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Entomological Survey for Sand Fly Fauna in Imamoglu Province (Cutaneous Leishmaniasis Endemic Region) of Adana, Turkey

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ABSTRACT Leishmaniasis, presenting in two clinical forms, cutaneous and visceral in Turkey, is widespread in most of the countries in the Mediterranean Basin. An average of 10 to 13% of cases are reported from Adana every year. This paper presents the results of an entomological survey in an endemic focus of cutaneous leishmaniasis in Imamoglu province of Adana in Turkey.

We collected 654 sand fly specimen using 100 light traps in 20 nights in August 2013 and July 2014. Several keys and previous drawings were used in the identification of the species. In total, six *Phlebotomus* species were identified; *Phlebotomus tobbi* (50.3%), *Phlebotomus papatasi* (34%), *Phlebotomus perfiliewi* (2.9%), *Phlebotomus sergenti* (0.4%), *Phlebotomus neglectus/syriacus* (0.8%), and *Sergentomyia* spp. (11.5%). In addition, the female/male rate was found to be 0.84.

Collected sand flies (44 pools of 1–10 individuals) were analyzed microscopically, and no promastigotes were found in the midgut specimens. Using a genus specific real time-polymerase chain reaction (PCR) assay for simultaneous detection of Old Word *Leishmania.* We detected only 3 of the 44 pools with *Leishmania* by genus-specific real-time PCR assay.

P. tobbi was found to be dominant species in spite of the differences in sand fly fauna composition. To the best of our knowledge, this is the first study for sand fly fauna including 15 villages where endemic focus of cutaneous leishmaniasis in Imamoglu, Adana Province, Turkey.

KEY WORDS psychodidae, diptera, sand flies

Phlebotomine sand flies are the arthropod vectors of the parasites that transmit *Leishmania* parasites in Old World. There are two clinical forms of leishmaniasis: visceral leishmaniasis or kala-azar, the most severe and fatal in almost all cases if left untreated, and cutaneous leishmaniasis (CL) which has a tendency towards spontaneous resolution (Yaman and Ozbel 2004). The previous studies carried out on sand fly fauna in leishmaniasis endemic areas showed that either one or both of the two forms of the disease epidemiology were related to sand fly fauna in 40 endemic areas in Turkey (Kavur 2011). Twenty-six species or subspecies raised to species level belonging to the *Phlebotomus* and *Sergentomyia* genera have recently been described within Anatolia with 16 of these species being proven or probable vectors of human leishmaniasis in the Old World (Houin et al. 1971, Alptekin et al. 1999, Volf et al. 2002, Sadlova et al. 2003, Yaman and Ozbel 2004, Çiçek et al. 2005, Deger and Yaman 2005, Dogan et al. 2005, Ertabaklar et al. 2005, Toprak and Ozer 2005, Davies 2006, Yaman and Dik 2006, Simsek et al. 2007, Toprak and Ozer 2007, Svobodová et al. 2009, Tok 2009, Erişöz 2010, Ermiş 2010, World Health Organization [WHO] 2010).

Leishmania infantum is responsible for both clinical forms occurred many areas of Turkey while *Leishmania tropica* causes CL especially southeastern and western parts of Turkey. *Phlebotomus tobbi* is the proven vector of CL caused by *L. infantum* in East Mediterranean parts of Turkey. *Phlebotomus sergenti* and *Phlebotomus similis* are the probable vectors of *L. tropica* in southeastern and western parts of Turkey, respectively (Depaquit et al. 1998, Alptekin 1999, Depaquit et al. 2002, Svobodová et al. 2009, WHO 2010). Between 2010 and 2012, 682 cases were recorded in Adana, 116 of whom were detected in Imamoglu province by the Ministry of Health (Ministery of Health Data System 2012).

Thus, in the present study, we aimed to determine sand fly fauna in fifteen villages to detect potential vector species of *Leishmana* with the conventional and molecular detection of *Leishmania* parasites in the sand flies collected in Imamoglu, Adana Province.

Materials and Methods

Study Area. The present study was carried out in the villages of Imamoglu, Adana Province, where CL cases had been reported over the past 3 yr

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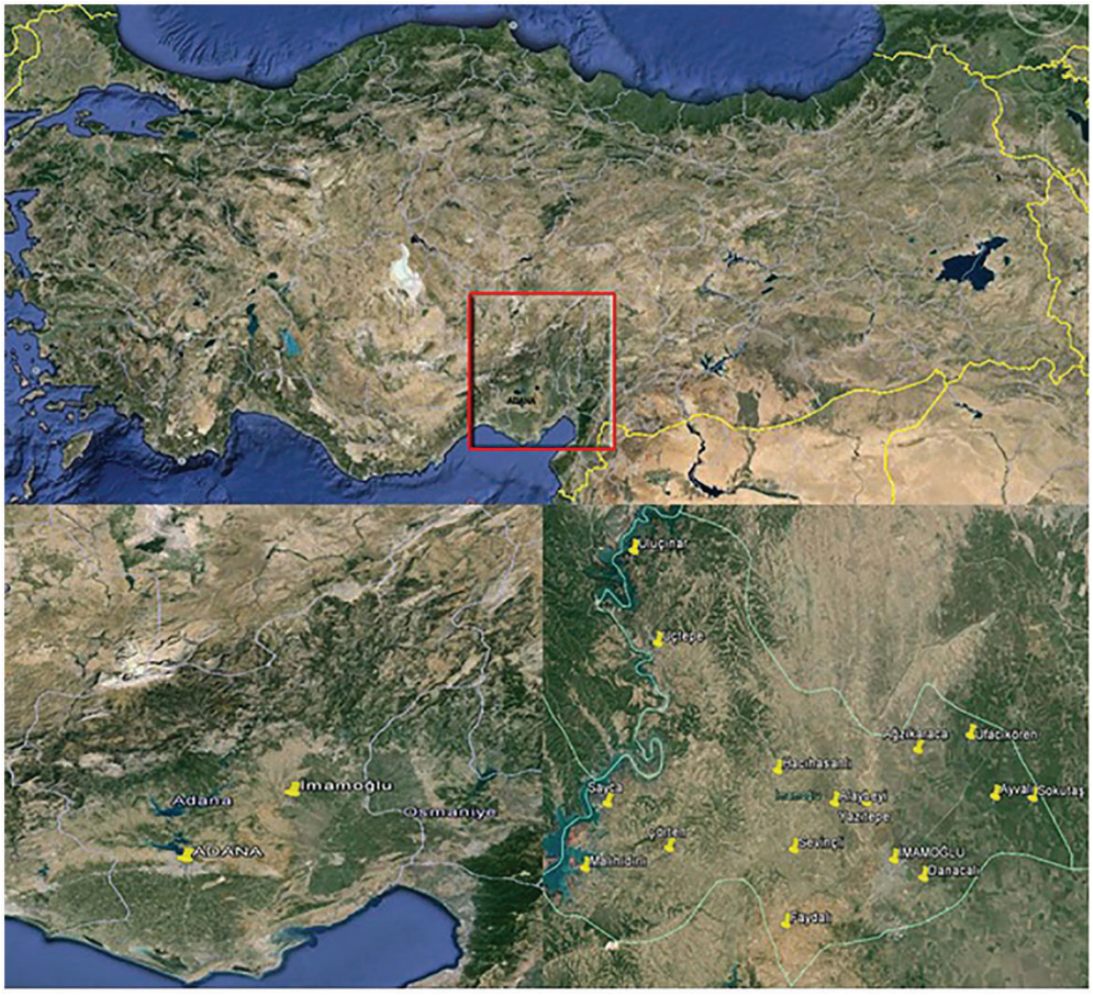


Fig. 1. Study area.

(Agzikaraca, Alaybeyi, Ayvali, Corten, Danacili, Faydali, Hacihasanli, Malihidirli, Sayca, Sevincli, Sokutas, Ufacikoren, Uctepe, Ulucinar, and Yazitepe). Adana basin is 14,032 km^2 in area, and it is located in the East Mediterranean district of Turkey (altitude 30 m., latitude 37°, 00′, 00.00″ N, longitude 35°, 19′, 16.80″ E). Imamoglu basin is 424 km^2 in area, and located in the south-east part of Adana (altitude 78 m., latitude 37°, 15′, 33.94″ N, longitude 35°, 39′, 41.34″ E; Fig. 1).

Sand Fly Collections and Identification. The field work was carried out in 100 stations using CDC light traps (Fig. 2). The first samples were collected from 50 stations between 24 August 2013 and 04 September 2013 and the second samples from the same stations between 28 June 2014 and 8 July 2014. In each area, during the collection period, traps were placed in under or close to animal (goat, cattle, sheep, or poultry) sheds. The animal sheds and nearby houses are usually made of brick, mud, wood, or stone. The midguts of 52 females were searched for live promastigotes in the field.

The obtained specimens were stored in 96% alcohol for morphological identification. Identifications were based on the morphology of male genitalia and female spermatheca and pharynges using the several keys (Theodor 1958, Lewis 1982, Killick-Kendrick et al. 1990). After identification, the sand flies caught with CDC light traps were removed to 70% ethanol for DNA isolation.

Extraction of DNA From Sand Flies. DNA extraction was performed using the QIAampDNA mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions for 44 pools of 1–10 individuals.

Genus-specific Real-time Polymerase Chain Reaction (PCR) assay. Forty-four sand fly midgut pool samples were analysed using the genus-specific real-time PCR assay. Genus-specific real-time PCR primers were designed on the basis of the alignment of the *Leishmania* minicircle kinetoplast (kDNA) gene region. The target gene kDNA was observed in copy numbers ranging from 10,000 to 20,000 in the *Leishmania* species.



Fig. 2. CDC light traps.

Table 1. Study area and geographical data

No	Villages	Coordinates	Altitude (m)	Temperature	Humidity
1	Agzikaraca	37° 19′23.68″ S 35° 40′52.89″ E	78	35.5	58.7
2	Alaybeyi	37° 17′51.69″ S 35° 36′43.08″ E	122	32.7	42.4
3	Ayvali	37° 17′35.16″ S 35° 44′11.72″ E	55	34.2	58.7
4	Corten	37° 15′40.61″ S 35° 28′59.71″ E	168	32.9	53.2
5	Danacili	37° 15′04.65″ S 35° 41′17.30″ E	67	35.1	62.2
6	Faydali	37° 13′30.35″ S 35° 34′47.18″ E	211	33.42	58.58
7	Hacihasanli	37° 18′20.16″ S 35° 34′27.96″ E	182	34.20	50.40
8	Malihidirli	37° 15′15.96″ S 35° 26′02.22″ E	177	31.58	59.58
9	Sayca	37° 17′09.31″ S 35° 26′56.96″ E	142	32.58	51.50
10	Sevincli	37° 15′52.45″ S 35° 35′17.67″ E	147	34.00	49.08
11	Sokutas	37° 17′34.60″ S 35° 45′48.71″ E	53	37.08	64.08
12	Ufacikoren	37° 19′43.92″ S 35° 43′30.42″ E	69	34.19	59.56
13	Uctepe	37° 22′26.40″ S 35° 29′09.71″ E	192	30.56	45.00
14	Ulucinar	37°25′21.14″ S 35° 27′19.93″ E	155	33.00	40.00
15	Yazitepe	37° 17′20.89″ S 35° 38′32.95″ E	124	36.25	45.31

Genus-specific real-time PCR assay amplification was carried out in a reaction mixture containing 10 ng of extracted DNA, 0.5 mM JW11 (5′-CCT ATT TTA CAC CAA CCC CCA GT-3′), 0.5 mM JW12 (5′-GGG TAG GGG CGT TCT GCG AAA-3′) and 1× Quanti-Fast SYBR Green PCR kit (Qiagen, Valencia, CA; 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 1 mM Rox dye, 2 U Taq polymerase, and 200 mM each dNTP; Nicolas et al., 2002). The reaction was brought to 20 ml total volume with PCR-grade water. The PCR was conducted using a Rotor Gene RG-3000 (Corbett Research, San Francisco, CA). The process was warming to 50°C for 2 min, initial denaturation at 95°C for 1.5 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min.

The cycle threshold (CT) is the fractional cycle number reflecting a positive PCR result. The CT values, therefore, were automatically determined by the Rotor-Gene 6.1.93 software using default parameters. The standard curves were also used to determine the detection limit of the assays and to assess the linearity (R2) and the efficiency (E) of the real-time PCR amplifications.

Data Collection and Statistical Analysis. Data from the environmental variables for each location including habitat, wall and site characteristics were collected using the Arzum v1.0 software (Istanbul, Turkey). At each location, a handheld Global Positioning System (Magellan Explorist 600) was used to record the coordinates. Variables included in the analyses are listed in Table 1.

Table 2. The results of the sand fly collection in Imamoglu province

Taxon	Subgenus	Species	VILLAGES: YAZITEPE	ALAYBEYİ	ÜÇTEPE	MALIHIDIRLI	ULUÇINAR	ÇÖRTEN	SAYCA	FAYDALI	UFACIKÖREN	AĞZIKARACA	SOKUTAŞ	AYVALI	DANACALI	HACIHASANLI	SEVİNÇLİ	♀	♂	♀/♂	Total (%)
Phlebotomus	*Phlebotomus*	*P. papatasi*	40	55	5	10	0	4	8	8	52	3	1	6	31	0	0	128	95	0.74	223 (34)
	Larroussius	*P. tobbi*	16	38	44	16	0	5	15	4	22	16	124	5	24	0	0	190	139	0.73	329 (50.3)
		P. perfiliewi	4	8	4	0	1	0	0	0	0	1	0	0	1	0	0	18	1	0.05	19 (2.9)
		P.neg/syr	0	0	0	1	0	1	1	0	0	0	0	0	2	0	0	4	1	0.25	5 (0.8)
	Paraphlebotomus	*P. sergenti*	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	2	1	0.5	3 (0.5)
	Sergentomyia	*Sergentomyia spp.*	9	4	3	0	3	0	2	0	1	0	1	0	52	0	0	13	62	4.76	75 (11.5)
	Total		**69**	**105**	**56**	**27**	**4**	**10**	**26**	**15**	**75**	**20**	**126**	**11**	**110**	**0**	**0**	**355**	**299**	**0.84**	**654 (100)**

Table 3. PCR Results of the Imamoglu female sand fly specimens

Species	Total specimens	Total pool number	*Leishmania* -positive pool no. (by real-time PCR)	*Leishmania*-positive villages
P. tobbi	131	20	2 (Pool 15 and Pool 29)	Uctepe, Ufacikoren
P. papatasi	94	18	1 (Pool 25)	Ufacikoren
P. perfiliewi	1	1	–	–
P. neglectus/ syriacus	–	–	–	–
P. sergenti	1	1	–	–
Sergentomyia spp.	62	4	–	–
Total	**289**	**44**	**3**	**–**

Results

Sand fly Fauna. The first field work of the study was carried out between 24 August 2013 and 04 September 2013, and the second was between 28 June 2014 and 8 July 2014, in the 15 villages in Imamoglu, Adana Province. We determined the sand fly fauna in the 15 villages in the north-east of Adana Province in Turkey. In total, 100 CDC light traps were placed in each of the 15 study areas, and 654 sand fly belonging to one *Sergentomyia* and five *Phlebotomus* species were collected. Specimens were identified as follows: *P. tobbi* (50.3%), *P. papatasi* (34.1%), *Phlebotomus perfiliewi* (2.9%), *Phlebotomus sergenti* (0.4%), *Phlebotomus neglectus/ syriacus* (0.8%), and *Sergentomyia* spp. (11.5%).

P. tobbi, appeared to be the most abundant species in 5 of the 15 study areas; Uctepe (78.6%), Malihidirli (59.3%), Corten (50%), Sayca (57.7%), and Sokutas (98.4%) villages. Similarly, in 5 of the 15 areas, *P. papatasi* were detected to be dominant species in Yazitepe (58%), Alaybeyi (52.4%), Faydali (53.3%), Ufacikoren (69.3%), and Ayvali (54.5%). *Sergentomyia* spp. was found to be dominant only in Danacali (47.3%) village (Table 2).

Leishmania-infected Sand Flies and PCR. No promastigotes were found in the midgut specimens obtained from 52 females and studied by microscopically after the dissection. *Leishmania* was observed in only 3 of the 44 pools, including female specimens DNA collected in Imamoglu, were detected using genus-specific real-time PCR assay (Table 3). The CT value of genus-specific real-time PCR assay was found to be between 20.1 and 37.1 with a median threshold of 35 cycles. The average R^2 value of the standard curve was 0.99 in all real-time PCR assays.

Discussion

In the present study, one *Sergentomyia* and five *Phlebotomus* species were identified. In the previous collection from Adana Province, Simsek et al. (2007) collected 11 *Phlebotomus* species (*P. papatasi*, *P. sergenti*, *Phlebotomus alexandri*, *P. tobbi*, *Phlebotomus syriacus*, *Phlebotomus neglectus*, *Phlebotomus perfiliewi*,

Phlebotomus galilaeus, *Phlebotomus Transcaucasicus*, and *Phlebotomus halepensis*) two *Sergentomyia* species (*Sergentomyia theodori* and *Sergentomyia dentata*), Kasap et al. (2008) detected four *Phlebotomus* species (*P. tobbi*, *P. papatasi*, *P. sergenti*, and *P. perfiliewi/galilaeus*) and one *Sergentomyia* species, Svobodová et al. (2009) identified six *Phlebotomus* species (*P. tobbi*, *P. syriacus*, *P. perfiliewi*, *P. papatasi*, *P. sergenti*, and *Phlebotomus simici*), Atakan et al. (2010) identified two *Phlebotomus* species (*P. papatasi* and *P. tobbi*) and Belen et al. (2011) identified eight *Phlebotomus* species (*P. tobbi*, *P. perfiliewi-galilaeus*, *P. papatasi*, *P. sergenti*, *P. syriacus-neglectus*, *P. alexandri*, *Phlebotomus brevis*, and *P. perfiliewi-perfiliewi*), two *Sergentomyia* species (*S. dentata* and *S. theodori*).

Imamoglu province is the endemic area for CL (Ministery of Health Data System 2013). Imamoglu region sand fly fauna in this study was first established and also *Phlebotomine* possible vector species were highlighted. The most abundant sand fly species identified in Imamoglu was *P. tobbi*, which is known as the dominant specific vector of *L. infantum* causing CL and visceral leishmaniasis in Cukurova and Adana Region (Svobodová et al. 2009, Belen et al. 2011).

P. tobbi female midgut specimens from Uctepe and Ufacikoren in two pools were found to be *Leishmania*-positive with genus-specific real-time PCR assay. In addition, *Leishmania* positive was observed in one pool, which involved *P. papatasi* female midgut specimens from Uctepe. *P. tobbi* was the primarily dominant species in Uctepe (78.6%), and the second dominant one in Ufacıkoren (29.3%). Despite the differences in Phlebotomine fauna composition, *P. tobbi* was the most dominant species in five focus group villages. Being a member of the *Larrousius* subgenus, *P. tobbi* is a specific vector of *L. infantum* (Svobodová et al. 2009). Our results support the *P. tobbi* vectorial capacity in Imamoglu; hence, we assume that *P. tobbi* might have transmitted the *Leishmania* agent in the Imamoglu province according to real-time PCR results. However, more work is needed for further information.

P. papatasi, which is a specific vector of *L. major* in Old World, is a dominant sand fly species in Turkey (Kavur 2011), yet there is no evidence about the possibility of its vectorial capacity in Turkey. *P. papatasi* was detected as dominant species in the five villages of Imamoglu. Furthermore, *Leishmania* was found to be positive in one PCR pool, including *P. papatasi* female abdomen from Ufacikoren villages, It might have been resulted from the *P. papatasi* females sucking the blood infecting other *Leishmania* species in Ufacikoren. Studies have reported that some *Leishmania* promastigotes are more susceptible to death by digestive enzymes of specific sand fly vector species after digestion of blood meal (Kamhawi et al. 2000).

P. perfiliewi, *P. sergenti*, *P. neglectus/syriacus*, and *Sergentomyia* species, on the other hand, were rarely collected in Imamoglu.

In conclusion, Imamoglu, Adana Province, has the richest sand fly fauna in Southern Anatolia. *P. tobbi* is called a dominant sand fly species and it could trigger CL transmission in Uctepe and Ufacikoren villages. We suggest that further studies the scope of which are different from the present one be conducted to make detailed investigations on the biology and ecology of *P. tobbi*, *P. papatasi*, *P. perfiliewi*, *P. sergenti*, *P. neglectus/syriacus*, and *Sergentomyia* spp.

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