Potential Transmission Cycles of *Leishmania tropica* in a Historic Disease Focus of Cutaneous Leishmaniasis in Southeast Tunisia[1]

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Abstract The transmission of *Leishmania tropica* (Wright) (Protozoa: Sarcomastigophora: Trypanosomatidae) was studied in a historic focus of the cutaneous leishmaniasis disease in southeast Tunisia. The sandfly *Phlebotomus sergenti* (Parrot) (Diptera: Psychodidae), the confirmed vector of *L. tropica* in humans, was the most abundant *Phlebotomus* species found in homes. *Phlebotomus chabaudi* s.l. (Croset) was the dominant species in the natural rocky habitats favored by the North African gundi, *Ctenodactylus gundi* (Rothman), which is a known putative rodent reservoir of *L. tropica*. *Leishmania tropica* MON-8 (Rioux, Lanotte and Pratlong) was the species isolated and identified from gundi, humans, and P. sergenti in the disease focus area. Based on these results, the North African gundi may serve at least as a maintenance host for *L. tropica* in this area of southeast Tunisia, even though *L. tropica* is commonly stated to be anthroponotic. These results also suggest that there may be two transmission cycles of *L. tropica* in this region, with *P. sergenti* transmitting *L. tropica* among humans inside and in peridomestic habitats and *P. chabaudi* s.l. transmitting the disease agent among gundi in their natural habitats. *Phlebotomus chabaudi* s.l. also may transmit to humans when humans venture into areas inhabited by gundi host reservoirs.

K**ey Words** cutaneous leishmaniasis, *Leishmania tropica*, anthroponotic, zoonosis, vectors, Tunisia

Cutaneous leishmaniasis (CL) is a widespread and resurging vector-borne disease caused by a protozoan parasite belonging to the genus *Leishmania* (Alvar et al. 2012). In Tunisia, there are three species of parasites including *L. infantum*, *L. major*, and *L. killicki* (synonymous with *L. tropica* MON-8 [Rioux, Lanotte and Pratlong]) that may cause CL. This last taxon was originally described in 1980 in southeast Tunisia (Rioux et al. 1986) and continues to occur in communities and villages next to the arid mountains of Tataouine (Bousslimi et al. 2010). When initially reported, sporadic cases were reported after that in different parts of the country (Bouratbine et al. 2005; Haouas et al. 2005; Kallel et al. 2005) with chronic skin lesions and low endemicity. The disease was also reported from neighboring Algeria and Lybia (Aoun et al. 2006; Harrat et al. 2009). Nevertheless, until recently,

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the epidemiology and the transmission cycles of the disease remain as not well known. *Leishmania tropica* is commonly stated to be anthroponotic (World Health Organization 1984). The probable zoonotic transmission of this parasite, with the gundi rodent *Ctenodactylus gundi* (Rothman) as the reservoir and the sandfly *Phlebotomus sergenti* (Parrot) as the vector, was suggested by Ben Ismail and Ben Rachid (1989) but requires further confirmation. In fact, the relative paucity of CL cases and their spatially separated distribution in Tunisia casts doubts that the disease is exclusively anthroponotic in the region and suggests that CL caused by *L. tropica* might be a zoonosis.

The putative reservoir host, the North African gundi, *C. gundi*, is extremely abundant in the mountainous area of Tataouine and also in all emerging Tunisian foci of CL caused by *L. tropica* (Bouratbine et al. 2005). While *P. sergenti* is considered as the main vector of *L. tropica* in Algeria, Morocco, and throughout the Middle East and Central Asia (Al-Zahrani et al. 1988; Boubidi et al. 2011; Guilvard et al. 1991; Killick-Kendrick 1990), is *P. sergenti* the same vector for both the human and the gundi transmission cycles in southeast Tunisia, or are other vectors involved? Indeed, Volf and Myskova (2007) recognized that some sandfly species may be "specific/restricted vectors" (i.e., vectors that support the development of only one *Leishmania* species) while others may be "permissive vectors" (i.e., vectors that support a range of *Leishmania* species). And, the same species of parasite may be transmitted by several species of vectors.

The purpose of this study was to assimilate previous reports and data on CL in a historic focus of the disease and to further determine the nature of the transmission cycle(s) of *L. tropica* in southeast Tunisia, where the occurrence of sporadic human cases in some foci suggests the concomitant presence of zoonotic transmission. Confirmation of these epidemiological factors is an important public health objective in management of this disease.

Materials and Methods

Study area. Data were collected in and around the village of Ghomrassen in the Governorate of Tataouine in southeastern Tunisia. Ghomarssen is 480 km south of the capital of Tunis, is surrounded by mountains, and occupies the site of an ancient desert oasis. The oasis and the majority of irrigation wells disappeared with urban development. Its population is 11,383 living in single family houses built on basalt rock and surrounded by gardens. The area is at 300 m above sea level and is characterized by an arid bioclimate (Gounot and Le Houerou 1959) with a mean temperature of 22°C and annual rainfall between 88 and 157 mm (National Institute of Meteorology – Tunisia; www.meteo.tn/htmlen/accueil.php [last accessed 11 March 2019]). This study area is a historic disease focus of *L. tropica* CL cases in humans, with the village and its homes serving as anthropogenic transmission sites and the nearby (approx. 6 km from the village) mountains inhabited by large populations of *C. gundi*, a suspected reservoir of *Leishmania* spp.

Sandfly collection and assay. Sand flies were collected in 2009 and 2010 using CDC Miniature Light Traps (John W. Hock Company, Gainesville, FL) in anthropogenic (i.e., inside and outside of homes, animal sheds) and in natural habitats (i.e., mountains). Traps were placed 2–3 h before sunset, operated

overnight, and retrieved the following morning. Head and genitalia of collected sandflies were used for identification according to morphological characters described by Boussaa et al. (2008), Croset et al. (1978), Depaquit et al. (1998), and Léger et al. (1983). Species diversity was quantified in habitats from which flies were collected.

To determine if sandflies were infected with *Leishmania*, live females were immobilized on ice, rinsed briefly in 96% ethanol, and dissected in 0.9% sterile saline. Guts were examined under 40× magnification and when found to contain promastigotes, they were cultured in Novy-MacNeal-Nicolle (NNN) medium at 26°C. DNA was extracted from the positive cultures using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two primers, LITSR and L5.8S, were used in polymerase chain reaction (PCR) amplification of the ribosomal internal transcribed spacer 1 region (Bousslimi et al. 2010). The amplified internal transcribed spacer 1 (ITS1) was digested using Hae III (Fermentas, Waltham, MA), and the products were electrophoresed in 3% agarose. These electrophoresis profiles were compared with those obtained in human samples from the same region as reported by Bousslimi et al. (2010). Positive cultures were also sent to the Pasteur Institute of Algeria (Dely Brahim, Algeria) for the characterization and identification of strains of *Leishmania* using multiloci enzyme electrophoresis.

Rodent collection and assay. Thirteen live gundis were captured in the summer of 2009 using metal rodent traps placed near burrows in rock crevices in mountain habitats near Ghomrassen. Each rodent was examined for skin lesions, and blood samples were obtained and placed in ethylenediaminetetraacetic acid (EDTA). DNA was extracted from 100-ml aliquots for these samples using the QIAamp DNA Blood Mini Kit. Kinetoplast DNA (kDNA) real-time PCR (Boussilim et al. 2012; Mary et al. 2004) and ITS-1 PCR (Boussilim et al. 2012; Schonian et al. 2003) were used for sample analysis. In cases where positive results were obtained, ITS1 sequencing and high-resolution melting (HRM) analysis of the 7SL RNA gene (Boussilim et al. 2012; Nasereddin and Jaffe 2010) were performed to identify *Leishmania* species.

Human sample collection and assay. For this study, 66 samples of skin lesions were collected from infected individuals living in Ghomrassen and neighboring villages between October 2008 and September 2009. Samples were collected by first cleaning the lesion and making a superficial incision on the inflammatory border of the lesion using a sterile cytology brush (Deltalab, Barcelona, Spain). The skin-brushed samples were placed individually into 200 μl of phosphate-buffered saline in sterile plastic tubes, stored at 4°C, and transported to the Parasitology Laboratory at the Pasteur Institute of Tunis for molecular analysis. Identification of *Leishmania* species in these samples was performed using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for the ITS-1 region as described by Bousslimi et al. (2010).

Results and Discussion

A total of 1,994 adult sandflies were collected in this study. These represented 10 species and 4 subgenera. Of the total, 1,337 were collected from anthropogenic

Table 1. Species abundance of phlebotomine sandflies in anthropogenic and natural sites in and around the village of Ghomrassen in southeast Tunisia.

Species	Habitat*			
	Sheds	R-Indoor	R-Outdoor	Natural
Phlebotomus alexandri Sinton	0.037	0.012	0.156	0.050
Phlebotomus chabaudi s.l. Croset	0.007	0.023	0.084	0.240
Phlebotomus longicuspis Nitzulescu	0.027	0	0.033	0.048
Phlebotomus papatasi Scopoli	0.623	0.172	0.284	0.090
Phlebotomus perniciosus Newstead	0.027	0.097	0.036	0.050
Phlebotomus sergenti Parrot	0.092	0.350	0.104	0.050
Sergentomyia antennata Newstead	0.036	0.026	0.023	0.072
Sergentomyia christophersi Sinton	0	0.003	0	0.030
Sergentomyia fallax Parrot	0.096	0.220	0.184	0.160
Sergentomyia minuta France Luberon	0.055	0.097	0.096	0.210

* Abundance of each species expressed as proportion of total sandfly captures within a single habitat (Sheds = animal sheds; R-indoor = inside residences; R-outdoor = outside residences; Natural = natural rocky habitats).

sites (605 from animal sheds, 636 from inside homes, and 88 from outside houses) while the remaining 657 were collected from rocky habitats in natural settings where gundis are known to reside (Table 1). Of the species collected, *Phlebotomus papatasi* was the dominant species (62%) collected in the animal sheds. A mixture of species was collected from the natural rocky habitats, but *Phlebotomus chabaudi* s.l. (Tabbabi et al. 2014) was most frequently collected (24%). *Phlebotomus sergenti* (35%), *Sergentomyia fallax* (22%), and *P. papatasi* (Scopoli) (17%) were collected from traps operated inside houses.

A total of 41 *P. sergenti* females collected from the anthropogenic habitats were dissected, examined, and tested for presence of the protozoan parasite. Of those, only one was found to have promastigotes in the gut tissue; those were determined by isoenzyme typing to be *L. tropica* MON-8. None of the other sandfly females examined were infected with the protozoan. PCR-RFLP analysis confirmed that 31 human patients were infected with *L. tropica*. Of the 13 rodents captured, six were infected with *Leishmania* (5 with *L. tropica*, 1 with *L. major*). Alignment of the ITS-1 DNA sequences and the 7S-HRM curves indicated that similar *L. tropica* genotypes were recovered from the humans, gundis, and sandfly in this survey. The intraspecific heterogeneity of *L. tropica* is recognized and reviewed (Schönian et al. 2001), but Aoun et al. (2008) determined that only *L. tropica* MON-8 (synonym *L. killicki*) occurs in Tunisia.

These results demonstrate that, in this historic focus of CL in southeast Tunisia, humans afflicted with CL are infected with *L. tropica* MON-8; and North African

gundi, *C. gundi*, inhabiting rocky habitats near the village and testing positive for *Leishmania*, are primarily infected with *L. tropica* MON-8. Although *C. gundi* is suspected as a reservoir host of *L. tropica* in Africa (Ben Ismail and Ben Rachid 1989), this is the first report confirming the natural infection of *C. gundi* with *L. tropica* in southeast Tunisia. Furthermore, the *L. tropica* isolated from the gundi population was identical to *L. tropica* isolated from humans and a single sandfly in the same focus area. The CL disease and its causative agent *L. tropica*, therefore, reside within the human population and the gundi population in this historic focus of the disease.

Phlebotomus sergenti is confirmed as a vector of *L. tropica* in North Africa (Boubidi et al. 2011; Guilvard et al. 1991; Jaouadi et al. 2012; Killick-Kendrick 1990; Tabbabi et al. 2011a,b); however, the abundance of *P. chabaudi* s.l. in Tunisian and Algerian foci of anthropogenic CL caused by *L. tropica* implicated *P. chabaudi* s.l. as a possible vector (Harrat et al. 2009; Izri et al. 2014; Rioux et al. 1986; Tabbabi et al. 2011a,b), although some authors did not find sufficient evidence as confirmation (Tabbabi et al. 2014). In the present study, *P. sergenti* was the prevalent species, with only a few specimens of *Paraphlebotomus* spp., trapped inside homes occupied by humans infected with CL caused by *L. tropica*. In contrast, *P. chabaudi* s.l. was the prevalent species, with only a few specimens of *Paraphlebotomus* spp., trapped in the rocky habitats where *C. gundi* infected with *L. tropica* resided. Although our efforts to test sandfly females for the presence of *Leishmania* promastigotes yielded only one specimen of *P. sergenti* as positive for *L. tropica*, we feel that our findings strongly suggest that there are two transmission cycles of *L. tropica* operating in the Ghomrassen locale. *Phlebotomus sergenti*, a highly endophilic species, transmits *L. tropica* among humans in anthropogenic habitats while *P. chabaudi* s.l. transmits *L. tropica* in the wild *C. gundi* reservoir populations in natural habitats.

We also propose that sandfly vectors may transmit the disease from the rodent reservoir to the human population. The gregarious, rock-dwelling gundis are common in the *L. tropica* foci in the Ghomrassen region and are likely efficient reservoirs of *L. tropica* in southeast Tunisia (Ashford 1996). They inhabit rock crevices and burrow under rock formations which are also suitable breeding sites for sandflies (Tabbabi et al. 2011a,b), and the sleeping gundis in these burrows are important blood meal sources for emerging, unfed phlebotomine females. Many sandfly species are permissive vectors for various *Leishmania* spp. (Sboui and Tabbabi, 2018; Volf and Myskiva 2007) that cause a spectrum of diseases, commonly referred to leishmaniasis, in vertebrate hosts including humans. Furthermore, *P. sergenti* has been suggested by Depaquit et al. (2002) as a species complex. Genetic and morphological differences among populations of the species might allow for enhanced vector competence and host range. *Phlebotomus chabaudi* s.l. and *P. sergenti* occur in both the rocky habitat of the gundi and in nearby animal shelters and human residences (Tabbabi et al. 2011a) and could transmit *L. tropica* from rodent reservoirs to humans.

The lack of efficacy of available CL management tactics is at least partially due to the complexity of the transmission cycle and our lack of sufficient knowledge of the epidemiology and the natural history of the disease. This study provides some additional information; yet, more research must be conducted to further elucidate

transmission cycles and infection mechanisms involved with this and related leishmaniasis diseases.

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