First Report on Natural Infection of *Phlebotomus sergenti* with *Leishmania tropica* in a Classical Focus of *Leishmania major* in Tunisia

Kaouter Jaouadi,^{1,2*} Jihene Bettaieb,^{1,2,3} Amira Bennour,¹ Sadok Salem,^{1,2} Mohamed Ridha Rjeibi,⁴ Sana Chaabane,^{1,2} Rihab Yazidi,^{1,2} Neila Khabouchi,⁵ Adel Gharbi,¹ and Afif Ben Salah^{1,2,3,6}

¹Laboratory of Medical Epidemiology, Institut Pasteur de Tunis (IPT), Tunis-Belvédère, Tunisia; ²University Tunis El Manar, Tunisia; ³Laboratory of Transmission, Control and Immunobiology of Infections (LTCl), LR11IPT02, Institut Pasteur de Tunis (IPT), Tunis-Belvédère, Tunisia; ⁴Faculty of Medicine of Tunis, University Tunis El Manar, Tunisia; ⁵Laboratoire de Parasitologie, Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Sidi Thabet, Tunisia; ⁶Pole Génomique-Plateforme Technique Institut Pasteur de Tunis (IPT), Tunis-Belvédère, Tunisia; ⁶Department of Family and Community Medicine, College of Medicine and Medical Sciences (CMMS), Arabian Gulf University (AGU), Manama, Bahrain

Abstract. In Tunisia, chronic cutaneous leishmaniasis due to *Leishmania tropica* is an important health problem. Its spreading has not been fully elucidated. Information on sandfly vectors, as well as their associated *Leishmania* species, is of paramount importance since vector dispersion is one of the major factors responsible for pathogen dissemination. Ninety-seven unfed females belonging to the genera *Sergentomyia* and *Phlebotomus* were collected between June and August 2015 using sticky paper traps. Polymerase chain reaction–restriction fragment length polymorphism analysis of the internal transcribed spacer 1 and sequencing were used for *Leishmania* detection and identification. In total, 650 sandflies were captured and identified (380 males and 270 females). Ninety-seven unfed females were tested for the presence of *Leishmania* parasite DNA. Six *Phlebotomus sergenti* were found positive for *L. tropica*. This novel finding enhances the understanding of the cycle extension of *L. tropica* outside its original focus of Tataouine.

Cutaneous leishmaniasis (CL) is one of the world's most neglected diseases affecting mostly developing countries, with about 1.2 million cases every year. Tunisia lies in the Mediterranean region, where three complexes of *Leishmania* infections are known to affect humans: 1) sporadic CL due to *Leishmania infantum* occurs in the north and the center.¹ The transmission cycle of this parasite is zoonotic but not completely elucidated. 2) Zoonotic CL due to *Leishmania major* has been described since the nineteenth century as the “bouton de Gafsa” by Deperet and Boinet² and is largely distributed in the center and the south. 3) Chronic CL caused by *Leishmania tropica* MON-8 occurs in southeastern Tunisia.³ The transmission cycle of this pathogen seems to be a zoonotic disease involving *Ctenodactylus gundi* in its life cycle. However, the isolation of the parasite from this rodent is crucial for the confirmation of this first result.^{4,5}

Among the three clinical CL forms, infection by *L. tropica* is a serious health problem since the treatment is difficult, the ulcers mainly being located in the face, its persistence for up to a year, and the risk of causing leishmaniasis recidivans.⁶ In Tunisia, this form of CL was described for the first time in Ghomrasen, a rural locality in Tataouine province in the southeast, on the basis of 29 strains isolated in 1980.³ Its proven vector is *Phlebotomus sergenti*.^{7,8} The emergence of human CL due to *L. tropica* is an increasingly important public health problem as reported in many foci in Tunisia.

Sidi Bouzid lies in the center of Tunisia. In this region, CL is endemic and, when the etiologic agent was identified, *L. major* has been the proven zoonotic parasite.⁹ In this focus, parasites are transmitted to humans by the bite of the vector *Phlebotomus papatasi*.⁹ In 2005, some human cases due to *L. tropica* were reported in the area.¹⁰ However,

because of these reports, no studies have demonstrated the natural infection of sandflies with this pathogen in the same region.

This study was designed to determine and detect *Leishmania* DNA within sandflies collected in the region. Here, we report the confirmation of *L. major* DNA in *Ph. papatasi* and the first detection of *L. tropica* DNA in *Ph. sergenti*.

Sandfly sampling was carried out from June to August 2015 in Souk Jdid (average altitude 310 m; N35°52' E9°30'), which is part of Sidi Bouzid (Figure 1). Verbal informed consent was obtained from residents of the region. The collections were done using sticky paper traps inserted into small cracks in the walls of room, which served as a depot, and in domestic animal shelters. The collected sandflies were then placed in 1.5-mL Eppendorf tubes with 96% ethanol, transferred to the laboratory, and all the sandflies were washed and subdivided into males, and unfed and fed females. The head and the posterior part of the abdomen of a single sandfly are cut off with single-use sterile equipment in a drop of ethanol then cleared in boiling Marc-André solution. Then mounted between slide and cover slide for morphological identification by examination of the head and spermathecae (females) or head and genitalia (males) using a morphological identification key.^{11,12} The remainder of the female body was stored sterile in 1.5-mL microtubes at –20°C until genomic DNA was extracted by using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instruction.

For all female sandflies, the leishmanial ribosomal internal transcribed spacer 1 (ITS1) region was amplified using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'), following the protocol described by Schonian and others.¹³ This polymerase chain reaction (PCR) was used to amplify a 300- to 350-bp fragment; then the ITS1-PCR products were digested with restriction endonuclease *HaeIII* enzyme for *Leishmania* species identification.¹³ The restriction profiles were analyzed by electrophoresis on 3% agarose gels stained with 10% GelRed™ (Biotium, Hayward, USA), and compared with

* Address correspondence to Kaouter Jaouadi, Department of Medical Epidemiology, Institut Pasteur de Tunis, 13, Place Pasteur, BP 74 1002, Tunis-Belvédère, Tunisia. E-mail: kaouter.jaouadi@pasteur.tn

FIGURE 1. Map of Tunisia showing Sidi Bouzid region and the Souk Jdid area of survey. This figure appears in color at www.ajtmh.org.

Leishmania reference strains used as positives controls: *L. major* MON-25 MHOM/TN/2009/S600, *L. tropica* MON-8 MHOM/TN/2011/MX, and *L. infantum* MHOM/TN/80/IPT1. A 100-bp DNA size marker was used. Negative controls (containing water, without DNA) were added to each PCR run. Before sequencing, the PCR products were purified using the Exonuclease I/Shrimp Alkaline Phosphatase. Sequencing was conducted using BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3500 DNA automated sequencer (Applied Biosystems). BLAST analysis of GenBank was used to assess the level of similarity with previously reported sequences (<http://blast.ncbi.nlm.nih.gov/>).¹⁴ Sequences were aligned using the MEGA 6.1 software¹⁵ based on the Saitou and Nei (1987) distance method with bootstrap analysis of 1,000 reiterations. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with the algorithm of MEGA program. Sequences were compared with those retrieved from GenBank.

In total, 650 sandflies were captured and identified (380 males and 270 females). According to morphological identification, four species belonging to genus *Phlebotomus* and two species belonging to genus *Sergentomyia* were identified: 272 *Phlebotomus* (*Phl.*) *papatasi* (Scopoli, 1786), 52 *Ph. (Paraphlebotomus) sergenti* (Parrot, 1917), 32 *Phlebotomus* (*Larroussius*) *longicuspis* (Nitzulescu, 1930), 10 *Phlebotomus* (*Larroussius*) *perniciosus* (Newstead, 1911), 138 *Sergentomyia minuta* (Rondani, 1843), and 146 *Sergentomyia*

fallax (Parrot, 1921). In this study, males were not considered regarding that only females were hematophagous. Indeed, fed females were excluded from the analysis because the eventual presence of *Leishmania* DNA in these specimens can be an immediate consequence of blood feeding on an infected host.

Only 97 unfed females belonging to *Ph. papatasi* ($n = 40$), *Ph. sergenti* ($n = 27$), *Ph. perniciosus* ($N = 11$), *Ph. longicuspis* ($N = 9$), *Se. minuta* ($n = 6$), and *Se. fallax* ($n = 4$) were tested for the presence of *Leishmania* parasite DNA, which should be considered as the result of the multiplication of the parasites in the gut of the sandfly.

The ITS1-PCR showed 15 positive specimens with a unique band of approximately 350 bp. The 15 positive samples were subjected to restriction fragment length polymorphism for species identification. Nine *Ph. papatasi* were found positive for *L. major* DNA and six *Ph. sergenti* were found positive for *L. tropica* (Figure 2).

To confirm results, ITS1-PCR products obtained in the sandfly-positive samples were subjected to sequencing analysis. The ITS1 DNA sequences obtained showed high similarity with *L. major* and *L. tropica* ITS1 sequences in GenBank (Figure 3).

The detection of *L. major* in *Ph. papatasi* was not unexpected as this species has already been implicated as a vector of *L. major* in Tunisia.⁹ Results are in agreement with the epidemiology of this disease, being generally transmitted by *Ph. papatasi* all over its distribution area. In Tunisia,

FIGURE 2. Restriction fragment length polymorphism products of the amplified internal transcribed spacer 1 fragment using *HaeIII* enzyme. L: 100-bp size marker (Invitrogen®), Lm: *Leishmania major* MHOM/TN2009/S600 (two fragments of 132 and 206 bp), Lt: *Leishmania tropica* MON-8/MHOM/TN/2011/MX (three fragments of 188, 57, and 26 bp), Li: *Leishmania infantum* MHOM/TN/80/IPT1 (three fragments of 187, 72, and 55 bp). Positive samples: ser1, ser2, ser3, ser4, ser5, and ser6. This figure appears in color at www.ajtmh.org.

FIGURE 3. Phylogenetic analysis of ITS1 sequences from human and sandflies (Tree obtained by the method of neighbor-joining, *Leishmania braziliensis* as an out-group); ser1 to ser6: *Ph. sergenti* infested by *Leishmania tropica*; pap1–pap 9: *Phlebotomus papatasi* infested by *Leishmania major*; *L. tropica** was isolated from human from Tataouine and *L. major** was isolated from human from Sidi Bouzid; **: Nucleotide sequences from GenBank. This figure appears in color at www.ajtmh.org.

L. tropica was first described in Ghomrassen, a rural locality in Tataouine Province in the southeast, in 1980³ and more recently in an emerging CL focus in Metlaoui, a locality of Gafsa in the southwest of Tunisia.⁷

To date, it is believed that *Ph. sergenti* is a vector of *L. tropica* in these foci.^{7,8} However, there are no data in the literature dealing with its possible infection in an area known endemic for only *L. major*. By sequence analysis, ITS1 fragments from *L. tropica* DNA detected in *Ph. sergenti* were grouped into one haplotype and showed perfect correlation with *L. tropica* strains isolated in a human CL case (*L. tropica**) and in *Ph. sergenti* (JN104588) from Tataouine (Figure 3).

The study reports the detection of *L. tropica* in *Ph. sergenti* from an endemic area of *L. major*. Females carrying *L. tropica* may result from the increase in the circulation of this pathogen in central Tunisia. Our finding is supported by a report from Morocco, where studies document the coexistence of *L. major* and *L. tropica* in Sidi Kacem¹⁶ and the coexistence of *L. infantum* and *L. tropica* in Sefrou Province.¹⁷

Regarding some risk factors such as increased agriculture and environmental changes, it is apparent that Sidi Bouzid region has preconditions for a stable cycle of *L. tropica* by altering the distribution of the vector and the reservoir host. For example, more than 10,000 wells were constructed during the last 10 years in the center for agriculture.¹⁸ Wells were shown to be a resting and possibly a breeding site for sandfly. In addition, the development of irrigation for agriculture in central Tunisia may offer a new suitable ecological niche for sandfly species which can lead to the emergence of new foci.¹⁹

The rodent *Ctenodactylus gundi* has been reported to have a role in transmission of *L. tropica* in south Tunisia.^{4,5} However, further studies must be conducted and infected gundis should be confirmed in this region. More epidemiological investigations are needed to demonstrate the role of *L. tropica* circulating in this area in the transmission of CL.

We report *L. tropica* identified from *Ph. sergenti* outside its original focus in Tataouine. This novel finding enhances the understanding of the transmission cycle of *L. tropica* parasites in central Tunisia. Without further study, it is not clear whether the parasite will spread to other surrounding areas and what barriers exist to prevent *L. tropica* from extending within the country from this newly documented focal point in central Tunisia.

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Authors' addresses: Kaouther Jaouadi, Amira Bennour, Sadok Salem, Sana Chaabane, Rihab Yazidi, Department of Medical Epidemiology, Institut Pasteur de Tunis, Tunis, Tunisia, and Laboratory

of Transmission, Control and Immunobiology of Infections (LR11IPT02), Institut Pasteur de Tunis, Tunis, Tunisia, E-mails: kaouther.jaouadi@pasteur.tn, bennouramira@hotmail.fr, sadok-salem@live.fr, sana.chaabane@pasteur.tn, and rihabyazidi@yahoo.fr. Jihene Bettaieb, Department of Medical Epidemiology, Institut Pasteur de Tunis, Tunis, Tunisia, Laboratory of Transmission, Control and Immunobiology of Infections (LR11IPT02), Institut Pasteur de Tunis, Tunis, Tunisia, and Faculty of Medicine of Tunis, University Tunis El Manar, Tunis, Tunisia, E-mail: bettaiebjihene@yahoo.fr. Mohamed Ridha Rjeibi, Laboratoire de Parasitologie, École Nationale de Médecine Vétérinaire de Sidi Thabet, Sidi Thabet, Tunisia, E-mail: medridharjeibi@yahoo.fr. Neila Khabouchi, Pôle génomique-Plateforme technique Institut Pasteur de Tunis, Tunis, Tunisia, E-mail: neila.khabouchi@pasteur.tn. Adel Gharbi, Department of Medical Epidemiology, Institut Pasteur de Tunis, Tunis, Tunisia, E-mail: adel.gharbi@pasteur.tn. Afif Ben Salah, Department of Medical Epidemiology, Institut Pasteur de Tunis, Tunis, Tunisia, Laboratory of Transmission, Control and Immunobiology of Infections (LR11IPT02), Institut Pasteur de Tunis, Tunis, Tunisia, Faculty of Medicine of Tunis, University Tunis El Manar, Tunis, Tunisia, and Department of Family and Community Medicine, College of Medicine and Medical Sciences (CMMS), Arabian Gulf University (AGU), Manama, Bahrain, E-mail: afif.bensalah@pasteur.tn.

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