Acta Tropica 145 (2015) 39–44

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

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

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ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 3 February 2015
Accepted 7 February 2015
Available online 18 February 2015

Keywords:
Leishmania donovani
L. tropica
Phlebotomine sand fly
Rodents
kDNA
ITS1

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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http://dx.doi.org/10.1016/j.actatropica.2015.02.006

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).

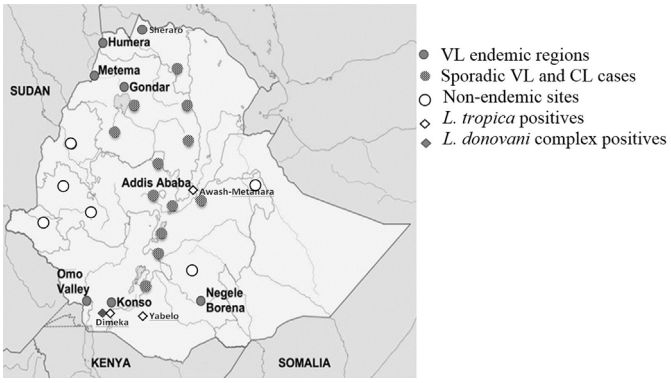

Fig. 1. Rodent trapping sites and their relation to human leishmaniasis foci in Ethiopia. (*Note*: The specific rodent trapping localities were indicated in the supplementary table.)

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.

Acknowledgements

We would like to thank Radim Sumbera and Josef Bryja for providing additional rodent sample; Yaarit Biala, Jana Radrova, and staffs of the leishmaniasis research and diagnostic laboratory (Medical school, Addis Ababa University) for their technical assistance. This project was funded by grants from the Bill and Melinda Gates Foundation Global Health Program (OPPGH5336), Grant Agency of the Charles University in Prague (GAUK 9108/2013), Czech Science Foundation (GACR P506-10-0983) and the EU grant 2011-261504 EDENext (the paper is cataloged as EDENext 319). The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of manuscript.

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