Visceral leishmaniasis in Ethiopia: Transmission and Variability

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Viscerální leishmanióza v Etiopii: přenos a variabilita

PhD Thesis / Disertační práce

Supervisor: Doc. RNDr. Jan Votýpka, PhD

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Praha, 21. října 2015

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I declare, that most of the works presented in this thesis are results of Aysheshm Kassahun Gelaglie or were obtained in the collaboration with the co-authors of the presented papers. Aysheshm has substantially contributed to the field and experimentally work, as well as on the writing of the manuscripts.

Prohlašuji, že většina výsledků prezentovaných v této práci byla dosažena Aysheshm Kassahun Gelaglie nebo byla získána ve spolupráci se spoluatory prezentovaných článků. Aysheshm se významně podílel na terénní i experimentální práci, rovněž tak i na sepsání prezentovaných publikací.

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Jan Votýpka

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Preface

This PhD work is a part of a major project funded and supported by the grant from the Bill and Melinda Gates Foundation Global Health Program (OPPGH5336) and entitled “Studies on Ecology and Transmission Dynamics of Visceral Leishmaniasis in Ethiopia”. The project aimed conducting thorough investigations of the epidemiology of visceral leishmaniasis (VL), aiming to tease apart the transmission cycle of the disease in important VL foci of Northwestern Ethiopia. The project was launched in 2009 and designed to quantify the relative contribution of different “drivers of transmission”, i.e., ecological, epidemiological and anthropogenic factors that impact the disease burden in human populations. It encompasses three major institutions, The Hebrew University of Jerusalem, Israel; The Addis Ababa University, Ethiopia and The Charles University in Prague, Czech Republic. Although this is a multi-lateral project, all parts are focused on one ultimate goal or interrelated tasks and subprojects to complement each other in filling of the crucial gaps in the understanding of VL problem in Ethiopia.

This PhD thesis was designed and avails the compiled researches in the framework of three major objectives designed in the aforementioned bigger project and six specific tasks. The first is to perform a comprehensive study aimed at elucidating the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology of VL in Northern Ethiopia. Under this objective, the thesis specifically reported the validation of PCR methods for detection of *L. donovani* infections and its uses for large-scale epidemiological studies. Then another objective on determination of variability and tropism in vector and parasite were conceived. In the thesis, two specific objectives were set and results of a study on genetic variability in *L. donovani* and variability study on *P. orientalis* on the biological, genetic and susceptibility parameters were investigated. Finally to solve the objective on the determination of non-human VL hosts in Ethiopia, three specific investigations were done. These include (i) determination of exposure to a parasite and sand fly infection in domestic animals and (ii) status and role of rodents and bats in the epidemiology of VL.

This PhD dissertation delivers several insights and recommendation for a better understanding of the disease. All the results and in-depth analysis of the findings are discussed in detail through published peer reviewed journals.

Abstract

Leishmaniasis, a protozoan infection caused by *Leishmania* parasites is a neglected disease affecting millions across the world. It is exhibited by diverse clinical presentations that broadly classified as visceral (VL) and cutaneous (CL) leishmaniasis. Both CL and VL are endemic to Ethiopia, which the later is generally considered as fatal, if left untreated. *Leishmania donovani* is the sole agent of Ethiopian human VL. In Africa, the worst VL affected regions are found in Sudan and Ethiopia. VL is considered as an endemic and at the same time emerging disease in north, northwest, south and southwest with sporadic cases in Eastern Ethiopia. The epidemiology is more or less associated with seasonal migration to endemic areas and HIV/AIDS. The transmission of CL in Ethiopia is known to involve zoonotic cycle while VL transmission isn't clearly understood despite traditional generalization of anthroponosis in East African platform.

The aim of this dissertation is to determine VL transmission cycle and study variability *L. donovani* and *P. orientalis* in Ethiopia. Studies on human and non-human hosts were conducted to determine the transmission dynamics. To assess the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology of VL, a community based cohort was conducted. As the study is ongoing, in-depth analysis of more data will accrue and in this thesis, result from protocol validation study is presented. Of 4,757 dried-blood samples tested by qRT-PCR, 680 samples (14.3%) had *Leishmania* kDNA and ITS1 sequences revealed 19 *L. donovani* and two *L. major* infections. To assess the involvement of non-human hosts, studies on domestic animals, rodents and bats were conducted. A total of 546 domestic animals (cow, dog, sheep, goat, donkey and camels) were tested for natural infection and 32 animals were positive on *L. donovani* DNA. Moreover, 19 % and 23% of the animals were seropositive for anti-*L. donovani* IgG and anti-*P. orientalis* saliva IgG respectively. A total of 586 rodents were tested by PCR. Fifty *Leishmania* kDNA positives were found and further ITS1 sequence revealed five *L. donovani* and five *L. tropica* infected animals. To investigate sylvatic involvement, 163 bat's DNA was tested and revealed eight kDNA positive; of which two were *L. tropica* and *L. major* positive through ITS1 sequences. Variability study on *L. donovani* isolates were performed using ITS1, cpb and k26 locus. The k26 target divide isolates in to two clusters: southern and northern Ethiopia based on the amplicon size. To identify the variability, if any, between *P. orientalis* colonies originating from different geographical locations, their biology, susceptibility to *Leishmania* infection and genetic profile were assessed. Despite variability on a few biological cues no significant genetic and susceptibility pattern difference was observed.

Generally this dissertation provides a new insight on the role of non-human host in VL epidemiology and existence of variability in the parasite between geography despite no difference in its respective vector was seen. Further studies in determining the level of infection through parasite isolation and xenodiagnosis is recommended for a better understanding of the animal's role in the *Leishmania* transmission. Moreover, the existence of polymorphism on the parasite population is evident and further action on the role of this tropism on transmission cycle and other phenotypic profiles needs to be investigated.

Abstrakt

Leishmanióza, opomíjené infekční onemocnění působené parazitickými prvoky rodu *Leishmania*, postihuje miliony lidí po celém světě. Onemocnění má různé klinické projevy, z nichž nejčastější dva jsou viscerální (VL) a kožní (CL) forma leishmaniózy, a obě tyto formy jsou v Etiopii endemické. *Leishmania donovani* je výhradním původcem lidské VL v Etiopii a pokud se neléčí, je považována za smrtelnou. V Africe jsou nejhůře postižené regiony v Súdánu a Etiopii, kde je VL považována za endemické a zároveň nově se objevující onemocnění na severu, severozápadě, jihu a jihozápadě země, rovněž tak jsou známy sporadické případy ve východní Etiopii. Epidemiologii onemocnění je více či méně spojené se sezónní migrací dělníků do endemických oblastí a rovněž s výskytem HIV/AIDS. Přenos kožní leishmaniózy je v Etiopii svázán se zoonotickým cyklem, zatímco přenos VL není dosud zcela objasněn a je v celé východoafrické oblasti spíše chápán v tradičním pojetí jako antroponóza.

Cílem předložené disertační práce bylo zjistit jaký je cyklus přenosu viscerální leishmaniózy v Etiopii, druhým cílem pak bylo sledovat variabilitu parazitů a jejich přenašečů. Abychom mohli stanovit dynamiku a způsob přenosu leishmanií, byly prováděny studie zaměřené na lidské i zvířecí hostitele. Kohortová studie zaměřená na místní komunitu lidí měla posoudit možnou roli symptomatických a asymptomatických osob infikovaných *L. donovani* v epidemiologii VL. Vzhledem k tomu, že tato studie stále probíhá, prezentuji v mojí disertační práci pouze dílčí data, související mimo jiné i s metodikou samotného provedení. Z 4,757 krevních vzorků testovaných pomocí qRT-PCR, bylo 680 vzorků (14,3%) pozitivních na leishmaniovou kDNA. Sekvenční analýza následně odhalila 19 infekcí *L. donovani* a dvě infekce *L. major*. Pro posouzení možného zapojení zvířecích rezervoárů do přenosu VL byly provedeny studie na domácích zvířatech i u volně žijících hlodavců a netopýrů. Celkem bylo testováno 546 domácích zvířat (krávy, psi, ovce, kozy, osli a velbloudi) na přirozenou infekci leishmaniemi a 32 zvířat bylo pozitivní na *L. donovani* kDNA. Kromě toho bylo 19% a 23% zvířat séropozitivní na protilátky proti *L. donovani* nebo na protilátky proti slinám flebotomů *P. orientalis*. Pomocí PCR bylo celkem testováno 586 hlodavců. U padesáti byla zjištěna leishmaniová kDNA a sekvence ITS1 prokázaly pět infekcí *L. donovani* a pět *L. tropica*. Rovněž jsme testovali 163 netopýrů, u nichž byla leishmaniová kDNA odhalena v osmi případech a následná sekvenace prokázala dvě infekce, *L. tropica* a *L. major*. Studie zaměřená na variabilitu izolátů *L. donovani* získaných z pacientů využívala tři nezávislé lokusy, ITS1, cpb a k26. Získané isoláty tvořili dvě, jasně oddělené skupiny korespondující s geografickým původem izolátů – jižní vs. severní Etiopie. Pro zjištění případné variability mezi přenašeči, flebotomy druhu *P. orientalis*, byly použity dvě kolonie pocházející z různých zeměpisných oblastí. Hodnocena byla jejich biologie, vnímavost na nákazu leishmaniemi a genetické rozdíly. I když byla zjištěna drobná variabilita v několika biologických znacích, nezjistili jsme mezi koloniemi žádné významné genetické rozdíly ani odlišnou náchylnost pro infekci parazity.

Tato disertační práce poskytuje nový pohled na roli zvířecích hostitelů jako možných rezervoárů v epidemiologii VL, odhaluje variabilitu parazitů v závislosti na geografickém původu a naopak poukazuje na podobnost mezi populacemi vektorů z endemických a neendemických oblastí. Pro hlubší pochopení možné úlohy zvířecích rezervoárů v přenosu leishmanií je do budoucna nutná izolace parazitů z těchto hostitelů a rovněž provedení základních xenodiagnostických pokusů. Rovněž tak je nutné provést další studie umožňující lepší porozumění vlivu existujícího polymorfismu parazitů na cirkulaci leishmanií a fenotypové projevy onemocnění.

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1. Introduction

The leishmaniases are a group of diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania* (family Trypanosomatidae). Human leishmaniasis is caused by about 20 species and subspecies of *Leishmania* parasite (Desjeux, 2004). The diseases have heterogeneous clinical forms ranging from self healing cutaneous lesion to mutilating and disfiguring mucosal ulcer and fatal visceral disease. It is transmitted by the bite of female Phlebotomine sand fly. Several reservoir animals were investigated and believed to play a role in the dynamics of the disease.

Leishmaniasis is prevalent in 88 countries across the world, affecting an estimated 12 million people with approximately 2 million new cases per year (WHO, 2015). More than 200 million people live in disease risk areas. The worst hit regions of the world are East African countries (mainly Sudan, Ethiopia and Kenya), Afghanistan, Iran, Saudi Arabia, India, Pakistan, Brazil and Peru that accounts for 90% of the global distribution (Desjeux, 2004). Moreover, in the past decades cases of leishmaniasis mainly, imported cases through tourism, military and immigration has increased in Europe (Ozbel et al., 1995; Di Muccio et al., 2015). Two geographical leishmaniasis foci, Old World and New World, exists that differ in species of the *Leishmania* parasite, vectors and reservoir system involved (Ashford, 2000). The New World leishmaniasis foci represent the South and Central America while the Old World encompasses the Asian, Mediterranean and African sites.

Manifestation of leishmaniasis is broadly classified in to two forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). VL, also known as Kala-azar (black fever) is the most serious form of the disease which is 100% fatal if left untreated. It is a generalized infection of the reticulo-endothelial system involving the spleen, liver, bone marrow and lymph nodes. The disease is characterized by irregular bouts fever, hepato-splenomegaly, and enlarged lymph nodes and tonsils. Patients often exhibit a wasting syndrome despite good appetite as the disease progresses. Other possible manifestations include edema, anemia, leucopenia, monocytoses, lymphocytoses, and thrombocytopenia. Some cases could represent a dermal rash usually after (or inadequate) treatment and this scenario is called post kala-azar dermal leishmaniasis (PKDL).

The annual global incidence of VL is believed to be as high as half million people which the Indian subcontinent, Bangladesh, East African region and Brazil alone account for 90% of the cases (Desjeux, 1991; 2004). *Leishmania donovani* complex is the sole agent of VL in Old World (*L. donovani* and *L. infantum*) and New World (*L. infantum* (syn. *chagasi*)) with sporadic cases due to *L. tropica* (Old World) and *L. amazonensis* (New World) has been reported (Ashford, 2000; Desjeux, 2004). In cases of human immunodeficiency virus (HIV), that is increasingly becoming an important disease in leishmaniasis epidemiology, dermotropic *Leishmania* parasites may result a fatal visceral disease (Gradoni et al., 1990).

Cutaneous leishmaniasis is the most common form of the disease with wide geographical distribution. It is represented by different clinical forms depending on the species of *Leishmania* parasite and sometimes with host factor (Moriearty and Grimaldi, 1983). Localized CL (LCL) is the most common manifestation of the disease and is characterized by benign self healing lesions that are generally painless and non-pruritic representing a localized infection at the site of the sand fly bite. In the Old World, *L. tropica*, *L. major*, *L. aethiopica*, *L. infantum*; and also sometimes dermotrohic *L. donovani* are the causative agents of LCL; while in the New World LCL is due to *L. mexicana* species complex (especially *L. mexicana*, *L. amazonensis*, and *L. venezuelensis*) and the *Viannia* subgenus (*L. braziliensis*, *L. panamensis*, *L. guyanensis*, and *L. peruviana*) and *L. infantum* (CDC, 2015). A rare form of LCL, called recidivans leishmaniasis, typically caused by *L. tropica* in Old World and few species like *L. panamensis* in the new World is a chronic distractive disease and usually difficult to treat (Calvopina et al., 2005; CDC, 2015). Diffuse cutaneous leishmaniasis (DCL) is characterized by disseminated nodular lesions that resemble lepromatous leprosy that causes disabilities and is difficult to cure. The lesions of DCL tend to be scaly and not ulcerated and could metastasize distally to cover large areas of the body (Desjeux 2004). DCL is caused by caused by *L. aethiopica* in Old World and *L. mexicana* complex in the New World (CDC, 2015). Some proportion CL patients develop skin lesions that metastasize through blood stream or lymphatics, particularly to the mucosae of the nose and mouth. This manifestation is characterized as mucocutaneous leishmaniasis (MCL). It is a chronic disease and at the same time recognized by active lesions after healing. The *Viannia* subgenus in the New World is mainly responsible for MCL; while *L. tropica*, *L. major* and *L. aethiopica* is distributed in the Old World (Hailu et al., 2006b; CDC, 2015).

1.1 The Life Cycle

The *Leishmania* are generally dimorphic parasites presenting two principal morphological forms: flagellated, motile extra-cellular promastigote in the phlebotomine sand flies and in culture medium, or as obligate intracellular non-flagellated amastigotes in mammalian host cells, mainly in macrophages.

During their life cycle, the promastigotes multiply intensively inside the gut of the sand fly, mainly in the mid gut of the subgenus *Leishmania* or in the hind- and mid-gut of the subgenus *Viannia* (Wilson et al, 2010). In both groups, the parasites migrate to the anterior part of the sand fly midgut where they are changed in to metacyclic promastigotes, the stage of *Leishmania* infective to the vertebrate host. Other than the human host several vertebrates have been suspected and found naturally infected with the *Leishmania* parasite that could represent their importance in the life cycle of a parasite in a sort of reservoir. Generally these animals could act as source and sinks of infection to sand flies (Ashford, 2000). The most commonly identified animals in endemic areas include rodents, hyrax and wild and domestic canines.

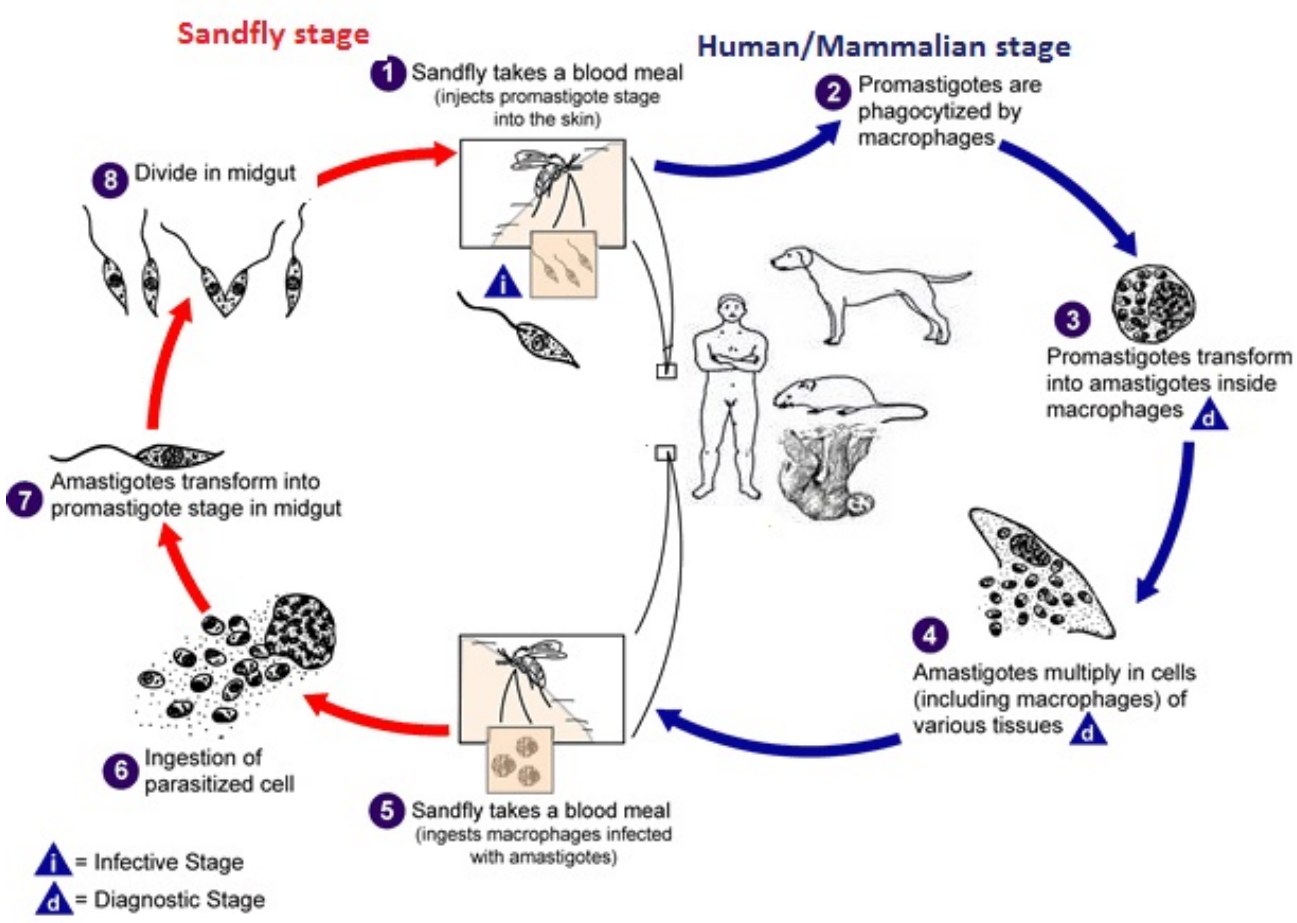

Figure 1. The life cycle of *Leishmania* spp. The sand flies inject the infective stage (1), metacyclic promastigotes, during blood meals. Promastigotes (2) that reach the puncture wound are phagocytized by macrophages and transform into amastigotes (3). Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species. This originates the clinical manifestations of leishmaniasis (4). Sand flies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes (5 and 6). In the sand flies midgut, the parasites differentiate into promastigotes (7), which multiply and migrate to the foregut (8). (modified from CDC, 2015)

1.2 Leishmaniasis in Ethiopia

Leishmaniasis is an important human disease in Ethiopia. All forms of the disease are known to exist with VL mainly distributed in lowland arid and semi-arid areas while the cutaneous forms, on the other hand, is widely spread throughout the country commonly in highlands and sporadic cases in lowland lying communities (Hailu et al., 2006b). The disease is regarded as 'disease of the poor' creating lots of social and economical burden in villagers who have limited health care access. Many species of sand flies occupy a vast area in the country and several species of the genus *Phlebotomus* are known to be abundant with a few species being identified as *Leishmania* vectors.

1.2.1 Cutaneous Leishmaniasis

Cutaneous leishmaniasis in Ethiopia remains a major neglected disease since its first report in 1913 and is a common public health problem in highland areas ranging from 1400 to 2700 m a.s.l. (Lemma et al., 1969; Ashford et al., 1973a; Hailu et al., 2006b). Ethiopian CL (ECL) represents various spectrums of clinical manifestations ranging from small cutaneous nodules to a full course of mucosal tissue destruction (Ashford et al., 1973a). The sole agent of ECL is *L. aethiopica*. However, rare cases due to *L. tropica* and *L. major* have been reported mainly in lowland regions and little is known about their epidemiological and transmission cycle (Hailu et al., 2006a; 2006b). The infection is transmitted by several species of phlebotomine sand flies with two members of the subgenus *Larroussius*: *P. longipes* and *P. pedifer* commonly identified as proven vectors of *L. aethiopica* from different parts of the county (Ashford et al., 1973a; Laskay et al., 1991). Moreover; *L. aethiopica* and *L. tropica* has been incriminated from two species in the subgenus *Paraphlebotomus*; *P. saevus* and *P. sergenti* from Awash Valley, northeastern Ethiopia (Gebre-Michael et al., 2004a). The only investigation of *L. major* natural infection in sand flies was reported in Aba Roba, southwestern Ethiopia from *Phlebotomus* (*P.*) *duboscqi* (Gebre-Michael et al., 1993). Studies to determine vectorial status and investigation of natural infection in other potential sand fly species that are prevalent in CL risk areas are ongoing (Table 2).

Ethiopian CL due to *L. aethiopica* is known to be zoonotic which the parasite is naturally harbored by two species of rock hyraxes (*Procavia capensis* and *Heterohyrax brucei*), however it has been believed that diverse groups of organisms could be involved in the epidemiology (Ashford et al., 1973a; Lemma et al., 2009).

The magnitude of ECL is usually unclear due to the attention given for this non-fatal disease and detailed information regarding its magnitude and epidemiology in the country is still incomplete. An extensive study has been conducted in the south and southwestern region of Ethiopia and along the rift valley in order to get complete mapping and epidemiological description of ECL in the country. The main transmission areas include the Ochollo highland focus in Gamo, southwestern; the Kutaber areas in Wello, eastern, Aleku area of Wollega, western; the Bale, Silti district and Sidamo highlands, southern; Sebeta area of Adiss Ababa, centeral; highland areas of Libo-kemkem and Damot district, northern; and Adigrat and eastern Tigray, northwestern Ethiopia (Hailu et al., 2006b). According to estimates from hospital records only, more than 20, 000 people per year are diagnosed for ECL all over the country. A recent outbreak in 2005 was reported in Silti district, which 4.8% prevalence was recorded exceeding the highest prevalence rate ever recorded in Ochollo in the 70's. The investigation of the Silti break describes a new focus of CL due to *L. aethiopica*, suggesting that parasite is possibly breaking out into new foci, spreading and establishing new endemic sites (Negera et al., 2008).

Another outbreak in the southern Ethiopia has been under investigation since its new cases of ECL detected in Sodo focus, with ecological profiles indicating the likelihood of *L. aethiopica* parasite being the cause of infection (Hailu et al., 2006b).

1.2.2 Visceral Leishmaniasis

Human VL, also known by local peoples as 'Kala-azar' in the North and as 'Goloba' or 'Lante' in southwestern Ethiopia, claims the lives of thousands in the past and still remains as one of the concern in many areas of the country. The disease is endemic throughout the lowlands of the country with various magnitude and transmission level. Alvar et al., (2012) estimated the annual incidence of VL in Ethiopia to be as high as 7000.

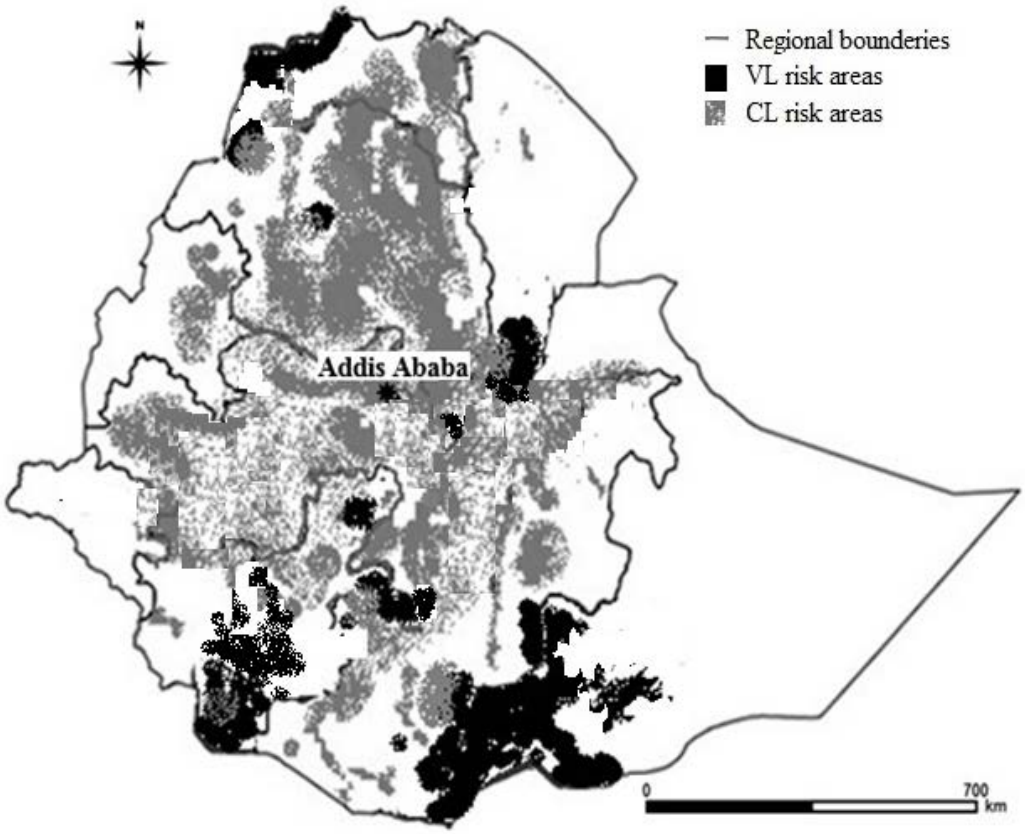

Figure 2. Visceral and cutaneous leishmaniasis risk regions of Ethiopia. Map modified and adapted from Leta et al., 2014; Seid et al., 2014; unpublished hospital records.

The main foci are found in the lowland areas of north, north-western, and south-western Ethiopia, with some sporadic cases in the central-east part of the country (Hailu et al., 2006b). Ethiopian VL is predominantly caused by *L. donovani* complex. The potential vectors of VL in the country include *P. orientalis*, *P. martini* and *P. celiae* (Gebre-Michael and Lane, 1996; Gebre-Michael et al., 2004b; Hailu et al., 2006a).

The southwestern Ethiopia is one of VL risk part of the country and remains endemic for the past couple of decades. It includes districts in the Omo plain, Segen- Weyto river Valley in Southern Nations and Nationalities People's Region (SNNPR) – all areas of lowlands with low rainfall. The Lower Omo plains are among the oldest areas in the southwest where VL has been reported for the first time. The level of exposure to the parasite in this region was reviled by Leishmanin

skin test (LST) and up to 80% of examined people were found positive (Hailu et al., 1996). Most of the population are nomadic pastoralist and develop certain sort of acquired immunity to the disease as indicated by high rate of LST.

Several surveys have been conducted in the Segen Valley since its first report in 1980's and it remains one of a major VL focus of the country. Most of the areas at risk of the disease transmission are characterized by termite mounds that are believed to have epidemiological importance in this region (Gebre-Michael and Lane, 1996). Children and young male are the most affected age groups in the district; showing a domestic and peri-domestic transmission of the disease (Ali and Ashford, 1994). Cases of VL in Aba-Roba peaks during the wet season from February to May and shortly after the end of the main rainy season from September to October (Gebre-Michael and Lane, 1996).

In Northwest the most important VL endemic areas are found in Metema and Humera lowlands, which accounts for approximately 60% of cases in the country. These endemic areas lay in Amhara and Tigray regional states, bordering to Sudan and Eritrea that are known in agro-ecological zones, with wide open plains covered by bush scrubs and *Acacia* woodland. The woodland cover is in process of being replaced by extensive commercial agriculture that produces sesame and cotton as the main cash crop (Gebre-Michael et al., 2010). Leishmanin skin test survey in Humera communities showed high positivity especially in farmers who stayed longer in the area. It is predominantly prevalent in young male workers, who are spending the night outdoors due to poor infrastructures and warm weather, thereby increasing their exposure to a possible sand fly bite (Fuller et al., 1976; Gebre-Michael et al., 2010). Generally, in the region a sharp rise in the number of VL cases has been attributed to the influx of seasonal temporary workers for the large-scale agricultural schemes and forced resettlements of populations from the neighboring highlands (Yared et al., 2014). A high HIV prevalence among seasonal workers has contributed to the rise in VL prevalence in this region. The highest HIV/VL co-infection rate in the world (~38%) was reported in this region (Hurisa et al., 2010). VL remains endemic in Humera-Metema foci and even in recent years Medecins Sans Frontieres (MSF) treatment centre treated 2000 to 5000 VL cases per year at one point in time. Recent spread beyond the Metema and Humera plains to villages in Tahtay Adiabo district and Sheraro

Kebeles, Welkaite and Armacheho districts in the Tigray and Amhara regional states has been reported. Especially in villages of Sheraro VL claimed the lives of hundreds and remains circulating since its first investigation. Returning daily laborers from VL endemic areas of Humera and Metema are believed to introduce the parasite in Sheraro focus (FMoH, 2013).

In 2005, a new focus of VL in the Libo Kemkem district, northern Ethiopia has been reported as an outbreak that claimed a large number of lives before its cause were identified. The disease appeared in Bura Kebele, one sub-district in Libo Kemkem that resulted 71% cases of the residents and rapidly spread to other neighboring villages (Alvar et al., 2007). In 2007 around 2450 primary cases has been reported since the beginning of the outbreak with majority of cases being treated at Addis Zemen health center, run by MSF (FMoH, 2013). The rapid spread of the disease was attributed through misdiagnosis of cases with drug-resistant malaria, which is endemic in the area (Herrero et al., 2009). Moreover, the focus is highland at about an altitude of 2000 a.s.l, where few cases of the disease had ever been reported in the area and never been endemic to VL. The better-known and classic endemic areas for VL in Ethiopia, such as the Omo, Segen and Abab Roba, Gelana and Woyto Valleys in the south and the Metema and Humera lowlands in the northwest, all lie at altitudes of 400 to 1400 m a.s.l. Even previous cases recorded in the neighboring Belessa Valley, in northern Ethiopia at an altitude of 1800 m a.s.l were believed to be travelers from the endemic areas of Metema and Humera lowlands (Ashford et al., 1973b). In early survey in Bura and Fogera villages, male migrant daily laborers who had travel history to Metema and Humera accounts 70% of the cases. Thus, these returnees from agricultural work from established transmission zones believed to introduce the disease to the Libo-Kemkem new foci (Bashaye et al., 2009; Herrero et al., 2009).

In wide areas of the northeastern Rift Valley of Ethiopia, sporadic VL cases have also been recorded, mainly in the Awash Valley and Afar regional states. The area, like the lowlands of the Humera-Metama endemic foci, is characterized by extensive agricultural development and influx of migrant laborers. The disease in this region is characterized and associated with HIV co-infection. The exposure rate as reviled by LST in Awash Valley in the 70's was as high as 59% which majority (95%) were young male (Ali, 1997). Subsequent surveys on disease exposure

revealed similar findings and it is unclear why the disease remains to be sporadic in this region in spite of high LST positivity (Hailu et al., 2006b).

Table 1. Distribution and epidemic records of VL transmitted by the three potential vectors of VL caused by *L. donovani* complex in Ethiopia.

Regional state	**Disease foci**	**Vector**	**Main outbreaks (area: year)**
SNNPR	- Omo plain - Segen Valley (Aba Roba focus) - Weyto Valley - Lake Abaya - Dawa Valley - Galena Valley	*P. orientalis* *P. martini* (confirmed) *P. celiae* (suspected)	
Oromia	- Moyale - Genale Valley	Not identified	
Somalia	- Afder & Liban districts	*P. orientalis*	In 2001 at border with Kenya and Somalia
Tigray	- Humera plains - Tahtay Adiyabo	*P. orientalis*	Humera: 1970s & 1996, now endemic Tahtay Adiyabo: 2005 – 2010
Amhara	- Metema plains - Libo Kemkem	*P. orientalis* (suspected)	Metema: 1970s & 1996 Libo Kemkem: 2003 – 2006
Afar	-Awash Valley	*P. orientalis* (suspected)	

1.3 Diagnosis of VL

Microscopic detection using Giemsa stain and in vitro culture of lymph node, spleen and bone marrow aspirates are still the gold standard diagnoses for VL in patients, but with low sensitivity and require invasive procedures and are difficult to repeat for follow-up of patients.

Currently, several serological diagnostic procedures, indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and, detection of anti-rK39 antibody using rK39 antigen strip test, direct agglutination test (DAT) (Kar, 1995; Srividya et al., 2012; FMoH, 2013), have come, even if these methods have their own limitations. Poor sensitivity particularly in case of HIV co-infected patients, not being helpful in diagnosing relapse, and lack of specificity as some of the *Leishmania* antigens cross-react with antigens of other organisms overlapping in VL endemic areas are some among the major drawbacks to use these serological tests (Chappuis et al., 2007).

More importantly, neither parasitological nor serological tests distinguish *Leishmania* species, and cannot detect multiple infections (if any) and low level of parasite, which is crucial, to determine distinct treatment regimes and for successful prediction of relapse or post kala-azar dermal leishmaniasis (PKDL) (Schonian et al., 2003).

The detection of parasite by PCR, thus, became a useful diagnostic technique. Detection by PCR has superior sensitivity of diagnosing or detecting very low number of parasites and diagnosing species responsible for VL (Cruz et al., 2002; Schoenian et al., 2003; Cortes et al., 2004). Besides, PCR has proved to be useful in identifying cases of PKDL and mixed infection of parasites, which rarely persists in culture (Ibrahim et al., 2002). Furthermore, PCR-based diagnosis is helpful for monitoring and follow-up especially in case of *Leishmania*/HIV co-infected patients (Cruz et al., 2002). Therefore, PCR-based diagnosis methods are useful to circumvent problems associated with both parasitological and serological methods. Nevertheless, simple rapid and low cost PCR-based diagnoses are not still available for developing countries where the disease costs the life of thousands.

Generally in the case of large scale epidemiological and community based cohort studies and to determine a role of asymptomatic infection pervious methods were complicated and usually involve invasive procedures (Chappuis et al., 2007). There are several PCR protocols that brought good result and among them real time PCR (qPCR) has been proven as a method of choice. Several target genes: kinetoplast DNA (kDNA) minicircles (Gramiccia at al.,1992; Nicolas et al., 2002; Selvapandiyan et al, 2008), the small subunit rRNA gene (van Eys et al., 1992), internal transcribed spacer 1 (ITS1) (Campino et al., 2000; Schoenian et al., 2003; Talmi-

Frank et al., 2010; Toz et al., 2013) and spliced leader sequence (Campino et al., 2000; Schoenian et al., 2003) existed and used to describe infection in host samples. However, some target gens were sensitive but couldn't identify the species of the parasite and thus more specific procedures and genes has been employed to fill the gap. Some of these procedures and techniques have been employed in our epidemiological studies and the details of a PCR protocol were dealt and presented in this thesis.

1.4 Etiology of VL and its diversity in Ethiopia

Three species of the genus *Leishmania* belonging to the same species complex, *L. donovani*, *L. infantum* and *L. archibaldi*, have been incriminated as ethologic agent of VL in Ethiopia and Sudan using isoenzyme analyses (Rioux et al., 1990). However, *L. archibaldi* is thought to be a synonym to *L. donovani* (Zemanova et al., 2007). Recent studies by RAPD (Zemanova et al., 2007), chitinase gene sequence analysis (Jamjoom et al., 2004) and ITS sequences analyses (Kuhls et al., 2005) did not support the placement of *L. archibaldi* as distinct species and suggested the necessity of a re-evaluation of the *L. donovani* complex taxonomy. Though much work has not been done in Ethiopia on characterization and identification of members of *L. donovani* complex as well as their degree of public health importance, *L. donovani* is thought to be the most frequently isolated species from VL patients in the southwest regions (that is, Lake Abaya, Konso and Omo river plains) and is principally anthroponotic (Chappuis et al., 2007). On the other hand, in northwest regions, Humera-Metema, including the new epidemic region, Lebo-Kemkem area, *L. infantum* is expected to be the most important and thought to produce zoonotic leishmaniasis (Alvar et al., 2007).

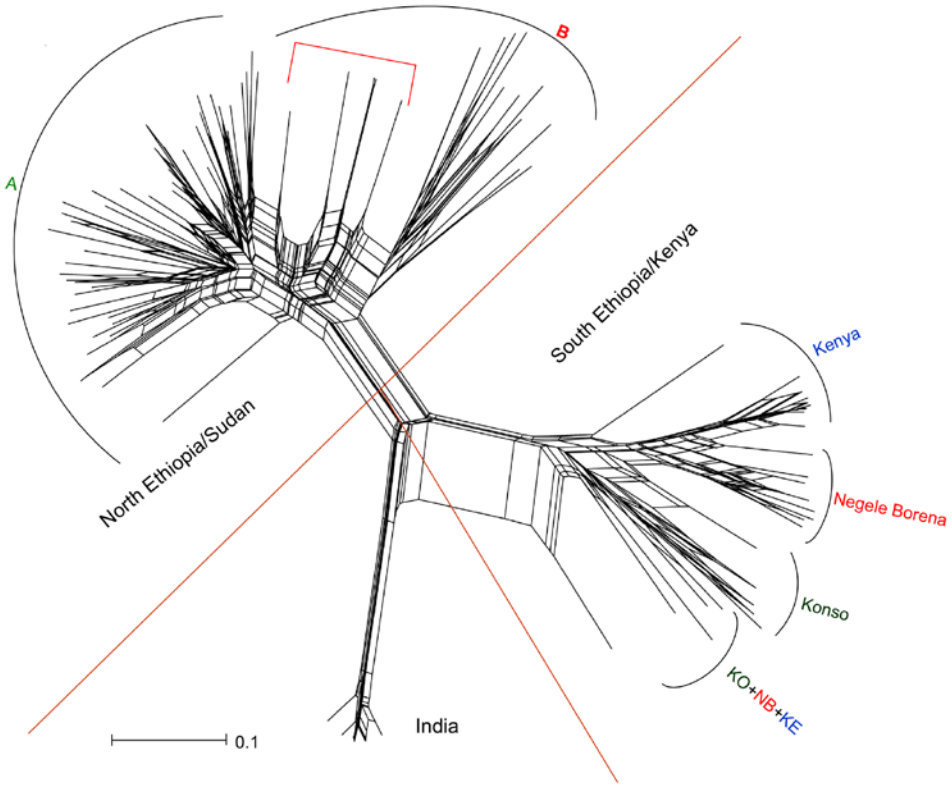

Figure 3. Population structure of East African *L. donovani* complex as revealed by using 14 highly polymorphic microsatellite markers. The phylogenetic network showed three main populations as well as the two subpopulations (A and B) in the North Ethiopia/ Sudan population and the four clusters, Konso, Negele Borena, Kenya and KO+KE+NB in the South Ethiopia/Kenya population. The reticulate patterns seen in the network indicates either hybridization or recombination events between similar or closely related genotypes. The putative hybrid genotypes are indicated by a red bar line on the top. They are located in between their hypothetical parental strains in NE/SD-A subpopulation A and in cluster B1 of the NE/SD subpopulation B. (Gelanew et al., 2010)

Using a highly discriminatory multilocus microsatellite typing (MLMT) typing approach, the east African *L. donovani* showed remarkably high genetic diversity. The parasite isolated from patients from various region of Ethiopia were compared among the East African strains and grouped into two genetically and geographically distinct populations comprising parasites from Southern Ethiopia and Kenya, and those from Northern Ethiopia and Sudan (Gelanew et al., 2010) (Fig 3).

Genetic heterogeneity using aforementioned molecular markers may correlate with phenotypic difference, which can be associated with a diversity of clinically important manifestations. For instance, the cpb and gp63 genes are associated with parasite virulence and thus they could probably be used to associate genetic polymorphism with clinical outcomes (Quispe Tintaya et al., 2004). Thus, further knowledge about the population structure of *L. donovani* parasites in this region is vital for the design of parasite-targeted control strategies which aim to eradicate VL in East Africa. A finding on the genetic variation between endemic regions based on a putative surface antigen of Ethiopian *L. donovani* isolate is shown and presented in this thesis.

1.5 Sand Fly Vectors and Eco-epidemiology of VL in Ethiopia

Studies on the status, eco-epidemiological importance of sand flies in Ethiopia starts back to 1930s mainly laying on identification based on morphological parameters. This led to the discovery of the first four species in various regions of Ethiopia: *P. orientalis* and *P. martini* (Parrot, 1936) and *P. longipes* and *P. saevus* (Parrot and Martini 1939). Since then at least 19 species in the genus *Phlebotomus* in six genera are so far known to exist in Ethiopia, some of which are proven vectors of CL and VL (Table 2). However, due to their leishmaniasis epidemiological role and importance, description and taxonomy of *Sergentomyia*, the most abundant genera of subfamily Phlebotominae; is by far limited. Several species of the genus *Sergentomyia* are known to exist in Ethiopia (Abonnenc and Minter, 1965; Gebre-Michael et al., unpublished). The recent experimental approach on colonies of Sergentomyia originated from VL endemic regions of Ethiopia proved that the genus is not involved in human *Leishmania* transmission (Sadlova et al., 2013).

1.5.1 Vectors in Major VL: Focus on *P. orientalis*

The main VL vectors in Ethiopia are *P. martini* and *P. celiae* in the south (Gebre-Michael and Lane, 1996) and *P. orientalis* in the SW (Hailu et al., 1995) and most likely a potential vector in northwest and northern Ethiopia (Gebre-Michael et al., 2010). The detailed entomological investigations in the Segen Valley focus showed that *P. martini* and it closely related species *P. celiae* were dominant species. Both species are proven vectors of *L. donovani* in the south and southwestern VL foci but *P. martini* is considered as a principal vector (Gebre-Michael and

Lane, 1996). In certain areas, especially the lower Omo river basin, there is an overlap of *P. martini*, *P. celiae* and *P. orientalis*, of which *P. orientalis* appeared to be dominant and incriminated as a vector (Hailu et al., 1995). The other region where overlap of these three species observed is in the Ethiopian central Rift Valley. Despite no cases of VL recoded, the importance of vectors was noted by high rate of LST positivity in the population (Hailu et al., 2006b).

Several studies implicate *P. orientalis* as the most probable vector in Humera and Metema endemic areas of northwest (Gemetchu et al., 1975; Gebre-Michael et al., 2010; Lemma et al., 2014), also being the main vector the neighboring country, Sudan (Elnaiem et al., 2011). The species is also highly suspected in the recent outbreak area of Libo district (Gebre-Michael et al., 2007) as well as in Belessa highland valley in the north (Ashford et al., 1973b). However, its role as a vector has not been proven in any of these endemic areas. *P. orientalis* has been assumed as anthropophilic, yet no evidence exists to refute the earlier assumption of zoonosis with occasional spill-over to human (Gebre-Michael and Lane, 1996). Blood meal analysis of *P. orientalis* in a Metema-Humera endemic locality showed zoophagic behavior of the vector and suggested its epidemiological role (Gebre-Michael et al., 2010; Lemma et al., 2014; Lemma et al., 2015). The species bites predominantly outdoors, especially in Acacia forest, forest edges as well as in farm fields near villages with cracking black cotton clay soil (vertisol). However, how and when people are mostly bitten remains unclear (Gebre-Michael et al., 2010). The extensive entomological survey in the Metema-Humera endemic site revealed several suggestive explanations on the behavior of the potential vector of *P. orientalis*. Due to the life-style of residents of the area *P. orientalis* is essentially exophagic and rarely enters to houses to feed (Gebre-Michael et al., 2010; Lemma et al., 2014; Lemma et al., 2015). People in the area either used to sleep outdoors to look after their cattle and/or due to the hot weather condition or during stay at home use smoke for cooking that could repeal the vector out. On the other hand sand flies could be distracted by the abundant cattle and reduce the man-sand fly contact. Thus, multiple factors must come into play to determine the feeding behavior *P. orientalis* in this focus (Gebre-Michael et al., 2010). In addition, the distribution of *P. orientalis* in Metema-Humera is positively influenced by the distribution and abundance of Acacia-Balanites woodland together with black cotton soil that cracks wide to provide shelter for developing and resting sand flies

(Gebre-Michael et al., 2010; Lemma et al., 2014). Agricultural daily laborers usually spent most of the night under these trees during crop harvesting periods that could significantly increase the sand fly-man contact in a per domestic setting (Fuller et al., 1973; Gebre-Michael et al., 2010). Large-scale agricultural extensions for sesame and cotton production substitute the already existing Acacia-Balanite vegetation and brought ecological changes, however, *P. orientalis* remains to be a major sand fly in the foci (Gebre-Michael et al., 2010; Lemma et al., 2014).

Table 2. Identified *Phlebotomus* species in Ethiopia and their vector status. Subgenus: *L. – Larroussius*; *P. – Phlebotomus*; *Pa. – Paraphlebetomus*; *S. – Synphlebotomus*; *Ad. – Adlerius*; *An. – Anaphlebomomus*. Vectorial status potentially suspected based on eco-epidemiological parameters (Dr. T Gebre-Michael; personal communication): "+" vectorial status proved; "-" vectorial status not proved; "?" no information about vectorial status.

Species	**Status**	**Disease**	**Agent**
P. (L.) oreintalis	+	VL	*L. donovani*
P. (L.) longipes	+	CL	*L. aethiopica*
P. (L.) pedifer	+	CL	*L. aethiopica*
P. (L.) aculeatus	-	-	-
P. (L.) ashfordi	?	CL	?
P. (L.) gibiensis	-	-	-
P. (L.) fantalensis	-	-	-
P. (P.) papatasi	-	-	-
P. (P.) duboscqi	+	CL	*L. major*
P. (P.) bergeroti	-	-	-
P. (Pa.) sergenti	+	CL	*L. tropica & L. aethiopica*
P. (Pa.) saevus	+	CL	*L. tropica*
P. (Pa.) alexandri	-	-	-
P. (Pa.) mireillae	-	-	-
P. (Pa.) gemetchi	-	-	-
P. (S.) martini	+	VL	*L. donovani*
P. (S.) celiae	+	VL	*L. donovani*
P. (Ad.) arabicus	+	?CL	*?*
P. (An.) rodhaini	-	-	-

The VL outbreak occurred in the highland region of Libo Kemekem highly hypothesized as the introduction of the parasite from endemic sites and establish local transmission with the already existing sand flies (Alvar et al., 2007; Bashaye et al., 2009). Entomological survey conducted in 2006 revealed the existence of *P. orientalis* as a dominant and abundant species among the other *Phlebotomus* species. It has been suggested that this species is a main or sole potential vector

involved during the outbreak. Unlike the Metema and Humera endemic sites, the risk factors associated with the disease transmission in Libo Kemkem is an indoor biting and is associated with dog ownership. Wide cracking, vertisol black cotton soil is evident in the district and claimed to provide a suitable habitat for *P. orientalis* (Gebre-Michael et al., 2007).

In the new VL focus of Sheraro, Tahtay Adiyabo district, northwest Ethiopia, entomological surveys revealed the dominance of *P. orientalis* that breed in deeply cracked vertisols commonly in open fields and tree-related habitats (Moncaz et al., 2014a). Blood meal analysis on *P. orientalis* caught indoor revealed high percentage of bovine blood meal (35%) next to human blood (26%). In villages, houses are built from stones usually un-plastered that are close to animal enclosures and are situated close from farm fields many of which are in vertisols. Emergence from gaps in stonewall in peri-domestic habitats could implicate that the house wall materials could serve as a resting or breeding habitat for *P. orientalis* (Gebresilassie et al., 2015b).

1.5.2 Ecology and Variability of *P. orientalis*

The ecological preference of sand flies differs accordingly with rainfall, humidity; temperature, soil type and moisture content, and land and vegetation cover type (Moncaz et al., 2012; Moncaz et al., 2014a; b). Thus, there distribution pattern is significantly associated with these ecological cue. However, for some species, like *P. orientalis*, no universal pattern has been established so far (Gebre-Michael et al., 2004b). In Ethiopia, *P. orientalis* has been observed in a range 600 to 1930 m a.s.l. (Ashford et al., 1973b). In some places it seems that its distribution is positively influenced by the presence of Acacia-Balanites vegetation and cracks in black cotton clay soil, for instance in Metema-Humera (Moncaz et al., 2014a; Shabtai et al., 2014). In the Omo and Awash river Valleys, and in the highlands of Belessa and Libo Kemkem, black cotton soil was also claimed to form the habitat for *P. orientalis* (Hailu et al., 2006b). However, in other places, like Tahtay Adiyabo foci, *P. orientalis* is associated with different microhabitat (Gebresilassie et al., 2015b)

Humera and Metema lie at altitudes of 500 to 700 m a.s.l. and ecological changes recently are observed where the previous dominant vegetation, *Acacia seyal – Balanites aegyptiaca* forest

been immensely cleared for large scale agricultural production of cash crops (cotton and sesame), and more recently and intensively for settlement (construction) and fire wood as well. Investors with large number of cattle, sheep and goats are also raised in the regions through agricultural extension programs (Gebre-Michael et al., 2010; Argaw et al., 2013). Despite all these major ecological changes *P. orientalis* is still a major potential vector of *L. donovani* in the area (Gebre-Michael et al., 2010). Observation of male sand flies with un-rotated genitalia suggested that deeply cracked black cotton soil is likely to be the most productive breeding habitat for this sand fly (Moncaz et al., 2014a; Lemma et al., 2014).

The new focus in the semi-arid lowlands of Tahtay Adiyabo district Tigray Regional State, northern Ethiopia became the most important focus in the epidemiology of VL transmitted by *P. orientalis* (Gebresilassie et al., 2015b). Villages at risk of VL transmission are located on hilly outcrops (peri-domestic) with sandy clay loam soil, that does not form cracks and are surrounded by large fields many of which are chromic vertisols soil, characterized by high contents of smectites clay minerals (Shabtai et al., 2014; Moncaz et al., 2014a). Agricultural fields are on the periphery of human residence with scattered and mixed trees of *Acacia-Balanites-Zyzpus-Combretum* trees and some scrub vegetation. However, similar to the Metema-Humera foci, the area was once covered with natural forest, but because of various human activities like cultivation, grazing, and wood cutting the natural vegetation have been reduced to a few scattered clumps of trees. Even though *P. orientalis* was reported in peri-domestic areas, high number of catch was found in agricultural fields (Moncaz et al., 2013; Moncaz et al., 2014a; b; Gebresilassie et al., 2015b).

Entomological studies at the highland VL focus of Libo Kemekem district showed that *P. orientalis* had potential to play a role for the establishment of infection mainly due to its high abundance and even in many sites being the only *Phlebotomus* species out of the catches (Gebre-Michael et al., 2007). Even though human activity changed the ecology of the areas similar to the lowland focus, few scattered clumps of acacia trees with the non-indigenous *Eucalyptus* trees are found around homesteads (Bashaye et al., 2009; Herrero et al., 2009). The district is mostly a flat plain characterized by black soil that get water-logged during rainy seasons and leave deep cracks in the soil surface when dried out. This potentially could serve as a breeding site for the

vector as observed in similar soil profile and where *P. orientalis* occur (Moncaz et al, 2014a). Being high land at an altitude of 1800 to 2000 m a.s.l., the region is known with cases of CL infection due to *L. aethiopica*. Thus, it seems possible that in certain localities of the district *P. longipes*, a proven highland *L. aethiopica* vector, and *P. orientalis*, a well known lowland species co-exist (Gebre-Michael et al., 2007).

In the southwestern VL endemic foci *P. orientails* overlap with the other two vectors *P. martini* and *P. celiae* (Hailu et al., 1995). In Omo Valley dominancy of *P. orientalis* has been observed and was also a dominant man-biter that has long been suspected as a vector of VL in the area (Fuller et al., 1976). The ecology of *P. orientalis* in this focus differs from the northern and its detailed ecological associations are drawn with *P. martini* habitat (Gebre-Michael et al., 2004b).

Generally, *P. orientalis* is a widely distributed species in the country that is found in variable habitats and associated with a wide range of vectorial capacity, feeding behavior and variable abundance in microclimatic changes. Generally, due to their poor dispersal capacity, populations of sand flies are expected to show some genetic structuring along their geographical range as a result of adaptation to local habitats and limited gene flow (Ready, 2011). Such genetic variability could play an important role in the epidemiology of the disease. In view of this, we studied if ecological and geographical difference has role in the genetic profile, biology and vector competence of *P. orientalis* colonies originating from two geographically distant Ethiopian localities, Addis Zemen and Melka Werer (with or without *L. donovani* occurrence respectively). The detailed procedures, findings and suggestive and scientific outlines are compiled and published in a peer reviewed journal and represented in this PhD dissertation.

1.6 Risk Factors for VL Infection in Ethiopia

Generally east African VL is more closely related to the movement of seasonal workers and new settlements to endemic or highly risk transmission zones (Desjeux 2004). This is hampered by poor working conditions and infrastructures provided for laborers (Herrero et al., 2009). It generally result humans into contact with natural vectors and increased infection rates. Studies in VL endemic regions have indicated that LST positivity has strong association with increasing age, occupation and sex (Fuller et al., 1976). Entomological and epidemiological studies also

revealed that outdoor biting and sylvatic transmission as major source of infection (Ali and Ashford, 1993). In addition to the soil (black cotton soil), vegetation type (Acacia-Balanite vegetation), presence of termite hills and migration to endemic foci for seasonal agricultural work, different behavioural, household and environmental factors have been implicated as risk factors for VL in Ethiopia (Fuller et al., 1976; Bashaye et al., 2009; Lemma et al., 2014;). All these factors increase the contact between man and sand flies. However the role of reservoir hosts in Ethiopian VL remains unclear and not given much emphasis as a risk factor. This arises from pervious general suggestions that East African VL caused by *L. donovani* is being considered as anthroponotic (Chapuis et al., 2007). Despite no clear evidences provided clear cut information on a different transmission route of VL, there are indications on the direction that animals' involvement may contribute a certain role in its cycle. In the Libo Kemekem areas one of the factor associated with clinical VL were dog ownership (Bashaye et al., 2009). In Humera endemic focus similar scenario documented that goat ownership and sleeping near dogs was among the odds for clinical VL (Yared et al., 2014). In neighboring country Sudan where VL due to *L. donovani* is one of a public health problem, dogs and rodents are considered as risk factors for disease transmission (Dereure et al., 2000; Dereure et al., 2003; Hoogstraal and Heyneman, 1969) These and similar studies so far implicated that animals in VL transmission could have risk or protective factor in its eco-epidemiology.

1.7 Reservoir Hosts

The suitability of a given mammalian hosts for the maintenance of *Leishmania* parasites depends on many factors, the most important of which are the host population density, the duration of the infection (and longevity of the host), the location of the parasites within the host, and the immune status of the host. The term 'reservoir host' should be restricted to the species that sustain the reservoir system in which a parasite survives indefinitely (Ashford, 2000).

The magnitude of the health problem represented by leishmaniasis combined with the complexity of its epidemiology make it necessary to clarify all of the links in transmission net, including non-human mammalian hosts, to develop effective control strategies. In many geographic areas where human leishmaniasis is found, infected people are not needed to maintain the transmission cycle of the parasite in nature; other infected animals (mammals),

along with sand flies, maintain the cycle. However, in some areas infected people are needed to maintain the cycle where effective treatment of individual patients can help control the spread of the parasite. For example in the cases of Post kala-azar dermal leishmaniasis (PKDL), is a complication of VL characterized by a rash in patients who has recovered or otherwise well from the disease, probably has an important role in interepidemic periods of VL, acting as a reservoir for parasites (Zijlstra et al., 2003; CDC, 2015).

Although studies have described dozens of mammalian species infected with *Leishmania* parasites, however only few are related their findings to the ecological scenario to indicate a possible role of that host in parasite maintenance and transmission (Ashford, 2000). Currently, it is accepted that a reservoir may be just one or a complex of several species responsible for maintaining the parasite in nature. A reservoir system should be considered unique on a given spatiotemporal scale. In fact, the transmission of *Leishmania* species in the wild still represents a complex scenario, as several links have not been identified.

So far 13 species of *Leishmania* infecting humans are known to have at least one or otherwise more species in the life cycle as a reservoir host (Gramccia and Gradoni, 2005; Ashford, 2000) (Table 3). The most important mode of transmission of VL caused by *L. donovani* is believed to be almost anthroponotic. While this scenario is still valid in India, the situation in East Africa has been changed in last decades. In recent years, dogs infected with *L. infantum* were recorded in a VL focus in Ethiopia (Alvar et al., 2007). In Eastern Sudan dogs infected with *L. donovani* and *L. infantum* were implicated as reservoir hosts for VL (Dereure et al., 2000; Dereure et al., 2003). Mongooses and rodent species were found infected with *L. donovani* in Eastern Sudan and suggested to comprise natural reservoirs of zoonotic transmission in uninhabited regions and the source of infection in people when encroaching upon uninhabited areas (Elnaiem et al., 2011; Hoogstraal and Heyneman, 1969). Sylvatic transmission is far less amenable to control interventions than domestic transmission (Gavgani et al., 2002). However, there may be means of distancing sylvatic foci from human settlements in order to reduce chances for introduction of parasites into human habitation and the establishment of anthroponotic foci.

***Leishmania* species**	**Geographical distribution**	**Vector**	**Reservoir host**	**Natural infections in mammals**	**Habitat**
L. tropica	Asia, Middle East, Israel, Turkey	*P. (Pa.) sergenti; P. (A.) arabicus*	Humans	Dog (Cutaneous leishmaniasis) Rodent: *Rattus rattus*) Rock hyraxes: Procavia capensis Golden jackals Red foxes	Densely populated cities
	Equatorial and southern Africa, Ethiopia, Kenya and Namibia	*P. (L.) guggisbergi* *P. (Pa.) sergenti* *P. (Pa.) saevus*	Probably rock hyraxes (*Procavia capensis*)	Rodents: *Acomys* spp., *Arvicanthis* spp., *Gerbillus nanus.* Bats	Rocky places in semi-arid areas
L. major	North Africa and south-west Asia, from Algeria to Saudi Arabia	*P. (P.) papatasi*	Fat sand-rat (*Psammomys obesus*) (epidemic maintained by *Meriones shawi*)	Rodents: *Gerbillus* spp., *Meriones shawi, M. libycus, M. crassus, Nesokia indica*	Saline depressions with Chenopodiaceae
	Central Asia from Iran to Uzbekistan	*P. (P.) papatasi*	Great gerbil (*Rhombomys opimus*)	Numerous desert mammals Rodents: *Tatera indica, Mus musculus,* and *R. rattus*	Alluvial fans with loess deposits
	West Africa to Kenya, Ethiopia	*P. (P.) duboscqi*	Relative importance of different hosts to be determined	Rodents: *Arvicanthis* spp., *Praomys* spp., *T. robusta, Aethomys kaiseri, Taterillus emini, Xerus rutilus, Cercopithecus aethiops* Bats	Sahel savannah
L. aethiopica	Highlands of Ethiopia and	*P. (L.) longipes* and *P.*	Rock hyraxes (*P. capensis*)	Rodents: *Cricetomys gambianus*	Cliffs and rocky areas, between 1500 and

	Kenya	*(L.) pedifer*	and *Heterohyrax brucei*		2600 m a.s.l.
L. donovani donovani (or *L donovani* complex)	Central and southern Sudan	*P. (L.) orientalis*	Presumably zoonotic but reservoir host uncertain	Rodents: *Arvicanthis niloticus, Acomys cahirinus, Praomys natalensis, R. rattus*, serval cat (*Felis serval*), genet (*Genetta genetta*)	Alluvial flat lands with forest of *Acacia seyal* and *Balanites aegyptiaca*
	Northern Kenya, Ethiopia	*P. (S.) martini* and possibly *P. (L.) orientalis*	Presumably zoonotic but reservoir host unknown	Rodents: *Arvicanthis Mastomys erythroleucus Gerbilliscus nigricaudus* Domestic animals	Semi arid bush, on laterite, with termitaria, Black cotton soil
	India, Bangladesh, Nepal	*P. (Eu.) argentipes*	Humans only	Domestic animals	Villages on alluvial plains
L. donovani infantum (syn. *chagasi*)	Southern France, Italy, Cevennes hills; Greece	*P. (L.) ariasi P. (L.) perniciosus*	Dog (Viscerocutaneous leishmaniasis)	Fox *Vulpes vulpes;* Rodents*: R. rattus; R. norvegicus*	Forested areas at middle altitude
	Central and western Mediterranean basin, Portugal, N. Africa	*P. (L.) perniciosus*	Dog (Viscerocutaneous leishmaniasis)	Fox *Vulpes vulpes*; Rodents: *R. rattus*; *M. musculus* and *R. norvegicus*	Villages and suburbs, with calcareous outcrops, in sub-humid bioclimatic zone
	Middle east; Iran	Various	Dog (Viscerocutaneous leishmaniasis)	Racoon dog (*Nyctereutes procyonoides*) foxes and jackals	Various
	Central and South America	*L. longipalpis; L. evansi*	Dog (Viscerocutaneous leishmaniasis)	Fox	Villages and homesteads in semi-arid areas

Table 3. Vectors and reservoir hosts that maintain the various *Leishmania* species in different habitats and geographical areas of Old World leishmaniasis foci (modified from Ashford, 2000).

1.7.1 Role of Domestic Animals in VL

Several studies showed the importance of domestic animals in leishmaniasis epidemiology. Dogs are the most important species among domesticated animals in the epidemiology of VL. Canine and human leishmaniasis caused by *L. infantum* has become a major zoonosis in Asia, the Mediterranean basin and the Americas which are potentially fatal to humans and dogs. Thus, dogs comprise the main reservoir of infection to humans transmitted by *Lu. longipalpis* in the New World and several *Phlebotomus* species including *P. perniciosus, P. ariasi, P. neglectus* in the Old World (Gramiccia and Gradoni, 2005).

In the east African VL endemic region dogs become very important risk factors of the disease. High seroprevalnce in endemic foci has been reported in Sudan and Ethiopia. According to a review by Dereure et al. (2003), in VL endemic focus Sudan, several investigations in the past has demonstrated *Leishmania* infection in domestic dogs. These repeated findings brought several suggestive hypotheses for the transmission dynamics of VL in Sudan as: man is responsible for the disease in dogs, dog is the reservoir of VL and as dog is an intermediate host between a possible sylvatic cycle and the anthroponotic cycle (Dereure et al., 2003).

Domestic dogs in many Ethiopian villages are almost entirely unrestrained, but most stay around human dwellings. These dogs frequently feed on garbage in the villages and take refuge under porches, granaries and other shelters. These characteristics are typical of village dog populations around the world; however, the overall density of dogs in Ethiopian villages is low when compared to many other countries (Ortolani et al., 2009). Thus, their role as reservoir hosts may be through the “introduction” of parasites from the natural environment where some type of transmission may be taking place. In Humera VL endemic site increased risk factor to VL has been associated with residents who slept with dogs (Argaw et al., 2013). In Libo Kemkem, a survey on dogs from owners with previous and/or active Human VL cases showed a seroprevalence rate of 3.8%. Molecular analysis of a bone marrow aspirate revealed *L. donovani* complex DNA in this village. Based on these and other factors association has been drown that animal ownership in Libo Kemekem resident especially dog ownership was linked to increased risk for VL (Bashaye et al., 2009).

According to Ashford (1996), a certain reservoir host must fulfill several criteria, including abundance, distribution, longevity, and close association with both human and vector. Following domestic animals, cattle, goat, sheep and donkey, in most African countries fulfill at least one of the criterions to have role in VL epidemiology. In most VL endemic foci of Africa domestic sanitary conditions, for example lack of waste management and open sewerage may increase sand fly breeding and resting sites, as well as their access to humans (WHO, 2015). Sand flies are attracted to crowded housing as these provide a good source of blood-meals.

Being a preferable host for *P. argentipes,* cattle were shown to play an undecided role in several epidemiological studies in the Indian subcontinent. For example, Ownership of cattle in Nepal and its density in Bangladesh were found to be protective (Bern et al., 2010). Whereas, increased risk of VL was found to be associated with the density of cattle or its ownership in India (Barnett et al., 2005). In a recent study in Nepal, *Leishmania* DNA was detected in several domestic animals including cattle from an endemic area (Bhattarai et al 2010). Serological evidences of anti-*L. donovani* antibodies in different domestic animals including cattle were reported in Sudan (Mukhtar et al., 2000). These and previous evidences from the endemic foci of Ethiopia showed domestic mammals (sheep, goats, cattle and donkey) in endemic area are possibly have role in the disease epidemiology. To explore the potential epidemiological status of domestic animals in VL, this thesis presents the published result of an extensive study in areas of active VL transmission in the northern Ethiopia.

1.7.2 Wild Animals in VL transmission

Several groups of wild mammals has been suspected and proved to be reservoirs of *Leishmania* parasite in different parts of the world (Table 3). Natural infections as revealed by different techniques suggested the epidemiological roles of these mammals in leishmaniasis cycle. Among them rodents are well-investigated both in the New World and Old World leishmaniasis foci. Several species of rodents are found to be reservoirs or at least naturally infected by the parasites. Wild canids and rock hyraxes are also epidemiologically proven reservoirs of several *Leishmania* species that are found repeatedly naturally infected (Ashford, 1996; 2000; Gramiccia and Gradoni, 2005). It has been indicated that other animals including hares, bats and marsupials

maintain the parasite and sustain sylvatic transmission in several countries where the diseases is endemic. Such findings open attentions for further studies to assess other possible sylvatic reservoirs of the parasite.

In view of these points, an investigation of the importance of zoonotic transmission as an “inter-epidemic” maintenance mechanism is definitely warranted. Within the framework of a project on the ecology and transmission dynamics of VL in Ethiopia we have investigated the role of both wild and domestic animals in Ethiopian VL epidemiology through measurement of their natural exposure to the parasite or sand fly. Detailed background and literature reviews of previous finding on the role of wild and domestic animals are reviewed in this thesis in our published articles.

2. Rationales of the research in the framework of this PhD

Leishmaniasis represents a complex and heterogeneous disease that emerged as epidemiologically important infection in Ethiopia. Over the years the burden is increasing and spreading out to new sites pausing several unanswered questions that make control of the disease more difficult (Hailu et al., 2006b). Visceral leishmaniasis is of a higher priority than CL as it is a fatal disease if left untreated (Desjeux, 2004). *Leishmania donovani* is regarded as the major cause of VL in Ethiopia that is transmitted by three major vectors, *P orientalis*, *P. martini* and *P. celiae*. The most important VL endemic areas in Ethiopia are found in the northwest (Metema-Humera lowland), which accounts for approximately 60% of cases, and in southwest Ethiopia (Lake Abaya, Omo river plains and Segen and Woito valleys) (Hailu et al., 2007). In recent years, VL has spread to new places like the highlands of Libo-Kemkem district (south Gondar) (Alvar et al., 2007; Gebre-Michael et al., 2007) and Tahta Adiyabo districts (FMoH, 2013) claiming the lives of hundreds. The spread of VL to unusual highland habitats and new foci was hypothesized to have been due to the introduction of the parasite, probably on multiple occasions, by migrant agricultural laborers returning to their villages from seasonal work from VL endemic areas (Alvar et al., 2007; Bashaye et al., 2009).

Rationale 1

Visceral leishmaniasis and post kala-azar dermal leishmaniasis (PKDL) patients and perhaps asymptomatic carriers, are the likely reservoir hosts of (at least partially) anthroponotic *L. donovani* (Thakur and Kumar, 1992). Parasites in VL patients are present in the blood making them available to biting sand flies (Rohrs, 1964; Sharma et al., 2000). PKDL is a complication of VL characterized by a macular, maculopapular, and nodular rash in patients who recover from VL. As many as 50% of the Sudanese VL patients treated become PKDL patients that harbor parasites in their skin where they are accessible to biting sand flies (Zijlstra et al., 2003). In Ethiopia, the rates of PKDL amongst treated patients are much lower but asymptomatic infections are common and may well be of importance as parasite reservoirs (Ali and Ashford, 1994; Chappuis et al., 2007). Although this assumption has not been demonstrated experimentally, in India amastigotes were found in the blood of asymptomatic persons living in

endemic regions (Sharma et al., 2000). In Sudan, *L. donovani* was demonstrated in the skin, causing a primary leishmanoma (Adler et al., 1966), and in Brazil asymptomatic carriers of *L. infantum* (the causative parasite of VL in Latin America and other regions) were shown to harbor parasites in their skins (Costa et al., 2002).

Approximately 30 to 50% of the patients with clinical symptoms of VL can be diagnosed using either parasitological or serological methods. The former relies primarily on microscopic examination of stained smears of spleen aspirates that gives a sensitivity up to 96% (Diro et al., 2007). For serological diagnosis of VL and PKDL, three simple tests are used: (i) Freeze Dried - Direct Agglutination Test (FD-DAT), (ii) rK39 strip test and (iii) latex urine agglutination (KAtex). A multi-center comparison of these assays demonstrated that while FD-DAT and rK39 tests are highly reliable in the Indian subcontinent, achieving >95% sensitivity and >90% specificity, they are less useful in Africa (Boelaert et al., 2008). In Africa, the FD-DAT showed higher sensitivities (86–99%) and specificities (82–98%) than the rK39 test (sensitivity 75–85% and specificity 70–92%). However there was considerable variation in these parameters depending on the origin of the patients (Ethiopia, Kenya or Sudan). The specificity and sensitivity of serological diagnosis can be improved if rK39 and DAT are used in series (ter Horst et al., 2009). However an urgent need exists for better diagnostic tests for VL in East Africa. An important part from the component, identification of putative parasite reservoirs in symptomatic and asymptomatic (sub-clinical) carriers is crucial through serological, molecular techniques and through xenodiagnosis (Costa et al., 2002).

Thus, as part of a study aimed at elucidating the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology VL in the new foci of the Tahtay Adiyabo district, prospective cohort study was designed to determine the VL infection dynamics in an endemic setting. In this PhD work a report of the experiment performed in order to validate the meaningfulness of RT-PCR results as indicators for infection with *L. donovani* in a large-scale community based survey is presented. The study is currently concluded, more data will accrue and the results of in-depth analysis will be reported in future publications.

Rationale 2

Molecular studies, which have been done so far, have shown the presence of a considerable genetic heterogeneity in isolates of *L. donovani* complex (Gelanew et al., 2010). The ability to distinguish *L. donovani* complex at subspecies level in East Africa is crucial in the development of diagnostic methods and prescribing treatment as well as in epidemiologic studies to determine possible control measure (Hailu et al., 2005). On the contrary, the presence of two species, *L. d. donovani* and *L. d. infantum*, as a cause for VL in East Africa including Ethiopia has been controversial (Alvar et al., 2007). However, using DNA fragment based typing of parasite isolates from VL cases from different parts of the country indisputably established that *L. donovani* is the causative agent of VL in Ethiopia. Microsatellite markers distinguished the circulating *L. donovani* in Ethiopia into two populations, the northern and southern Ethiopia, where the northern Ethiopia clusters with the Sudan isolates, and the southern Ethiopia and with Kenya/Uganda strain. The difference between the two populations is congruent with a difference in sand fly vector (Gelanew et al, 2010). To support previous result on the population variability of *L. donovani* by microsatellite markers it is crucial to demonstrate the reproducibility by typing of a parasite using simple PCR target and subsequent determination of the fragment size.

Rationale 3

Phlebotomus orientalis has been recorded in various ranges of altitudes, latitudes and well distributed in most VL endemic regions caused by *L. donovani*. Gelanew et al., (2010) suggested the of sand fly vectors for the existence geographically and genetically isolated populations of *L. donovani* in Ethiopia. A potential vector of *L. donovani* in north is *P. orientalis* while *P. martini* and *P. celiae* are vectors in southern Ethiopia *L. donovani* foci. However, *P. orientalis* is also a potential candidate at least in some foci in the south (Hailu et al., 1995). From this, one could hypothesize that at least two populations of *P. orientalis* complex with different vector potential could exist corresponding to the different micro-geographical scale. But this remains to be proven by analyzing species of *P. orientalis* from the two geographical ecotypes of VL in Ethiopia. Therefore we found it crucial to study the biology and genetics profile of two

populations of *P. orientalis* characterized by contrasting environmental conditions and geographical dimensions.

Rationale 4

In the neighboring VL endemic foci of Sudan, there are pretty good evidences for residual zoonotic reservoirs (Hoogstraal and Heyneman, 1969; Elnaiem et al., 2011; Dereure et al., 2000, 2003). *Leishmania* parasites of the same complex, and sometimes of the same zymodemes, were present in humans (VL and PKDL) and dogs simultaneously, suggesting the possibility of zoonotic transmission in Eastern Sudan (Dereure et al., 2003). We can hypothesize a similar scenario in Ethiopian context due to geographical proximity of the two countries. Moreover, in Ethiopia, Libo Kemkem districts, a study demonstrated as domestic animals as risk factors of VL (Basheye et al., 2009). However most of the studies use similar approaches and determination of animal's role by far is limited to detection. Therefore, further studies involving various procedures are required to elucidate the role of domestic animals in VL epidemiology in Ethiopian context.

Moreover, it has been indicated that among the wild animal rodents represent an eco-epidemiological important groups in leishmaniasis due to their proximity to humans and sand fly vectors. In VL endemic districts of Sudan natural infection with *L. donovani* in rodents were found and are suspected as reservoirs (Hoogstraal and Dietlein 1969; Elnaiem et al., 2011). Several studies in other countries revealed natural infections and signified the importance of rodents in leishmaniasis epidemiology. Moreover, the finding of natural infection in sylvatic system open insights for further action to look many other possible mammals having eco-epidemiological relation with the parasite and sand flies. For example bats recently attract the attention in the New World leishmaniasis foci. For better understanding and effective control strategy in the Ethiopian leishmaniasis platform such investigation would add knowledge in determination of potential hosts role.

Thus, part of this PhD research was designed to highlight the status of domestic animals, rodents and bats to the context of natural infection by *Leishmania* parasite and exposure to sand fly bite.

3. Specific Objectives

- To elucidate the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology of VL in Northern Ethiopia
- To validate the meaningfulness of the qRT-PCR results as indicators for infection with *L. donovani* and to evaluate its use for large-scale community based survey
- To describe the population structure of Ethiopian strains of *L. donovani* and to track its polymorphism
- To study the potential population variability in *P. orientalis* in different Ethiopian VL epidemiological settings
- To determine whether *L. donovani* is maintained zoonotically
    - To investigate natural infection in rodents, domestic animals and bats
    - To determine exposure of domestic animals to sand fly bite

4. Publications

1. Abbasi I, Aramin S, Hailu A, Shiferaw W, **Kassahun A**, Belay S, Jaffe C, Warburg A, 2013. Evaluation of PCR procedures for detecting and quantifying *Leishmania donovani* DNA in large numbers of dried human blood samples from a visceral leishmaniasis focus in Northern Ethiopia. BMC Infect. Dis. 13:153-161
2. Seblova V, Volfova V, Dvorak V, Pruzinova K, Votypka J, **Kassahun A**, Gebre-Michael T, Hailu A, Warburg A, Volf P, 2013. *Phlebotomus orientalis* sand flies from two geographically distant Ethiopian localities: Biology, genetic analyses and susceptibility to *Leishmania donovani*. PLoS Negl. Trop. Dis. 7(4): e2187
3. Zackay A, Nasereddin A, Takele Y, Tadesse D, Hailu W, Hurissa Z, Yifru S, Weldegebreal T, Diro E, **Kassahun A**, Hailu A, Jaffe CL, 2013. Polymorphism in the HASPB repeat region of east African *Leishmania donovani* strains. PLoS Negl. Trop. Dis. 7(1): e2031.
4. Rohousova I, Talmi-Frank D, Kostalova T, Polanska N, Lestinova T, **Kassahun A**, Yasur-Landau D, Maia C, King R, Votypka J, Jaffe CL, Warburg A, Hailu A, Volf P, Baneth G, 2015. Exposure to *Leishmania* spp. and sand flies in domestic animals in northwestern Ethiopia. Parasite Vector, 8:360.
5. **Kassahun A**, Sadlova J, Dvorak V, Kostalova T, Frynta D, Aghova T, Yasur-Landau D, Lemma W, Hailu A, Baneth G, Warburg A, Volf P, Votypka J, 2015. Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents. Acta Trop. 145: 39-47.
6. **Kassahun A**, Sadlova J, Kostalova T, Benda P, Warburg A, Hailu A, Baneth G, Volf P, Votypka J, 2015. Natural infection of bats with *Leishmania* in Ethiopia. Acta Trop. 150:166-170.

5. Summary and Conclusions

5.1 Validation of *L. donovani* Detecting PCR Methods for Large-scale Epidemiological Study

For a purpose of detection of *L. donovani* in large-scale survey requires appropriate procedures that yield reliable and reproducible results. PCR amplification can be considered to be a powerful alternative method for the diagnosis of *Leishmania* since it showed the maximum sensitivity for the reliable detection in their natural habitats where the commonly used detection tools are often inefficient (Cruz et al., 2002; Talmi-Frank et al., 2010).

Our study was designed to elucidate the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology of VL in Northern Ethiopia. We used quantitative real-time kinetoplast DNA PCR (qRT-kDNA PCR) for detecting *L. donovani* in dried-blood samples of volunteers living in an endemic focus in the Sheraro District of Tigray.

Of 4,757 blood samples, 680 (14.3%) were found positive for *Leishmania* kDNA, but most of those (69%) had less than 10 parasites/ml of blood. Samples were re-tested using identical protocols and only 59.3% of the samples with 10 parasites/ml or less were qRT-kDNA PCR positive for the second time. Furthermore, 10.8% of the PCR negative samples were positive in the second test. Most samples with higher parasitaemia remained positive upon re-examination (55/59 = 93%). DNA sequencing of ITS1 PCR products showed that 20/22 samples were of *L. donovani* complex, while two had ITS1 sequences homologous to *Leishmania major*. The finding of *L. major* infection in this study was unexpected for a reason that none in the study population (11,000 inhabitants from three villages) did have record of cutaneous manifestations. Being a dermotropic parasite, finding of *L. major* in blood is unusual; however experimental infections in rodents showed early dissemination a parasite to internal organs (Papadogiannakis et al., 2010). Recent entomological investigation revealed the presence of three sympatric species that are proven vectors of *L. major*, *P. papatasi*, *P. bergeroti* and *P. duboscqi*, in the same villages where the survey was conducted (Gebresilassie et al., 2015b). Although qRT-kDNA PCR is a highly sensitive test, the dependability of low positives remains questionable. It is crucial to correlate between PCR parasitaemia and infectivity to sand flies. While optimal

sensitivity is achieved by targeting kDNA, it is important to validate the causative species of VL by DNA sequencing.

We also compared three different methods for DNA preparation. Phenol-chloroform was more efficient than sodium hydroxide or potassium acetate. It has provided, by far a satisfactory and consistently producing good quality template for qRT PCR assay. Moreover the protocol gave us a low cost method that could have been prohibitively expensive to use a commercial kit for such large-scale community based cohort study.

5.2 Parasite Polymorphism

East African VL due to *L. donovani* complex continues and remains as a major health problem affecting the poorest who have limited otherwise no access to up-to-date treatment regimens and subsequent clinical managements. This is hampered by variation on epidemiological, ecological and clinical features of the disease between VL foci. For instance, based on the phlebotomine sand fly species involved in the transmission cycle of *L. donovani*, two markedly different ecological situations in Ethiopia have been recognized: (i) the north Ethiopian where VL is transmitted by *P. orientalis* and (ii) the south Ethiopian where VL is associated with *P. martini* and *P. celiae* (Gebre-Michael et al., 2004b). Moreover previous studies on population structure of *L. donovani* parasites showed a remarkably high genetic diversity in Ethiopian isolates (Gelanew et al., 2010). Thus, population genetic studies of the parasite together with certain epidemiological variables strengthen and add insights to design parasite-targeted control strategies. Paying attention to the fundamental importance of parasite population genetics, we designed further study on isolate of *L. donovani* from VL and HIV-VL co-infected patients in north and south Ethiopia and characterized using k26 PCR, and high resolution melt (HRM) analysis. Several strains from Kenya, Sudan and India were also included for comparison. In this study isolates were characterized by PCR targeting the ITS1, cpb E⁄F and k26. PCR targeting ITS1 were unable to differentiate strains in the *L. donovani* complex. Thus, we analyzed these strains using a modified cpbE/F PCR and differentiate the complex as *L. donovani* and *L. infantum*. Similarity between the *L. infantum* cpbE and *L. donovani* cpbF genes was observed except for a 39 bp insert that only present in the latter species. Further digestions with restriction

enzymes differentiate strains as *L. donovani* (gave a 400 bp PCR product), and *L. infantum* strains (gave a shorter 361 bp product).

To study genetic polymorphism of *L. donovani*, PCR using a suitable target, k26 gene, which amplifies the HASPB was used. HASPB is an orthologous gene expressed only by metacyclic promastigotes and amastigotes stage of the *Leishmania* parasite. It is characterized by amino acid repeats, which are inter- and intra-species specific and has domains attributed for parasite polymorphism. The repeat region of *L. donovani* and *L. infantum* HASPB protein, also known as k26, is previously recognized and has been used as a putative target in serodiagnostic procedures of VL in human and canine sera (Bhatia et al., 1999; Mohapatra et al., 2010). The k26 PCR, differentiate among *L. donovani* complex strains based on the size of a PCR product. Strains from northwestern Ethiopia produced a 290 bp product with few giving a 410 bp amplicon. Strains that gave a 410 bp product were isolated from patients with HIV-VL co-infected patients. Strains from the southern Ethiopian VL foci produce a 450 bp amplicon with three strains having smaller products (290 or 360 bp). Sudanese strains produced amplicons identical (290 bp) to those found in northwestern Ethiopia; while Kenyan strains gave larger PCR products (500 and 650 bp). From this, we could hypothesize that at least two genetically distinct populations of parasites of the *L. donovani* complex should be present in Ethiopia corresponding to each geographical focus that is certainly played by host factors.

Moreover, the finding of variability within similar geographical cluster; for instance southern Ethiopian strains that gave a 290 bp and 450 bp PCR product is not clear understood and we couldn't claim to represent a third group in the region. We can't rule out the possibilities including human migration, or a parasite vector factor due to the overlap of *P. orientalis* and *P. martini* in the region (Gebre-Michael et al., 2004b). Thus, more work is needed to determine whether there is a direct correlation between the parasite vector and k26 genotype, as HASPB plays a role in parasite differentiation and localization in the sand fly (Sadlova et al., 2010). Paying attention to the fundamental of *L. donovani* HASPB repeat domain variation with the geographic origin of the strain, our finding is a prerequisite for the development of effective control strategies. Moreover using this finding and analysis from epidemiological questions we

demonstrated the potential effect of k26 polymorphism on the use of HASPB as serodiagnostic tools and a potential vaccine candidate.

5.3 Comparison of Two *P. orientalis* Colonies

To study the potential population variability, the comparative laboratory-based studies on life cycle parameters, susceptibility to leishmania infection, and genetic profile of two reared colonies of sand fly vector species *Phlebotomus orientalis* obtained from two locations with different epidemiological status in northern Ethiopia were implemented. In East Africa, *P. orientalis* serves as the main vector of *L. donovani*, the causative agent of VL. In Ethiopia, *P. orientalis* is widely distributed while VL is known to occur in varying magnitudes in several isolated foci. To find out whether the difference in epidemiology of VL is due to phenotypic, biological or genetic polymorphism of the different populations of *P. orientalis*, we studied colonies initiated from sand flies trapped in Addis Zemen (endemic area, AZ) and Melka Werer (non-endemic area, MW). We studied life cycle parameters of colonies, their susceptibility to *L. donovani*, and genetic variability between the two geographical demarcations. We found marked differences between the two colonies in life-cycle parameters, including different requirements for larval food and humidity during pupation.

The level of exposure to the parasite in MW surroundings as revealed by LST (the leishmanin skin test) has been found to be as high as 60% while subsequent surveys showed sporadic disease distribution of the disease mostly associated with HIV/AIDS co-infection (Hailu et al., 1996). On the other hand, AZ focus has been characterized by stable transmission and the locality remains endemic with full-blown VL disease. The potential vector in both regions is *P. orientalis* (Gebre-Michael et al., 2007; Gebre-Michael et al., 2004b). This scenario was tested in laboratory condition and both MW and AZ *P. orientalis* colonies were highly susceptible to Ethiopian *L. donovani* strains; with infection rate of around 90% using a standard infection doses. Even the small number of parasites was able to initiate heavy infection in *P. orientalis* females; the lowest infective dose tested ($2x10^3$ per ml) was sufficient for successful establishment of *L. donovani* infection in about 50% *P. orientalis*. As the average volume of blood meal taken by *P. orientalis* females is about 0.7 microliters, this infective dose is equivalent for one or two *L. donovani* promastigotes per sand fly

female only. *Leishmania* development in *P. orientalis* was fast; the presence of metacyclic promastigotes in the thoracic midgut and the colonization of the stomodeal valve by haptomonads were recorded in most *P. orientalis* females by day five post-blood meal. However, the development of *L. donovani* was similar in both *P. orientalis* colonies; therefore, we suppose that the absence of visceral leishmaniasis in non-endemic area cannot be attributed to different susceptibility of local *P. orientalis* populations to *L. donovani*.

However, morphological and genetic analysis did not reveal any significant differences between the two colonies. Comparison of the populations using RAPD PCR and DNA sequencing of cytB and COI mitochondrial genes revealed no significant genetic differences. We successfully demonstrated the ability of laboratory colonies arising from these populations to crossbreed (MW male / AZ female and AZ male / MW female) by obtaining F1 and F2 progeny with hybrids similar or even higher fecundity than parental colonies.

5.4 Studies on Animal Reservoirs of VL in Ethiopia

Generally, transmission of VL due to *L. donovani* in Ethiopia and neighboring East African countries has been assumed to be entirely anthroponotic (Chappuis et al., 2007). However, recent studies in the region revealed the importance of both domestic and wild animals in the disease epidemiology. Thus found it crucial to determine and assess the role animals for the Ethiopian VL epidemiology. Based on previous findings in other VL endemic countries and their epidemiological associations we investigate domestic (goats, sheep, cattle, dogs and donkeys) and wild (rodents and bats) animals for natural infection and degree of exposure to sand fly bite.

5.4.1 Exposure of Domestic Animals to *Leishmania* and Sand Fly Bite

For animals to have a role in disease epidemiology, their distribution, longevity and their close association to a vector and other host should be taken to account (Ashford, 1996). Domestic animals especially in east African countries more-or-less meet all these condition and brought an insight in the role of VL transmission. Domestic animals sanitary conditions in Africa, for example lack of waste management and open sewerage system may increase sand fly breeding and serve as resting sites. In addition, the close proximity between animals and humans could

increase the contact with sand flies in such places. Thus, sand flies are attracted to crowd housing places as these provide a good source of blood-meals (WHO, 2015). In several endemic VL places, including in Ethiopia, the status of domestic animals has been determined but their clear involvement remain unclear despite shown to be important risk factors. In view of this, we conducted a study on domestic animals (goats, sheep, cows, dogs, and donkeys) in three *L. donovani* foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and for anti-*L. donovani* IgG. Serum anti-sand fly saliva antibodies were used as a marker of exposure to a *P. orientalis* bite.

A total of 546 animal blood samples were tested using qRT-PCR targeting kDNA and 32 (5.9 %) were positive for *Leishmania* DNA. All the kDNA positive samples were re-tested using a species-specific target, ITS1, and nine were determined as *L. donovani* complex. The majority of *Leishmania*-positive animals (30 out of 32) were found in Humera, with the highest prevalence observed in cows (18.8 %). At the other localities, only one donkey in Sheraro and one dog in Addis Zemen were found to be positive for *Leishmania* DNA. The finding of these animals in this regions support the previous suggestion, that these animals could be one of the risk factors (Argaw et al., 2010). Similarly, dogs infected with *L. infantum* were recorded in Addis Zemen (Alvar et al., 2007). In neighboring country Sudan dogs were found infected with *L. donovani* and *L. infantum* that were implicated as reservoir hosts for VL (Dereure et al., 2000; Dereure et al., 2003).

Seroprevalence of *L. donovani* infection in sera of the domestic animals was determined by ELISA through the measurement of a specific anti-*L. donovani* IgG. A total of 19% of the animals were seropositive for anti-*L. donovani* IgG, with the highest seroprevalence observed in dogs and sheep. Moreover, similar ELISA procedure was used to estimate the exposure of domestic animals to *P. orientalis*, anti-saliva IgG antibodies. A total of 126 (23%) were identified to be exposed to *P. orientalis* bite. In Addis Zemen and Sheraro, the highest seroprevalence was observed in dogs, while in Humera; donkeys, dogs, and sheep were the highest. A positive correlation was found between anti-*P. orientalis* saliva and anti-*L. donovani* IgGs in cows, goats and sheep.

Our finding and further investigations therefore could put the previous assumption of anthropontic transmission of *L. donovani* in Africa. The possible involvement of domestic animals as sources of blood for vector sand flies should therefore be considered in VL control strategies. However, the direct involvement of domestic animals in the transmission cycle of *L. donovani* warrants further investigation, most importantly through xenodiagnoses and parasite isolation and cultivation to determine their transmissibility competence.

5.4.2 *Leishmania* Natural Infection in Rodents

Rodents are one the most important groups of mammals for leishmaniasis epidemiology, as are widely distributed in many geographic ranges and close habitat to many sand flies (Ashford, 2000). *Leishmania* infection in rodents has been investigated in many endemic countries including Sudan (Hoogstraal and Heyneman, 1969; Elnaiem, 2011), Portugal (Helhazar et al., 2013), Italy (Gradoni et al., 1983), Greece (Papadogiannakis et al., 2010) and Iran (Davami et al., 2014). The finding of *Leishmania* infection in rodents has been long waited and we have demonstrated natural infection using PCR.

A total of 581 rodent's spleen collected from various region of Ethiopia was used to determine natural infection. All samples were screened using real time PCR targeting kDNA of *Leishmania* and then positive samples retested with a species-specific primer for ITS1 and species determination was done after sequencing of the PCR products. Fifty rodent samples were found to be positive for *Leishmania* kDNA. Since PCR results based on kDNA doesn't determine the species of the parasite a suitable target; ITS1 testing was employed on the positive samples (Nicolas et al., 2002; Nasereddin et al., 2008). Sequencing of ITS1 revealed five *L. tropica* and five *L. donovani* infections. All of the positive rodents were found from areas where infection in humans and/or sand fly was reported, that signify their importance. All except one kDNA positive rodents were from southern Ethiopian that the scenario could be explained with the variability of the parasite genetics and nature *P. orientalis* between the two geographical areas.

Our investigations suggest that wild rodents in Ethiopia could play an important epidemiological role in the transmission cycle of two *Leishmania* species, *L. donovani* and *L. tropica*. Further

studies are recommended focusing on parasite isolation, experimental infection, and xenodiagnosis to prove their epidemiological role.

5.4.3 *Leishmania* Natural Infection in Bats

Investigation of *Leishmania* natural infection other than the traditionally known organism (e.g. rodents and dogs) has diverted attentions to other possible sylvatic reservoir hosts in endemic leishmaniasis foci. Of these bats became an optional focus by epidemiologist especially in the New World. The general ecological and innate behavioral details highlight their prime importance in the reservoir system of infectious diseases such as Ebola virus (Leroy et al., 2005) and various kinetoplastids transmitted by vectors (Lord and Brooks, 2014). Moreover, *Leishmania* natural infection of bats in the New World foci is evident while in the Old World attempts were not successful or limited to outdated methods. In view of these facts we carried out a *Leishmania* DNA survey in 163 Ethiopian bats collected from various regions. Out of eight kDNA positive samples, two were verified as *L. tropica* and *L. major* by ITS1 sequences. The *L. tropica* positive bat was from Awash region, where human, sand fly and rodent natural infection of *L. tropica* are reported. Similarly, the *L. major* positive bat was found in area where potential vectors occur.

Bats could have adequate features to be naturally infected by *Leishmania* parasites and could subsequently to play a role in its epidemiological cycle. The present study revealed natural *Leishmania* infection of Old World bats, in areas both endemic and non-endemic for human leishmaniasis. The wide geographical distribution of *Leishmania* parasite in the country could imply the existence of different modes of transmission and our finding might indicate the importance of bats in the disease cycle. However, to play a role in *Leishmania* cycles it is required to investigate the host's pathogenic features and being infectious to vectors; which were not covered in our study. Thus, further studies on persistence of the *Leishmania* parasite in bats and its interaction with sand fly vectors are recommended for the better understanding of their epidemiological involvement.

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Paper I

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Evaluation of PCR procedures for detecting and quantifying *Leishmania donovani* DNA in large numbers of dried human blood samples from a visceral leishmaniasis focus in Northern Ethiopia

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Abstract

Background: Visceral Leishmaniasis (VL) is a disseminated protozoan infection caused by *Leishmania donovani* parasites which affects almost half a million persons annually. Most of these are from the Indian sub-continent, East Africa and Brazil. Our study was designed to elucidate the role of symptomatic and asymptomatic *Leishmania donovani* infected persons in the epidemiology of VL in Northern Ethiopia.

Methods: The efficacy of quantitative real-time kinetoplast DNA/PCR (qRT-kDNA PCR) for detecting *Leishmania donovani* in dried-blood samples was assessed in volunteers living in an endemic focus.

Results: Of 4,757 samples, 680 (14.3%) were found positive for *Leishmania* k-DNA but most of those (69%) had less than 10 parasites/ml of blood. Samples were re-tested using identical protocols and only 59.3% of the samples with 10 parasite/ml or less were qRT-kDNA PCR positive the second time. Furthermore, 10.8% of the PCR negative samples were positive in the second test. Most samples with higher parasitemias remained positive upon re-examination (55/59 =93%). We also compared three different methods for DNA preparation. Phenol-chloroform was more efficient than sodium hydroxide or potassium acetate. DNA sequencing of ITS1 PCR products showed that 20/22 samples were *Leishmania donovani* while two had ITS1 sequences homologous to *Leishmania major*.

Conclusions: Although qRT-kDNA PCR is a highly sensitive test, the dependability of low positives remains questionable. It is crucial to correlate between PCR parasitemia and infectivity to sand flies. While optimal sensitivity is achieved by targeting k-DNA, it is important to validate the causative species of VL by DNA sequencing.

Keywords: Asymptomatic infections, Cohort study, DNA extraction, Ethiopia, Visceral Leishmaniasis, *Leishmania donovani*, kDNA-PCR

Background

Visceral leishmaniasis (VL) known as Kala-Azar, is a disseminated protozoan infection caused by eukaryotic intracellular parasites belonging to the *Leishmania donovani* complex. An estimated 390,000 VL cases occur annually, over 90% of which are concentrated in the Indian sub-continent, East Africa and Brazil [1,2]. Distinct modes of transmission characterize the two causative parasite species responsible for VL. *L. infantum* in Europe, the Middle East and North Africa and *L. donovani. chagasi* in Latin America are transmitted zoonotically with dogs serving as reservoir hosts while *L.* d *donovani* in the Indian subcontinent as well as East Africa is considered anthroponotic and transmitted between humans [3].

In Africa, the worst affected region is southern Sudan with an estimated average of 15,000-20,000 cases per year [4,5]. The most important VL endemic areas in Ethiopia are found in the northwest (Metema-Humera lowland),

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which accounts for approximately 60% of the cases, and in the southwest (Lake Abaya, Omo river plains and Segen and Woito valleys) [6]. In recent years VL has spread to the highlands of Libo-Kemkem district (south of Gondar), claiming the lives of hundreds of patients [7,8].

Patients with clinical symptoms of VL are routinely diagnosed using either parasitological or serological methods. The former method relies primarily on microscopic examination of stained splenic aspirate smears (96% sensitive). For serological diagnosis of VL and PKDL, two simple tests are used Freeze Dried - Direct Agglutination Test (FD-DAT) and rK39 strip test. A multi-center comparison of these assays demonstrated that while FD-DAT and rK39 tests are highly reliable in the Indian Subcontinent, >95% sensitivity and >90% specificity, they are less useful in Africa [9]. In general, the FD-DAT showed higher sensitivities (86 – 99%) and specificities (82 – 98%) than the rK39 test in Africa (sensitivity 75 – 85% and specificity 70 -92%). However, there was considerable variation in these parameters depending on the origin of the patient (Ethiopia, Kenya or Sudan). The specificity and sensitivity of serological diagnosis can be improved if rK39 and DAT are used in series [10]. However an urgent need exists for better diagnostic tests for VL in East Africa.

PCR-based diagnostic assays are more sensitive than traditional methods including immunoassays [11]. There are several PCR protocols for detecting and diagnosing *Leishmania* infections in humans. These include; kinetoplast DNA (kDNA) minicircles [12,13], the small subunit rRNA gene [14] internal transcribed spacer 1 (ITS1) [15] and spliced leader sequence [14,16]. These PCR systems are genus-specific but do not separate the different *Leishmania* species. Further analysis of the PCR amplicon is required for species identification. For example, restriction cut analysis following PCR amplification of the ITS1 [15,17], high resolution melt analysis of the kDNA / PCR amplicon or the 7SL gene [12,18,19].

As part of a study aimed at elucidating the role of symptomatic and asymptomatic *L. donovani* infected persons in the epidemiology of Kala Azar, we are conducting a thorough study of persons living in the endemic district of Tahtay Adiabo in Northern Ethiopia. An important component of the project is the identification of putative parasite reservoirs in VL and PKDL patients as well as asymptomatic (sub-clinical) carriers. Some 4,900 individuals living in 18 villages were screened for infection or exposure to *L. donovani* by physical and laboratory tests; Leishmanin Skin Test (LST), Direct Agglutination Test (DAT) and kDNA / RT-PCR. Of the 4,757 dried-blood samples tested by RT-PCR, 680 samples (14.3%) were found positive for *Leishmania* k-DNA (Hailu et al. in preparation). The experiments reported here were performed in order to validate the meaningfulness of the RT-PCR results as indicators for infection with *L. donovani*.

Methods

Ethical considerations

Informed consent was obtained from all the adults who participated in the study. Consent for inclusion of young children, was obtained from parents or guardians. The study was reviewed and approved by the ethical review committee at the Medical Faculty, Addis Ababa University and the National Research Ethics Review Committee (NRERC) at the Ethiopian Ministry of Science and Technology.

Samples

As part of a prospective cohort study on the transmission dynamics of VL, blood samples were collected from around 4,900 villagers in the Tahtay Adiabo district of northern Ethiopia. Whole families were selected randomly based on a census comprising over 11,000 individuals.

Four drops (approximately 50 μl each) of venous blood were spotted on Whatman 3MM filter paper. All blood samples were identified by an ID number and processed blindly. To minimize the possibility of contaminating parasite DNA in these PCR procedures all DNA extractions were performed in a room into which, live cultured *Leishmania* were never introduced. The paper punches were washed and sterilized using bleach between different samples. Every RT-PCR run included a negative control (no DNA) and several positive controls with known numbers of parasites (for the standard curve). Only disposable plastic ware (tubes, and pipette tips) was used in all these procedures.

For VL screening DNA was extracted from two paper punch disks (r = 3 mm, calculated to have been saturated with approximately 10 μl of blood each), using a phenol-based DNA extraction method [20]. The results reported in the current publication were derived from re-testing of the original samples (Hailu et al., in preparation).

Quantitative real-time kinetoplast DNA PCR (qRT-kDNA PCR)

Real-Time hot-start PCR was performed with Absolute Blue qPCR kit (Thermo scientific, Surrey, UK) based on SYBR green detection using a real time PCR thermo cycler (Rotor-Gene 6000, Qiagene, Hilden, Germany). The qPCR reaction (total volume of 20 μl) was prepared by mixing 10 μl of the 2x concentrated absolute blue solution with 1 μM of each kDNA minicircle specific primers JW11 and JW12 (Table 1) and template DNA (2 μl) [12]. For fluorescence signal acquisition, time and temperature profile were set as follow: holding step at 95°C for 15 minutes for enzyme activation,

Table 1 PCR systems and primer sets used for the real time kDNA and ITS1 PCR amplification

PCR system	Primers	DNA sequence	Amplicon size (bp)	Ref.
kDNA minicircle	JW11	CCTATTTTACACCAACCCCCAGT	120	[12]
	JW12	GGGTAGGGGCGTTCTGCGAAA		
ITS1 PCR	L5.8S	TGATACCACTTATCGCACTT	320	[15]
	LITSR	CTGGATCATTT-TCCGATG		

40 cycles starting in denaturation step at 95°C for 10 seconds, annealing at 58°C for 10 seconds and lastly extension step at 72°C for another 10 seconds. The qPCR kDNA results were viewed and analyzed by the Rotor-Gene's real time software (Rotor-Gene 6000; Corbett Life Science, Sydney).

To achieve accurate quantitation, *L. donovani* cultured promastigotes were diluted into heparinized human blood at 10^6, 10^5, 10^4, 10^3, 10^2, 10, 0 parasites /ml. These parasite dilutions were spotted on Whatman 3MM filter paper and allowed to dry. For every RT-PCR run, two control discs from each concentration were included and the results used to form calibration curves (Figure 1).

ITS1 polymerase chain reaction (PCR)

PCR reactions were carried out in a volume of 25 μl using ready mix PCR tubes (Syntezza, Jerusalem, Israel). For each reaction 20 pmoles of the two *Leishmania* specific ITS-1 primers (LITSR and L5.8S, Table 1) were added followed by 5 μl of the template DNA [15]. The thermal profile comprised 5 min at 95°C, followed by 35 cycles starting at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 min, a final elongation step at 72°C for 10 min. PCR results were analyzed by running 10 μl of the PCR amplicon on 1.2% agarose gels with known controls.

DNA preparation

Fifty nine blood samples that were found positive for *Leishmania* in the cohort study were divided into three categories: 16 samples with 11–100 parasites/ml (low), 24 samples with 100–1000 parasites/ml (medium) and 19 samples with over 1000 parasites /ml (high). Two discs with dry blood (6 mm diameter, Whatman 3MM blotting paper) were cut from each sample with a standard paper- punch. DNA from these discs was prepared using three methods: 1) Phenol/chloroform DNA extraction (repetition of the approach used in the initial study). 2) NaOH based DNA extraction. 3) Potassium acetate DNA extraction method. Precipitated DNA from all samples was suspended in 100 μl of DNAase/RNAase free double distilled water.

Phenol based DNA extraction method

The blood/paper discs were incubated in a microfuge tube with 200 μl of lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl pH 7.4, 1% triton X-100, and 200 μg/ml of proteinase K) at 60°C for 2 hours. Equal volumes of TE-saturated phenol (pH 8) were added to the aqueous solution, the mixture was vortexed for few seconds and then centrifuged for 2 minutes at 14,000 rpm. The upper aqueous layer was removed to a new micro centrifuge tube and the DNA was precipitated by adding NaCl to a concentration of 0.2 M (addition of 8 μl of 5 M

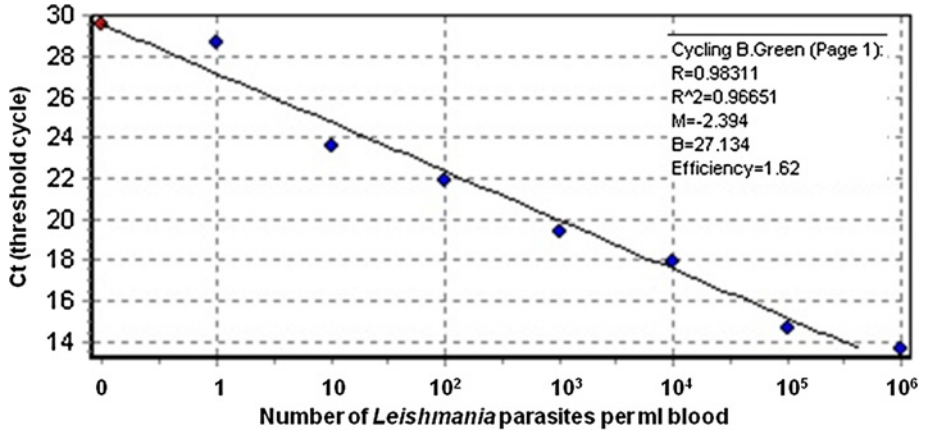

Figure 1 A standard curve for qRT-kDNA PCR of *Leishmania donovani* promastigotes in blood. Human blood was mixed well, and dripped onto Whatman 3MM filter papers. On average, each drop (~50 μl) covered an area equivalent to 5 paper punch discs (r = 3 mm). Two discs were used for extracting DNA per reaction (~20 μl of blood). Standard curves were run with every batch of qRT-kDNA PCR and the number of parasites in tested samples was extrapolated from it.

NaCl to 200 μl aqueous solution) and 2.5 volumes of 100% cold ethanol. DNA was incubated at −20°C overnight and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was dried in speed-vac.

Sodium hydroxide DNA extraction method

The blood/paper discs were incubated in a microfuge tube with 200 μl of lysis buffer (1 N NaOH, 0.1% SDS) at 60°C for 2 hour. The solution was neutralized with concentrated (36%) HCl solution by adding about 18 μl to reach pH of 5–7 as measured using pH-detection strips. Removal of denatured debris was achieved by centrifugation for 10 minutes at high speed in a micro centrifuge. The DNA was further purified by ethanol precipitation as described above

Potassium acetate DNA extraction method

Was performed as described by [21]. The blood/paper discs were incubated in a microfuge tube with 200 μl of lysis buffer (1% sodium dodecyl sulfate, 25 mM NaCl, 25 mM EDTA), and samples were placed at 65°C for 2 hours. 100 μl of 3 M potassium acetate (pH 7.2) were added, the mixture was incubated on ice for 30 min and centrifuged at high speed for 15 min in a micro centrifuge. DNA from the supernatant was precipitated by the addition of 600 μl of 100% ethanol.

Results

Re-examination of blood samples from the cohort study

The qRT-kDNA PCR results of the cohort study indicated that 69% of the positive samples had 1–10 parasites /ml of blood. These comprised almost 10% of the volunteers. Notably too, the qRT-kDNA PCR values corresponding to parasite concentrations of 10^6-10^2 *L.donovani* pros/ml of blood in the calibration curves, fit squarely on the linear logarithmic curve, while the lower concentrations below 10 pros/ml deviated significantly (Figure 1). Results were interpreted as showing that low concentrations were less consistent and, therefore not as robust as the higher parasite concentrations. Based on these observations we decided to re-examine some of the samples using the same methodology as that used during the cohort study, namely phenol-based DNA extraction and qRT-kDNA PCR to assess for the possibility of false positives. Results show that 96% to 100% of the samples with high infections (100–1000 and over 1000 parasites /ml, respectively) remained positive on retesting. However, only 85.4% of the samples with 11–100 parasites /ml and 59.3% of the samples with 1–10 parasites /ml were positive again during repeat examination. In addition, 8.4% of the previously negative samples tested as low positives upon repeat PCRs (Table 2).

Efficiency of DNA extraction

In order to determine the efficacy of simple inexpensive DNA preparation protocols for detecting *Leishmania* DNA in dried blood samples, DNA was prepared using phenol, sodium hydroxide or potassium acetate. The purified DNA was used as template for ITS-1 PCR amplification. The phenol-based method yielded the best template, allowing detection of 10 parasites /ml of blood (Figure 2A arrow). DNA prepared using the sodium hydroxide-based method was 3 fold less sensitive requiring a minimum of 10^3 parasites /ml of blood (Figure 2B arrow). DNA prepared using the potassium acetate-based method proved the least sensitive detecting only 10^5 parasites /ml of blood (Figure 2C arrow).

In subsequent experiments we re-examined positive blood samples from some of the volunteers from the cohort study. DNA was prepared using the above three methods and tested by ITS1-PCR as well as qRT-kDNA PCR. Tested samples included all infection categories. Here again, DNA preparation using the phenol-based method proved superior and more consistent than the other two techniques (Table 3).

DNA sequencing of the ITS1-PCR amplicon

To validate the identity of the *Leishmania* DNA in the blood of the naturally-infected volunteers in the cohort study, 64 samples with differing parasite loads were amplified by ITS1-PCR (Figure 3). The amplified PCR products from 16 samples exhibiting moderate to strong ITS1 bands were sequenced by-automated fluorescent

Table 2 Reexamination of qRT-kDNA PCR results from the cohort study

1	2	3	4	5
Category parasites /ml	1st cohort study kDNA RT-PCR	Retested by kDNA RT-PCR	kDNA RT-PCR + (current study)	Level of uniformity
0	4,076	107	9	91.6%
1-10	468	108	64	59.3%
11-100	93	48	41	85.4%
101-1000	96	24	23	95.8%
Above 1000	23	19	19	100%

Some of the qRT-kDNA PCR results from the cohort study (Column 2) were reexamined using the same t2:2 protocols (Column 3) the samples that were positive upon re-examination depicted in Column 4. Levels of uniformity (Column 5) indicate the percentage of samples that gave the same result in both tests. Negative and high positive samples were highly consistent. Very low positive samples (1–10 parasites/ml) less so.

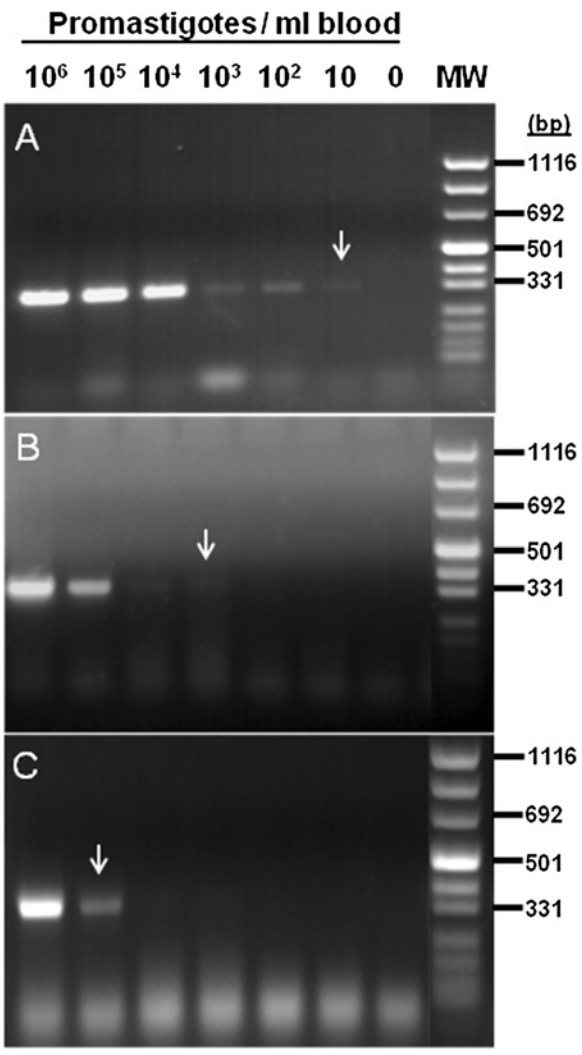

Figure 2 Comparison of ITS1/PCR results using template DNA prepared by: A) The phenol-based method. B) The sodium hydroxide-based method. **C**) The potassium acetate method. DNA was prepared from two Whatman 3MM filter paper discs with dried blood containing *Leishmania donovani* promastigotes. 1 ml human blood was spiked with different numbers of promastigotes: lane 1 = 10^6, lane2 = 10^5, lane 3 = 10^4, lane 4 = 10^3, lane 5 = 10^2, lane 6 = 10, lane 8 = 0.

DNA sequencing using ABI PRISM 377 sequencer (PE Biosystems, Foster City, California). To improve ability to sequence low parasitemias, ITS1 PCR products from a further 6 samples with weaker bands, were cloned into CloneJet PCR cloning kit (Fermentas, Vilnius, Lithuania). DNA from the produced recombinant plasmids was purified using miniprep purification kit (Qiagene, Hamburg, Germany) and sequenced. The sequences were compared for their homology to known sequences in the GenBank data base using BLAST online service provided through the PubMed /US National Institute of health. Of the 22 samples sequenced, 20 revealed complete homology with *L. donovani* ITS1, the other two samples were found to be homologous to *L. major* (Table 4).

Discussion

Large-scale cohort studies on infectious diseases in rural areas of Africa are labor intensive and time consuming. Therefore, the samples collected are extremely valuable and the data derived from them warrants rigorous validation. An optimal combination of a sensitive PCR assay with an efficient DNA extraction method is crucial for the success of DNA-based epidemiological studies. There is a wide range of available commercial kits for DNA extraction most of which depend on proteoloytic tissue digestion followed by DNA binding and elution through glass membranes. Although these efficiently produce clean DNA, they are prohibitively expensive when large numbers of samples require processing. We tested three simple DNA extraction methods (costing less than 10% of the cost of commercial kit.) and found that phenol based DNA extraction was by far the most satisfactory, consistently producing good quality template for our qRT-kDNA PCR diagnostic assays.

PCR -based methods for detecting parasites are highly sensitive and have the added advantage that they may be performed on dry specimens without the need for cold-storage [22,23]. In order to optimize our accomplishments from the current cohort studies, we experimented with primers for ITS1 and 7SL RNA gene [17,18]. However, the levels of sensitivity were inadequate (data not shown). Therefore, we resorted to kDNA RT- PCR which is the most sensitive method for detecting *Leishmania* since there are 10,000 kDNA minicircles per parasite [19]. In our hands the limit of detection of the qRT-kDNA PCR was around 10 parasites per ml (Figure 1). For DNA extraction we routinely used 2 punch-disks containing approximately 20 μl of blood (0.2 parasites). The DNA solution was diluted into 100 μl of which only 2 μl were used for each kDNA RT-PCR reaction. Thus, the detection threshold was approximately 0.004 parasites per reaction. This sensitivity is comparable with that previously reported for kDNA RT- PCR in dried blood [12,24]. Since PCR amplification of kDNA using the primers JW11 and JW12 does not discern between *Leishmania* species [12], we amplified and sequenced the ITS1 gene of select samples. As expected, most were shown to be *L. donovani*. However, two of the 21 ITS1 PCR sequences were homologous with *L.major* (Table 4). This result was surprising for several reasons. Firstly, in a preliminary census of more than 11,000 inhabitants of the Sheraro region, we did not record any cutaneous leishmaniasis cases. Secondly, our entomological studies, which have been going on for over 2 years, have identified only very few specimens of *Ph. papatasi*, the vector of *L. major*. Lastly, *L. major* is

Table 3 Comparison of the efficiency of three DNA preparation methods (phenol, sodium hydroxide and potassium acetate) for detection of *Leishmania* DNA in dried blood spots

Infection category	Number of samples	DNA preparation method	kDNA/RT-PCR +	ITS1/PCR+	kDNA/ITS1 (shared positives)
Low (1–100)	16	Phenol	13	13	10
		NaOH	12	3	3
		Potassium acetate	3	0	0
Medium (100–1000)	24	Phenol	23	12	11
		NaOH	20	6	5
		Potassium acetate	1	0	0
High (above 1000)	19	Phenol	19	13	13
		NaOH	13	9	8
		Potassium acetate	8	6	6
Totals	59	Phenol	55	38	34
		NaOH	45	18	16
		Potassium acetate	12	6	6

Results for ITS1 were obtained on gels following standard PCR. Data for kDNA was obtained by qRT-PCR.

essentially a skin parasite and is not normally found in the blood. It is important to note that all PCRs were performed in a "clean room" (i.e. containing no possible source of *Leishmania* DNA contamination) and that validation of these findings included repeat extraction of DNA and repeat PCR reactions.

The ITS1 DNA sequences of the different *Leishmania* species are well characterized and available in GenBank. Many authors have submitted these sequences from different parts of the world. There is a significant sequence difference [exceeding 10%] between *L. major* and *L. donovani*. The ITS sequences obtained from our samples showed complete homology with either *L. donovani* (19 samples), or *L. major* (2 samples). The amplified ITS1 sequence was 330 bp and for such short sequence the possible introduced amplification errors caused by DNA polymerase are minimal and would not affect the fidelity of species identification.

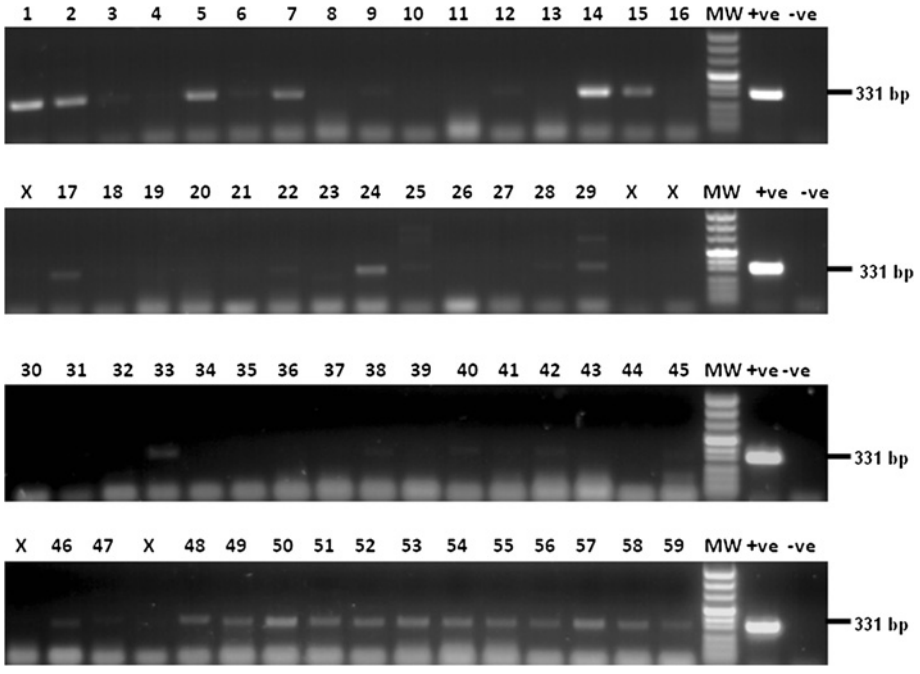

Figure 3 ITS1 PCR targeting leishmanial DNA extracted from 64 dry blood samples previously shown positive by qRT-kDNA PCR. DNA from two 6 mm punch discs per specimen, was prepared by the phenol-based method. Twenty-two samples were clearly positive and these were sequenced to determine the *Leishmania* species. Note: low molecular weight bands represent primer-dimers.

Table 4 ITS-1 sequencing for validation of *Leishmania* species identity in kDNA / RT-PCR positive samples

Number	Parasite /ml	Identified species	Notes
1	56	*L.major*	Sequencing of cloned ITS1 amplicon
2	87	*L. donovani*	
3	65	*L. donovani*	
4	36	*L. donovani*	
5	23	*L. donovani*	
6	1993	*L. donovani*	
7	69	*L. donovani*	Direct sequencing
8	552	*L.major*	
9	577	*L. donovani*	
10	584	*L. donovani*	
11	643	*L. donovani*	
12	1022	*L. donovani*	
13	1180	*L. donovani*	
14	1314	*L. donovani*	
15	1397	*L. donovani*	
16	8923	*L. donovani*	
17	11735	*L. donovani*	
18	11753	*L. donovani*	
19	11973	*L. donovani*	
20	11988	*L. donovani*	
21	30770	*L. donovani*	
22	47851	*L. donovani*	

Twenty two samples with differing parasite loads were selected for amplification and sequencing. 20/22 samples proved to be *L. donovani* while two were *L. major* infections.

Having achieved extremely high sensitivity, it became crucial to validate the repeatability of our qRT-kDNA PCR assay. Only 59% of the samples originally found to contain 1–10 parasites were positive in repeat qRT-kDNA PCR tests (Table 2). This lack of consistency is not surprising since these numbers are very close to the detection threshold of the qRT-kDNA PCR (Figure 1). Indeed, when we randomly re-tested negative samples, over 8% showed up as low positives (Table 2). On the other hand, the results of this study confirmed the overall robustness of qRT-kDNA PCR for detecting *Leishmania* infection in dried blood spots. All 19 samples with high numbers of parasites were confirmed positive upon reexamination using the same protocols. Similarly, of the medium infections, 23 of 24 (96%) were consistently positive. Even lower parasitemias of 11–100 parasites/ml of blood were 85.4% repeatable (Table 2).

As seen in Table 2 the probability of inaccuracies increases around the detection threshold. The first type of error would be a false negative (i.e., missing parasites that do exist in the sample). Such errors could arise from the fact that in the first sample there are no parasites, while in the repeated sample there is parasite DNA. A second type of error is false positive where PCR indicates presence of parasite DNA where there is none. Performing several repeat PCRs on a large number of samples would enable the application of statistical tests to estimate the exact rate of both type of errors and to recalculate the infection rates more rigorously. Since we do not have the material to repeat the tests, we necessarily limit our inferences to the current observation - low qRT-kDNA PCR results (1–10 parasites per ml) are less dependable than either negative or high-positive ones (Table 2).

In terms of disease transmission the most relevant question is which of these PCR positive individuals are infectious to sand flies that imbibe 1.0 μl of blood or less [25,26]. Therefore, to be likely of picking up 1 parasite per meal, they would require infections of 1,000 parasites or more per ml of blood. Although we do not know what is the amastigote dose required for infecting sand flies in nature, laboratory infections of *Ph. orientalis*, the vector of *L. donovani* in Ethiopia and Sudan required some 2×10^4 *L. donovani* promastigotes per ml of blood to obtain a high rate of infection (Seblova et al., in press). Thus, it seems likely that only the very high qRT-kDNA PCR positive individuals actually serve as effective reservoirs for infecting sand flies. This would be consistent with xenodiagnostic data on *L. d. chagasi* from Brazil indicating that only patent VL cases were

infectious to *Lutzomyia longipalpis* sand flies while asymptomatic carriers were not [27].

In India *L. donovani* amastigotes were found in the blood of asymptomatic persons living in endemic regions [28]. In Sudan, *L. donovani* was demonstrated in the skin, causing a primary leishmanoma [29]. Asymptomatic infections are thought to be common in Ethiopia as well and may serve as parasite reservoirs [3,30]. However, blood parasitemias may be misleading in that *Leishmania* spp are not "true" blood parasites and may potentially be more abundant in the skin and internal organs. Sand flies macerate the skin to obtain blood. Thus, they may pick up parasites not only from the blood they imbibe but also from resident macrophages in the skin itself. In fact, low amounts of *Leishmania* DNA in the blood may indicate heavy infections elsewhere in the body. To gain an improved understanding of the possible significance of such findings, we plan to test skin samples as well as blood from volunteers in future cohort sampling.

Conclusions

Our results so far indicate that the detection of very low blood parasitemias is not a reliable parameter for determining infections with *L. donovani*. Current studies are focused on following PCR positive volunteers over time to detect possible correlations between the levels of blood parasitemias and the probability of a person developing VL. The cumulative data analyzed using sophisticated statistical methods and examined with a dynamical VL model should help to determine the probable pathogenetic course of asymptomatic *L. donovani* infections, either becoming sick or recovering with time.

Competing interests

The authors declared that they have no competing interest.

Authors' contributions

Field work in Ethiopia collection of samples – AH, WS, AK, SB. Conceptual development and optimization of the PCR methodology IA, CJ, AH, AW. Performance of the Real time KDNA assays – IA, SA, WS. Analyses and interpretation of the results - IA, CJ, AH, AW. Writing of the manuscript – IA, SA, AH, AW. All authors read and approved the final manuscript.

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Paper II

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Polymorphism in the HASPB Repeat Region of East African *Leishmania donovani* Strains

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Abstract

Background/Objectives: Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a major health problem in Ethiopia. Parasites in disparate regions are transmitted by different vectors, and cluster in distinctive genotypes. Recently isolated strains from VL and HIV-VL co-infected patients in north and south Ethiopia were characterized as part of a longitudinal study on VL transmission.

Methodology/Principal Findings: Sixty-three *L. donovani* strains were examined by polymerase chain reaction (PCR) targeting three regions: internal transcribed spacer 1 (ITS1), cysteine protease B (cpb), and HASPB (k26). ITS1- and cpb - PCR identified these strains as *L. donovani*. Interestingly, the k26 - PCR amplicon size varied depending on the patient's geographic origin. Most strains from northwestern Ethiopia (36/40) produced a 290 bp product with a minority (4/40) giving a 410 bp amplicon. All of the latter strains were isolated from patients with HIV-VL co-infections, while the former group contained both VL and HIV-VL co-infected patients. Almost all the strains (20/23) from southwestern Ethiopia produced a 450 bp amplicon with smaller products (290 or 360 bp) only observed for three strains. Sudanese strains produced amplicons identical (290 bp) to those found in northwestern Ethiopia; while Kenyan strains gave larger PCR products (500 and 650 bp). High-resolution melt (HRM) analysis distinguished the different PCR products. Sequence analysis showed that the k26 repeat region in *L. donovani* is comprised of polymorphic 13 and 14 amino acid motifs. The 13 amino acid peptide motifs, prevalent in *L. donovani*, are rare in *L. infantum*. The number and order of the repeats in *L. donovani* varies between geographic regions.

Conclusions/Significance: HASPB repeat region (k26) shows considerable polymorphism among *L. donovani* strains from different regions in East Africa. This should be taken into account when designing diagnostic assays and vaccines based on this antigen.

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Introduction

Parasites belonging to the *Leishmania donovani* complex, *L. donovani* and *L. infantum* (synonym = *L. chagasi*), are the main causative agents of visceral leishmaniasis (VL), also known as kala-azar. This disease is invariably fatal if not properly diagnosed and treated. The World Health Organization (WHO) estimates that the yearly incidence of VL is between 2–400,000 cases, resulting in 20–40,000 deaths annually with the majority of cases, >90%, occurring in Brazil, the Indian subcontinent and east Africa [1]. VL in the latter region is found primarily in Sudan, South Sudan and Ethiopia where an estimated 30,000–57,000 cases occur each year [1,2,3]. In East Africa and India, VL is primarily caused by *L. donovani*, and believed to be an anthroponosis, while in other regions, where VL is caused by *L. infantum*, this disease is a zoonosis with dogs and wild canids acting as reservoir hosts [4].

In Ethiopia, VL is distributed throughout the lowlands with the most important foci found in northwestern and southwestern parts of the country. However, the ecology, vectors responsible for parasite transmission, and epidemiology of VL differ between these regions. Northwestern Ethiopia (NW) accounts for ~60% of the VL cases [3], and a majority of the HIV - VL co-infections, with the disease focused in the Metema - Humera region near the Sudanese border. This is a semi-arid region, with extensive commercial monoculture, and scattered Acacia - Balanite forests. *Phlebotomus orientalis* is the suspected vector responsible for transmission [3,5]. The recent large increase in VL in NW

Author Summary

HASPB belongs to a hydrophilic repeat-containing surface antigen family found in *Leishmania*. The *L. infantum*/*L. donovani* protein has been used for diagnosis of visceral leishmaniasis, and is a putative vaccine candidate for this disease. Visceral leishmaniasis is a fatal disease, and approximately one third of the cases are found in East Africa. The k26 – PCR, which amplifies the repeat region of HASPB, produced different amplicon sizes for recent Ethiopian *L. donovani* depending on the strain's geographic origin. Further analysis showed that the number and order of the peptide motifs, either 13 or 14 amino acids long, comprising the *L. donovani* repeats varies between endemic regions of East Africa. Polymorphism in the amino acid sequence of the peptides was also observed. In addition, the 13 amino acid peptide motifs prevalent in *L. donovani* are rare in *L. infantum*. The observed polymorphisms in the HASPB repeat region suggests that custom antigens may be needed for diagnosis or vaccination in distinct endemic foci.

Ethiopia has been correlated with agricultural development, and the large influx of seasonal workers ([6,7]. Migrant workers returning from this area to the non-endemic highlands appear to be responsible for introducing the VL into the latter regions, as typified by the recent outbreak that occurred in Libo-Kemkem, South of Gondar [6]. In southwestern Ethiopia (SW), VL foci are mainly located in the Omo River plains, Segen and Woito Valleys, and near the border with Kenya [3,8]. These regions include savannah and forest, and *P. martini* and *P. celiae* have been implicated as vectors [3,9,10]. Disease in Southern Ethiopia appears to be sporadic and stable occurring most frequently among children or young adults [8].

Analysis of parasites belonging to the *L. donovani* complex using multiple molecular markers that included DNA sequences of protein coding, non-coding and intergenic regions, microsatellites (MLMT) and other techniques, resulted in a revised taxonomy [11]. East African strains, previously split into *L. donovani*, *L. archibaldi* or *L. infantum* by multilocus enzyme electrophoresis (MLEE) are now classified in one group as *L. donovani s.s.* This large study confirmed several earlier publications using individual molecular techniques [12,13,14]. Several of these studies identify genetically distinct populations among the *L. donovani* complex associated with different geographic regions [13,14]. Recently, analysis using 14 unlinked microsatellite markers of 90 East African strains, including 63 new isolates from Ethiopia, showed that *L. donovani* can be divided into two genetically distinct populations, Sudan plus NW, and Kenya plus SW. These major groups could also be further divided into several subpopulations [15]. Although MLMT easily distinguishes between two main *L. donovani* genotypes in Ethiopia, NW and SW type, and can produce individual parasite genetic pedigrees, it is relatively expensive, requires more sophisticated analysis, and not available in most laboratories working on *Leishmania*.

HASPB (hydrophilic acylated surface protein B) belongs to a family of orthologous genes, originally called the LmcDNA16 locus, found in Old and New World *Leishmania* species [16]. The protein is expressed only by metacyclic promastigotes and amastigotes, and is characterized by amino acid repetitive domains that show both inter- and intra-species polymorphism [17,18,19]. A recent study using *L. major* LmcDNA16 locus null mutants, and parasites complemented for either HASPB or the whole locus showed that this protein is involved in metacyclogenesis and promastigote localization in the sand fly vector [20]. The repeat region of the *L. donovani* and *L. infantum* HASPB protein, also known as k26, is recognized by human and canine VL sera, and has been used with varying success for serodiagnosis [19,21,22,23,24,25,26]. In addition, HASPB has been shown to be a potential vaccine candidate [27,28,29].

A specific PCR targeting the *L. donovani* complex HASPB repeat region (k26 – PCR) was shown to distinguish between *L. donovani* and *L. infantum* strains grouping them according to the size of the amplicon [30]. However, only a few East African strains from Sudan (n = 6) and Ethiopia (n = 2) isolated between 1954 and 2000 were examined. More recently, Gadisa et al. [31] characterize five clinical isolates from VL patients in Ethiopia by k26 - PCR. Only a single PCR fragment was observed, all the same size as the WHO reference strain LV9 (MHOM/ET/67/HU3).

In this study, we characterized 63 recent *L. donovani* strains from Ethiopia using k26 - PCR, and high resolution melt (HRM) analysis. Several strains from Kenya, Sudan and India were also included for comparison. Analysis by these techniques split the Ethiopian strains into groups that are correlated with the geographic origin of the parasite strain. DNA sequencing of the amplicons showed that the number and organization of the peptide motifs comprising the *L. donovani* HASPB repeat domain varies with the geographic origin of the strain. Potential effect of k26 polymorphism on use of HASPB for serodiagnosis and vaccination is discussed.

Materials and Methods

Ethical considerations

This study was conducted according to the Helsinki declaration, and was reviewed and approved by the Institutional Review Board (IRB), Medical Faculty, Addis Ababa University. Written informed consent was obtained from each study participant.

Clinical isolates and reference strains used in the study

Leishmania strains (n = 63) recently isolated from patients with VL or HIV - VL co-infections in northwestern (n = 40) and southern Ethiopia (n = 23), see Figure 1, were cultured in M199/Hepes pH 6.8 medium supplemented with 10% fetal calf serum and antibiotics [32]. DNA extraction was carried out using the Gentra DNA extraction kit (Gentra system, Minneapolis, MN). In addition, DNA from *L. donovani* strains, Ethiopian (n = 24) and Kenyan (n = 7) previously examined by MLMT [15], and from Sudan (n = 2) and India (n = 2) was also analyzed. The strains used in this study are described in Table S1.

Polymerase chain reaction (PCR) and high resolution melt (HRM) analysis

Internal transcribed spacer 1 (ITS1) - PCR followed by restriction fragment length polymorphism (RFLP) analysis was carried out as described [33]. A modified, "short" cpbE/F - PCR was used to distinguish between *L. infantum* and *L. donovani*, and was carried out using the primers 5-GTTATGGCTGCGTGGC-TTG-3 (this study) and 5-CGTGCACTCGGCCGTCTT-3 [34]. DNA (50–100 ng) was added to a PCR - Ready Supreme reaction mix (Syntezza Bioscience, Jerusalem, Israel) in 25 µL total reaction, and performed as follows: Initial denaturation 4 min at 95°C; followed by 35 cycles with each cycle consisting of denaturation 30 s at 94°C, annealing 15 s at 50°C, and extension 60 s at 72°C. Final extension step was carried out for 10 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized

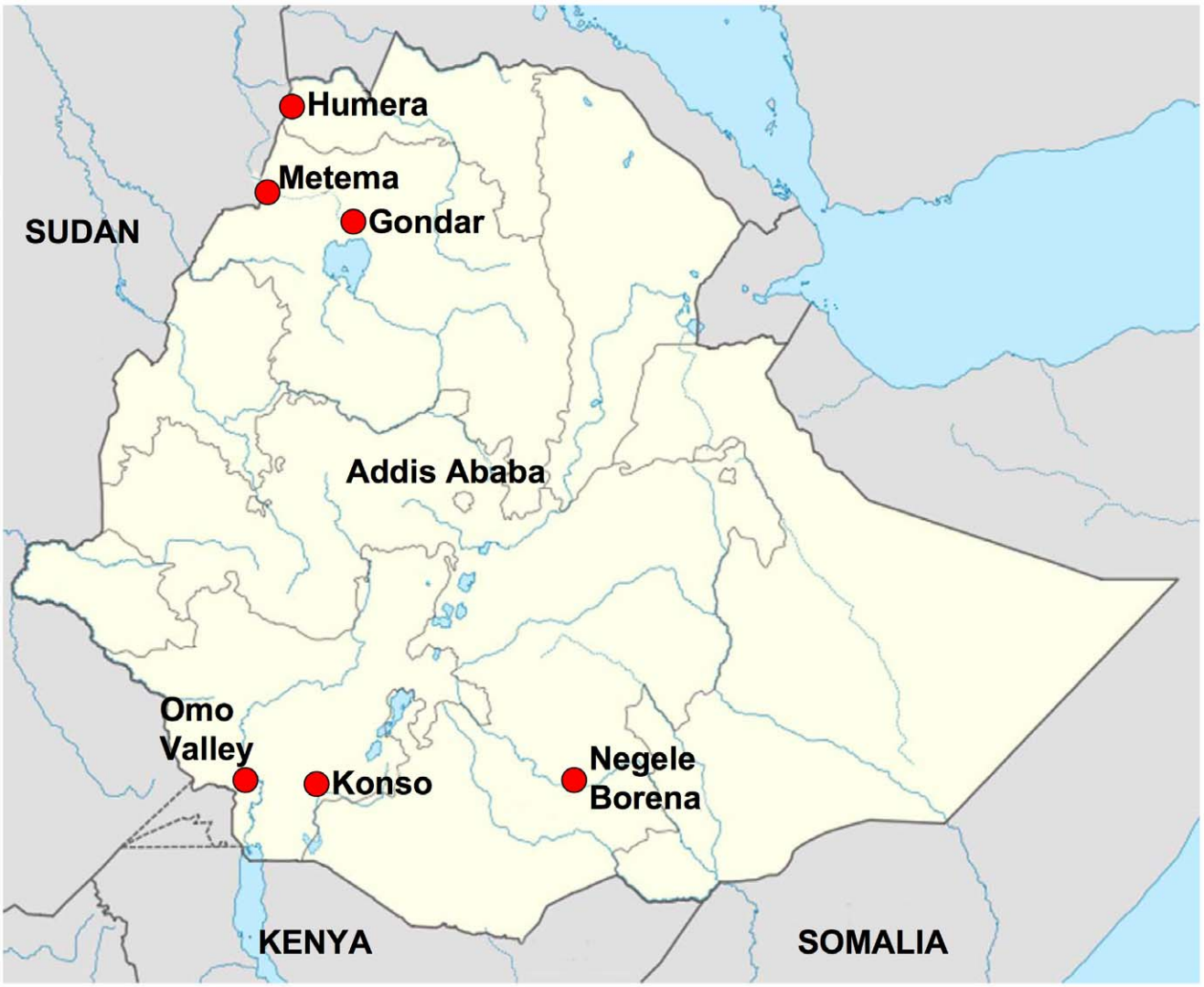

Figure 1. Origin of Ethiopian *Leishmania donovani* strains used in this study. Specific details on all strains are provided in Table S1.
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using UV light. *L. infantum* gives a 361 bp product, while *L. donovani* give a 400 bp product in the short cpbE/F PCR.

K26 - PCR was carried out as described [30], and analyzed by agarose gel electrophoresis as above. HRM analysis of the k26 amplicons was carried out as follows: DNA (20 ng) or no DNA control was added to Type-it HRM PCR Kit reaction mix (12.5 µl, QIAGEN GmbH, Germany) containing the k26 primers (1 µM each final concentration), and ultra-pure PCR-grade water (final volume 25 µl/PCR). Amplification conditions were as follows: 10 min denaturation at 95°C, followed by 40 cycles of denaturation 5 s at 95°C; annealing 10 s at 55°C; and extension 20 s at 72°C. HRM ramping was carried out at 0.2°C/s from 70 to 95°C. HRM PCR and analysis were performed using a Rotor-Gene 6000 real-time thermal analyzer (Corbett Life Science, Australia). Positive-control (reference strain DNA, 20 ng/reaction) and negative-control reactions were included in each experiment. A normalized melt window, ~85 to 90°C, was used in analyzing the HRM curves.

DNA sequencing and analysis

For direct sequencing, the PCR products were purified using Wizard SV gel and PCR clean-up system purification kit (Promega, WI, USA). The eluted DNA was sequenced at the Center for Genomic Technologies, The Hebrew University of Jerusalem, and the sequences submitted to GeneBank at NCBI. Peptide sequences were obtained using the ExPASy Translate Tool (http://web.expasy.org/translate/). DNA and peptide sequences were aligned using CLUSTAL 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2), and linear B-cell epitopes predicted using BepiPred and ABCpred (http://www.cbs.dtu.dk/services/BepiPred and http://www.imtech.res.in/raghava/abcpred/index.html, respectively) [35] [36].

Results and Discussion

Characterization of recent *Leishmania* isolates from Ethiopian patients with visceral leishmaniasis

DNA was purified from 63 *Leishmania* strains isolated from Ethiopian patients presenting with either VL or HIV-VL co-infections. As an initial step the DNA's were first examined by ITS1 - PCR RFLP, and shown to belong to the *L. donovani* complex (data not shown). Since it can be difficult to distinguish between *L. infantum* and *L. donovani* using the ITS1 - PCR RFLP

[33], we also analyzed these strains using a modified cpbE/F – PCR based on the procedure described by Hide and Banuls [34]. The *L. infantum cpbE* and *L. donovani cpbF* genes are similar except for a 39 bp insert only present in the latter species. This difference is more easily observed by gel electrophoresis using the short cpbE/F – PCR where the amplicon size is 361 bp for *L. infantum* and 400 bp for *L. donovani*, rather than 702 and 741 bp, respectively, in the original procedure [34], since the relative size difference between the two short PCR products is larger. This alleviates the need for additional treatments, such as digestion with restriction enzymes [31,37], which can facilitate species identification. Using the short cpbE/F – PCR all 39 new Ethiopian VL patient strains gave 400 bp PCR products typical of *L. donovani* (Figure 2, and data not shown), and are identical to the Sudanese reference strain (MHOM/SD/1962/1S cl2, lane Ld). As expected, the *L. infantum* reference strains (MCAN/IL/2000/LRC-L792 – lanes Li1 and MHOM/TN/1980/IPT1 - Li2) gave a shorter 361 bp product.

Analysis of k26 repeat region of the HASPB gene in *L. donovani*

The k26 - PCR, a *L. donovani* complex specific assay, targets the repeat region of the *HASPB* gene, and was shown to differentiate among *L. donovani* strains based on the size of the PCR product. *L. donovani* strains from East Africa gave products <430 bp, and Indian isolates showed significantly larger products (~660 bp) [30,31]. Strains previously examined from Sudan (n = 6) and Ethiopia (n = 2) gave two main products, ~284 and ~430 bp, with one Ethiopian isolate in each group. These strains were isolated between 11 to 49 years ago, and mutations in the *HASPB* gene may have occurred over time, or due to repeated passage in culture. In a recent report where five clinical isolates from Ethiopia were examined only one product, ~290 bp, was observed [31]. Therefore, we decided to examine a large number, n = 63, of recent *L. donovani* strains isolated from VL and HIV – VL co-infected patients in different geographic regions of Ethiopia. Interestingly, four different amplicon sizes were observed: ~290, ~360, ~410 and ~450 bp (Figure 3). The PCR product sizes for all the strains examined are summarized in Table S1. Surprisingly, there was a good correlation between geographic origin and amplicon size with strains isolated from patients in northwestern Ethiopia giving either ~290 or ~410 bp products, and all the strains isolated in southern Ethiopia, except for three, giving ~450 bp products. Interestingly, the four strains in the k26-410 cluster were isolated from 3 HIV – VL co – infected patients. Two of the strains were obtained from the same patient, one before drug treatment (LDS 373), and one following relapse (DM376). Prior to drug treatment, the parasites cultured from the spleen or bone marrow of the same patient (LDS 373) gave different k26 amplicon sizes, k26-290 or k26-410 respectively, when examined by PCR. The remaining 11 NE strains isolated from HIV – VL patients all grouped in the k26-290 cluster together with all of the strains isolated from HIV negative VL patients.

Endemic regions for VL in northwestern and southern Ethiopia extend into neighboring Sudan and Kenya, respectively. For this reason, it was interesting to see whether AM553 (k26-360), which gave a unique amplicon different from the other southern strains, represented a second group. This strain is from Negele-Borena close to the border with northwest Somalia and northeast Kenya. Seven *L. donovani* strains from Kenya were screened by k26 – PCR. All of the Kenyan strains produced amplicons larger than the Ethiopian *L. donovani* strains examined here. Of these, 6/7 Kenyan strains gave products ~500 bp and 1/7 strains gave a product of ~650 bp. Both Sudanese reference strains examined in this study gave a 290 bp PCR fragment, similar to that previously reported [30], and belong to the k26-290 cluster (data not shown).

HRM analysis is a rapid and inexpensive method for detecting polymorphisms in double stranded DNA that can potentially distinguish between single base differences. This technique was used in conjunction with k26 - PCR to examine the Ethiopian strains. Typical results are shown in Figure 4. These results show that this technique can be used to rapidly and easily distinguish between the groups found in Ethiopia (k26-290, -360, -410 and -450).

The k26 - PCR and HRM results suggested that there is little size and DNA sequence variation within each Ethiopian *L. donovani* geographic cluster. This was confirmed by DNA sequencing of 15 amplicons (Genbank accession Nos.: JX088380 - JX088392, JX294866, JX294867) from samples belonging to the four Ethiopian clusters. Analysis of the amino acid sequences (Figure 5) showed that the HASPB repeat region for each *L. donovani* group in Ethiopia is comprised of two motifs, A and B, 14 and 13 amino acids long respectively. These motifs are further distinguished by the amino acids *GHTQK* and *DHAH* present in the central region of each peptide (shown in italics). Two peptides, A3 (PKED*GHTQK*NDGDG) and B2 (PKED*DHAH*NDGGG), comprise 81% of the peptides found in the repeat region, and represent 62.5 and 92.3%, respectively, of the A (Figure 5, yellow) and B (Figure 5, blue) motifs observed in the Ethiopian strains. Several amino acid substitutions, primarily at positions 5, 12–14 of peptide A3 or positions 3 & 12 of peptide B2, also occur in each of the motifs (Figure 5A). As expected, the number of repeats correlates with the size of the PCR amplicon (Figure 5B), however the organization of the peptide repeats is different for each cluster, and doesn't appear to be due to simple DNA duplication or deletion. The order of the peptide motifs observed for each of the Ethiopian cluster can be thought of as a bar code specific for that region.

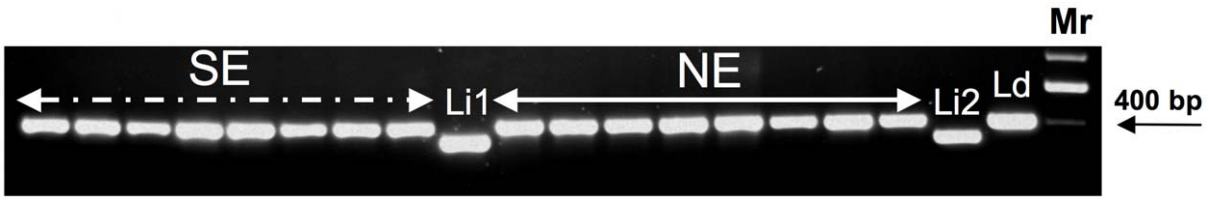

Figure 2. Characterization of Ethiopian parasite strains from patients with visceral leishmaniasis by short cpbE/F - PCR. Amplicons were separated by electrophoresis on 2% agarose gel and staining with ethidium bromide. Reference DNA samples for *Leishmania infantum* are indicated by Li1 (MCAN/IL/2000/LRC-L792) and Li2 - (MHOM/TN/1980/IPT1), and for *L. donovani* by Ld (MHOM/SD/1962/1S cl2). Mr −100 bp molecular weight marker. Representative parasite DNA samples examined by short cpbE/F - PCR from left to right Southern Ethiopia (SE): AM546, AM548, AM551, AM552, AM553, AM554, AM560, AM563 and Northern Ethiopia (NE): GR284, GR353, GR356, GR358, GR361, GR378, GR379, GR383.
doi:10.1371/journal.pntd.0002031.g002

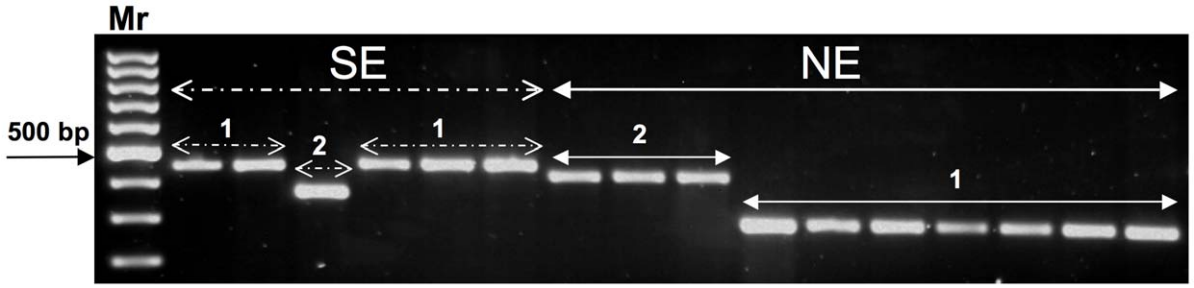

Figure 3. Analysis of Ethiopian *Leishmania donovani* strains by k26 - PCR and agarose gel electrophoresis. PCR products were separated by electrophoresis in 2% agarose gels and stained with ethidium bromide. Southern Ethiopian (SE): 450 bp amplicon −1 and 360 bp amplicon −2. Northern Ethiopian (NE): 290 bp amplicon −1 and 410 bp amplicon −2. A 100 bp molecular weight marker (Mr) is shown on either side of the gel. DNAs from *L. donovani* examined by k26 – PCR in order from left to right: DM290, DM317, AM553, DM283, DM291, AM546, DM256, DM257, DM376sp, GR284, DM14, DM297, DM259, DM287, DM299a, DM389.
doi:10.1371/journal.pntd.0002031.g003

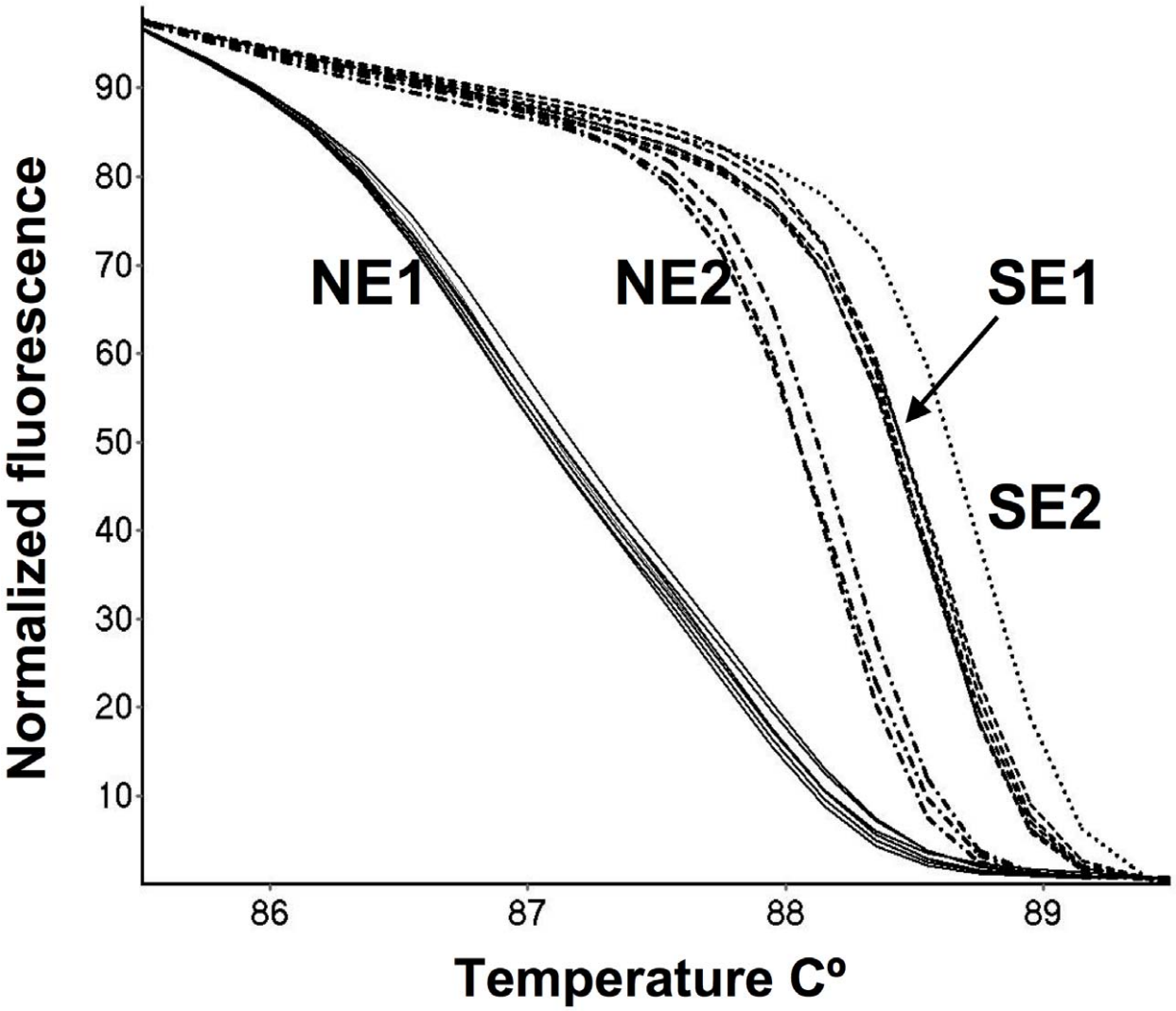

Figure 4. High resolution melting (HRM) curves for k26 - PCR amplicons of Ethiopian *Leishmania donovani*. Plot of normalized fluorescence versus temperature of strains from southern Ethiopia: SE1, 450 bp and SE2, 360 bp. Northern Ethiopia, NE 1, 290 bp and NE2, 410 bp. Strains shown in figure: SE1 – DM283, DM290, DM291, DM317, AM546 ; SE2 - AM553; NE1 - DM259, GR284, DM287, DM 297, DM 299a and DM14 and NE2 - DM256, DM257, DM376spl.
doi:10.1371/journal.pntd.0002031.g004

A.

Leishmania donovani		*Leishmania infantum*	
ID	**Sequence**	**ID**	**Sequence**
A3.	PKEDGHTQKNDGDG	A3.	PKEDGHTQKNDGDG
A0.	. . . . . G.	A9.	. . . . . A
A14.	. . . . D	A16.	. . . . . DG.
A20.	. . . . . GA		
A21	L.		
A23.	. . . . . CGV		
A24.	. . . . . GV		
a10.	. . . . . R.	a10.	. . . . . R. . . . .
		a17	. . . . . R. . . . . DG.
		a18.	. . . . . R. . . . N. . .
B2.	. . . . D . AH- . . . G.	a19	. . . . . R. . . . . G.
B4.	. . . . D . AH-		
B22.	. . D . D . AH- . . . G.		

B.

K26 cluster	Repeat Peptide Organization											
	1	2	3	4	5	6	7	8	9	10	11	12
Leishmania donovani												
ET-290	A3	A14	B2									
ET-360	A3	B2	B2	B2	B2							
ET-410	A21	A20	A3	B4	B2	B2						
ET-450	A3	B2	B2	A3	B2	B2	B2					
KE-500	A3	B2	B2	A3	B2	B22	B2	A23				
KE-650	A0	B2	B2	A3	B2	B2	B2	A3	a10	A3	A0	A24
IN-600	A0	B2	A3	B4	B2	B4	A3	B4	B2	B2	B2	B2
*Leishmania infantum**												
1a	A3	A16	A3	a18	A9	a10	a10	a10	a10	A3	a19	
1b	A3	a17	A3	a18	A9	a10	a10	a10	a10	A3	a19	
2**	A3	A3	a10	a18	A9	a10	a10	a10	a10	A3	?	?

Figure 5. Amino acid sequences and organization of the *Leishmania donovani* complex HASPB repeat region. Panel A. Comparison of peptide repeats motif sequences found in *L. infantum* and *L. donovani*. Panel B. Bar code of peptide motif organization for the k26 repeat region of HASPB in *Leishmania donovani* complex from different geographic regions of the Old World. Peptides A (bright yellow) −14 amino acid peptides found both in *L. donovani* and/or *L. infantum*; Peptides a (banana yellow) −14 amino acid peptides found primarily in *L. infantum* containing the arginine (R) substitution at position 6; Peptides B (blue) −13 amino acid peptides found in *L. donovani*. Peptide A3 found in both species was chosen as the reference sequence to which the amino acid sequences of all the other peptides are compared: Single amino acid abbreviations in blue indicates a substitution, in red a deletion, and in black conserved; (−) missing amino acid, (.) conserved amino acid. Peptides numbers from 0–19 have amino acid sequences identical to those reported by Maroof et al. [29]; those with numbers ≥ 20 are new sequences described in this study.
doi:10.1371/journal.pntd.0002031.g005

Kenyan and Indian *L. donovani* strains produce larger k26 -PCR amplicons than the Ethiopian strains (this study and [30,31]). As such it was interesting to sequence these products and determined the peptide composition and organization of the HASPB repeat region (Genbank accession No.: JX294868–JX294870). This region in the Kenyan and Indian *L. donovani* strains is also comprised of the same peptide motifs, A and B, found in the Ethiopian strains. Several amino acid substitutions (A0, a10, A23, A24 and B22), not observed in HASPB of the Ethiopian strains, are found in these parasites (Figure 5), but A3 and B2 still comprise a majority of the sequences observed. Together, these two peptides comprise 75 and 66.6% of the sequences found in the Kenyan and Indian strains, respectively. The combined percentage of peptides A3 and B2 for the Indian *L. donovani* strain described here is similar to that reported for other Indian isolates, 59.7% [29], even though additional peptide sequences, not observed in our study, were found in the latter isolates (Table S2). However, if the motif A (yellow) or B (blue), rather than the specific peptide sequence, is examined, then a similarity in organization of the repeats, ABBABBB, in the Kenyan and Ethiopian-450 k26 clusters is readily apparent.

The repeat region of the *L. chagasi* (syn = *L. infantum*) HASPB gene was previously characterized and cloned; and has been used in serological assays for VL with mixed results

[19,21,22,23,24,25,26]. Sequences for *L. infantum* strains from Brazil, France, Greece, Iran, and Spain (Genbank accession Nos.: AF131228.1, EF504256.1, EF504255.1, EF504258.1, EF504257.1, DQ192034.1, and FR796455.1) show that the HASPB repeat region is only comprised of 14 amino acid peptide repeats. Two peptides A3 (PKED*GHTQK*NDGDG) and a10 (PKED*GRTQK*NDGDG) comprise a majority of the *L. infantum* k26 repeats. Peptide A3 is identical to the peptide found in the *L. donovani* repeat region, while peptide a10 only differs from peptide A3 by substitution of arginine for histidine at position 6 (underlined), and should be considered a member of the peptide A archetype family. However, the latter peptide, a10, does not appear to be very common in East African *L. donovani*, appearing only once among all the parasites examined to date. Conversely, the *L. donovani* 13 amino acid peptide B archetype family, exemplified by PKED*DHAH*NDGGG (peptide B2) and other B peptides (Figure 5 and Table S2), was not present in any of the seven *L. infantum* sequences examined above, as well as six additional strains from Israel (data not shown). However, peptide B8 (Table S2) belonging to the B family archetype appears once in a *L. infantum* strain previously analyzed [29]. HASPB repeat region in fifteen *L. infantum*/*L. chagasi* strains contained almost exclusively peptides belonging to the A family archetype. The organization of peptide motifs was very similar for all the *L. infantum* strains where sequence data was available (Figure 5). However, most of the isolates analyzed belong to clusters 1a and 1b [30] which both give 626 bp amplicons by k26 - PCR.

The HASPB repeat region of *L. donovani* and *L. infantum* strains is predicted to contain multiple linear B-cell epitopes using two different programs (Figure 6, and data not shown [35,36]). Most of the predicted epitopes (16 amino acids long, threshold $\geq$ 0.8 out of 1.0) in the *L. donovani* k26 clusters (East Africa and India) span motif junctions (A|A, A|B, B|A or B|B, 84%) with a unique *L. donovani* sequence, K/HNDGD/GG | PKEDDHAHND, accounting for 32/50 (64%) of these epitopes (Figure 6). This sequence is even more predominant, 80–100% of the predicted epitopes, in the southern Ethiopian, Kenyan and Indian *L. donovani* k26 clusters which contain multiple B motifs. This epitope is not seen in the *L. infantum* k26 repeat region, as the B motif is rarely observed in this species. Instead most of the predicted B-cell epitopes, 75%, contain the complete 14 amino acid A motifs, with only a few centered at the A | A motif junctions. Several of the predicted *L. infantum* B-cell epitopes are also found in *L. donovani*.

In this study we examined 63 recent strains isolated from Ethiopian VL patients in different regions of the country. All the parasites were shown to be *L. donovani* by three techniques, confirming previous findings that this species, not *L. infantum*, is responsible for VL in Ethiopia. Interestingly, we found that

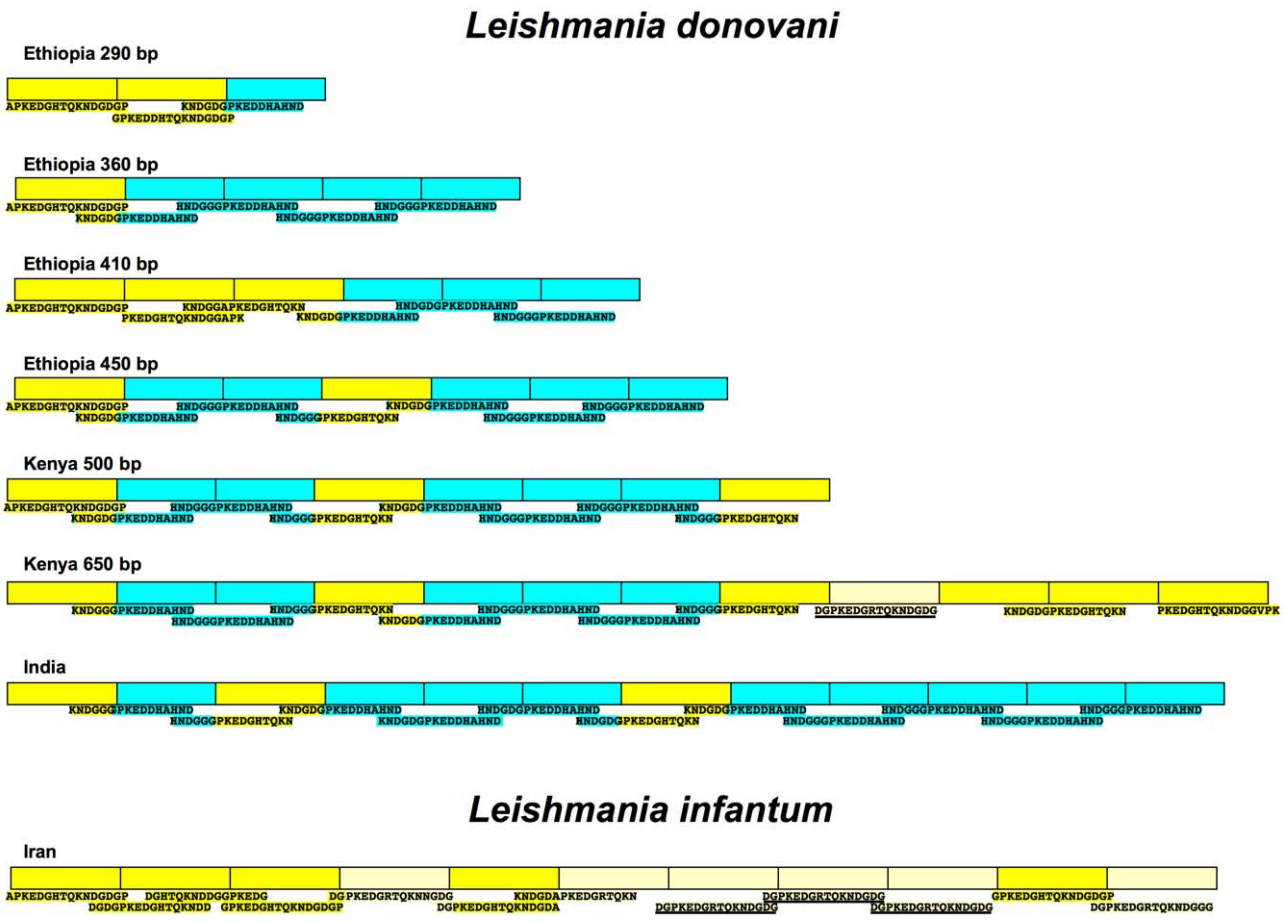

Figure 6. Predicted B-cell epitopes in HASPB repeat regions of *Leishmania donovani* and *L. infantum*. Bar codes, see Figure 5B for legend, showing the A(a) or B motif organization of the repeat region for East African and Indian *L. donovani*, and for *L. infantum* cluster 1a. Non-overlapping B-cell epitopes, 16 amino acids long with a threshold $\geq$ 0.8, were predicted using a recurrent artificial neural network (ABCpred server [36]). The position of each epitope is indicated under the respective k26 – PCR product cluster bar code. The B-cell epitope recognized by infected canine visceral leishmaniasis sera, DGPKEDGRTQKNDGDG, is underlined.
doi:10.1371/journal.pntd.0002031.g006

parasites from northwestern and southern Ethiopia could be easily distinguished based on the size of the k26 – PCR amplicons or their corresponding HRM curves. A similar clustering into two major populations by geographic origin was first reported using multiple microsatellite markers that grouped Sudanese and northwestern (Metema, Humera and Belessa) strains separately from Kenyan and southern strains (Negele-Borena and Konso) [15]. Clustering into genetically separate populations is perhaps, expected, since the primary sand fly vectors, *P. orientalis* and *P. martini*, and habitats are different for the two regions. Other differences between parasites isolated from patients in these two regions, such as sensitivity to paromomycin, have been reported [38]. Interestingly, parasites from northwestern Ethiopia could be divided into additional groups based on the k26 amplicon size, 290 bp and 410 bp. All the Sudanese parasites examined so far gave PCR products similar in size to parasites from northwestern Ethiopia (this study, [13,30], and data not shown). While the k26-290 group contained isolates from both VL (n = 25) and HIV-VL co-infected (n = 11) patients, the k26-410 group only contained strains from HIV-VL co-infected patients (n = 3). Of the latter isolates, 3/4, were previously analyzed using microsatellite markers [15], and belong to subpopulation B2. Interestingly, this subpopulation was postulated to represent one parent strain of putative hybrid/mixed genotypes.

Different k26 – PCR products were also found when parasite strains from southern Ethiopia were analyzed, k26 −290, −360 and −450. All of the strains examined except three (AM422, AM452, and AM553) produced a 450 bp amplicon. Since microsatellite analysis grouped southern Ethiopian and some Kenyan parasites together [15], and *P. martini* is the primary vector involved in the *L. donovani* transmission in these regions [9], we decided to examine several Kenyan strains by k26 – PCR. Surprisingly, the k26 amplicons for all the Kenyan parasites tested were larger (~500 and ~650 bp) than those found for the south Ethiopian isolates, and similar in size to Indian *L. donovani* parasites (this study and [30]). Thus, there doesn't seem to be a direct correlation between the size of the k26 amplicon, and the microsatellite cluster to which the strain belongs. It is not clear whether the two southern Ethiopian strains that gave the 290 bp PCR product represent a third group present in this region, are a result of human migration or are due to culture contamination. The k26 DNA sequence for these strains is identical to the other 290 bp Sudanese and northern Ethiopian strains examined (Table S1, and data not shown). Interestingly, one of the strains, AM422, originates from the Omo Valley where transmission by both vectors may occur, and is close to Sudan. More work is needed to determine whether there is a direct correlation between the parasite vector and k26 genotype, as HASPB plays a role in parasite differentiation and localization in the sand fly [20]. At this time it is not clear why *L. donovani* strains from different regions in East Africa show variations in the k26 – PCR fragment size, or the factors responsible for the size polymorphism, however this technique appears to be useful for rapid mapping of strain origin on a large scale.

The HASPB1 protein is a potential vaccine candidate, as well as a diagnostic antigen [19,21,22,23,24,25,26,27,28,29,39,40]. However, serodiagnostic assays using the HASPB1 protein or k26 repeat region as antigen have produced conflicting results. While assays using sera from canine or human VL caused by *L. infantum* give consistently high sensitivity (94–100%) and specificity (100%) [23,24,39], similar assays using VL sera from patients in India and Sudan showed variable sensitivity (India −21.3 and 38%; Sudan −92 and 93.5%) [21,22,25,26]. Assay specificity in latter studies was consistently high (80–100%). Interestingly, the assays showing low sensitivity in Indian VL patients used the *L. infantum* k26 antigen [21,22], while assays demonstrating high sensitivity in Sudanese VL patients used the *L. donovani* antigen [25,26]. The B-cell epitopes recognized by serum antibodies in the HASPB1 repeat region have not been extensively analyzed, though one study reported that the 17 amino acid peptide, *G*DGPKEDGRTQKNDGDG from *L. infantum* reacted strongest with canine VL sera [41]. Interestingly, when putative linear B-cell epitopes in the *L. infantum* k26 repeat region were predicted (Figure 6) using a recurrent artificial neural network (ABCpred server [36]) a peptide, DGPKEDGRTQKNDGDG, 16 amino acids in length, and identical in 16/17 amino acid residues to the peptide recognized by canine sera above, ranked first with a score of 0.88 out of 1.0. This peptide includes the 14 amino acid motif (a10 – PKEDGRTQKNDGDG) frequently found in *L. infantum* (Figures 5 and 6), but rarely in *L. donovani* strains (this study and [29]). The a10 motif was predicted to be a B-cell epitope (score = 0.81). On the other hand, none of the peptide motifs (B2, B4 and B22; PKE/DDDHAHNDGG/DG) unique to *L. donovani* rk26 are found in *L. infantum*, and combinations of these motifs generated *L. donovani* B-cell epitopes giving the highest scores (e.g., KNDGDGPKEDDHAHND, 0.88; HNDGGGPKEDDHAHND, 0.87; HNDGDGPKEDDHAHND, 0.87; and data not shown). It will be interesting to see if better sensitivity and specificity can be obtained using either single antigen or mixtures of recombinant k26 antigens produced from the *L. donovani* strains responsible for local disease in Ethiopia and Sudan. This work is in progress.

HASPB1 is differentially expressed by metacyclic promastigotes and intracellular amastigotes [42]. Immunization of BALB/c mice with *L. donovani* HASPB1, even in the absence of adjuvant, generates a protective CD8+ T-cell response via an immune complex-mediated complement activation involving natural antibodies against a challenge with this parasite [27,28]. The CD8+ T-cell epitopes were shown to reside in both the conserved and repeat regions of the protein [29]. While a role for HASPB in the development of metacyclic promastigotes was demonstrated [20], the function of these proteins in amastigotes is not yet clear. Interestingly, an orthologous protein, O-HASP, from *L. (Viannia) braziliensis* showed considerable genetic polymorphism in the repeat region among clones isolated from individual patients [43], and it was postulated that genetic variation may play a role in immune recognition. A similar phenomenon appears to occur in Old World *Leishmania* causing VL, as one report suggests that clonal variation is present in HASPB of Indian *L. donovani* strains [29]. However, DNA sequencing of 21 clones from four Ethiopian strains (k26-290 bp) did not identify any polymorphism in the repeat region of this protein (data not shown).

In summary, we show that the number, order and arrangement of the *L. donovani* k26 repeat region of the HASPB protein varies among strains from different geographic regions, and that the repeat motifs are different from those observed for *L. infantum*. The role that this genetic variation plays in the interaction with the host and vector is not clear and should be investigated further.

Supporting Information

Table S1 *L. donovani* strains used in this study.
(DOCX)

Table S2 Summary of peptides found in the HASPB repeat region of parasites belonging to the *Leishmania donovani* complex.
(DOC)

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Author Contributions

Conceived and designed the experiments: AZ AN CLJ. Performed the experiments: AZ AN. Analyzed the data: AZ CLJ. Contributed reagents/materials/analysis tools: YT DT WH ZH SY TW ED AK AH AZ AN CLJ. Wrote the paper: AZ CLJ AN AH.

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Paper III

Seblova V, Volfova V, Dvorak V, Pruzinova K, Votypka J, **Kassahun A**, Gebre-Michael T, Hailu A, Warburg A, Volf P, 2013. ***Phlebotomus orientalis*** **sand flies from two geographically distant Ethiopian localities: Biology, Genetic Analyses and Susceptibility to** ***Leishmania donovani*****.** PLoS Negl. Trop. Dis. 7: e2187

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Phlebotomus orientalis Sand Flies from Two Geographically Distant Ethiopian Localities: Biology, Genetic Analyses and Susceptibility to *Leishmania donovani*

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Abstract

Background: *Phlebotomus orientalis* Parrot (Diptera: Psychodidae) is the main vector of visceral leishmaniasis (VL) caused by *Leishmania donovani* in East Africa. Here we report on life cycle parameters and susceptibility to *L. donovani* of two *P. orientalis* colonies originating from different sites in Ethiopia: a non-endemic site in the lowlands - Melka Werer (MW), and an endemic focus of human VL in the highlands - Addis Zemen (AZ).

Methodology/Principal Findings: Marked differences in life-cycle parameters between the two colonies included distinct requirements for larval food and humidity during pupation. However, analyses using Random Amplified Polymorphic DNA (RAPD) PCR and DNA sequencing of cytB and COI mitochondrial genes did not reveal any genetic differences. F1 hybrids developed successfully with higher fecundity than the parental colonies. Susceptibility of *P. orientalis* to *L. donovani* was studied by experimental infections. Even the lowest infective dose tested (2×10^3 per ml) was sufficient for successful establishment of *L. donovani* infections in about 50% of the *P. orientalis* females. Using higher infective doses, the infection rates were around 90% for both colonies. *Leishmania* development in *P. orientalis* was fast, the presence of metacyclic promastigotes in the thoracic midgut and the colonization of the stomodeal valve by haptomonads were recorded in most *P. orientalis* females by day five post-blood feeding.

Conclusions: Both MW and AZ colonies of *P. orientalis* were highly susceptible to Ethiopian *L. donovani* strains. As the average volume of blood-meals taken by *P. orientalis* females are about 0.7 µl, the infective dose at the lowest concentration was one or two *L. donovani* promastigotes per sand fly blood-meal. The development of *L. donovani* was similar in both *P. orientalis* colonies; hence, the absence of visceral leishmaniasis in non-endemic area Melka Werer cannot be attributed to different susceptibility of local *P. orientalis* populations to *L. donovani*.

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Introduction

Visceral leishmaniasis (VL, kala-azar) caused by the protozoan parasite *Leishmania donovani* is a deadly disease occurring mainly in the Indian subcontinent and Africa. In Africa, VL is endemic in the eastern part of the continent; the Horn of Africa and adjacent countries, namely Sudan, South Sudan, Kenya, Somalia, Uganda, Erithrea and Ethiopia. In Ethiopia, the main endemic areas are located in the lowlands of the southwestern Ethiopia (e.g. Omo river plains and Segen/Woito valleys) and Metema-Humera plains in the northwest [1]. Three sand fly species, *Phlebotomus orientalis*, *P. celiae* and *P. martini* have been implicated as vectors [2,3]. *Phlebotomus celiae* Minter and *Phlebotomus martini* Parrot (both belonging to the subgenus *Synphlebotomus*) are limited to the south of the country, often being associated with termite hills, which provide suitable breeding sites. In the rest of Ethiopia, however, *P. (Larroussius) orientalis* seems to be the only vector.

Most biological information regarding habitat, seasonality and feeding preferences of *P. orientalis* was acquired thanks to demanding field studies in Sudan [4,5,6].The distribution of this species seems to be affected by the vegetation type, with preference for Acacia – Balanites forests and cracks of black cotton clay soil

Author Summary

Phlebotomus orientalis is the main vector of *Leishmania donovani* in East Africa and is, therefore, a sand fly species of high importance. We studied various properties of *P. orientalis* populations from both endemic (Addis Zemen) and non-endemic (Melka Werer) areas in Ethiopia. We successfully demonstrated the ability of laboratory colonies arising from these populations to crossbreed by obtaining 1st and 2nd generation hybrid progeny. Hybrids had similar or even higher fecundity than parental colonies. Comparison of the populations by sequencing of two genes (cytB and COI) and by RAPD (a multilocus method) revealed no genetic differences. We demonstrated that both populations are highly susceptible to experimental infection with *L. donovani* and even small numbers of parasites are able to initiate heavy infections in *P. orientalis* females. As the development pattern of *L. donovani* was similar for females from both colonies, we deduce that the absence of visceral leishmaniasis in the non-endemic area of Melka Werer cannot be attributed to different susceptibility of local *P. orientalis* populations to *L. donovani*.

[7,8,9]. Additional important information, like actual breeding sites of this species, remains unknown. Despite several attempts of colonization of this species [10,11] the life cycle and behaviour of *P. orientalis* in laboratory colonies has not been reported in detail and *P. orientalis* has a reputation of being difficult to colonize and maintain.

In this study, we focused on *P. orientalis* from two geographically distant Ethiopian localities, Addis Zemen (AZ) and Melka Werer (MW). Addis Zemen is located in the highlands of the Amhara Region in northwestern Ethiopia at altitude of 1800–2000 m where in 2005 and 2008, outbreaks of VL resulted in 2,500 cases and initially a very high mortality [12]. On the other hand, Melka Werer is a non-endemic area situated in Awash National game reserve in Rift Valley at an altitude of approximately 800 m, 200 km East of Addis Ababa.

Here, we compare individuals of both colonies by Random Amplification of Polymorphic DNA (RAPD) and sequencing analysis. The two populations were also tested for ability to produce viable hybrids in cross-mating studies. Different biological aspects of the two colonies found during the study allowed us to optimize the conditions for laboratory maintenance of both *P. orientalis* colonies, which appeared to be a fundamental prerequisite for the major goal of this work: experimental infections and comparison of susceptibility of both colonies to infections with *L. donovani*.

Materials and Methods

Ethical statement

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments (including sand fly feeding) were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University in Prague and were performed under the Certificate of Competency (Registration Number: CZU 327/99, CZ 00179). All samples were anonymized.

Rearing sand fly colonies and life-cycle analysis

Both of *P. orientalis* colonies Addis Zemen (AZ) and Melka Werer (MW) were established in 2008 and reared for about ten generations at the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia. For larval food, dried and ground hyrax faeces were used, females were fed on rabbits. Both the larvae and the adults were kept at 26°C. After transfer to Prague the sand flies were adapted to the conditions and the larval food routinely used in our laboratory [13]. Briefly, the larvae of both colonies were fed on a composted mixture of rabbit faeces and rabbit pellets. The suitability of autoclaved and non-autoclaved larval food was tested and compared. Adult sand flies were maintained on 50% sugar solution at 26–27°C. In the first generation after arrival to Prague, females were offered a bloodmeal on rabbit or human arm (co-author PV served as volunteer), and within several generations they were adapted to feeding on anesthetized mice. The life-cycle details (length of egg development, each larval instar etc.) were collected from 12168 (AZ) and 8751 (MW) ovipositing females and recorded for over 20 months. Data monitoring the effect of nutrition on the life cycle of two *P. orientalis* colonies originate from the offspring of about 4,600 ovipositing females (2,200 MW and 2,400 AZ) during a three month period.

Hemoglobin assay for measuring the blood-meal size

Due to massive prediuresis during bloodfeeding the classical weighing of bloodfed sand fly females leads to underestimation of the volumes of bloodmeals [14]. Therefore, the colorimetric method developed by Briegel *et al.* [15] for measuring the hemoglobin concentration in blood-fed mosquitoes was adopted. Females of *P. orientalis*, 3–6 days old, were fed through a chick-skin membrane on rabbit blood. Individual midguts of blood-fed females were dissected 1 h after blood-feeding, transferred to tubes containing 200 µl 0.15 mM NaCl and homogenized. Gut homogenates (50 µl) or diluted rabbit blood (5 µl rabbit blood/1000 µl 0.15 mM NaCl) were mixed with 200 µl of Drabkin's reagent (Sigma) in the dark for 30 min. Absorbance was measured in 96-well plates in doublets at 540 nm. Human hemoglobin (Sigma) in concentrations from 3.1 to 100 µg/well was used as standard. The bloodmeal volume was calculated from 40 midguts of fully bloodfed *P. orientalis* (MW) females.

Cross-mating study

For the cross-mating study we slightly modified the method described by Dvorak *et al.* [16]. Briefly, individual pupae from each parental colony were separated into glass vials to obtain virgin adult flies. Virgin females from one colony were grouped with virgin males from the other colony (MW male/AZ female = Hybrids 1, AZ male/MW female = Hybrids 2) in an approximate 1:1 ratio of sexes and allowed to feed on a human arm (PV served as a volunteer). Blood-fed females were separated and five days post blood-meal (PBM) transferred to moist oviposition pots to lay eggs. The egg production of hybrids was compared with both parental colonies (20 ovipositing females in each group). The parental and hybrid colonies were reared under identical conditions and their developmental life cycles were recorded (see Table 1). Adult F1 hybrids were used for F2 brother-sister mating to verify that F2 progeny were viable and develop similarly to parental lines.

Genetic analyses

The two *P. orientalis* colonies were compared by RAPD and by DNA sequencing of two mitochondrial genes, cytochrome B (cytB)

Table 1. Life-cycle of two Ethiopian *P. orientalis* colonies and their hybrid F1 and F2 progeny.

			Life cycle in days PBM*							Egg production**		
			Eggs	Larvae			Pupae	Adults		Host	Eggs	
				L1	L2	L4		From	To		Total	Mean per female
Parental colonies***	AZ	mean	6.5	13.5	19.1	28.4	36.9	46.6	105.3	mouse	544	27.2
		range	5–9	11–19	16–29	23–34	31–47	39–69	61–147	human	975	48.75
	MW	mean	7.9	14.9	20.6	28.3	35.3	45.5	83.9	mouse	641	32.05
		range	4–12	12–20	18–24	24–32	29–41	40–52	54–110	human	693	34.65
Hybrids 1 ♂MW/♀AZ	F1		7	14	18	25	30	39	91	human	852	42.6
	F2		7	14	18	25	31	42	nd	human	846	42.3
Hybrids 2 ♂AZ/♀MW	F1		7	14	18	25	30	39	91	human	806	40.3
	F2		7	14	18	25	31	42	nd	human	812	40.6

*Days represent an interval between the female took a bloodmeal and the first offspring reached the respective instar.
**In the egg production study 20 ovipositing females were used in each group.
***In the parental colonies the life cycle data were collected from 12,168 (Addis Zemen, AZ) and 8,751 (Melka Werer, MW) ovipositing females within the period from VIII/2010 to IV/2012. Each cell contains the mean and the range of values.
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and cytochrome oxidase I (COI). For RAPD analysis, eight specimens from each colony (four males and four unfed females) were selected randomly. Two other sand fly species were added into the analysis as outgroups: *Phlebotomus* (*Larroussius*) *tobbi* Adler and P*hlebotomus* (*Phlebotomus*) *bergeroti* Parrot. DNA was extracted using High Pure PCR Template Preparation Kit (Roche, France). Of 60 decamer random primers previously tested (OPA 1–20, OPD 1–20, OPF 1–20, by Operon Technologies Inc, USA), five were used: OPE16, OPI 12, 13, OPL5, OPO20. The PCR reaction was subjected to 45 amplification cycles in 25 µl volumes, with a temperature profile: 94°C for 1 min, 35°C for 2 min and 72°C for 3 min. An initial denaturation step of 94°C for 4 min and a final extension step of 72°C for 10 min were added. After PCR amplification, electophoretic bands were transformed into a binary matrix and genetic distances were computed from Nei-Li's coefficient of similarity [17]. Phylogenetic trees were constructed by the unweighted pair-grouping analysis (UPGMA) [18]. PC program FreeTree [19] was used for computations of genetic distances and construction of trees.

For sequencing analysis COI and a part of cytB genes were chosen. Templates for direct sequencing were amplified by PCR in a 50-µl volume using primers and conditions previously published [20,21]. PCR products were sequenced in both directions using the same primers as for the DNA amplification on 3100 Avant Genetic Analyser (Applied Biosystems, USA). All PCR products were cleaned by QIAquick PCR Purrification Kit (Qiangen, Germany) prior to the sequencing. Obtained DNA sequence data were compared with those in the GenBank database. The sequences were aligned using ClustalX 1.81 and the resulting alignment was manually edited by BioEdit.

Experimental infection of *P. orientalis*

Two *L. donovani* strains, GEBRE-1 (MHOM/ET/72/GEBRE1) and GR374 (MHOM/ET/2010/DM-1033) originating from VL patients in northern Ethiopia and kept in cryobank of the Department of Parasitology, Charles University were used for experimental infection of *P. orientalis*. Parasite strains were maintained at 23°C on medium 199 (Sigma) supplemented with 10% fetal calf serum (Gibco), 1% BME vitamins (Sigma), 2% human urine and amikin (250 µg/ml). Females of both colonies (~–7 day old) were fed through a chick-skin membrane on a suspension of promastigotes (from 4-days-old *Leishmania* culture) mixed 1:10 with heat-inactivated rabbit blood (Bioveta, Ivanovice na Hane, Czech Rep.). If not stated otherwise, an infective dose of 10^5 promastigotes per ml of blood was used. To test dose-dependent differences in *Leishmania* development, GR374 cultures were used at the following concentrations: 2×10^3, 2×10^4, 10^5 and 5×10^5 promastigotes/ml of blood. Furthermore, the accurate number of parasites ingested by individual females (N = 8) was determined using Q-PCR immediately after the experimental feeding (details below).

Blood-fed females were separated immediately after feeding and kept at 26°C with free access to 50% sugar solution. One group of females was dissected for microscopical observations at different intervals PBM, the second group was placed into the plastic tubes filled with 100 µl of elution tissue buffer (from DNA isolation kit) on day 0 and 10 PBM and stored at −20°C for the following *Leishmania* DNA extraction, see below.

On days 2, 5–6, 8–11 PBM females were dissected in drops of saline solution. The individual guts were checked for presence and localization of *Leishmania* promastigotes under the light microscope, special emphasis was given to colonization of the stomodeal valve as the prerequisite for successful transmission [for review see 22]. Levels of *Leishmania* infections were graded into four categories according to Myskova *et al.* [23]: negative, light (<100 parasites/gut), moderate (100–1000 parasites/gut) and heavy (>1000 parasites/gut). Data were evaluated statistically by means of $\chi 2$ test using the S-PLUS 2000 program.

The number of *Leishmania* promastigotes in individual females was estimated by Q-PCR the SYBR Green detection method (iQ SYBR Green Supermix, Biorad, CA). The total DNA was isolated using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according manufacturer's instruction. Kinetoplast DNA was chosen as the molecular target with primers described by Mary *et al.* [24]. Q-PCR was calibrated using serial dilutions of *L. donovani* DNA extracted from known number of promastigotes. Two microliters of eluted DNA was used per individual PCR reaction - 3 min at 95°C followed by 45 cycles of: 10 s at 95°C, 10 s at 56°C, and 10 s at 72°C. Results from Q-PCR were statistically evaluated using Kruskal-Wallis H-test.

Results

Life cycle of *P. orientalis* and differences between colonies

The developmental data of both *P. orientalis* colonies are summarized in Tables 1 and Figure 1. The life cycle beginning with egg development in blood-fed females to eclosion of the adult sand fly (including egg, larval and pupal stages) ranged from seven to sixteen weeks in MW and from seven to twenty-one weeks in AZ (Figures 1A, B). In contrast to most other sand flies maintained in our laboratory *P. orientalis* larvae and adults (including blood-fed females) prefer relatively high humidity. However, AZ and MW colonies differ in humidity demands during pupation: while MW pupae concentrated close to the upper edge of the rearing pot, the AZ larvae pupated mainly in the substrate on the bottom of the pot. Different pupation strategies might reflect dissimilar humidity demands of the two *P. orientalis* populations adapted to different microclimatic conditions.

Development of both colonies was affected considerably by the quality of larval food. On non-autoclaved food the emerging adults peaked at eight and nine weeks PBM for MW and AZ, respectively, and most of the adults (>90% in MW and >60% in AZ) emerged within ten weeks (Figure 1A). On autoclaved food the differences between colonies were more obvious as the development of AZ colony was significantly delayed. Peak of emerging offspring was nine and thirteen weeks PBM for MW and AZ colony, respectively. Only 16% of individuals of AZ colony achieved the adult stage within ten weeks PBM (Figure 1B). The quality of food affected mainly the fourth instar larvae where significant proportion of larvae stopped feeding and went into dormant phase, while the early larval stages were unaffected. In AZ colony, the non-synchronized larval development and tendency to diapause (predictive dormancy) occurred even on the non-autoclaved food. The growth of the L4 larvae was slightly improved by supplementation with TetraMin (aquarium fish food) (data not shown).

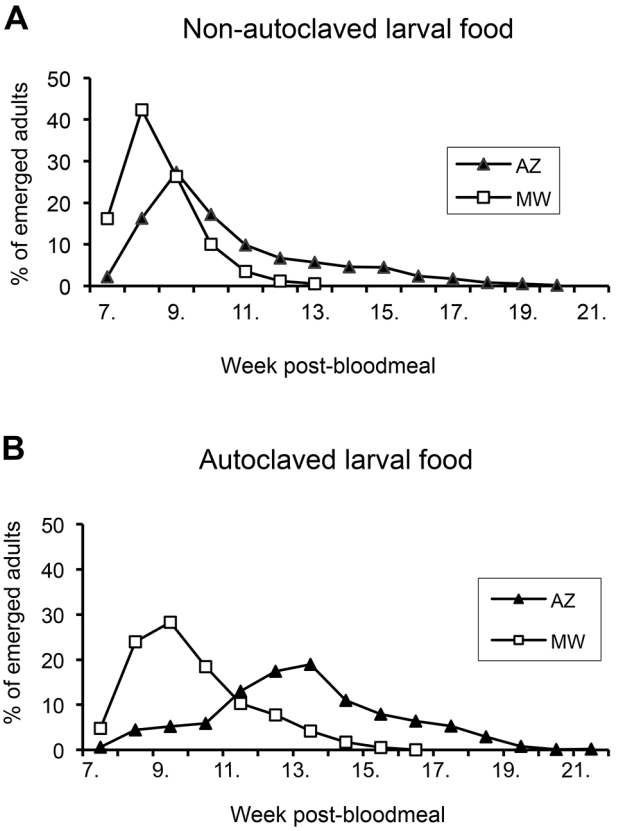

Figure 1. Effect of nutrition on the life cycle of two *P. orientalis* colonies. Data originate from the offspring of about 4,600 ovipositing females (2,200 MW and 2,400 AZ) during a 3 month period. **1A**: On the non-autoclaved food the number of adults emerging from pupae peaked on week 8 PBM in MW, and week 9 PBM in AZ. All individuals completed the life cycle within 13 and 20 weeks for MW and AZ, respectively. **1B**: On the autoclaved food the life cycle was prolonged and the larval growth appeared less synchronized in both colonies. The impact was more significant in the AZ colony: emergence of AZ adults peaked on week 13 (four weeks later than on non-autoclaved food).
doi:10.1371/journal.pntd.0002187.g001

Cross-mating study

Reciprocal hybridization crosses of both colonies resulted in successful mating and insemination, and produced viable F1 and F2 progeny. Hybrids had very high fecundity and developed successfully. In the F1 generation, the mean number of eggs per female was 42.6 and 40.3 for hybrids 1 (MW male/AZ female) and hybrids 2 (AZ male/MW female), respectively, and 42.3 and 40.6 in F2 generation. This egg production was even higher than in parental colonies (see Table 1). Immature larval stages of hybrids developed similarly or even faster than the parents. In both hybrid colonies egg development took 7 days and the whole life cycle from egg laying to eclosion from pupae lasted 32 days and 35 days in F1 and F2 generations, respectively (Table 1).

Genetic analyses

No morphological differences were found between *P. orientalis* colonies. Five decamer random primers were used for the RAPD analysis (Figure 2). A total number of 58 fragments, ranged from 100 to 1000 bp, were amplified. The band pattern given by amplification with each primer was reproducible and stable. The UPGMA analysis of these data revealed a position of two distinct clades, each containing specimens exclusively from one colony. None of the specimens fell into a clade of the other colony. A similar grouping pattern was also obtained by the neighbor-joining method (data not shown).

All analyzed CytB and CO-I sequences of several specimens belonging to both colonies were identical and no differences were observed. Sequences were submitted to GenBank (Accession numbers KC204965-KC204968).

Development of *L. donovani* in *P. orientalis*

The susceptibility of both *P. orientalis* colonies to *L. donovani* was demonstrated first using GEBRE-1 strain. On day 2 PBM, parasites were located inside the intact peritrophic matrix as procyclic promastigotes and showing high intensity of infection in 75% of females. On day 6 PBM, all females had defecated and the infection rate was 78%. Elongate nectomonads were located mainly in the abdominal midgut while short promastigotes and metacyclic forms migrated forward to the thoracic midgut; in 62% of the infected females promastigotes colonized the stomodeal valve. Subsequently, on day 9 PBM, mature infection with high parasite burdens and colonization of the stomodeal valve were found in the majority (84%) of females (data not shown).

Accurate determination of potential differences in vector competence of the two *P. orientalis* colonies was assessed by infections with *L. donovani* strain GR374. In the early stage of infection (on day 2 PBM) parasites developed similarly in both *P. orientalis* colonies (P>0.05). On day 5–6 PBM, the infection rates

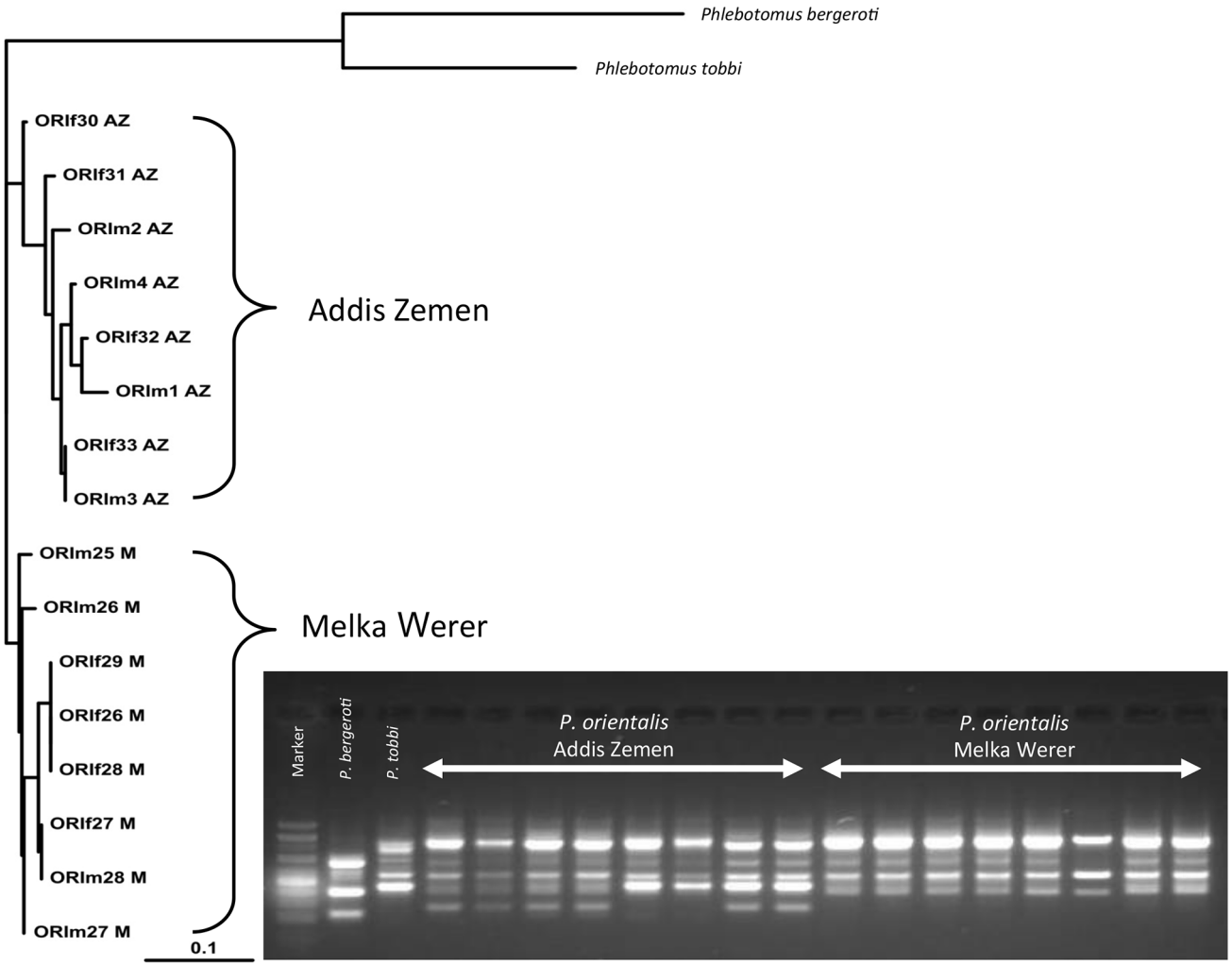

Figure 2. RAPD analysis of two *P. orientalis* colonies. RAPD analysis was based upon PCR results using five random primers (OPI12, 13, OPO20, OPE16, OPL5; in total 58 characters), electrophoretogram for OPL5 is shown as an example. Dendrogram was constructed by the Neighbor-joining method.
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were high (around 90%) in both colonies and the intensity of infection was slightly higher in AZ colony ($P = 0.048$). Abundant metacyclic promastigotes (more than 50%) and colonized stomodeal valves were observed as early as 5 days PBM. On day 8–11 PBM, high infection rates (94% for MW and 86% for AZ) and similar intensities of infection were found in both colonies ($P>0.05$) (Figure 3A). Similarly, the Q-PCR revealed no significant differences ($P>0.05$) in total parasite numbers in sand fly midguts on day 10 PBM (MW vs. AZ; $N = 50$ engorged females) (Figure 3B).

The effect of initial infective dose on total parasite numbers in sand fly gut during late stage infection was tested in *P. orientalis* (MW) infected by *L. donovani* (GR374) (Figure 4A,B). In fully bloodfed females of *P. orientalis* the average bloodmeal volume was 0.69 µl ($SD = 0.1$) ranging from 0.43 to 0.99 µl. It indicates that females infected of 5×10^5, 10^5, 2×10^4 and 2×10^3 promastigotes/ml of blood took on average 350, 70, 14 and 1–2 promastigotes, respectively. These results were confirmed by Q-PCR detecting accurate numbers of parasites from individual females immediately after blood feeding (data not shown). Despite the fact, that infection of sand flies was initiated with significantly different numbers of ingested promastigotes, the differences in infection rates were found only in group infected with 2×10^3 promastigotes/ml. In this group the late stage infections (on days 6 and 10 PBM) were found only in 30–45% of females while in other three groups the positivity of females reached 75–95% (Figure 4A). However, the location of parasites during late stage infections was similar in all four groups tested and colonization of the thoracic midgut and the stomodeal valve was observed as early as on day 5 PBM. Even in the group infected with the lowest dose (2×10^3 promastigotes/ml) numerous parasites colonizing the stomodeal valve were found in the majority (71%) of positive females on day 10 PBM.

The Q-PCR showed no significant differences in parasite loads at late stage infections (day 10 PBM) between groups of females infected with 5×10^5, 10^5 and 2×10^4 promastigotes. In contrast, the significantly lower parasite loads ($P<0.05$) were found in group infected with 2×10^3 promastigotes/ml of blood (Figure 4B); however, even this lowest dose was high enough to infect about 50% of females.

Discussion

Sequencing analysis of cytB and COI genes as well as RAPD confirmed the high degree of similarity between the MW and AZ

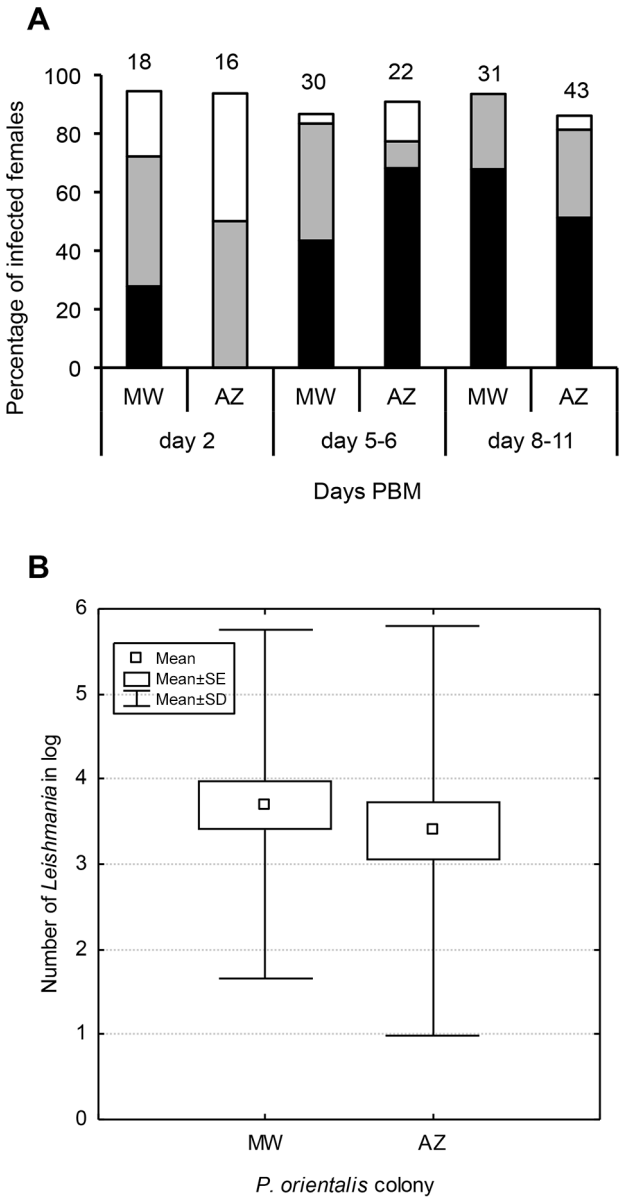

Figure 3. Development of *L. donovani* (GR 374) in females of two *P. orientalis* colonies. Sand flies were infected by feeding on a suspension of 10^5 promastigotes/ml of blood and kept at 26°C. **3A**: Infected females of *P. orientalis* were examined microscopically 2, 5–6 and 8–11 days post-bloodmeal (PBM). The infection intensities were classified into three categories according to their intensity: heavy (more than 1,000 parasites per gut [black]), moderate (100–1,000 parasites [grey]) and light (1–100 parasites [white]). Numbers above the bars indicate the number of dissected females. **3B**: Parasite numbers from 40–50 individual females were quantified by Q-PCR targeted on amplification of *Leishmania* kDNA 10 days PBM.
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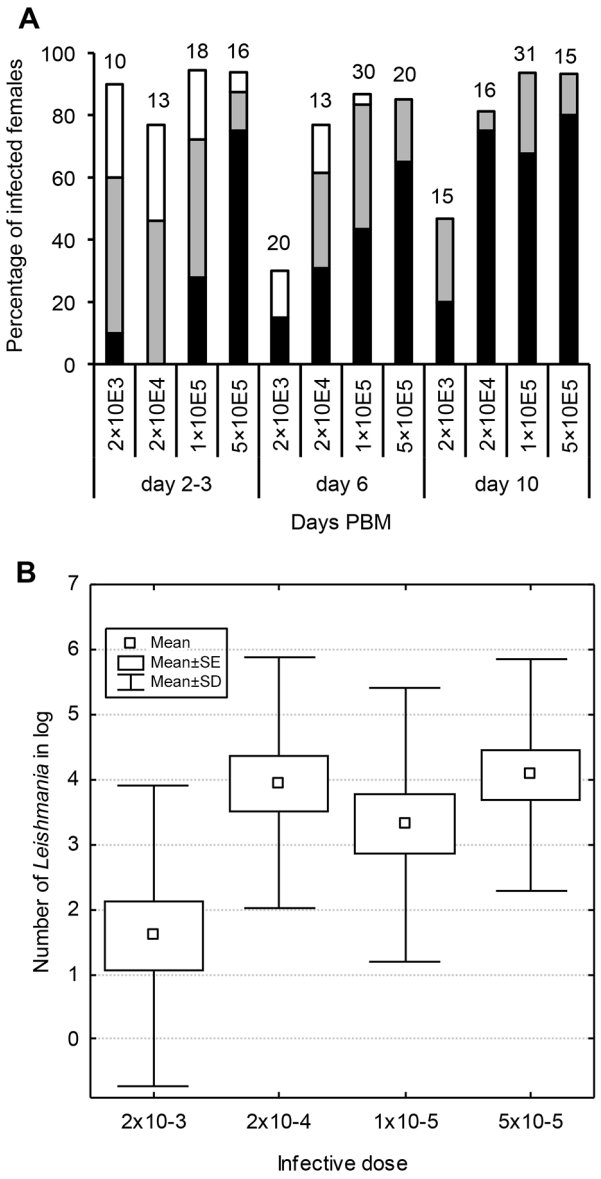

Figure 4. Effect of initial infective dose on development of *L. donovani* (GR 374) in *P. orientalis*. 4A: Infected females of *P. orientalis* (MW colony) were examined microscopically 2–3, 6 and 10 days post-bloodmeal (PBM). The infection intensity was classified as described in Fig. 3. **4B**: Parasite numbers were determined using Q-PCR at 10 days PBM. Twenty females were used per group.
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colonies originating in geographically distant areas and different altitudes. Despite this fact, obvious differences were found in certain life-cycle parameters of these populations.

The critical factor affecting larval development was the quality of larval food; autoclaved food resulted in a high proportion of dormant larvae and prolonged the generation time with the AZ colony being more sensitive to this change. Diapause of 4th instar larvae has been described in some Palaearctic species, whereas species from warmer, wetter habitats are expected to diapause at the egg stage [25]. Our findings, as well study by Schmidt [26], proved the presence of diapause in the fourth larval stage in *P. orientalis* populations. The diapause and the non-synchronized larval development in the AZ population might be explained as an adaptation to more challenging natural conditions of the highland area, and probably assure that at least some of the population will survive through periods with challenging climatic conditions. A significant proportion of fourth instar AZ larvae diapaused despite of being maintained under a constant temperature of 27°C. This finding is in contrast with observations on other sand fly species where higher temperatures decreased the tendency of larvae to diapause [27].

The results of blood-meal analysis in females from endemic sites in Ethiopia showed bovines as preferred hosts of *P. orientalis* in natural conditions (about 92% of tested females) with a low proportion of females fed on humans [28]. In laboratory conditions an alternative bloodmeal source has to be adopted for the long term colonization. The AZ colony was less adaptable for substituting of blood-meal source than the MW colony. After arrival to the laboratory in Prague, females of both colonies were bloodfed on rabbits. MW females fed readily despite the initial small size of the colony and were adapted to anesthetized mice relatively easily within two or three generations (about six months). On the other hand, AZ females originally refused feeding even on rabbits and had to be offered a human arm. Adaptation for feeding on mice took more than ten generations (almost two years). To date, adaptation has not been 100% successful yet, and AZ females must be fed alternatively on rabbits and mice. Differences between the two colonies were also noted during experimental membrane feeding: AZ females were more reluctant to feed through a chick-skin membrane. Data on egg production seem to be in accord with requirements of AZ for blood source; AZ females fed on mouse produced less than 60% of eggs than those fed on human arm (see Table 1). For more robust conclusions a study on a larger sample would be needed.

The susceptibility of *P. orientalis* to *L. donovani* is the crucial factor for the epidemiology of visceral leishmaniases. Natural infections of *P. orientalis* with *L. donovani* were repeatedly reported from various foci in East Africa [1,4,11,29], but only once in the south-west Ethiopia [30]. In Sudan, the susceptibility of *P. orientalis* to *L. donovani* has also been demonstrated by feeding on patients with kala-azar [10,31] or by feeding infected blood through mouse-skin membranes [11]. These pioneering studies were, however, done using a limited number of *P. orientalis*.

In our study both tested strains of *L. donovani* developed very well in *P. orientalis* females and colonized anterior parts of the midgut and the stomodeal valve. Parasite development at 26°C was relatively fast as the presence of metacyclic promastigotes and colonization of stomodeal valve by haptomonads was observed already on day 5 PBM. On day 10 PBM, the infection rates in both colonies were very high (93% [MW] and 81% [AZ]) and the Q-PCR revealed that females from the two colonies did not differ in total numbers of parasites in their midguts.

The volume of *P. orientalis* blood-meals measured by hemoglobinometry was on average 0.7 µl of blood. This is about one half of the volume reported for *L. longipalpis* using the same technique [32]; the difference can be easily explained by body size as *P. orientalis* is a smaller sand fly.

Experimental infections revealed that even the lowest infective dose tested (2×10^3 *L. donovani* promastigotes per ml of blood) was sufficient for high infection rates and successful establishment of late stage midgut development of this parasite in about 50% of females. Taking into account the average bloodmeal size of *P. orientalis* this concentrations is equivalent to infective dose between one and two *L. donovani* promastigotes per fly. This finding suggests extremely high susceptibility of *P. orientalis* for *L. donovani*; at present, the similar study using amastigotes is underway in our laboratory. Due to technical difficulties similar studies using amastigotes have not been performed yet in *P. orientalis*, however, in *L. longipalpis* Freitas *et al.* [33] demonstrated that promastigote-initiated *L. infantum* infections are fully comparable to amastigote-initiated ones.

In summary, this study describes in details behavioural and life-cycle parameters of two laboratory colonies of *P. orientalis* originating from Ethiopia and advances the knowledge of *P. orientalis* biology. We showed that demands for laboratory maintenance may significantly differ between two sand fly colonies of the same species. Therefore, the conditions of sand fly rearing should not be considered uniform and have to be optimized individually for each colony. Importantly, the study brings the first detailed description of *L donovani* development in *P. orientalis* under laboratory conditions. It proves that *P. orientalis* is a highly susceptible vector and only very low parasites are needed for establishment of experimental infections in this sand fly species. In view of our findings, we deduce that non-endemicity of visceral leishmaniases in Melka Werer cannot be explained by low susceptibility of local *P. orientalis* to *L. donovani*.

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Author Contributions

Conceived and designed the experiments: VS VV PV. Performed the experiments: VS VV VD KP JV. Analyzed the data: VS VV VD KP JV PV. Contributed reagents/materials/analysis tools: AK TGM. Wrote the paper: VS VV VD AH AW PV.

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Paper IV

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Exposure to *Leishmania* spp. and sand flies in domestic animals in northwestern Ethiopia

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Abstract

Background: Human visceral leishmaniasis caused by *Leishmania donovani* is considered an anthroponosis; however, *Leishmania*-infected animals have been increasingly reported in *L. donovani* foci, and the role of these animals as reservoirs for human *L. donovani* infection remains unclear.

Methods: We conducted a study of domestic animals (goats, sheep, cows, dogs, and donkeys) in three *L. donovani* foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and for anti-*L. donovani* IgG. Serum anti-sand fly saliva antibodies were used as a marker of exposure to the vector sand fly, *Phlebotomus orientalis*.

Results: Of 546 animals tested, 32 (5.9 %) were positive for *Leishmania* DNA, with positive animals identified among all species studied. Sequencing indicated that the animals were infected with parasites of the *L. donovani* complex but could not distinguish between *L. infantum* and *L. donovani*. A total of 18.9 % of the animals were seropositive for anti-*L. donovani* IgG, and 23.1 % of the animals were seropositive for anti-*P. orientalis* saliva IgG, with the highest seroprevalence observed in dogs and sheep. A positive correlation was found between anti-*P. orientalis* saliva and anti-*L. donovani* IgGs in cows, goats, and sheep.

Conclusions: The detection of *L. donovani* complex DNA in the blood of domestic animals, the reported seroprevalence to the *L. donovani* antigen, and the widespread exposure to sand fly saliva among domestic animals indicate that they are frequently exposed to *Leishmania* infection and are likely to participate in the epidemiology of *Leishmania* infection, either as potential blood sources for sand flies or possibly as parasite hosts.

Keywords: Visceral leishmaniasis, Ethiopia, Domestic animals, Serology, PCR, *Phlebotomus orientalis*, *Leishmania donovani*, Sand fly saliva

Background

Leishmaniasis, a protozoan disease that is transmitted by sand flies (Diptera: Phlebotominae) and caused by parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), is a neglected tropical and subtropical disease endemic to 98 countries worldwide. In East Africa, life-threatening human visceral leishmaniasis (VL) is caused by *Leishmania donovani* and primarily affects the poor due to the lack of preventive measures and reduced access to health care facilities [1].

The optimal strategy for controlling this disease depends on understanding the epidemiology of VL, including its local transmission cycles. Leishmaniasis caused by *L. donovani* is believed to be an anthroponosis. However, in Latin America and the Mediterranean Basin, the closely related species *L. infantum* causes a zoonosis for which canids are the main reservoirs [2]. Controlling zoonoses involving domestic or sylvatic transmission requires a more complex intervention than would be necessary if humans were the only hosts. Several

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Leishmania-infected animals have been previously reported in *L. donovani* foci, including wild and domestic animals [3–5]. However, the role of these animals as parasite hosts or, possibly, as reservoirs for human *L. donovani* VL remains unclear and requires further examination.

Our study focused on the detection of *Leishmania* infections in domestic animals in three VL foci in northwestern Ethiopia. Domestic animals were screened for *Leishmania* DNA and anti-*L. donovani* IgG in their peripheral blood to detect infection and exposure to *Leishmania*, respectively. Additionally, anti-sand fly saliva antibodies were used as a marker of exposure [6] to *Phlebotomus orientalis*, the suspected vector of *L. donovani* in northwestern Ethiopia [7, 8]. The findings from this study could be used to further study the involvement of domestic animals in the transmission cycle of VL.

Methods

Study sites and sample collection

Animal blood and serum samples were collected in Addis Zemen, Humera, and Sheraro, three localities in northwestern Ethiopia endemic to human VL. In the Humera district (Tigray region), several outbreaks of VL have been recorded since 1970. Addis Zemen (Amhara region) and Sheraro (Tigray region) are sustained VL foci characterized by a local transmission cycle supported by migrant agricultural laborers returning from Humera [1].

Animal surveys were conducted during two field studies. In October 2010, 266 samples were collected in Addis Zemen and Sheraro, and in November 2010, an additional 280 samples were obtained in Humera (Table 1). For DNA extraction, samples of whole blood (with anticoagulant) were transported to the Hebrew University of Jerusalem (Israel), where extraction was performed. For serological testing, serum samples treated with a 1 % azide solution were transported to Charles University in Prague (the Czech Republic) and stored at −70 °C.

Table 1 Serum samples collected from October to November 2010 in Ethiopian VL foci

	Addis Zemen	Sheraro	Humera	Total
Cow	62	26	16	104
Dog	19	7	8	34
Donkey	3	11	6	20
Goat	0	106	133	239
Sheep	27	5	117	149
Total	111	155	280	546

Ethical approval

The study was approved by the Ethiopian National Research Ethics Review Committee (NRERC), under approval no. 3.10/3398/04. Consent was obtained from the owners of the domestic animals for the collection of blood samples by a veterinarian. International animal experimentation guidelines were followed.

DNA extraction and PCR amplification

DNA was extracted from whole blood using the guanidine thiocyanate technique [9]. DNA was tested for *Leishmania* spp. infection via kDNA real-time PCR as previously described [10, 11]. Samples that tested positive were further tested by *Leishmania* internal transcribed spacer 1 (ITS1) real-time PCR and high-resolution melt analysis (ITS1-HRM PCR) [12]. Samples that tested positive by ITS1-HRM PCR were further assessed via conventional PCR to amplify a larger segment of ITS1 [13]. All samples were tested in duplicate, and the results were compared with positive controls: *L. infantum* (MCAN/IL/2002/Skoshi), *L. tropica* (MHOM/IL/2005/ LRC-L1239), and *L. major* (MHOM/TM/1973/5ASKH) promastigotes. The negative controls included blood samples obtained from five Israeli dogs that had tested negative for *Leishmania* by PCR. All positive PCR products were submitted for DNA sequencing to the Center for Genomic Technologies at the Hebrew University of Jerusalem. The derived DNA sequences were compared with sequences in GenBank using the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST). The percentage of positive animals for each species was calculated based on positive kDNA PCR results followed by sequencing. Samples were considered positive for *Leishmania* only if their kDNA sequence demonstrated the closest BLAST match to *Leishmania* and was at least 80 % identical. A species was considered to be identified only when its ITS1 sequence shared 99 to 100 % identity with an existing GenBank sequence.

Discrimination between *Leishmania infantum* and *Leishmania donovani*

As ITS1-HRM PCR does not discriminate between *L. infantum* and *L. donovani* infections [12], samples that tested positive for the *L. donovani* complex were further evaluated using conventional PCR to determine the species. Two independent PCR assays were carried out to amplify fragments of the *Leishmania* cysteine protease B (CPB) gene [14, 15]. Furthermore, amplification of the heat shock protein 70 (HSP70) gene, followed by restriction fragment length polymorphism analysis was also attempted for species discrimination [16]. The same positive and negative controls used for ITS1-HRM PCR were employed.

A phylogenetic analysis was carried out using Kalign (www.ebi.ac.uk/tools/msa/kalign/) and BioEdit softwares. Only well-defined ITS sequences that were unambiguously assigned to the species *L. donovani* or *L. infantum* were downloaded from the GenBank database and used in the analysis (Additional file 1). The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for maximum likelihood (ML); the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 software and bootstrapped with 1000 replicates.

Anti-*Leishmania donovani* IgG antibodies

An ELISA was used to measure specific anti-*L. donovani* IgG. Wells (CovaLink NH, Nunc) were coated with *L. donovani* promastigotes (Ethiopian strain MHOM/ET/67/HU3, 10^5 cells per well) in 20 mM carbonate-bicarbonate buffer (pH 9.25) overnight at 4 °C and incubated with 6 % blocking solution for 60 min at 37 °C. Serum samples were diluted in 2 % blocking solution and incubated in duplicate for 60 min at 37 °C. Thereafter, peroxidase-conjugated secondary antibodies were added, followed by 45 min of incubation at 37 °C. For details on the blocking solutions, sample dilutions, and conjugates employed in these assays, see Additional file 2. Absorbance was measured using a Tecan Infinite M200 microplate reader (Schoeller) at 492 nm.

Hyperimmune sera from laboratory-bred mice experimentally infected with *L. donovani* served as positive controls. Negative serum samples were obtained from healthy cattle (n = 33), horses (as controls for the donkeys; n = 9), goats (n = 21), and sheep (n = 32) from the Czech Republic, which is a sand fly- and *Leishmania*-free country. Canine-negative (n = 15) and canine-positive (n = 2) control sera were obtained during a previous study [17] from laboratory-bred beagles with no history of exposure to sand flies or *Leishmania* or from *Leishmania*-positive dogs, respectively.

Anti-sand fly saliva IgG antibodies

To estimate the exposure of domestic animals to *P. orientalis*, anti-saliva IgG antibodies were measured via ELISA. The same protocol applied for anti-*Leishmania donovani* IgG was used, with the following modifications: wells were coated with a salivary gland homogenate (corresponding to 0.2 gland/well, prepared as previously described [18]), and serum samples were incubated in duplicate for 90 min at 37 °C. Hyperimmune sera from laboratory-bred mice exposed solely to *P. orientalis* served as a positive control. The same negative controls employed for the anti-*L. donovani* ELISA were also used here.

To assess the possible cross-reactivity of *P. orientalis* salivary gland homogenate with IgG antibodies against the saliva of other sand fly species, sera from mice and dogs that were experimentally exposed to a single sand fly species were used. Canine sera positive for anti-*P. perniciosus* and anti-*L. longipalpis* IgG antibodies were available from previous experiments in laboratory-bred beagles exposed solely to *P. perniciosus* [17] and *L. longipalpis* [18], respectively, the two proven vectors of *L. infantum*. The ELISA protocol described in Additional file 2 was applied with one modification: the sera were diluted 1:500. For the murine sera, the applied ELISA protocol was modified as follows: low-fat, dry milk (Bio-Rad) was used as a blocking solution and diluent for the serum samples (1:200), and goat anti mouse IgG:HRP (AbD SEROTEC, STAR120P) diluted 1:1000, was used as a secondary antibody. The serum samples were obtained from BALB/c mice subjected to more than ten repeated exposures solely to *P. orientalis* (Ethiopia), *P. papatasi* (Turkey), *P. duboscqi* (Senegal), *P. arabicus* (Israel), or *Sergentomyia schwetzi* (Ethiopia). The experiments were approved by the Committee on the Ethics of Animal Experiments of Charles University in Prague (Permit Number: 24773/2008-10001) and were performed under a Certificate of Competency (Registration Number: CZU 934/05), in accordance with an Examination Order approved by the Central Commission for Animal Welfare of the Czech Republic.

Statistical analysis

For seroprevalence, cut-off values were calculated by the addition of three standard deviations to the mean optical density (OD) of the control sera. The differences in antibody levels between localities were analyzed using the nonparametric Wilcoxon Rank-Sum Test for Differences in Medians. Spearman's rank correlation matrix was used to assess the correlation between the variables. Statistical analyses were performed using NCSS 6.0.21 software, and the p-value was set at 0.05.

Results

Prevalence of *Leishmania* infection

The overall prevalence of *Leishmania* DNA detected via PCR was 5.9 % (32/546) (Table 2, Additional file 3). None of the 546 tested domestic animals presented visible clinical signs associated with leishmaniasis. Of the 32 animals that tested positive by kDNA PCR, nine were also positive for ITS1 PCR (Table 2, Additional file 3). The majority of *Leishmania*-positive animals (30 out of 32) were found in Humera, with the highest prevalence observed in cows (18.8 %). At the other localities, only one donkey in Sheraro and one dog in Addis Zemen were found to be positive for *Leishmania* (Table 2, Additional file 3).

Table 2 *Leishmania* PCR positivity in samples from Ethiopian animals

	Leishmania kDNA positive/total animals sampled (% positive)				*Leishmania* ITS1 positive (% positive)
Species	Addis Zemen	Sheraro	Humera	Total	Total
Cow	0/62	0/26	3/16 (18.8 %)	3/104 (2.9 %)	1 (1 %)
Dog	1/19 (5.3 %)	0/7	1/8 (12.5 %)	2/34 (5.9 %)	1 (2.9 %)
Donkey	0/3	1/11 (9.1 %)	1/6 (16.7 %)	2/20 (10.0 %)	0
Goat	0/0	0/106	16/133 (12.0 %)	16/239 (6.7 %)	3 (1.3 %)
Sheep	0/27	0/5	9/117 (7.7 %)	9/149 (6.0 %)	4 (2.7 %)
Total	1/111 (0.9 %)	1/155 (0.6 %)	30/280 (10.7 %)	32/546 (5.9 %)	9 (1.6 %)

A total of nine ITS1 DNA sequences, 265 bp long and 99 % identical to *L. infantum*/*L. donovani* sequences, were obtained via ITS1-HRM-PCR. None of the animal samples yielded positive PCR results when targeting the CPB and HSP70 genes. A DNA sequence was obtained for only a single longer ITS1 amplicon from one sheep originating in Humera. This sequence (314 bp, [GenBank:KJ010540]) shares 100 % identity with sequences from both *L. infantum* and *L. donovani* with 100 % coverage, and its phylogeny did not permit discrimination between these two closely related species (Fig. 1).

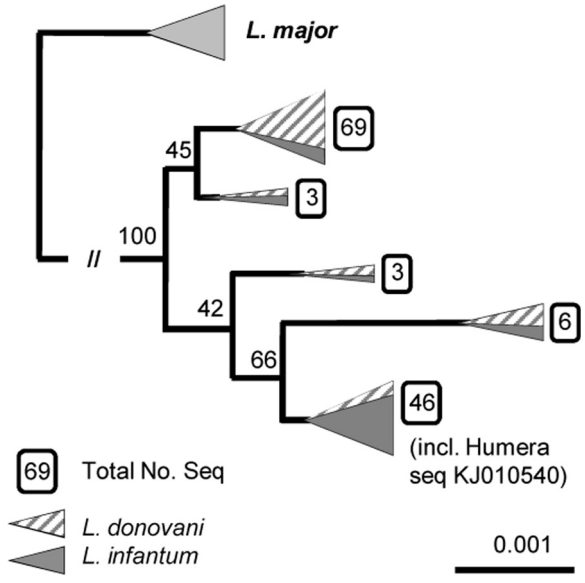

Fig. 1 Phylogenetic analysis of the *Leishmania* ITS1 sequence in a sheep from Humera, Ethiopia. Maximum likelihood (ML) phylogenetic analysis of a 286 bp *Leishmania* sequence amplified from the blood of a sheep from Humera, Ethiopia [GenBank:KJ010540]. Only well-defined ITS sequences that could be unambiguously assigned to a species (*L. donovani* or *L. infantum*) were downloaded from the GenBank database and used for the following analysis (Additional file 1). The ITS sequences were aligned using Kalign (www.ebi.ac.uk/Tools/msa/kalign/), and the resulting alignments were edited manually using the BioEdit software program to remove unambiguous positions. The final alignment included 286 characters and is available upon request. Phylogenetic analyses of the ITS datasets were performed with PhyML for ML (the best-fitting model [GTR + *I* + Γ] of sequence evolution was assessed using Modeltest 3.7 and bootstrapped with 1000 replicates; likelihood: loglk = −404.139)

Anti-*Leishmania donovani* IgG antibodies

Seropositive animals were found for every species tested. The overall seroprevalence of anti-*L. donovani* IgG in the Ethiopian samples was 18.9 % (103/546) (Table 3; Fig. 2). Across all localities tested, the highest seropositivity was observed in dogs (overall 55.9 %) and the lowest in cows and donkeys (Table 3). Of the 32 animals that tested positive for *Leishmania* DNA, 12 animals also demonstrated seropositivity for the *L. donovani* antigen: 1 donkey, 3 goats, and 8 sheep (Additional file 3).

Apart from the cows, all of the Ethiopian animal species exhibited significantly higher levels of anti-*L. donovani* IgG compared with control animals (Fig. 2). Geographically, significantly higher levels of anti-*L. donovani* IgG were observed in all animal species from Humera and in dogs, goats, and sheep from the other localities tested, when compared with control animals (Fig. 2).

Anti-*Phlebotomus orientalis* saliva IgG antibodies

The seroprevalence of anti-*P. orientalis* IgG in Ethiopian animals was 23.1 % (126/546) (Table 4). Seropositive animals were identified for every species and at every locality tested. In Addis Zemen and Sheraro, the highest seroprevalence was observed in dogs (57.9 and 57.1 %, respectively), whereas in Humera, the highest seroprevalence was among donkeys, dogs, and sheep (66.7, 62.5, and 57.3 %, respectively) (Table 4).

Apart from cows, all of the animal species from Ethiopia exhibited significantly ($p < 0.05$) higher anti-*P. orientalis* IgG seroreactivity compared with control animals (Fig. 2). Geographically, elevated levels of anti-*P. orientalis* IgG were observed in dogs, donkeys, and sheep from Humera and in dogs, donkeys, and goats from Sheraro. In Addis Zemen, only dogs exhibited significantly higher seroreactivity than control animals. The seroreactivities in the bovine

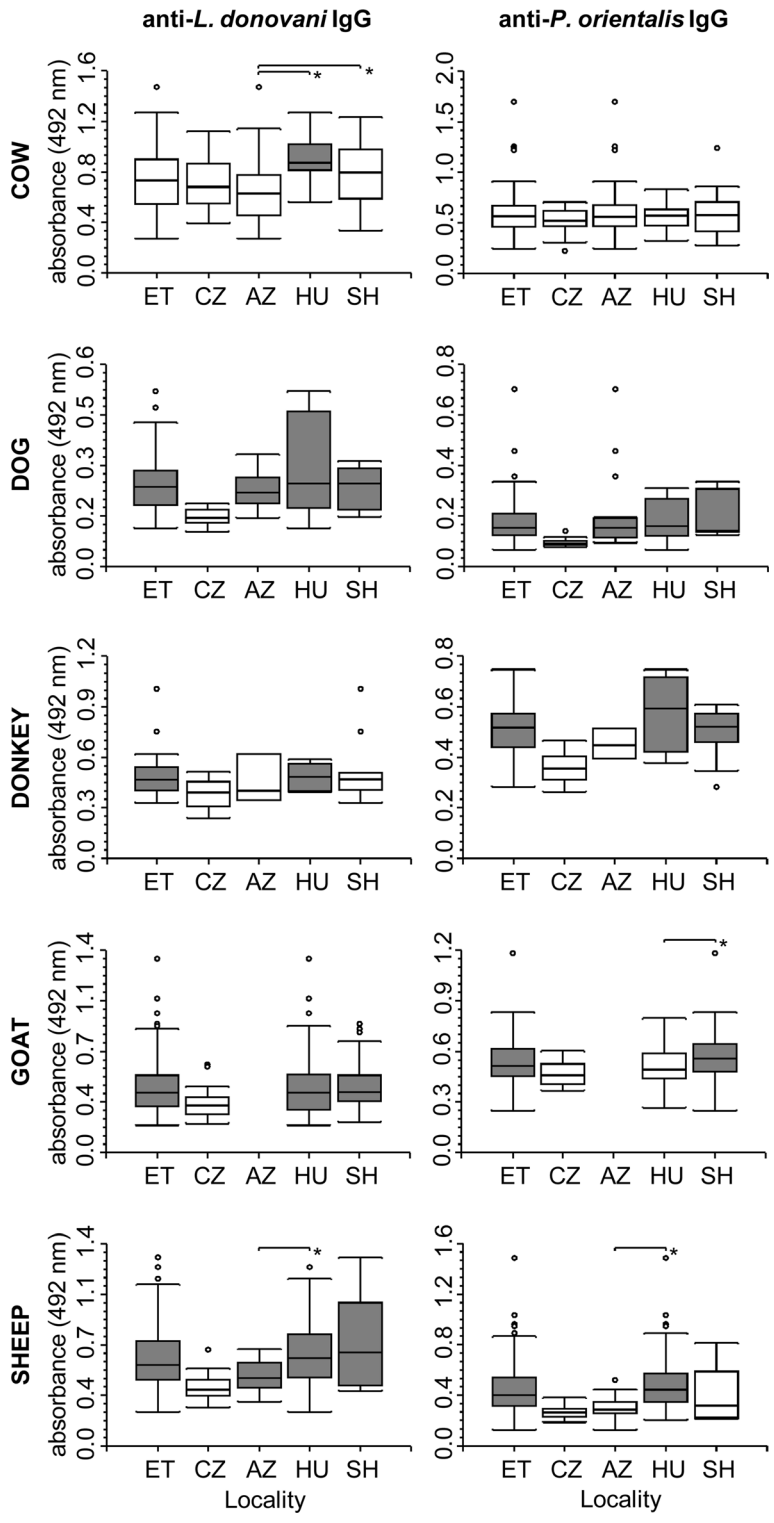

Fig. 2 Serological survey of domestic animals in Ethiopia. IgG antibodies against *Leishmania donovani* promastigotes or *Phlebotomus orientalis* saliva in all serum samples collected from domestic animals in Ethiopia (ET) from Addis Zemen (AZ), Humera (HU), and Sheraro (SH). Significant differences compared with the Czech controls (CZ) are highlighted in gray. The asterisk represents differences between the 3 localities (AZ, HU, and SH) in Ethiopia

Table 3 Seropositivity of Ethiopian animals for *Leishmania donovani* IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*L. donovani* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	1.298	1/62 (1.6 %)	0/26 (0 %)	0/16 (0 %)	1/104 (1.0 %)
Dog	0.223	9/19 (47.4 %)	5/7 (71.4 %)	5/8 (62.5 %)	19/34 (55.9 %)
Donkey	0.652	0/3 (0 %)	2/11 (18.2 %)	0/6 (0 %)	2/20 (10.0 %)
Goat	0.675		10/106 (9.4 %)	15/133 (11.3 %)	25/239 (10.5 %)
Sheep	0.648	1/27 (3.7 %)	3/5 (60.0 %)	52/117 (44.4 %)	56/149 (37.6 %)
Total		11/111 (9.9 %)	20/155 (12.9 %)	72/280 (25.7 %)	103/546 (18.9 %)

samples were similar to those in control animals, regardless of the locality (Fig. 2).

To verify the specificity of the anti-*P. orientalis* saliva antibodies we used sera from dogs and mice that had been experimentally exposed to a single sand fly species. In dogs, the reactivity of anti-*P. perniciosus* and anti-*Lutzomyia longipalpis* sera against *P. orientalis* salivary gland homogenate (SGH) was similar to that for sera from non-exposed dogs (Fig. 3a). However, all of the selected canine sera of Ethiopian origin reacted strongly to *P. orientalis* SGH (Fig. 3a). In mice, the *P. orientalis* salivary antigen reacted strongly only to the homologous IgGs (Fig. 3b). The reactivities of all heterologous antigen-antibody combinations were similar to those for sera from non-exposed mice (Fig. 3b).

Correlation analysis of serological results

A positive correlation was found between the levels of anti-*P. orientalis* and anti-*L. donovani* IgG in Ethiopian cows ($\rho = 0.37$, $p = 0.0001$), goats ($\rho = 0.37$, $p < 0.0001$), and sheep ($\rho = 0.65$, $p < 0.0001$) (Table 5). This correlation remained significant even when the locality was considered, except for the cows from Humera, for which the correlation was only slightly outside of the level of significance ($\rho = 0.48$, $p = 0.057$). No significant correlation was found for the canine and donkey sera (Table 5).

Discussion

Visceral leishmaniasis is considered to be an anthroponosis in northwestern Ethiopia, but in nearby Sudanese foci, zoonotic transmission has also been suspected, with dogs and mongooses serving as possible reservoirs [3–5, 19]. With regard to domestic animals, sleeping near dogs, cattle, goats, or donkeys has been associated with an increased risk of VL in migrants and residents of Humera [20]. Understanding the mode of disease transmission, whether anthroponotic or zoonotic, is critical for the planning and implementation of effective VL control programs. Thus, one of the main goals of our study was to screen domestic animals for *Leishmania* DNA and discuss their possible involvement in the epidemiology of VL in Ethiopia as possible parasite hosts.

We evaluated two parameters associated with the ability of an animal to be a host for *Leishmania* parasites [21, 22]: (1) exposure to a sand fly vector as a source of blood and (2) the presence of *Leishmania* DNA in the animal's peripheral blood.

In northwestern Ethiopia, the sand fly vector species of *L. donovani* has not yet been identified. However, *Phlebotomus orientalis* is the most probable vector given that it has been found to be infected with *L. donovani* in nearby Sudanese foci [7] and its susceptibility to this *Leishmania* species has been demonstrated experimentally [8]. Exposure to *P. orientalis* was assessed using anti-sand fly saliva antibodies as a marker [6]. Anti-

Table 4 Seropositivity of Ethiopian animals for *Phlebotomus orientalis* saliva IgG. The cut-off value was calculated as the mean optical density in the control animals plus 3 standard deviations (details provided in the Methods)

		Anti-*P. orientalis* IgG positive/total animals sampled (% seropositive)			
Species	Cut-off	Addis Zemen	Sheraro	Humera	Total
Cow	0.876	4/62 (6.5 %)	1/26 (3.8 %)	0/16 (0 %)	5/104 (4.8 %)
Dog	0.143	11/19 (57.9 %)	4/7 (57.1 %)	5/8 (62.5 %)	20/34 (58.8 %)
Donkey	0.550	0/3 (0 %)	3/11 (27.3 %)	4/6 (66.7 %)	7/20 (35.0 %)
Goat	0.685		17/106 (16.0 %)	6/133 (4.5 %)	23/239 (9.6 %)
Sheep	0.410	3/27 (11.1 %)	1/5 (20.0 %)	67/117 (57.3 %)	71/149 (47.7 %)
Total		18/111 (16.2 %)	26/155 (16.8 %)	82/280 (29.3 %)	126/546 (23.1 %)

saliva IgG antibodies were found in all of the animal species tested, which is indicative of the opportunistic feeding behavior of *P. orientalis* [23], thus meeting one criteria for the possible zoonotic transmission of *L. donovani*. Feeding preferences, together with other ecological constraints such as the localization of vector breeding sites [24] or vector susceptibility to harboring *Leishmania* infection [8] may help us to understand the complex picture of the ecology and transmission dynamics of VL in Ethiopia.

The presence of *Leishmania* DNA in animal peripheral blood and *Leishmania* seropositivity serve as reliable epidemiological markers for assessing infection. PCR positivity indicates the presence of the parasite [25, 26]. Although this technique cannot prove the intact integrity of the parasite, viability of the detected *Leishmania* is highly probable given that its DNA degrades shortly after parasite death [27]. Seropositivity, on the other hand, is considered a marker of exposure to *Leishmania* infection [28]. The majority of *Leishmania*-positive animals were found in Humera, indicating dynamic transmission to domestic animals in this well-known active focus. However, many *L. donovani*-seropositive animals were found in all the three surveyed localities, suggesting that exposure to *Leishmania* parasites also occurred in the foci of Addis Zemen and Sheraro.

The fact that only one-third of the PCR-positive animals were positive for both kDNA and ITS1-HRM PCR, is not surprising because the ITS1 region has a considerably lower copy number [11, 12]. Due to the small amount of parasite DNA available in blood samples, distinguishing between the closely related species *L. donovani* and *L. infantum* is notoriously difficult [15]. Moreover, distinction within the *L. donovani* complex in East Africa is controversial; strains that were previously split into *L. donovani*, *L. archibaldi* or *L. infantum* have now been classified into one group: *L. donovani* s.s. [29].

The most suspected animal reservoirs for *L. donovani* are dogs, which are known to play a key role as reservoir hosts in the transmission cycle of the closely related *L. infantum* [2, 30]. Several authors have reported PCR-positivity or seropositivity of dogs in *L. donovani* foci [3, 4, 19, 31–35], including Humera and Addis Zemen in Ethiopia [36–39]. In the present study, dogs demonstrated the highest *Leishmania* seroprevalence out of all the species tested at all study sites, with two PCR-positive dogs identified in Humera and Addis Zemen. As a suspected reservoir species, dogs are also highly attractive to the vector [35], which is supported by our findings that dogs exhibited the highest seroprevalence of anti-*P. orientalis* antibodies among the tested animal species. Most importantly, the same *Leishmania* strains have been recovered from dogs and VL patients [3, 4, 19] and have been shown to persist in dogs for years [19]. Dogs have been recognized as a risk factor for human VL [20, 37, 39], and as the most probable reservoir hosts, their involvement in disease transmission should be addressed in control strategies for VL caused by *L. donovani*.

Almost 38 % of *Leishmania*-positive animals have also been found to be seropositive, indicating these domestic animals (donkeys, goats, sheep) as putative host species in local VL foci. Nevertheless, it is important to mention that neither PCR-positivity nor seropositivity indicates that an animal is able to maintain the parasite for a long period of time. This must be primarily demonstrated by the follow-up of infected animals. Several studies of naturally or experimentally infected non-canine domestic animals have demonstrated their different capabilities to maintain *Leishmania* infection. Cerqueira *et al*. [40] experimentally infected four donkeys with *L. chagasi* (syn. *L. infantum*). These donkeys remained seropositive until the end of the study, which lasted 12 months; however, the donkeys were able to overcome the infection and failed to infect the vector [40]. A PCR survey reported by Bhattarai *et al*. indicated that *Leishmania* infection in goats can persist for at least seven months [41]. On the other hand, *L. donovani* infection in sheep is likely time-limited because only one out of six experimentally infected sheep was shown to develop measurable amounts of anti-*L. donovani* antibodies and the transient presence of amastigotes in sampled tissue in a study that included 244 days of monitoring [42]. Thus, the 37.6 % seropositivity detected in our study may indicate a high infection rate among Ethiopian sheep, further supported by the significantly higher levels of anti-*L. donovani* IgG antibodies among *Leishmania*-positive sheep (Additional files 3 and 4).

The fact that many animals were seropositive for *Leishmania* while PCR-negative in the blood, and, on the other hand, that out of 32 PCR-positive animals, 20 animals were seronegative, could be explained by several possible mechanisms. Seropositivity and PCR-negativity might be attributable to infection in hosts that have resolved the infection but retain high titers of specific antibodies [40, 43]. Another possibility is that seropositive animals might carry the infection in their tissues without parasitemia and are therefore negative according to blood PCR [44]. The reverse situation with PCR-positivity and seronegativity could be attributable to the delayed development of a detectable antibody response in early infection [45], or due to an infection in animals whose B-cells are unresponsive to *Leishmania* antigens, as found in some asymptomatic hosts [2, 43, 44].

The role of other domestic animals as hosts or potential reservoirs for *L. donovani* is still unclear. The

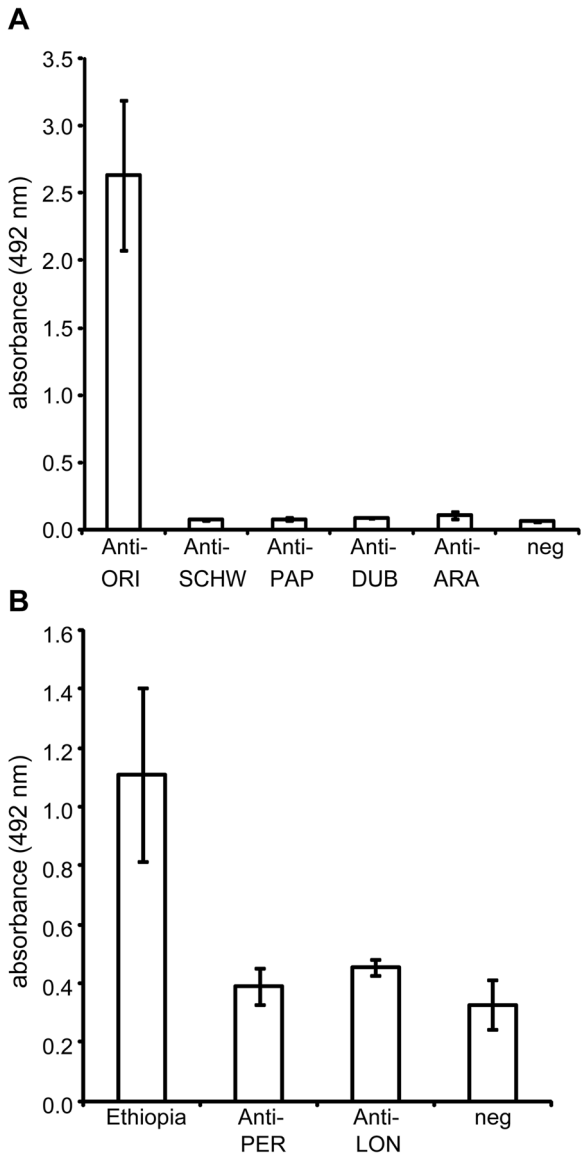

Fig. 3 Specificity of the anti-sand fly saliva IgG antibody response. The reactivity of *Phlebotomus orientalis* salivary gland homogenates (SGH) with sera from mice (**a**) and dogs (**b**) repeatedly exposed to a single sand fly species was measured via ELISA. In graph A, SGH was incubated with sera from mice exposed to *P. orientalis* (Anti-ORI), *Sergentomyia schwetzi* (Anti-SCHW), *P. papatasi* (Anti-PAP), *P. duboscqi* (Anti-DUB), or *P. arabicus* (Anti-ARA). Each bar represents the mean for two serum samples ± the standard error. The values for the positive controls (the sera of mice incubated with homologous antigen) were as follows: *S. schwetzi* = 1.48 ± 0.43, *P. papatasi* = 2.38 ± 0.32, *P. duboscqi* = 2.87 ± 0.60, and *P. arabicus* = 1.86 ± 0.24. In graph B, the SGH was incubated with the sera from dogs exposed to *P. perniciosus* (Anti-PER) or *Lutzomyia longipalpis* (Anti-LON). Seropositive Ethiopian dogs (Ethiopia) and dogs that had never been exposed to sand flies (neg) were used as positive and negative controls, respectively. Each bar represents the mean of five serum samples ± the standard error. The absorbencies of the sera incubated with the homologous antigen were 2.42 ± 0.06 for *L. longipalpis* and 1.73 ± 0.13 for *P. perniciosus*

Table 5 Correlation analysis of serological results

Species		Ethiopia	Addis Zemen	Sheraro	Humera
Cow	ρ	0.37***	0.38**	0.43*	0.48
	n	104	62	26	16
Dog	ρ	0.12	0.15	−0.46	0.36
	n	34	19	7	8
Donkey	ρ	0.31	0.50	0.52	−0.03
	n	20	3	11	6
Goat	ρ	0.37***		0.36***	0.37***
	n	239		106	133
Sheep	ρ	0.65***	0.67***	1.00***	0.61***
	n	149	27	5	117

Results from the Spearman-Rank Correlation Matrix test for anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG
ρ correlation coefficient, *n* number of serum samples tested
Asterisk (*) indicate significant correlations: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

present study is the first to report PCR-positive cattle, donkeys, goats, and sheep in Ethiopia. These animals, especially cattle, serve as sources of blood for *L. donovani* vectors [23, 46]. Even if these species do not serve as reservoir hosts for the parasite, they still attract large numbers of blood-questing female sand flies and may, therefore, act as a protective barrier in the case of resistant or refractory mammal species or as a risk factor in the case of susceptible species [30, 37, 47]. Prediction of the role of domestic animals in the amplification or dilution of VL risk might be possible using a recently described mathematical model for multi-host infectious diseases by applying relevant data [48].

In addition to the maintenance of persistent infection, the transmissibility competence, e.g. infectivity for the sand fly vector, is an important prerequisite for any mammal to serve as a *Leishmania* reservoir [28, 49]. These two criteria, among other aspects, can distinguish between a reservoir host and an incidental host that is not capable of infecting the vector [25]. Validation of these prerequisites for domestic animals in northwest Ethiopia, however, requires further investigation.

Conclusions

In conclusion, leishmaniasis caused by *L. donovani* is traditionally considered to be an anthroponosis in East Africa. However, the present study revealed widespread exposure to *L. donovani* and sand fly vector bites among domestic animals. The possible involvement of domestic animals as sources of blood for vector sand flies should therefore be considered in VL control strategies. However, the direct involvement of domestic animals in the transmission cycle of *L. donovani* warrants further

investigation, most importantly by xenodiagnosis to determine their transmissibility competence.

Additional files

Additional file 1: Accession numbers for *Leishmania* ITS sequences downloaded from the GenBank database and used for the phylogenetic analysis presented in Fig. 1.

Additional file 2: Details of the ELISA methods.

Additional file 3: Detailed list of Ethiopian animals positive for *Leishmania* DNA.

Additional file 4: Differences in the levels of anti-*Leishmania donovani* IgG and anti-*Phlebotomus orientalis* saliva IgG between *Leishmania*-positive (full circle) and *Leishmania*-negative (open circle) animals in the Humera region (the majority of PCR-positive animals are from this locality: 30 out of 32). Significant differences are marked by the probability level on the X-axis.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay; ITS1: Internal transcribed spacer 1; kDNA: Kinetoplast deoxyribonucleic acid; *L.*: *Leishmania* or *Lutzomyia*; OD: Optical density; *P.*: *Phlebotomus*; PBS: Phosphate-buffered saline; PBS-Tw: Phosphate-buffered saline with Tween; PCR: Polymerase chain reaction; SGH: Salivary gland homogenate; VL: Visceral leishmaniasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IR participated in the study design and field sample collection, carried out *Leishmania* serology, performed the statistical analysis, and drafted and finalized the manuscript. DTF and DYL carried out the *Leishmania* PCR and sequencing. TK, NP, and TL performed sand fly serology. AK, CM, RK, CLJ, and AW participated in field sample collection. JV carried out the sequence alignment and phylogenetic analysis. JV, AW, AH, and PV participated in the study design and coordination. GB conceived and designed the study, coordinated and participated in field work, and drafted and finalized the manuscript. DTF and TK contributed equally to the paper. All authors read and approved the final manuscript.

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Paper V

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Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents

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ABSTRACT

Human visceral (VL, also known as Kala-azar) and cutaneous (CL) leishmaniasis are important infectious diseases affecting countries in East Africa that remain endemic in several regions of Ethiopia. The transmission and epidemiology of the disease is complicated due to the complex life cycle of the parasites and the involvement of various *Leishmania* spp., sand fly vectors, and reservoir animals besides human hosts. Particularly in East Africa, the role of animals as reservoirs for human VL remains unclear. Isolation of *Leishmania donovani* parasites from naturally infected rodents has been reported in several endemic countries; however, the status of rodents as reservoirs in Ethiopia remains unclear. Here, we demonstrated natural *Leishmania* infections in rodents. Animals were trapped in 41 localities of endemic and non-endemic areas in eight geographical regions of Ethiopia and DNA was isolated from spleens of 586 rodents belonging to 21 genera and 38 species. *Leishmania* infection was evaluated by real-time PCR of kinetoplast (k)DNA and confirmed by sequencing of the PCR products. Subsequently, parasite species identification was confirmed by PCR and DNA sequencing of the 18S ribosomal RNA internal transcribed spacer one (ITS1) gene. Out of fifty (8.2%) rodent specimens positive for *Leishmania* kDNA-PCR and sequencing, 10 were subsequently identified by sequencing of the ITS1 showing that five belonged to the *L. donovani* complex and five to *L. tropica*. Forty nine kDNA-positive rodents were found in the endemic localities of southern and eastern Ethiopia while only one was identified from northwestern Ethiopia. Moreover, all the ten ITS1-positive rodents were captured in areas where human leishmaniasis cases have been reported and potential sand fly vectors occur. Our findings suggest the eco-epidemiological importance of rodents in these foci of leishmaniasis and indicate that rodents are likely to play a role in the transmission of leishmaniasis in Ethiopia, possibly as reservoir hosts.

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1. Introduction

Leishmaniasis, a group of diseases ranging from self-healing localized cutaneous (CL) to the life threatening visceral form (VL or Kala-azar), is widely distributed in over 88 countries with up to 1.6 million new cases annually (WHO, 2010). Humans are infected by twenty species of the genus *Leishmania* that are transmitted by the bite of phlebotomine sand fly females. The source of infection for humans and parasite circulation is either anthroponotic (transmitted between humans) or zoonotic, where animals serve as reservoir hosts (Desjeux, 2004).

Leishmania species differ in the degree to which they are associated with different host species and reservoirs, among which rodents are considered to be of most importance. However, their role in the transmission cycle as a reservoir host and source of infection for humans differs significantly. For example *Leishmania turanica* is highly infectious and pathogenic to rodents, but human cases are very rare (Guan et al., 1995). In *L. major*, the parasites circulate under natural conditions in rodent populations; nevertheless, they are equally infective to humans and rodents that represent a natural source (reservoir) for human populations (Ashford, 1996, 2000). Cutaneous leishmaniasis caused by *L. tropica* was generally considered to be anthroponotic; however, in some areas hyraxes and rodents could play a role in zoonotic transmission (Jacobson, 2003; Svobodova et al., 2003; Svobodova et al., 2006).

The etiological agent of human VL in the Old World is represented by two closely related parasite species belonging to the *L. donovani* complex: *L. infantum* which circulates as a zoonosis with domestic dogs and wild canids as the main reservoirs (Baneth and Aroch, 2008; Quinnell and Courtenay, 2009), and *L. donovani*, which is believed to be anthroponotic and mainly transmitted among humans (Chappuis et al., 2007).

Visceral leishmaniasis caused by *L. donovani* has claimed the lives of thousands of people in Ethiopia. The main foci are found in the lowland areas of north, northwestern, and southwestern Ethiopia, with some sporadic cases in the central-east part of the country (Hailu and Formmel, 1993; Hailu et al., 2006a). The main potential vectors of VL include *P. orientalis, P. martini*, and *P. celiae* (Hailu et al., 1995; Gebre-Michael and Lane, 1996). The transmission dynamics of VL in Ethiopia and neighboring East African countries is generally believed to be anthroponotic (Chappuis et al., 2007); however DNA of *L. donovani* complex has recently been detected in both wild and domestic animals (Bashaye et al., 2009) and in certain districts of Sudan, rodents are suspected to be reservoirs of the parasite (Chance et al., 1978; Le Blancq and Peters, 1986; Elnaiem et al., 2001). The closely related species, *L. infantum*, has been detected in rodents in Euro-Asian leishmaniasis foci including Portugal (Helhazar et al., 2013), Italy (Gradoni et al., 1983), Greece (Papadogiannakis et al., 2010), and Iran (Davami et al., 2014). In addition, our recent study demonstrated presence of *L. donovani* complex DNA in blood specimens of various domestic animals in the VL endemic foci of north and northwestern Ethiopia (Rohousova et al., unpublished).

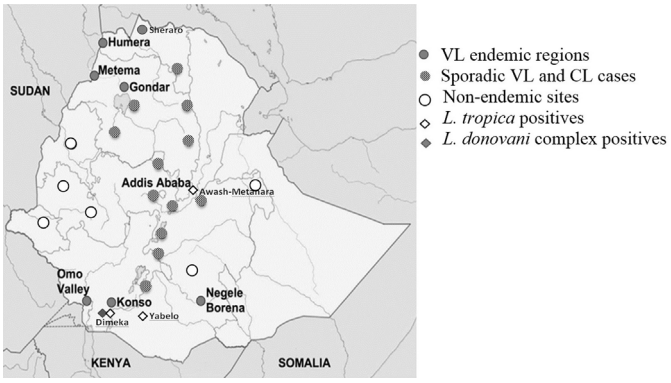

Fig. 1. Rodent trapping sites and their relation to human leishmaniasis foci in Ethiopia. (*Note*: The specific rodent trapping localities were indicated in the supplementary table.)

In Ethiopia, the search for *L. donovani* infection in wild rodents has been going on for many years. Here we focused on the detection of natural *Leishmania* spp. infections in rodents using PCR that targets the kinetoplast (k)DNA and internal transcribed spacer one (ITS1).

2. Materials and methods

2.1. Sample collection

Rodents were trapped in 41 localities (between 2010 and 2013) selected based on altitude, the occurrence of Kala-azar (9 endemic, 18 sporadic and 14 non-endemic), the abundance of sand flies, and the presence of microhabitat features related to *Leishmania* transmission (Fig. 1; Supp. Table S1). Permission to trap rodents was obtained from the Ethiopian Wildlife Conservation Authority (EWCA), Government of Ethiopia.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.02.006.

Rodents were trapped using Sherman live traps and snap traps baited with a piece of bread with peanut butter or sardine. The traps were placed over-night near houses, animal shelters, around burrows, caves, agricultural fields, termite mounds, under trees, and in other habitats deemed suitable for sand flies. Trapped rodent was photographed and weight, sex, characteristics, and external measurements (lengths of body, tail, hind foot, and ear) were recorded. Rodents captured by live traps were first immobilized in a plastic bag and then humanely euthanized by intra peritoneal injection of ketamine and xylazine, dissected, and a sample of spleen was kept in pure ethanol for subsequent DNA extraction. After removing the viscera, the remaining body was kept in denatured alcohol for further morphological identification.

2.2. DNA extraction

DNA was extracted from spleen samples by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions or by the guanidine thiocyanate technique (Hoss and Paabo, 1993) with slight modification. Briefly, 10 mg of spleen tissue was homogenized by a grinder in 2 ml eppendorf tube and suspended in 1 ml extraction buffer containing 10 M GuSCN, 0.1 M Tris–HCl (pH 6.4), 0.02 MEDTA (pH 8.0) and 1.3% Triton X-100 and left for overnight agitation in a 56 °C shaker incubator. Then the tissue was boiled for a maximum of 10 min at 94 °C. After centrifugation at 14,000 rpm for 3 min, the supernatant was transferred to a new tube and 1 ml of freshly prepared NaCl solution with 1 μl silica and 1 μl linear acrylamide was added and kept on ice for 1 h with 15 min interval of vortexing. Then the mix was centrifuged at 5000 rpm for 30 s and supernatant discarded. The pellet was washed with washing buffer and then with ethanol and left to air dry. Finally, the pellet was re-suspended in ultra-pure water.

2.3. Host and parasite detection and determination

Confirmation of the morphological identification of hosts was provided by sequencing a fragment of the cytochrome b gene (900 bp). PCR was performed using the following primers: L14723 (forward, 5′-ACC AATGACATGAAAAATCATCGTT-3′) and H15915 (reverse, 5′-TCTCCATTTCTGGTTTACAAGAC-3′) (Lecompte

Table 1
Number of trapped rodents and *Leishmania* infections in different geographical regions of Ethiopia.

Geographic region of Ethiopia	No. of animals sampled	*Leishmania* DNA positive	
		kDNA (%)	ITS1 (*Leishmania* species)
Central	29	–	–
Central-east	53	10 (1.7)	3 (*L. tropica*)
East	72	6 (1.0)	–
North	61	–	–
Northwest	34	1 (0.2)	–
South	97	1 (0.2)	–
Southwest	144	31 (5.3)	7 (5 *L. don.* complex; 2 *L. tropica*)
West	96	1 (0.2)	–
Total	586	50 (8.5)	10 (5 *L. don.* complex; 5 *L. tropica*)

et al., 2002). The PCR product was purified using calf intestine alkaline phosphatase and exonuclease I (New England Biolabs) for sequencing (GATC Biotech company, Germany). All sequences were assigned to genus using BLAST search (https://blast.ncbi.nlm.nih.gov) and species determinations were performed through phylogenetic analysis of our recent unpublished materials.

For the purpose of *Leishmania* detection in the rodent's tissues and species determination, a combination of a mini circle kDNA real time (RT)-PCR and sequencing followed by ITS1-PCR and sequencing was used. PCR targeting fragments of about 116 bp of the kDNA is considered to be highly sensitive due to the presence of thousands of target copies in each parasite cell and has been used for screening of *Leishmania* in various vertebrate hosts (Selvapandiyan et al., 2008; Abbasi et al., 2013). However, as sequencing of kDNA does not identify the *Leishmania* species (Anders et al., 2002; Nicolas et al., 2002; Nasereddin et al., 2008), a more appropriate target, the internal transcribed spacer one (ITS1) gene, was introduced as a specific marker for each species (Schoenian et al., 2003; Talmi-Frank et al., 2010). Primers: JW11 (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′) and JW12 (reverse, 5′-GGGTAGGGGCGTTC TGCGAAA-3′) were used to amplify the mini-circle kDNA of *Leishmania* (Nicolas et al., 2002); while primers ITS-219F (forward, 5′-AGCTGGATCATTTTCCGATG-3′) and ITS-219R (reverse, 5′-ATCGCGACACGTTATGTGAG-3′) amplified a 265 to 288-bp product of the ITS1 region of the *Leishmania* rRNA operon (Talmi-Frank et al., 2010). The RT-PCR conditions for kDNA and ITS1 were as described by Nicolas et al. (2002) and Talmi-Frank et al. (2010). For each set of reactions, a standard positive DNA extracted from 100 μl of *L. infantum* (strain MHOM/TN/1980/IPT1), *L. tropica* (ISER/IL/2002/LRC-L90), and *L. major* (MHOM/TM/1973/5ASKH) promastigote cultures [5×10^2 parasites/μl] and non-template controls (NTC) were used. All *Leishmania* kDNA- and ITS1-PCR positive samples underwent direct sequencing of the target amplicons.

3. Results

During a period of four years, a total of 586 rodents belonging to 17 genera and 34 species were caught from 41 trapping locations grouped in eight geographical regions (Table 1). The following six rodent genera were abundant (each represent at least five percent of the total catches): *Acomys* (24.1%), *Mastomys* (20.0%), *Stenocephalemys* (15.2%), *Lophuromys* (10.6%), *Mus* (8.0%), and *Arvicanthis* (7.8%) (Table 2). Based on cursory inspection of the captured animals, none of the rodents had visible clinical signs that could be attributed to CL. Fifty (8.5%) of the analyzed rodents were kDNA-RT-PCR positive for the presence of *Leishmania* spp. Presence of *Leishmania* DNA was confirmed by subsequent sequencing of the kDNA-RT-PCR amplicon. At least one kDNA *Leishmania*-positive was found in nine rodent genera and in the following five, kDNA-positive samples were detected repeatedly: *Mastomys* (18 kDNA-RT-PCR positive animals out of 117 tested; 15.3%), *Acomys* (14/141; 9.9%), *Arvicanthis* (8/46; 17.4%), *Aethomys* (4/10; 40.0%), and *Gerbilliscus* (2/26; 7.7%). The kDNA-RT-PCR positive rodents were classified generally as "infected with *Leishmania*

Table 2
Total number of trapped rodents according to genera (listed in alphabetical order) and *Leishmania* kDNA (kDNA+) and/or ITS1 (ITS1+) positivity as obtained by (RT)-PCR and subsequent sequencing.

Genus[a]	Number[b] (%)	kDNA+	ITS1+	*Leishmania* species (by ITS1)
Acomys (3)	141 (24.1)	14	3	*L. tropica*
Aethomys (2)	10 (1.7)	4		
Arvicanthis (6)	46 (7.8)	8	2	1 *L. don.* complex and 1 *L. tropica*
Dendromus (1)	5 (0.8)			
Desmomys (1)	3 (0.5)			
Gerbilliscus (4)	26 (4.4)	2	1	*L. donovani* complex
Gerbillus (1)	5 (0.8)	1	1	*L. tropica*
Graphiurus (1)	6 (1.0)	1		
Lophuromys (1)	62 (10.6)			
Mastomys (3)	117 (20.0)	18	3	*L. donovani* complex
Mus (4)	47 (8.0)			
Myomyscus (1)	5 (0.8)	1		
Rattus (1)	19 (3.2)			
Saccostomus (1)	3 (0.5)	1		
Stenocephalemys (1)	89 (15.2)			
Tachyoryctes (1)	1 (0.2)			
Taterillus (1)	1 (0.2)			
Total	586 (100)	50	10	

[a] The number of species per genus is presented in brackets.
[b] Total number and percentage of trapped rodents.

Table 3
Rodent species found ITS1-positive for *Leishmania* parasites and their trapping sites.

Leishmania species	Rodent species	Locality	Geographic region
L. donovani complex	*Arvicanthis* sp.	Alduba	Southwest Ethiopia
	Mastomys erythroleucus	Alduba	
	Mastomys erythroleucus	Dimeka	
	Mastomys erythroleucus	Dimeka	
	Gerbilliscus nigricaudus	Dimeka	
L. tropica	*Acomys* sp.	Sorobo, Konso	South Ethiopia
	Arvicanthis sp.	Derito, Yabelo	
	Acomys cf. *mullah*	Awash-Metahara	Central-east Ethiopia
	Acomys cf. *mullah*		
	Gerbillus nanus		

spp." (Table 2). Only one rodent specimen (*Acomys* sp.) was found positive for *Leishmania* kDNA in the northern part of the country; while the rest were either from the southern or eastern parts of Ethiopia (Table 1).

All of the 50 rodent specimens positive for *Leishmania* spp. by kDNA-RT-PCR and confirmed by sequencing of the kDNA amplicons were further tested and re-screened by amplification of the *Leishmania* ITS1 gene followed by DNA sequencing of the amplicon. A total of ten rodent specimens from the following five genera were positive for ITS1-PCR (Table 2): *Acomys*, *Arvicanthis*, *Gerbilliscus*, *Gerbillus*, and *Mastomys*. The sequencing revealed that five samples belonged to *L. tropica* and five to the *L. donovani* complex. As our sequences of ITS1 are unable to separate *L. donovani* from *L. infantum*, the positive samples of these species are represented here as *L. donovani* complex.

The *L. tropica* positive rodents, represented by *Arvicanthis* sp., *Gerbillus nanus*, and three specimens of *Acomys* spp., were caught in Konso and Yabello in southern Ethiopia and in Awash-Metahara in central-east Ethiopia. On the other hand, rodents positive for *L. donovani* complex are represented by *Arvicanthis* sp., *Gerbilliscus nigricaudus*, and three specimens of *Mastomys erythroleucus*, originated from the south western part of Ethiopia in the locality of Dimeka and Alduba (Fig. 1; Table 3).

4. Discussion

Eighty-four different species of rodents have been identified in Ethiopia so far. These include rodents belonging to species in the genera *Acomys*, *Mastomys*, *Arvicanthis*, and *Mus* which are the most common (Bekele and Leirs, 1997). This is in agreement with our collections in which *Acomys* (24.1%) and *Mastomys* (20.0%) are the predominant species. This was probably due to the location of the trapping sites as the majority of our traps were set in domestic and peri-domestic areas and in fields where these rodents are abundant and considered as agricultural pests (Bekele and Leirs, 1997; Chekol et al., 2012).

Correct species identification of Ethiopian rodents remains tricky due to the presence of several cryptic species where identification by morphological parameters alone is not sufficient. The need for molecular identification is crucial; however, the reference species found in Gene Bank or the BOLD (The Barcode of Life Data Systems: http://www.boldsystems.org/) database for analyzing unknown sequences is still limited (Galan et al., 2012). Although we identified all the trapped rodents to the species level; in the present study, we presented the number of rodent species per genus and/or species of ITS1 positive specimens only.

Sharing the same ecological niche and nocturnal activity facilitates the frequent contact between sand flies and rodents and may lead to infection with a *Leishmania* parasite transmitted by a bite. In this study, PCR positive rodents belonged to those genera and species that are common in arable lands and nests in cracks, burrows, or dig holes with multiple entrances (Kingdon et al., 2013; Bekele and Leirs, 1997) which in turn could be resting and breeding sites for sand flies. In addition to this, *Arvicanthis* is one of the rodent genera commonly found around termitaries (Kingdon et al., 2013) and could be a preferred blood source for *P. martini* and *P. celiae*, the two potential vectors of *L. donovani* in southern Ethiopia which are associated with termite mounds (Gebre-Michael and Lane, 1996).

All five rodent specimens infected with *L. donovani* complex were captured in the localities of Dimeka and Alduba, southwestern Ethiopia (Table 2) which is considered an important Kala-azar focus (Hailu et al., 2006b) and where the suspected vector species, *P. orientalis*, *P. martini*, and *P. celiae*, exist sympatrically (Hailu et al., 1995; Gebre-Michael and Lane, 1996). The infected rodents we found belong to *Arvicanthis* sp., *M. erythroleucus*, and *G. nigricaudus*. Natural infections of *Arvicanthis* (*A. niloticus*) and mongoose (*Herpestes ichneumon*) with *L. donovani* were previously reported in the Aethiopian geographical region (Chance et al., 1978; Le Blancq and Peters, 1986; Elnaiem et al., 2001). During our field study we found a fresh body of a white-tailed mongoose (*Ichneumia albicauda*) which was hit by a car in the locality of Alduba and sample taken from this mongoose was positive for *Leishmania* kDNA, and ITS1-PCR revealed *L. donovani* complex (data not shown). Our finding corresponds with the previous ascertainments and therefore could signify the existence of natural infection of wild animals in the whole region.

Three of the rodents infected with *L. tropica* (*G. nanus* and two *Acomys* spp.) were found in the Awash valley, central-east Ethiopia. Previous investigations in this region demonstrated human cases of cutaneous leishmaniasis due to *L. tropica* and sand flies including *P. saevus* and *P. sergenti* were found harboring this parasite (Gebre-Michael et al., 2004; Hailu et al., 2006a). However, no *L. tropica* infections were reported in south and southwestern Ethiopia.

Although leishmaniasis due to *L. tropica* results mainly in cutaneous manifestations in humans, we detected the presence of this parasite in the studied rodents based on PCR of their spleen tissue samples. Experimental infections of rodents demonstrated early dissemination of parasites to internal organs including the spleen (Papadogiannakis et al., 2010). We did not find any visible clinical signs that could be attributed to CL in the *L. tropica*-positive rodents. Although symptomatic cases of disease are the most important in human and veterinary medicine, asymptomatic hosts may be much more abundant and, therefore, crucial sources of infection for sand flies, playing a significant role in the epidemiology and transmission dynamics of the diseases. Asymptomatic and subclinical infections of leishmaniasis have been well documented in humans (Abbasi et al., 2013; Picado et al., 2014), dogs (Baneth et al., 2008; Miro et al., 2008) and rodents (Svobodova et al., 2003). From the epidemiological point of view, asymptomatic hosts contribute to the parasite transmission cycle. Previous studies on subclinical dogs and rodents infected with *L. infantum* and *L. tropica*, respectively, have demonstrated their competence to infect sand fly vectors (Svobodova et al., 2003; Laurenti et al., 2013; Sadlova et al., unpublished).

Only one *Leishmania* kDNA positive rodent was found in the northern part of Ethiopia, in the locality of Mai-Temen, Western Tigray, northwestern Ethiopia, even though we captured almost one hundred rodents in areas with established human VL transmission. The explanation for this finding could be evaluated from different perspectives. Studies on the genetic structure of Ethiopian *L. donovani* isolates have revealed polymorphism with geographical clusters in northern and southern Ethiopian foci (Gelanew et al., 2010; Zackay et al., 2013). Moreover, the fauna of potential sand fly vectors responsible for the transmission of VL in the north and south Ethiopian foci varies: the southern foci are dominated by two proven vectors, *P. martini* and *P. celiae* with sporadic *P. orientalis* while in the north, *P. orientalis* is the sole potential vector and the other two species are not present (Gebre-Michael and Lane, 1996; Hailu et al., 2006b). Thus, our finding could suggest differences in the transmission cycle including vector and reservoir hosts in these two geographical regions exist. Further studies; with special attention to the feeding habits of sand flies particularly on rodents are recommended.

In conclusion, VL caused by *L. donovani* in Eastern Africa is traditionally considered to be anthroponotic. However, our investigations suggest that wild rodents in Ethiopia could play an important epidemiological role in the transmission cycle of two *Leishmania* species, *L. donovani* and *L. tropica*. Further studies focusing on parasite isolation, experimental infection, and xenodiagnosis should be accomplished to prove their epidemiological role.

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Paper VI

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Natural infection of bats with *Leishmania* in Ethiopia

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ABSTRACT

The leishmaniases, a group of diseases with a worldwide-distribution, are caused by different species of *Leishmania* parasites. Both cutaneous and visceral leishmaniasis remain important public health problems in Ethiopia. Epidemiological cycles of these protozoans involve various sand fly (Diptera: Psychodidae) vectors and mammalian hosts, including humans. In recent years, *Leishmania* infections in bats have been reported in the New World countries endemic to leishmaniasis. The aim of this study was to survey natural *Leishmania* infection in bats collected from various regions of Ethiopia. Total DNA was isolated from spleens of 163 bats belonging to 23 species and 18 genera. *Leishmania* infection was detected by real-time (RT) PCR targeting a kinetoplast (k) DNA and internal transcribed spacer one (ITS1) gene of the parasite. Detection was confirmed by sequencing of the PCR products. *Leishmania* kDNA was detected in eight (4.9%) bats; four of them had been captured in the Aba-Roba and Awash-Methara regions that are endemic for leishmaniasis, while the other four specimens originated from non-endemic localities of Metu, Bedele and Masha. *Leishmania* isolates from two bats were confirmed by ITS1 PCR to be *Leishmania tropica* and *Leishmania major*, isolated from two individual bats, *Cardioderma cor* and *Nycteris hispida*, respectively. These results represent the first confirmed observation of natural infection of bats with the Old World *Leishmania*. Hence, bats should be considered putative hosts of *Leishmania* spp. affecting humans with a significant role in the transmission

1. Introduction

In Ethiopia, leishmaniases, caused by protozoan parasites of the genus *Leishmania* and transmitted by the bite of female sand flies, are diseases of significant public health importance. The country is endemic for two human disease presentations: cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL, kala-azar). Cutaneous leishmaniasis is widely distributed and usually prevalent in highland areas with occasional reports in the lowland regions of Omo (south) and Awash (central east) (Hailu et al., 2006a). The annual incidence of CL ranges from 20,000 to 50,000 cases, but this is probably an under-estimate (Alvar et al., 2012), with over 28 million people residing in regions with risk of transmission (Seid et al., 2014). The main causative agent of CL in Ethiopia is *Leishmania aethiopica*, however, infections due to *Leishmania tropica* and *Leishmania major* were also reported in the country (Hailu et al., 2006a,b; Abbasi et al., 2013). Visceral leishmaniasis affecting up to 7400 people annually in the country is the most severe form and is fatal, if left untreated. The VL foci lie in the south-west lowland savannah and the north-west semi-arid plains of the country with sporadic cases in highland areas of the Libo Kemkem district (north), the Awash valley (center) and further in the east of the country, bordering Kenya and Somalia (Leta et al., 2014; Hailu et al., 2006a). The causative agent of human VL in Ethiopia is *Leishmania donovani* (Hailu et al., 2006a).

Cutaneous leishmaniasis caused by *L. aethiopica* and *L. major* is commonly zoonotic (Ashford et al., 1973; 2000; Lemma et al., 2009 Lemma et al., 2009). Although being the agent of anthro-

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ponotic CL in urban endemic settings, *L. tropica* has been strongly suspected to be zoonotic in some foci (Sang et al., 1994; Jacobson, 2003; Svobodova et al., 2003). In Ethiopia, rock hyraxes were found infected with *L. aethiopica*, suggesting a zoonotic cycle of the parasite (Ashford et al., 1973; Lemma et al., 2009). Recently, *L. tropica* DNA was detected in spleens of rodents in areas where human cases have been reported (Kassahun et al., 2015). However, no study in Ethiopia demonstrated natural infection in animals by *L. major*.

Most reports agree that like the Indian sub-continent, VL in East Africa is assumed to be anthroponotic (Chappuis et al., 2007). Nevertheless, there is evidence for the possible involvement of zoonotic transmission with uncertain reservoir hosts (Ashford, 2000). Recently, natural infections of dogs (Bashaye et al., 2009), domestic animals (Rohousova et al., 2015) and rodents (Kassahun et al., 2015) with *L. donovani* complex were reported in Ethiopia.

Natural infections by various *Leishmania* species have been repeatedly reported in domestic, peridomestic and wild animals, which dogs and rodents being the most commonly investigated animals and traditionally considered reservoirs (Baneth and Aroch, 2008). However, recent investigations of *Leishmania* parasites in animals including hares (Jimenez et al., 2013), and marsupials (Roque and Jansen, 2014) have diverted attention to other possible sylvatic reservoir hosts in endemic leishmaniasis foci.

Bats ecology and innate behavioral details highlight their prime importance in the reservoir system of infectious diseases such as Ebola virus (Leroy et al., 2005) and various kinetoplastids transmitted by vectors (Lord and Brooks, 2014). Bats were also suggested as possible natural blood source for sand flies after laboratory feeding procedure (Lampo et al., 2000) and known to host several trypanosomes transmitted by sand flies (McConnell and Correa 1964; Williams, 1976). Importantly, being cave-dwelling organisms, bats and sand flies frequently share living habitats where ample opportunity exists for sand flies to feed on bats (Feliciangeli, 2004). Natural *Leishmania* infection in bats has been reported in New World leishmaniasis foci and the findings suggested their possible epidemiological involvement in the transmission cycle (Lima et al., 2008; Savani et al., 2010; Shapiro et al., 2013; Berzunza-Cruz et al., 2015). Despite the attempts else where (Millan et al., 2014; Rotureau et al., 2006; Rajendran et al., 1985; Mutinga, 1975; Morsy et al., 1987), the extent of *Leishmania* natural infection in the Old World bats remains uncertain, and cases of Chiropteran *Leishmania* infections have not been documented in Ethiopia until now. In view of these facts we carried out a *Leishmania* DNA survey in Ethiopian bats.

2. Materials and methods

2.1. Sample collection

Bats were collected as a part of an extensive ecological and faunistic study in Ethiopia. Permission for trapping was obtained from the Ethiopian Wildlife Conservation Authority (EWCA), government of Ethiopia. Here, we reported results for the 163 specimens collected in leishmaniasis endemic (44 bats) and non-endemic (119 bats) areas of Ethiopia (Fig. 1). Bats were captured at presumed flyways using a standard mist-net between 18:00 and 22:00 h. Bats were removed from the net, anesthetized by intra peritoneal injection of ketamine and xylazine. All the necessary external morphological characters including size, color of hair and naked parts, length of forearm, shape of snout, shape of ear and type of membrane concerning the form of tail were recorded and the identification of each particular bat was confirmed based on the keys by Happold and Happold (2013). Then bats were sacrificed and their spleens were removed and kept in ethanol for the subsequent DNA extraction.

2.2. DNA extraction, parasite detection and determination by PCR

All the techniques, materials and procedures: DNA isolation, primers, real time polymerase chain reaction (RT-PCR) procedure, target genes (kinetoplast DNA (kDNA) and 18S rRNA internal transcribed spacer one (ITS1)) and post PCR evaluation and parasite determination, were performed as described in our previous work on rodents (Kassahun et al., 2015). Briefly, for the purpose of *Leishmania* detection and identification, we tested extracted DNA using RT-PCR targeting kDNA of *Leishmania* and positivity was confirmed by direct sequencing of amplicons. Real time PCR targeting kDNA gene is generally considered to be highly sensitive (Selvapandiyan et al., 2008; Selvapandiyan et al., 2008) but sequence does not identify the *Leishmania* species (Nicolas et al., 2002; Nasereddin et al., 2008). Therefore, all the kDNA positive specimens were re-analyzed by RT-PCR of the ITS1 locus and positive samples underwent sequencing of amplicons (Schoenian et al., 2003; Schoenian et al., 2003).

3. Results and discussion

A total of 163 bats, belonging to 25 species of 18 genera (Table 1), were collected. The dominant species in our collection were *Pipistrellus hesperidus* (18%), *Miniopterus africanus* (11%) and *Scotoecus hirundo* (11%).

Amongst the 163 samples, *Leishmania*–kDNA positivity was confirmed by sequencing of a parasite DNA from eight bats belonging to six species. Out of the eight *Leishmania* kDNA PCR positives, the ITS1-PCR and subsequent sequencing revealed infection of *L. tropica* in one specimen of *Cardioderma cor* and *L. major* in one specimen of *Nycteris hispida* (Table 1). We were unable to amplify ITS-1 sequences for the six additional *Leishmania* kDNA positive samples. There was a similar scenario in our previous work (Kassahun et al., 2015). PCR targeting kDNA fragment is considered to be highly sensitive due to the high number of target copies in each parasite cell. Even though ITS-1 based PCR determines the species of the *Leishmania* parasite, the level of sensitivity is lower than that of kDNA PCR (Abbasi et al., 2013) which does not provide sufficient information for species determination.

Leishmaniasis due to *L. tropica* and *L. major* generally cause dermal lesions in humans; however none of the bats had visible dermal signs resembling cutaneous leishmaniasis. It is well know that *Leishmania* species dermotropic for humans could migrate to visceral organs of other animal hosts (Laskay et al., 1995). Moreover, early dissemination of *Leishmania* parasites to the spleen has been reported in asymptomatic animals (Schilling and Glaichenhaus, 2001). Such scenarios may explain our finding of parasite DNA in the spleens of infected bats thus validating our experimental approach for an epidemiological study.

Our finding represents a confirmed first report of natural *Leishmania* infection of bats in the Old World. Previous studies conducted in the Old World (e.g. Spain (Millan et al., 2014), France (Rotureau et al., 2006), India (Rajendran et al., 1985) and Kenya (Mutinga, 1975)) did not yield any positive specimens. Moreover, the attempts in Egypt (Morsy et al., 1987) were using old methods and the detection procedure was speculative with specificity and parasite species characterization. However, bats in the New World were repeatedly investigated and found infected with *Leishmania* species pathogenic to humans. In our study, the prevalence reached 5% (8 out of 163) corresponds with the infection rates of bats recorded in Sao Paulo, Brazil (4%) (Savani et al., 2010); while higher prevalence has been detected in Venezuela (9%) (Lima et al., 2008); Mexico (9.8%) (Berzunza-Cruz et al., 2015) and Mato Grosso do Sul, Brazil (40%) (Shapiro et al., 2013).

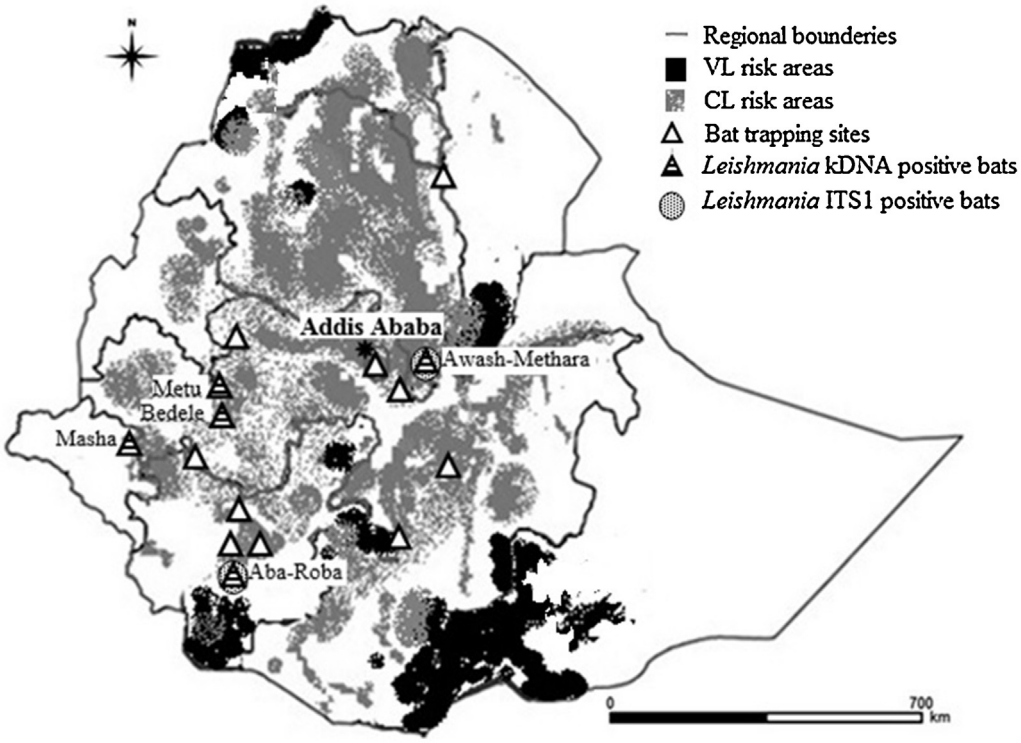

Fig. 1. Map of leishmaniasis distribution in Ethiopia (modified and adapted from Leta et al., 2014; Seid et al., 2014 and unpublished hospital records) and trapping localities with respective *Leishmania* DNA detection results.

Table 1
Bats collected in different trapping localities[a] in Ethiopia and examined for *Leishmania* DNA by RT-PCR. The number of *Leishmania* kDNA positive bats appears in square brackets.

Bat species	BCH	ABR	MSH	BDL	DDS	KNS	TPI	GOB	AMR	ALM	WLT	MTU	SFO	SOR	MNG	Σ (%)
Cardioderma cor	–	–	–	–	–	–	–	–	1 [1][b]	–	–	–	–	–	–	1 (0.6)
Glauconycteris variegata	–	1	–	3[1]	–	–	–	-	–	–	–	4	–	–	–	8 (4.9)
Laephotis wintoni	-	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2 (1.2)
Micropteropus pusillus	-	–	–	2	–	–	–	–	–	–	-	2	–	2	–	6 (3.7)
Miniopterus arenarius	–	–	2[1]	–	–	–	–	–	–	–	–	–	–	–	–	2 (1.2)
Miniopterus africanus	–	–	–	–	–	–	–	–	–	–	–	–	18	–	–	18 (11.0)
Mops condylurus	–	–	–	–	–	–	–	–	–	–	–	9	–	–	–	9 (5.5)
Myotis scotti	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	2 (1.2)
Myotis tricolor	–	–	–	–	–	–	–	–	–	–	–	–	11	–	–	11 (6.7)
Neoromicia somalica	–	2[2]	–	–	–	2	–	–	–	1	–	–	–	–	1	6 (3.7)
Neoromicia guineensis	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	3 (1.8)
Neoromicia nana	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1 (0.6)
Nycteris hispida	–	–	–	–	–	–	–	–	1 [1][c]	–	–	–	–	–	–	1 (0.6)
Nycticeinops schlieffenii	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	3 (1.8)
Otomops martiensseni	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	3 (1.8)
Pipistrellus hesperidus	–	–	2	–	–	–	–	4	–	–	–	9	–	2	13	30 (18.4)
Pipistrellus rusticus	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	1 (0.6)
Rhinolophus fumigatus	–	–	–	–	–	–	–	–	–	–	1	–	5	–	–	6 (3.7)
Scotoecus hirundo	–	3	–	1	–	14	–	-	–	–	–	–	–	–	–	18 (11.0)
Scotophilus colias	–	–	–	1	–	–	–	–	–	–	–	11[2]	–	–	-	12 (7.4)
Stenonycteris lanosus	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.6)
Tadarida sp.	–	–	–	–	8	–	–	–	–	–	–	–	1	–	–	9 (5.5)
Triaenops afer	–	–	–	–	–	–	–	–	–	–	–	–	11	–	–	11(6.7)
Total	1	9	4	12	8	16	1	4	2	1	1	35	49	4	17	163

[a] Abbreviation of localities: BCH-Bechu, ABR-Aba-Roba, MSH-Masha, BDL-Bedele, DDS-Dedesa, KNS-Konso, TPI-Tepi, GOB-Goba, AMR-Awash-Methara, ALM-Alemata, WLT-Welenchiti, MTU-Metu, SFO-Sof Omar caves, SOR-Sorr, MNG-Menagesha.
[b] *L. tropica* positive bats.
[c] *L. major* positive bats.

Four of the positive bats were captured in the Aba-Roba and Awash-Methara leishmaniasis endemic foci while the other four specimens originated from non-endemic localities of Metu, Bedele and Masha (Fig. 1). The Awash-Methara foci are known for *L. tropica* infections in humans (Hailu et al., 2006a), phlebotomine sand flies (Gebre-Michael et al., 2004) and recently rodents (Kassahun et al., 2015). Our results corroborate these findings as one specimen of *C. cor* captured in this area was found infected with *L. tropica*. Although *L. tropica* is regarded to be anthroponotic, infections in dogs (Baneth et al., 2014), golden jackal and red foxes (Talmi-Frank et al., 2010) and rodents (Svobodova et al., 2003; Talmi-Frank et al., 2010) have been well documented generally in zoonotic foci (Sang

et al., 1994). The finding of this parasite both in bats and in our previous study of rodents (Kassahun et al., 2015) points to the possibility of zoonotic transmission in the particular area.

The specimen of *N. hispida* infected with *L. major* was trapped in the same area, Awash-Methara. The findings of *L. major* in Ethiopia are rare but natural infections in humans (Abbasi et al., 2013) and sand flies (Gebre-Michael et al., 1993) were recorded in North and South-west Ethiopia, respectively. No previous *L. major* infection was reported in Awash-Methara region; however our unpublished preliminary entomological survey in this area revealed the presence of *Phlebotomus papatasi* and *Phlebotomus duboscqi*, both being considered as a potential vectors of *L. major* (Dostalova and Volf, 2012).

The finding of four *Leishmania*–kDNA positive bats in the non-endemic localities could be explained by the fact that the geographical distribution of the parasite in Ethiopia is much wider than anticipated. Moreover, bats have a potential to migrate from place to place and we could hardly rule out the possibility that bats from *Leishmania* endemic areas could move to non-endemic areas.

No *L. donovani* complex DNA was detected in our bats sample. It is obvious that *L. donovani* is the sole agent of human VL in Ethiopia with wide geographical areas (Hailu et al., 2006a). The recent finding of DNA in rodents (Kassahun et al., 2015) and domestic animals (Rohousova et al., 2015) could also determine its host range. However, the absence of this species in bats doesn't reflect being refractory or the parasite's specificity.

Generally, to determine the role of a given host in a reservoir system it should fulfill some criteria among others: overlap of geographical distribution of vectors and hosts; forming large biomass, being gregarious and long lived in addition to being found naturally infected and subsequently being infective for transmitting vectors (Ashford, 1996). Some of these conditions work with bats and their ability to fly long distances and colonize places could make them suitable bridge hosts for leishmaniasis. Moreover, most colonies of bats live and rest in caves and cracks that are assumed to provide ambient temperatures and relative humidity suitable for sand fly breeding and diurnal resting (Feliciangeli, 2004). Laboratory feeding experiments on *Lutzomyia longipalpis*, most widely distributed vector of New World VL, was capable of feeding from different families of bats that suggested the importance of bats as a possible natural blood source of sand flies (Lampo et al., 2000). In addition to this, bats are well known hosts of *Trypanosoma* transmitted by sandflies (McConnell and Correa, 1964; Williams, 1976; Lord and Brooks, 2014), which is closely related to the genus *Leishmania*.

In conclusion, bats could have adequate features to be naturally infected by *Leishmania* and could subsequently to play a role in its epidemiological cycle. The present study revealed natural *Leishmania* infections of Old World bats, in areas both endemic and non-endemic for human leishmaniasis. The wide geographical distribution of *Leishmania* parasite in the country could imply the existence of different modes of transmission and our finding might indicate the importance of bats in the disease cycle. However, to play a role in *Leishmania* cycles it is required to investigate the host's pathogenic features and being infectious to vectors; which were not covered in this paper. Thus, further studies on persistence of the *Leishmania* parasite in bats and its interaction with sand fly vectors are recommended for the better understanding of their epidemiological involvement.

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