, presumably due to a low parasitemia.

Sand fly activity

Human landing catches A total of 161 sand flies were captured with HLC, of which 93% were identified as *P. pedifer*, while the remaining 7% belonged to the subgenus *Sergentomyia*, which were removed from further analysis. *P. pedifer* was found to bite humans both indoors and outdoors, but the overall the probability of indoor biting was significantly higher (p = 0.003, Fig 3.4A).

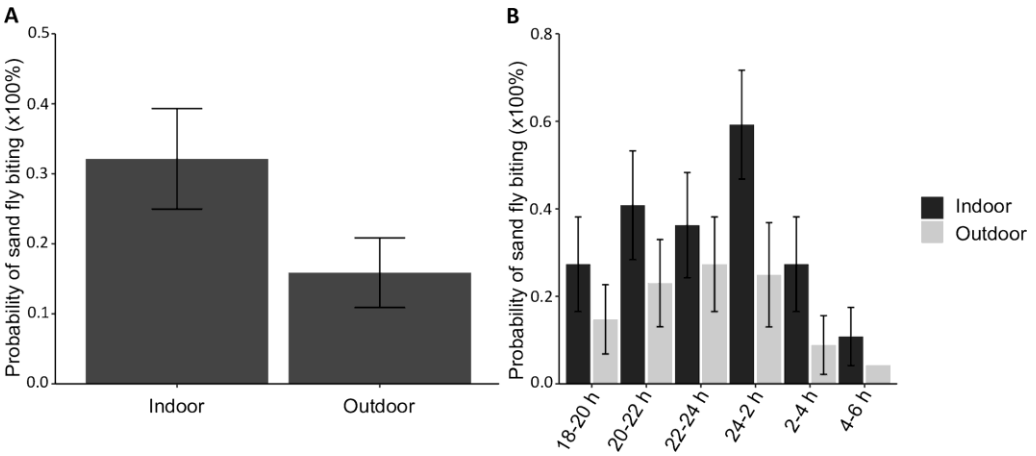

Fig 3.4 Indoor and outdoor human biting rhythms of *Phlebotomus pedifer* by human landing catches. (A) Average probability (%) of sand fly biting indoors and outdoors. (B) Average probability of temporal sand fly biting (%) indoors (dark grey bars) and outdoors (light grey bars). Error bars represent the standard error of the response variable.

Sand fly collections showed a similar temporal biting pattern indoors and outdoors ($p = 0.912$, Fig 3.4B). There was a substantial probability of sand fly biting in the early evening, which increased during the night, reaching a its maximum around midnight. After that, a drop was observed, with the lowest biting probability just before sunrise. Although Fig 3.4B shows a clear pattern in the activity, comparison of the biting activity at the different time intervals provided no significant differences between neighboring intervals (Additional file 3.2: Table S2).

CDC light trap captures

A total of 821 sand flies were captured with CDC light traps during seven trapping nights, of which 711 were female and 110 male. The hourly activity pattern of female and male sand flies is depicted in Fig 3.5. Significantly more female than male sand flies were captured ($p < 0.001$). The activity of female sand flies between 19h-20h was significantly lower compared to the other hours, except for 24h-1h (Additional file 3.3: Table S3). Other time intervals were not significantly different from each other. Overall, the activity pattern of female sand flies shows that there was considerable activity in the early evening, which increased over time, reaching its maximum at 22h-23h. For male sand flies, no clear trend could be distinguished.

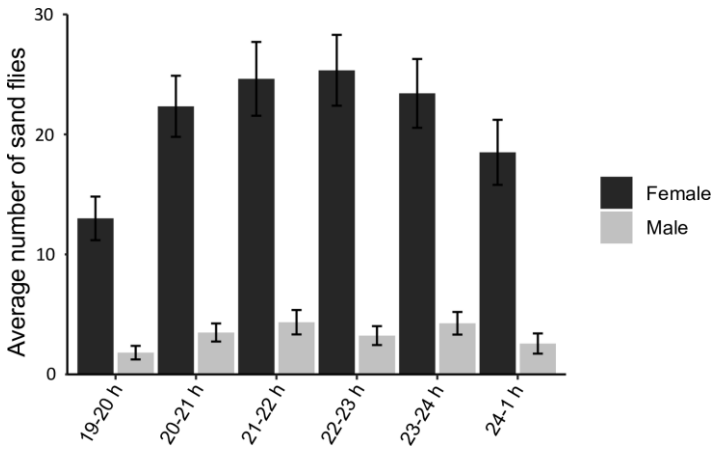

Fig 3.5 *Phlebotomus pedifer* activity pattern based on CDC light trap captures. The left, dark grey and the right, light grey bars are respectively the average number of female and male sand flies at a certain time interval. Error bars represent the standard error of the response variable.

Host preference

A total of 716 female *P. pedifer* sand flies were used in the host choice experiment, of which in total 65 sand flies were found blood fed over the eight repeats of the experiment (Additional file 3.4: Table S4).

The *Cyt B* fragment was successfully amplified and sequenced for all freshly engorged sand flies. All sand flies included in the experiment were *P. pedifer*. Fig 3.6 shows that sand flies were biting both hosts, but significantly more sand flies fed on hyraxes (61.5%) than on humans (38.5%, $p = 0.009$). The position of the host had no effect on the host choice ($p = 0.776$).

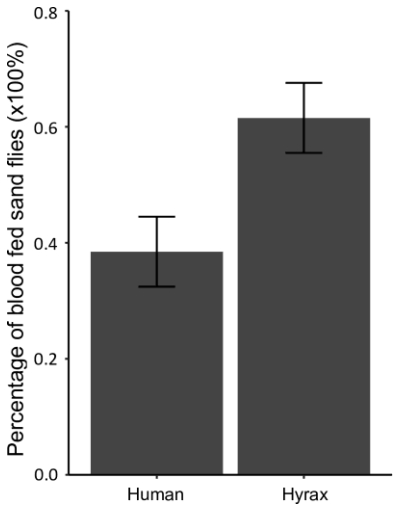

Fig 3.6 Host choice preference of *Phlebotomus pedifer* in an experimental set-up. Average percentage of sand flies that fed on a human or hyrax host during the host choice experiment. Error bars represent the standard error of the response variable.

Discussion

We gathered novel insights in the biting behavior and activity of *P. pedifer,* which can be used as a guidance in disease control programs; and studied the role of livestock in transmission of CL in southwestern Ethiopia.

We identified the blood meal sources of sand flies in Ochollo indoors, outdoors and in caves. Sand flies acquired their blood meals from hosts of 12 different genera, which is a wider variety compared to the results of Ashford *et al.* (1973) from Ochollo, who found only hyraxes and humans as blood meal sources in caves and indoors [48]. This may be linked to our larger sample size and the availability of more sophisticated analysis methods.

Overall, the majority of sand flies fed on humans and 4.7% of these sand flies were *Leishmania* DNA positive. Additionally, previous research showed that there is a high infection prevalence in humans in Ochollo and a study in Kenya demonstrated that *L. aethiopica* transmission from a human CL lesion to *P. pedifer* is very efficient [100,129]. These combined data suggest that humans are probably more than just an accidental host in the transmission cycle [17,100,129].

Only 10.6% of the sand fly blood meals were derived from hyraxes, however, the low representation of hyraxes in the blood meals may be biased by the higher proportion of specimens captured indoors in the analysed material. None of the sand flies that fed on hyraxes were found kDNA positive, which is an interesting result because we recently documented that 20% (5/25) of the hyraxes captured in Ochollo were *Leishmania* DNA positive [163]. Although CL in Ethiopia has only been reported as zoonotic with hyraxes serving as the only reservoir host [17,47,114,173], this study suggests that human-to-human transmission may be involved in southwestern Ethiopia. Hence, control should focus on humans, whether or not with additional reservoir control. Notably, the kDNA positive blood fed sand flies should be interpreted with care, because sand flies could have been infected before the current blood meal was acquired.

Some sand flies fed on rodents, in particular on different spiny mouse species (*Acomys spp.*), thicket rat (*Grammomys sp*.) and grass rat (*Arvicanthis sp*.). Several researchers have focused already on rodents as potential reservoirs of CL in Ethiopia. In a previous study that we carried out in Ochollo (2019), only a single African pigmy mouse (*Mus mahomet*) out of 192 rodents of eight different species was found kDNA positive. Despite the large trapping effort in that study, *Acomys* and *Grammomys spp.* were not captured [163].

In another study carried out all over Ethiopia by Kassahun and his colleagues (2015), 141 *Acomys spp.* were collected, of which 14 (9.9%) were found kDNA positive and three of these could be further identified as *L. tropica* infections [116]. A giant rat (*Cricetomys sp*.) and a ground squirrel (*Xerus rutilus*) have been found naturally infected with *L. aethiopica*. The latter was found in Aba Roba (1200 m), a visceral leishmaniasis (VL) endemic area in Ethiopia, where human CL cases have never been reported [118,119]. Except these observations, *L. aethiopica* has to our knowledge never been found in rodents before, despite the various sampling efforts that have been undertaken previously [47,48,103,119]. This suggests that rodents are probably not a reservoir for *L. aethiopica* and hence do not play an important role in the transmission dynamics.

Acomys spp. in Ethiopia are known to inhabit rocky slopes and rock crevices, but in our study, five out of six sand flies that fed on this species were found indoors [142]. Likewise, three sand flies that were captured indoors had fed on hyraxes. This result suggests that sand flies might rest indoors after have taken their blood meal elsewhere,

which could be further investigated with i.e. fluorescent powder on sand fly wings to demonstrate their dispersal [174,175].

Remarkably, the blood meal analysis from cave collected sand flies demonstrated that sand flies feed on humans as much as on hyraxes, while hyraxes are abundant and live inside the caves. This could be interpreted as an increased preference for biting humans. We tested this by a host choice experiment, in which human and hyrax were both available. Surprisingly, while sand flies did feed on both hosts, there was a significant preference for hyraxes, which contradicts the previous hypothesis. The result that humans are equally dominant as hyraxes as sand fly host meals in caves is probably not due to blood meal preference, but potentially to an increased availability of humans during the peak sand fly activity hours.

Previously, Ashford *et al.* recommended complete hyrax elimination by shooting or biological control, such as release of predators [31]. Other researchers suggested hyrax elimination near human settlements (about 1 km) as a possible intervention against *L. aethiopica* transmission [34,173]. This rises the concern that *P. pedifer*'s preferred blood meal host would not be available anymore, resulting in a shift towards biting humans, thereby increasing their exposure to sand fly bites and accordingly their risk of infection.

A study of Svobodova *et al.* (2006) showed that asymptomatically infected hyraxes were infectious to *P. arabicus*, but with a low success rate [176]. Additional research remains necessary to establish the transmission efficiency of parasites from infected hyraxes to the current vector to deliberate whether elimination of hyraxes should be included in control programs. The fact that that sand flies captured from caves obtained a similar proportion of blood meals from humans as from hyraxes implies that humans are accessible as blood source in proximity to the hyrax habitats.

Many specimens in the blood meal analysis did not provide a successful PCR or sequence according to the previously set requirements, while host sequences could be determined from all freshly fed sand flies in the host choice experiment. It has been shown that the success rate of host DNA analysis is negatively correlated with the time-course after the blood meal was taken [164,177–179]. We did not record the estimated days post-feeding, but sand flies with partially digested blood were included in the blood meal analysis, which explains the success rate of the blood meal analysis.

To unravel the complex CL cycle in southwestern Ethiopia, it is important to assess all players of transmission. This study demonstrates that livestock accounts for 21.1% of the blood meal sources of *P. pedifer*, but in ear and nose biopsies from goats and bovines, we found only a single goat nose biopsy positive for kDNA and SL-RNA with a high Ct value. This points to a relatively low parasitemia, although persistence and transmission of the parasites are not guaranteed [170]. Overall, it should be considered that some animals in the current study might have had parasites in their skin, which remained undetected due to the collection of only a small tissue biopsy [180].

Studies have already found DNA or antibodies indicating the presence of VL parasites in livestock, also in northern Ethiopia [121,181–183]. Research investigating the role of livestock in CL transmission is rather scarce. A study conducted during a CL outbreak in a non-endemic village in Venezuela found suspected active CL lesions in seven out of 29 (24%) donkeys in hairless areas (ear, tail, etc.), of which six lesion samples contained *Leishmania* parasites [184]. In a similar research conducted in a CL endemic area in Kenya, one goat was found with lesions and detectable levels of *L. aethiopica* DNA in the skin and other organs [122].

Based on our results, gathered from a large sample size collected from areas with different ecological features and screened with highly sensitive assays, we conclude that domestic animals in similar ecological areas in southwestern Ethiopia are likely not to play a considerable role in transmission. However, many sand flies acquired their blood meal from these animals and it was observed that livestock is living close to or even inside human settlements in Ochollo.

It has been suggested to keep livestock close to human settlements to divert vector biting from humans (zooprophylaxis) or to use them as baits for vector attraction to insecticide-treated livestock [185–188]. In contrast, other researchers assert that this could increase the vector population near humans (zoopotentiation) or augment the vector infectivity if blood meal sources are readily available [12,182,186,188]. More research is necessary to determine whether domestic animals could serve as protection against contraction of leishmaniasis.

Understanding the vector's biting behavior gives an indication about when and where *Leishmania* transmission occurs, and at which time and place control strategies would be most effective.

Both activity experiments showed that sand flies are predominantly active around midnight and the majority of the sand flies were captured indoors with HLC. Therefore, insecticide-treated bed nets or indoor residual spraying are potentially effective control strategies to manage the peak transmission at night [79,189–191].

Considerable activity was also observed in the early evening with about 30% of the sand flies captured outdoors by HLC. During the fieldwork, children were collecting water near caves and rock crevices and adults were performing outdoor activities in the early evening (e.g. dinner preparation and washing), thereby increasing their risk of exposure to potentially infectious sand fly bites. This was also shown in a study by Sang *et al.* in a CL endemic area in Kenya, where almost all CL cases admitted that they often visit caves [192]. Hence, improvement of community knowledge and attempts to decrease the vector population densities near places of outdoor activity could contribute to a reduction of residual transmission [49].

The activity of the CL vectors in Ethiopia has never been studied so far, but similar studies were carried out on *P. orientalis* in different VL foci in northern Ethiopia and Sudan [193–198]. These studies found various activity patterns for this vector species, indicating that the activity of a single species can differ between regions. Research on sand fly behavior in each ecologically different setting is accordingly necessary to accomplish efficient vector control.

In conclusion, this study shows that sand flies in Ochollo often feed on humans and, therefore, human-to human transmission of *L. aethiopica* should be considered. Hyraxes are the preferred blood meal source when hosts are equally accessible, so the efficiency of parasite transmission from *H. brucei* to *P. pedifer* should be investigated before including them in control programs. Livestock appears an important blood meal source for sand flies, but does probably not play a significant role in transmission of CL in southwestern Ethiopia. *P. pedifer* is mainly active at night indoors, but there is also considerable outdoor activity, suggesting that combined measures are required for efficient disease control.

Acknowledgements

We are very grateful to the village head, agricultural extension worker, volunteers and field workers of Ochollo, who made the sample collection possible. Also, we want to thank Dr. Simon Shibru of Arba Minch University for his support in Ethiopia. Our special

thanks goes out to Natalie Van Houtte of the Evolutionary Ecology Group (University of Antwerp, Belgium) for her excellent technical assistance in the laboratory.

Funding

This work was supported by the University of Antwerp and the Norwegian Programme for Capacity Development in Higher Education and Research for Development (NORHED). MP is a fellow of the Flemish Interuniversity Council (VLIR-UOS, NDOC2016PR003).

Additional files

Additional file 3.1: Table S1 Comparison of different blood meal sources of *Phlebotomus pedifer* overall and in each habitat (indoor, outdoor, cave) separately.

Habitat	Comparison	Estimate	Standard error	p-value
Overall	Human-hyrax	1.555	0.352	<0.001
	Human-livestock	1.070	0.319	0.007
	Human-rodent	2.384	0.511	<0.001
	Human-other	2.339	0.466	<0.001
	Hyrax-livestock	-0.485	0.421	0.778
	Hyrax-rodent	0.829	0.580	0.609
	Hyrax-other	-0.783	0.541	0.596
	Livestock-rodent	1.314	0.560	0.131
	Livestock-other	1.269	0.520	0.105
	Other-rodent	0.045	0.655	1.000
Indoors	Human-hyrax	3.344	0.584	<0.001*
	Human-livestock	1.041	0.211	<0.001*
	Human-rodent	2.497	0.391	<0.001*
	Human-other	3.056	0.509	<0.001*
	Hyrax-livestock	-2.303	0.602	0.001*
	Hyrax-rodent	-0.847	0.686	0.731
	Hyrax-other	-0.288	0.759	0.996
	Livestock-rodent	1.455	0.417	0.005*
	Livestock-other	2.015	0.529	0.001*
	Other-rodent	-0.560	0.623	0.898
Outdoors	Human-hyrax	1.254	0.802	0.522
	Human-livestock	0.154	0.556	0.999
	Human-rodent	1.954	1.069	0.362
	Human-other	1.254	0.802	0.522
	Hyrax-livestock	-1.109	0.816	0.663
	Hyrax-rodent	-0.693	1.225	0.980
	Hyrax-other	0.000	1.000	1.000
	Livestock-rodent	1.799	1.080	0.460
	Livestock-other	1.109	0.816	0.663
	Other-rodent	0.693	1.225	0.980
Cave	Human-hyrax	0.069	0.372	0.999
	Human-livestock	2.015	0.753	0.057
	Human-rodent	2.708	1.033	0.066
	Human-other	2.708	1.033	0.066
	Hyrax-livestock	1.946	0.756	0.075
	Hyrax-rodent	2.639	1.035	0.080
	Hyrax-other	2.639	1.035	0.080
	Livestock-rodent	0.693	1.225	0.979
	Livestock-other	0.693	1.225	0.979
	Rodent-other	0.000	1.414	1.000

Additional file 3.2: Table S2 Comparison of hourly differences in biting behavior of *Phlebotomus pedifer* sand flies indoors and outdoors by human landing catches.

Sex	Comparison	Estimate	Standard error	p-value
Indoors	18h-20h - 20h-22h	-0.606	0.639	0.999
	18h-20h - 22h-24h	-0.414	0.643	1.000
	18h-20h - 24h-2h	-1.352	0.642	0.619
	18h-20h - 2h-4h	-0.001	0.661	1.000
	18h-20h - 4h-6h	1.133	0.785	0.955
	20h-22h - 22h-24h	0.193	0.620	1.000
	20h-22h - 24h-2h	-0.746	0.616	0.988
	20h-22h - 2h-4h	0.605	0.639	0.999
	20h-22h - 4h-6h	1.739	0.786	0.501
	22h-24h - 24h-2h	-0.938	0.622	0.639
	22h-24h - 2h-4h	0.413	0.643	1.000
	22h-24h - 4h-6h	1.547	0.771	0.689
	24h-2h - 2h-4h	1.351	0.642	0.620
	24h-2h - 4h-6h	2.485	0.772	0.058
	2h-4h - 4h-6h	1.133	0.785	0.955
Outdoors	18h-20h - 20h-22h	-0.548	0.745	0.999
	18h-20h - 22h-24h	-0.777	0.732	0.996
	18h-20h - 24h-2h	-0.652	0.797	0.999
	18h-20h - 2h-4h	0.575	0.956	1.000
	18h-20h - 4h-6h	1.359	1.185	0.993
	20h-22h - 22h-24h	-0.229	0.676	1.000
	20h-22h - 24h-2h	-0.104	0.748	1.000
	20h-22h - 2h-4h	1.123	0.916	0.987
	20h-22h - 4h-6h	1.907	1.154	0.889
	22h-24h - 24h-2h	0.125	0.735	1.000
	22h-24h - 2h-4h	1.353	0.906	0.943
	22h-24h - 4h-6h	2.137	1.146	0.782
	24h-2h - 2h-4h	1.227	0.951	0.981
	24h-2h - 4h-6h	2.011	1.182	0.868
	2h-4h - 4h-6h	0.784	1.293	1.000

Additional file 3.3: Table S3 Comparison of hourly differences in activity of male and female *Phlebotomus pedifer* sand flies by CDC light trap captures. *statistically significant

Sex	Comparison	Estimate	Standard error	p-value
Female	19h-20h - 20h-21h	-0.542	0.138	0.005*
	19h-20h - 21h-22h	-0.639	0.149	0.001*
	19h-20h - 22h-23h	-0.668	0.141	< 0.001*
	19h-20h - 23h-24h	-0.588	0.144	0.003*
	19h-20h - 24h-01h	-0.353	0.167	0.618
	20h-21h - 21h-22h	-0.098	0.124	1.000
	20h-21h - 22h-23h	-0.126	0.115	0.995
	20h-21h - 23h-24h	-0.047	0.119	1.000
	20h-21h - 24h-01h	0.189	0.145	0.980
	21h-22h - 22h-23h	-0.029	0.127	1.000
	21h-22h - 23h-24h	0.051	0.132	1.000
	21h-22h - 24h-01h	0.286	0.150	0.758
	22h-23h - 23h-24h	0.079	0.123	1.000
	22h-23h - 24h-01h	0.315	0.149	0.618
	23h-24h - 24h-01h	0.236	0.154	0.932
Male	19h-20h - 20h-21h	-0.657	0.362	0.809
	19h-20h - 21h-22h	-0.876	0.373	0.442
	19h-20h - 22h-23h	-0.580	0.379	0.933
	19h-20h - 23h-24h	-0.855	0.364	0.443
	19h-20h - 24h-01h	-0.351	0.438	1.000
	20h-21h - 21h-22h	-0.219	0.297	1.000
	20h-21h - 22h-23h	0.077	0.304	1.000
	20h-21h - 23h-24h	-0.199	0.286	1.000
	20h-21h - 24h-01h	0.306	0.375	1.000
	21h-22h - 22h-23h	0.296	0.318	0.999
	21h-22h - 23h-24h	0.021	0.301	1.000
	21h-22h - 24h-01h	0.505	0.384	0.970
	22h-23h - 23h-24h	-0.276	0.308	0.999
	22h-23h - 24h-01h	0.229	0.584	1.000
	23h-24h - 24h-01h	0.505	0.079	0.975

Additional file 3.4: Table S4 Overview of the sand fly blood meal sources in the host choice experiment. For each of the eight iterations of the experiment, the number (%) of sand flies that were used for the experiment, that eventually took a blood meal and which host they were found to feed on are presented.

Experiment number	Total amount of female sand flies	Total amount of blood fed female sand flies (%)	Total amount of sand flies fed on hyrax (%)	Total amount of sand flies fed on human (%)
1	95	5 (5.3%)	1 (20.0%)	4 (80.0%)
2	118	13 (11.0%)	9 (69.2%)	4 (30.8%)
3	65	11 (16.9%)	6 (54.6%)	5 (45.5%)
4	80	2 (2.5%)	0 (0.0%)	2 (100.0%)
5	34	4 (11.8%)	2 (50.0%)	2 (50.0%)
6	108	6 (5.6%)	5 (83.3%)	1 (16.7%)
7	106	18 (16.0%)	13 (72.2%)	5 (27.8%)
8	110	6 (5.5%)	4 (66.7%)	2 (33.3%)
Total	716	65 (9.0%)	40 (61.5%)	25 (38.5%)

CHAPTER 4

High-resolution habitat suitability model for *Phlebotomus pedifer*, the vector of cutaneous leishmaniasis in southwestern Ethiopia

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Research article

Published in Parasites & Vectors, 2020, 13(467)

DOI 10.1186/s13071-020-04336-3

Abstract

Phlebotomus pedifer is the vector for *Leishmania aethiopica* causing cutaneous leishmaniasis (CL) in southwestern Ethiopia. Previous research on the transmission dynamics of CL resulted in recommendations for vector control. In order to target these interventions towards affected areas, a comprehensive understanding of the spatial distribution of *P. pedifer* at high spatial resolution is required. Therefore, this study determined the environmental predictors that facilitate the distribution of *P. pedifer* and created a map indicating the areas where conditions are suitable for survival of the vector in southwestern Ethiopia with high spatial resolution.

Phlebotomus pedifer presence points were collected during two entomological surveys. Climate, vegetation and topographic variables were assembled. Climate variables were interpolated with variables derived from high-resolution digital elevation models to generate topoclimatic layers representing the climate conditions in the highlands. A Maximum Entropy model was run with the presence points, predicting variables and background points, which were selected based on a bias file.

Phlebotomus pedifer was the only captured *Phlebotomus* species in the study area and was collected at altitudes ranging between 1685 and 2892 m. Model projections indicated areas with suitable conditions in a 'belt' surrounding the high mountain peaks. Model performance was high, with train and test AUC values being 0.93 and 0.90, respectively. A multivariate environmental similarity surface (MESS) analysis showed that the model projection was only slightly extrapolated for some of the variables. The mean annual temperature was the environmental variable, which contributed most to the model predictions (60.0%) followed by the seasonality in rainfall (13.2%). Variables representing steep slopes showed very low importance to model predictions.

Our findings indicate that the suitable habitats for *P. pedifer* correspond well with the altitudes at which CL was reported previously, but the predictions are more widely distributed, in contrast with the description of CL to occur in particular foci. Moreover, we confirm that vector distribution is driven by climate factors, suggesting inclusion of topoclimate in sand fly distribution models. Overall, our model provides a map with a high spatial resolution that can be used to target sand fly control measures in southwestern Ethiopia.

Background

Phlebotomine sand flies (Diptera, Phlebotominae) are tiny, hematophagous insects that occur in tropical and subtropical regions. In Africa, the genera *Phlebotomus* and *Sergentomyia* occur and some species of the former genus are the vector of *Leishmania* protozoa, causing leishmaniasis in humans [54]. The infection can manifest in three major clinical forms: cutaneous (CL), mucocutaneous (MCL) and visceral (VL) leishmaniasis, which are all three occurring in Ethiopia [2,28,106]. The most common form is CL, which is caused by *Leishmania aethiopica*. This parasite species is transmitted by *Phlebotomus pedifer* in southwestern and *P. longipes* in central and northern Ethiopia [123,124].

In great contrast to the 878 CL cases that were reported to the WHO in 2018, it is estimated that the incidence of the infection lies between 20,000 to 50,000 cases yearly, reflecting the severe underreporting of the infection in Ethiopia [105,106]. CL particularly occurs in foci on the mountain slopes of the Ethiopian Rift Valley, ranging from North to Southwest and South to Northeast in the country. The described foci are all situated at altitudes ranging between 1,700 m and 2,700 m and are located in four regional states: Southern Nations, Nationalities and Peoples' Region (SNNPR), Amhara, Tigray and Oromia, and Addis Ababa city administration [47,48,100–104].

Ochollo is a well-known CL focus at about 2,100 m in southwestern Ethiopia and is considered a model village for research investigating the transmission dynamics of CL [48,100,199]. The area has a rough topography and is characterized by steep slopes, many rocks and basalt cliffs with caves, providing the ideal habitat for *P. pedifer* and the animal reservoir of the infection, hyraxes [163]. According to findings on the transmission cycle in Ochollo, suggestions have been made for vector control and disease prevention in the area [48,100,199].

Effective and efficient implementation of integrated vector control programs and resource allocation requires a comprehensive understanding of the spatial distribution of *P. pedifer*. Besides from Ochollo and an outbreak in Silte woreda, neither *P. pedifer* nor CL has been reported in the surrounding areas, even though the topography and ecology appear similar in some areas [48,100,102,163,199]. However, a recent study indicated many of these areas to be at high-risk for CL based on environmental parameters (rainfall, altitude and slope), yet no (entomological) surveys have been conducted here [111].

It is quite novel that species distribution models (SDMs) are being implemented to predict the distribution of a vector to optimize control measures [200]. SDMs are sophisticated, dynamic tools that can identify areas that are suitable for the survival of a particular species. It integrates species occurrence data and information about environmental conditions at these locations to characterize the niche of the species and project it into the geographic space, resulting in a map that predicts the species' potential distribution [201,202].

Commonly, bioclimatic variables are applied in SDMs at 30 arc-second resolution (1 km^2) or coarser, which represent free-air conditions that were averaged over the past 30 years [203–205]. Although these layers are probably adequate for flat terrains, they may not be sufficient for representation in mountainous areas with a variable topography [206–209]. Due to this vertical dimension, organisms experience microclimatic conditions, which can vary noticeably over a short distance. This is attributed to several topographic factors, such as slope angle, aspect, solar radiation, distance to the ocean etc. [210]. An additional issue of these macroclimatic data is that other layers with a higher spatial resolution need to be resampled, which can lead to loss of important details.

However, macroclimatic data can be downscaled with variables derived from high-resolution digital elevation models (DEMs) to generate a statistical relationship that results in higher resolution climatic data [206,207]. Integration of these high resolution climatic variables was demonstrated to significantly improve the predictive power of SDMs [211,212].

In this study, we used topoclimatic variables in an SDM to determine the environmental predictors that facilitate the presence of *P. pedifer* and assessed areas that are suitable for the survival of the vector with high spatial resolution in five zones of the SNNPR. The generated maps can be implemented by policymakers for guidance of targeted vector control programs to reduce the burden of CL in this area in southwestern Ethiopia.

Methods

Site description

The study was conducted in the SNNPR, in southwestern Ethiopia (Fig 4.1a). The area has a variable topography with an altitude ranging from 340 to 3433 m (Fig 4.1b). The south and west of the area comprise flat lowlands, whereas the north and east are mountainous. *P. pedifer* occurs in mountainous areas and the model village for research on CL transmission, Ochollo, is situated near Arba Minch, in Gamo zone. Therefore, we selected Gamo zone, three additional surrounding administrative zones, including Gofa, Wolaita and Dawuro, and Dherashe area for sample collection (Fig 4.1c). Together, the area covers approximately 22,000 km^2 and is inhabited by 4.7 million people.

The study area consists of mountains and valleys with an altitude ranging between 550 and 3,390 m. Due to its topography, it has a temperate climate, with an average yearly temperature ranging from 9.3 °C to 25.5 °C and rainfall varying between 630 mm and 2,280 mm respectively in the low- and highlands. Because the study area covers a wide topographical range, the seasons vary from place to place, but generally the dry season lasts from October to April and the wet season from May to September. In recent years, the area has been subjected to ecological modifications related to human activities, like urbanization, agriculture and deforestation.

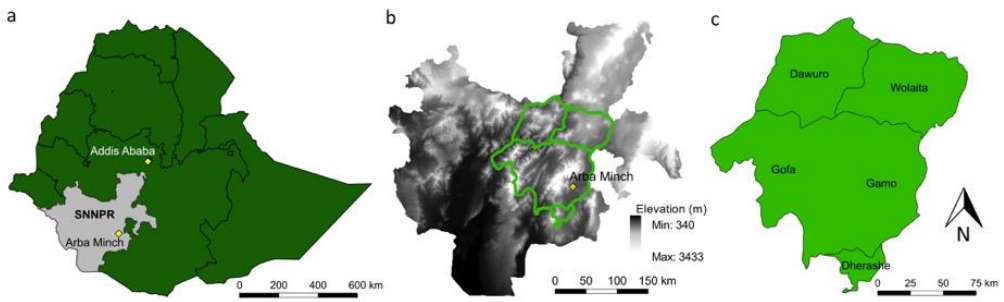


Fig 4.1 Location of the study area [166,167]**. a** The SNNPR in southwestern Ethiopia, with the capital city of Gamo Zone, Arba Minch, situated in the east. **b** Magnification of the topography (elevation, m) of the SNNPR and the study area indicated in green. **c** Magnification of the study area: four zones (Dawuro, Wolaita, Gofa and Gamo) and Dherashe area. *Abbreviation:* SNNPR, Southern Nations, Nationalities and Peoples' Region.

Workflow

An overview of the research data and methods are summarized in Fig 4.2 and explained in detail below.

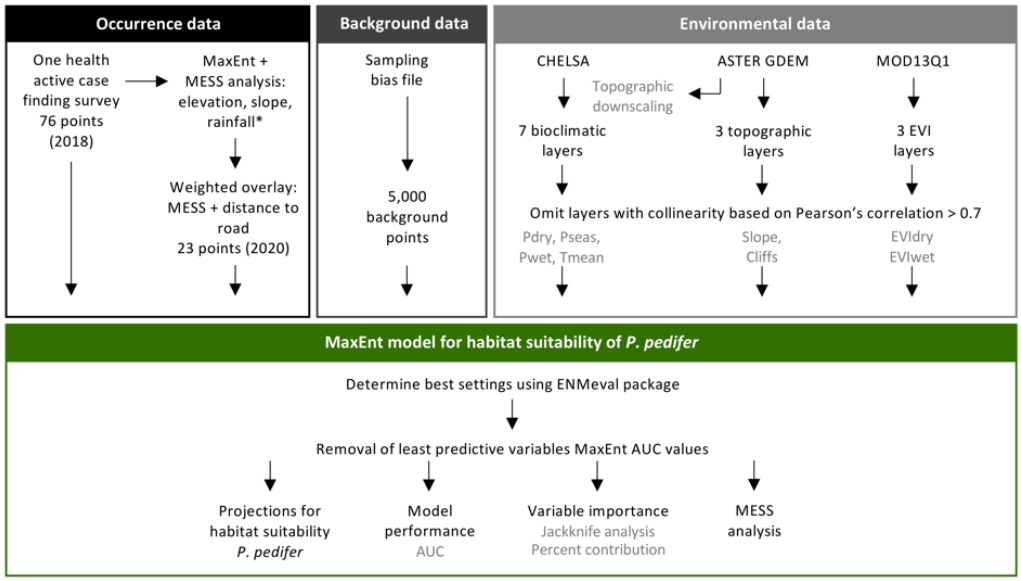


Fig 4.2 Overview of the research data and methods. See Methods section for details. *Abbreviations:* MaxEnt, Maximum Entropy; MESS: multivariate environmental similarity surface; CHELSA, Climatologies at high resolution for the earth's land surface areas; ASTER, advanced spaceborne thermal emission and reflection radiometer; GDEM, global digital elevation model; MOD13Q1, terra moderate resolution imaging spectroradiometer vegetation indices; EVI, enhanced vegetation index; Tmean, mean temperature; Pseas, precipitation seasonality; EVIdry, enhanced vegetation index in the dry season; Pdry, precipitation in the driest months; Pmean, mean precipitation; Cliffs, ordinal categorical values indicating cliffs between 20-40% and above 40%; EVIwet, enhanced vegetation index in the wet season; AUC, area under the curve.

Occurrence data

Occurrence points of *P. pedifer* were collected during two consecutive entomological surveys (Fig 4.2 black frame, Fig 4.3).

First, 76 presence points were assembled during an active case finding survey carried out from May to July 2018. The survey was performed under guidance of a neglected tropical diseases (NTD) focal person and health extension workers, and by questioning community members about the presence of CL patients supported by pictures of lesions and hyraxes. When a suspected CL case was found, a CDC miniature light trap

(John W. Hock Company, Gainesville, Florida, USA) was set in the late afternoon inside the patient's dwelling or in a nearby cave or rocky area where hyraxes were present. Sand flies were collected the next morning, mounted in CMCP-10 high viscosity mounting medium (Polysciences Europe, Herschberg, Germany) and the species was determined according to relevant morphological keys [124,125,156].

Second, an elementary Maximum Entropy (MaxEnt) model was developed using the *P. pedifer* presence points collected during the active case finding survey in 2018 and environmental layers that were found to predict the presence of CL in Ethiopia in a study of Seid *et al.* (2014): altitude, slope and rainfall [111]. A multivariate environmental similarity surface (MESS) analysis was integrated, measuring the extent of the projected data which was not within the range with the training variables (thus causing model extrapolation). We intended to keep the extent of extrapolation low as it informs on the credibility of the model output. Therefore, a new sampling approach was designed based a weighted overlay of the MESS analysis (70%) and the distance to the road (30%), in order to reduce the degree of extrapolation by additional sampling in accessible places. This entomological survey was carried out in the dry season (January and February 2020), when sampling sites were better accessible and a higher sand fly abundance was expected [163]. During this survey, we searched for suspected CL cases for nearby sand fly trapping. If CL cases were absent, traps were placed in other potential sand fly breeding or resting sites, because the area could still be at risk for an outbreak if the vector would be present. Collected specimens were processed as described above, leading to 23 additional *P. pedifer* presence points.

Sampling bias file

Most of the sampling effort was performed within a certain distance to the roads and towns (approximately 10 km), which was necessary to ensure access to the sampling areas, particularly at rainy days. Moreover, case finding and sand fly trapping were never attempted at altitudes under 1,400 m. This is because neither *P. pedifer* nor CL have been observed at altitudes under 1,700 m. Additionally, we applied a buffer of 300 m in altitude to avoid missing sites where P. pedifer could be present on the one hand and prevent putting to much effort in sites where the vector cannot be found on the other hand.

In order to diminish spatial autocorrelation of the sampled presence points without reducing the predictive power of the model, a sampling bias file was designed to match

the sampling effort and avoid overfitting of the model (Fig 4.2 dak grey frame, Fig 4.3) [213]. Therefore, a weighted overlay was performed with increasing weights for proximity (< 2.5 km, 2.5 - 5 km, 5 km - 10 km, > 10 km) to a town and road (50/50%). All areas under 1,400 m were given the lowest weight and the final raster file was used for selection of background points with an increased probability in areas with a high sampling effort (explained below).

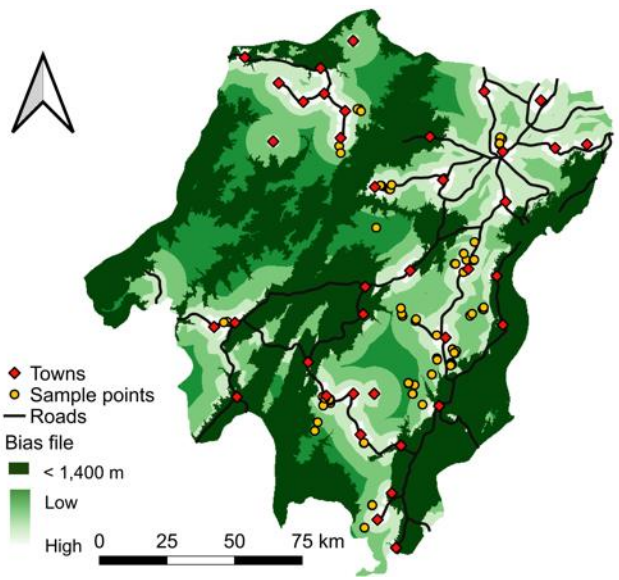


Fig 4.3 *Phlebotomus pedifer* occurrence points collected during the two surveys and the sample bias file [166,167]**.** The design of the bias file is based on a higher weight for areas nearby the roads and towns to match with the sampling effort of the study.

Environmental data

Collection of environmental data

A wide range of environmental layers was acquired as candidate explanatory variables for the model (Fig 4.2 light grey frame). Specifics and sources of the variables and the range in our study site are demonstrated in Table 4.1. All manipulations of the variable layers were carried out in ArcGIS version 10.4.1.

Table 4.1 Environmental layers acquired as candidate explanatory variables to predict the habitat suitability of *Phlebotomus pedifer*. CHELSA layers at 30 arcseconds were downscaled and USGS Slope and Cliffs layers were resampled, all to a 250 m spatial resolution. *Abbreviations:* CHELSA, Climatologies at high resolution for the earth's land surface areas; USGS: United States Geological Survey.

Name	Explanation	Source	Original spatial resolution	Data range study area
Tmean	Annual Mean Temperature (Bio1)	CHELSA	30 arcsec	8 – 34 °C
Tmax	Max Temperature of the Warmest Month (Bio5)	CHELSA	30 arcsec	14 – 45 °C
Tmin	Min Temperature of the Coldest Month (Bio6)	CHELSA	30 arcsec	2 – 31 °C
PrecMean	Annual Precipitation (Bio12)	CHELSA	30 arcsec	526 – 3216 mm
PrecWet	Precipitation of the Wettest Month (Bio13)	CHELSA	30 arcsec	90 – 369 mm
PrecDry	Precipitation of the Driest Month (Bio14)	CHELSA	30 arcsec	5 – 68 mm
PrecSeas	Precipitation Seasonality (Bio15)	CHELSA	30 arcsec	21 – 94 %
Slope	Hill slope calculated from elevation DEM	USGS	30 m	0 – 77 %
Cliffs	Ordinal categorical value for number of slopes exceeding 20% and 40% per pixel	USGS	30 m	1 – 9
EVImean	Average EVI from January 2017 until December 2019 – MOD13Q1	USGS	250 m	-0.13 – 0.59
EVIdry	Average EVI for the dry season (January to March) – MOD13Q1	USGS	250 m	-0.99 – 0.60
EVIwet	Average EVI for the wet season (July to September) – MOD13Q1	USGS	250 m	-0.16 – 0.70

Because temperature and precipitation are relevant drivers for the distribution of *P. pedifer*, bioclimatic variables were derived from 'Climatologies at high resolution for the earth's land surface areas' (CHELSA, https://chelsa-climate.org.bioclim/) with a spatial resolution of 30 arcsec (~1 km) [163]. A subset of seven out of 19 available bioclimatic variables were considered ecologically relevant to the species and selected, in particular annual averages and extrema (minimum and maximum) for both temperature and precipitation and a variable describing the annual rainfall variation as a measure for seasonality.

The vector is breeding in caves on cliff walls, where hyraxes are living. Therefore, an ASTER digital elevation model (DEM) with 30 m spatial resolution was acquired from U.S. Geological Survey (USGS, earth explorer, https://earthexplorer.usgs.gov/), of which the slope (percentage) was computed. To avoid losing the information about cliffs while resampling to a resolution of 250 m, an additional ordinal categorical layer was created using a weighted overlay analysis, indicating number of slopes between 20 - 40% (25% weight), and > 40% (75% weight) per 250 m pixel.

Sand flies require vegetation through which they can move, forage and reproduce [214]. Hence, vegetation layers were included as potential predictors from USGS. The Moderate Resolution Imaging Spectroradiometer (MODIS) enhanced vegetation index (EVI) quantifies the vegetation density. The MOD13Q1 product (https://earthexplorer.usgs.gov/) is produced on a 16 days interval base and corrects for particular atmospheric conditions and canopy background noise. Indices were derived for the annual and seasonal averages over the past three years (2017-2019) with 250 m spatial resolution. All environmental layers, the occurrence points and bias file were projected in the same spatial reference system, World Geodetic System 84 (WGS84 EPSG:4326).

Topographic downscaling of climate layers

The bioclimatic layers were downscaled on the basis of topographic variables to produce topoclimate (local climate at a particular topography) at high resolution as functionally relevant predictor variables [215]. We opted for a resolution of 250 m because it formed an appropriate balance between a feasible spatial resolution to guide implementation of vector control measures and the computational capacity required for the downscaling process. Downscaling followed a Geographically Weighted Regression (GWR) approach [216] outlined by Lenoir *et al.* [217] and was based on elevation, slope, northness, eastness, distance from the ocean, and potential solar radiation. These predictor variables have shown good results for predicting temperature and precipitation data in previous studies [217–223].

Data were prepared for downscaling in R version 3.5.2 [224] using the *raster* package [225]. The area was subdivided into 16 sections to make the computation time for downscaling feasible for the size of our study site. Topographic variables were derived from the ASTER DEM at 250 m resolution. Distance from the ocean was downloaded from http://www.soest.hawaii.edu/pwessel/gshhg/ at 1 arc-minute resolution [226].

The potential incoming solar radiation was calculated for each grid cell of the DEM for the spring equinox (March 21st) with a 6-hour resolution using the SAGA GIS 6.3.0 tool *Potential Incoming Solar Radiation* [227]. The downscaling was performed on resources provided by the NTNU IDUN/EPIC computing cluster using R version 3.6.0 and the *spgwr* package [228]. The 16 sections were mosaicked together and checked for correspondence to CHELSA values. Single outliers due to small bandwidth of the GWR were removed and missing data were interpolated using the *Close Gaps* tool of SAGA.

Variable preparation

Overall, 12 environmental layers were considered to potentially predict the habitat suitability of *P. pedifer* (Table 4.1). All layers were resampled to match a 250 m spatial resolution. For aggregation of the slope variable, the maximum values were retained to prevent the loss of information on slope steepness, while for all other layers, average values were calculated.

Apart from ecological relevance, multi-collinearity among candidate predictor variables was assessed with a Pearson's correlation (Fig 4.2 light grey frame, Additional file 4.1: Fig S1). If the absolute coefficient exceeded 0.7, one of the pair variables was omitted for inclusion in the model. This resulted in eight remaining candidate predictor variables: Tmean, Pmean, Pdry, Pseas, Slope, Cliffs, EVIdry and EVIwet.

MaxEnt model implementation

A model predicting the habitat suitability of *P. pedifer* was developed by a MaxEnt model using the *dismo* package in R version 3.3.1 [229]. The optimal settings for the MaxEnt model were determined using the *ENMeval* R package, in which the random 10-fold cross-validation data partitioning method was used (Fig 4.2 green frame) [230,231]. The function compares all possible model setting combinations and calculates the Akaike information criterion (AIC) value for each combination. The lowest AIC value was found for a model with a regularization multiplier of 0.5, including linear and quadratic features and a combination of these classes. Hence, these settings were used to fit the model, which was run using a 10-fold cross-validation method, with 75% of the presences used for training and 25% for testing. Additionally, 5,000 background points were assigned based on the bias file (Fig 4.3).

A second round of variable selection was carried out by an iterative removal of the least predictive variables by the area under the curve (AUC) values of the receiver operator

characteristic (ROC) of the MaxEnt model to maximize the model performance and minimize overfitting. Yet, all variables contributed considerably to a better model AUC, so the final model consisted of the following eight variables: Tmean, Pmean, Pdry, Pseas, Slope, Cliffs, EVIdry, EVIwet.

In order to assess the robustness of the final model, it was run 200 times, including new random background points in each run. Model accuracy was evaluated by calculating the average training and testing AUC values over 200 runs.
A MESS analysis was performed to indicate areas where model projections were extrapolated (Fig 4.2 green frame).

The relative importance of the variables to predict the habitat suitability of *P. pedifer* was assessed using the jackknife estimates and percent contributions.

Results

Entomological survey

The only *Phlebotomus* species that was captured during both entomological surveys was *P. pedifer*. The species was collected at altitudes ranging between 1685 and 2892 m and most were captured inside human dwellings, which was in most cases due to excessive rainfall impeding outdoor trapping.

Prediction of suitable habitats for P. pedifer

The predicted habitat suitability for *P. pedifer* based on the MaxEnt model is shown in Fig 4.4a. The predictive performance of the model was high, with average training and testing AUC values being 0.93 (SD $\pm$ 0.01) and 0.90 (SD $\pm$ 0.02) respectively.

Generally, the conditions are predicted highly suitable for the vector in a 'belt' surrounding the main mountain ranges (Fig 4.1b, Fig 4.4a). This is most pronounced in Gamo zone and and the eastern part of Gofa zone, where the highest mountain peaks are situated (Fig 4.1b and c, Fig 4.4a). In the central and western part of Gofa, Wolaita and Dawuro zones and Dherashe area, where mountains are generally lower, the predicted suitable habitats are more evenly distributed. In our study site, an area of 720 km^2 (3.6%) was indicated with very suitable conditions for the presence of *P. pedifer* (> 0.6), 674 km^2 (3.4%) showed a habitat suitability value between 0.4 - 0.6 and 1174 km^2 (5.2%) between 0.2 - 0.4.

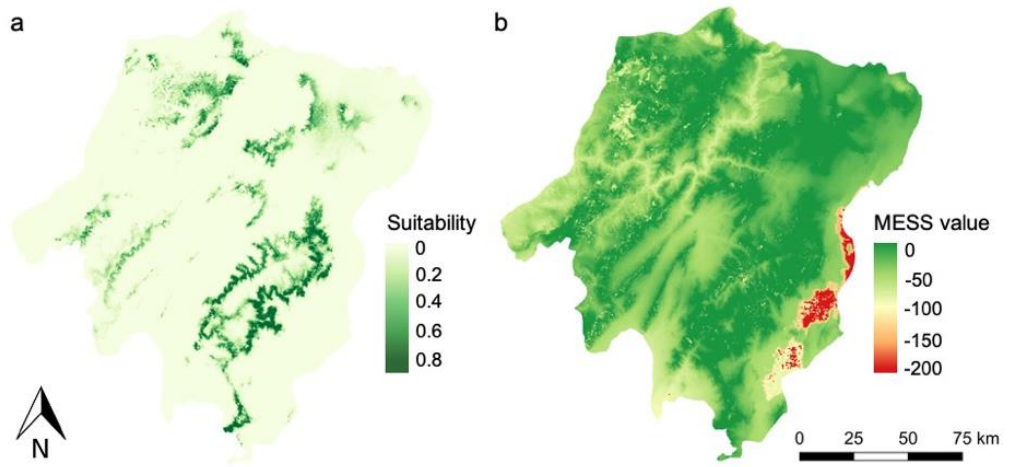


Fig 4.4 Predicted suitable habitats for *Phlebotomus pedifer* using a MaxEnt model (a) and MESS analysis outcome (b). In the MESS map, values below zero are predictions slightly out of the range of the training variables. Areas indicated in red are the Abaya and Chamo lakes surrounding Arba Minch. *Abbreviations: MaxEnt, Maximum Entropy; MESS, multivariate environmental surface similarity.*

The MESS analysis (Fig 4.4b) demonstrates that almost none of the predicted suitable areas were projections out of the range of the training variables (no extrapolation), supporting the credibility of the model. Negative values were observed particularly in the lowlands, where *P. pedifer* was not found during the entomological surveys.

Environmental variables associated with vector presence

The percent contributions (Fig 4.5a, Additional file 4.2: Table S1) and jackknife test estimates (Fig 4.5b) indicated that the most important variable to predict the habitat suitability of *P. pedifer* was the mean annual temperature variable, which had an average relative contribution (± SD) of 60.0% (± 3.0%) to the model. The regularized training gain of the model (± SD) with only and without the mean annual temperature were respectively 0.83 (± 0.06) and 0.80 (± 0.05) of the total model training gain of 1.47 (± 0.07). The second most important variable was precipitation seasonality (13.2, ± 2.1), followed by the Enhanced Vegetation Index in the dry season, mean annual precipitation and precipitation in the dry season. The mean annual precipitation was slightly correlated with the precipitation seasonality (Additional file 4.1: Fig S1), causing the jackknife estimate for this variable only to be low. Cliffs and Slope variables had very low importance in the model.

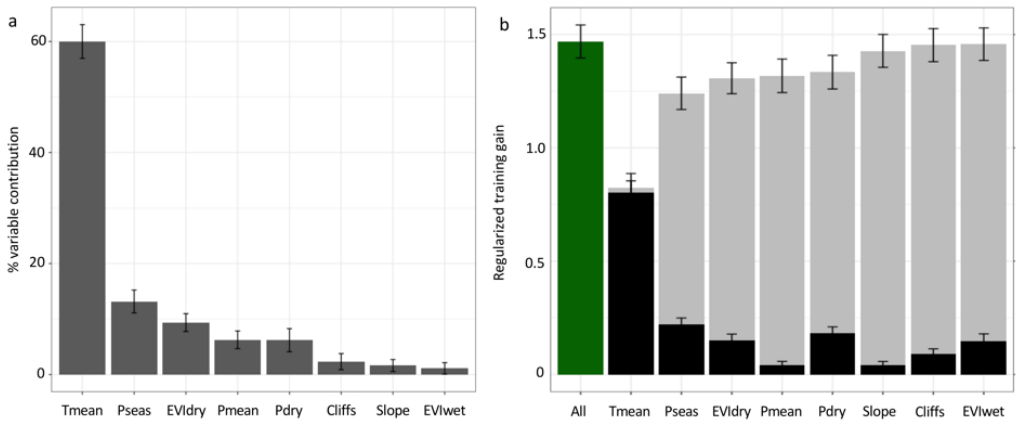


Fig 4.5 Percent variable contribution (a) and jackknife estimates (b) indicating the relative importance of variables to predict the habitat suitability of *P. pedifer*. The green bar is the total regularized training gain, grey bars are the model training gain without the variable and black bars with only the indicated variable. *Abbreviations:* Tmean, mean temperature; Pseas, precipitation seasonality; EVIdry, enhanced vegetation index in the dry season; Pdry, precipitation in the driest months; Pmean, mean precipitation; Cliffs, ordinal categorical values indicating cliffs between 20-40% and above 40%; EVIwet, enhanced vegetation index in the wet season.

The way the prediction depends on the two most important variables (mean annual temperature and precipitation seasonality) and their correlation with the other variables is presented in Fig 4.6 (other variables in 4.3: Fig S2). The mean annual temperature variable indicated suitable habitats for yearly average temperatures ranging approximately between 12 and 20 °C, reaching an optimum at about 16°C. A similar pattern was observed for the precipitation seasonality variable with an optimum at 50% precipitation variability.

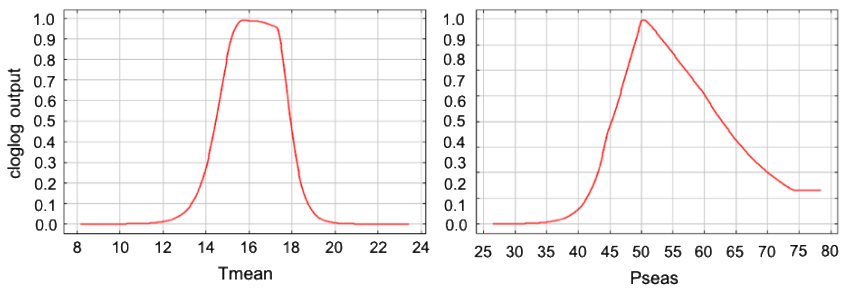


Fig 4.6 Dependence of the predicted suitability on two most contributing variables. The curves show how the prediction changes as each environmental variable is varied, keeping all other environmental variables at their average sample value. The cloglog value provides an estimate between 0 and 1 of probability of presence. *Abbreviations:* Tmean, mean temperature; Pseas, precipitation seasonality.

Discussion

Identifying the vector distribution is pivotal for guidance of targeted integrated vector control, because places where *P. pedifer* occurs are either burdened by CL or vulnerable for a disease outbreak [232]. In this study, we designed a MaxEnt model resulting in a practical, high-resolution map indicating areas suitable for the presence of *P. pedifer* in five zones in southwestern Ethiopia.
Previous studies pointed out that *P. pedifer* is the only vector for transmission of *L. aethiopica* in Ochollo village [48,61,163]. Our entomological surveys confirm this finding in a much larger area, as this was the only *Phlebotomus* species captured in the five zones.

Our model predicts that suitable habitats for *P. pedifer* are situated in a 'belt' surrounding the slopes of the high mountain peaks, whereas it is more evenly distributed in lower mountainous areas with similar altitudes. This is in contrast with previous studies which describe the distribution of CL in Ethiopia to occur in foci [17].
Although the variables selected for our model were thoughtfully selected, there could potentially be an additional micro-ecological variable that was not included but could predict this focal distribution. Another likely explanation could be that our model predictions are accurate and the considered patchy distribution is a result of underreporting of CL because of various reasons, like misdiagnosis, lack of diagnostics and understanding of the importance of reporting cases etc. [17,233].

Therefore, it is sometimes suggested to perform a field validation study to evaluate the accuracy of the model [232,234–236]. However, it should be taken into account that not all individuals of a species live in optimal conditions, so it is possible to find the species outside the predicted suitable habitat [237]. Moreover, a species distribution can be constrained by dispersal limitations [238]. Also, even though generally the environmental conditions are permissive for the vector, it could be that there are no available blood or sugar sources or there are no niches for resting, breeding and survival of the vector within its flight range [239]. Therefore, neither finding some sand flies in areas that are not suitable for a species nor not being able to capture sand flies in certain suitable habitats means the prediction is unreliable.

The obvious 'belt' around the higher mountains indicates that the environment is unsuitable for the presence of the vector up to and as of a certain height. In many studies, elevation is included as a response variable in the model [111,202,240–242].

However, this variable can have different environmental characteristics in different areas (depending on the slope, aspect, wind, etc.) and may thus result in overfitting of the model. Therefore, we used elevation, slope, aspect and distance to ocean as indirect measures of topoclimate.

We demonstrate that the mean annual temperature is by far the most important predictor for the presence of *P. pedifer*. The seasonality in precipitation also contributed considerably to the predictions. This means that lowland areas have too high temperatures and little variation in precipitation, while at high altitudes it is too cold and excessively raining in the wet season compared to the dry season for the vector to survive (Fig 4.6). The importance of the climate variables is consistent with other studies mapping the distribution of leishmaniasis and its vectors [111,239,241,242].

Our previous findings in Ochollo village show that the abundance of the vector population is similarly correlated with temperature and humidity (as a proxy for rainfall) [163]. Ochollo lies at an altitude of 2,100 m with annual temperatures ranging between 17.0 and 22.6 °C. *P. pedifer* is present in the village during the whole year, but less abundant in the wet season and infected sand flies were continuously present inside caves [163]. In villages at lower or higher altitudes, however, there is a distinct climate, thus the seasonality of the (infected) sand fly population probably differs from what is observed in Ochollo. Periods without infections in sand flies can occur because *Leishmania* requires particular temperatures for development in the vector [157]. Furthermore, sand fly larvae can diapause, waiting for several months for favorable environmental conditions to develop to an adult stage, resulting in months without any sand flies [56,72]. This phenomenon has been observed for *P. orientalis*, the main VL vector in Ethiopia, in the wet season [243]. Hence, we expect that the seasonality found in our previous paper would lead to periods without sand flies in the wet season in highland areas while sand flies can possibly only survive in the wet season in the lower highlands.

The importance of these climate variables could also indicate that the distribution of the vector could alter when the climate changes [112,232,244]. For Ethiopia, it is predicted that the temperature will rise and rainfall will become erratic with flood and drought events likely to increase [245]. We hypothesize that therefore there could be a shift of *P. pedifer* presence towards the highlands. If these are places where people have no immunity due to parasite exposure yet and hyraxes are present to serve as

reservoirs, this could lead to new outbreaks. It would be interesting for future studies to make a model with only microclimate variables and project the vector's potential niche to the future to assess what would happen to the distribution of the vector.
The variables Slope and Cliffs showed a low relative importance for the model. This was unexpected, as rock crevices in cliffs are the main breeding sites of *P. pedifer* and CL is positively correlated with proximity to caves and hyrax habitats [17,104,132,163]. This could potentially be a result of sampling that was mainly performed inside human dwellings instead of in outdoor sand fly breeding sites to avoid decreased trapping efficiency due to excessive rainfall. However, the selected houses for sand fly collection were often nearby potential sand fly and hyrax habitats. The study of Seid and colleagues that predicted the area at risk for CL in Ethiopia found slopes > 7.45 degrees to be highly associated with CL presence [111], which corresponds with a slope > 13%. Our model focused on steep slopes (20 – 40% and > 40%) to represent cliffs as hyrax and sand fly habitats, which presumably explains why it was not important in our model.

In our previous study in Ochollo, we demonstrated that sand flies are mainly present inside caves, but considerable numbers and infected sand flies can also be found in stone fences around houses or in cracks of large boulders [163]. During the present entomological surveys, *P. pedifer* was trapped in some sites where no typical basalt cliffs with caves were observed, suggesting that it may indeed not be a crucial environment for sand fly presence as the model suggests but rather enhance the abundance of the vector population. Knowledge on the distribution of basalt cliffs and caves at high resolution could perhaps provide a better insight into the importance of the cliffs for the presence of *P. pedifer*.

Previous distribution maps were already made for the distribution of CL cases and VL vectors (*P. orientalis* and *P. martini*) in Ethiopia [111,239]. The former was designed by Seid *et al.* in 2014 using a multivariate logistic regression analysis to assess the most important predictor variables and a probabilistic and weighted overlay analysis to generate a risk map for CL [111]. They found elevation, rainfall and slope as the most important predictors of CL distribution and the map indicates more than one fifth of the country at high or highest risk for CL. Even peak highlands (> 2,650 m) were indicated at highest risk and lowland areas were still medium to low risk areas. This deviates from our results, where only about 7% of the mountainous study area had favorable conditions (suitability > 0.4) for survival of *P. pedifer*.

Although other methods were applied in that study, logically their map should overlap with ours of the distribution of the vector. We have visited the lowland and peak highland areas, but *P. pedifer* was only found between 1,685 and 2892 m. Our results correspond with the reported CL endemic sites which were never situated at such high or low altitudes [47,48,100–104]. The authors indicate in their paper that the predictions at these altitudes are indeed odd but might be due to a recent change in vector behavior [111]. However, our study demonstrates that the vector does also not occur there and therefore suggests that their map overestimates the distribution of CL drastically.

Other studies modeling the distribution of vectors commonly use a 1 km spatial resolution because climate layers are only available at this coarse resolution [241,242,246–248]. Because climate variables are often the most important predictors for SDMs of disease vectors, these data should be very detailed [241,242,244,246]. Moreover, recently SDMs are being used for optimization of vector control, so it is beneficial for coordination of resource allocation for targeted control measures to have smaller grids indicating the suitable habitats of the vector [200]. To our knowledge, our study is the first to implement downscaled climate variables (topoclimate) to model the distribution of sand flies in a mountainous area at fine resolution.

Overall, this study indicates that the mean annual temperature is the most important predictor for the spatial distribution of *P. pedifer*. We demonstrate that about 7% of the study area is suitable for the presence of the vector and show with a high-resolution map the localities that should be focused on for implementation of integrated vector control measures, which are mainly located at mid-highland altitudes.

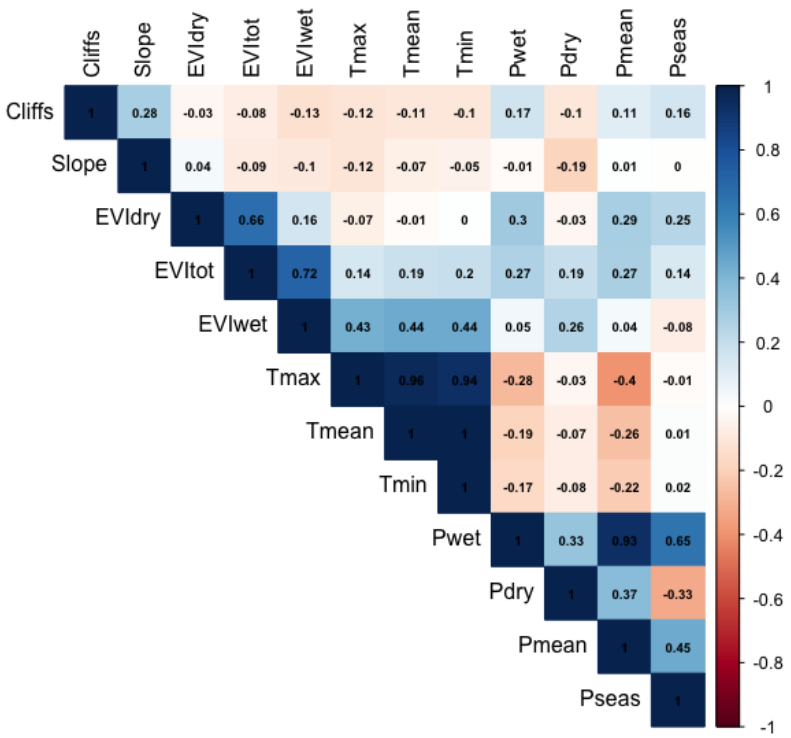
Acknowledgements

We are very grateful to all NTD focal persons and health extension workers for their guidance and support during the fieldwork. We also thank Arba Minch University, zonal and district health offices for their arrangements to accomplish the fieldwork. Furthermore, we acknowledge Jonas Lembrechts from the University of Antwerp for his advice on the model analyses.

Funding

MP is a PhD fellow in the VLADOC program of the Flemish Interuniversity Council VLIR-UOS. HL is part of the University of Antwerp Center of Excellence VAX-IDEA. The study was supported by VLIR-UOS through the Inter-University Cooperation (IUC) agreement with Arba Minch University.

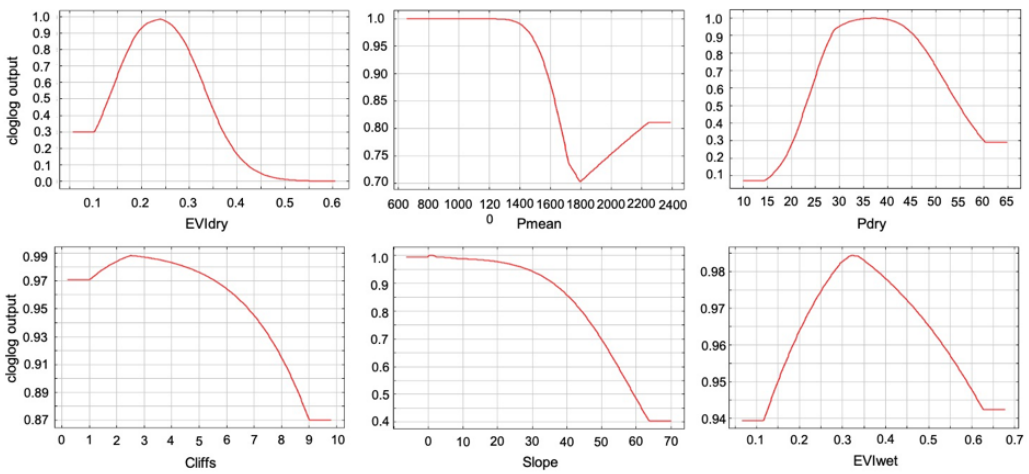
Additional files



Additional file 4.1: Fig S1 Output of the Pearson's correlation analysis to reduce multi-collinearity of the variables.

Additional file 4.2: Table S1 Percent variable contribution and jackknife estimates indicating the most important variables for the model. *Abbreviations:* SD, standard deviation; Tmean, mean temperature; Pseas, precipitation seasonality; EVIdry, enhanced vegetation index in the dry season; Pdry, precipitation in the driest months; Pmean, mean precipitation; Cliffs, ordinal categorical values indicating cliffs between 20-40% and above 40%; EVIwet, enhanced vegetation index in the wet season.

Variable	Contribution % (SD)	Model gain without this variable (SD)	Model gain with only this variable (SD)
Tmean	60.0 (3.0)	0.83 (0.06)	0.80 (0.05)
Pseas	13.2 (2.1)	1.32 (0.07)	0.22 (0.03)
EVIdry	9.4 (1.6)	1.31 (0.07)	0.15 (0.03)
Pdry	6.2 (2.1)	1.33 (0.07)	0.18 (0.03)
Pmean	6.2 (1.6)	1.32 (0.07)	0.04 (0.02)
Cliffs	2.3 (1.4)	1.45 (0.07)	0.09 (0.02)
Slope	1.6 (1.1)	1.43 (0.07)	0.04 (0.02)
EVIwet	1.1 (1.0)	1.46 (0.07)	0.15 (0.03)



Additional file 4.3: Fig S2 Dependence of the predicted suitability on the six least contributing variables. The curves show how the prediction changes as each environmental variable is varied, keeping all other environmental variables at their average sample value. The cloglog value provides an estimate between 0 and 1 of probability of presence. *Abbreviations:* EVIdry, enhanced vegetation index in the dry season; Pdry, precipitation in the driest months; Pmean, mean precipitation; Cliffs, ordinal categorical values indicating cliffs between 20-40% and above 40%; EVIwet, enhanced vegetation index in the wet season.

CHAPTER 5

Detection of cutaneous leishmaniasis foci in south Ethiopia

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Short report

Submitted to American Journal of Tropical Medicine and Hygiene

Abstract

Cutaneous leishmaniasis (CL) is a major public health problem in Ethiopia. The disease is endemic in Ochollo, a village in southern Ethiopia, but there are no reports of CL in the wider area, although it is ecologically very similar. We conducted a rapid assessment survey in the South-Ethiopian Rift Valley and found 100 parasitologically-confirmed CL cases in 38 villages not reported endemic for CL. About half of the cases were children (57%) and most lesions occurred on the face (78%) and were older than six months (77%). Only 2% of the people was aware about the mode of transmission and 9% sought modern treatment at a hospital. These preliminary data indicate that CL is much more widespread than reported and that the disease might have a large socio-psychological impact, hence this study calls for larger surveys across the Ethiopian highlands. Additionally, health education and treatment capacity need to be implemented.

Short report

Cutaneous leishmaniasis (CL) is a parasitic disease transmitted by the bite of phlebotomine sand flies. CL is characterized by slowly growing nodular or crusted lesions, typically healing with scars. Particularly lesions occurring on the face can be disfiguring and stigmatizing.[1] CL has diverse clinical forms; localized CL (LCL), which is limited in size and generally self-healing; mucocutaneous leishmaniasis (MCL) that affects the mucous membranes of the nose, mouth and throat; and diffuse CL (DCL) a chronic and progressive type, often non-responsive to the common antileishmanial drugs [54].

All three forms occur in Ethiopia, where CL poses a major public health problem. Most cases are attributed to *Leishmania aethiopica,* while there are few reports of *L. tropica* [2,99,249]. Hyraxes are the apparent reservoirs of *L. aethiopica*, living in close proximity to the vectors, *Phlebotomus pedifer* and *P. longipes* [48]. The World Health Organization (WHO), Armauer Hansen Research Institute (AHRI) and Ethiopian Federal Ministry of Public Health estimated that yearly there are 20,000 to 50,000 CL cases in Ethiopia, while only 878 cases were reported to the WHO in 2018, suggesting severe underreporting of the disease [105,106]. Several endemic areas have been identified across the country, in particular in the central highlands [249].

One of the main CL foci in the South-Ethiopian Rift Valley is Ochollo village (Fig 5.1, right panel) [48,100]. A prevalence of more than 30% of active CL lesions/scars has been documented for several decades, mainly amongst children [100,101]. While geographically very similar, none of the villages in the wider area (with a surface of > 30,000 km^2 and a population of > 4 million) are reported to be CL endemic. However, this area has been largely defined as at high risk for CL in a modelling study based on environmental parameters such as rainfall, altitude and slope [111]. Importantly, no surveys have been conducted in this area. The aim of this study was to 1) conduct a rapid assessment for the presence of CL in villages not reported endemic for CL (up to 350 km from the Ochollo focus) in the South-Ethiopian Rift Valley; 2) collect pilot data among the local population on the basic knowledge, attitude and health-seeking behaviour related to CL.

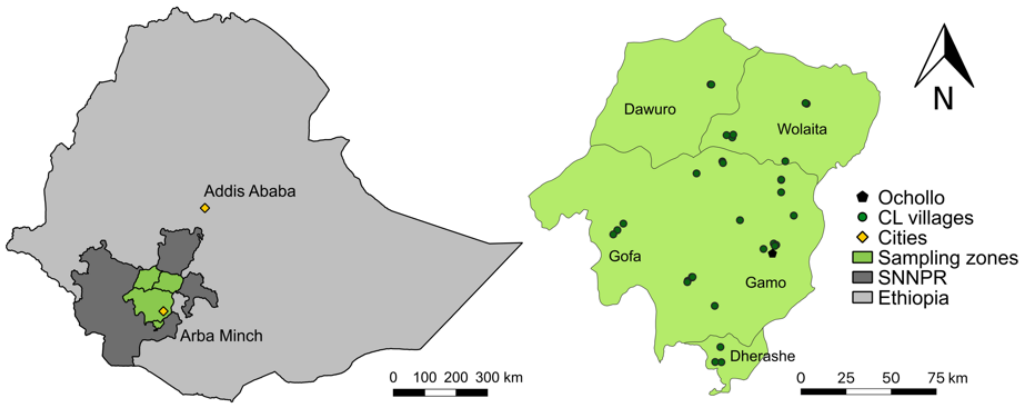

Fig 5.1 Study site and sample points. Left panel: the Southern Nations, Nationalities and Peoples' Region (SNNPR) in southwest Ethiopia with the sample site presented in green. Right panel: five zones where the survey was carried out and dark green points indicating the villages where parasitologically confirmed CL cases were found.

Between 20 May and 21 July 2018, we performed a survey in the highlands of southwestern Ethiopia, within the zone where Ochollo is situated (Gamo) and four neighboring zones (Gofa, Wolaita, Dawuro and Dherashe, all in the Southern Nations, Nationalities, and Peoples' Region (SNNPR) in South-West Ethiopia, Fig 5.1 left panel). The study sites were chosen based on environmental characteristics (altitude and annual rainfall) indicating districts that were potentially ecologically suitable for CL. In the district, we were supported by the neglected tropical diseases (NTD) district focal person who lead us to villages that might potentially have CL cases. Guided by pictures of CL lesions, community members were questioned about the presence of individuals with lesions compatible with CL, and then these individuals were visited.

Skin scrapings were collected from a maximum of five CL suspects per village participants were queried about their lesions, treatment and knowledge on disease transmission. Although there are no established features to characterize CL lesions in Ethiopia, an expert dermatologist identified LCL as one or more lesions, each originating from a single sand fly bite, DCL as multiple lesions occurring on different body parts and MCL as lesions with any kind of mucosal involvement (Fig 5.2). Skin scrapings were analysed for parasitological confirmation by microscopy (according to WHO guidelines) and real-time PCR [170,250]. Additionally, species typing based on the ITS-1 gene was performed for five samples, obtained from Gamo, Gofa and Wolaita zones. All diagnostic tests were conducted as described by Merdekios and Pareyn *et al.* (under review).

The study was approved by the Arba Minch University Institutional Ethical Review Board (CMHS/1167/111), Ethiopia. Written informed consent was collected from all participants.

Fig 5.2 Lesions due to *Leishmania aethiopica*. A and B present localized CL, and C and D mucocutaneous CL.

A total of 111 CL suspects were identified across the 38 villages (Fig 5.1, right panel, Supporting file 5.1: Table S1), with 100 CL cases confirmed by microscopy and/or PCR. A detailed description of the diagnostics results is presented in Merdekios and Pareyn *et al.* (under review). Species typing revealed only *L. aethiopica* infections. About half of the 100 confirmed CL cases were male and 57 were children (< 15 years of age, Table 5.1). The majority of the lesions were on the face (78%) and were present for more than six months (77%). Three quarters of the lesions were between one and five centimetres in size; multiple lesions were seen in 23 subjects. There were 76 cases of LCL, no DCL and 24 cases of MCL (Fig 5.2). The majority of the lesions (74.0%) were crusted plaques.

Table 5.1. Demographic characteristics and clinical features of 100 individuals with parasitologically-confirmed cutaneous leishmaniasis (CL).

Characteristics	N
Sex	
Male	52
Female	48
Age in years	
≤ 5	20
6 – 10	19
11 – 15	18
16-40	23
> 40	20
Site of the lesion	
Face	78
Hands & legs	10
Multiple sites	12
Type of CL	
Localized CL	76
Diffuse CL	0
Mucocutaneous leishmaniasis (MCL)	24
Type of Lesion	
Nodulo-ulcerative	74
Ulcerative plaques	15
Nodular	6
Papular	5
Duration of the lesion (months)	
< 6	23
6 – 12	51
13 – 24	15
> 24	11
Size of the largest lesion (cm)	
< 1	17
1 - 5	78
> 5	5
Number of lesions	
One	77
Two	13
Three & above	10

Most CL suspects - or their guardian for children - (94%) recognized and namd the disease on a picture with CL lesions that was shown to them and stated that CL (88%) and hyraxes (94%) had been present in the village for a long time. Only 2 individuals could correctly explain the mode of transmission of *Leishmania*. It was reported that individuals typically go for traditional medicine for CL treatment, with only 9 individuals who mentioned seeking modern treatment at a hospital.

Our findings have several important public health implications. First, although the applied sampling strategy does not allow estimating the exact burden in each village and area, this rapid assessment allowed to identify CL in 38 villages not reported endemic for CL. Consequently, our findings should be seen as a wake-up call, requiring standard surveys in risk areas in Ethiopia and strengthened NTD surveillance programs because CL numbers are obviously much higher than currently reported. Second, because the infection mainly presents in children and results in large, chronic lesions mainly occurring in the face, thorough qualitative studies should be conducted to assess the psycho-social impact of CL. This will allow determining to what extent CL should be prioritized by policy makers and public health experts amongst the various NTDs present in the country. Third, we observed a low tendency to seek for treatment in health care facilities and limited understanding on disease transmission. Combined with community health education and information campaigns, increased CL treatment capacity should be established within affected areas.

Acknowledgements

We would like to acknowledge all the community members of the study area for their participation in this study. We are very thankful to Arba Minch University, zonal and district health offices for their arrangements of the field work.

Funding

MP is a PhD fellow in the VLADOC program of the Flemish Interuniversity Council VLIR-UOS. HL is part of the University of Antwerp Center of Excellence VAX-IDEA. The study was supported by VLIR-UOS through the Inter-University Cooperation (IUC) agreement with Arba Minch University.

Additional files

Additional file 5.1: Table S1 Villages (and according GPS coordinates) where cutaneous leishmaniasis was found during the survey in 2018.

N°	Zone	District	Village	Coordinates	
				E	N
1	Dawuro	Ella	Ella	37.28616	7.01018
2	Dawuro	Lemmo	Gessa Chere	37.28217	7.01073
3	Dawuro	Tarcha	Tarcha	37.10200	7.09120
4	Gamo	Bonke	Sime	N/A	N/A
5	Gamo	Chencha	Bilala	37.61034	6.20795
6	Gamo	Chencha	Tutusha	37.59784	6.21482
7	Gamo	Chencha	Shaye	37.60353	6.20279
8	Gamo	Zadha	Goza	37.42918	6.33164
9	Gamo	Mirab Abaya	Egirsa wakie	37.69813	6.35455
10	Gamo	Mirab Abaya	Morode	37.69813	6.35464
11	Gamo	Mirab Abaya	Layo Tirga	37.65592	6.62639
12	Gamo	Boreda	Zefine 01	37.63513	6.47120
13	Gamo	Boreda	Dondo Anono	37.63541	6.53281
14	Gamo	Bonke	Geresse	37.30289	5.90311
15	Gamo	Kemba	Lae	37.18905	6.04803
16	Gamo	Kemba	Fudale	37.16772	6.02860
17	Gamo	Kemba	Geta	37.16592	6.02195
18	Gamo	Kemba	Kemba01	37.19070	6.04522
19	Gamo	Kemba	Kemba zirko	37.19220	6.04771
20	Gofa	Geze Gofa	Bulki Parcha	36.81564	6.28125
21	Gofa	Geze Gofa	Bulki-Kencho Weiza	36.81612	6.28211
22	Gofa	Demba Gofa	Dakisho Subbo	36.84606	6.31457
23	Gofa	Demba Gofa	Gachigalla	36.79558	6.26056
24	Gamo	Kucha	Kodo Lade	37.21194	6.56565
25	Gamo	Kucha	Weide Weshe-Kontoayo	37.34324	6.62094
26	Gamo	Kucha	Weide Weshe	37.34115	6.62557
27	Gamo	Chencha	Shama	37.34429	6.61774
28	Gamo	Chencha	Belle	37.54680	6.18775
29	Gamo	Geresse	Geresse-Sime	37.36205	6.75703
30	Dherashe	Derashe	Bussakilla	37.33147	5.69714
31	Dherashe	Derashe	Nallo	37.30518	5.62251
32	Dherashe	Derashe	Yayibe	37.33626	5.62143
33	Wolaita	Kindo didaye	Kindo didaye-Wamura	37.2529	6.4618
34	Wolaita	Sodo Area	Damota Waja	37.63545	6.53462
35	Wolaita	Sodo Area	Waraza Lasho	37.76260	6.91405
36	Wolaita	Sodo Area	Gurmo Woyide	37.75668	6.91730
37	Wolaita	Kindo Didaye	Bossa Borto	37.39055	6.74532
38	Wolaita	Kindo Didaye	Sharuma	37.39558	6.75945

PART III
DIAGNOSTICS

CHAPTER 6

Evaluation of conventional and four real-time PCR methods for the detection of *Leishmania* in field-collected samples in Ethiopia

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Research article

Submitted to PLoS Neglected Tropical Diseases

Abstract

In most low-resource settings, microscopy still is the standard method for diagnosis of cutaneous leishmaniasis, despite its limited sensitivity. In Ethiopia, the more sensitive molecular methods are not yet routinely used. This study compared five PCR methods with microscopy on two sample types collected from patients with a suspected lesion to advise on optimal diagnosis of *Leishmania aethiopica*.

Between May and July 2018, skin scrapings (SS) and blood exudate from the lesion spotted on filter paper (dry blood spot, DBS) were collected for PCR from 111 patients of four zones in Southern Ethiopia. DNA and RNA were simultaneously extracted from both sample types. DNA was evaluated by a conventional PCR targeting ITS-1 and three probe-based real-time PCRs: one targeting the SSU 18S rRNA and two targeting the kDNA minicircle sequence (the 'Mary kDNA PCR' and a newly designed 'LC kDNA PCR' for improved *L. aethiopica* detection). RNAs were tested with a SYBR Green-based RT-PCR targeting spliced leader (SL) RNA. Giemsa-stained SS smears were examined by microscopy.

Of the 111 SS, 100 were positive with at least two methods. Sensitivity of microscopy, ITS PCR, SSU PCR, Mary kDNA PCR, LC kDNA PCR and SL RNA PCR were respectively 52%, 22%, 64%, 99%, 100% and 94%. Microscopy-based parasite load correlated well with real-time PCR Ct-values. Despite suboptimal sample storage for RNA detection, the SL RNA PCR resulted in congruent results with low Ct-values. DBS collected from the same lesion showed lower PCR positivity rates compared to SS.

The kDNA PCRs showed excellent performance for diagnosis of *L. aethiopica* on SS. Lower-cost SL RNA detection can be a complementary high-throughput tool. DBS can be used for PCR in case microscopy fails and when the lesion has a difficult to sample location.

Background

Cutaneous leishmaniasis (CL) is a vector-borne disease caused by parasites of the genus *Leishmania*, which are transmitted by the bite of infected female phlebotomine sand flies. CL is endemic in more than 80 countries globally with an estimated 0.7-1.2 million CL cases each year, predominantly in 4 countries of the New World and 6 of the Old World (including Ethiopia) together accounting for 70 to 75% of global CL incidence [106]. More than 20 different *Leishmania* species can cause CL with some that geographically coexist. *Leishmania major* and *L. tropica* are most common in the Old World. In Ethiopia, there is a unique dominant species, *L. aethiopica,* which is mainly found in the highlands putting nearly 29 million populations at risk and has an annual burden of an estimated 20,000 to 50,000 cases per year [106,136,251].

CL is characterized by slowly growing nodular or crusty lesions, typically healing with scars. While not life-threatening, lesions can be disfiguring and stigmatizing, particularly those occurring in the face [252]. Localized CL is the most common clinical form, predominantly affecting the face, but also mucocutaneous CL and to a lesser extent diffuse CL are regularly reported in Ethiopia. In contrast to New World CL, *L. aethiopica* typically causes crusty lesions with a patchy distribution and local edema that slowly develop and heal eventually (requiring approximately one to three years). However, sometimes the infection may progress to more severe, chronic and complicated forms [17,19,26].

Like in many other resource-constrained countries, microscopic examination of Giemsa stained skin scrapings (SS) is still the cornerstone for CL diagnosis in Ethiopia. However, it is increasingly recognized that its sensitivity is suboptimal, ranging from around 17-83% [19,253,254], and is heavily dependent on technical expertise, staining quality, lesion type, and reference test used to determine the sensitivity. Molecular methods, such as polymerase chain reaction (PCR), combine high sensitivity with high specificity. Even in resource-constrained settings, user-friendly PCR platforms are nowadays well-established for routine diagnosis and surveillance of tuberculosis [255] and HIV [256]. Although such molecular tools have the potential to be used for other neglected diseases like leishmaniasis as well [21,257], there is still a long way to go before implementation in routine care.
Ideally, PCR testing should facilitate both diagnosis and surveillance. For the latter, easier tools for sample collection and/or storage would be useful. For example, filter

paper is increasingly used for sample collection in remote settings with subsequent centralized analysis at a later stage [258–261].

PCRs designed in either conventional or probe-based formats can target different regions of the *Leishmania* genome for parasite detection at the genus, complex or species level [262,263]. Typically, these PCRs target nuclear DNA like the small subunit (SSU) 18S ribosomal RNA or internal transcribed spacer (ITS) ribosomal regions [145,264] or the mini-exon spliced leader (SL) gene repeat [265,266]. Another commonly used target is the extra-chromosomal minicircle kinetoplast DNA (kDNA), which is present in several thousands of copies, resulting in a considerably higher sensitivity [144,250].

Alternatively, the parasite RNA can be detected which is considered as a marker for viable parasites, such as with the recently developed RT-PCR targeting the SL RNA sequence. This molecular target is conserved in *Leishmania* and performed well on *L. infantum* infected hamsters, spiked human blood and clinical samples from visceral leishmaniasis patients [170], but is not yet evaluated on CL patients.

With several targets and diagnostic methods available, it can be difficult to select the PCR method that is optimal for a particular setting. In the context of CL in Ethiopia, the PCR method must be applicable on different clinical sample types, capable of detecting *L. aethiopica* and more sensitive than microscopy.

The aim of this study was to compare microscopy with five different molecular methods on two different sample types collected from skin lesions of suspected CL patients in the south of Ethiopia and to discuss their potential as diagnostic and surveillance tool in endemic settings.

Methods

Ethics statement

This survey was ethically approved by the Institutional Ethical Review Committee of the College of Medicine and Health Sciences of Arba Minch University, Ethiopia (Letter Ref No: CMHS/1167/111 18th April 2018). Samples were collected from all volunteers who gave their oral consent to participate in the study.

Sample Collection

An active case finding survey was conducted between May and July 2018, in which participants suspected of CL were conveniently selected from the 38 rural kebeles in four zones (Gamo Gofa, 27; Wolaita, 5; Dawuro, 3 and Dherashe area, 3) of the Southern Nations Nationalities and Peoples' Regional State of Ethiopia. Samples were collected from 111 suspected CL patients who gave their oral consent to participate in the survey. For collection of the samples, one small incision was made with the point of a surgical blade at the margin of the lesion after it was cleaned with 70% denatured alcohol. Two types of samples were subsequently collected: *(i)* a skin scraping (SS) collected along the cut edge of the incision of which one part was stored in 97% ethanol at -20°C for PCR analysis and the second part was smeared onto two glass slides for microscopy, and *(ii)* a blood exudate from the same lesion spotted on filter paper, further referred to dry blood spot (DBS). The blood exudate for DBS collection was immediately taken from the SS incision with a capillary and dropped onto two Serobuvard calibrated pre-punched filter paper disks (LDA, Zoopole, Ploufragan, France) until saturation (approximately 5 µl/disk).

Microscopy

After dried completely, the skin smears were fixed with 100% methanol, dried again and stained with 5% Giemsa for microscopic examination [34]. The slides were observed under a light microscope with a 1000× magnification. The examination of duplicate smears was carried out blindly by two experienced staff members, and thereafter, results were compared to each other. In case of a discordant result between the two readers, a third expert observed the slides and a consensus result was reached by a two out of three observers' agreement. Parasite load was graded from +1 to +6 according to WHO parasite grading standard operating procedure [34].

DNA/RNA extraction

Before the extraction procedure, the SS were centrifuged, the ethanol was removed and the remaining tissue was left to dry. DNA and RNA were simultaneously extracted from the SS pellet and DBS using the NucleoSpin RNA kit and an additional NucleoSpin RNA/DNA buffer set (Macherey Nagel, Germany). This protocol enabled sequential elution of DNA and RNA from a single sample. The isolation from DBS was done slightly different than mentioned in the standard manufacturer's protocol: one pre-cut circle

was incubated in β-mercaptoethanol (for RNase inactivation) and lysis buffer for 3 hours at room temperature with frequent vortexing to elute the blood from the filter paper. Eventually, RNA was eluted in 60 µl nuclease-free water while DNA was eluted in 100 µl DNA elute. Both extracts were stored at -20°C until further analysis in the laboratories of Arba Minch University by conventional PCR and Gondar University by real-time PCR.

Conventional ITS-1 PCR

DNA isolates of the SS samples were subjected to a conventional PCR targeting a 350 bp fragment of the ITS-1 gene ("ITS PCR"), based on El Tai *et al*. [145] as described before [163]. In short, the samples were screened in duplicate with a 15 µl reaction mix consisting of 0.5 µM of each primer (LITSR 5'-CTGGATCATTTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGCACTT-3' (Invitrogen, Life Technologies, Belgium)), 0.2 mM dNTP (GE Healthcare Lifescience, Belgium), 1X QIAGEN PCR Buffer (Qiagen, Belgium), 0.04 U/µl HotStarTaq DNA polymerase (Qiagen) and 1.5 µl of 1/10 diluted DNA extract. The reaction was carried out on a Biometra T professional gradient Thermocycler (Biometra, the Netherlands) and amplicons were visualized on a 1.5% agarose gel. A negative (no template) and positive (*L. aethiopica* infected *Phlebotomus pedifer* DNA extract) control were used for each run.

The ITS-1 amplicons were also used to identify the *Leishmania* species in a selection of positive samples from six areas: five from Gamo Gofa (Zadha, Kemba, Demba Gofa, and Kucha woredas) and one from Wolaita (Kindo-Didaye woreda). Amplicons were sent to Vlaams Instituut voor Biotechnologie (VIB) at the University of Antwerp (Belgium) for Sanger sequencing. The obtained sequences were aligned in GenBank using the BLAST tool and the Leishmania species was identified if query coverage and identity exceeded 98%. and the species in all samples was identified as *L. aethiopica* with a query coverage and identity of > 98% (BLAST). Therefore, *Leishmania* will be further referred to as *L. aethiopica*.

Real-time PCR assays

DNA extracts were also tested with three TaqMan probe-based real-time PCRs: one targeting the SSU 18S rRNA gene (referred to as 'SSU PCR') and two targeting the kDNA minicircle sequences (the first here so-called 'Mary kDNA PCR' that was originally designed by Mary *et al.* for *L. donovani* complex species for VL [250]; and the second

further called 'LC kDNA PCR' that was newly designed to improve *L. aethiopica* detection).

The SSU PCR used primers (18S-L-F and 18S-L-R; 0.4 µM) as described by Deborggraeve *et al*. [267] with an additional 18S probe (0.1 µM) as described before [268] and the Mary kDNA PCR was performed with the primers (0.6 µM of each primer) and hydrolysis probe (0.4 µM) as described [250]. The LC kDNA PCR makes use of primers from Nuzum *et al.,* [257] that were adapted to forward primer LC-F (5'-TATTTTACACCAACCCCCAGT-3'; 1 µM) and reverse primer LC-R (5'-GGTAGGGGCGTTCTGC-3'; 1µM) with a newly designed FAM-labeled LC-probe (5'-CAGAAAYCCCGTTCAAAAAATGGC-3'; 0.4 µM).

Technical validation of the LC kDNA PCR was performed by testing reactivity with all Old World *Leishmania* species (*L. aethicopica, L. tropica, L. major, L. infantum, L. donovani*) and New World reference strains (*L. braziliensis, L. mexicana, L. amazonensis, L. peruviana, L. panamensis, L. guyanensis, L. lainsoni*). The analytical sensitivity was determined based on serial dilutions of four *L. aethiopica* strains. Cross-reactivity was also assessed for *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense, Mycobacterium leprae*, *Mycobacterium lepromatosis*, and *Plasmodium falciparum.* In addition, analytical specificity was tested on whole blood samples of 25 endemic controls (from Ethiopia) and 10 healthy non-endemic controls (from Belgium).

The three PCRs were run with HotStarTaq Master mix kit (Qiagen) in a total volume of 25 µL containing 1x master mix, primers and probes (Integrated DNA Technologies (IDT), Leuven, Belgium), 4.5 mM $MgCl_2$ (SSU PCR only), 0.01% BSA (Roche, Vilvoorde, Belgium), and 5 µL DNA. The PCR programme consisted of an initial activation step of 15 min at 95°C, followed by 50 cycles of denaturation for 5 sec at 95°C, annealing for 20 sec at 58°C, and elongation for 30 sec at 72°C on the RotorGeneQ cycler (Qiagen). A fixed and stringent fluorescent threshold (0.2) was used to determine the cycle threshold (Ct) value.

Real-time RT-PCR assay

RNA extracts were diluted 1/10 and subjected to the SYBR green-based reverse transcriptase (RT)-PCR targeting the spliced leader RNA sequence (SL RNA PCR) as described [170]. The SL RNA PCR was also run on the RotorGeneQ cycler. In the absence of a melt curve analysis, a stringent Ct-value cut-off of 32.9 was applied for the positive identification of SL RNA amplicons. This cut-off was established based on a ROC analysis

on historical RT-PCR data (82 negatives and 81 VL-positive samples), providing 98% sensitivity and specificity (not published).

In each real-time PCR run, two no-template negative PCR controls (PCR-grade water and elution buffer) were used to monitor for contamination, and a positive PCR control (*L. donovani*, 100 pg/reaction) was included twice to check the PCR performance. All PCR runs were valid meaning that all positive controls were positive with Ct-values in the expected range and that all negative controls were negative (no Ct-values detected within 50 cycles). Ct-values are an indirect measure for parasite load, with low Ct values indicating high parasite loads and high Ct-values indicating low parasite loads.
Whenever a high Ct-value (> 38) was detected in a clinical sample for a single real-time PCR test, the sample was retested to confirm the positive result.
In case the clinical sample was negative for all real-time PCRs, a HBB PCR (targeting the human beta-globin gene) was done to detect human DNA to control for PCR inhibition, insufficient material or inefficient extraction as described before [269].

Statistical analysis

The PCR data were entered into an Excel spreadsheet and analysed with R software (version 3.5.2, "Eggshell Igloo"- R Core Team) [270]. A CL suspected patient was identified as a true positive case if at least two of the six diagnostic tests (microscopy, ITS PCR, SSU PCR, Mary kDNA PCR, LC kDNA PCR and SL RNA PCR) were positive, which was used as the composite reference test, similarly to as described before [259]. Confidence intervals (CIs) for sensitivity and specificity were constructed using the Clopper-Pearson formula. The association between parasitic load and Ct-value was tested with ordinal ANOVA, using the ordPens package and illustrated with smoothers constructed with the mgcv package [271]. A type-I error (α) of 5% and equivalent 95% coverage for CIs was used for all analyses. Correlation between Ct-values of different methods was calculated using Pearson's correlation coefficient and expressed as its associated R^2 (which is the squared correlation, the percentage of variance explained or in common).

Results

Pre-analysis validation

The LC kDNA PCR validation showed reactivity for all Old and New World *Leishmania* species, except *L. major*. No New World *Leishmania* species were detected and no cross-reactivity was observed for *T. b. gambiense*, *T. b. rhodensiense*, *M. leprae*, *M. lepromatosis* and *P. falciparum*. The analytical sensitivity of the assay was assessed to be at least 1 fg/reaction. Endemic and healthy non-endemic controls were all negative (*e.g*. no Ct-value detected up to 50 cycles).

The species was identified as *L. aethiopica* based on ITS-1 sequences found in the selected samples from six different area's. Therefore, *Leishmania* will be further referred to as *L. aethiopica*.

Comparison of different CL diagnostic tests on SS samples

First, the positivity rate of SS samples was determined for each of the six diagnostic tests individually (microscopy and ITS, SSU, Mary kDNA, LC kDNA and SL RNA PCRs) (Table 6.1). Microscopy identified 46.8% of the subjects as *Leishmania* positive, after re-examination by a third reader due to 19.9% inter-observer discordant results. The molecular methods showed higher positivity rates, except the ITS PCR (19.8%). The SSU PCR resulted in 57.7% positive subjects while the Mary and LC kDNA PCRs showed a higher positivity rate of 89.2% and 90.1% respectively. The SL RNA PCR identified 85.6% positive SS RNA extracts.

Table 6.1 Overview of index tests with the number of positive and negative samples, and sensitivity and specificity compared to the composite reference (any two tests positive).

Test	Positive n (%)	Negative n (%)	Sensitivity % (95% CI)	Specificity % (95% CI)
Microscopy	52 (46.8)	59 (53.2)	52 (42-62)	100.0 (72-100)
ITS PCR	22 (19.8)	89 (80.2)	22 (14-31)	100.0 (72-100)
SSU PCR	64 (57.7)	47 (42.3)	64 (54-73)	100.0 (72-100)
Mary kDNA PCR	99 (89.2)	12 (10.8)	99 (95-100)	100.0 (72-100)
LC kDNA PCR	100 (90.1)	11 (9.9)	100 (96-100)	100.0 (72-100)
SL RNA PCR	95 (85.6)	16 (14.4)	94 (87-98)	91 (59-100)
Composite reference	100 (90.1)	11 (9.9)	NA	NA

Due to the lack of a gold standard reference test, the sensitivity and specificity of each method were calculated with a composite reference (Table 6.1), with a sample defined as truly positive if positive by at least two of the six index tests. This resulted in sensitivity for microscopy of 52% (95% CI, 42%-62%). The ITS PCR had the lowest sensitivity (22%; 95% CI, 14%-31%). The SSU, Mary and LC kDNA PCRs had a sensitivity of 64% (95% CI, 54-73%), 99% (95% CI, 95%-100%) and 100% (95% CI, 96%-100%) respectively. The SL RNA PCR displayed a sensitivity of 94% (95% CI, 87%-98%). With keeping in mind the limitation of having only a small set of samples that were negative with the composite reference test (n=11), the specificity of all index tests was 100% except for the SL RNA PCR (91%) because the latter test identified one sample as positive at low Ct-value, which was not confirmed by the other assays.

When comparing overall, 10 out of the 111 SS samples were negative with all diagnostic methods, with one sample that was positive with only one test (the SL RNA PCR). In five of these negative samples, no human DNA was detected by the HBB PCR, indicating that PCR inhibition, insufficient sample start material or inefficient extraction cannot be excluded in these samples. All other 100 samples were confirmed as positive with at least one additional test (Table 6.2). Of these, four samples were positive by two tests, 20 by three tests, 29 by four tests, 35 by five tests and 12 were positive by all six tests.

Table 6.2 An overview of a number of tests and the number of skin scraping samples that gave a positive result for all observed combinations of index tests.

Nr of + tests	Nr of SS samples	Microscopy	ITS	SSU	Mary kDNA	LC kDNA	SL RNA
0	10						
1	1						+
2	3				+	+	
2	1	+				+	
3	18				+	+	+
3	2	+			+	+	
4	17			+	+	+	+
4	10	+			+	+	+
4	2		+		+	+	+
5	27	+		+	+	+	+
5	8		+	+	+	+	+
6	12	+	+	+	+	+	+

The two kDNA PCRs showed the highest agreement among each other with the same results except for one sample. The samples that were positive with the less sensitive tests (see Table 6.1), were all confirmed by tests with higher sensitivity, except one sample that was microscopically positive which was confirmed by the LC kDNA PCR only. This cumulative trend of samples being positive in more than one diagnostic test can also be seen.

The range of Ct-values for SS samples positive in all four real-time PCRs (n=64, blue boxplots) are shown in Fig 6.1. Among these samples, the SSU PCR showed the highest median Ct-value (34.2), whereas Mary and LC kDNA PCRs had lower median Ct-values of 28.5 and 25.6 respectively. The SL RNA PCR assay had the lowest median Ct-value (17.7). Samples that were negative for the SSU PCR but positive with kDNA PCRs and SL RNA PCR (n=30; pink boxplot), showed higher median Ct-values in the Mary kDNA (37.8), LC kDNA (34.8) and SL RNA (25.7) PCR assays than their counterparts that were also positive with SSU PCR. The five samples only positive by the two kDNA PCRs (red boxplots), gave median Ct-values of 41.0 for the Mary kDNA PCR and 36.7 for the LC kDNA PCR.

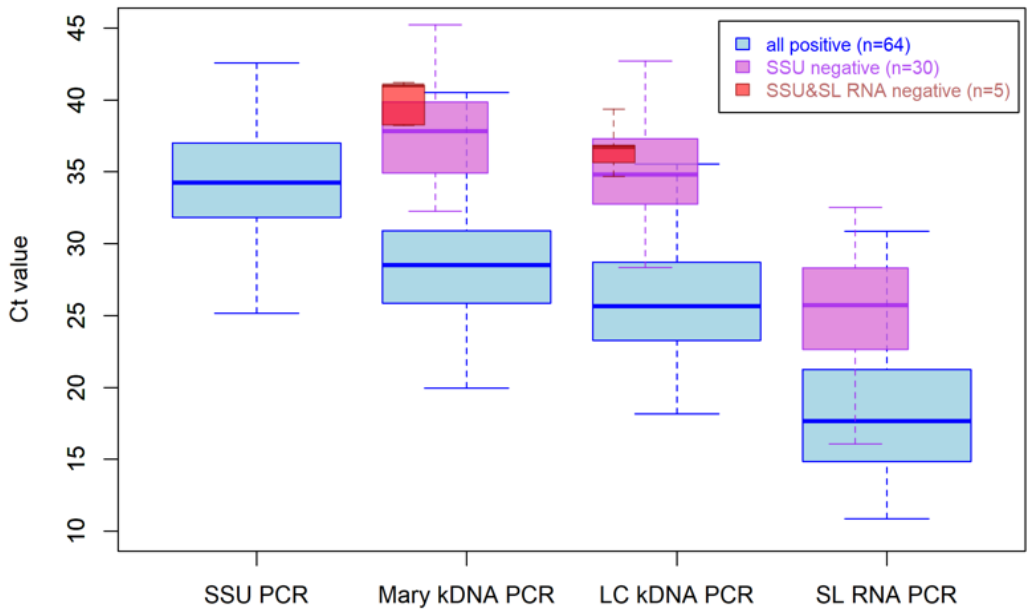


Fig 6.1 Range and median Ct-values for samples positive with the real-time PCR assays.

Fig 6.2 shows the R^2 correlation between Ct-values detected on SS DNA and RNA extracts with the different real-time PCR methods. The Mary kDNA and LC kDNA had the strongest relationship (R^2=0.943, n=99). The SSU PCR Ct-values were slightly less correlated with the ones from the LC kDNA (R^2=0.872, n=64) and Mary kDNA (R^2=0.853, n=64) assays. The correlation of the SL RNA PCR with the various DNA PCRs was lower: LC kDNA (R^2=0.721, n=94), Mary kDNA (R^2=0.691, n=94) and SSU PCR (R^2=0.383, n=64).

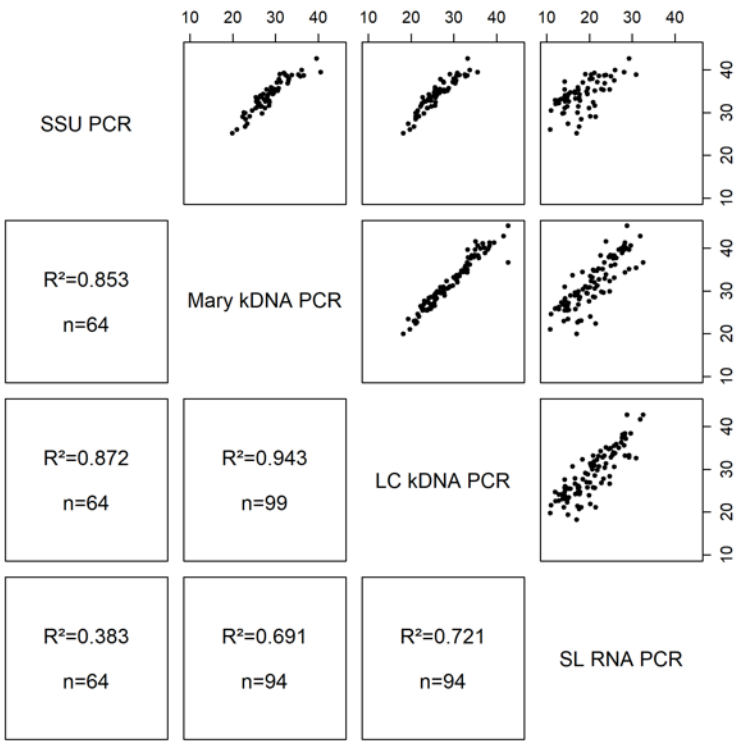


Fig 6.2 The R-square (R^2) of Ct-values for the real-time PCR assays on positive skin scraping DNA and RNA extracts. n = number of samples positive in both PCR methods that were compared.

Ct-values of the real-time PCR assays were also compared with the parasitic load determined by microscopy (Fig 6.3). Overall, a clear trend was observed between the parasite load and the median Ct-values. The higher the parasite load, the lower the Ct-values and samples that were microscopy negative had the highest Ct-values in all PCRs. Statistical analysis showed that Ct-values were significantly associated with the parasite load for the SSU PCR (Fig 6.3A, p-value=0.0253), and were more significant for the kDNA PCRs (Fig 6.3B, p-value: 0.0003; 6.3C p-value: 0.0004) and SL RNA PCR (Fig 6.3D, p-value: 0.0001).

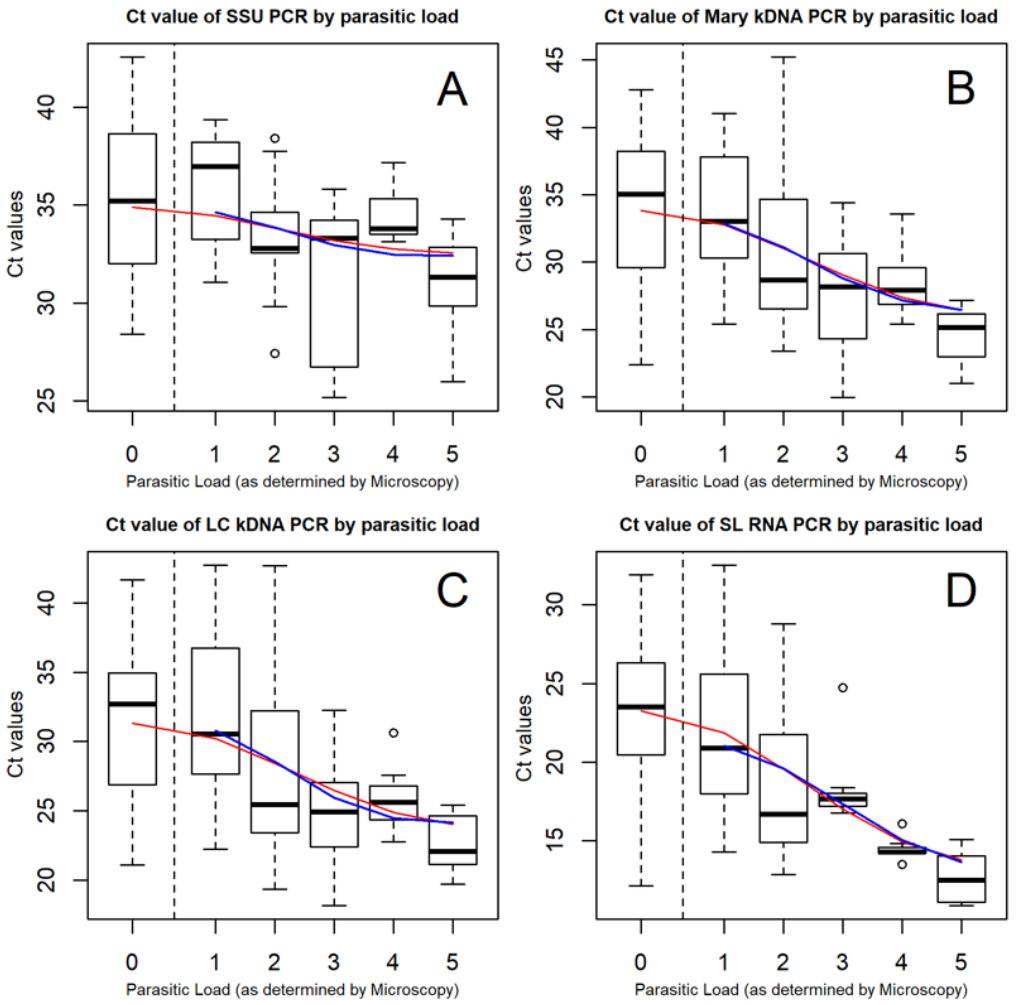


Fig 6.3 Boxplot for comparison of Ct-values with microscopy parasite load. The thick horizontal lines in the box represent the median; the bottom and top line of the box are the 25th and 75th percentile respectively. The red fitted line shows the trend of Ct-values of samples which were identified as positive by PCRs, including those negative by microscopy. The blue line shows the trend of Ct-values of the PCR by parasite load as of +1 in microscopy.

Comparison of SS and DBS sample types

To compare the two sampling methods, the PCRs with the highest sensitivity were also evaluated on DBS samples. The comparison of DBS and SS samples tested by the LC kDNA PCR is displayed in Fig 6.4 and those by the Mary kDNA PCR and SL RNA PCR are shown in Additional file 6.1: Fig S1. The DBS, collected from the same incision of the

lesion, generally showed a lower positivity rate (76/111; 68.4%) compared to the SS. In particular, 28 subjects that were identified as positive by the LC kDNA PCR on SS (with Ct-values ranging between 21.4 and 42.6) were negative based on the DBS sample. Seven individuals were identified as negative by both sampling methods and 72 (65%) subjects were identified as positive in both sample types with Ct-values that were generally higher on DBS (ranging between 18.2 and 39.2 on SS and 27.1 and 42.0 on DBS). On the contrary, four subjects that were negative on SS were additionally identified as CL case based on the DBS sample with the LC kDNA PCR with Ct-values between 27.1 and 43.3. Of these four DBS samples, three were positive with the Mary kDNA PCR and two with the SL RNA PCR as well. Additionally, one more case was only detected on the DBS sample and not on the SS sample by the Mary kDNA PCR and SL RNA PCR (Additional file 6.1: Fig S1)

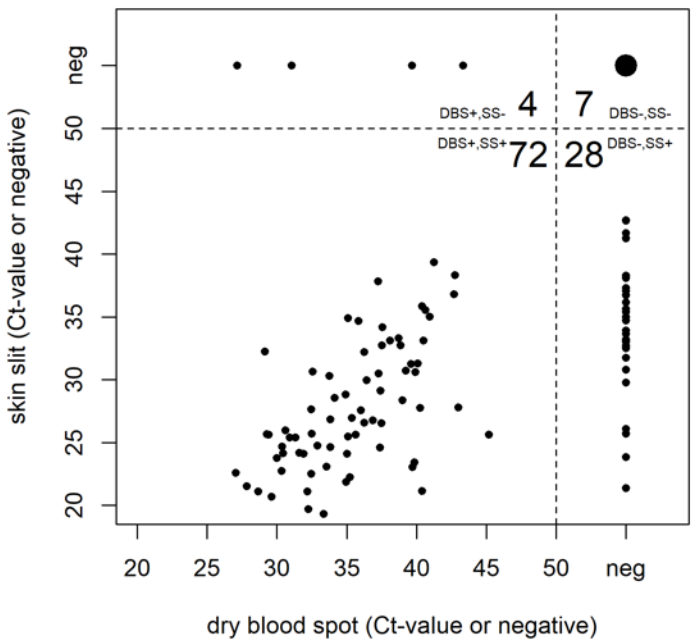


Fig 6.4 Comparison of Ct-values and concordance of Dry Blood Spots (DBS) versus Skin Scraping (SS) for the LC kDNA PCR.

Discussion

For neglected tropical diseases such as leishmaniasis, laboratory confirmation of clinical suspicion is mostly done by traditional methods (microscopy). Due to lack of sensitivity

of this approach, diagnosis and treatment can be hampered. We therefore investigated the added value of PCR to accelerate its implementation in routine practice. We compared microscopy with five PCR assays including conventional and real-time formats with different gene targets on DNA or RNA extracts of two sample types from patients suspected of CL from a wide geographical area in the South of Ethiopia. The two sample types (SS and DBS) were collected from the same lesion and the DNA and RNA were simultaneously extracted from the same starting material. This approach avoids inter-lesional differences and reduces deviations due to different extraction methods which make the comparison as fair as possible.

Six samples from different zones were identified up to species level and were all *L. aethiopica*. However, this does not rule out that there were no *L. tropica* or *L. major* cases among the samples. As there are several reports of other *Leishmania* species isolated from sand flies and rodents in the country, more large-scale molecular studies in different parts of Ethiopia are required to determine which species are causing CL, DCL and MCL.

Direct identification of amastigotes by microscopy on Giemsa-stained skin scraping smears is still the standard method for the diagnosis of CL in Ethiopia. Especially in endemic regions, it is widely available and the first-choice method [272] being familiar to lab staff and not expensive. In this study, the positivity rate of microscopy was 46.8% (Table 6.1) and lies within the range of 40% to 75% seen in other Old World CL endemic countries [273–277]. The high inter-observer disagreement observed in this study demonstrates again that microscopy can be technically challenging [273] and requires the presence of a relatively high number of intact parasites [278]. This can be problematic in chronic lesions when patients present late [279] or with complex mucocutaneous CL [273], as parasite loads in these lesions are generally low [280]. Hence, PCR is reported to be superior to other methods for chronic lesions [281].

In contrast to microscopy, it is well-known that molecular tools can provide rapid, sensitive, accurate detection, quantification, and species identification depending on the target and design used [282–284]. In this study, we, therefore, compared the performance of well-known (SSU, kDNA) and less common (SL) PCR targets for CL detection. Since microscopy could not be used as the reference method, we applied a composite reference, similar to as described before [259,285] to judge on the sensitivity of the different methods. Specificity was presented for completeness, but this result should be interpreted with care due to the low number of negative samples.

Of the five molecular assays in this study, the sensitivity of the conventional ITS PCR was lowest and unexpected, even lower than microscopy (Table 6.1). Literature showed various sensitivities of ITS for CL diagnosis ranging from 69.2% up to 96.6% [253,263,286]. This poor performance might be explained by the use of 1/10 diluted DNA, the lower PCR reaction volume and the possibility of the parasite load in the samples of our study being at the limit of detection of the ITS assay. Moreover, the copy number of the ITS gene (20-200 copies) is much lower compared to the kDNA and SL RNA targets [20,253] as demonstrated before [263]. However, when the ITS PCR does not provide the desirable sensitivity, a subsequent nested PCR could be performed to increase its performance [268]. Based on our experience, we would advise against the use of conventional or nested PCR formats for routine diagnostics, due to the higher workload and risk of post-PCR contamination. On the other hand, the amplicons of conventional PCRs are generally longer, and the universal ITS PCR therefore allows species identification by RFLP, sequencing or high-resolution melt technology [20,44].
The SSU PCR targets a 115 bp-long highly conserved region [264] allowing broad use in *Leishmania* detection at the genus level, but without the ability of species discrimination. Although the SSU gene has similar copy numbers (20-400), the SSU PCR performed better than the ITS PCR as demonstrated here and before [287], probably due to its real-time format and shorter amplicon length. The PCR can amplify up to one single parasite in human blood [267], which relates to clinical disease [288]. However, in our study, the SSU PCR had a lower sensitivity than the kDNA PCRs as has been reported before [262,263].

In this study, both kDNA PCRs identified the same subjects as CL positive, except one (Table 6.2). Studies done in Old World CL countries demonstrated high sensitivities for kDNA targeting PCRs ranging between 91.7% up to 100% [253,263,284,289]. The kDNA minicircle sequence is by far the most often used target in studies on visceral [250,257,288] or New World leishmaniasis [262,290]. With over 10,000 copies of minicircles per parasite, this PCR is more sensitive than the SSU [287] and ITS PCRs [253]. The Mary kDNA PCR was designed for species of the *L. donovani* complex group and some of the CL causing *Leishmania* species are not well detected [250]. We, therefore, designed a new PCR, the LC kDNA PCR, to improve amplification of *L. aethiopica* based on primers described first by Nuzum *et al.* [257] for symptomatic VL and used before by Nicolas and colleagues [144] for Old and New World CL species in mice and for differentiation of Old World CL species by melt curve analysis [291]. With its new probe-based format, the LC kDNA PCR showed the highest sensitivity among all PCRs, with lower Ct-values than the Mary kDNA PCR for *L. aethiopica* detection in CL

suspected cases (Fig 6.1), illustrating its potential in Old World CL diagnosis. The potential of the kDNA PCR as a quantitative tool for treatment follow-up for (M)CL patients [280] is also of interest in clinical practice.

The skin slit RNA extracts were subjected to the pan-*Leishmania* SL RNA PCR [170] which performed well for CL diagnosis despite sample storage at -20°C without RNA stabilizing reagents and the use 1/10 diluted RNA. The assay performed better than the SSU and ITS PCRs, probably due to the high copy number and very short amplicon (39 bp). Only six out of 99 SS samples that were positive by both kDNA PCRs were not detected (Table 6.2). Overall, Ct-values were low compared to the other PCR assays as described earlier (Fig 6.1) [170]. Although more stringent storage conditions are generally needed for RNA [292,293], it did not compromise assay performance in this study of field collected samples. RNA detection is also considered as a marker for viable parasites [294] although it has been demonstrated that longer targets are more indicative of viability than shorter amplicons [295]. The intercalator dye-based format of the SL RNA PCR assay is substantially cheaper than probe-based assays and thus beneficial for use in high throughput testing and epidemiological research.

One of the added values of PCR methods, in general, is that they can be applied to different types of clinical specimens [296–300]. The standard sample for CL diagnosis is a punch biopsy or skin scraping but less invasive sample collection methods have been studied [284,300]. Lesion aspirates showed lower sensitivities compared to biopsies [281,301] while filter paper lesion impression has a high sensitivity for ulcerative lesions and looks promising in New World CL [259,285]. Collection of samples with filter paper is relevant for use in field conditions and to simplify transport.

In this study, a total of 102 of the 111 suspected cases were confirmed by PCR, 100 on SS and two additional ones on DBS. CL could not be diagnosed in only eight patients that were negative by all PCRs on both sample types, of which only a part could be explained by PCR inhibition, insufficient starting material or inefficient nucleic acid extraction [301]. We also found that the DBS performed less than SS and had a 28% lower positivity rate with the LC kDNA PCR and that the filter paper storage conditions were not ideal for RNA stability as demonstrated by the 38% lower positivity rate with the SL RNA PCR. Of note, the performance of PCR on DBS was still better than microscopy. It would be interesting to evaluate other sample collection methods like lesion impressions on filter paper or a tape stripping sampling method in future studies, which are also easy to perform in the field [302,303].

Overall, all four real-time PCR formats performed better than microscopy and the conventional ITS PCR, and Ct-values correlated well with the parasite load, making them valid for monitoring parasite quantities during follow-up [302]. The new LC kDNA PCR proved to be an excellent assay for CL diagnosis in Ethiopia. The lower-cost SL RNA detection represents a complementary tool, which can be useful for high throughput studies. SS samples performed much better than DBS, but regarding sensitivity, PCR on DBS is still preferred above microscopy.

In Ethiopia, there is currently no comprehensive diagnostic algorithm that includes molecular methods and it would require additional infrastructure and training at centers nearby CL endemic sites. Therefore, on the basis of our results, we propose that at the primary health care level, microscopy can still be the first diagnostic method followed by treatment when positive. In case that microscopy is negative, the SS sample can be sent to the referral health facility where a kDNA PCR method is available. With this study, we therefore advocate for the implementation of PCR in routine care for CL diagnosis, and at least at the referral hospital level.

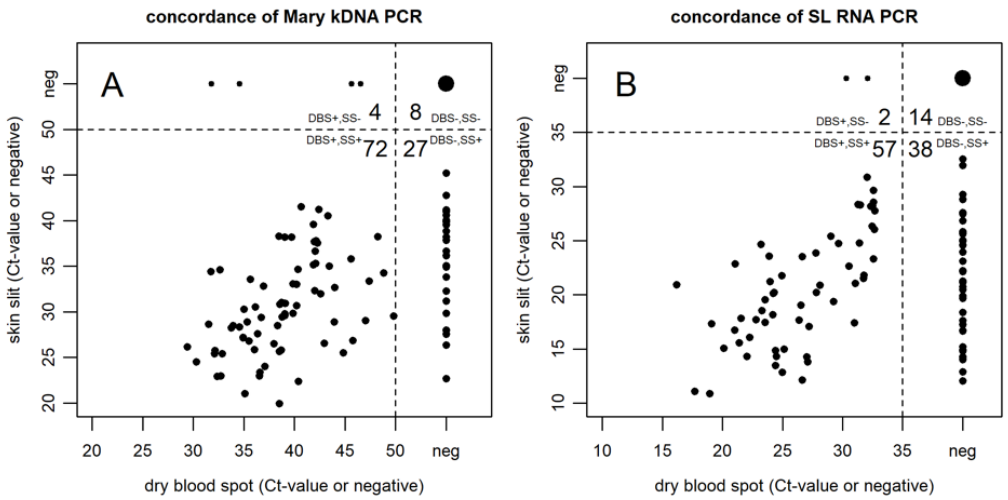
Acknowledgements

We would like to thank all study subjects volunteered to participate in this study. We are also thankful to the zonal and district health offices for their cooperation and to Arba Minch University, and the University of Gondar for their lab support.

Funding

MP is a PhD fellow in the VLADOC program of the Flemish Interuniversity Council VLIR-UOS. HL is part of the University of Antwerp Center of Excellence VAX-IDEA. The study was supported by VLIR-UOS through the Inter-University Cooperation (IUC) agreement with Arba Minch University. We thank Ilse Maes and dr. Gert Van der Auwera for providing kDNA alignments and testing analytical sensitivity.

Additional files



Additional file 6.1 Fig S1 Comparison of Ct values of DBS vs SS concordance for Mary kDNA and SL RNA PCRs. The scatterplots show the Ct-values of DBS vs SS samples screened by the Mary kDNA and SL RNA PCRs. On top and on the right side of the graph, the Ct values are shown for patient samples of which only one out of two tests was positive (indicated by "neg" on their respective axes). The number of patient samples in each of the pos/pos, pos/neg, neg/pos, neg/neg combos are shown in the upper right corner.

CHAPTER 7

Evaluation of a pan-*Leishmania* SL RNA qPCR assay for parasite detection in laboratory-reared and field-collected sand flies and reservoir hosts

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Research article

Published in Parasites & Vectors, 2020, 13(276)

DOI 10.1186.s13071-020-04141-y

Abstract

In eco-epidemiological studies, *Leishmania* detection in vectors and reservoirs is frequently accomplished by high-throughput and sensitive molecular methods that target minicircle kinetoplast DNA (kDNA). A pan-*Leishmania* SYBR green quantitative PCR (qPCR) assay which detects the conserved spliced-leader RNA (SL RNA) sequence was developed recently. This study assessed the SL RNA assay performance combined with a crude extraction method for the detection of *Leishmania* in field-collected and laboratory-reared sand flies and in tissue samples from hyraxes as reservoir hosts.

Field-collected and laboratory-infected sand fly and hyrax extracts were subjected to three different qPCR approaches to assess the suitability of the SL RNA target for *Leishmania* detection. Nucleic acids of experimentally infected sand flies were isolated with a crude extraction buffer with ethanol precipitation and a commercial kit and tested for downstream DNA and RNA detection. Promastigotes were isolated from culture and sand fly midguts to assess whether there was difference in SL RNA and kDNA copy numbers. Naive sand flies were spiked with a serial dilution of promastigotes to make a standard curve.

The qPCR targeting SL RNA performed well on infected sand fly samples, despite preservation and extraction under presumed unfavorable conditions for downstream RNA detection. Nucleic acid extraction by a crude extraction buffer combined with a precipitation step was highly compatible with downstream SL RNA and kDNA detection. Copy numbers of kDNA were found to be identical in culture-derived parasites and promastigotes isolated from sand fly midguts. SL RNA levels were slightly lower in sand fly promastigotes (ΔCt 1.7). The theoretical limit of detection and quantification of the SL RNA qPCR respectively reached down to 10^{-3} and 10 parasite equivalents. SL RNA detection in stored hyrax samples was less efficient with some false-negative assay results, most likely due to the long-term tissue storage in absence of RNA stabilizing reagents.

This study shows that a crude extraction method in combination with the SL RNA qPCR assay is suitable for the detection and quantification of *Leishmania* in sand flies. The assay is inexpensive, sensitive and pan-*Leishmania* specific, and accordingly an excellent assay for high-throughput screening in entomological research.

Background

Leishmaniasis is a vector-borne disease caused by protozoans of the genus *Leishmania*, which are transmitted during the blood-feeding of female phlebotomine sand flies. The infection can be manifested in three major clinical forms, cutaneous (CL), mucocutaneous, and visceral leishmaniasis [2]. In Ethiopia, *Leishmania aethiopica* is the predominant species causing CL and its vectors are *Phlebotomus longipes* and *P. pedifer* [47,48,136]. Hyraxes (*Heterohyrax brucei* and *Procavia capensis*) have been found asymptomatically infected with *L. aethiopica* in large numbers, indicating that they are major animal reservoirs in Ethiopia [47,48,115].

For eco-epidemiological research, which is often covering large sample sizes, there is a need for low-cost, sensitive, high-throughput methods to identify and quantify *Leishmania* parasites in (potential) vectors and hosts [304]. The golden standard for parasite detection in sand flies and animal tissues is microscopy examination. This method allows to confirm the presence of viable parasites, but is time consuming and requires a substantial level of expertise [21]. These drawbacks resulted in a shift towards sample screening with molecular assays. Procedures generally start with nucleic acid extraction for which efficient, but expensive kits are commercially available. Low-cost methods, like organic (i.e. phenol-chloroform) or chelex extractions, are widely utilized, but have disadvantages. The former method is very time consuming and often involves toxic chemicals while the latter only yields low amounts of genomic DNA [305]. Extraction approaches with lysis buffers containing SDS, EDTA, Tris-HCl and NaCl have been applied successfully to various tissues [143], although this crude procedure may lead to inhibition in downstream molecular applications [305].

A variety of (real-time) PCR methods targeting different gene fragments has been described, many of which remain to be validated on multiple *Leishmania* species and different tissues, or have issues regarding quantification [20,306]. The most commonly used PCR assay for *Leishmania* detection in sand flies [307,308] and small mammals [116,117,144] is targeting the minicircle kinetoplast DNA (kDNA). Because of the high kDNA copy number (10^4 minicircles per parasite), very low numbers of parasites can be detected [21]. However, the nucleotide sequence and copy number sometimes differ among *Leishmania* species, impeding consistent quantification [250,309]. Another concern is that it sometimes results in false positive assay results due to its high

sensitivity, even though all preventive measures to avoid contamination are taken [310–312].

Few studies investigated the use of RNA targets for parasite detection, although these may be more informative than DNA targets given the ability to discriminate viable parasites [295]. Recently, a pan-*Leishmania* SYBR Green quantitative PCR (qPCR) assay has been developed, targeting the highly conserved mini-exon encoded 39 bp spliced-leader RNA (SL RNA) sequence, which shows excellent sensitivity and specificity. The assay was able to detect eight Old- and New-World *Leishmania* species with equal threshold cycle (Ct) values and was validated on tissue samples of *L. infantum*-infected hamsters, promastigote spiked human blood and blood nucleic acid extracts from visceral leishmaniasis patients. It appeared that the limit of detection (LoD) of the SL RNA qPCR was one log lower than the LoD of a TaqMan duplex assay targeting kDNA [170].

In this study, we aimed to evaluate the SL RNA qPCR assay in combination with a crude extraction procedure for detection and quantification of *Leishmania* parasites in field- and laboratory-collected (infected) sand flies and hyrax tissue samples collected in Ethiopia.

Methods

Ethics approval and consent to participate

The used chicken skins were obtained from day-old male chicks of a layer breed (Verpymo, Poppel, Belgium). The euthanasia of the chicken and use of laboratory rodents were carried out in strict accordance with all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version). All animal handling was approved by the Ethics Committee of the University of Antwerp, Belgium (UA-ECD 2016–54 (2-9-2016)).
Hyrax trapping and sample collection in Ethiopia were conducted with authorization of the appropriate institutional authorities. Handling of the animals was carried out according to the 2016 Guidelines of the American Society of Mammalogists for use of small mammals in research and education.

Parasites

The *L. major* strain MHOM/SA/85/JISH118 used in this study was cultivated *in vitro* at 26 °C in HOMEM promastigote medium (Gibco, Life Technologies, Ghent, Belgium), supplemented with 10% inactivated fetal calf serum (Invitrogen, Merelbeke, Belgium) and was sub-cultured twice weekly.

Sand flies

Lutzomyia longipalpis sand flies were maintained at the insectary of the Laboratory of Microbiology, Parasitology and Hygiene, Antwerp, Belgium. The colony was kept at 25–26 °C, 75% relative humidity and 12:12 h light:dark photoperiod. A 30% sugar source was permanently provided to adult sand flies. Depending on the experiment, naive or experimentally infected *L. longipalpis* were used. For laboratory infection, the sand flies were starved 12 h prior to feeding through a chick-skin membrane on heparinized (100 U/ml blood) heat-inactivated mouse blood spiked with *L. major* procyclic promastigotes (5×10^6 promastigotes/ml blood). Engorged females were separated 24 h post-blood meal and were continuously provided with 30% sugar solution.

Phlebotomus pedifer sand flies were captured in a previous study in Ochollo (6°11'N, 37°41'E), a village in southwestern Ethiopia where CL is endemic [161,163]. Sand flies were captured between March 2017 and February 2018 using CDC light traps and sticky traps. Specimens were stored in 97% ethanol at -20 °C until nucleic acid isolation was carried out in March 2018 (as described in 'Sand fly nucleic acid isolation and purification' below and in [163]). *Leishmania* DNA positive sand flies were all *P. pedifer* infected with *L. aethiopica* [163]. Nucleic acid extracts were stored at -20 °C until analysis for the current study.

Hyraxes

Hyraxes had been captured in Ochollo using traditional trapping methods in 2017. Nose and ear samples were collected and stored in 97% ethanol at -20 °C until further handling. Molecular analyses revealed that all hyraxes were *H. brucei* infected with *L. aethiopica*. The original tissue samples in 97% ethanol were stored at -20 °C until analysis [163].

SL RNA qPCR evaluation on field and laboratory-infected sand flies and hyraxes

Sand fly nucleic acid isolation and purification

Experimentally infected (*L. major*) *L. longipalpis* were collected six days after infection for dissection of the thorax and abdomen (*n* = 96). Nucleic acids of these specimens were isolated with a crude extraction buffer and purified using an ethanol precipitation approach as described previously [163].

In short, individual sand fly specimens were incubated overnight in 50 µl extraction buffer (10 mM Tris-HCl pH 8, 10 mM EDTA, 0.1 % SDS, 150 mM NaCl) and 0.5 µl proteinase K (200 µg/ml) without maceration. The next day, 25 µl nuclease-free water was added and the samples were heated for 5 min at 95 °C. For nucleic acid precipitation, 20 µl of the extract was supplemented with 1/10th volume 3 M NaOAc (pH 5.6) and 2 volumes 97% ethanol (chilled at -20 °C). This suspension was left overnight, after which the samples were centrifuged for 15 min at 21,000× *g* at 4 °C. The supernatant was removed and 500 µl chilled 70% ethanol was added, followed by centrifugation under the same conditions. The supernatant was removed, and the pellet was air-dried for 15 min in a heating block at 50 °C followed by resuspension in 20 µl nuclease-free water.

Additionally, 37 *P. pedifer* nucleic acid extracts were selected from our previous study (see section 'Sand flies'), of which 17 were identified as *L. aethiopica* positive (kDNA and ITS1) and 20 as negative (kDNA) [163].

DNA/RNA extraction from hyrax samples

Seven *L. aethiopica* DNA positive (kDNA and ITS-1) and 15 negative hyrax tissue samples were selected from our previous study (see section 'Hyraxes') [163]. DNA and RNA were simultaneously extracted from the original tissue samples with the NucleoSpin RNA kit (Macherey Nagel, Düren, Germany) and additional reagents from the NucleoSpin RNA/DNA buffer set (Macherey Nagel) according to the manufacturer's instructions.

Molecular screening

Nucleic acid extracts of the sand fly and hyrax samples were subjected to three different real-time PCR approaches, targeting four markers: (i) kDNA and *18S* DNA in a

multiplex TaqMan probe assay (further referred to as 'MP kDNA' and '*18S* DNA', respectively); (ii) kDNA in a SYBR Green assay with an alternate set of primers ('JW kDNA'); and (iii) SL RNA in a SYBR Green assay ('SL RNA'). The primers for the JW kDNA qPCR were adopted from Nicolas et al. [144] and the assay was carried out as explained in our previous study [163], while the other assays were performed as described by Eberhardt et al. [170]. All extracts were diluted 1:10 prior to qPCR to prevent inhibition of the polymerase enzyme. All assays were run on a Step One Plus real-time qPCR system (Applied Biosystems, Life Technologies) and the threshold was set at 1 for each qPCR.

Copy number and comparison of extraction methods

Promastigote isolation from sand fly midgut and culture

We assessed whether there is a potential copy number difference of kDNA and SL RNA between parasites isolated from sand fly midguts and *in vitro* cultures in HOMEM.
First, promastigotes were harvested from midguts of *L. major* experimentally infected *L. longipalpis* (see section 'Sand flies'). Sand flies were collected six days after feeding on a blood meal containing parasites, and the midguts were dissected under a dissection microscope. Pools of midguts were macerated with a pestle in 100 µl Dulbecco's phosphate-buffered saline (DPBS; Gibco, Thermo Fisher Scientific, Ghent, Belgium) to release the parasites.
Second, *L. major* promastigotes from a culture were counted using a KOVA chamber to determine parasite concentration. An excess volume was taken for further washing steps. Both suspensions were washed twice in 100 µl DPBS with intermediate centrifugation steps of 1 min at 21,300× *g*. The pellet was resuspended in 100 µl DPBS. Parasite concentrations were determined in a KOVA chamber and used to prepare two replicates of 10^6, 10^5 and 10^4 parasites in 20 µl DPBS from promastigotes isolated from the sand fly midguts and from culture.

DNA/RNA isolation and molecular screening

To determine whether the crude extraction buffer in combination with ethanol precipitation is suitable for efficient nucleic acid isolation and subsequent downstream RNA and DNA detection, nucleic acids from three different concentrations of promastigotes, isolated from either sand fly midguts or culture medium, were extracted using (i) a Nucleospin RNA kit and additional RNA/DNA buffer set (Macherey

Nagel) and (ii) the crude extraction buffer and ethanol precipitation approach. For the latter, the complete volume of the nucleic acid extract was used for ethanol precipitation. The final elution volumes were equalized to ensure the same relative DNA and RNA yields for both methods. All extracts were subjected in duplicate to the qPCRs targeting JW kDNA and SL RNA.

Contribution of RNA *versus* DNA in the SL RNA qPCR assay

Ten *L. major*-infected *L. longipalpis* nucleic acid isolates that were used for the qPCR assay comparison experiment were selected. These samples were subjected in duplicate to the SL RNA qPCR assay with and without the use of a reverse transcriptase enzyme to demonstrate how much of the fluorescent signal originates from RNA *versus* DNA in the crude sand fly extracts. For the assay without reverse transcriptase, the volume of the enzyme was replaced by nuclease-free water. The percentage of DNA detected by the SL RNA qPCR assay was calculated by $\frac{100\%}{2^{(\text{Ct with RT - Ct without RT})}}$ based on the assumption that each PCR cycle doubles the number of amplicons.

Sand fly spiking

Promastigotes (*L. major*) were harvested from a stationary-phase culture (see section 'Parasites') and washed with DPBS. The number of promastigotes was determined in a KOVA counting chamber and the pellet was stored at -20 °C until extraction. Naive laboratory-reared *L. longipalpis* sand flies were spiked with a 10-fold serial dilution of *L. major* promastigotes, ranging from 1.6×10^{7} to 1.6×10^{-6} parasites. The samples were extracted with the crude extraction buffer and ethanol precipitation approach, and subsequently subjected in duplicate to the SL RNA qPCR.

Data analysis

Analyses were carried out using GraphPad Prism version 8 (GraphPad Software, La Jolla California, USA). The correlation between the Ct values of the qPCRs targeting SL RNA and the other three markers was determined by a Pearson's correlation test. This analysis was performed using the infected field-collected sand flies because of the broad range of Ct values. A standard curve with linear regression and PCR efficiency was generated to determine the theoretical LoD and limit of quantification (LoQ) of the SL RNA qPCR.

Results

Evaluation of the SL RNA qPCR for *Leishmania* detection

Of the 96 *L. major*-infected laboratory *L. longipalpis* sand flies, two samples were negative and 82 were positive assays (Fig 7.1a, Additional file 7.1: Table S1). Among the samples that were positive by all qPCRs, the *18S* DNA marker provided the highest mean Ct value (30.3 ± 2.3), followed by MP kDNA (17.3 ± 1.4), JW kDNA (14.6 ± 1.4) and SL RNA (13.8 ± 0.9). Ten samples were not positive for the *18S* DNA marker, but were detected by all other markers. These samples had higher mean Ct values of 21.4 ± 4.4, 17.5 ± 3.1) and 17.1 ± 2.4 for the MP kDNA, JW kDNA and SL RNA markers, respectively. Two sand fly specimens with the highest Ct values for the JW kDNA (27.1 ± 0.2) and SL RNA (25.7 ± 0.7) qPCRs were not positive for the MP kDNA target. Overall, the JW kDNA and SL RNA qPCRs provided concordant results on the laboratory-infected sand flies.

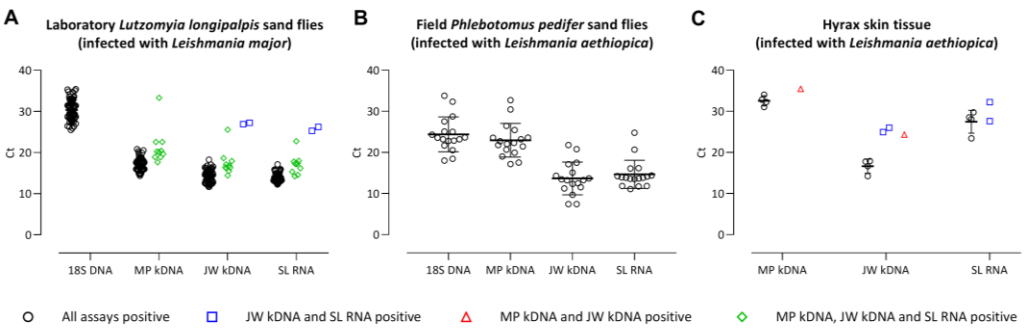

Fig 7.1 qPCR Ct values of laboratory- (a) and field-infected (b) sand flies, and infected hyrax tissue samples (c). Mean Ct values and error bars (standard deviation) are presented for the samples that were positive in all assays that they were tested for. Due to technical issues, the analysis of the *18S* DNA qPCR on hyrax tissue samples was not included.

Among the field-collected, ethanol stored sand fly specimens, 20 were negative and 17 positive for all assays (Fig 7.1b, Additional file 7.1: Table S1). Mean Ct values of the JW kDNA and SL RNA qPCRs were similar (13.7 ± 3.9 and 14.7 ± 3.4, respectively) and consistently lower than the Ct values obtained by the duplex assay (*18S* DNA: 24.2 ± 4.2 and MP kDNA: 22.9 ± 4.2). The difference in Ct values between the MP kDNA and JW kDNA markers was larger for *P. pedifer* infected with *L. aethiopica* than for *L. longipalpis* infected with *L. major* (Fig 7.1a *versus* 7.1b).

Seven out of 22 long-term stored hyrax tissue samples tested positive for two or more markers (Fig 7.1c, Additional file 7.1: Table S1). Four samples were positive in all assays, resulting in the lowest Ct values for JW kDNA (16.6 ± 1.7), compared to SL RNA (27.5 ± 2.8) and MP kDNA (32.6 ± 1.2). Two samples with high Ct values in the JW kDNA and SL RNA qPCRs were negative for the MP kDNA marker, while one sample was positive for the MP kDNA and JW kDNA targets with high Ct values, but not by the SL RNA qPCR.

Overall, Pearson's correlation showed that the Ct values for the SL RNA target correlated quite well with the Ct values of the JW kDNA (Fig 7.2a; $R^2 = 0.82$, $n = 17$), MP kDNA (Fig 7.2b; $R^2 = 0.90$, $n = 17$) and *18S* DNA markers (Fig 7.2c; $R^2 = 0.88$, $n = 17$) based on field-collected sand flies. For all comparisons, the confidence intervals increased towards the higher Ct values, which could be due to slight inhibition of the SL RNA qPCR.

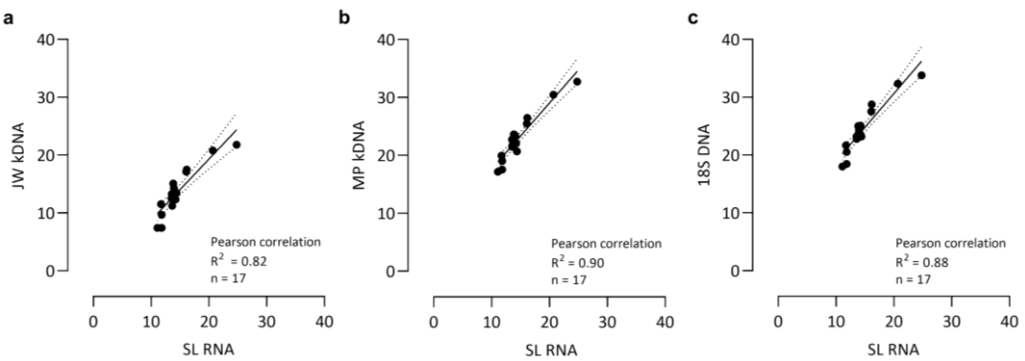

Fig 7.2 Correlation between Ct values obtained by the different qPCR assays. Correlations between Ct values of the SL RNA qPCR and the JW kDNA qPCR (a), MP kDNA qPCR (b) and *18S* DNA qPCR (c). Pearson's correlation analysis of the results obtained with the different assays. Linear regression and 95% confidence intervals (dotted lines) are shown in the graphs.

Extraction method comparison and copy number difference

The crude extraction buffer with ethanol precipitation and column purification (respectively referred to as 'crude' and 'column' in Fig 7.3) methods showed similar extraction efficiencies for kDNA, with comparable Ct values obtained for the standardized concentrations of promastigotes isolated from culture or sand fly midguts. Likewise, both methods performed well for SL RNA extraction, although the RNA yield appeared even slightly higher (on average 1.5 lower Ct values) with the crude method.

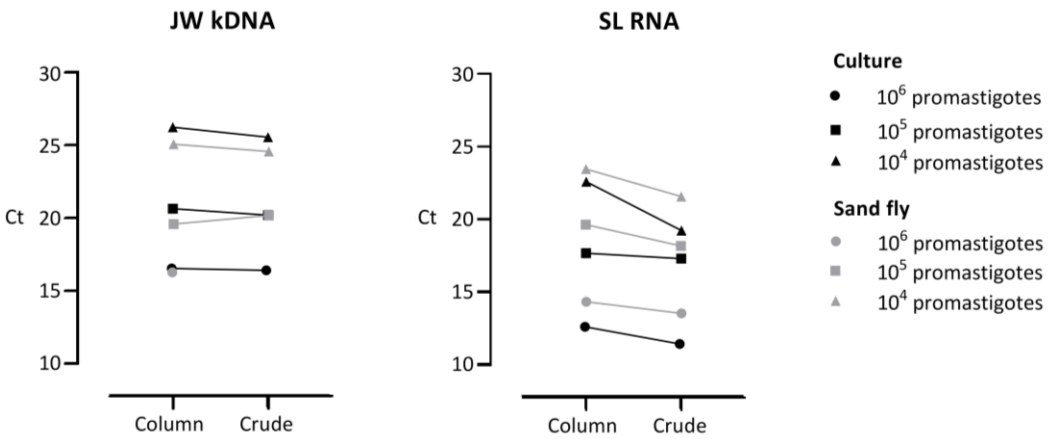

Fig 7.3 Extraction method and copy number comparison. Ct values of promastigotes isolated from culture (black symbols) and sand fly midguts (grey symbols) that were extracted with a commercial column extraction ('column') or crude high-salt extraction buffer ('crude') and subjected to JW kDNA and SL RNA qPCRs. Each symbol presents the assay result for a standardized concentration of promastigotes that was used for the comparisons.

The Ct values for kDNA were similar for promastigotes isolated from culture and sand fly midguts (Fig 7.3, grey *versus* black symbols). For SL RNA, both extraction methods revealed that the Ct values for sand fly derived promastigotes were slightly but consistently higher (Ct on average 1.7) than those for culture-derived promastigotes. The JW kDNA qPCR reaction suffered inhibition in both runs for 10^6 promastigotes isolated from sand fly midguts (Fig 7.3, lacking grey circle for 'crude').

Contribution of RNA *versus* DNA in the SL RNA qPCR assay

The differences in Ct values of the ten *L. major*-infected *L. longipalpis* nucleic acid extracts screened by the SL RNA qPCR with and without reverse transcriptase are presented in Table 7.1. When the assay was performed without reverse transcriptase, the Ct values were on average 5.1 ± 1.1 higher than when the enzyme was used, meaning that only 3.8% (± 2.7%) of the fluorescence produced by the SL RNA qPCR assay was because of DNA amplification, while the remaining signal originated from SL RNA.

Table 7.1 Ct values of the SL RNA qPCR assay with and without reverse transcriptase enzyme.

With reverse transcriptase (RNA + DNA)	Without reverse transcriptase (DNA)	Cq difference	% DNA
15.0	19.7	4.8	3.7
27.4	30.8	3.4	9.8
23.6	27.7	4.2	5.6
14.8	21.5	6.7	1.0
14.0	19.2	5.2	2.7
15.6	20.9	5.3	2.6
16.3	20.7	4.3	5.0
13.7	20.2	6.5	1.1
16.1	20.4	4.3	5.0
14.4	20.7	6.3	1.3
Mean ± SD		5.1 ± 1.1	3.8 ± 2.7

LoD and LoQ of the SL RNA qPCR assay

Based on the serial dilution of *L. longipalpis* sand flies spiked with *L. major* promastigotes, the theoretical LoD of the SL RNA qPCR was 10^{-3} parasite equivalents (Fig 7.4a). For 1.6×10^{7} promastigotes, the assay did not provide a result in any of the two independent runs, implying that there was PCR inhibition at this concentration. The assay showed a very good PCR efficiency of 105% for the serial dilution down to 10 parasites, representing the theoretical LoQ. The Pearson's correlation demonstrated an excellent inter-run stability for the two independent runs of the SL RNA qPCR on the serial dilution (Fig 7.4b; $R^2 = 0.99$, $n = 10$).

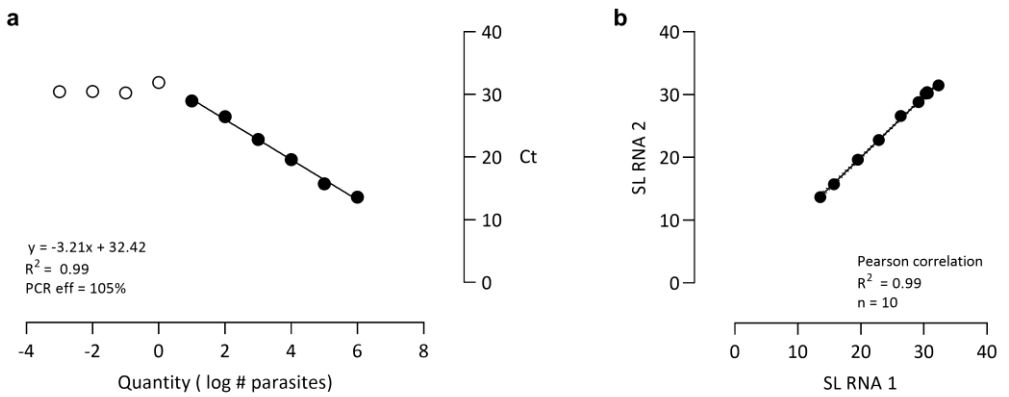

Fig 7.4 Performance of the SL RNA qPCR on a serial dilution of sand flies spiked with promastigotes. A) Standard curve with linear regression and qPCR efficiency. The open symbols depict all concentrations that were detected by the assay, while the filled symbols are the parasite concentrations that show a linear correlation. B) Inter-run variability of the SL RNA qPCR in two replicates, analyzed by Pearson's correlation and linear regression analysis. Dotted lines indicate the 95% confidence intervals.

Discussion

For eco-epidemiological surveys, a large number of sand fly and potential reservoir samples need to be screened in order to find some *Leishmania*-positive specimens, because the infection prevalence is overall quite low, even in endemic areas [163,251]. Therefore, researchers currently opt for sensitive, low-cost, high-throughput molecular screening methods for *Leishmania* detection in vectors and potential hosts. These molecular methods are often expensive TaqMan probe assays that target DNA sequences, which may persist for quite some time after parasite death [313].

In our study, we evaluated whether the recently developed SL RNA qPCR assay by Eberhardt et al. [170] enables *Leishmania* detection in sand flies and skin tissue from CL-infected animals. The targeted 39-bp SL RNA sequence is conserved amongst *Leishmania* species and fulfils an essential function in RNA trans-splicing and polyadenylation processes [314]. To our knowledge, this study is the first to evaluate the use of an RNA target for *Leishmania* detection in vectors and field-sampled tissue of reservoir hosts and to combine it with a low-cost extraction method. Since RNA quickly decays after death of the infectious agent, it is considered as a promising detection marker for viable *Leishmania* parasites [295,315,316], although the half-life of SL RNA as a small nuclear RNA molecule remains to be determined.

We assessed the performance of the SL RNA qPCR assay in comparison with two other molecular diagnostic assays on field-collected and laboratory-infected sand flies that were extracted with the crude method and hyrax tissue samples. The JW kDNA [144,163] and SL RNA qPCRs [170] showed concordant results using the laboratory *L. major*-infected *L. longipalpis* and field-collected *L. aethiopica*-infected *P. pedifer* sand flies. Both assays identified the same positive and negative samples, indicating that they have a similar analytical sensitivity and specificity. It was surprising that the SL RNA qPCR performed very well on field-collected sand flies, considering that these samples had not been preserved under favorable conditions for RNA, which may relate to the short amplicon length [317,318]. These observations indicate that SL RNA could be an interesting target for *Leishmania* detection in vectors collected during entomological surveys.

On the contrary, the MP kDNA and *18S* DNA targets could not identify all positive laboratory-infected sand flies. One reason is the low copy number of the *18S* rDNA fragment (50–200 copies per *Leishmania* genome) [319] compared to the much higher copy number of SL RNA (a single *Trypanosoma* cell contains about 8600 copies [320]) and kDNA (a *Leishmania* parasite contains approximately 10,000 copies [321]). Whereas the Ct values for kDNA were fairly similar in promastigotes isolated from sand flies and from culture, Ct values for SL RNA were more distinct, suggesting that this marker is potentially slightly less abundant in parasites isolated from sand flies. This may indicate a reduced transcriptional activity of the vector-derived parasite pool (containing various life-cycle stages) as compared to culture-derived parasites.

The duplex assay targeting the MP kDNA marker could not identify some of the laboratory-infected sand flies that were positive by the JW kDNA and SL RNA qPCRs and Ct values for the MP kDNA target were generally slightly higher than for the JW kDNA assay. This is most probably because multiplex qPCRs are commonly slightly less sensitive than uniplex assays and due to the intrinsic difference in fluorescent signal development between SYBR Green and TaqMan probe assays [322,323]. Additionally, the qPCR targeting the MP kDNA marker resulted in higher Ct values on the field-collected sand flies (*L. aethiopica*-infected) compared to laboratory-infected sand flies (*L. tropica*), which most probably relates to mismatches of the reverse primer with the *L. aethiopica* kDNA fragment (Fig 7.5) [250]. Earlier observations of a lower sensitivity for *L. tropica* (genetically very similar to *L. aethiopica*) and *L. mexicana* [170] corroborates the limitations of this MP kDNA target that was originally described by Mary et al. [250] for detection of *L. donovani*. The SL RNA qPCR provided equal Ct values

for various *Leishmania* species, demonstrating its suitability as a pan-*Leishmania* assay [170].

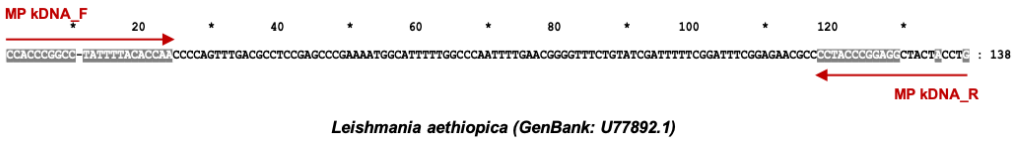

Fig 7.5 Annealing of MP kDNA qPCR primers to the *L. aethiopica* kDNA fragment (GenBank: U77892.1).

Although only a few positive hyrax tissue samples were tested, the JW kDNA qPCR could identify most of the true-positive samples under the used sample storage conditions. A sample was considered positive if identified by two different assays. The SL RNA assay identified one false-negative sample and showed generally higher Ct values compared to the JW kDNA PCR than for sand fly screening, which is probably due to the fact that the samples had been stored in ethanol for two years before DNA/RNA extraction was performed. Most likely, proper RNA storage conditions and/or immediate RNA isolation would result in a substantially improved performance of the SL RNA qPCR on tissue samples [318,320]. Favoring this viewpoint, the SL RNA qPCR showed excellent analytical sensitivity in laboratory-infected (*L. infantum*) mouse spleen and liver samples, detecting down to 10^{-3} parasite equivalents per mg tissue [170].

Considering the large sample size that needs to be screened in search for positive field specimens, a low-cost, efficient nucleic acid extraction method is preferred [143]. We found that a crude extraction buffer in combination with an ethanol precipitation step is as efficient as a commercial column extraction for downstream DNA and RNA detection in sand flies. Ct values tended to be even slightly lower when the extraction was carried out with the crude method, suggesting that there is some nucleic acid loss on the silica columns, or in addition, that some DNA is detected. Other important advantages of this crude extraction method are the low-cost and reduction in sample processing time as maceration is not required [143,305]. The latter is compensated by a more time-consuming ethanol precipitation step. However, because of the low prevalence in field-collected sand flies, individual extracts can be pooled to reduce the number of samples for purification and PCR [163]. This method extracts all nucleic acids, including RNA and DNA. Nevertheless, we found that only a fraction of the generated signal of the SL RNA qPCR originates from DNA amplification, which is quite low considering the storage conditions and crude extraction method used. The actual

contribution of RNA *versus* DNA in positive samples can be easily assessed by comparison with a no-reverse transcription control.

Determination of the parasite load in sand flies can be highly informative, especially for studies that investigate, e.g. the vectorial capacity. Previously, the LoD of the SL RNA qPCR on cultured promastigotes has been established at 0.0002 parasite equivalents [170]. We assessed the theoretical LoD of the SL RNA qPCR based on sand flies spiked with a serial dilution of *L. major* promastigotes. The determined theoretical LoD of 10^{-3} parasite equivalents per reaction of our assay is similar to findings of Bezerra-Vasconcelos et al. [308], who could detect 10^{-3} parasites per reaction with a kDNA qPCR assay on *L. infantum*-spiked *L. longipalpis* sand flies. This substantiates that the sensitivity of qPCR assays targeting SL RNA and kDNA are comparable, which corroborates the comparative assessment performed in the present study. Moreover, congruence of the assays appears very good, indicating that both can achieve reliable quantification. Based on the standard curve, it can be concluded that SL RNA qPCR can quantify down to 10 parasites per sand fly with high PCR efficiency, which is sufficient for determination of biologically relevant parasite loads.

Overall, to the best of our knowledge, this study shows for the first time that the SL RNA target can be used for detection and quantification of *Leishmania* parasites in field-collected and laboratory-infected sand flies, even in combination with a crude, low-cost extraction method. The SL RNA qPCR assay is inexpensive, sensitive and pan-*Leishmania* specific, which can be a major advantage for eco-epidemiological studies including identification of vectors and reservoirs.

Acknowledgement

We are grateful to Dr Gert Van der Auwera (Institute of Tropical Medicine, Antwerp, Belgium) for his advice on the interpretation of the experiments. We would also like to thank the village head and field workers in Ochollo for the possibility to collect the sand fly and hyrax samples.

Funding

MP is a fellow supported by the Flemish Interuniversity Council (VLIR-UOS, NDOC2016PR003, https://www.vliruos.be/). The work was funded by a VLIR Global Minds Small Research Grants project, the Research Fund Flanders (FWO scholarships

1136417N and 12I0317N, https://www.fwo.be) and a research fund of the University of Antwerp (TT-ZAPBOF 33049).

Additional files

Additional file 7.1: Table S1 qPCR results of sand fly and hyrax samples from the laboratory and field in four different assays. For each assay the mean quantification cycle threshold value (± standard deviation) are presented.

# positive tests	# samples	MP 18S qPCR	MP kDNA qPCR	JW kDNA qPCR	SL-RNA qPCR
A) Laboratory *Lutzomyia longipalpis* sand flies (infected with *Leishmania major*)					
0	2				
1	0				
2	2			27.1 (± 0.2)	25.7 (± 0.7)
3	10		21. 4 (± 4.4)	17.5 (± 3.1)	17.1 (± 2.4)
4	82	30.3 (± 2.3)	17.3 (± 1.4)	14.6 (± 1.4)	13.8 (± 0.9)
B) Field *Phlebotomus pedifer* sand flies (infected with *L. aethiopica*)					
0	20				
1	0				
2	0				
3	0				
4	17	24.4 (± 4.2)	22.9 (± 4.1)	13.7 (± 3.9)	14.7 (± 3.4)
C) Hyrax skin tissue (infected with *L. aethiopica*)					
0	15	-			
1	0	-			
2	1	-	35.5	24.4	
	2	-		25.5 (± 0.8)	29.9 (± 3.3)
3	4	-	32.6 (± 1.2)	16.6 (± 1.7)	27.5 (± 2.8)

+: positive; -: not tested with this assay; MP 18S qPCR: Multiplex TaqMan probe real-time PCR targeting 18S DNA; MP kDNA qPCR: Multiplex TaqMan probe real-time PCR targeting kDNA; JW kDNA qPCR: SYBR Green real-time PCR assay targeting kDNA; SL-RNA qPCR: SYBR Green real-time PCR targeting SL-RNA.

PART IV
METHODS IN SAND FLY RESEARCH

CHAPTER 8

An integrative approach to identify sand fly vectors of leishmaniases in Ethiopia by morphological and molecular techniques

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Accepted at Parasites & Vectors

Abstract

Ethiopia is affected by human leishmaniases caused by several *Leishmania* species and transmitted by a variety of sand fly vectors of the genus *Phlebotomus*. The sand fly fauna in Ethiopia is highly diverse and some species are closely related and similar in morphology, resulting in difficulties with species identification that requires deployment of molecular techniques. DNA barcoding entails with high costs, required time and lack of reference sequences for many Ethiopian species. Yet, proper species identification is pivotal for epidemiological surveillance as species differ in their actual involvement in transmission cycles. Recently, protein profiling using MALDI-TOF mass spectrometry has been introduced as a promising technique for sand fly identification.

In our study, we used an integrative taxonomical approach to identify most of the important sand fly vectors of leishmaniases in Ethiopia, applying three complementary methods: morphological assessment, sequencing analysis of two genetic markers and MALDI-TOF MS protein profiling.

Although morphological species identification resulted in some inconclusive determinations, both DNA- and protein-based techniques performed well, providing a similar hierarchical clustering pattern for the analyzed species. Both methods generated species-specific sequences or protein patterns for all species except for *Phlebotomus pedifer* and *P. longipes*, the two presumed vectors of *Leishmania aethiopica*, suggesting that they may present a single species, *P. longipes* Parrot & Martin. All three approaches also revealed that the collected *Adlerius* sp. specimens differ from *P. (Adlerius) arabicus*, the only *Adlerius* species currently reported in Ethiopia, and molecular comparisons indicate that it may represent a yet undescribed new species.

Our study uses three complementary taxonomical methods for identification of taxonomically challenging and y et medically important Ethiopian sand flies. The generated MALDI-TOF MS protein profiles resulted in unambiguous species identifications, hence we suggest that this technique is suitable for sand fly species identification. Furthermore, our results contribute to the currently inadequate knowledge of the sand fly fauna of Ethiopia, a country that is severely burdened with human leishmaniases.

Background

Phlebotomine sand flies (Diptera, Phlebotominae) are hematophagous insects of great medical importance as the females of some species are the vectors of *Leishmania* protozoa, which are transmitted during blood feeding on a vertebrate host. Human leishmaniasis can manifest in three major clinical forms: visceral (VL), cutaneous (CL) and mucocutaneous (MCL) leishmaniasis [2,54].
All three forms affect people in Ethiopia, particularly the poorest part of the population living in rural areas [28,106]. VL is generally endemic in the lowlands widespread in the country and is caused by *Leishmania donovani*. Proven vectors of VL in different regions in Ethiopia are *Phlebotomus orientalis, P. martini* and to a lesser extent *P. celiae* [60,187,194,239,324]. Localized (LCL) and diffuse (DCL) forms of CL and MCL occur mainly at mid-highland altitudes, on the mountain ridges of the Ethiopian Rift Valley. The main causative parasite species is *L. aethiopica*, which is transmitted by *P. longipes* in northern and central Ethiopia and *P. pedifer* in southwestern Ethiopia [17,47,99,100,104,136,163].

It is pivotal for entomological and eco-epidemiological research to accurately identify the sand fly species that act as vectors in a particular area and assess their ecology and behavior, as this information is a prerequisite for implementation of efficient, targeted control programs [199].
Morphological identification is done by mounting the head and abdomen of the sand flies, which include the main distinctive characteristics for classification (cibarial and pharyngeal armature, genitalia). However, this taxonomic approach is labor intensive, demands high proficiency and morphological keys are often obsolete and incomprehensive, leading to incorrect identifications [58,72,325].
To overcome these drawbacks, studies are frequently shifting to molecular techniques, which allow simultaneous processing of samples, provide reliable identifications and have good reference sequences available for many species. Yet, this method is technically demanding, considered costly for large-scale studies and its validity depends on the genetic variability of the target locus [326,327].

Recently, an alternative method, protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has been introduced for species identification in different arthropod families, including sand flies [58,328–333]. This approach provides unique protein profiles that allow unambiguous species identification and sample processing is rapid, simple and cost-effective.

However, it still needs validation on field specimens and requires a centralized database to approve its use for routine species identification of sand flies [58,334]. As the method utilizes only certain body parts, in sand flies particularly the thorax in standardized protocols [58], a coordinated sample preparation allows simultaneous or later application of other techniques, including morphological analysis of the mounted head and genitalia and DNA-based techniques using template DNA isolated from the sand fly abdomen.

The sand fly fauna in Ethiopia is very diverse, comprising remarkable numbers of species in both genera *Sergentomyia* and *Phlebotomus*, within which species of at least six subgenera were recorded, many of them proven or suspected vectors of several *Leishmania* species and thus of great medical significance. Among these, some closely related sand fly species are remarkably similar in morphological characteristics, resulting in many difficulties for conclusive species identification [125,156,335,336]. Especially females of some species are challenging to distinguish from each other as species-specific morphological features are poorly described or undefined. These species, however, often do not play the same role in *Leishmania* transmission which is hindering sand fly ecology studies and implementation of control measures accordingly, indicating that more sophisticated techniques are necessary for species differentiation [30,31]. DNA barcoding could be an appropriate alternative, although very few reference sequences of the Ethiopian sand fly species are currently available in genetic databases and analyses are rather too costly to process large sample sizes.

Our study presents for the first time an integrative taxonomical approach to identify most of the important sand fly vectors of leishmaniases in Ethiopia, applying three complementary methods: morphological assessment, sequencing analysis of two genetic markers and MALDI-TOF MS protein profiling. We aimed to demonstrate that MALDI-TPF MS protein profiling could be a suitable taxonomical technique, providing unambiguous sand fly species identification in Ethiopia. Furthermore, our results contribute to the currently still inadequate knowledge of the sand fly fauna in Ethiopia, a region among those most affected by the burden of human leishmaniases.

Methods

Sand flies

Field specimens were captured in May and September 2019 from different CL (Hagere Selam in the north, Saris in the center and Ochollo in the southwest) and VL (Aba Roba, Dimeka and Turmi in the south) foci in Ethiopia (Fig 8.1). Sand flies were captured with CDC miniature light traps (John W. Hock Company, Florida, USA), which were set at 6 p.m. and collected at 7 a.m. the next morning. In order to capture CL vectors in the mid-highlands, traps were placed in caves or around rocky areas, whereas to capture VL vectors in the lowlands, traps were set nearby termite hills and human dwellings [48,163,337]. Collected sand flies were preserved in 70% ethanol stored at -20°C [338].

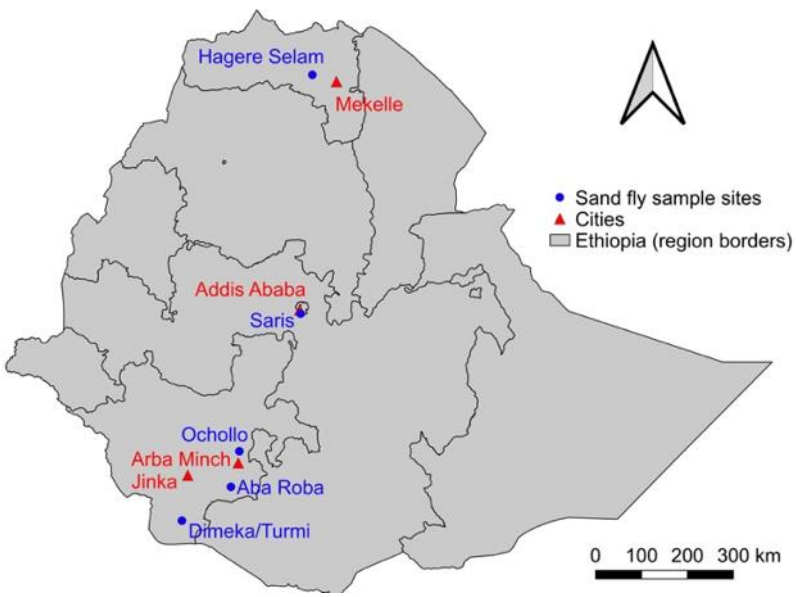


Fig 8.1 Sampling locations of field collected sand flies [166,167]**.** Blue dots represent the places where field collected sand flies came from: CL foci Hagere Selam (Tigray Region), Saris (Addis Ababa city administration) and Ochollo (Southern Nations, Nationalities and Peoples' Region, SNNPR) and VL foci Aba Roba, Dimeka and Turmi (SNNPR). The cities nearby the sample sites are displayed by red triangles.

Morphological identification of sand fly species

The head and terminal segments of the abdomen of the sand fly specimens were mounted using CMCP-10 high viscosity mounting medium (Polysciences, Hirschberg, Germany) and species identification was done according to relevant morphological

keys [124,125,156,197,335,339–342]. Slide-mounted specimens were observed using a light microscope Olympus BX51 with a camera system Olympus D70. Morphological characters were measured using the QuickPHOTO MICRO 3.0 software.

Molecular identification of sand fly species

After mounting, the remaining part of the abdomen was used for molecular identification of the sand fly species. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). Samples were subsequently subjected to two PCRs targeting *(i)* a fragment of the cytochrome oxidase subunit I (COI) with LCO 1490 (5'-GGT CAA ATC ATA AAG ATA TTG G-3') and HCO 2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') primers in a total reaction volume of 25 µl as described by Folmer *et al.* (2012) [343]; and *(ii)* the ND4 gene with ND4ar (5'-AAR GCT CAT GTT GAA GC-3') and ND4c (5'ATT TAA AGG YAA TCA ATG TAA-3') primers in a reaction volume of 50 µl based on Soto *et al.* (2001) [344]. Amplicons were visualized on a 1% agarose gel and obtained products were purified with the QIAquick PCR Purification Kit (Qiagen). These were sent to Vlaams Instituut voor Biotechnologie (VIB, University of Antwerp, Belgium) or BIOCEV OMICS genetika (Charles University, Czech Republic) for Sanger sequencing in two directions with the primers used for DNA amplification.

Phylogenetic analyses

The obtained chromatograms were edited and consensus sequences were made for each specimen, which were compared with reference sequences in GenBank using BLAST. Multiple sequence alignment of the COI and ND4 sequences was done by the Clustal W tool implemented in the MEGA X 10.1 software and primers were trimmed to have sequences with an equal length of 658 bp and 597 bp respectively [345,346]. The nucleotide compositions and sequence divergences were calculated with the Kimura two-parameter model (K2P) and a distance matrix was generated [347,348]. A Neighbor-joining (NJ) tree of the K2P distances was created using a bootstrapping method with 1,000 replicates for a graphic presentation of the clustering pattern of the sand fly species [346,349]. To make a NJ tree of our specimens of the *Adlerius* subgenus in combination with other species of the same subgenus, COI sequences were derived from GenBank. Based on the NJ trees, groups of species were indicated to assess the inter- and intra-species distances. All sequences are made available in GenBank (http://www.ncbi.nlm.nih.gov/genbank).

MALDI-TOF MS analysis of sand fly species

The MALDI matrix was prepared fresh as an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid (30 mg/ml; Bruker Daltonics). Specimens stored in 70% ethanol were air-dried, dissected and thoraces were manually ground in 10 µl of 25% formic acid by a BioVortexer homogenizer (BioSpec, Bartlesville, USA) with sterile disposable pestles. After a short centrifugation of the homogenate (10 000 *g* for 15 s), 2 µl was mixed with 2 µl of MALDI matrix and 1 µl was deposited and air-dried on a steel target plate (Bruker Daltonics, Bremen, Germany) in duplicate. Protein mass spectra were measured in a mass range of 4-25 kDa by an Autoflex Speed MALDI-TOF spectrometer (Bruker Daltonics) and calibrated externally with the Bruker Protein Calibration Standard I. Each spectrum was acquired as a sum of 4000 manually adjusted laser shots (20×200 shots from different positions of the target spot) and visualized by FlexAnalysis 3.4 software.

For cluster analysis, the protein profiles were processed (normalization, smoothing, baseline subtraction, peak picking) using MALDI Biotyper 3.1. The peak picking parameters for generation of a main spectrum (MSP) were to include maximum of 100 peaks, which had a signal-to-noise ratio greater than 3 and a relative intensity of minimum 1% of the most intense peak. The desired peak frequency for MSP reference spectra was set to 60%. For MSP dendrogram creation, a correlation distance measure and average linkage parameters were applied. The dendrograms were generated using the individual MSPs or MSP references created for each sand fly species. In addition to six field-caught Ethiopian species, MSP references from our sand fly database were included (country origin is given between brackets): *P. ariasi* (France), *P. kandelakii* (Georgia), *P. longicuspis* (Morocco), *P. neglectus* (Croatia), *P. orientalis* (Ethiopia), *P. perfiliewi* (Macedonia), *P. perniciosus* (Spain), *P. tobbi* (Northern Macedonia), *P. arabicus* (Israel), *P. balcanicus* (Georgia), *P. creticus* (Crete), *P. halepensis* (Georgia), *P. simici* (Macedonia). *P. arabicus* and *P. orientalis* originated from colonies maintained in Prague, the others were field-collected.

Results

Morphological species identification

Morphological analysis identified six sand fly species of four subgenera. From VL endemic sites in the south (Dimeka, Turmi and Aba Roba), *P. (Phlebotomus) duboscqi*,

P. (Synphlebotomus) celiae and *P. (Sy.) martini* were identified. *Phlebotomus (Larroussius) pedifer* was obtained from Ochollo and *P. (La.) longipes* from Saris and Hagere Selam. A main feature to differentiate the two species of the *Larroussius* subgenus is based on the bending of the tip of the males' aedeagus [124,156]. However, our specimens showed a varying range of tip endings within a single species (Fig 8.2) that did not allow clear differentiation between the species-specific morphological arrangements of the two species. Furthermore, we counted overlapping numbers of inner surface coxite hairs, being 39 (31-48, n = 43) for *P. pedifer* from Ochollo, 31 (29-36, n = 13) for *P. longipes* from Hagere Selam and 44 (40-48, n = 2) for *P. longipes* from Saris. Therefore, species determination of the two *Larroussius* species was mainly based on previous geographical presence records [47,48,163].

Females of the species with the subgenus *Synphlebotomus* as well as subgenus *Larroussius* could often not be separated to species level based on morphological features [124,125,350,351].

Fig 8.2 Aedeagi of male *Phlebotomus longipes* from Hagere Selam and Saris (left two columns 1 and 2) and *P. pedifer* from Ochollo (right column 3).

Table 8.1 Morphometric analysis of the *Adlerius* sp. from Hagere Selam, in comparison with other species of the *Adlerius* subgenus. Measurements were collected from: [1] four males from Ethiopia; [2] one from Ethiopia and four from Yemen [339]; [3] for coxite hairs, 20 specimens and for other measurements seven males from Israel; All measurements are in µm. ! We speculate that the authors attributed the numbers of the coxite hairs incorrectly.

Species (country, reference)	Ascoid	Style	Coxite	N° coxite hairs	Position coxite hairs	Aedeagus length	Aedeagus tip to tooth
Adlerius sp. [1] (Ethiopia, Pareyn)	2/3-7, 1/8-15	173 (158-188)	366 (355-387)	45 (36-52)	0.56 (0.51-0.57)	186 (181-195)	13 (11-15)
P. davidi (Yemen & Ethiopia, Artemiev 1980)	2/3-7, 1/8-15	157 (156-160)	315 (300-328)	46 (38-59)	0.59 (0.55-0.64)	162 (152-172)	12 (10-14)
P. arabicus (Israel, Petr Volf, unpublished)	2/3-7	189 (176-206)	386 (370-428)	64 (55-76)	-	183 (176-206)	20 (17-25)
P. arabicus TYPE[2] (Saudi Arabia, Lewis & Büttiker 1982)	2/7 (8)	190	360	57	0.59	190	-
P. arabicus HESUA[3] (Saudi Arabia, Lewis & Büttiker 1982)	2/7 (8)	-	-	54 (42-69)	(0.50-0.59)	-	(12-22)
P. Naqben species (Saudi Arabia, Lewis & Büttiker 1982)	2/7	-	-	54-98	0.58	-	(15-22)
P. naqbenius (Saudi Arabia, Lewis & Büttiker, 1986) !				53 (41-69)			
P. naqbenius holotype (Petr Volf, unpublished)	2/7	185	357	60	-	172	16
P. naqbenius syntype (Petr Volf, unpublished)	2/7	196	381	80	0.6	185	21

Seven males and one female of an additional species were captured from a cave nearby Hagere Selam (N 13°40'14", E 39°07'29"). and identified as a species of the *Adlerius* subgenus (further referred to as '*Adlerius* sp.'). Morphometric analysis of four male *Adlerius sp.* specimens (Table 8.1) indicates that our sand flies show high similarity in number and position of coxite hairs and the aedeagus tip to tooth distance as described for *P. davidi* [339]. The style, coxite and aedeagus length of our specimens, however, are longer than reported for *P. davidi*, but shorter than measured and described for *P. arabicus* and fit more to *P. naqbenius*. Accordingly, no conclusive species identification could be done for *Adlerius* sp..

Sand fly species identification by molecular techniques

Most specimens tested gave reproducible MALDI-TOF MS spectra with a high number of intense signals (Fig 8.3a). Except for the protein profiles of *P. pedifer* and *P. longipes*, each species generated a heterogeneous spectrum with species-specific peaks allowing unambiguous species identification. The dendrogram of the specimen's protein profiles (Fig 8.3b) indicates that all subgenera cluster on distinct branches. The closely related *P. martini* and *P. celiae* were clearly distinguished from each other, whereas the specimens of the *Larroussius* subgenus from all three sites grouped in a single cluster. The *Adlerius* sp. formed its own separate branch.

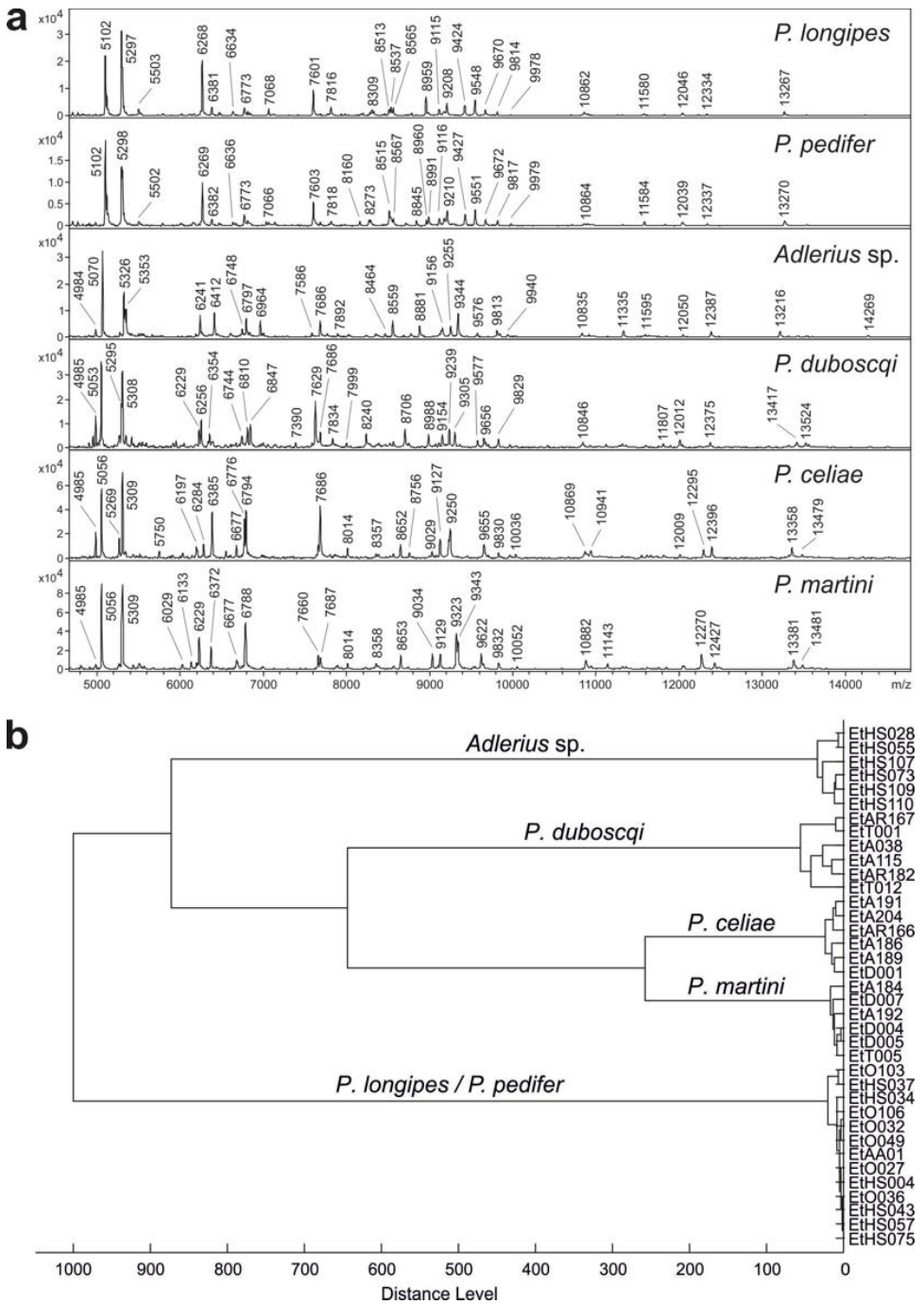


Fig 8.3 MALDI-TOF mass spectra (a) and according dendrogram generated by cluster analysis of the protein profiles (b) of sand fly species from Ethiopia. Zoomed mass range of 4 to 15 kDa is shown in panel a and distances in panel b are displayed in relative units.

Taxonomic clustering of the COI and ND4 sequences in the NJ tree (Fig 8.4a and b, respectively) substantiates the hierarchical clustering of the protein profiles (Fig 8.3b), separating all species except for the two *Larroussius* species. The mean intra-group K2P distances within the *P. longipes* and *P. pedifer* cluster was only 0.3% (SD 0.1) for COI and 0.1% (SD 0.1) for ND4 genes (Additional file 8.1: Table S1), indicating that there is no genetic difference between the two presumable species. The K2P distance between *P. martini* and *P. celiae* was only 1.6% (SD 0.4) for COI and 1.2 (SD 0.3) for ND4 genes, yet the species clearly clustered in distinct clades (Additional file 8.2: Table S2).

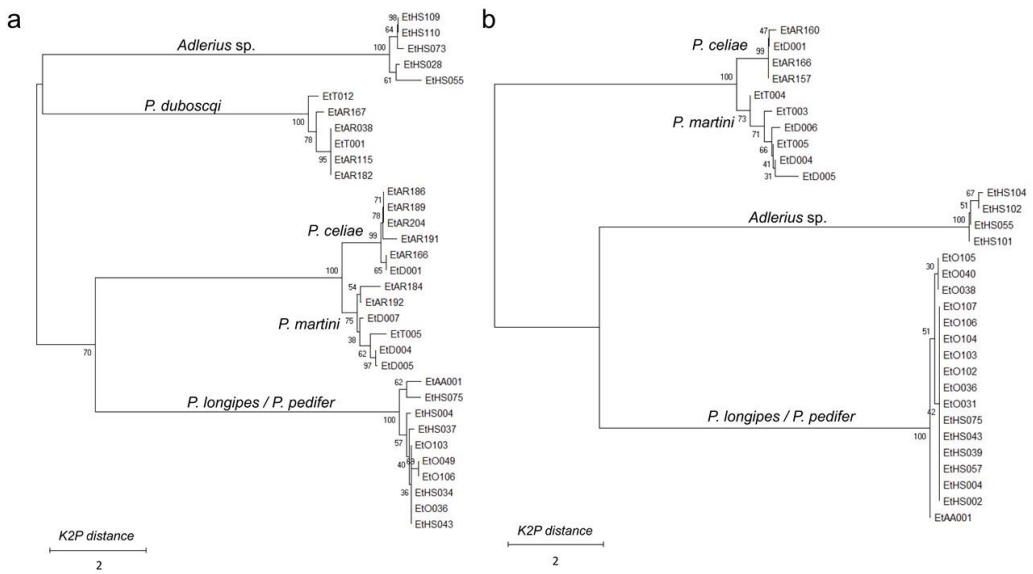


Fig 8.4 Neighbor joining tree based on K2P distances of COI (a) and ND4 (b) sequences of Ethiopian sand flies. *Abbreviations:* K2P, Kimura two-parameter model.

The dendrogram including the MSP reference spectra of *P. pedifer*, *P. longipes*, *Adlerius* sp. and other available species of the *Larroussius* and *Adlerius* subgenera from our library (Fig 8.5) demonstrates that only the two Ethiopian CL vectors cannot be distinguished from each other, while the protein profiling could clearly differentiate all other analyzed species, including closely related *P. tobbi* and *P. perfiliewi* that provide highly similar, yet species-specific protein spectra. The dendrogram depicts that the *Adlerius sp*. is closely related with *P. arabicus* from Israel but some distinct peaks were found in their protein profiles (Additional file 8.3: Fig S1). Moreover, there is a large relative distance between the MSP reference spectra of the two species (Fig 8.5), demonstrating that *Adlerius* sp. is definitely not *P. arabicus*.

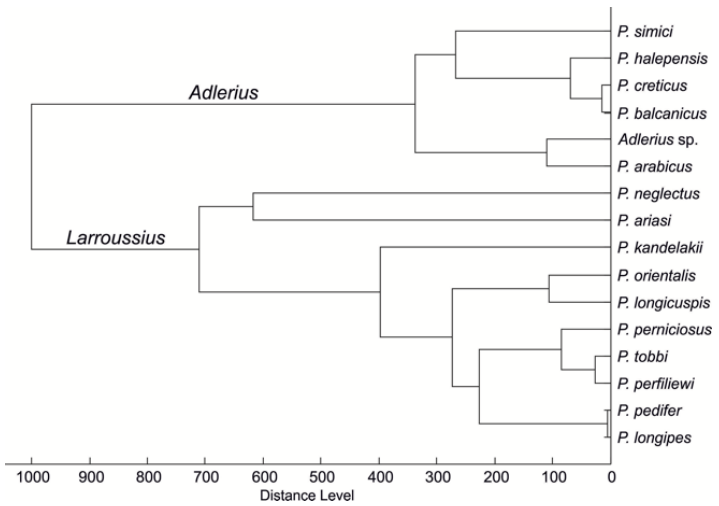


Fig 8.5 Dendrogram of MSP reference spectra of *P. pedifer, P. longipes* and *Adlerius* sp. with MSP references of other species of the *Larroussius* and *Adlerius* subgenera from our library. The origin of the species in given in the methods section. Specifically, *P. arabicus* was derived from the sand fly facility at Charles University, Czech Republic, which originated from northern Israel. Distances are displayed in relative units. *Abbreviations:* MSP, main spectrum.

Furthermore, the COI sequence profile of the *Adlerius* sp. was compared with available GenBank sequences of other species of the *Adlerius* subgenus (Fig 8.6), which supports the MSP dendrogram (Fig 8.5), indicating that the species is closely related to *P. arabicus* but constitutes a distinct branch with a K2P distance of 6.5% (SD 1.2, Additional file 8.4: Table S3).

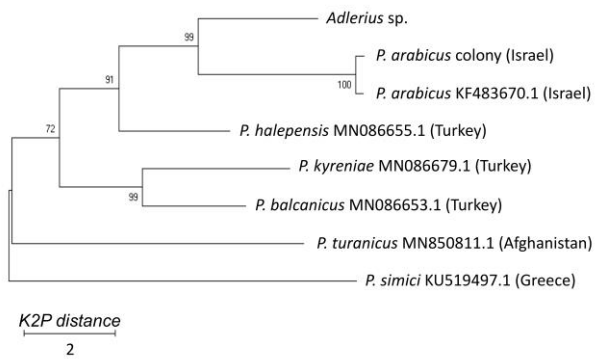


Fig 8.6 Neighbor-joining tree based on K2P distances of the COI gene of *Adlerius* sp. from Hagere Selam, Ethiopia and other members of the *Adlerius* subgenus. Accession codes of the species retrieved from GenBank are displayed after the species. K2P, Kimura two-parameter. The *P. arabicus* colony was obtained from Israel.

Discussion

Since East Africa accounts worldwide among the regions most affected by both cutaneous and visceral leishmaniasis, phlebotomine sand flies occurring in the Horn of Africa have been extensively studied due to their exclusive role as vectors in the transmission cycles. Unfortunately, whereas considerable amount of knowledge regarding the diverse sand fly fauna of Ethiopia was gradually gathered, the last comprehensive morphological key for species identification has not been updated for more than five decades [342], leaving the attempt to identify the specimens collected in field surveys very tedious and challenging. This further urges the need to deploy alternative molecular techniques that have recently emerged and were successfully adopted for species identification of various medically significant arthropods. In this study, we applied an integrative taxonomical approach to identify most CL and VL vectors in Ethiopia using a combination of morphological assessment, sequencing analysis of two genetic markers and MALDI-TOF MS protein profiling, and demonstrate some novelties in the complex sand fly fauna of Ethiopia.

The three vectors of *L. donovani* in Ethiopia are *P. orientalis*, *P. martini* and *P. celiae*. The former is a species of the subgenus *Larroussius*, whereas the latter two belong to the *Synphlebotomus* subgenus and occur sympatrically in Aba Roba focus in southwestern Ethiopia [335,337]. While to our knowledge this is the only place where *P. celiae* has been identified in Ethiopia, *P. martini* is more widespread, serving as a vector also in the northwestern and southeastern parts of the country, where it sometimes cohabitates with *P. orientalis* [65,239,352].

Males of *P. martini* and *P. celiae* can be easily distinguished based on the morphology of the lateral lobes on their external genitalia, but females of these two species were previously regarded as indistinguishable until Gebre-Michael and Lane differentiated them based on their labrum length and labrum-to-wing length ratio [335]. Moreover, their spermathecal ducts vary in length, but this characteristic cannot be used for routine identifications, as it requires dissecting out the fragile ducts. However, still a few field-caught specimens in their study could not surely be identified by these characters and the measurement ranges do not match with findings from Davidson *et al.* in Egypt [353] and the original description of *P. martini* by Parrot *et al.* in Dire Dawa (about 600 km from Aba Roba in eastern Ethiopia) [354]. This could be due to the geographical distance or environmental conditions but as a result these measurements

can presumably not be extrapolated to a larger area without leading to misidentifications.

The two species have a different distribution, infection prevalence and abundance, and thus their contribution to disease transmission varies [337]. Therefore, proper species determination is pivotal and requires novel determination techniques. Both genetic and MALDI-TOF MS analyses in this study pointed out that DNA marker sequences and protein profiles were similar and enable rapid and conclusive species identification, indicating that this is a suitable approach for species identification.

Sand flies were also captured from different CL endemic sites in Ethiopia, where we found two species of the *Larroussius* subgenus, identified morphologically as *P. pedifer* and *P. longipes*. The latter species was first described by Parrot *et al.* in 1939 in Ethiopia [123], but the species complex was re-examined by Lewis, Mutinga and Ashford in 1972 [124], who compared presumed *P. longipes* specimens from Kenya and South Sudan with *P. longipes* from different places in northern, western and central Ethiopia. The species from Kenya appeared smaller than the ones from Ethiopia, although this was probably a geographical difference. Moreover, the authors describe that male specimens from Kenya had an up-turned foot-like aedeagus, whereas it was only slightly up-turned in the Ethiopian species. Therefore, the Kenyan specimens were described as a new related species, *P. pedifer*, which was later also found as the CL vector in southwestern Ethiopia (Ochollo) [124]. Killick-Kendrick *et al.* later confirmed that *P. pedifer* and *P. longipes* males can be distinguished based on this slight difference in the aedeagus shape and additionally also the number of inner surface coxite hairs, being 50 to 60 for *P. pedifer* and 35 to 50 for *P. longipes* [156]. In contrast, females of the two species are considered indistinguishable [124,125], except for a slight difference in the base of the spermathecal ducts, which often still results in inconclusive identifications [156].

Results of the current study show that the tips of the aedeagi of the Ethiopian 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 the two species and seems more geographically dependent than species-specific. Accordingly, we conclude that these morphological characters are not appropriate for species determination. Moreover, sequences of two genetic markers and protein profiles of *P. pedifer* and *P. longipes* were found identical, whereas all other species of the *Larroussius* subgenus in our database could be easily distinguished using

MALDI-TOF MS. This includes also *P. orientalis* protein profiles generated by colony specimens originating from an Ethiopian VL endemic site, Melka Werer. Even though *P. orientalis* is morphologically closely related to *P. pedifer* and *P. longipes* [351], our results show that the species can easily be distinguished based on MALDI-TOF MS protein profiling. Among other analyzed *Larroussius* species, the method clearly differentiated also between *P. perfiliewi* and *P. tobbi* which are very similar morphologically and have a similar geographical distribution [355–357], demonstrating the discriminatory power of the approach. Furthermore, the ecology of *P. pedifer* and *P. longipes* is comparable, as both occur in close association with hyraxes, inhabiting caves, basalt cliffs, cracks in boulders and gorges at mid-highland altitudes [47,48,163]. Collectively, our results suggest that the two species incriminated in the transmission cycle of *L. aethiopica* may actually represent a single species, *P. longipes,* which was described first.

Phlebotomus duboscqi was found in the Aba Roba focus and could easily be identified by MALDI-TOF MS as it matched with *P. duboscqi* from Senegal in our reference database. Two other species of the *Phlebotomus* subgenus were recorded elsewhere in Ethiopia, namely *P. papatasi* and *P. bergeroti*, which are sympatric with *P. duboscqi* in some areas [196,358]. In these places, taxonomic tools like MALDI-TOF MS protein profiling can be very useful for entomological studies to differentiate similar species and to thoroughly investigate their potential roles in *Leishmania* transmission.

Another *Phlebotomus* species was found in Hagere Selam, which belonged to the *Adlerius* subgenus according to the morphology of the collected specimens. The only other currently reported *Adlerius* species in Ethiopia is *P. arabicus*, which has been described in the upper Awash Valley, where it was captured from rocky valleys and found infected with *Leishmania* parasites, although the species could not be determined due to contamination [130]. In Israel, *P. arabicus* is known as an efficient vector of *L. tropica* in rocky places colonized by hyraxes [45]. Our specimens were also captured in caves together with *P. longipes.* Morphometric and molecular analyses in this study, however, show that the captured species is definitely not *P. arabicus*.

In 1980, Artemiev described four males and one female sand fly from Yemen and one male and female sand fly from Ethiopia as *P. davidi* [339]. The Ethiopian specimens were derived from the area around Langano Lake, approximately 200 km south of Addis Ababa. Our measurements of several morphological parameters, namely number of coxite hairs and the distance from the aedeagus tip to tooth, fit best with the

description of *P. davidi* [339]. However, the coxite, style and aedeagus length were slightly larger than described by Artemiev, which can be due to small sample sizes and geographical variation. Moreover, the author described that the antenna of the male specimen from Ethiopia were missing and that the female specimen from Ethiopia had a different pharynx than the one from Yemen, requiring more specimens from both countries to determine the true taxonomy [339]. This suggests that the species from Ethiopia was potentially different from the holotype collected in Yemen.

Lewis and Büttiker (1982) questioned the validity of *P. davidi* as a species based on doubts about the value of the ascoid formula that also distinguished it from *P. arabicus*. While not addressing apparently different numbers of coxite hairs and emphasizing the overlapping values of wing lengths and possible same or varying ascoid formulae, they concluded that specimens from Ethiopia previously identified as *P. davidi* were a geographical variant of *P. arabicus* and regarded *P. davidi* as its synonym [359]. Interestingly, in the same study, the authors collected specimens of another *Adlerius* species in several localities in Saudi Arabia, provisionally named *P.* Naqben species, with a remarkably wide range in number of coxite hairs (54-98) [359]. In a later study, they formally described it as *P. naqbenius*. However, for this new species they do not provide the morphological characters regarded as important for the subgenus *Adlerius* by Artemiev (ascoid formula, length of style, coxite and aedeagus tip to tooth) and they base its description on the number of coxite hairs, which they nevertheless now find significantly lower compared to *P. arabicus* [360]. The authors do not further discuss this abrupt change in their understanding of *P. arabicus* and *P. naqbenius* morphology and we speculate that the authors attributed the number of the coxite hairs incorrectly. It shall be noted that while the validity of *P. naqbenius* was later never formally challenged, there are no recent records of this species in faunistic surveys from Saudi Arabia in the last decades and no molecular reference data are available. We hypothesize that, based on scarce morphological data, our *Adlerius* sp. do not represent an Ethiopian population of *P. naqbenius*. On the contrary, they are morphologically more similar to the Ethiopian specimens described as *P. davidi* by Artemiev and together they represent a different species from specimens collected in Yemen. This suggests that investigation on its taxonomy, distribution and potential role in *Leishmania* transmission is required.

In conclusion, MALDI-TOF MS protein profiling and DNA barcoding provided similar results in this study, demonstrating that methods of molecular taxonomy provide a viable alternative to often poorly characterized and minuscule morphological

characters required for traditional species identification. An integrated approach is especially useful in regions where the sand fly fauna is highly diverse. Because DNA barcoding is much more expensive, time consuming and sequences of many Ethiopian species are not available in online genetic databases, we suggest to develop a centralized MALDI-TOF MS protein profile database and use this approach for routine identification of sand fly specimens from field surveys.

Conclusion

This study demonstrates for the first time that MALDI-TOF MS protein profiling is a suitable taxonomical approach for cost-effective, unambiguous species identification of Ethiopian sand flies. DNA- and protein-based molecular techniques as well as morphometric analysis suggest that the vectors of *L. aethiopica*, *P. pedifer* and *P. longipes*, may represent a single species. We also report that the *Adlerius* species we found differs from *P. arabicus*, the only *Adlerius* species reported in Ethiopia tp date. This *Adlerius* species probably represents a new species. Collectively, our results contribute to the understanding of the complex sand fly fauna in Ethiopia, a region heavily burdened with human leishmaniases.

Acknowledgements

We are very grateful to Arba Minch University and all staff of the regional, zonal and woreda (district) offices for their cooperation and coordination of the field work. Furthermore, we would like to thank dr. Meshesha Belkew for his advice on trapping places and sand fly identifications.

Funding

MP is a PhD-fellow in the VLADOC program of the Flemish Interuniversity Council VLIR-UOS and her research stay at Charles University was supported by an Infravec2 Grant (Agreement No. 731060). HL is part of the University of Antwerp Center of Excellence VAX-IDEA. This research was supported by the projects BIOCEV CZ.1.05/1.1.00/02.0109 from the European Regional Development Fund. Additional support from the Czech Infrastructure for Integrative Structural Biology (LM2015043 funded by MEYS CR) is gratefully acknowledged.

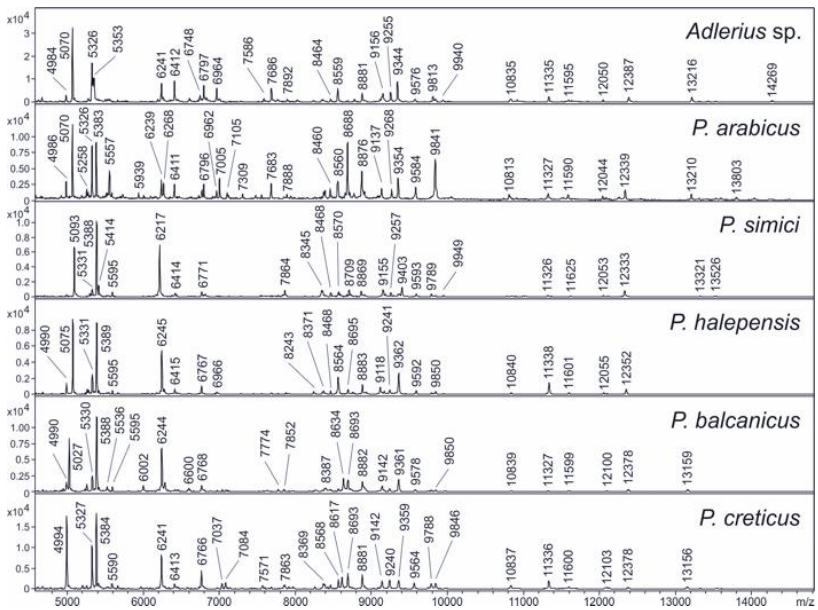
Additional files

Additional file 8.1: Table S1 Intraspecies K2P distances of COI and ND4 genes of Ethiopian sand flies. *Abbreviations:* K2P, Kimura two-parameter Model; SD, standard deviation.

Sand fly species	K2P COI (SD)	K2P ND4 (SD)
P. Adlerius sp.	0.5 (0.2)	0.1 (0.1)
P. longipes/P. pedifer	0.3 (0.1)	0.1 (0.1)
P. duboscqi	0.3 (0.1)	-
P. celiae	0.2 (0.1)	0.1 (0.1)
P. martini	0.6 (0.2)	0.4 (0.2)

Additional file 8.2: Table S2 Interspecies K2P distances of COI and ND4 genes of Ethiopian sand flies. The lower left quadrant presents the K2P distances (SD) of the COI gene, the upper right quadrant of the ND4 gene. The ND4 gene of *P. duboscqi* specimens was not included in the analysis*.

Sand fly species	*P. Adlerius sp.*	*P. longipes/P. pedifer*	*P. duboscqui*	*P. celiae*	*P. martini*
P. Adlerius sp.	-	14.9 (1.6)	*	15.6 (1.6)	16.0 (1.7)
P. longipes/P. pedifer	15.4 (1.6)	-	*	15.1 (1.6)	15.1 (1.6)
P. duboscqi	14.9 (1.6)	17.5 (1.7)	-	*	*
P. celiae	17.1 (1.8)	13.7 (1.5)	14.7 (1.6)	-	1.2 (0.3)
P. martini	16.2 (1.7)	13.8 (1.5)	14.0 (1.5)	1.6 (0.4)	-



Additional file 8.3: Fig S1 Comparison of MALDI-TOF MS protein profiles of Ethiopian *Adlerius* sp. with five other species of the subgenus *Adlerius*. Zoomed mass range of 4 to 15 kDa is depicted.

Additional file 8.4: Table S3 Interspecies K2P distances of COI gene of *Phlebotomus* sand flies of the *Adlerius* subgenus.

Sand fly species	1	2	3	4	5	6	7
1. *P. turanicus* Afghanistan, MN850811.1							
2. *P. kyreniae* Turkey, MN086679.1	13.4 (1.7)						
3. *P. simici* Greece, KU519497.1	14.2 (1.8)	13.1 (1.6)					
4. *P. halepensis* Turkey, MN086655.1	11.1 (1.6)	8.6 (1.3)	12.9 (1.7)				
5. *P. arabicus* Israel, KF483670.1	12.5 (1.6)	12.5 (1.6)	16.3 (1.9)	7.5 (1.2)			
6. *P. balcanicus* Turkey, MN086653.1	11.8 (1.5)	5.7 (1.1)	12.9 (1.7)	7.7 (1.2)	11.2 (1.6)		
7. *P. Adlerius sp.* Ethiopia, Hagere Selam	12.5 (1.7)	11.4 (1.6)	15.4 (1.9)	7.1 (1.3)	6.5 (1.2)	9.1 (1.4)	
8. *P. arabicus* Israel, colony	13.0 (1.7)	12.5 (1.7)	16.3 (2.0)	7.5 (1.3)	0.3 (0.2)	11.2 (1.6)	6.1 (1.1)

PART V

DISCUSSION

CHAPTER 9

General discussion and conclusions

The general aims of this PhD project were to explore the transmission dynamics of *L. aethiopica* and map its distribution in southwestern Ethiopia. Moreover we assessed the most suitable diagnostic assays to detect *L. aethiopica* in a variety of clinical, sand fly and animal samples and determined whether MALDI-TOF MS protein profiling can by applied for identification of Ethiopian sand flies.

Transmission dynamics of CL and its distribution in southwestern Ethiopia

Transmission of *L. aethiopica* caused by *P. pedifer* is restricted to a relatively small area in southwestern Ethiopia and some parts of Kenya. Ashford and colleagues already identified *P. pedifer* as the main vector of *L. aethiopica* and assigned hyraxes as the reservoir hosts (Fig 1.4), but many elements of disease transmission remained to be revealed in order to be able to provide guidance for disease management [31,48].

In this thesis, we explored different aspects of the ecology and behavior of *P. pedifer* and the role of potentially important mammals in disease transmission in a model village of CL, Ochollo. We found that the vector is mostly abundant in the dry season and that most infected sand flies reside inside caves, where they are infected throughout the whole year (chapter 2). Moreover, we show that *P. pedifer* sand flies are mainly endophagic, although they are biting outdoors as well. The peak biting hours are at midnight, but there is also considerable activity in the early evening. In contrast to previous reports [48,159], *P. pedifer* acquired its blood meal mainly from humans and some of these blood meals contained parasite DNA, indicating that there is most probably also considerable human-to-human transmission (Fig 9.1, chapter 3). We confirmed the earlier suggestion that many hyraxes are abundant in CL foci and that a high proportion of the population is infected, and found that rodents and livestock are not important for transmission of *L. aethiopica* (Fig 9.1, chapter 2 and 3).

To our knowledge, there has been no other place where the eco-epidemiology of *L. aethiopica* has been investigated this thoroughly. However, this thesis does not completely resolve the transmission dynamics yet and there are still some aspects to discover.

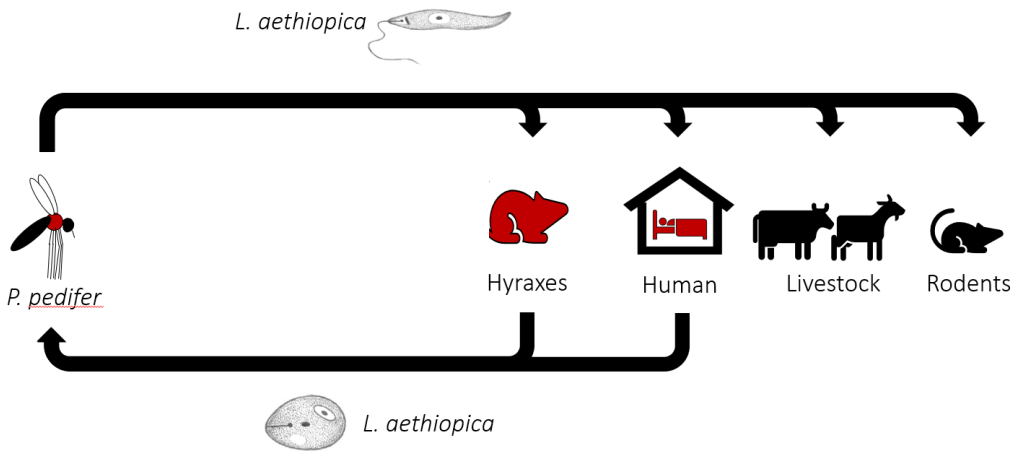


Fig 9.1: Transmission cycle of cutaneous leishmaniasis in southwestern Ethiopia. *Phlebotomus pedifer* is the only vector transmitting *Leishmania aethiopica* parasites. Besides from hyraxes as the animal reservoir, humans do also play a major role in the transmission cycle. They are mostly exposed to the (infected) vector indoors, at night. Livestock and rodents are sometimes bitten by sand flies, but are end hosts for the parasite.

The results in chapter 2 and 3 show that most (infected) sand flies are found inside caves, while many blood fed sand flies were captured indoors. However, it should be considered that in chapter 2 we did not assess the prevalence of sand flies indoors, which could have provided valuable information. This was not done because we initially thought transmission mainly happened inside caves, as various studies indicated that human dwellings or activities in proximity to hyrax colonies are a major risk factor for acquiring CL [29,48,159,361]. Based on the findings we gathered, we hypothesize that sand flies are mainly breeding inside the caves, where hyrax feces are present for larval development, and fly towards houses if hyraxes are not available in the caves to acquire their blood meal from. Although the activity pattern of *Heterohyrax* is described to be diurnal, we most often saw them moving around in the early evening and at night in the dry season, which could be due to the warm temperatures, making them unavailable for a blood meal [113]. Furthermore, we speculate that sand flies mainly rest inside the houses after their blood meal and move back to the caves for breeding. In order to test this, the flight behavior of the sand flies should be investigated, which can be done by several approaches (e.g. fluorescent powder on the wings or colored sugar meals) [78,362]. If this hypothesis can be proven, vertical insecticide treated barriers could be an effective sand fly control measure (chapter 1) [78,86]. Although research is again required to assess the collateral harm to non-target insects, like bees,

which are very important for honey production in the highlands in southern Ethiopia. Moreover, if we could identify a bait for hyraxes, insecticide-treated baits (chapter 1) would kill the vector feeding on the hyrax, but also the larvae feeding on the hyrax feces [92].

Hyraxes are present in high numbers and a high proportion is infected asymptomatically. The prevalence of 20% estimated in chapter 2 is probably even an underestimation, as it was assessed on ear samples and we later on found that most parasites are present in the nose of the animals. These findings all indicate that hyraxes are a good reservoir for *L. aethiopica*. However, even though they are the preferred blood meal source of *P. pedifer* and many are infected, the vector is not predominantly feeding on them and *Leishmania* parasites could not be detected in blood meals derived from hyraxes (chapter 3), raising the question whether they can efficiently transmit parasites to the vector. Svobodová and colleagues showed that after feeding of *P. arabicus* on *P. capensis* infected with *L. tropica* under laboratory conditions, only a few sand flies got infected [176].

We aimed to conduct a similar experiment under natural circumstances in Ochollo [171]. *P. pedifer* sand flies were captured in a cave with CDC miniature light traps and placed in a cage inside a cave for starvation (Fig 9.2 A). In the meantime, hyraxes were captured and a nose sample was collected for *Leishmania* detection by PCR in order to find an infected animal (Fig 9.2 B). For the continuation of the experiment, sand flies were subdivided in a treatment and control group. The infected hyrax was exposed to the sand flies of the treatment group for blood meal acquisition (Fig 9.2 C). Sand flies of both groups were continuously provided with 10% sugar solution and kept inside the cave. The idea was to dissect the sand flies of both groups for *Leishmania* detection and species identification two and twelve days after feeding to assess whether they picked up the parasites from the hyrax and were able to develop metacyclic promastigotes. Eventually, we aimed to compare the control and treatment groups to assess the transmission efficiency of *L. aethiopica* from *H. brucei* to *P. pedifer*.

Although the experiment was unfortunately unsuccessful (probably due to weather conditions), this research remains important to investigate how efficient hyraxes can transmit parasites to *P. pedifer* and should therefore be carried out again in the beginning of the dry season.

Figure 9.2 Experimental transmission experiment. A) transfer of captured sand flies to a cage; B) hyrax captured for nose sample collection to test it for the presence of *Leishmania* DNA; C) sand flies feeding on sedated hyrax. *Pictures by Myrthe Pareyn.*

Additionally, it would be interesting to investigate whether their infection continues throughout the year or is seasonal. Chapter 2 demonstrates that sand flies are infected with *L. aethiopica* during the whole year. This could give an indication of whether humans and/or hyraxes are fueling this continuous infection in sand flies and sustain the transmission cycle.

Because many sand flies are actually feeding on livestock, but none of the animals was found infected, it could be assessed whether livestock could perhaps be used to reduce the vector population by topical or systemic insecticides or just by distracting sand flies from biting humans (chapter 1) [185–188].

Several studies have already been carried out during the past decades in Ochollo that indicate the impact of the disease on the population, in particular on young children [48,100,101]. Besides from Ochollo, an outbreak in Silti (in the North of the SNNPR) and some scarce reports at Arba Minch Hospital, there is very limited knowledge on the distribution of the disease in southern Ethiopia [102]. However, chapters 4 and 5 show

that in fact the distribution of the infection and vector are much more widespread than indicated in previous reports. This is probably due to many reasons, like the lack of adequate surveillance systems, misdiagnosis or the misunderstanding of the importance of reporting non-fatal diseases.

Chapter 4 provides a robust, dynamic model that indicates the geographical space that is suitable for the vector. It should be taken into account, however, that CL cases do not occur at each place that is indicated suitable for the vector. It would have been valuable to have additional species distribution maps indicating sites where the reservoirs (hyraxes and/or humans) are present. Stacking these maps could probably provide a better indication of where CL can present.

Our map is an improved version of the one produced by Seid and colleagues [111], because it was based on a sophisticated ecological niche modeling approach and has a much higher resolution, which is useful for guiding interventions. Moreover, based on our field work and the model, we find that the previous predictions of the number of people at risk for CL are an overestimation of the true distribution [111].

However, the question still remains whether the results obtained in Ochollo can be extrapolated to these other areas. Even though the same vector is present in all the other affected villages, different ecological circumstances could change the behavior of the vector. Unlike in Ochollo, where human dwellings are surrounded by caves, in some villages there are no caves or they are far from the village and sand flies were only captured inside the houses during the survey. Here, sand flies were captured inside human dwellings, while hyrax colonies were only present at a distance. Therefore, a comparative study should be carried out in ecologically different settings with the same vector to evaluate whether the main characteristics of transmission are similar to the situation in Ochollo.

Although there is still research to be done, our results already lay groundwork for guidance of disease control, focusing on limitation of the human - sand fly contact, reducing the vector population and early diagnosis and treatment of human CL cases.

We suggest that insecticide treated bed nets should be provided to reduce indoor transmission at night and that the residual outdoor transmission in the early evening should be tackled by personal protection and community knowledge. These interventions would be most effective if implemented at the end of the wet season,

when the vector population reaches its lowest density and the weather allows accessibility. Moreover, humans were found to be more than simply an incidental host for *L. aethiopica*. Therefore, it is important to detect CL cases early and provide them the appropriate treatment to considerably reduce the time they remain infectious and accordingly avoid that they can fuel transmission.

These suggested control measures should be evaluated for their efficacy. To decide which insecticide to use for the bed nets, susceptibility studies should be carried out for *P. pedifer*. Although a low chance of resistance is expected, because no malaria interventions have ever been carried out in the highlands in southern Ethiopia. Subsequently, a cluster randomized control trial in Ochollo and neighboring affected villages with a similar ecology could be carried out with entomological and clinical endpoints to assess the efficacy of the use of impregnated bed nets and community education. Furthermore, to be able to know which drug to use for treatment of *L. aethiopica* infections and to evaluate which effect is has on the transmission dynamics, a clinical trial could be conducted comparing SSG, cryotherapy, paromomycin and miltefosine [26].

In case a combination of particular interventions would be effective in Ochollo and the eco-epidemiology appears similar in other CL endemic sites, the high-resolution habitat suitability map of the vector can be used to guide vector control in other potentially affected areas or places at risk for infection in five zones surrounding Arba Minch.

It is key for the suggested interventions that the local community has a good understanding on disease transmission. However, it can be concluded from chapter 5 that the majority of the population has misconceptions on the transmission of CL and is only seeking treatment from healthcare facilities when infections are severe and chronic. It is crucial for the community to understand how a change in behavior and adequate use of the bed nets with high coverage could protect them from CL infection. In order to achieve this, health promotion campaigns should be organized (oral information, leaflets, posters and/or visual media) to stimulate the use of bed nets and self-protection. Besides from the community, also people working at local health centers should be trained and made aware of the importance of reporting cases to referral centers, as a good reflection of the magnitude of the infection is crucial to convince policymakers to invest in CL control.

Leishmania detection methods

Many approaches and targets are available for detection of *Leishmania* in different sample types. However, which method to apply for a certain purpose remains unclear and an additional challenge in Ethiopia is that CL is caused by a unique species, *L. aethiopica,* for which most assays have not been validated. It was our objective to evaluate different diagnostic assays for detection of *L. aethiopica* in a variety of clinical sample types, field- and laboratory-infected sand flies and reservoir hosts.

Early diagnosis and treatment of human CL cases are pivotal to reduce the risk of scar formation and stigmatization and to reduce the longevity of infectivity of the human host that may fuel transmission (chapter 1 and 3). In resource-constrained countries, microscopy remains the golden standard for parasite detection in human samples, because it is inexpensive, requires limited resources and detects viable parasites [363]. However, chapter 6 and other studies demonstrate that there is high inter-observer disagreement, reflecting the difficulties that are faced with this approach, leaving many cases undetected [364]. Hence, routine usage of this technique is challenging and results in underdiagnosis, especially when people only seek health care when their lesions are chronic and severe, in which case the parasite load is considerably lower (chapter 5) [302].

Based on our findings in chapter 6, we present a diagnostic algorithm for diagnosis of *L. aethiopica* infections (Fig 9.3). When a suspected CL case has been clinically identified at the kebele (village) health post (HP), the person should be sent to the primary health care unit (PHCU) of the woreda (district). Here, skin scraps should be collected: two for microscopy and one stored in ethanol, to avoid that samples need to be taken at different time points. If a CL case is identified by microscopy by at least two readers, the person should immediately be sent to a referral hospital for treatment. The number of confirmed cases and demographic information of the patients should additionally be reported to this referral hospital every month, who will then report it to the zonal health department and higher up, like it is being done for other diseases as well (chapter 5).

When a suspected CL case appears microscopy negative, we suggest in chapter 6 to use the skin scrap sample in ethanol for screening in a referral center. This should be done by the TaqMan probe-based LC kDNA qPCR, which was specifically developed for detection of *L. aethiopica* kDNA, as this assay performed with the highest sensitivity.

The assay could also be used to evaluate the treatment outcome, because its Ct values correlate well with the parasite load estimated by microscopy. In case the patient's sample is also negative by qPCR, a differential diagnosis should be considered.
The suggested diagnostic algorithm will require additional training of the health care staff. The map generated in chapter 4 could be implemented to target the right people for education, as it indicates areas suitable for *P. pedifer*, and subsequently at risk for CL.

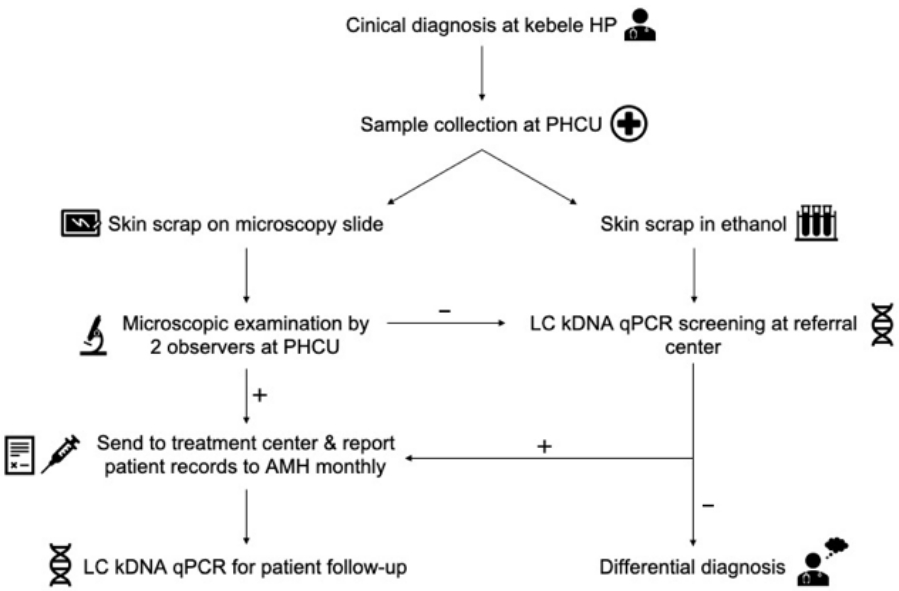


Fig 9.3 Diagnostic algorithm for *L. aethiopica* detection in clinical samples. HP: health post; PHCU: primary health care unit; LC kDNA qPCR: TaqMan-probe based qPCR developed for *L. aethiopica* detection specifically; AMH: Arba Minch Hospital.

For high-throughput screening or large-scale eco-epidemiological research, however, the LC kDNA qPCR might be very costly. In this case, researchers prefer a less expensive, yet highly sensitive assay. Therefore, the pan-*Leishmania* SYBR Green SL RNA qPCR could be a good alternative [170]. For *L. aethiopica* detection in both skin scrap samples (chapter 6) and sand flies (chapter 7), this assay showed a very good performance, high sensitivity and low Ct values. For sand fly screening, chapter 7 presents that the assay can even be used in combination with a cheap, crude extraction method, which considerably lowers the price and work load per sample. This was not tested on human samples, but the assay would most probably have suffered from inhibition (e.g. by

proteins in blood) [365]. Even though the assay worked very well with sub-optimal sample storage conditions, we advise to store the samples in RNA stabilizing reagents, which will probably even improve the performance of the assay.

Unfortunately, we did not include the LC kDNA qPCR for the evaluation of the SL RNA qPCR in chapter 7, because we were not in contact yet at that time with Cnops and colleagues (Clinical Sciences Department, Institute of Tropical Medicine, Antwerp, Belgium), who developed this assay. However, we expect that it would have worked better than the SYBR Green kDNA and TaqMan probe duplex 18S/kDNA qPCRs that were applied, because these generally have an impaired performance because of the SYBR Green dye and the multiplex design and because the LC kDNA qPCR was specifically designed for *L. aethiopica* detection [322,323].

However, the aim of chapter 7 was not to compare the different assays to assess which one works best, but rather to evaluate the suitability of the SL RNA qPCR in combination with a crude extraction buffer for *Leishmania* detection in sand flies; an objective for which the current approach and included assays were appropriate.

A drawback of screening hosts by molecular approaches is that the above-mentioned assays do not provide information on the viability of parasites, in contrast to cultivation and microscopy. One of the criteria for inclusion of reservoir hosts is that parasites must be viable and present in high numbers [31].

The presence of *Leishmania* DNA in an animal sample does not mean that viable parasites are present and that the animal can be considered as a reservoirs. The signal generated by the SL RNA qPCR when used in combination with the crude extraction buffer is for approximately 95% due to SL RNA detection, so it could give an indication of the presence of viable parasites, because SL RNA is pivotal for RNA trans-splicing and polyadenylation processes (Chapter 7) [314]. However, generally longer fragments are preferred for detection of viable agents [295].

A promising method to detect specifically viable cells is the implementation of an incubation step with propidium monoazide (PMA) prior to *Leishmania* specific PCR amplification [366]. The approach relies on the permeability of the membrane of cells. The plasma membrane of dead cells is compromised, in contrast to the membrane of viable cells, enabling PMA to penetrate the cell upon which it will intercalate in the

DNA, due to its azide group. This complex will inhibit DNA amplification in dead cells and considerably reduce the amplification signal compared to viable cells.
This method has already been applied to detect viable bacteria [367], viruses [368], parasites [369] and fungi [370], although incomplete signal suppression was observed in some studies. PMA was evaluated for its use to differentiate viable from unviable *Trypanosoma cruzi.* The method performed very good, except at low concentrations (10 parasites/mL) [371].

Recent advances show the ability of a new, improved version PMAxx to completely suppress the amplification signal [372]. This indicates that the applicability of PMAxx for detection and quantification of viable *Leishmania* parasites would be an interesting research track.

Furthermore, parasites acquired by sand flies during blood feeding may sometimes not be able to establish an infective metacyclic form in the sand fly. In this case, DNA from dead or dying parasites may still remain detectable. Hence, it would be highly informative to be able to specifically detect metacyclic promastigotes, because only this form eventually leads to transmission. Even more interesting would be to be able to quantify the number of metacyclic promastigotes to assess the number of infective parasites injected into the host when the sand fly is feeding.

Several studies demonstrate that small hydrophilic endoplasmatic reticulum-associated protein (SHERP) and hydrophilic acylated surface protein B (HASPB) that are encoded on a single locus on chromosome 23 are crucial for metacyclogenesis of *Leishmania* parasites. Both proteins were predominantly expressed in cultured metacyclic *Leishmania* parasites [373,374].

Based on this information, Giraud and colleagues recently tested multiple targets, among others SHERP and HASP, for detection and quantification of metacyclic promastigotes in sand flies by real-time quantitative PCR. They found that only the SHERP target has sufficient specificity and sensitivity to discriminate infective forms of *L. mexicana* and *L. infantum* from other promastigotes forms [375]. This novel target seems very promising and could be very useful for both laboratory and field purposes, but requires validation on other species first.

MALDI-TOF protein profiling for identification of Ethiopian sand flies

Unambiguous, high-throughput sand fly identification is important for entomological and eco-epidemiological research that investigates the ecology and behavior of *Leishmania* vectors. The currently available species identification approaches are based on morphological features or genetic markers.

The first method is time-consuming and as identification keys are obsolete or incomprehensive, it often leads to inconclusive species identifications, especially for females, which are important for transmission [125,350]. This was proven in chapter 8, in which we show that we had difficulties to identify females and even males within certain subgenera and our morphometric analysis did not match with published records [335,351].

The second available approach offers a solution to this, because the genetic markers that we used could easily distinguish the different species, except for the two presumed species of the *Larroussius* subgenus, *P. longipes* and *P. pedifer*. This could have been due to lack of genetic variability in the two mitochondrial markers that we used (COI and ND4 genes), so it would have been interesting to include an additional ribosomal marker to check for variability between the two presumed species. However, we tried to amplify the ITS-2 gene with C1a and JTS3 of Depaquit *et al.* (2002) [376] but although specific clear amplicons were obtained, sequences were not good and therefore useless for phylogenetic analyses. Additional issues of DNA barcoding are that many species captured from Ethiopia were not found in GenBank for identification and that it is expensive and requires a high level of expertise.

Recently, MALDI-TOF MS protein profiling has been introduced as a rapid, less expensive method for conclusive species identification. We demonstrate in chapter 8 that the technique performed equally well as DNA barcoding for identification of Ethiopian sand flies. Although we included all Ethiopian human leishmaniasis vectors and some additional *Phlebotomus* species in the analysis, more Ethiopian sand flies should be incorporated to be able to use this approach for large field studies. As this technique is much faster, cheaper and easier than DNA barcoding, we suggest investing in a centralized database with protein spectra for sand fly species identification.

Recent advances showed that the blood meal sources of engorged sand flies can also be determined by a novel MALDI-TOF MS based method that targets host specific hemoglobin peptides [377]. The method is even capable of determining mixed blood meal sources up to 48 hours post blood meal. This is very useful in order to reveal which hosts may potentially play a role in transmission, like we did by sequencing host blood DNA in chapter 3. Protein profiles are already available in centralized databases, although for site-specific hosts, like for instance hyraxes, first animal blood samples should be included in the reference database.

Furthermore, MALDI-TOF MS protein profiling has shown to enable detection of *Plasmodium* parasites in *Anopheles* mosquitoes [378]. Hence, it would be very valuable for epidemiological surveillance if the technique could also determine the infection status of sand flies. Researchers already reported that the method can identify and distinguish different *Leishmania* species [379,380], but to our knowledge there are no reports yet of *Leishmania* detection within sand flies.

Overall conclusions

In this PhD thesis, we present new insights in the transmission dynamics, distribution and diagnostics of *L. aethiopica* transmitted by *P. pedifer* in southwestern Ethiopia. The sand fly population is highest in the dry season and most infected sand flies are found in caves. The vector is mostly active at night and mainly endophagic. Most sand flies feed on humans, although they also take their blood meals from livestock, hyraxes and rodents. There is most probably also human-to-human transmission and besides from hyraxes, no other animals are involved in transmission. The occurrence of CL is much more widespread that initially thought, which should be a wake-up call for policymakers. These main findings suggest that impregnated bed nets could be an effective intervention to reduce the human-sand fly contact and subsequently disease transmission. Additionally, early diagnosis (by microscopy and the LC kDNA qPCR assay) and appropriate treatment of CL cases are required to reduce the longevity of the infectivity in humans to avoid that they can fuel transmission. Overall, community knowledge is pivotal to implement these control measures and achieve a successful integrated control program. If we can extrapolate the results from Ochollo to ecologically different areas, the habitat suitability map could be used to target specific areas for vector and disease control and community education. However, it should be taken into account that not all aspects of the transmission cycle have been elucidated and that the control measures can only be implemented at the locations indicated on the habitat suitability map if the transmission dynamics appears similar in ecologically different areas where *P. pedifer* occurs. To distinguish sand flies that are similar in morphology, MALDI-TOF MS protein profiling showed excellent performance on Ethiopian sand flies, showed that *P. pedifer* and *P. longipes* represent a single species and identified a new species of the *Adlerius* subgenus. For future entomological field studies, we suggest that the crude extraction buffer in combination with the SL RNA qPCR can be used for inexpensive, high-throughput *Leishmania* detection in sand flies and MALDI-TOF MS protein profiling can be applied to determine the sand flies species.

CAPACITY BUILDING

Development of a molecular laboratory at Arba Minch University

An important aim of the VLIR-UOS IUC project was to contribute to capacity building in laboratory skills and research to empower AMU. Several project members indicated the need to apply molecular techniques for sample processing. Many conventional and real-time PCR machines were already available at AMU, however, due to the lack of a sophisticated laboratory with required instruments, consumables and trained staff, the PCR machines could not be used for research. Therefore, we engaged ourselves to establish a molecular laboratory with trained staff to perform requested analyses.

First, we reorganized the Medical Entomology laboratory in order to separate the different handlings and avoid contamination, resulting in four rooms (extraction, pre-PCR, PCR and post-PCR). Second, required laboratory instruments (centrifuges, vortexes, thermoshakers, pipets, an RNA cabinet, etc.) were purchased with support of the NORHED project. Third, three laboratory technicians from AMU were selected for a six-weeks hands-on training at the Evolutionary Ecology Group laboratory at the University of Antwerp, during which we taught them how to perform DNA and RNA extractions, conventional and real-time PCRs and sequence analyses.

Currently, the molecular laboratory is operative and the technicians are capable to design and perform molecular analyses for a variety of research purposes. We still provide consumables and support for development of standard operating procedures, but they can perform the remaining work independently. This is an important step forward with regard to capacity building at AMU, leading to research that can include molecular processing of samples for a variety of purposes.

Molecular laboratory at Arba Minch University. Panel A and B present the DNA extraction room, panel C and D the pre-PCR room and panel E and F the post-PCR room. *Pictures by Nigatu Girma.*

ACKNOWLEDGEMENTS
DANKWOORD

Gedurende de afgelopen vier jaar heb ik met heel veel plezier en passie aan dit doctoraat gewerkt. De realisatie van dit proefschrift was echter één groot goepswerk en daarom zou ik enkele mensen expliciet willen bedanken die hiertoe hebben bijgedragen.

Eerst en vooral wil ik mijn promotor, Herwig, oprecht bedanken. Hij maakte me al nieuwsgierig voor onderzoek naar de eco-epidemiologie van infectieziekten door zijn gepassioneerde uitleg tijdens de lessen. Tijdens mijn doctoraat gaf hij me veel wetenschappelijke vrijheid en opportuniteiten om naar internationale congressen te gaan, wat ontzettend heeft bijgedragen tot deze thesis en het opbouwen van mijn netwerk. Ook al was het waarschijnlijk niet altijd even gemakkelijk om me te begeleiden 'met mijn zandvliegen', toch stond hij altijd achter me wanneer dat nodig was en heeft hij me geholpen bij het maken van juiste beslissingen of wanneer ik bepaalde inzichten nodig had. Of ik ooit het verschil tussen een *Ficus* en *Acacia* boom of een muis en rat zal weten betwijfel ik, maar hopelijk vergeet je dit 'kleefkruid' niet snel en vind je me toch een beetje bioloog-waardig ☺. Heel erg bedankt voor de ondersteuning en leuke momenten de afgelopen jaren!

Furthermore, there are two very important people without whom this all would not have been impossible.

Eerst en vooral verdient Natalie een ereplaats. In het labo heeft ze me geholpen met het verwerken van enorme hoeveelheden celebrations en stalen met veel perfectie en structuur, wat leidde tot mooie resultaten. Ze was enthousiast en toegewijd over elk onderwerp waar ze mee kon helpen. Met een lach denk ik terug aan al haar trucjes om alles zo efficiënt en nauwkeurig mogelijk te doen en altijd toch weer net een minuutje sneller te zijn. Bovendien is ze ook een enorme hulp geweest bij het begeleiden van zowel Belgische als Ethiopische

studenten in het labo en heeft ze daarmee veel druk van mijn schouders genomen. Daarnaast is Natalie ook altijd vrolijk en opgewekt en heeft ze daardoor voor ontzettend veel plezier gezorgd tijdens mijn doctoraat. Ondanks het door velen benoemde 'geschater en getater' in het labo, hebben we samen toch heel erg veel werk verzet en leute gehad, waarvoor een dikke merci molleke!

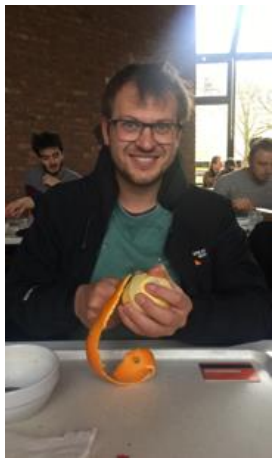
I also want to express my enormous gratitude to Nigatu. Since my first field visit in Ethiopia, he has been by my side as a field assistant and as my best friend. He was always happy to perform the field work and did it with a lot of care and enthusiasm, even when I was not around. He climbed steep mountains with heavy traps, stayed awake sitting on a rock outside the whole night for sand fly trapping and dissected thousands of specimens in the laboratory. I really appreciate that he cared so much about this research. When I was in Belgium, he always took great care of the master students that we sent to Ethiopia. Overall, he did a lot of work for this research, which I am very grateful for. Moreover, we had a lot of laughter and fun together, both during the field work and while going out to enjoy ourselves. He also taught me the essentials of Amharic, including some very useless words, leading to hilarious conversations. I really value that he and his family treated me as part of their family and gave me the opportunity to really get to know the Ethiopian culture. Monkey, thank you so much for everything. I really hope we can work together in the future and that I can see you again very soon. Betame amesigenalew!

Furthermore, I would like to thank Behailu, with whom I share the same passion for research on neglected tropical diseases and who has become a really good friend over the past years. Like many people say, we are the perfect example of 'synergy'. I feel like Behailu and I complement eachother and that we make a superb team. He really amazed and inspired me with his great enthusiasm and helped me with the field work. He made me laugh a lot, provided great support

in Ethiopia and he and his wife Aster have been very hospitable. Behailu, I could not have been happier with anybody else to work so closely together with. Thank you very much for being my friend and co-worker. I truly hope that our research can be useful for the communities and that we can continue working and spending more time together in the future!

Omiiiid, my dearest buddy, I really enjoyed being in the office with you for three years. I liked nagging to you, fooling around with you and I appreciate that you always took care of my plants when I was in Ethiopia. Even though I hate it when you call me fatty and you left me earlier than expected, you were the best office buddy I could have imagined! Thanks for everything!

Ik wil ook graag Brambo en Anneleentje bedanken, waar ik de afgelopen jaren heel veel plezier mee heb gehad, heb kunnen brainstormen en die me beide met hun expertise geholpen hebben met mijn onderzoek. Bovendien heeft Bram me de kneepjes van het appelsienen schillen geleerd en heeft Anneleen me vaak doen lachen met haar onbeschrijfbare onhandigheid. Merci voor alles maatjes!



Stijn hielp me geduldig bij het behandelen van vaak ingewikkelde administratie en op Frank kon ik steeds rekenen bij het verzamelen een voorbereiden van veldmateriaal, waarvoor dank. Furthermore, I would also like to thank my fellow lab members for their help and support when needed and for creating a good atmosphere at work. Thank you Laura, Omid, Vincent, Joachim, Bram, Anneleen, Natalie, Nathalie, Mats, Gerardo, Joris, Luci, Lisa, Luc, Frederik, Constance, Anna-Pia, Dieter, Frank, Stijn, Herwig, Erik V, Erik M, and Stefan.

Over de jaren heb ik een goede band opgebouwd met master studenten, waaronder Emma, Luca en Wim. Ik wil hen graag bedanken voor de fijne tijd samen en om zo vlijtig aan hun thesis te werken en te helpen bij het verzamelen en analyseren van de data die in deze thesis verwerkt zijn.

I am also very grateful to the field assistants and community in Ochollo for their participation in this research. In particular Melkamu, Zewdeh and Mirte have been crucial for accomplishment of the fieldwork. I will always remember the hilarious moments we had, like running after escaping hyraxes. 'Tossimo' you guys for the great work, funny jokes, laughter and the way you took care of me and the master students.

I would also very much like to thank my supervisor in Ethiopia, Simon, who provided me the opportunity to perform my PhD at AMU and has been very hospitable during my field trips. I am grateful to both him and Fekadu for their support and supervision of my field projects and for taking care of Belgian master students.

Ook in België kreeg ik ondersteuning van toppers in het vak.

Eerst en vooral wil ik het komische duo Guy en Louis bedanken, die me de kans gaven om in het LMPH labo alles te leren over zandvliegen en om experimenten uit te voeren. Ik stel het heel erg op prijs dat ze me mee begeleid hebben en vond onze samenwerking heel aangenaam. Het was zo aangenaam dat we zelfs een deel van onze reis in de Galapagos samen doorbrachten en dat ik mee mocht met jullie naar Toledo. Beide, heel erg bedankt voor de leute en wetenschappelijke samenwerking.

Door het feit dat ik af en toe op LMPH mocht werken, kreeg ik ook de kans om met de onderzoekers van het labo een wetenschappelijke en vriendschappelijke band op te bouwen. Naast alle andere leuke collega's wil ik graag Dorien en Lieselotte bedanken voor de fijne babbels en het plezier en Sarah, Rik en Eline voor hun advies en de vlotte samenwerking. Heel veel succes allemaal met jullie verdere carrière!

Daarnaast wil ik ook graag twee experts van het ITG bedanken. Gert ben ik enorm dankbaar voor zijn advies en sturing voor labo experimenten in het begin van mijn doctoraat. Het was een van de moeilijkste periodes, waar ik dankzij jou een goede oplossing voor gevonden heb. Merci! Daarnaast wil ik ook Lot bedanken voor de leuke samenwerking en begeleiding. Ze is een top-madam zowel op wetenschappelijk als sociaal vlak waar ik enorm naar op kijk. Bedankt voor de leuke tijd samen Lot en hopelijk tot snel.

I am also grateful to Petr and Vit for the opportunity to come to visit their lab to learn about setting up a sand fly colony and to perform laboratory experiments. I really appreciate that they offered me this great opportunity and would like to thank them for their exceptional support during the sample analysis and writing process.

I would also like to thank all people in Ethiopia who have treated me like a friend or family, among others Behailu, Aster, Nigatu G, Nigatu E, Girum, Gemechu, Tirhas, Hibist, Dio, Aba, Butu, Belete, Tegenu, Addis, Gisachuw, Chane, Dagimawie, Zewdneh, Yenu, Wondemeneh, Fasil, and many others. Thank you all so much for making my stay in Ethiopia each time so much fun!

Tine, samen zijn we gestart aan onze studies negen jaar geleden en op dezelfde dag dienen we onze thesis in en zijn we er klaar mee. Al die jaren hebben we samen gelachen, gezeurd, gestudeerd en mekaar gesteund. Super bedankt voor alles aapke, maar laten we nu toch maar eens aan een echte job beginnen! ☺

Ook mijn andere liefste vriendjes Laura, Isabeau, Kathleine, Lonne, Merel, Dorien, Stef, Ward, mijn zusje Renske en de waterballerina's wil ik bedanken om in het weekend mijn uitlaatklep te zijn en me mee te amuseren.

Een heel speciaal plekje is er voor mijn ouders en omaatje. Hen wil ik heel hard bedanken in de eerste plaats om het mogelijk te maken dat ik zo lang kon studeren en om altijd zo goed voor me te zorgen. Een super dikke merci voor alle steun en om altijd achter me te staan!



Allerlaatst, maar misschien wel het allerbelangrijkst gedurende dit hele proces was mijn favorietje en sinds kort mijn man, Dennis, of in Ethiopië ook wel 'Danny' genoemd. Ik weet dat het voor hem niet gemakkelijk is om me keer op keer weer richting Afrika te zien vertrekken, maar ik apprecieer enorm hoe veel vrijheid hij me geeft om mijn dromen te vervullen en dat hij me (bijna) altijd volgt, zelfs met mijn gekste ideeën. Hij luisterde altijd naar mijn verhalen, hielp me waar hij kon, ondersteunde me tijdens de moeilijkere momenten en deed me elke dag lachen. Lieve flappie, dankjewel om zo een ongelofelijk lieve man te zijn voor me. Ik hou van je!



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