

Natural infection of bats with *Leishmania* in Ethiopia

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ABSTRACT

The leishmaniasis, 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

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1. Introduction

In Ethiopia, leishmaniasis, 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 possibleylvatic 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 (Felicangeli, 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 elsewhere (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 known that *Leishmania* species dermatropic 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).

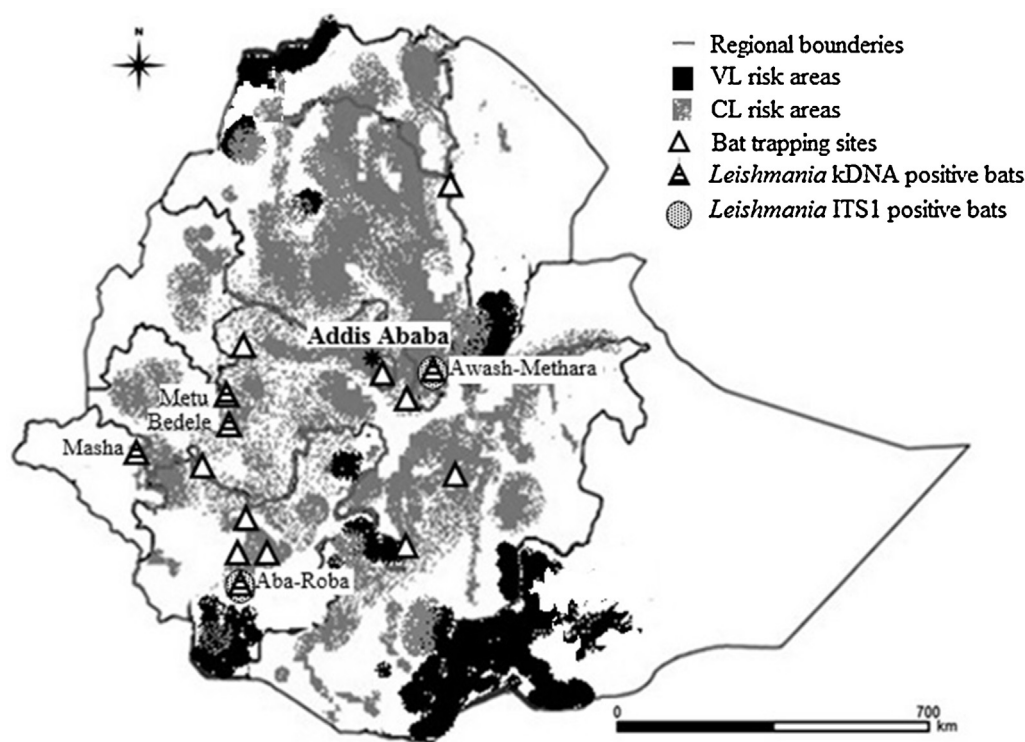


Fig. 1. Map of leishmaniasis distribution in Ethiopia (modified and adapted from Leta et al., 2014; Seid et al., 2014 and unpublished hospital records) and trapping localities with respective *Leishmania* DNA detection results.

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 (%)
<i>Cardioderma cor</i>	-	-	-	-	-	-	-	-	1 [1] ^b	-	-	-	-	-	-	1 (0.6)
<i>Glauconycteris variegata</i>	-	1	-	3 [1]	-	-	-	-	-	-	-	4	-	-	-	8 (4.9)
<i>Laephotis wintoni</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2 (1.2)
<i>Micropteropus pusillus</i>	-	-	-	2	-	-	-	-	-	-	-	2	-	2	-	6 (3.7)
<i>Miniopterus arenarius</i>	-	-	2 [1]	-	-	-	-	-	-	-	-	-	-	-	-	2 (1.2)
<i>Miniopterus africanus</i>	-	-	-	-	-	-	-	-	-	-	-	-	18	-	-	18 (11.0)
<i>Mops condylurus</i>	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	9 (5.5)
<i>Myotis scotti</i>	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2 (1.2)
<i>Myotis tricolor</i>	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	11 (6.7)
<i>Neoromicia somalica</i>	-	2 [2]	-	-	-	2	-	-	-	1	-	-	-	-	1	6 (3.7)
<i>Neoromicia guineensis</i>	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	3 (1.8)
<i>Neoromicia nana</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1 (0.6)
<i>Nycteris hispida</i>	-	-	-	-	-	-	-	-	1 [1] ^c	-	-	-	-	-	-	1 (0.6)
<i>Nycticeinops schlieffenii</i>	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	3 (1.8)
<i>Otomops martiensseni</i>	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	3 (1.8)
<i>Pipistrellus hesperidus</i>	-	-	2	-	-	-	-	4	-	-	-	9	-	2	13	30 (18.4)
<i>Pipistrellus rusticus</i>	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1 (0.6)
<i>Rhinolophus fumigatus</i>	-	-	-	-	-	-	-	-	-	-	1	-	5	-	-	6 (3.7)
<i>Scotoecus hirundo</i>	-	3	-	1	-	14	-	-	-	-	-	-	-	-	-	18 (11.0)
<i>Scotophilus colias</i>	-	-	-	1	-	-	-	-	-	-	-	11 [2]	-	-	-	12 (7.4)
<i>Stenonycteris lanosus</i>	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1 (0.6)
<i>Tadarida</i> sp.	-	-	-	-	8	-	-	-	-	-	-	-	1	-	-	9 (5.5)
<i>Triadenops afer</i>	-	-	-	-	-	-	-	-	-	-	-	-	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-Bede, 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 (Felicangeli, 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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