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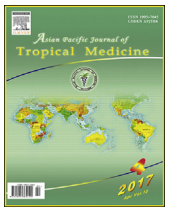

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Preliminary study on investigation of zoonotic visceral leishmaniasis in endemic foci of Ethiopia by detecting *Leishmania* infections in rodents

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ABSTRACT

Objective: To investigate the zoonotic visceral leishmaniasis (ZVL) by identification of the most probable reservoir hosts using parasite isolation and analysis of a possible transmission dynamics of the disease in extra-domestic agricultural fields and rural villages.

Methods: Rodents were collected from selected study sites in kala-azar endemic areas based on information for localities of kala-azar cases for screening of *Leishmania* infections using parasitological, serological and polymerase chain reaction (PCR) from March, 2013 to January, 2014. Ketamine (Clorketam Veterinary) was used to anaesthesize the rodents according the prescribed dosage (average 2 mg/kg for intra-venous route). The blood obtained using sterile needle was dropped into sterile filter paper and allowed to air dry before sealing in plastic bags. The tissues from liver, spleen and skin were macerated in Locke's solution before transferring them into NNN medium. Blood and touch smears of liver, spleen, skin and bone marrow were prepared for fixing using methanol and staining by Giemsa stain for microscopy. These tissues were also used for DNA extractions and PCR amplification of *Leishmania* infection.

Results: A total of 335 rodents (13 species) were analyzed by sampling internal organs. The infection rate by PCR was 11.1% (6/54) for *Arvicanthis nilothicus* compared to 17.6% (3/17) and 12.5% (2/16) for *Acomys cahirinus* and *Tarera* (*G*) *robustus* respectively. Almost all the infections were found from bone marrow samples (8/48 or 16.7%) compared with 1/91 (1.1%) liver, 2/87 (2.2%) spleen and 0/87 (0%) skin. In all study sites with past human VL cases, rodents and proved vectors shared similar habitats.

Conclusions: *Leishmania donovani* might circulate among different species of rodents in kala-azar endemic lowlands and valleys of Ethiopia by *Phlebotomus orientalis* and *Phlebotomus martini*. Detailed studies to substantiate the preliminary data on the possible role of these rodents are urgently needed.

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1. Introduction

In Ethiopia, the annual incidence of visceral leishmaniasis (VL) is around 4000–7000 from the estimated 3.2 million people at risk for getting this disease [1,2]. The disease is believed to have a zoonotic transmission except during the man to man epidemics situation in Libo-Kemkem district [2–4]. Intrusion into a zoonotic cycle in extra-domestic environments has been indicated as the source VL infections in the major foci in Southern and northwestern Ethiopia [1,3–7]. However, the reservoir hosts of kala-azar have not been found conclusively. Direct agglutination test (DAT), enzyme linked immuno sorbent assay (ELISA) and polymerase chain reaction (PCR) indicated the presence of *Leishmania donovani* (*L. donovani*) infection and salivary protein of *Phlebotomus orientalis* (*P. orientalis*) (vectors of VL) in serum of cow, dogs, sheep, goats and donkeys in the northwestern Ethiopia [8,9]. The role of domestic animals such as cow, sheep, goat, donkey, camel and dogs in northwest Ethiopia might be related only to blood meal sources for the vector of VL [7–9]. A study in Sudan suggested Egyptian mongoose as probable reservoir host [10] while other studies considered rodents as possible reservoirs [11–14]. The fact that this carnivorous mongoose feed on rodents might lead to rodents' origin of the *Leishmania* infection. *Leishmania* screening of the rodents and bats in Ethiopia, including those samples for the study of animal wildlife in the country, indicated only rodents in the VL endemic areas harbored *L. donovani* infections [14,15].

Experimental *L. donovani* infection in *Arvicanthis nilothicus* (*A. nilothicus*) in Sudan indicated the susceptibility of this species to *Leishmania* infection but parasitemia decreased with time indicating that rodents may not be the reservoir host of VL [16]. Hoogstraal and Heyneman [11], on the other hand, found chronic natural of the infection in *A. nilothicus* and considered this rodent species as the most probable reservoir host of VL in Sudan, where the *Leishmania* parasites isolated from the rodents were found to be identical with parasites from VL patients and *P. orientalis* vectors. The objective of this study was to describe zoonotic visceral leishmaniasis in Ethiopia by investigating the role of rodents as reservoir hosts of VL in endemic lowland areas of Ethiopia.

2. Materials and methods

2.1. Study area

In Western Tigray Zone (Kafta Humera district), extra-domestic agricultural fields and tickets of *Acacia seyal* near Baeker (14°01′N, 36°59′E) and May Kadra towns (Gelanzeraf) (13°59′N, 036°31′E) were used for trapping the rodents in addition to agriculture fields in Adijamus village in Welkit districts. In Tahtay Adiyabo district (around Shiraro town; Western Tigray Zone), rodents were sampled from Ademiti, Chameskebet and Mayhas villages. In Raya Azebo district, southern Tigray zone, rodents were collected from pre-domestic areas around houses with previous VL cases in a village located 7 km away from Mehoni town, where the administrative center of the zone was located. In southern Ethiopia rodents were trapped from Galga-village (900–1300 m.a.s.l) and Segen Valley (<700 m.a.s.) in Aba-Roba kebele (5°15′0″N; 37°16′0″E) in Konso district. In Guji Zone of the Oromia Region, Negele Borana (or Neghelle) (5°20′N39°35′E, 5.333°N 39.583°E; 1475 m altitude) is the largest town in the zone. Rodents were sampled from Gofe Ambo (Enso) and Kobadi villages that were located at about 20 km west of Negele Borana town. The inhabitants in these two villages were semi-pastoral communities. The surroundings of clustered huts (tukuls) were bushy with big termite mounds serving as habitats for rodents and *Phlebotomus martini*, the proved vector of kala-azar in southern Ethiopia [17], similar to Galga village. Algude and Cherkeka (026°34′E; 05°65′N; 550 m altitude) villages were the other sites for rodent sampling in Omo valley in Hamer district of South Omo zone. These villages had similar vegetation with Segen valley, Galga and Negele Borana areas.

2.2. Study design

The rodent sampling sites in villages and extra-domestic habitats were selected based on information on document obtained from VL patients in treatment centers in Humera, Gondar, Arbaminch and Negele Borana Hospitals. Rodents were trapped for screening of *Leishmania* infection by parasitological, serological and molecular techniques before the species of the rodents were identified by morphological characters.

2.3. Study period

Rodents were sampled from sampling sites in Humera-Shiraro lowlands (March, April September and December, 2013), Mehoni lowland (June, 2013), Galga village (July, October, 2013), villages in Hamer (August, 2013), villages in Negele Borana (August, 2013) and Segen Valley (January, 2014).

2.4. Rodent trapping and tissue sampling techniques

Rodents were captured by Sherman live traps baited with peanut butter and placed overnight in the sampling sites. The rodents were anaesthetized for tissue biopsies from liver, spleen, bone marrow and tip of the nose (skin) for screening of *Leishmania* infections. Ketamine (Clorketam Veterinary) was used to anaesthesize the rodents according the prescribed dosage (average 2 mg/kg for intra-venous route). The blood obtained using sterile needle was dropped into sterile filter paper and allowed to air dry before sealing in plastic bags. The tissues from liver, spleen and skin were macerated in Locke's solution before transferring them into NNN medium. Blood and touch smears of liver, spleen, skin and bone marrow were made on microscope slides and allowed to air dry before fixing using methanol and staining by Giemsa stain for microscopy. Rodents were trapped from the sampling sites after permission was obtained from Ethiopian Wildlife Conservation Authority (EWCA), Government of Ethiopia.

2.5. Serological test of the blood samples on filter paper

Five mm disks from the dried blood samples on the filter papers were punched out before eluting in 125 μL DAT buffer in each well of a micro-plate row B which was incubated for overnight at 4 °C. Fifty micro-liter DAT diluent (physiological saline (0.9% NaCl) containing 0.78% β-mercaptoethanol) was dispensed into every well of the vertical rows A, B, C, D, E, F, G

and H except into row B. Freeze dried serum diluted in PBS (100 μL) was used as positive control and dispensed into well 12C while 100 μL Freeze dried solution of bovine albumin was added (negative control) in 11C. Serial dilution, using a multi-channel pipette, was performed from Row B until Row H by transferring 50 μL and mixing five times. Mixed and re-suspended DAT-antigen (50 μL) was pipetted to every well except row B. Finally, the micro-plate was sealed with an adhesive plate sealer and shacked gently and incubated for overnight. The end point titre was determined by visual inspection of the agglutination reactions with reference of the negative controls. The cut-off value of the DAT was set at >1:800 so as to increase sensitivity of the test in detecting *Leishmania* infection.

2.6. Morphological data

Morphological data relevant to the systematics of rodents such as pelage colour, length of body, head, tail, soles of the feet and ear size were obtained when the rodents were anaesthetized for tissue biopsies. Each rodent has been photographed before dissection and the head was mounted in 95% ethanol. Each head sample was individually boiled to remove the flesh before taking skull morphometric characters for species identification.

2.7. DNA extraction and PCR

DNA was extracted from spleen, liver, bone marrow and skin samples and PCR reaction performed according to Abbasi *et al.* [18]. In brief, 25 μL final volume was prepared from 20 μL master mix (1 μL forward primer, 1 μL backward primer, dNTPs and 18 ddH_2O) and 5 μL DNA sample for PCR reaction. 18S rRNA internal transcribed spacer one (*ITS1*) was amplified using primers *LITSR* (5′-CTG GAT CAT TTT CCG ATG-3′) and *L5.8S* (5′-TGA TAC CAC TTA TCG CAC TT-3′) for 35 cycles. The thermal profile of the PCR comprised 5 min at 95 °C, followed by 35 cycles starting at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, a final elongation step at 72 °C for 10 min. The *ITS1* PCR product, sized ca 330 bp, was analyzed by gel electrophoresis at 120 V in 1× TAE buffer in 1.5% agarose gels to find *Leishmania* spp. specific bands. The fragments were visualized using ethidium bromide up on exposure to UV light.

3. Results

A total of 335 rodents were trapped. Of these, a total of 164 rodents were collected from Northern and 171 from southern Ethiopia (Table 1). Rodents were found in pre-domestic and extra-domestic areas. Fences and bushes around houses in pre-domestic areas, agricultural fields, termite mounds and grasses in sparse tickets of *Acacia seyal* in extra-domestic habitats were the habitats for the rodents.

Of the total 91 rodents [*Arvicanthis niloticus* (*A. niloticus*; *n* = 54), *Tarera* (*G*) *robustus* (*T. robustus*; *n* = 16), *Acomys cahirinus* (*A. cahirinus*; *n* = 17), *Mylomys albegena* (*n* = 2), *Xerus erythropus* (*n* = 2)] collected from extra-domestic habitats of Baeker and Gelanzeraf (Kafta Humera district) in March and April, 2013 and analyzed by PCR using skin, spleen, liver and bone marrow samples, 6/54 (11.1%) of *A. niloticus* were positive compared to infection rates in *A. cahirinus* (3/17 or 17.6%) and *T. robustus* (2/16 or 12.5%). Only 48 bone marrow samples

Table 1
The number of different species of rodents sampled from the different sampling sites in VL endemic foci villages of Ethiopia, from March, 2013 to January, 2014.

Species	Sample site						Total
	A	B	C	D	E	F	
A. niloticus	68	27	–	–	–	–	95
A. somalicus	–	–	–	25	21	31	77
A. cahirinus	25	1	16	7	5	2	56
T. robustus	21	3	–	–	–	–	24
Mastomys erythroleucus	3	5	25	5	10	7	55
Taterillus emini	–	–	11	–	–	–	11
Mylomys albipes	2	–	–	–	–	–	2
Rattus ratus	5	–	–	–	–	–	5
Paraechimus aethiopicus (hodgehog)	1	–	1	–	–	–	2
Crocidura macmillani (Elephant shrew)	–	–	2	–	–	–	2
Hetrocephalus glaber (Nacked mole rat)	–	–	1	–	–	–	1
Xerus erythropus (striped ground squirrel)	3	–	–	–	1	–	4
Civettitis civetta (African civet)	–	–	–	1	–	–	1
Total	128	36	56	38	37	40	335

Sample site A: Humera (Gelanzeraf); B: Mekoni and Shiraro; C: Galga (Aba-Roba); D: Segen valley; E: Hamer (Algude and cherkaka villages); F: Negelle Borena (Gofe Ambo and Enso villages).

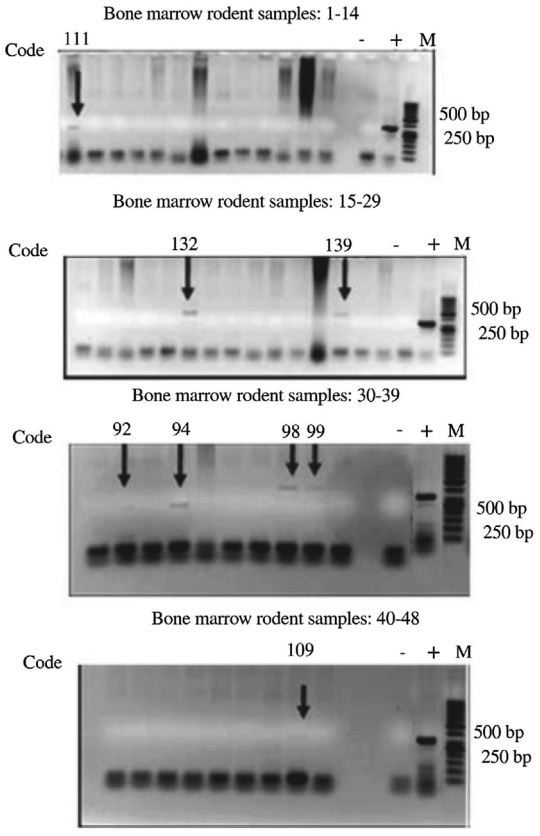

Figure 1. Representative samples of PCR amplifications of *ITS1* of *Leishmania* parasites from bone marrow samples from rodents collected in March and April, 2013 in extra-domestic habitats of Baeker and Gelan zeraf areas in Kaft – Humera district.
Lane M: MW marker; Lanes 1 and 45 (code 111 and 109): *T. robustus* collected from Gelanzeraf in April; Lanes 20 and 27 (Code H132 and H139): *A. cahirinus* collected from Baeker and Gelanzeraf; Lanes 31, 33, 37 and 38 (Code H92, H94, H98 and H99): *A. niloticus* from Baeker.

were found enough for DNA extraction to run PCR. Almost all the infections were found from these bone marrow samples (8/48 or 16.7%) compared with 1/91 (1.1%) liver, 2/87 (2.2%) spleen and 0/87 (0%) skin (Figure 1). Different organs of the same rodent were not found infected. DAT of blood on the filter papers for 91 rodents from northern Ethiopia showed all negative. Only 1 *A. cahirinus* in Galga village from all 171 rodents collected from southern Ethiopia were DAT positive. Microscopy and NNN medium were negative for all rodents except 2 spleen samples NNN medium positive *A. niloticus*. Bone marrow samples were not used for NNN medium.

4. Discussion

All the rodents including those found infected in this study such as *A. niloticus*, *T. robustus, Mastomys erythroleucus* (*M. erythroleucus*) and *A. cahirinus* share a habitat (cracks of black soil) with *P. orientalis*. Getting infected rodents and human VL cases near rodents, which share a habitat with the proved vectors, could give clue for vectors – rodents – vectors *L. donovani* cycle in extra-domestic agricultural fields and rural areas with incidental human infections (vectors – rodents – vectors – humans). Rodent species (*A. nilothicus*, *T. robustus* and *A. cahirinus*) in Kafta Humera lowlands were found with 12.5%–17.6% infection rate similar to infection rate of rodents in southern Ethiopia by Kassahun *et al.* [14].

Most probably, rodents play important role in maintaining *L. donovani* cycle during rainy season when *P. orientalis* population declines [6]. Identification of *L. donovani* complex from *Arvicanthis* spp., *Gerbilliscus nigricaudus*, and *M. erythroleucus*, using real time PCR in the Southern kala-azar endemic lowlands of Ethiopia [14] and in northwest Ethiopia by this study could prove the zoonotic nature of VL transmission. Intrusion of humans into lowlands and valleys and settlement in rural areas, where zoonotic parasite cycle is maintained, could be the main reason for incidence of VL in Ethiopia.

Infection rates obtained in this study using ordinary PCR were ranged from 0 to 2.2% for skin, liver and spleen samples compared with 16.7% in bone marrow. For Kassahun *et al.* [14], bone marrow samples were not analyzed; all the infections were obtained from spleen samples. In this study, bone marrow samples were not used for NNN-medium. Only 2 spleen samples were positive by NNN-medium from the total 335 rodents analyzed (skin, liver and spleen). Future study should take into considerations about the type of techniques and tissue samples used during the screening of infections in rodents. Unlike the common thought of the sequestration of *L. donovani* amastigotes in the spleen [19], bone marrow has also been shown, in this study, as a major source of *L. donovani* infection. Our finding was supported with previous experiment on BALB/c mice which confirmed bone marrow as site of persistent infection in addition to a striking similarity with the spleen in the time of onset of rapid amastigote accumulation [20].

In conclusion, *A. niloticus, A. cahirinus, Gerbilliscus nigricaudus* and *M. erythroleucus* could play a role in the transmission cycle of zoonotic kala-azar infection due to *L. donovani* in endemic areas of Ethiopia. Further studies are required for *L. donovani* isolation from rodents in the endemic areas. Additional evidences relating to infectivity of sand flies using xenodiagnosis should be sought before considering these rodents as potential reservoir hosts of *L. donovani*.

Conflict of interest statement

We declare that we have no conflict of interest.

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