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First molecular detection and identification of *Leishmania* species in small wild rodents from Turkey

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Research Article

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Abstract

Leishmaniasis is a parasitic disease infecting animals and humans. Two clinical forms (Visceral and cutaneous leishmaniasis) and four species are reported to be present in Turkey. Several studies have investigated canine and human leishmaniasis in Turkey but no study was performed to screen the infection among wild rodents, so far. The present study aims to investigate the role of small wild rodents as reservoir animals for *Leishmania* spp. in different regions of Turkey. Formalin-preserved tissue samples (spleen, liver, lung) of 712 rodents from 30 provinces were screened for the presence of *Leishmania* spp. DNA. Before DNA extraction, tissues were dried, rehydrated, and homogenated. *Leishmania* screening in rodent tissues and species determination was performed with a combination of real-time kDNA and ITS1 polymerase chain reaction protocols. Eight (1.12%) out of 712 animals were found to be positive for *Leishmania* spp. DNA and species typing revealed five *L. infantum*, two *L. tropica* and one *L. major* among positives. *Leishmania major* and *L. infantum* DNA were detected in *Apodemus* spp. from Zonguldak province located in the Western Black Sea Region, while *L. tropica* DNA was found in *Meriones* sp. and *Gerbillus dasyurus* from Adana and Hatay provinces located in Eastern Mediterranean Region of Turkey. The present study is first to report natural infection of *L. infantum, L. major* and *L. tropica* in small wild rodents in Turkey, suggesting their possible roles as reservoirs. Further studies are needed for planning epidemiological studies and also for developing rodent control measures in risky endemic areas to break the transmission cycle.

Introduction

Both visceral (VL) and cutaneous leishmaniasis (CL) are endemic diseases in several countries located in Mediterranean Basin as well as in Turkey (Dujardin *et al.*, 2008). Each year around 2000 CL cases are officially reported in Turkey and more than 95% of them were from nine endemic provinces mainly located in Mediterranean and Southeastern Regions (Gürel *et al.*, 2012). *Leishmania infantum* MON-1 is the main causative agent for zoonotic human and canine visceral leishmaniasis while anthroponotic *L. tropica* is the main species causing CL in all regions of Turkey. Besides these agents, *L. tropica* and *L. donovani* in VL patients while *L. infantum*, *L. major* and *L. donovani* in CL patients were also reported (Koltas *et al.*, 2014; Özbilgin *et al.*, 2019).

Dogs are considered as main reservoirs of *L. infantum* in Europe as well as in Turkey. So far, canine leishmaniasis (CanL) and reservoir role of the dogs have been studied comprehensively in different VL endemic areas of Turkey. The prevalence of CanL was detected as between 1.45% and 27.5% according to the endemic area (Toz *et al.*, 2009). The possible role of the cats in the transmission of leishmaniasis is another debating topic in endemic countries. The presence of *Leishmania* DNA is not the conclusive finding to incriminate cats as a reservoir even though their infectiousness to sand fly was shown (Maroli *et al.*, 2007). Up to day, several studies performed to screen *Leishmania* spp. among cats in Turkey and the positivity were ranging from 7% to 33.3% (Paşa *et al.*, 2015; Can *et al.*, 2016; Karakuş *et al.*, 2019). The possible role of the rodents was also investigated in Italy by sampling the spleen and various tissues and of the sampled rodents, 45% ($n = 20$) were found to be infected with *L. infantum* (Di Bella *et al.*, 2003). The presence of *L. infantum* in natural rodent populations was also investigated in Portugal and the infection rate was found to be 33.3% (Helhazar *et al.*, 2013).

There are several studies performed in the Old-World countries to investigate the role of rodents in the *Leishmania* transmission cycle especially for zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major*. Due to the high endemicity of ZCL in Iran, numerous studies related to rodents were performed. The first study was done in the 1950s by Ansari and Mofidi in Iran and followed by several reports up to today stating their importance in the

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transmission of ZCL caused by *L. major* (Ansari and Mofidi, 1950; Akhoundi *et al.*, 2013). *Leishmania tropica* was also demonstrated to infect the Ethiopian wild rodent populations (Kassahun *et al.*, 2015).

According to those findings, four different *Leishmania* species present in Turkey was reported to be found in sampled tissues of rodents in different countries. The prevalence rate of *Leishmania* in various populations of humans, dogs and even cats was investigated in Turkey. However, yet no study was performed to screen leishmaniasis infection in small wild rodents even though at least 65 species are present belonging to seven families in Turkey (Kryštufek and Vohralík, 2005, 2009; Yiğit *et al.*, 2006, 2016; Wilson *et al.*, 2016, 2017). Therefore, the potential role of rodents in the *Leishmania* infection cycle was investigated in the present study.

Materials and methods

Sample collection and DNA preparation

The samples collected during epidemiological Hantavirus studies in various provinces of Turkey were used in the present study. For these studies, the wild rodents were collected using Sherman live traps placed with consecutively 10–15 m intervals, and placement points were recorded by GPS. Species identification of rodents was performed according to the phenotypic characteristics of the animals. After taking body measurement of animals, they were sacrificed by cervical luxation method, and tissue samples were preserved in formalin. A total of 906 different tissue samples (spleen, liver, and lung) obtained from 712 rodents caught from 29 provinces were screened. The samples were collected from the individuals belonging to 23 rodent genera and were transferred to *Leishmania* Laboratory in the Department of Parasitology, Faculty of Medicine of Ege University for molecular screening studies to detect the *Leishmania* DNA. Samples obtained from *Apodemus* 431 (47.5%), 68 (7.5%) *Mus* sp., 68 (7.5%) *Myodes* sp., 55 (6.0%) *Spalax* sp., 51 (5.6%) *Nannospalax* sp., 45 (4.9%) *Microtus* sp., 42 (4.6%) *Meriones* sp. and 32 (3.5%) *Rattus* sp. samples were enrolled in the study. These eight genera made up most of the total samples (87.1%).

Before the DNA extractions, all tissue samples were first cut in small pieces to speed up the evaporation step and dried using Eppendorf Concentrator™ (Thermo Scientific, UK) to remove the formalin. Dried tissue samples were rehydrated using 200-µL of Qiagen® tissue lysis buffer (Qiagen GmbH, Hilden, Germany) and Proteinase K (20-µL) and incubated at 56°C overnight. Tissue samples were later transferred to tubes containing ZR Bashing Bead™ tubes (Zymo Research Corp., USA) and homogenated using Magna Lyser (Roche Molecular Diagnostics, Germany) at 7000 ***g*** for 90 s. Homogenates were used in DNA extractions following the manufacturer's instructions of DNeasy Blood & Tissue Kit (Qiagen GmbH).

Realtime kDNA PCR and realtime ITS1 PCR

The detection of *Leishmania* in rodent tissues and species determination was performed with a combination of Realtime kDNA and ITS1 PCR protocols. The first step was to screen the presence of *Leishmania* in tissue samples and due to its high copy number, the minicircle region of the kDNA was targeted using genus-specific primers (JW11/JW12) as reported in recent studies (Kassahun *et al.*, 2015). A Realtime PCR was performed using SYBR Green I Master Kit (Roche Diagnostic, France) and positivity determined according to genus-specific melt peaks.

Leishmania spp. positive samples were used in a second step PCR, which was performed to determine the species of the causative agent. Primers targeting the ITS1 region rRNA gene (LITSR/ITS1R-TR1) was used and species determination were made in *Leishmania* positive samples using melt analysis. A Real-time PCR protocol was applied by the previously published paper (Toz *et al.*, 2013).

Results

During the years of 2005–2017, 906 tissue samples (spleen, liver, and lung) were obtained from 712 rodent species belonging to 23 genera and 41 species. Sampling was done in 73 different locations and samples were divided according to their geographical locations. The majority of animals (56%) were obtained from urban residential locations in CL endemic areas. Based on the sampled rodents, the majority of them ($n = 432$; 47.6%) were belonging to *Apodemus* genera. The mean of whole-body length was 188 mm with a range of 171–220 mm.

Of the analysed animal samples, eight (8/712; 1.12%) of them were found to be positive for *Leishmania* spp. DNA by realtime kDNA-PCR (Suppl Fig. 1). The species typing by real-time ITS1 PCR revealed five *L. infantum,* two *L. tropica* and one *L. major* among the positive samples (Suppl Fig. 2).

Leishmania major DNA was detected in the specimen belonging to *Apodemus* genus from Zonguldak province located in the Western Black Sea Region of Turkey. *Leishmania infantum* DNA was detected in three different rodent species, *Apodemus filavicollis, Apodemus mystacinus* and *Apodemus* sp. (four positive rodent specimens could not be detected in species level) from the same province, Zonguldak. *Leishmania tropica* DNA was detected in *Gerbillus dasyurus* (the mean of whole-body length was 84 mm with a range of 73–94 mm) from Hatay province and *Meriones tristrami* (the mean of whole-body length was 120 mm with a range of 100–155 mm) from Adana province. Both provinces are located in the Eastern Mediterranean Region (Table 1; Figure 1). *Leishmania infantum* positive rodents were all male while *L. major* and *L. tropica* positives were female.

Discussion

Small rodent species in The New World and The Old World are suspicious reservoir hosts for different *Leishmania* species. Despite the important roles of small rodents for spreading of leishmaniasis, the studies are relatively in low number and mainly carried out in the Middle East and North Africa countries such as Iran, Tunisia, Morocco and Algeria where *L. major* is the dominant causative agent for CL (Ghawar *et al.*, 2011 ; Echchakery *et al.*, 2017; Foroutan *et al.*, 2017; Pourmohammadi *et al.*, 2017; Othman *et al.*, 2018). Also, some reports have indicated the infection to *L. donovani, L. infantum* and *L. tropica* in some rodents such as *Gerbillus* spp., *Rattus norvegicus* and *Mus musculus* (Helhazar *et al.*, 2013; Kassahun *et al.*, 2015; Navea-Perez *et al.*, 2015) suggesting their potential role to act as a reservoir for another *Leishmania* species.

The comprehensive studies on canine leishmaniasis were done for many years in Turkey, and results are always showing higher prevalence than human infection in VL endemic areas. *Leishmania infantum* was found as the main causative agent of CanL as well as human VL in these areas (Toz *et al.*, 2013; Sarı *et al.*, 2015). Because of *Leishmania* species having zoonotic life cycle have been found in Turkey and no study was carried out on rodents so far (Koltas *et al.*, 2014; Özbilgin *et al.*, 2016), it was necessary to conduct studies for revealing the reservoir potential of the small wild rodents. In the present study, we have investigated the presence of *Leishmania* DNA in the tissue samples collected from different rodent species in different provinces of Turkey using molecular techniques. Since we had rodent tissue

Table 1. The locations where eight positive small wild rodents were caught and PCR results

No	Sample Code	Province	Town / Village	Long/Lat	Altitude	Total Samples (Province/ Town-Village)	Rodent species	Tissue	Real time kDNA PCR	Real time ITS1 PCR
1	8604	Adana	Yumurtalık	36.772877; 35.789730	18	41 / 8	*Meriones tristrami*	Spleen	POS	*L. tropica*
2	7780	Hatay	Kırıkhan/ KaletepeKöyü	36.651196; 36.554549	226	90 / 3	*Gerbillus dasyurus*	Spleen	POS	*L. tropica*
3	689	Zonguldak	Kozlu/OlukyanıKöyü	41.389786; 31.778748	365	491 / 88	*Apodemus* sp.	Spleen	POS	*L. major*
4	640	Zonguldak	Kozlu/OlukyanıKöyü	41.389786; 31.778748	365	491 / 88	*Apodemus* sp.	Spleen	POS	*L. infantum*
5	311	Zonguldak	Kozlu/Değirmenağzı	41.418801; 31.722165	43	491 / 15	*Apodemus mystacinus*	Spleen	POS	*L. infantum*
6	399	Zonguldak	Merkez/Kurtköy	41.493872; 31.985017	185	491 / 70	*Apodemus flavicollis*	Spleen	POS	*L. infantum*
7	359	Zonguldak	Çaycuma/ TemenlerKöyü	41.518497; 32.065252	170	491 / 18	*Apodemus* sp.	Spleen	POS	*L. infantum*
8	328	Zonguldak	Çaycuma/ SarmaşıkKöyü	41.547210; 32.122129	80	491 / 73	*Apodemus* sp.	Spleen	POS	*L. infantum*

samples that were previously collected and kept in formalin, the microscopical examination of their smears could not be performed for these samples. Our results revealed the presence of two *Leishmania* species (*L. infantum, L. major*) in the collected *Apodemus* spp. Specimens from one province, Zonguldak located in the Western Black Sea Region of Turkey. DNA of *L. tropica* was also found in two specimens (*Meriones tristrami* and *Gerbillus dasyurus*) from two different provinces, Adana and Hatay, located in the East Mediterranean Region of Turkey.

Although *L. tropica* is known as anthroponotic species, it is found in different mammalian species as dogs, cats and wild mammals, which play a reservoir role in nature (Töz *et al.*, 2013; Paşa *et al.*, 2015; Echchakery *et al.*, 2017; Baneth *et al.*, 2017). However, confirmation of these hosts as reservoirs requires xenodiagnostic studies as performed in Italy and Spain for *L. infantum* (Maroli *et al.*, 2007; Jimenez *et al.*, 2014). Adana and Hatay provinces are highly endemic areas for CL caused by *L. tropica* and *L. infantum* (Serin *et al.*, 2005), and recently *L. major* and *L. donovani* were also reported as causative agents in both provinces (Koltas *et al.*, 2014; Özbilgin *et al.*, 2019). According to the Ministry of Health records, a total of 616 and 207 CL cases were reported from Adana and Hatay provinces between 2013 and 2017, respectively (Özbel Y, personal comm). In a recent study, 21 out of 25 strains (84%) from Adana province were detected as *L. tropica*, and there were only two isolates from Hatay province and one of them is also found as *L. tropica* by Realtime ITS1 PCR (Özbilgin *et al.*, 2019). Dog studies carried out in these provinces revealed a high prevalence of CanL as 27.18% by IFAT and 41.74% by conjunctival swap nested PCR in rural areas of Adana (Karakuş *et al.*, 2015). We analyzed 41 and 90 rodent tissue samples from Adana and Hatay, respectively. Nineteen out of 41 were from *Meriones tristrami*, caught in five different towns, and one (5.26%; 1/19) of them was found positive for *L. tropica*. For Hatay province, 12 out of 90 samples from *Gerbillus dasyurus*, caught in four different towns, and one (8.33%; 1/12) of them was found positive for *L. tropica*. Although the number of samples included in the present study is not very high, the detection of *L. tropica* in small rodents in Adana and Hatay provinces will make the control of CL more complicated in these endemic areas. Applying control measures for small wild mammals are more difficult because of the geographical and climatic conditions in the area. More studies are needed to understand better its epidemiological importance. Hatay and Adana provinces are among the six highly endemic provinces in Turkey. Because of four Old World *Leishmania* species causing CL and suitable vector sand fly species (Alten *et al.*, 2015) are present in both provinces, small wild rodents need to be included to the epidemiological studies related to reservoirs planned in these areas.

Among 65 rodent species recorded in Turkey (Kryštufek and Vohralík, 2005, 2009; Yiğit *et al.*, 2006, 2016; Wilson *et al.*, 2016, 2017), six of them belonging to genus *Meriones* (*M. persicus, M. tristrami, M. vinogradovi, M. crassus, M. libycus, M. dahli*) playing important role as reservoir animal in different countries (Saliba and Oumeish, 1999), are available in most regions of Turkey except Black Sea and Marmara regions (Yiğit *et al.*, 2006). One endemic species classified in *Acomys* genus (*A. cilicicus*) lives only around Silifke and Erdemli towns of Mersin province (Çetintaş *et al.*, 2017), where CL caused by *L. tropica* is endemic. The widespreading of these small wild mammals in many areas enhancing the risk for human and canine leishmaniasis, and keeping the circulation of the parasite in nature.

Leishmaniasis tropica was detected in five (0.85%) out of 586 specimens belonging to *Gerbillus* genus by molecular techniques in Ethiopia (Kassahun *et al.*, 2015). We also found *L. tropica* DNA in *Gerbillus dasyurus* collected from Hatay province,

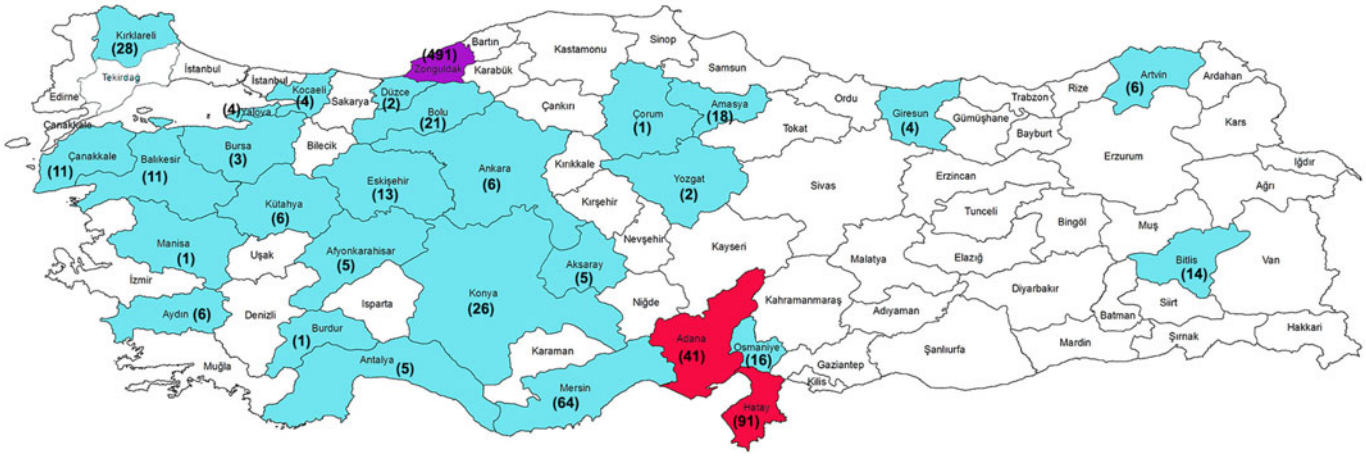

Fig. 1. The map showing sampling provinces with negative (Blue) and positive results (Red: *L. tropica*; Purple: *L. infantum* and *L. major* DNA positive rodents). Numbers in the bracelets are showing the samples number for each province.

where CL caused by *L. tropica* and *L. infantum* is highly endemic (Table 1).

In Central Tunisia, it was reported that the prevalence of *L. major* in *Psammomys obesus* and *Meriones shawi* species ranged from 5% to 33% according to the methods used (Ghawar *et al.*, 2011). Three *Leishmania* species (*L. infantum, L. major, L. tropica*) coexist in Morocco and the small rodent *Meriones shawi* is found to be a rodent reservoir for *L. major* (Kahime *et al.*, 2014). In a recent study carried out in western Morocco, 18 (9.13%) out of 197 animals belonging to ten species were found positive for *Leishmania* DNA. Sixteen (six *Rattus rattus*, nine *Mus musculus,* and one *Rattus norvegicus*) and two (*Mus musculus*) positive samples were identified as *L. infantum* and *L. tropica*, respectively (Echchakery *et al.*, 2017). In the present study, we used Realtime kDNA PCR for *Leishmania* DNA detection and a second Realtime PCR targeting ITS1 region was performed only for positive samples. Eight positives (0.88%) were found among 906 samples. Only one *Meriones* specimen *(M. tristami)* was found positive while none of the *Mus* and *Rattus* specimens was found positive (Table 1).

Besides dogs are the main reservoirs for *L. infantum*, CanL itself is an important veterinary health problem in southern European countries. The studies on the reservoir potential of rodents for leishmaniasis are very limited in European countries.

In Greece, 97 small rodents in urban and rural areas were collected and liver, spleen, and blood samples were taken for investigation by parasitological, serological, and molecular techniques. Sixteen (16/66; 24%) *Mus musculus* and 3 (3/12; 25%) *Rattus rattus* were found positive by molecular and serological tests while they were negative in smears. The researchers pointed out that the parasite load could be very low in the tissues (Tsakmakidis *et al.*, 2017). In Portugal, the presence of *L. infantum* DNA in *M. musculus* and *R. norvegicus* with infection rates of 33.3% in both animals was reported. The authors pointed out that *Leishmania* DNA was found especially in the skin samples taken from the ear lobe and this was a suitable place for sand flies to get infected (Helhazar *et al.*, 2013). In a similar study in Italy, the positivity rate was reported as 15.5% among 78 captured *R. rattus* samples in Montecristo island (Zanet *et al.*, 2014). In our study, among 100 specimens belonging to *Mus* and *Rattus* genera, all of them were found negative. Because of 56% of these animals were from urban sites of CL endemic areas, the possibility to detect *Leishmania* DNA was very low. On the other hand, the rodent species of both genera were found positive in different countries, are very wild spread in all regions of Turkey, and this should be considered for the risk estimations and management. In rural areas, considering that the rodent burrows are one of the most suitable living and breeding places for sand flies, it should be known that the parasite is constantly circulated in the environments that can allow limited interventions, and it has a zoonotic cycle among small rodents.

In a study conducted in Granada, Spain, the tissue samples (liver, spleen, blood, skin and bone marrow) from 37 rodent specimens were investigated and ten (27%) animals were found positive by parasitological and/or molecular techniques. Besides three *R. rattus* and two *M. musculus* specimens, five *Apodemus sylvaticus* were also reported as positive (Navea-Perez *et al.*, 2015). In the present study, 431 *Apodemus* spp. (62 *A. mystacinus*) were included in the analysis and only 66 (44 *A. mystacinus)* of them were from CL endemic areas. However, five *L. infantum* and one *L. major* positive *Apodemus* sp. samples were from CL non-endemic but VL sporadic province, Zonguldak. Only eight CL cases were reported from this province in the last 22 years (species and origin of the cases were unclear, Özbel Y, personal comm) while sporadic human VL cases have been reporting for decades. The prevalence of CanL was also reported as 8% from that region previously (Özbel *et al.*, 2002). In Turkey as in other endemic countries, VL circulation among humans continues together with higher percentages of CanL. Therefore, this area is accepted as endemic for CanL. The positivity rate was 1.39% (6/431) in total and 1.70% (6/351) among the samples collected only in Zonguldak province suggesting *Apodemus* species probably has a role as reservoir animal of *Leishmania* spp. in the region; even it needs to be supported by further studies such as isolation of the parasites and experimental studies for showing reservoir potentials.

Concluding remarks

This study reports for the first time in Turkey, the natural infection of *L. infantum, L. major* and *L. tropica* in small wild rodents, suggesting these animals possibly have a role as reservoirs in the life cycle of all three *Leishmania* species in Turkey. The molecular detection of three *Leishmania* species in the present study in highly endemic areas for CL such as Adana and Hatay provinces and also in an endemic area for VL such as Zonguldak shows the possible zoonotic cycle including small wild rodents in addition to the zoonotic cycle of *L. infantum* in the dogs. Infected rodents with *Leishmania* may increase the risk not only for humans but also for dogs in rural and urban areas. And this suggests the possible involvement of other wide-spread rodent species in the studies that will be carried out in other endemic areas in Turkey.

Further studies are needed not only for planning epidemiological studies but also for developing rodent control measures in potential endemic areas to break the transmission cycle.

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Conflicts of interest. The authors declare there are no conflicts of interest.

Ethical standards. Ethical approval was obtained from Dokuz Eylül University Animal Ethical Committee under the registration number of 2011-24-28/12 at the date of December 28, 2011.

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