Natural Infection of *Phlebotomus sergenti* by *Leishmania tropica* in Libya

Mostafa Ramadhan Dokhan,^{1,2†} Kaouther Jaouadi,^{3,4**} Sadok Salem,^{3,4} Osama Zenbil,¹ Jean Paul Gonzalez,⁵
Afif Ben Salah,^{3,4,6,7} and Badreddin Bashir Annajar^{1,8}

¹Libyan National Centre for Disease Control (NCDC), Tripoli, Libya; ²Department of Zoology, Faculty of Science, University of Sabratha, Sabratha, Libya; ³Laboratory of Medical Epidemiology, Institut Pasteur de Tunis, Tunis, Tunisia; ⁴Laboratory of Transmission, Control and Immunobiology of Infections (LR11IPT02), Institut Pasteur de Tunis, Tunis, Tunisia; ⁵Center of Excellence for Emerging and Zoonotic Animal Disease, Kansas State University, Manhattan, Kansas; ⁶Faculty of Medicine of Tunis, University of Tunis El Manar, Tunis, Tunisia; ⁷Department of Family and Community Medicine, College of Medicine and Medical Sciences (CMMS), Arabian Gulf University (AGU), Manama, Bahrain; ⁸Public Health Department, Faculty of Medical Technology, University of Tripoli, Tripoli, Libya

Abstract. Cutaneous Leishmaniasis (CL) is a public health concern caused by *Leishmania* (*L.*) *major* and *L. tropica* in Libya. Information on sandfly vectors, as well as their associated *Leishmania* species, is of paramount importance because vector dispersion is one of the major factors responsible for pathogen dissemination. A number of 515 sandflies (275 males and 240 females) were collected during June–November 2012 using the Centers for Disease Control miniature light traps from Al Rabta, northwest of Libya. Two hundred and forty unfed females were identified; *Phlebotomus* (*Ph.*) *papatasi* (*N* = 97), *Ph. sergenti* (*N* = 27), *Ph. longicuspis* (*N* = 32), *Sergentomyia* (*Se.*) *minuta* (*N* = 38), and *Se. fallax* (*N* = 46). These flies were screened for *Leishmania* DNA using the polymerase chain reaction–restriction fragment length polymorphism analysis of the internal transcribed spacer 1 and sequencing. Two *Ph. sergenti* were found positive to *L. tropica* DNA. This finding should be considered for any further vector surveillance and epidemiological studies of CL in endemic areas across Libya.

INTRODUCTION

Cutaneous Leishmaniasis (CL) is one of the world's most neglected diseases generally affecting developing countries. In North Africa, CL incidence has been increasing since 1980s with an expansion in its geographical distribution.¹

In Libya, the estimated total number of cases between 1971 and 2013 was more than 60,000 with an estimated 20,000 cases that occurred from 2004 to 2009 only.² In Libya, both types of Leishmaniasis are known to affect humans: zoonotic CL due to *Leishmania* (*L.*) *major*³ and anthroponotic CL caused by *L. tropica*.⁴ Among the two clinical CL forms, infection by *L. tropica* is a serious health problem because the treatment is difficult, and parasite persistence for up to a year, and finally, the possible risk of leishmaniasis recidivans.^{5,6} Cutaneous leishmaniasis ulcers occur in several regions of the body; however, it is more common in the face which is in exposure to the sandfly bites. In Libya, *L. tropica* was described for the first time in the district of Misrata (northeast of Libya) in 2006.⁴ In 2012, Amro et al.⁷ reported the first molecular typing of the *L. tropica* identified from Murqub, Al Jabal Al Gharbi, and in addition in Nalut districts.⁸ To date, it is believed that *Phlebotomus* (*Ph.*) *sergenti* is the vector of *L. tropica* in Tunisia,⁹ Algeria,¹⁰ Morocco,¹¹ and Egypt.¹² However, no studies have been carried out to demonstrate the natural infection of sandflies with *L. tropica* in Libya. The objective of the study includes sandfly vector surveillance and identifying the natural infections of *Leishmania* parasites in these flies for vector incrimination in Al Rabta district.

MATERIALS AND METHODS

The study was carried out in two villages in Al Rabta area: Al Rabta East (RE) and Al Rabta West (RW) (Figure 1). Al Rabta

(32°9'46.59" N, 12°50'50.65" E) is a rural area in the foothills of the Nafusa Mountains. It has an altitude of about 300 m above the sea level. The area is characterized by a warm and dry climate with an average annual rainfall of 16 mm. The mean annual temperature of the area is 21°C. The main components of local economy of the study area are agriculture and pastoralism.

Sampling of sandflies was carried out in four outdoor resting sites (T1 and T2 in RW and T3 and T4 in RE). Specimens were collected from outside of caves and abandoned old settlements during June–November, 2012, using the CDC miniature light traps (Model 512; John W. Hock Co., Gainesville, FL). The traps were set before sunset and collected after sunrise next morning. The collected sandflies were then placed in 1.5-mL Eppendorf tubes with 70% ethanol. All the sandflies were washed and subdivided into males and unfed 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 and then cleared in boiling Marc-André solution. All flies were mounted for species identification via local morphological keys.¹³ These keys examined the morphological characters of head and spermathecae in females or head and genitalia in males. The remaining parts of the female body were stored in 1.5-mL sterile microtubes at –20°C until genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

The internal transcribed spacer 1 (ITS1) region was amplified in positive flies using the primers LITSR (5'-CTGGATCATTT TCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'), following the protocol described by Schönian et al.¹⁴ This polymerase chain reaction (PCR) was used to amplify a 300–350-base pair (bp) fragment of the gene along with a 100-bp DNA size marker. The MHOM/TN/80/IPT1 *Leishmania* reference strain was used as positive control. Negative control (i.e., PCR mix without DNA) was added to each PCR run.

Then, the ITS1-PCR products were digested with restriction endonuclease *HaeIII* enzyme for *Leishmania* species identification.¹⁴ The restriction profiles were analyzed by electrophoresis

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

† These authors contributed equally to this work.

FIGURE 1. Satellite maps showing the sandfly sampling sites in Al Rabta East (RW) and Al Rabta West (RE) villages in the northwestern region of Libya. This figure appears in color at www.ajtmh.org.

on 3% agarose gels stained with 10% GelRed™ (Biotium, Fremont, CA) and compared with *Leishmania* reference strains used as positives controls: *L. major* MON-25 MHOM/TN2009/S600, *L. tropica* MON-8 MHOM/TN/2011/MX, and *L. infantum* MHOM/TN/80/IPT1. A 100-bp DNA size marker was used.

Before sequencing, the amplicon products were purified using exonuclease I/shrimp alkaline phosphatase. Sequencing was conducted using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3500 DNA-automated sequencer (Applied Biosystems). Basic Local Alignment Tool analysis of GenBank was used to assess the level of similarity with previously reported sequences (<http://blast.ncbi.nlm.nih.gov/>).¹⁵ A phylogenetic tree was constructed using the neighbor-joining algorithm available via the MEGA software (v. 6.0).¹⁶

RESULTS

A total of 515 sandflies were captured in this study (275 males and 240 females). Three species belonging to the genus

Phlebotomus and two species belonging to the genus *Sergentomyia* (Se.) were identified: 97 *Ph. papatasi* (Scopoli, 1786), 27 *Ph. (Paraphlebotomus) sergenti* (Parrot, 1917), 32 *Ph. (Larroussius) longicuspis* (Nitzulescu, 1930), 38 *Se. minuta* (Rondani, 1843), and 46 *Se. fallax* (Parrot, 1921) (Table 1). Males were not considered regarding that only females were hematophagous.

All of the 240 females were tested for the presence of *Leishmania* parasite DNA.

The ITS1-PCR showed two positive specimens with a unique band (Figure 2A). The two positive samples were subjected to restriction fragment length polymorphism analysis for species identification and were found positive to *L. tropica* (Figure 2B). To confirm results, ITS1-PCR products obtained in the sandfly-positive samples were subjected to sequencing analysis.

Regarding the ITS1 gene, an amplicon product sequence of 250 bp was used for phylogenetic analysis. All sequenced ITS1 gene amplicons, including the Libyan strain of *L. tropica* (accession KP691595) and the Tunisian strain of *L. tropica* isolated from *Ph. sergenti* (GenBank accession JN104588), were grouped into a unique clade of *L. tropica* haplotype (Figure 3).

TABLE 1
Number of M and F sandflies collected in RE and RW villages in the northwest of Libya

		<i>Ph. papatasi</i>		<i>Ph. sergenti</i>		<i>Ph. longicuspis</i>		<i>Se. minuta</i>		<i>Se. fallax</i>	
		M	F	M	F	M	F	M	F	M	F
RW	T1	34	27	17	08	04	08	14	10	14	21
	T2	31	29	13	07	07	10	15	11	05	03
RE	T3	29	22	12	06	04	09	16	13	10	18
	T4	24	19	07	06	05	05	08	04	06	04
Total		118	97	49	27	20	32	53	38	35	46

F = females; M = males; *Ph.* = *Phlebotomus*; RE = Al Rabta East; RW = Al Rabta West; and *Se.* = *Sergentomyia*.

FIGURE 2. (A) Polymerase chain reaction (PCR) amplification of *Leishmania* (*L.*) DNA using internal transcribed spacer 1 (ITS1) from sandfly females. MW = Molecular Weight marker 100-bp (Invitrogen®, Carlsbad, CA), Lane 1 = negative control (PCR mix without DNA), Lane 2 = *L. major* MHOM/TN2009/S600, Lane 3 = *L. tropica* MON-8 /MHOM/TN/2011/MX, Lane 4 = *L. infantum* MHOM/TN/80/IPT1, Lane 5-6 = *Leishmania* ITS1 sequence amplified from *Ph. sergenti*. (B) Restriction Fragment Length Polymorphism products of the amplified ITS 1 fragment using HaeIII enzyme. MW = 100-bp size marker (Invitrogen®, Carlsbad, CA), Lane 1 = *L. major* MHOM/TN2009/S600 (two fragments of 132 and 206 bp), Lane 2 = *L. tropica* MON-8/MHOM/TN/2011/MX (three fragments of 188, 57, and 26 bp), Lane 3 = *L. infantum* MHOM/TN/80/IPT1 (three fragments of 187, 72, and 55 bp). Lanes 4-5 = Positive samples: *L. tropica* 1*, *L. tropica* 2*. This figure appears in color at www.ajtmh.org.

DISCUSSION

Al Rabta is identified as an endemic foci where CL is clinically documented from 1977 to 1980 and from 2004 to 2012.² In spite of this situation, the area was surveyed for sandflies only by Annajar¹⁷ and Dokhan et al.²

Five sandfly species (three *Phlebotomus* and two *Sergentomyia*) were identified in the present study. This presents a species diversity lower than that described by Dokhan et al.,² in the same foci where nine species were reported. The most likely factor that may explain this discrepancy is the sample size because these authors have identified 5,605 specimens.

In Libya, *L. major* infecting *Ph. papatasi*, is in agreement with the epidemiology of the disease. To our knowledge, this is the first evidence of potential risk of infection by *Ph. sergenti* in Libya.¹⁸ Indeed, from the literature, only *Ph. papatasi* was identified as a potential vector of *Leishmania* in Libya.^{17,19}

Also, the ITS1-PCR showed that the two positive *Ph. sergenti* of our study presented a unique characteristic banding pattern of the genus *Leishmania*. In the present study, *Ph. sergenti* specimens harboring *L. tropica* DNA have been caught in natural biotopes of Al Rabta and confirmed by ITS1-PCR amplicon sequencing analysis. Jaouadi et al.⁹ identified *Ph. sergenti* infected by *L. tropica* in the same mountain biotope of Metlaoui (southwestern Tunisia).⁹ More recently, the same result has been reported in Sidi Bouzid, a classical focus of *L. major* in central Tunisia.²⁰ The high infection rate among sandflies (2/27)

FIGURE 3. Phylogenetic analysis of internal transcribed spacer 1 sequences from sandflies (tree obtained by the neighbor-joining method, *Leishmania* (*L.*) *braziliensis* chosen as an out-group); *L. tropica* 1* and *L. tropica* 2* were isolated from *Phlebotomus* (*Ph.*) *sergenti* sampled in this study; and **: nucleotide sequences from GenBank.

may come from the higher infection rates among human cases. Similar findings of sandfly infections were reported in previous regional studies in Tunisia^{9,20} and Algeria.¹⁰

The identification of the natural hosts of *Leishmania* species is essential to understand the epidemiology of the disease. In North Africa, several rodent species have been implicated in the transmission of *L. tropica*.^{21,22} *Ctenodactylus gundi* has been proposed as a reservoir host in Tunisia,²³ whereas in Algeria, *L. tropica* is considered a zoonotic form with *Mas-soutiera mzabi* as a reservoir.¹⁰ In Egypt, *Gerbillus pyramidum floweri* has been infected with *L. tropica*.¹² In Libya, parasite isolation has been reported, yet it needs to be documented for a better understanding of the epidemiology of CL in Libya.

Since February 2011, the ongoing armed conflict in Libya has largely impacted the health-care system and disrupted the national and international control programs of CL. In addition, climate change scenarios could eventually affect sandfly vectors distribution and CL dispersion in Libya.¹⁸ Herein, the first natural infection of *Ph. sergenti* by *L. tropica* is reported. This novel finding enhances the understanding of the *L. tropica* transmission cycle in Libya. Altogether and 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 further expansion from Al Rabta districts to other new foci across the country.

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Authors' addresses: Mostafa Ramadhan Dokhan, Libyan National Centre for Disease Control (NCDC), Tripoli, Libya, and Department of Zoology, Faculty of Science, University of Sabratha, Sabratha, Libya, E-mail: mustsun@yahoo.com. Kaouther Jaouadi and Sadok Salem, Laboratory 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 and sadok-salem@live.fr. Osama Zenbil, Libyan National Centre for Disease Control (NCDC), Tripoli, Libya, E-mail: oys2016.ly@gmail.com. Jean Paul Gonzalez, Center of Excellence for Emerging and Zoonotic Animal Disease, Kansas State University, Manhattan, KS, E-mail: jpgonzalez2808@gmail.com. Afif Ben Salah, Laboratory 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. Badreddin Bashir Annajar, Libyan National Centre for Disease Control (NCDC), Tripoli, Libya, and Public Health Department, Faculty of Medical Technology, University of Tripoli, Tripoli, Libya, E-mail: bbannajar@yahoo.com.

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