

Natural Infection of North African Gundi (*Ctenodactylus gundi*) by *Leishmania tropica* in the Focus of Cutaneous Leishmaniasis, Southeast Tunisia

Nadia Bousslimi, Soumaya Ben-Ayed, Imène Ben-Abda, Karim Aoun, and Aïda Bouratbine*
Laboratoire de Recherche 05SP03, Laboratoire de Parasitologie, Institut Pasteur de Tunis, Tunis, Tunisia

Abstract. North African gundis (*Ctenodactylus gundi*) were trapped in the *Leishmania* (*L.*) *tropica* focus of cutaneous leishmaniasis, situated in southeast Tunisia and evaluated for *Leishmania* infection by real-time kinetoplast DNA polymerase chain reaction (PCR). Species identification was performed by internal transcribed spacer one (ITS1)-PCR-restriction fragment length polymorphism (RFLP) and high-resolution melting (HRM) analysis of the 7SL RNA gene. Real-time PCR on blood was positive in 6 of 13 (46.2%) tested gundis. *Leishmania tropica* was identified in five infected gundis and *Leishmania major* in one specimen. Alignments of the ITS-1 DNA sequences and 7S-HRM curves analysis indicated that similar genotypes were present in humans, a sandfly, and gundis from the same region suggesting a potential role of this rodent as reservoir host of *L. tropica* in southeast Tunisia.

INTRODUCTION

Leishmania (*L.*) *tropica* is widely distributed in north and east Africa, the Middle East, and large parts of Asia. It is a genetically heterogeneous species including multiple genotypes.¹ Cutaneous leishmaniasis (CL) caused by *L. tropica* is usually considered as an anthroponosis and *Phlebotomus* (*P.*) *sergenti* the acting vector.² However, the occurrence of sporadic human cases in some foci suggests the concomitant presence of zoonotic transmission.³ The rock hyrax is the proven reservoir and its reservoir role was confirmed even experimentally.^{3–6} This animal species is found throughout sub-Saharan Africa and north-east Africa, being discontinuously distributed from Senegal through southern Algeria, Libya, and Egypt to central and southern Africa. It also extends to the Arabian Peninsula and to the Middle-East, but is not found in Tunisia. *Rattus rattus* is the suspected rodent reservoir of *L. tropica* as asymptomatic rats were infectious for *P. sergenti* many months after *L. tropica* inoculation.⁷

The CL caused by *L. tropica* MON-8 (syn *Leishmania killicki*) is endemic in the southeast of Tunisia.⁸ It occurs as scarce scattered cases and prevails in communities living in the rocky mountainside of the Tataouine region.⁸ Recently, new foci have emerged in the Central part of the country.⁹ Until now, little data concerning the transmission routes of CL caused by *L. tropica* in Tunisia have been available. The relative paucity of CL cases and their spatial distribution excludes the anthroponotic character of the disease and suggests that CL caused by *L. tropica* might be a zoonosis^{8,9}; the putative reservoir host is the North African gundi (*Ctenodactylus gundi*). In fact, this rodent is extremely abundant in all Tunisian *L. tropica* foci, where it is found in natural and peri-domestic environments.^{8–10} On the other hand, natural infection of *P. sergenti* with *Leishmania* promastigotes was reported in the CL focus in southeastern Tunisia.¹¹ Likewise, *P. sergenti* has been recently identified as the *L. tropica* host in a sylvatic site populated by gundis (*Massoutiera mzabi*) of a neighboring region, namely Ghardaïa, south Algeria.¹²

The purpose of this study was to assess the *Leishmania* infection rate in North African gundis of the Tataouine region,

to identify *Leishmania* species infecting this wild rodent, and to compare the genetic relatedness between parasites characterized in gundis, humans, and sandflies of the same region.

MATERIALS AND METHODS

Study area. The study was carried out in the surrounding of the village of Ghomrassen, governorate of Tataouine, southeast Tunisia. This village is situated in a desert region presenting arid rock outcrops. It is built on a rocky mountainside at moderate altitude (300 m a.s.l.) and is a well-known focus of *L. tropica* CL.⁸ *Ctenodactylus gundi* is the most prevalent wild rodent living in the stony mountains around the village.

Sample collection. Thirteen gundis were live-trapped in October 2009 in natural sites located 6 km from the village, in the rocky mountain at c. 310 m a.s.l. The gundis were manually caught by the local population during the twilight hours near the entrances of caves and crevices inhabited by the rodent and then kept alive in cages. Animals were physically examined. Blood samples were extracted from the 13 gundis into tubes containing EDTA. Biopsies were performed only on three rodents. Nasal biopsies were taken from the three animals, whereas a piece of spleen was taken from only one. Biopsies were stored in a sterile microtube (Deltalab S.L., Spain) at –80°C until DNA extraction. The study was conducted after the authorization of the Ministry of Agriculture and Environment, Forest directory, Tunisia.

Polymerase chain reaction (PCR) assays. The DNA was extracted from 100 µL of blood using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The DNA extraction from tissue homogenates was performed using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. All samples were analyzed by kinetoplast DNA (kDNA) real-time PCR¹³ and internal transcribed spacer 1 (ITS1) PCR,¹⁴ and if positive, species were identified by ITS1-restriction fragment length polymorphism (RFLP),¹⁴ ITS 1 sequencing, and high-resolution melting (HRM) analysis of the 7SL RNA gene.¹⁵

Briefly, kDNA real-time PCR was conducted in a final volume of 25 µL by using a TaqMan universal master mixture (Roche, Palo Alto, CA) containing 100 µM of direct primer (5'-CTTT-TCTGGTCCCTCCGGGTAGG-3'), 100 µM of reverse primer (5'-CCACCCGGCCCTATTTTACACCAA-3'), 50 µM of probe (FAM-5'-TTTTTCGAGAACGCCCTACCCGC-3'-TAMRA),

*Address correspondence to Aïda Bouratbine, Laboratoire de Parasitologie, Institut Pasteur de Tunis, 13 Place Pasteur, BP 74, 1002 Tunis Belvédère, Tunisia. E-mail: aida.bouratbine@pasteur.rns.tn

and 1 μ L of DNA extract.¹³ The DNA was amplified in an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) for 50 cycles at 95 and 60°C. Each sample was tested in duplicate, and each run included both positive and negative controls. Samples were checked for inhibition by being retested at a 1:10 dilution and by spiking these samples with 1 μ L of the positive control. A standard curve, plotted from a dilution series of *Leishmania* DNA (extracted from 10⁶ *Leishmania infantum* promastigotes), allowed parasite quantification by PCR. The volume or the weight of collected samples and the dilution rates applied during DNA extraction and amplification were taken into account to estimate the parasite load in each collected specimen.

Concerning ITS1-PCR, LITSR and L5.8S primers were used to amplify the genes encoding the small subunit and 5.8S rRNA.¹⁴ The positive ITS1-PCR products were digested by the *Hae*III restriction enzyme following the protocol described earlier.¹⁴ The ITS1 products were also purified and sequenced in both directions with the forward and reverse primers on an ABI Prism 377 DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. The consensus sequences were deposited in GenBank and compared with nucleotide sequences of 12 *L. tropica* complex strains and 5 *Leishmania major* strains. These 17 reference strains were isolated in the study site (three isolates from humans and one from *P. sergenti*) and in other geographical regions. A phylogenetic tree was generated using MEGA5 software. Relationships were inferred based on genetic distances by the neighbor-joining option. The evolutionary distances were computed using the Kimura 2-parameter method with *L. mexicana* as an out-group and 2,000 bootstraps.

The 7SL-HRM PCR assay using the primers CJ7SLF (5'-ACG TGG ACC AGC GAG GGT-3') and QRT7SLR (5'-CGG TTC CCT CGC TTC AAC-3') was performed in 20 μ L, final volume of the PCR mix, containing 2 \times SsoFast EvaGreen supermix (Bio-Rad), 0.5 μ M of each primer, and 2 μ L of DNA extract.¹⁵ Amplification conditions were as follows: 15 min of denaturation at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C; annealing for 30 s at 63°C; and a final extension for 2 min at 72°C. The HRM ramping was carried out at 0.2°C/s from 65 to 95°C. The HRM PCR and analysis were performed by a CFX96 real-time PCR detection system (Bio-Rad) and the Precision Melt Analysis Software (Bio-Rad). Positive and negative control reactions were included in each experiment. The PCR efficiency was evaluated using the threshold cycle (C_q), and a normalized melt window, 88 to 90, allowed the analysis of HRM curves. The sensitivity, specificity, and discrimination between *L. major* and *L. tropica* species were ana-

lyzed according to strains promastigotes, isolated from human CL cases living in the same region, and typed by iso-enzyme analysis. Sensitivity was determined using 10-fold dilutions of each DNA (data not shown).

RESULTS

All trapped gundis have no lesions. Using kDNA real-time PCR, 6 of the 13 gundis had *Leishmania* DNA in their blood (46.2%). Among these six positive rodents, spleen and/or nose biopsies, taken from two specimens, were positive (Table 1). Samples quantification yielded low parasite loads, ranging from 0.5 to 5.6 parasites per 100 μ L of blood samples and 6.7 to 9.4 parasites per 100 mg of nose and spleen tissues. All these samples were also found positive using 7SL-HRM, allowing the species identification (Table 1). The average melting temperatures (T_m) \pm SD for 40 7SL-HRM runs were as follows: *L. major*, 88 \pm 0.04; *L. tropica*, 89 \pm 0.02. The HRM analysis typed correctly eight samples as *L. tropica*, and one as *L. major* compared with reference strains (Figure 1, Table 1).

Using ITS1 PCR, 5 out of 13 gundis had *Leishmania* DNA in blood (38%). Among these five positive rodents, spleen and/or nose biopsies, taken from two specimens, were positive. Among the five positive blood samples, only three had successful species identification by sequencing and/or RFLP analysis. However, among the three tissue specimens only one had successful species identification (Table 1).

Species characterization of all samples correlated 100% using the three assays (Table 1). Three samples infected by *L. tropica* or *L. major* parasites from three different infected gundis were analyzed by the ITS1 sequencing. Phylogenetic analysis showed that ITS1 DNA sequences of *L. tropica* parasites isolated from gundis were highly similar to those isolated from human CL cases and the incriminated peri-domestic vector *P. sergenti* of the same region (Figure 2). On the other hand, sequences of *L. major* parasites isolated from one gundi were also highly similar to those isolated from human CL cases of the Tataouine region (Figure 2).

DISCUSSION

Ctenodactylus gundi ranges from eastern Morocco, through Algeria and Tunisia, to western Libya. It is found in deserts with arid outcrops where it shelters in fissures and rock crevices, and under rock formations. In Tunisia, North African gundis are extremely common in the *L. tropica* foci in the Tataouine region, where they inhabit crevices within the rocky mountainside surrounding the villages. It was shown in

TABLE 1
Diagnosis of *Leishmania* infection in gundis by different PCR assays

Specimen	Sample	Parasite load*	ITS PCR	ITS PCR RFLP	Sequencing	7SL HRM PCR
GK 01	Blood	0.72	+	<i>L. tropica</i>	JF719996	<i>L. tropica</i>
	Nose	6.8	+	NO		<i>L. tropica</i>
GK 05	Blood	5.6	+	<i>L. major</i>	JN242001	<i>L. major</i>
GK 07	Blood	0.65	+	NO		<i>L. tropica</i>
GK 09	Blood	0.66	+	NO		<i>L. tropica</i>
GK 12	Blood	0.5	-	NO		<i>L. tropica</i>
GK 13	Blood	0.9	+	<i>L. tropica</i>		<i>L. tropica</i>
	Nose	6.78	+	<i>L. tropica</i>	JF719995	<i>L. tropica</i>
	Spleen	9.4	+	NO		<i>L. tropica</i>

*Number of parasites per 100 μ L of blood or per 100 mg of tissue (nose and spleen).

PCR = polymerase chain reaction; ITS = internal transcribed spacer; HRM = high-resolution melting; NO = non observed.

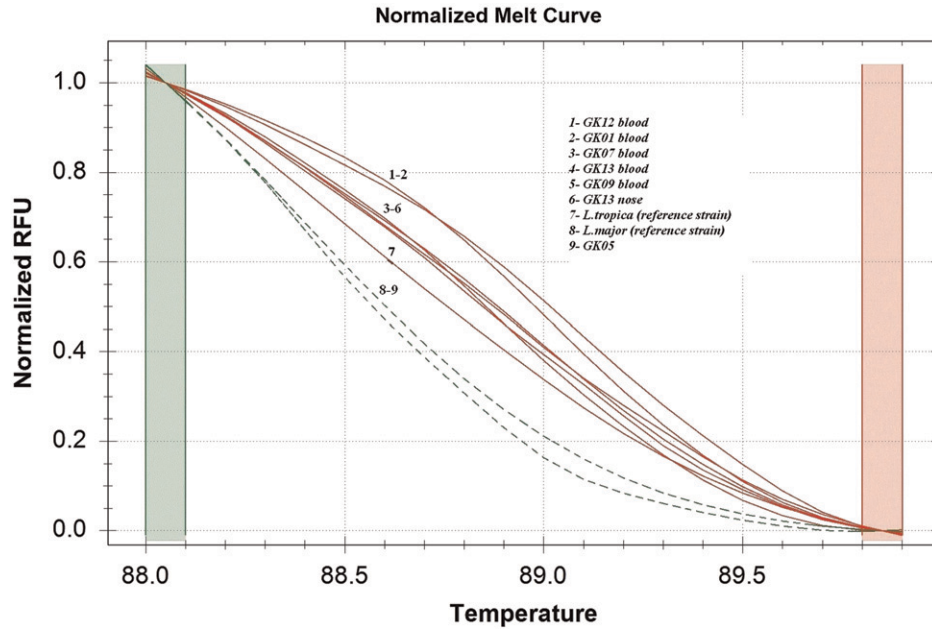


FIGURE 1. Comparison of normalized high-resolution melting (HRM) curves of the 7SL polymerase chain reaction (PCR) amplicon obtained for Tunisian *Leishmania* references species (*Leishmania tropica* MON8 and *Leishmania major*) and seven gundi samples (GK01, GK05, GK07, GK09, GK12, GK13 blood and GK13 nose).

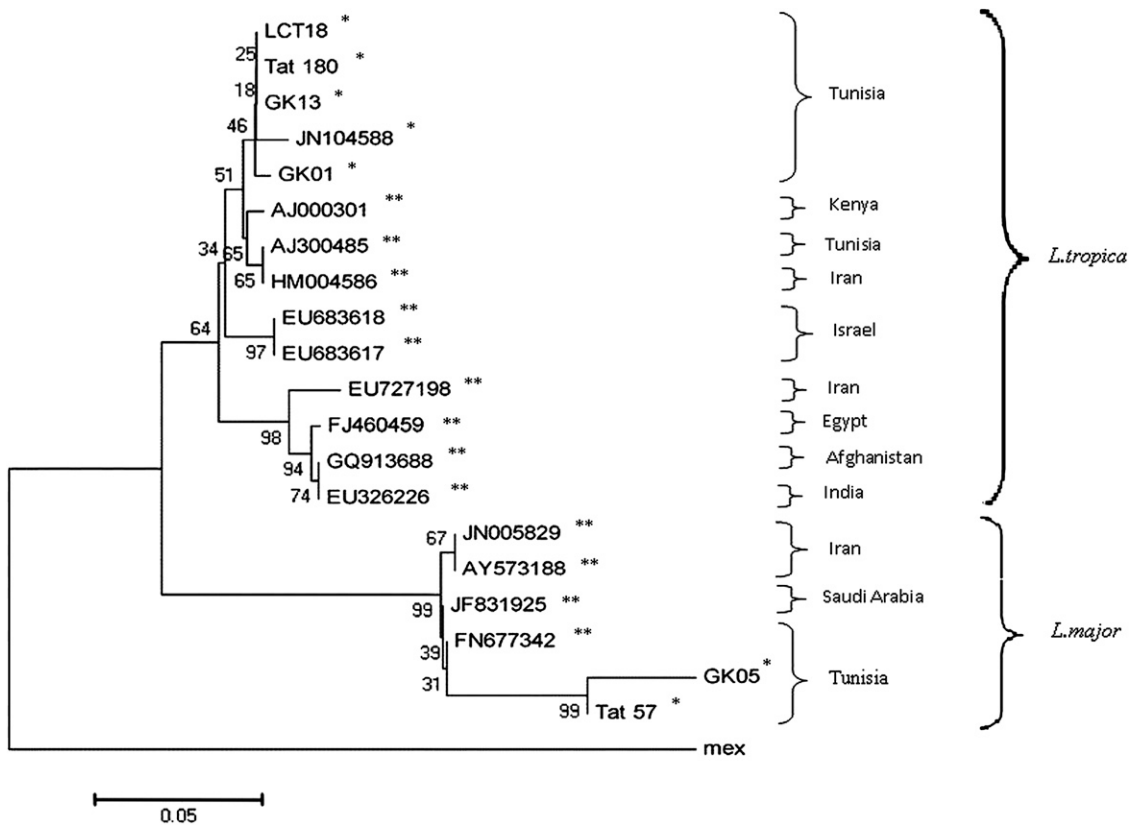


FIGURE 2. Phylogenetic analysis of internal transcribed spacer one (ITS1) sequences from human, gundi, and *Phlebotomus sergenti* (Tree obtained by the method of neighbor-joining, *Leishmania mexicana* as an out-group, bootstrap values based on 2,000 replicates). *Our samples: GK13, GK01, and GK05 were from gundis, LCT18, Tat180, and Tat57 were from humans and JN104588 was from *Phlebotomus sergenti*. **Nucleotide sequences from GenBank.

previous studies that this habitat affords suitable breeding sites for sandflies.¹⁰ Thus, as gregarious diurnal mammals, sleeping gundis are an easy blood source available for night-questing phlebotomine females. Furthermore, gundis live for 5–6 years in the wild and may survive through any non-transmission season. Accordingly, they have all the behavioral and ecological requirements of good reservoir hosts for *L. tropica* CL in southeast Tunisia.¹⁶

The detection of *Leishmania* in naturally infected rodents in endemic areas is of utmost importance to involve these rodents as reservoir hosts.¹⁶ To our knowledge, this is the first study that implements quantitative PCR for detection of *Leishmania* infection in North African gundis. Our results revealed a high rate of *L. tropica* infection in asymptomatic animals (38.5%). Interestingly, the transmission of *L. tropica* from black rats without any visible cutaneous changes was previously reported and suggested that an asymptomatic host would be of great epidemiological significance in field conditions.⁷ On the other hand, the low parasitic load observed in all specimens (a mean of 0.69 parasites per 100 μ L of blood) was already reported in hyraxes.⁴ This may explain the difficulty of parasite isolation by culture (data not shown).

The predilection sites of *Leishmania* may differ according to reservoir host.⁶ Indeed, in hyraxes, *L. tropica* is usually found in the skin above the nose where sandfly vectors prefer to feed.^{3,6} The presence of *L. tropica* DNA in nose biopsies of infected gundis suggests that the nose may correspond to a potential feeding site for local sandfly vectors. On the other hand and as reported for hyraxes, the presence of *L. tropica* DNA in gundi blood demonstrated in the current study could be caused by either the circulation of live amastigotes present in phagocytes or to degraded parasites.⁴ The *Leishmania* DNA positivity observed in one spleen sample suggests that *L. tropica* infection may visceralize. However, the possibility of infection involving visceral organs and circulatory blood dissemination in gundis requires substantiation in further studies.

On the other hand, sequence analysis of ITS1 fragments showed perfect correlation with *L. tropica* strains isolated in human CL cases and in *P. sergenti* of the same region. Therefore, *Ctenodactylus gundi* could be considered, as a potential reservoir of *L. tropica* in this region. However, further studies must be conducted to show that gundis are experimentally susceptible to *L. tropica*. Moreover, infected gundis should be confirmed as an infective feeding for potential vectors, namely *Phlebotomus riouxi* and *P. sergenti*.^{10,11}

Recently, zoonotic CL caused by *Leishmania (L.) major* has emerged likewise in the Tataouine region. A previous study showed that cases of *L. major* are clustered at the margin of some villages where the gerbil reservoir hosts (*Meriones* species) are widespread.⁸ The presence of *L. major* infection, with relatively high parasitic load, in one infected gundi stresses the need of further experimental investigation in this wild rodent. However, it is highly probable that these animals are not reservoir hosts, and they might be just considered as incidental hosts because the infection is scarce and the relative abundance of the proven vector of *L. major* and *Phlebotomus papatasi* in gundis' habitats is low.¹⁰

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Authors' addresses: Nadia Bousslimi, Soumaya Ben-Ayed, Imène Ben-Abda, Karim Aoun, and Aïda Bouratbine, Laboratoire de Parasitologie, Institut Pasteur de Tunis, Tunis Belvédère, Tunisia, E-mails: nguetai@gmail.com, Soumayatba@yahoo.fr, benabda.imen@gmail.com, karim.aoun@pasteur.rns.tn, and aida.bouratbine@pasteur.rns.tn.

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