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Research

Entomological Survey for the Detection of Sand Fly Fauna and Vector Species in the Cutaneous Leishmaniasis Endemic Area in East Mediterranean Region of Turkey, Mersin Province

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Abstract

Cutaneous (CL) and visceral (VL) forms of leishmaniasis, transmitted by sand flies, are seen in all countries located in Mediterranean Basin including Turkey. In this study, we aimed to conduct an entomological survey for the detection of sand fly fauna and vector species in Mersin province, one of the important endemic areas for CL in Turkey. In total, 912 sand fly specimens were collected in 2010 and 2011 using CDC light traps. Nine *Phlebotomus* (Diptera: Psychodidae) and three *Sergentomyia* (Diptera: Psychodidae) species were detected. Of the collected *Phlebotomus* sand flies, *P. sergenti* Parrot, 1917 (30.1%) was the most dominant followed by *P. alexandri* Sinton, 1928 (18.2%), *P. neglectus/syriacus* Tonnoir Adler (12.0%), *P. tobbi* Adler & Theodor, 1930 (11.7%), and *P. papatasi* Scopoli, 1786 (10.2%), while *S. minuta* Rondani, 1843 (11.3%) was the dominant species among *Sergentomyia*. During the field work in 2011, female specimens (n = 81) were screened for the presence of *Leishmania* promastigotes by midgut dissection, and all were found negative. The rest of the collected female specimens (n = 334) were pooled according to species (*P. alexandri, P. neglectus/syriacus, P. papatasi, P. sergenti, P. simici*, and *P. tobbi*) and location (Mut, Silifke, and Anamur). In total, 29 pools were generated and real-time ITS1 PCR assay was performed to detect and identify natural *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatida) infection. Two pools, both from Mut town, containing *P. sergenti* specimens were found positive and *Leishmania tropica* Ross, 1903 was identified as an infectious agent for both pools. In conclusion, the sand fly fauna was determined in an endemic area for CL. The detection of *L. tropica* DNA in *P. sergenti* specimens showed the possible vectorial role of this species in Mersin province.

Key words: leishmaniasis, cutaneous, vector, real-time ITS1 PCR

Leishmaniasis is a group of protozoan disease caused by the members of genus *Leishmania*, and transmitted by Phlebotomine (Diptera: Psychodidae) sand flies. Vector arthropods such as sand flies have great importance due to their transmission ability of pathological agents from/to humans and animals. In Turkey, *L. infantum* Nicolle, 1908 (Kinetoplastida: Trypanosomatida) is the main causative agent for visceral (VL), while cutaneous (CL) caused by *L. tropica*, *L. infantum*, and *L. major* Yakimoff and Schokhor, 1914 are the main causative agents for CL. Recently, *L. donovani* Ross, 1903 was also reported as a causative agent of CL in Turkey (Özbilgin et al. 2017). Previous studies related to sand fly fauna in Turkey were conducted in more than 45 provinces and 28 sand fly species belonging to *Phlebotomus* and *Sergentomyia* genera were identified (Kasap et al. 2019). At least 18 of these species are proven or probable vectors of human/canine leishmaniasis in the Old World (Volf et al. 2002, Sadlova et al. 2003, Yaman and Özbel 2004, Ertabaklar et al. 2005, Toprak and Özer 2007, Svobodova et al. 2009, WHO 2010). *Phlebotomus tobbi* is the proven vector of CL caused by *L. infantum* in East Mediterranean parts of Turkey. *Phlebotomus sergenti* and *P. similis* Perfil'ev, 1963 are the probable vectors of

L. tropica in south-eastern and western parts of Turkey, respectively (Alptekin et al. 1999, Depaquit et al. 2002, Svobodova et al. 2009, WHO 2010).

Phlebotomine sand flies are biological vectors harboring different developmental stages of *Leishmania* parasites from amastigotes to infective metacyclic promastigotes. The detection of these stages by microscopical examination of the midguts is an important evidence for the vectorial status of a particular species in an endemic area (Killick-Kendrick 1990). The biggest advantage of this technique is isolation of the parasite strain by culture; however, the midgut dissection takes relatively long time and needs skilled personnel, and limited in sensitivity. PCR methods targeting different gene regions are also in use for detecting the *Leishmania* DNA in sand flies. As these techniques are not able to determine the developmental stages of the parasite in sand fly, the use of these methods is not sufficient to determine vectorial role of the sand fly. However, positive result in PCR indicates the parasite circulation and natural infection in wild caught sand flies in a particular study area.

Cutaneous leishmaniasis has posed a public health problem in Mersin province. In total, 420 (66.9%) out of 627 cases were recorded in three towns of Mersin between 1994 and 2001; Anamur (36.5%), Mut (26.1%), and Silifke (4.3%) (Baz et al. 2002, Kokturk et al. 2002, Ministry of Health [MoH] 2014). Additionally, 1,578 CL cases were also recorded between 1990 and 2010 (Gürel et al. 2012). Therefore, we aimed to carry out an entomological survey in endemic areas for CL in Mersin province for the determination of sand fly fauna and possible vector species.

Materials and Methods

Study Area

The present study was carried out in eight villages belonging to three towns in Mersin province where CL cases had been reported over the past 10 yr; Anamur (Çarıklar, Malaklar), Mut (Ortaköy, Kelce, Balabanlı, Topluca), and Silifke (Keben, Ortaören) (Table 1). Mersin province is 15.853 km^2 in area, and it is a major port city located in the southern part of Mediterranean coast (latitude 36°48′0″N, longitude 34°38′0″E; (Fig. 1). The province has 321 km coastline and flatland along the coast, but the mountainous area covers 87% of the land. The altitude is changing between sea level and >3,500 m. The coastlines of the province usually have Mediterranean vegetation while there are forests in higher areas. The climate is typical of the Mediterranean; very hot and very humid in summer, warm and wet in winter. In total, 65 small mammal or rodent species including 6 *Meriones* species that are well-known reservoirs especially for *L. major* were reported in Turkey. Beside *Meriones* species, endemic species *Acomys cilicicus* and *Apodemus mystacinus* were recorded in Mersin province (Çetintaş et al. 2017).

Sand Fly Sampling and Midgut Dissection

Sand fly specimens were collected between July 2010 and August 2011 in selected villages in three towns where CL cases had been reported over the past 10 yr. One field work in July 2010 and two field works in July and August 2011 were carried out. Six or eight CDC miniature light traps (John W. Hook Comp.) per night per village were set up mainly in animal shelters, poultries, dog shelters, and houses in the evening nearby to the CL patient's houses (17:00–18:00) and collected very early in the morning (05:00–06:00).

Alive specimens were transferred to the laboratory using a plexiglass cage for midgut dissection. The specimens were immobilized on ice, rinsed briefly in 70% ethanol and the antibiotic solution containing gentamycin and penicillin, and then dissected in 0.9% sterile saline using the digestive tract dissection technique (Volf et al. 2002). The head and genitalia were used for species identification, and the gut was examined microscopically for the presence of promastigotes. The dead flies were stored in 96% alcohol for morphological identification. The species identification was done based on the morphology of male genitalia and female spermatheca and pharyngeal armature using the several published written keys for Mediterranean sand flies (Theodor 1958, Lewis 1982, Artemiev 1991, Killick-Kendrick 1990). The head and genitalia were cut and mounted in the Swan solution, and remaining body parts were kept in 96% ethanol in 4°C for further molecular analysis.

Extraction of *Leishmania* DNA From Sand Flies and Real-Time ITS1 PCR

For the detection of *Leishmania* DNA, Zymo insect DNA kit (Zymo Research Corp., Irvine, CA) was used for extracting DNA from sand flies following the manufacturer's instructions. In total, 334 specimens were used to generate monospecies pools using 3–19 individuals for each pool and a total of 29 pools were generated (Table 3). Additionally, a negative control group was created using male sand flies. The real-time ITS1 PCR method was performed using Old World species-specific primers and probes. All procedure was done as described previously (Töz et al. 2013). Four WHO reference strains were used as positive controls; *L. infantum/chagasi* (MHOM/XX/99/LRC-L774), *L. donovani* (MHOM/IN/80/DD8), *L. tropica* (MHOM/IL/90/ LRC-L590 and MHOM/IL/96/LRC-L691), and *L. major* (MHOM/IL/2000/LRC-L779).

Results

Sand Fly Fauna and Midgut Dissection

Throughout the field works, a total of 912 sand fly specimens were captured using 52 CDC light traps in three towns of Mersin province where 637 CL leishmaniasis cases were reported between 2005 and 2011. The 90% of trap locations were open and closed animal shelters. In total, 9 *Phlebotomus* species, *P. sergenti* 30.15%,

Table 1. The villages and towns selected as study area in Mersin province

District/village	District	Altitude (m a.s.l.)	Latitude	Longitude	No. of light traps/nights (in total)
Keben	Silifke	218	36°25′50.86″N	33°44′01.00″E	4/2
Ortaören	Silifke	261	36°25′32.03″N	33°41′50.20″E	6/1
Çarıklar	Anamur	69	36°06′44.69″N	32°52′39.14″E	6/1
Malaklar	Anamur	77	36°07′45.25″N	33°41′50.20″E	3/2
Balabanlı	Mut	251	36°39′25.14″N	33°24′52.29″E	6/1
Kelceköy	Mut	489	36°39′33.63″N	33°28′04.01″E	4/2
Ortaköy	Mut	180	36°40′36.53″N	33°25′12.48″E	6/1
Topluca	Mut	486	36°42′37.23″N	33°28′24.18″E	6/1

P. alexandri 18.20%, *P. neglectus/syriacus* 12.06%, *P. tobbi* 11.73%, *P. papatasi* 10.20%, *P. simici* Nitzulescu, 1931 3.18%, *P. halepensis* Theodor, 1958 0.55%, *P. mascittii* Grassi, 1908 0.88%, *P. brevis*, Theodor & Mesghali 1964, 0.11%, and three *Sergentomyia* species, *S. minuta* 11.29%, *S. dentata* Sinton 1933, 1.21%, *S. fallax* Parrot, 1921 0.44%, were identified in the study area (Table 2 and Fig. 2). *Phlebotomus sergenti* was found as dominant species in the province level. *Phlebotomus sergenti, P. alexandri*, and *S. minuta* were identified as dominant species in Mut, Silifke, and Anamur, respectively. The male/female ratio was 1.19 in the total capture.

The midguts of 81 female sand fly specimens were checked microscopically for natural *Leishmania* infection, and no promastigotes were detected.

In Mut town, *P. sergenti* was found as dominant species with 45.25%, while *P. tobbi* (12.5%), *P. alexandri* (12.32%), and *P. papatasi* (11.79%) were other dominant species. All nine species detected in this study were only present in Mut town. In Silifke town, the ratio of *P. sergenti* was only 8.27% and *P. alexandri* was the dominant species with 55.17%. In Anamur town, *S. minuta* was the dominant species with 42.78% while *P. neglectus/syriacus* (21.39%)

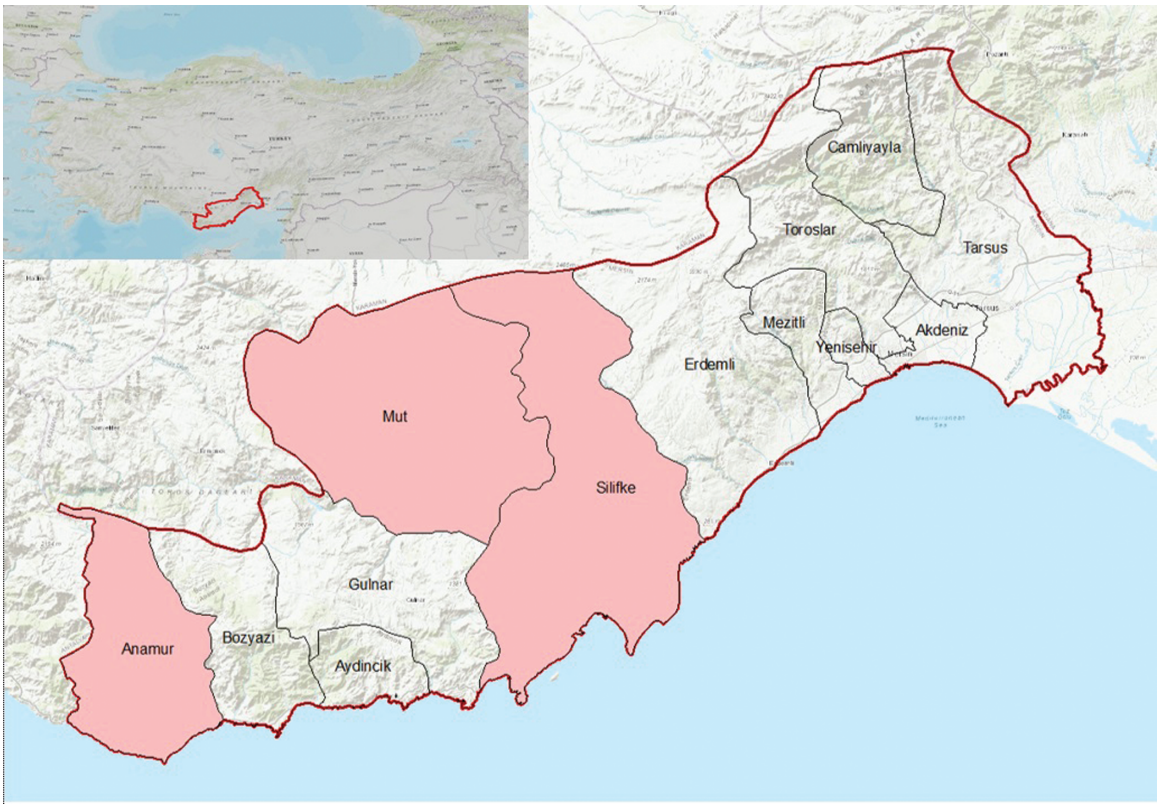

Fig. 1. The location of Mersin province in Turkey and geographical towns of the province (the study area was shown in red color, for color figure refer online version).

Table 2. The sand fly species identified in the study area

		Anamur				MUT				Silifke				Total			
Subspecies	Species	M	F	T	%	M	F	T	%	M	F	T	%	M	F	T	%
Paraphlebotomus	*P. sergenti*	3	3	6	3.2	158	99	257	45.2	4	8	12	8.2.	165	110	275	30.1
Paraphlebotomus	*P. alexandri*	10	6	16	8.5	53	17	70	12.3	44	36	80	55.1	107	59	166	18.2
Phlebotomus	*P. papatasi*	–	6	6	3.2	39	28	67	11.8	1	19	20	13.8	40	53	93	10.2
Larroussius	*P. neglectus/ syriacus*	30	10	40	21.4	23	14	37	6.5	28	5	33	22.7	81	29	110	12.0
Larroussius	*P. tobbi*	15	15	30	16.0	26	45	71	12.5	2	4	6	4.1	43	64	107	11.7
Larroussius	*P. mascitti*	–	–	–	–	2	–	2	0.3	6	–	6	4.1	8	–	8	0.8
Adlerius	*P. simici*	–	–	–	–	7	22	29	5.1	–	–	–	0.0	7	22	29	3.1
Adlerius	*P. halepensis*	–	–	–	–	3	2	5	0.8	–	–	–	0.0	3	2	5	0.5
Adlerius	*P. brevis*	–	–	–	-	1	–	1	0.1	–	–	–	0.0	1	–	1	0.1
Sergentomyia	*S. minuta*	22	58	80	42.7	9	14	23	4.0.	–	–	–	0.0	31	72	103	11.3
Sergentomyia	*S. dentata*	5	–	5	2.6	6	–	6	1.0	–	–	–	0.0	11	–	11	1.2
Sergentomyia	*S. fallax*	–	4	4	2.1	–	–	–	0.0	–	–	–	0.0	–	4	4	0.4
Total		85	102	187	100.0	327	241	568	100	81	64	157	100	497	415	912	100

and *P. tobbi* (16.04%) were second and third dominant species (Table 2).

Leishmania DNA in Sand Flies

In total, 29 pools were generated using 334 female specimens. Specimens were categorized as unfed, blood-fed, gravid, semi blood-fed, and gravid, and they were pooled according to town and species for the examination of *Leishmania* DNA. Parasite DNA was detected only in two pools (Pool 2 and Pool 6) containing *P. sergenti* females. Both parasites were identified as *L. tropica* by real-time ITS1 PCR (Fig. 3a–c). One of the positive pools was from Mut town and was containing 3 gravid, 13 blood-fed, and 4 blood-fed and gravid *P. sergenti* specimens while the other one was from Anamur town and containing only 10 unfed *P. sergenti* specimens (Table 3).

Discussion

Sand fly fauna studies have been mainly performed in both cutaneous and visceral leishmaniasis endemic areas in Turkey. Mersin province has divided into 13 administrative towns and 627 CL cases were reported from 10 towns between 1994 and 2001. In total, 420 (66.9%) out of 627 cases were recorded in three towns; Anamur (n = 229; 36.5%), Mut (n = 164; 26.1%), and Silifke (n = 27; 4.3%) (Baz et al. 2002, Köktürk et al. 2002). Additionally, 1,578 CL cases were also recorded between 1990 and 2010 (Gürel et al. 2012). For this reason, to detect sand fly fauna and better understand the possible vector species, these three towns were selected as the study area and sand fly collections were carried out in three different time intervals between 2010 and 2011.

Fauna studies, in Turkey, revealed 23 and 5 species belonging to *Phlebotomus* and *Sergentomyia* genera, respectively. In the present study, 9 *Phlebotomus* and 3 *Sergentomyia* species were identified and Mut town had the richest species diversity among three areas. In Turkey, sand fly species belonging to *Larroussius* (Diptera: Psychodidae) subgenus such as *P. tobbi* have usually seen in the regions that having Mediterranean climate and species belonging to *Adlerius* Nitzulescu (Diptera: Psychodidae) subgenus such as *P. halepensis*, usually seen in higher altitudes. Mut district resembles the middle of a bowl between the rising mountains. Although the Mediterranean climate is observed in Mut district, the climate is terrestrial due to the mountainous and hilly terrain structure as it goes toward the inner parts. Two villages selected within Mut town are in almost 500 m altitudes while the other two are around 200 m altitude. The proven vector species of *L. tropica is P. sergenti* and was found to be dominant with 45.2% in Mut town followed by, *P. tobbi* (12.5%), which is the proven vector species of *L. infantum* (Svobodova et al. 2009) and *P. halepensis* (12.3%). Besides *P. sergenti*, *P. halepensis* is also probable vector species for *L. tropica* (Sadlova et al. 2003, Kavur et al. 2018). The presence of the members of both subgenera can be increased the possibility of transmission of the parasite among people. The high number of the CL cases reported between 1994 and 2001 (Köktürk et al. 2002) is also supporting this phenomenon. Since the causative *Leishmania* species for CL in the study area is not known, we can only speculate on the basis of *Leishmania* species seen in neighboring provinces such as Adana and Antalya, where the agents are found as *L. tropica* and *L. infantum* in human (Svobodova et al. 2009) as well as in dogs (Balcıoğlu İC et al. 2009, Karakuş et al. 2015).

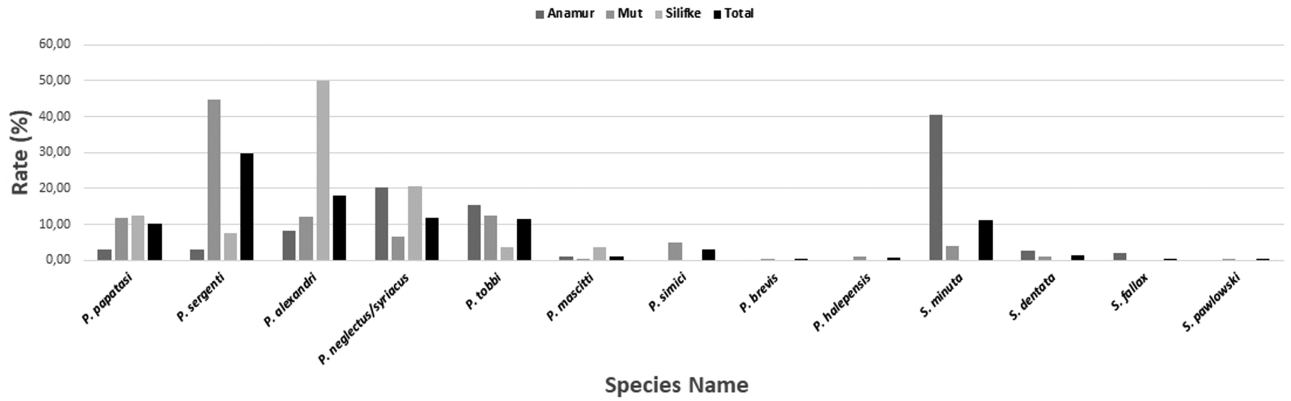

Fig. 2. The sand fly species collected in three towns.

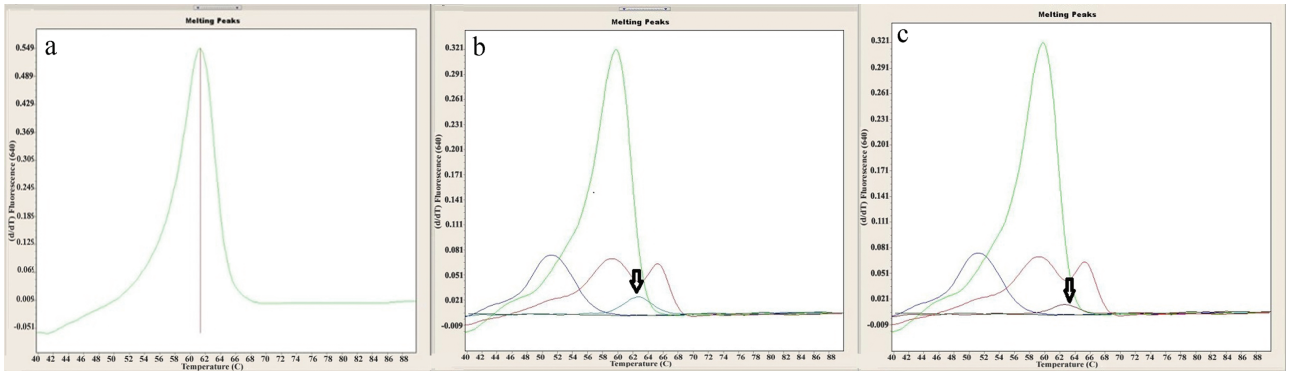

Fig. 3. Melting curves of (a) *L. tropica* positive control, (b) pool 2 and (c) pool 6. (Arrows indicate the pick of sample).

Table 3. The details about 29 sand fly pools and real-time ITS1 PCR results

Species	Town	No of pools	BF	Non-BF	G	BFG	Total SF	ITS1 PCR
P. alexandri	Silifke	2	3	15	8	0	26	NEG
	Mut	1	1	13	1	3	18	NEG
	Anamur	1	2	11	0	2	15	NEG
P. neglectus/syriacus	Mut	3	6	14	2	5	27	NEG
P. papatasi	Mut	3	9	17	6	7	39	NEG
	Silifke	1	3	7	0	4	14	NEG
P. sergenti	Mut	9	19	76	8	6	109	POS (Pools 2 and 6)
P. simici	Mut	3	4	17	1	0	22	NEG
P. tobbi	Mut	6	12	46	4	2	64	NEG
Total		29	59	216	30	29	334	

N (normal; nonblood-fed; nongravid), G (gravid), BF (blood-fed), BFG (semi blood-fed and gravid), SF (sand fly).

In Silifke town (around 200 m a.s.l), *P. alexandri* that is reported as vector species of *L. infantum* and *L. donovani* in Iran (Azizi et al. 2006) and Xinjiang Uygur Autonomous Region (XUAR) of China (Li-Ren et al. 1986), respectively, was found to be the dominant species with the ratio of 55.17% (Table 2). It can be emphasized that *P. alexandri* seems to be a permissive vector like other vectors of VL such as *P. argentipes* (Annandale and Brunetti 1908) (Pimenta et al. 1994). In Turkey, *P. alexandri* was detected in many CL and VL endemic areas, but it was not reported as dominant species before (Simsek et al. 2007). However, *P. sergenti* (8.27%) is present in the town and causative agent is not known yet. Although *P. alexandri* could be probable vector species and causative agent(s) may be *L. infantum* and/or *L. donovani* (Özbilgin et al. 2017), new studies on the identification of *Leishmania* strains and additional entomological surveys are needed to clarify the vectorial status of the species.

Even though similar trapping locations were selected in Anamur town, interestingly *S. minuta* was detected as a dominant species with 42.7% (Table 2). The second and third dominant species were belonging to *Larroussius* subgenus, *P. neglectus/syriacus* (21.3%) and *P. tobbi* (16.0%), in this area. Anamur town is located in the Mediterranean coast in the westernmost part of Mersin province and sand fly fauna of this area was found to be similar to the neighboring town Gazipaşa where is an endemic area for CL, especially caused by *L. infantum* (Ser and Çetin 2013). Because of 229 CL cases were reported between 1994 and 2001, we can speculate that *P. tobbi* can be a vector species and the primer causative agent is *L. infantum* in this particular area.

The entomological study including midgut dissection was performed in the Cukurova region and resulted in 13 successful isolation of *L. infantum* from *P. tobbi* specimens (Svobodova et al. 2009). Then, *L. infantum* was detected in *P. tobbi* specimens by microscopic examination and its DNA was determined by real-time ITS1 PCR assay in the survey carried out in a VL and canine leishmaniasis focus in western Turkey (Özbel et al. 2016). In the present study, no promastigotes were found in the midguts of 81 dissected female sand fly specimens. On the other hand, two pools containing *P. sergenti* specimens in Mut town were found to be positive for *L. tropica* DNA (Fig. 3). One of the positive pools (no. 2) was containing gravid (n = 3) plus blood-fed (n = 13) plus blood fed/gravid (n = 4) *P. sergenti* specimens while the other one (no. 6) was containing 10 unfed *P. sergenti* specimens (Table 3). Although positivity with any kind of PCR is not sufficient for the incrimination as vector species, *P. sergenti* is a very well-known and proven vector species in many CL endemic areas of the Old World countries (WHO 2010) as well as in Turkey (Demir and Karakuş 2015).

It is hypothesized that *L. infantum* has two possible cycles in the neighboring province in the east Adana, zoonotic and/or anthroponotic, which needs to be clarified by further studies on other possible vector species (Karakuş et al. 2015). *Leishmania tropica* transmitted by *P. sergenti* is a dominant causative agent for CL, and additionally CL cases caused by *L. major* were also reported from this province recently (Koltaş et al. 2014). For this reason, several *Leishmania* parasites can also be present in Mersin province because it has a similar climate and vegetation to Adana province.

In the present study, the results showed that *P. sergenti* is probable vector species for CL transmission in Mersin province, especially in highly endemic Mut town, as well as *P. tobbi* and *P. halepensis* can also be involved in the parasite transmission, especially for *L. tropica*, in the province. For better understanding, further studies related to the causative agent(s) is necessary. The detection of certain or probable vector species can be helpful for applying control measures in particular endemic areas.

In conclusion, this study provides new data on the sand fly fauna of the southern Mediterranean coast of Turkey and the possible vectorial role of *P. sergenti* in the area. The variation observed in the Phlebotomine fauna composition among the towns are probably depending on geographical differences in the province. Our results showed that the dominant species *P. sergenti* is a probable vector for CL transmission in Mersin, especially in highly endemic Mut town. *Phlebotomus tobbi*, *P. neglectus/syriacus*, and *P. alexandri* may also be involved in the parasite transmission in the province. The presence of these possible vector species and high frequency of human movements coming from south-eastern region where different *Leishmania* parasites were identified suggests that more than one *Leishmania* species may be involved in the transmission of CL in Mersin. In order to apply effective control measures in the study area. Further epidemiological studies related to the identification of causative *Leishmania* species and vectorial role of *P. tobbi*, *P. neglectus/syriacus*, and *P. alexandri* is needed for better understanding of the transmission dynamics of *Leishmania* and the detection of possible vector species.

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