Blood Meal Analysis of Phlebotomine Sandflies (Diptera: Psychodidae: Phlebotominae) for *Leishmania* spp. Identification and Vertebrate Blood Origin, Central Tunisia, 2015–2016

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Abstract. During the time periods of June 2015 and from July to August 2016, sandflies were collected among seven collection sites of the three leishmaniasis endemic villages of Sidi Bouzid, Tunisia. A total of 690 sandflies were captured and identified (380 males and 310 females). Four species belonging to genus *Phlebotomus* (*Ph.*) and two species belonging to genus *Sergentomyia* were identified. *Leishmania* DNA was detected in four out of 310 females (one *Ph. sergenti* and three *Ph. papatasi*). The overall sensitivity of the *Prepronociceptin* gene detection reached 76%. The concurrent presence of *Ph. papatasi* and *Ph. sergenti* vectors, the analysis of blood-meals, together with the detection of *L. major* in *Ph. papatasi*, confirms the ultimate conditions for the transmission of the disease in center Tunisia. These results expand the known epidemiological area of distribution of leishmaniasis and its vectors in this part of Tunisia, highlighting the need for ongoing entomological and parasitological surveillance.

INTRODUCTION

Leishmaniasis is endemic throughout the Mediterranean Basin. In Tunisia, three complexes of *Leishmania* (*L.*) infections are known to affect man: *L. infantum*, *L. major*, and *L. tropica* MON-8. Transmission occurs when a female sandfly acquires infection during feeding on an infected host.¹ Efforts to identify reservoir hosts for vector-borne zoonotic pathogens have historically been labor-intensive exercises, repeatedly requiring the capture of potential wildlife hosts.² Because of this complexity, it can be difficult to identify all the mammalian reservoir hosts involved. Therefore, an efficient solution has emerged in the form of host blood-meal identification. By doing so, it is not only possible to provide significant information about many still unknown reservoir host species but also to obtain information about the host feeding pattern of each vector species, thus opening the possibility to identify new mammalian reservoirs or cryptic reservoirs present in its vicinity and the dynamics of their interaction.

This study was carried out in Sidi Bouzid that lies in the center of Tunisia where zoonotic cutaneous leishmaniasis is endemic and, *L. major*, was identified and proven to be a zoonotic parasite and aims to 1) provide information on the phlebotomine sandflies species trophic preferences and 2) the prevalence of *Leishmania* infection using molecular techniques.

MATERIALS AND METHODS

The sandflies were collected in three neighboring areas in the province of Sidi Bouzid governorate, central Tunisia (Figure 1A). Sandfly collections were carried out in June 2015 and from July to August 2016 (Table 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 rooms which served as a depot, as well as in back yard and in animal shelters.

DNA was extracted from engorged individual sandflies using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The DNA was eluted in a final volume of 200 µL of Acetate-EDTA buffer.

For all sandfly females, 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.³ Sequences of primers used for the detection of host DNA are based on specific amplification and sequencing of the blood-meal-derived single copy *Prepronociceptin* (*PNOC*) gene using the primers *PNOC-F*: (5'-GCA TCC TTG AGT GTG AAG AGA A-3') and *PNOC-R*: (5'-TGC TCA TAA ACT CAC TGA ACC-3').^{4,5} Two negative controls (containing water, without DNA or DNA of male sandfly (which do not feed on blood) were added to each polymerase chain reaction (PCR) run to detect possible contaminations of both the extraction and amplification steps.

Sequences for *PNOC* gene identified in this study have been deposited in GenBank under the following accession numbers: KY713603–KY713604 (*Homo sapiens*), KY827042 (*Rattus norvegicus*), KY827040–KY827041 (*Mus musculus*), KY827043–KY827044 (*Bos taurus*), KY827045–KY827046 (*Oryctolagus cuniculus*), KY827047–KY827048 (*Ovis aries*), and KY827049–KY827050 (*Capra hircus*).

RESULTS

In total, 690 sandflies were captured and identified (380 males and 310 females). According to morphological identification, four species belonging to genus *Phlebotomus* (*Ph.*) and two species belonging to genus *Sergentomyia* (*Se.*) were identified: 575 *Ph. papatasi*.

Twenty *Ph. (Paraphlebotomus) sergenti*, 10 *Ph. (Larrousius) longicuspis*, 32 *Ph. (Larrousius) perniciosus*, 28 *Se. minuta*, and 25 *Se. fallax* (Table 1).

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FIGURE 1. (A) The geographic source of isolates. Study sites, Sidi Bouzid Governorate, Tunisia. Caption: Red circle = Sites a, b and c located in Souk Jedid; green triangle = Sites d and e of West Sidi Bouzid; yellow square = Sites f and g of East Sidi Bouzid. (B) Maximum likelihood Phylogenetic tree (midpoint rooted) of *Leishmania* (*L.*) ITS1 sequences, including that amplified from *Phlebotomus* (*Ph.*) *papatasi* and *Ph. sergenti* collected in Sidi Bouzid Tunisia. (*L. braziliensis* as an out-group); *L. tropica* GU015 was from human originated of Tataouine and *L. major* S028: was from human originated of Sidi Bouzid; **: Nucleotide sequences from GenBank. This figure appears in color at www.ajtmh.org.

All of the 310 females were tested for the presence of *Leishmania* parasite DNA. The ITS1-PCR showed four positives specimens with a unique band of approximately 350 bp (Figure 2A). The four positive samples were subjected to sequencing analysis for species identification. Three engorged *Ph. papatasi* were found positive for *L. major* DNA and one unfed *Ph. sergenti* was found positive for *L. tropica*. The ITS1 DNA sequences obtained showed high similarity with *L. major* and *L. tropica* ITS1 sequences in GenBank (Figure 1B).

DNA was extracted from 129 individual blood-fed females belonging to four sandflies species (93 *Ph. papatasi*, 15 *Ph. perniciosus*, 14 *Ph. sergenti*, and 7 *Ph. longicuspis*) and used as a template in the mammal-specific *PNOC* gene PCR amplification. A 330 pb PCR product was detected (Figure 2B). Overall, 76% of the blood-fed specimens showed positive results in the blood-meal PCR assay. All positive PCR products amplifying the *PNOC* gene were sequenced. Edited sequences were compared with the GenBank database and were successfully identified to species level. Alignment of these sequences revealed their sequence heterogeneity (Figure 2C). Among the identified blood-meal samples, the majority were from humans (*Homo sapiens*, 42.85%), followed by rodents (*Mus musculus*, 13.26% and *Rattus norvegicus*, 5.1%), cattle (*Bos taurus*, 15.3%), rabbits (*Oryctolagus cuniculus*, 11.22%), sheep (*Ovis aries*, 9.18%), and goats (*Capra hircus*, 3.06%).

No superposed fluorograms were detected to suggest a mixed blood-meal.

DISCUSSION

Six sandfly species were observed in the region under study. *Ph. papatasi* was the predominant species (83, 33%), thus providing evidence in favor of its role as a vector in Tunisia. Even more interestingly is that a single of *Ph. sergenti* was found infected with *L. tropica*. Sequence analysis of ITS1 fragments from *L. major* DNA detected in *Ph. papatasi* were grouped into one haplotype and showed perfect correlation with *L. major* strains isolated in a human cutaneous leishmaniasis (CL) case (*L. major* S028). As well, sequence analysis of ITS1 fragments from.

L. tropica DNA detected in *Ph. sergenti* was grouped into one haplotype and showed perfect correlation with *L. tropica* strains isolated in a human CL case (*L. tropica* GU015) and in *Ph. sergenti* (JN104588) from Tataouine (Figure 1B).

Our finding showed that the rate of *Leishmania* infection is higher in engorged compared with unfed females, maybe because the eventual presence of cutaneous leishmaniasis (CL) DNA in these specimens can be an immediate consequence of blood feeding on an infected host.

To the best of our knowledge, this is the first study that investigated the source of the blood-meal of sandflies in central Tunisia. Seven vertebrates were identified that provide blood-meal sources for four sandfly species. The vector *Ph. papatasi* transmitting *L. major* feeds on humans, rodents, cattle, sheep, and goats, with a trophic preference for humans and rodents.

The source of blood obtained in the three engorged *Ph. papatasi* infected with *L. major* is from humans which are not

TABLE 1
Geographical and ecological characteristics of sites where blood-fed sandflies were collected in Sidi Bouzid governorate

Geographic and Ecological Characteristics of Sites Where <i>Ph. papatasi</i> and <i>Ph. longicuspis</i> were Collected in the Bouzid Governorate										
Site code	Village	Geographic coordinate	Collection date	Prevalent animal species on site	Trap site	Species captured	Males	Female		Total
								Unfed	Fed	
Site A	Souk Jedid	34°49'20.76", 9°33'39.97"	June 15, 2015	Sheep, rabbit, cow	Indoor, goat stable	<i>Ph. papatasi</i>	128	37	40	205
Site B	Souk Jedid	34°49'10.54", 9°33'6.57"	June 16, 2015	Cow	Garden house, cow stable	<i>Ph. sergenti</i>	1	1	8	10
Site C	Souk Jedid	34°49'32.79", 9°33'43.22"	July 6, 2016	Sheep, goat	Garden house, inside animal shelter	<i>Ph. longicuspis</i>	1	1	3	5
Site D	S. Bouzid west	35°3'42.79", 9°22'52.24"	July 13, 2016	Cow	Indoor, cow stable	<i>Ph. perniciosus</i>	6	2	7	15
						<i>Se. minuta</i>	6	4	0	10
						<i>Se. fallax</i>	4	3	0	7
						<i>Ph. papatasi</i>	80	29	31	140
Site E	S. Bouzid west	35°2'58.05", 9°22'3.57"	August 16, 2016	Chicken, sheep	Inside animal shelter	<i>Ph. sergenti</i>	2	0	2	4
Site F	S. Bouzid east	35°0'10.13", 9°30'34.87"	August 22, 2016	Chicken, dog	Indoor, inside animal shelter	<i>Ph. longicuspis</i>	0	0	2	2
						<i>Ph. perniciosus</i>	3	0	5	8
						<i>Se. minuta</i>	5	3	0	8
						<i>Se. fallax</i>	4	3	0	7
						<i>Ph. papatasi</i>	120	88	22	230
Site G	S. Bouzid east	35°1'42.18", 9°32'12.96"	September 12, 2016	Rabbit, sheep, goat, dog	Inside animal shelter	<i>Ph. sergenti</i>	1	1	4	6
Site H	S. Bouzid east	35°1'42.18", 9°32'12.96"	September 12, 2016	Rabbit, sheep, goat, dog	Inside animal shelter	<i>Ph. longicuspis</i>	1	0	2	3
						<i>Ph. perniciosus</i>	5	1	3	9
						<i>Se. minuta</i>	6	4	0	10
						<i>Se. fallax</i>	7	4	0	11
						Total	380	181	129	690

L. = *Leishmania*; Ph. = *Phlebotomus*; Se. = *Sergentomyia*.

reservoirs. It seems that the infection was obtained from a blood meal on an infected host. Our result supports the notion that *Ph. papatasi* is an opportunistic feeder and its host preference depends on the availability and abundance of the blood sources.⁶ A previous study reported in Iran that combined blood-meals of human-dogs and human-cattle were observed in *Ph. papatasi*.^{7,8} The same species in Sanliurfa (Turkey) feed on domestic animals and wild rodents.⁹ Thus, *Ph. papatasi* sandflies may be adapted to feed on available vertebrates hosts and may take eventually multiple

blood-meals on different host while disturbed or challenged with less accessible capillary veins.^{10,11}

Interestingly, we highlighted for the first time the blood-source preferences of *Ph. sergenti* in central Tunisia, that appears to feed on human and rabbit. In Morocco, *L. tropica* was isolated from dogs in Azilal, a southern CL focus.^{2,12} No *Ph. sergenti* were found to feed on dog in our study. In Tunisia, *Ctenodactylus (C.) gundi* was suggested as a potential reservoir host of *L. tropica*.¹³ However, no *C. gundi* blood was detected in our specimen tested, suggesting that *Ph. sergenti*

FIGURE 2. (A) Polymerase chain reaction (PCR) amplification of *Leishmania (L.)* DNA using ITS1 from sandfly females. MW: Molecular Weight marker 100-bp (Invitrogen®, Carlsbad, CA), Lane 1: *L. major* MHOM/TN2009/S600, Lane 2: *L. tropica* MON-8/MHOM/TN/2011/MX, Lane 3: *L. infantum* MHOM/TN/80/IPT1, Lane 4: negative control (distilled H₂O), Lane 5: ITS1 sequence amplified from *Ph. sergenti*, Lanes 6–8 *L. ITS1* sequences amplified from *Ph. papatasi*. (B) Electrophoresis gel of *Prepronociceptin (PNOC)* gene PCR amplification (333 bp). MW: Molecular Weight marker 100-bp (Invitrogen®), Lane 1 and Lane 20: negative controls (DNA of male sandfly which do not feed on blood), Lane 2 and Lane 26: positive control (Rabbit genomic DNA), Lane 3–Lane 19 and Lane 21–Lane 25: positive samples. (C) *PNOC* gene sequences alignment from engorged sandflies with Tunisian haplotype. Using GENTle software (v.1.9.4). This figure appears in color at www.ajtmh.org.

the main blood sources for this opportunistic sandfly species feed on other more abundant vertebrates (human and rabbits).

The blood-meal analysis of the engorged *Ph. perniciosus* revealed that this species fed on cattle and sheep; however, nothing is known about the susceptibility of *Bos taurus* and *Ovis aries* to leishmanial infection in Tunisia. Even more, three *Ph. perniciosus* were found to feed on *Oryctolagus cuniculus* addressing the question of the wild rabbits as a potential host for *Leishmania* in Sidi Bouzid., a hypothesis sustained by Benito-De Martín and others.¹⁴ A study reporting that rabbits are highly attractive blood-source for *Ph. perniciosus* and could contribute to maintain high population levels of sandflies.¹⁵

Dogs have been clearly defined as a proven reservoir of *L. infantum* in Tunisia.^{16,17} Interestingly, no dog blood was detected in blood-fed *Ph. perniciosus*. No blood-fed female *Sergentomyia* was identified in the present study, also it is not clear if *Sergentomyia* transmit or not of *Leishmania* parasites.¹⁸

The methodology based on *PNOC* gene amplification constitute an important tool for identifying the blood-meals sources of female sandflies in endemic areas of CL, although in our hand the overall sensitivity of the *PNOC* gene detection reached 76%, Baum and others¹⁹ and de Paternina and others,²⁰ respectively, reported 29% and 7% of amplified DNA host, showing such discrepancies more likely associated to the digestion stages of studied specimen.⁹

Although, to the best of our knowledge, this is the first study on sandfly's blood-meal sources in central Tunisia; therefore, from our finding, the role of cattle, rabbits, sheep, and goats in the epidemiology of CL requires a more detailed and systematic investigation for better understanding the zoonotic transmission of sandflies borne pathogens.

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