



Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi* ☆

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ABSTRACT

Transmission of cutaneous leishmaniasis (CL) caused by *Leishmania infantum* was studied in South Anatolia, Turkey. Small, non-ulcerating lesions prevailed and patients were negative in rK39 tests for antibody detection for human visceral leishmaniasis (VL). The most abundant sand fly species, *Phlebotomus tobbi*, was found positive for *Leishmania* promastigotes with a prevalence of 1.4% (13 out of 898 dissected females). The isolated strains were identical with those obtained from patients with CL and were typed as *L. infantum*. Phylogenetic analysis revealed similarity to MON-188 and a clear difference from the MON-1 clade. Blood-meal identification showed that *P. tobbi* feeds preferentially on cattle and humans. This finding, the high number of CL patients and relative scarcity of dogs in the focus, suggests that the transmission cycle could be anthroponotic.

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1. Introduction

Leishmaniasis are diseases with a wide spectrum of clinical forms, from relatively mild cutaneous lesions to life-threatening visceral diseases. Their causative agents, protozoans of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), are transmitted by phlebotomine sand flies (Diptera: Psychodidae).

In Turkey, visceral leishmaniasis (VL) is caused by *Leishmania infantum* and affects mainly children (Ozcel et al., 1999; Ok et al., 2002; Tanir et al., 2006). Cutaneous leishmaniasis (CL) is endemic in several regions of Turkey. It has been attributed almost exclusively to *Leishmania tropica* although sporadic occurrence of *Leishmania major* has been also reported (Akman et al., 2000). The biggest outbreak with over 11,000 reported cases occurred recently in Sanliurfa (Ok et al., 2002) and the parasite has been typed as *L. tropica* (Gramiccia et al., 1991; Waki et al., 2007). However, several other foci exist including Cukurova, a part of the Mediterranean region with Adana being the capital. Since 1985, thousands of new CL cases have emerged there and it has been suggested that the causative agent is *L. tropica* (Ok et al., 2002; Uzun et al., 1999).

In parallel, vectors of *Leishmania* have been investigated in CL foci in Turkey (Volf et al., 2000; Simsek et al., 2007) but infected sand flies were never found.

We performed a study on humans and sand flies in the CL focus in Cukurova region where, according to the local health centers, hundreds of human cases continue to occur every year. Surprisingly, the causative agent of human CL was identified as *L. infantum*. We also found 13 *Phlebotomus tobbi* infected with *L. infantum*, and demonstrated that human isolates of *L. infantum* from the study area are identical to isolates from *P. tobbi*.

2. Materials and methods

2.1. Study area

The study was performed in two adjacent areas in the North part of Cukurova region, south Anatolia, during 2005–2007. South Anatolia is bound by the West Taurus Mountains, the mountain ranges of Taurus and anti-Taurus to the North, and the Amanos Mountains to the East (Fig. 1). The Western part of the focus comprises five villages approximately 55 km northeast of Adana city with an altitude of 150–280 m above sea level: Tepecikören (37°21'46"N, 35°37'40"E), Zerdali, Camili, Aydın and Otluk. The local centre of the Eastern part of the focus is Boyali (37°17'56"N, 36°21'33"E) situated 30 km northeast of Osmaniye

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under Accession Nos. EU545236–EU545255.

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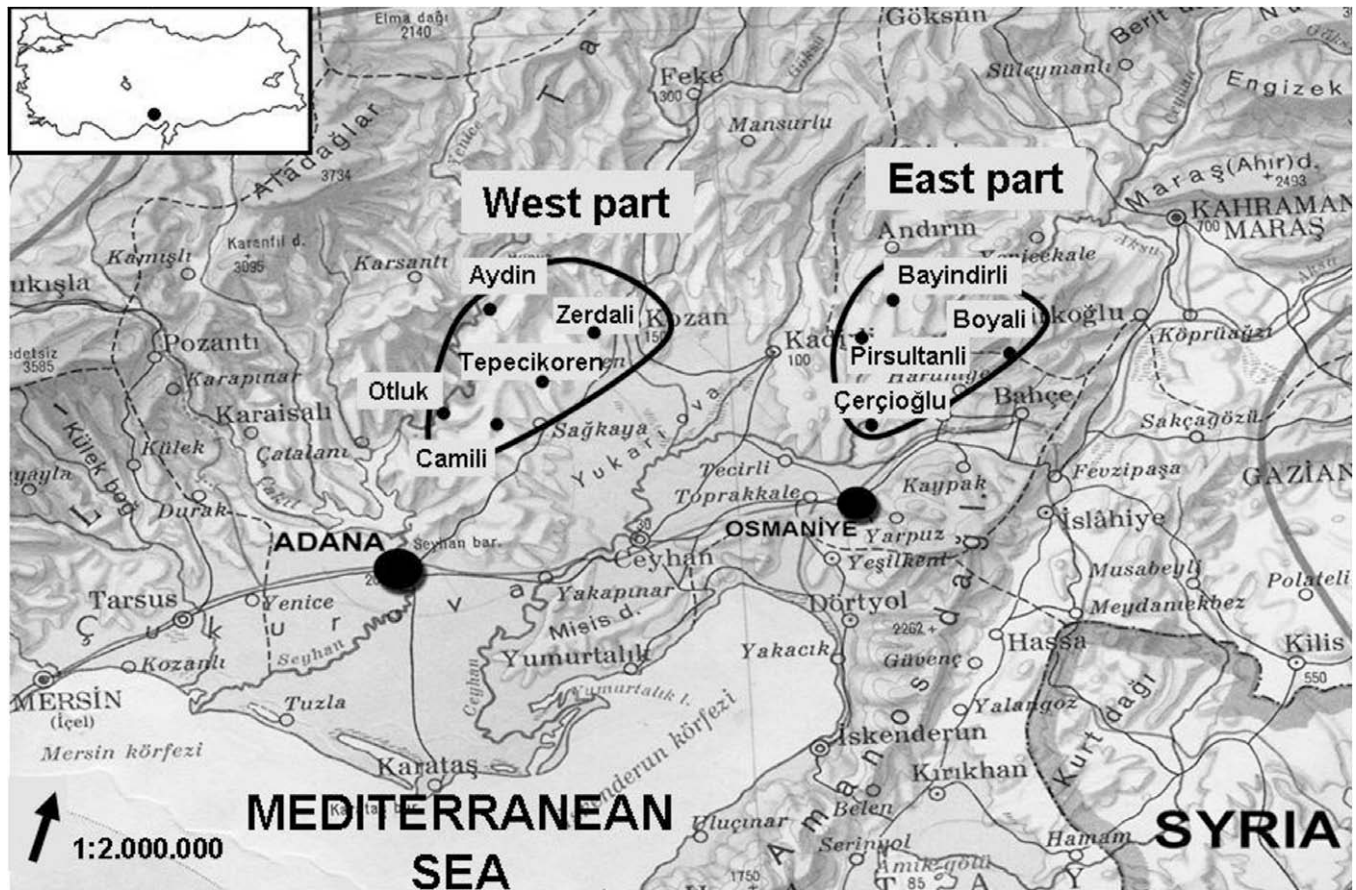


Fig. 1. Map of western and eastern parts of the study area.

city and surrounded by several other villages (Alibozlu, Bayandirli, Cercioğlu, Oluklu, Pirsuntanlı and Karakuyu). The altitudes range from 205 to 320 m above sea level. Most of the area in both parts of the focus is fertile (“mollisol” soil) and used for agricultural activities, but young *Pinus* (pine-tree) and *Abies* (fir-tree) forests are also cultivated. Citrus orchards and cotton fields are more common in the Eastern part. The mean annual precipitation is 636.8 mm (West) and 761.3 mm (East) with 66% relative humidity and the mean annual temperature is 18.7 °C (West) and 19.6 °C (East). Residents live in single-family houses built from briquette, adobe, stone and cement, surrounded by gardens with henhouses and sheep or cattle sheds. Treatment of patients with CL is provided in regional health centres (Kozan in the West and Duzici in the East) by multiple weekly injections of Glucantime (meglumine antimoniate) for at least 2 months.

2.2. Sand fly collection, dissection and identification

Sand flies were collected in September 2005, 2006 and 2007, mostly using Centres for Disease Control (CDC) light traps (John W. Hock, USA) placed inside the houses, animal shelters and in house yards. A small proportion of sand flies inside the houses and shelters were collected by hand aspirators. Dead females and all males were stored in 70% ethanol for morphological identification. In males of *Phlebotomus* cf. *syriacus*, the thorax was used for DNA extraction and molecular identification based on sequencing of internal transcribed spacer 2 (ITS2), 18S rRNA, and cytochrome *b* (*cyt b*) genes (see below).

Live sand fly females were immobilized on ice, rinsed briefly in 96% ethanol, washed and dissected in 0.9% sterile saline. The head and genitalia were used for identification and the gut was examined microscopically for the presence of promastigotes. In blood-fed females the gut with the rest of the blood-meal was smeared on a piece of filter paper (Whatman 3; Whatman, Brentford, UK), allowed to dry and then stored at –20 °C until use.

Upon microscopical detection of *Leishmania* infection, the material from the slide was divided into two parts. The first was inoculated into glass vials (2.5 ml) containing SNB-9 blood agar made from defibrinated rabbit blood and overlaid with 1:1 RPMI 1640/Schneider insect medium, supplemented with 10% FCS (Sigma or Gibco), 2% sterile human urine, 10,000 IU penicillin (Biotika), 100 µg/ml amikacin (Bristol-Myers Squibb) and 1500 µg/ml 5-fluorocytosine (Sigma). The second was soaked on a piece of filter paper (Whatman 3) for DNA extraction and *Leishmania* identification as a backup in case of unsuccessful cultivation.

2.3. Sampling from patients

Patient sampling was performed in cooperation with local medical doctors. Tissue samples were obtained from lesions by insulin syringe aspiration and immediately inoculated into 2.5 ml glass vials with SNB-9 (as above). In addition, blood plasma was taken from 26 patients with well-developed lesions to detect the presence of antibodies against causative agents of VL by DiaMed-IT LEISH (DiaMed, Switzerland) immuno-chromatographic dipstick test using the recombinant antigen K39. Two sera of VL-positive

patients kindly provided by J.-C. Dujardin were used as positive controls.

2.4. DNA extraction

Extraction of total DNA from the material preserved in ethanol (sand fly thorax), filter papers (midguts of blood-fed or infected females), and from successfully established cultures (exponential phase of growth) was performed using a DNA extraction kit (Roche, France) according to the manufacturer's instruction. For the analysis of sand fly blood-meals, the total DNA from the filter papers was extracted using standard proteinase K digestion (20 mg/ml; 55 °C overnight). The lysate was extracted with Tris-buffered phenol (pH 8), followed by extraction with phenol–chloroform. Nucleic acid from the aqueous phase was precipitated with ethanol and resuspended in 100 µl of redistilled water.

2.5. Leishmania typing

PCR-restriction fragment length polymorphism (PCR-RFLP) was performed with LITSR and L5.8S primers (El Tai et al., 2000) to obtain the ITS1 region. The PCR were performed in 50 µl total volumes of reaction mix (Combi PPP Master Mix, Top-Bio, Czech Republic) using the following conditions: initial denaturation at 94 °C for 2 min followed by 32 amplification cycles (94 °C for 20 s, 53 °C for 30 s, 72 °C for 1 min); and 72 °C for 6 min. The PCR products were restricted by *Hae*III enzyme for 1 h in 60 °C and visualised by electrophoresis with 2% Metaphor agarose gel (Schönian et al., 2003). The following reference strains were used: *L. infantum* (MCAN/IL/1999/LRC-L760, MHOM/TR/2000/OG-VL, MHOM/TR/2003/Adana(dot), *L. tropica* (MHOM/SU/1974/K27) and *L. major* (MHOM/IL/1967/LRC-L137 Jericho II).

PCR-based multilocus sequence typing (MLST) was used to distinguish between *L. infantum* and *Leishmania donovani* and for more accurate identification of *Leishmania* strains (Zemanová et al., 2007). We focused on sequence diversity of five enzymes: isocitrate dehydrogenase (*icd*), cytosolic malic enzyme (*me*), mannose phosphate isomerase (*mpi*), glucose-6-phosphate dehydrogenase (*gpi*) and fumarate hydratase (*fh*). Our isolate from a patient from the Western part of the focus (MHOM/TR/2005/CUK1) was compared with one sand fly isolate from the Western part (ITOB/TR/2005/CUK2) and one isolate from the Eastern part (ITOB/TR/2007/CUK10). In addition, a viscerotropic isolate MHOM/TR/2000/OG-VL originating from Hatay, South Anatolia, was sequenced. PCR products of expected size were purified from the gel using QIAquick gel extraction kits (Qiagen, Germany) according to the manufacturer's instructions and sequenced directly on an automated DNA sequencer (310 Genetic Analyzer; ABI Prism) using the BigDye 3.1 kit (Applied Biosystems, USA). The obtained sequences were deposited in GenBank under the following Accession Nos. EU545236–EU545255. Partial sequences were assembled manually using Seq-Man (DNASStar). Maximum likelihood (ML),

maximum parsimony (MP) and neighbor-joining (NJ) were calculated by PhyML.win32 (Guindon and Gascuel) and PAUP* 4.0b10 (Swofford) phylogenetic software.

2.6. Blood-meal identification

In 2005 and 2006, blood-meals were identified using the modified vertebrate-universal specific primers *cytB1-F* (5'-CCA TCC AAC ATY TCA DCA TGA TGA AA-3') and *cytB2-R* (5'-GCH CCT CAG AAT GAT ATT TGK CCT CA-3') (Kocher et al., 1983; Malmqvist et al., 2004) to amplify a 305 bp segment of the *cyt b* gene from host mtDNA. PCR was performed in a 25 µl vol. using a reaction mix. PCR products were checked on a 1.5% agarose gel; amplified fragments were isolated and sequenced as described above. Sequence analyses were performed using DNASStar software; sequences were compared with sequences deposited in the GenBank database using standard nucleotide BLAST searches.

2.7. Identification of *P. syriacus*

The identification of captured specimens of *P. syriacus* was based on sequencing of a 450 bp variable region of 18S rRNA and complete sequences of ITS2 and *cyt b*. A variable region of the 18S rRNA gene (Forward primer 5'-GTGCGGGTTTGCATTACTT-3', Reverse primer 5'-AACATCCTTGCAAAATGTT-3'), ITS2 (Depaquit et al., 2002) and *cyt b* (Forward primer 5'TATGTACTACCATGAGG ACAAATATC-3', Reverse primer 5'-GCTATTACTCCYCTAACTTRTT-3', modified from (Essegheir and Ready, 2000)) were amplified from 10 randomly selected specimens of *P. cf. syriacus*. Six specimens from Israel (*P. syriacus*) and four from Italy (*Phlebotomus neglectus*) were used for comparison. PCR was performed in a 50 µl vol. using a reaction mix with the following cycling profile for SSU: initial denaturation at 94 °C for 2 min followed by 25 amplification cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min). PCR product was sequenced as described above. Obtained sequences were aligned and compared by ML and MP methods calculated by PhyML and PAUP with known sequences of *P. neglectus* (GenBank Accession Nos. AJ244367–74, AF161188–91 and AF205524) and *P. syriacus* (GenBank Accession No. AJ244375–6) and with sequences obtained from control specimens originating from Israel (*P. syriacus*) and Italy (*P. neglectus*).

3. Results

3.1. Phlebotomine fauna

In total, seven sand fly species were identified (five in the Western part and four in the Eastern part of the focus), in both parts the most abundant being *P. tobbi* (Table 1). Interestingly, *Phlebotomus papatasi* and *Phlebotomus perfiliewi* were found only in the Western part while *Phlebotomus simici* was present only in the Eastern part of the focus. For *P. cf. syriacus* the morphological examination and

Table 1
Phlebotomine sand flies in the study area

| Species | Western part (2005) | | Eastern part (2006–2007) | |
|---|---------------------|-------------|--------------------------|-------------|
| | n (%) females | n (%) males | n (%) females | n (%) males |
| <i>Phlebotomus (Larrousius) tobbi</i> | 428 (77.7) | 604 (86.4) | 470 (81.2) | 572 (81.5) |
| <i>Phlebotomus (Larrousius) syriacus</i> | 3 (0.5) | 16 (3.3) | 24 (4.1) | 52 (7.4) |
| <i>Phlebotomus (Larrousius) perfiliewi</i> | 3 (0.5) | 6 (0.9) | 0 | 0 |
| <i>P. (Larrousius) sp.</i> (unidentified) | 28 (5.1) | 0 | 16 (2.8) | 0 |
| <i>Phlebotomus (Phlebotomus) papatasi</i> | 85 (15.4) | 67 (9.6) | 0 | 0 |
| <i>Phlebotomus (Paraphlebotomus) sergenti</i> | 4 (0.7) | 6 (0.9) | 67 (11.6) | 71 (10.1) |
| <i>Phlebotomus (Adlerius) simici</i> | 0 | 0 | 2 (0.3) | 7 (1.0) |
| Total | 551 | 699 | 579 | 702 |

measurements of male genitalia did not result in identification as the specimens were of intermediate phenotype between *P. syriacus* and *P. neglectus* (length of coxite: average 0.37, continuous range 0.32–0.43 mm; length of style: average 0.17, continuous range 0.15–0.21 mm). Phylogenetic and statistical analysis of 18S rRNA, ITS2 and *cyt b* genes revealed that all studied samples from Cukur-ova were significantly closer to *P. syriacus* (genetic *p*-distance means were 0.00, 0.01 and 1.15, respectively) than to *P. neglectus* (*p*-distance means were 0.01, 5.45 and 3.55, respectively).

3.2. *Leishmania*-infected flies

Out of 1,130 females dissected, 13 (1.1%) had promastigotes in their gut (two out of 551, six out of 228, and five out of 351 dissected females in 2005–2007, respectively). Nine (one in 2005, five in 2006, and three in 2007) of these infections were mature with parasites localised on the stomodeal valve; however in one case the midgut contained fresh blood from the subsequent blood-meal. In four (one in 2005 and 2006, and two in 2007) blood-fed females *Leishmania* parasites were found within the blood-meal. Isolates were obtained from 11 females, two from the Western (in 2005) and nine from the Eastern (five in 2006 and four in 2007) part of the focus. All infected females belonged to *P. tobbi*. *Leishmania* prevalence in *P. tobbi* was 0.7%, 2.8% and 1.9% in 2005–2007, respectively.

3.3. Human isolates

We obtained inocula from 128 people with putative CL (93 and 35 from Western and Eastern parts, respectively). In six cultures, promastigotes appeared in subsequent microscopical controls; however, only one isolate resulted in a thriving culture. In total, 26 patients with well-developed lesions were tested by dipstick test; however no positive result was obtained. Lesions occurring in patients within the study area are presented on Fig. 2. In most cases, lesions were non-ulcerating and relatively small (1–2 cm in diameter). Questioning the patients revealed that lesions lasted for at least 2 years and approximately one-third of patients had more than one lesion. In a few cases, ulceration or spontaneous healing was observed (Fig. 2).

3.4. Parasite typing

PCR-RFLP of ITS1 was successfully used for typing of a human isolate, 11 isolates from sand flies, and two DNA samples from promastigote-positive sand fly females. All samples showed the same pattern on electrophoresis and belonged to the *L. donovani*/*L. infantum* group (Fig. 3). The MLST assay determined parasites as *L. infantum* and sequences of five analysed genes of all three strains (CUK1, CUK2 and CUK10) were identical. Comparison with

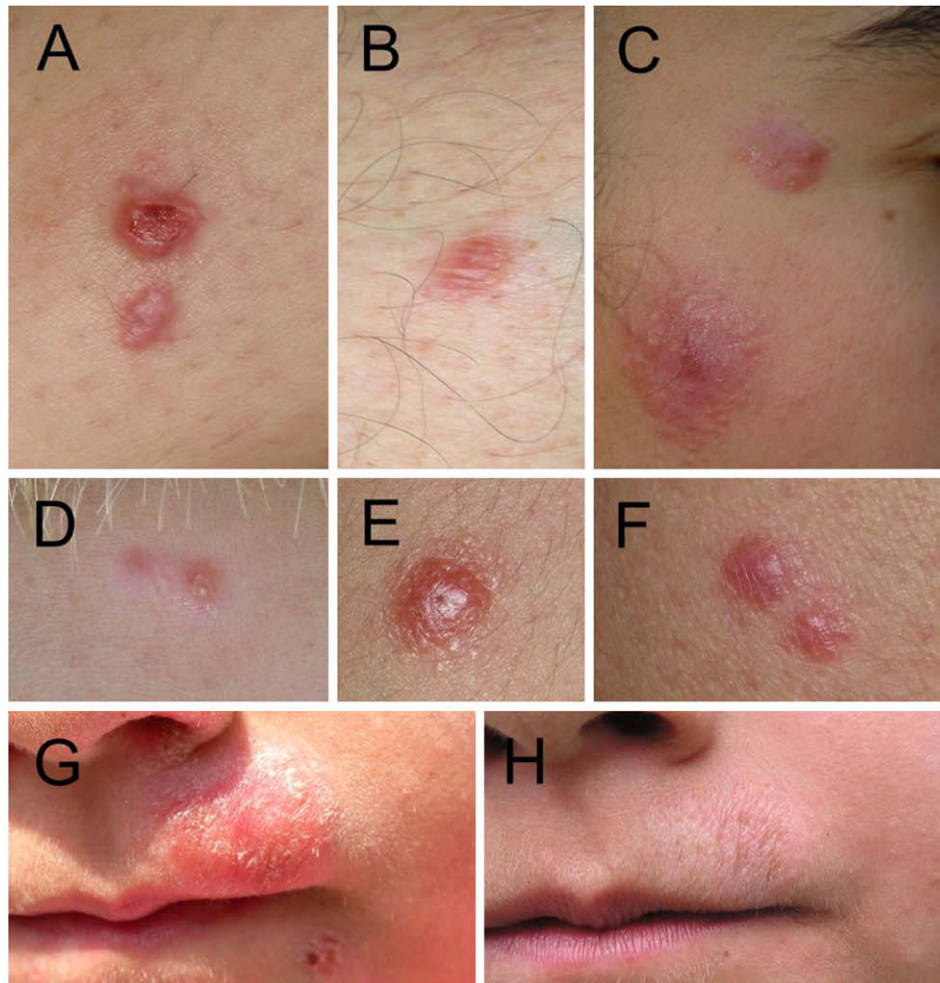


Fig. 2. Lesions occurring in patients in both parts of the study area: (A) active protuberant lesions with ulceration on left shin of 16 year old girl; (B) flat lesion about 2-years-old on left shin of 43-year-old woman; (C) atypical large lesion on right cheek and temple of 14-year-old girl; (D and E) lesions from patients culture-positive for *Leishmania* promastigotes on left thigh of 11-year-old boy and on forehead of 5-year-old boy; (F) typical lesions without ulceration on left calf of 54-year-old woman; (G and H) healing of expanded lesion in 10 year young girl, during and 2 years after treatment.

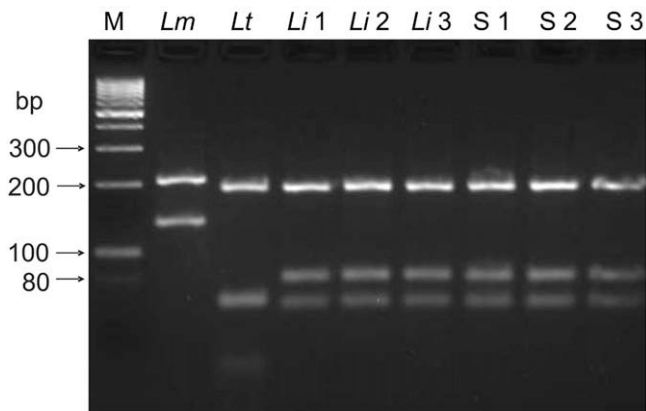


Fig. 3. PCR-restriction fragment length polymorphism of ITS1 fragments of DNA digested with HaeIII. M, 100 bp DNA Ladder Plus (Fermentas); Lm, *Leishmania major* (MHOM/IL/1967/LRC-L137 Jericho II); Lt, *Leishmania tropica* (MHOM/SU/1974/K27); Li 1, *Leishmania infantum* (MCAN/IL/1999/LRC-L760); Li 2, *Leishmania infantum* (MHOM/TR/2004/CL); Li 3, *Leishmania infantum* (MHOM/TR/2000/OG-VL); S1, (MHOM/TR/2005/CUK1); S2, (ITOB/TR/2005/CUK2); S3, (ITOB/TR/2007/CUK10).

other *L. infantum* strains typed by Zemanová et al. (2007) and Turkish isolate MHOM/TR/2000/OG-VL typed by us revealed highest similarity of CUK's strains to *L. infantum* strain MHOM/IT/93/ISS800 (MON-188) (Fig. 4).

3.5. Blood-meal identification

In 2005 and 2006, out of 779 dissected females of five sand fly species, 267 individuals (34%) were engorged with blood. Sequenc-

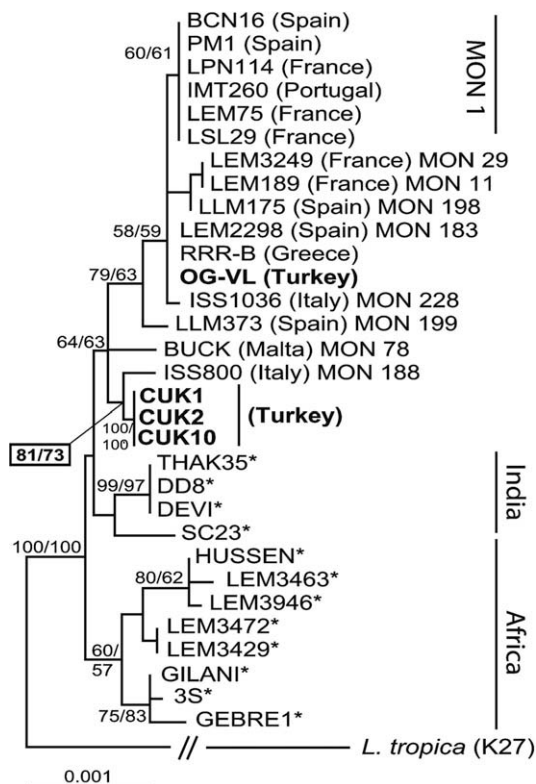


Fig. 4. Phylogenetic analysis of available strains belonging to the *Leishmania donovani* complex based on sequences of the *me*, *mpi*, *g6pdh*, *icd* and *fh* genes. The maximum likelihood (ML) tree (GTR model) was constructed from a concatenated dataset (7614 characters, 161 parsimony-informative) of 32 strains. Numbers above branches represent bootstrap support (ML/maximum parsimony). Strains considered to be *L. donovani sensu stricto* are marked by *. Strains in bold were sequenced by us.

Table 2

Identification of sand fly blood-source in 2005–2006

| Sand fly host | TOB | PAP | LAR | SER |
|----------------|-------------------------|-----|-----|-----|
| Cattle | 159 (70.7%) | 11 | 6 | 0 |
| Human | 23 ^a (10.2%) | 8 | 0 | 0 |
| Chicken | 5 (2.2%) | 2 | 2 | 1 |
| Goat | 1 (0.4%) | 0 | 0 | 0 |
| Mouse | 1 (0.4%) | 0 | 0 | 0 |
| Vole | 1 ^b (0.4%) | 0 | 0 | 0 |
| Not identified | 35 (15.6%) | 8 | 3 | 1 |
| Total | 225 | 29 | 11 | 2 |

TOB, *Phlebotomus tobbi*; PAP, *Phlebotomus papatasi*; LAR, *Phlebotomus* sp. (*Larrousius*); SER, *Phlebotomus sergenti*.

^a Include three double meals: cattle–human.

^b One double meal: cattle–vole.

ing of the amplified part of the *cyt b* gene was found to be a very sensitive method with an efficiency level of almost 85% and the source of the blood-meal was determined in 220 specimens. Five mammalian and one avian species were identified as hosts: cattle, human, goat, mouse (*Mus* sp.), vole (*Microtus* sp.) and chicken. The identity of blood-meals of four sand fly species is presented in Table 2. In four females of *P. tobbi*, mixed blood-meals of human-cattle (three specimens) and vole-cattle (one specimen) were observed. The major blood sources for *P. tobbi* were cattle (70%) and humans (10%). Furthermore, in four *Leishmania*-infected females of *P. tobbi* (one with fresh and three with extensively digested blood) human blood was detected.

4. Discussion

The etiological agent of CL in the Cukurova region was identified as *L. infantum*. Although we have attempted to isolate *Leishmania* from several dozens of patients, only in six samples were promastigotes observed in cultivation medium and one isolate has been established in vitro. *Leishmania infantum* is notoriously difficult to cultivate from cutaneous lesions (Gramiccia et al., 1991). This parasite species has been incriminated for the first time as a causative agent of human CL in France (Rioux et al., 1980). Since then, several other countries in the Mediterranean were added to the list (del Giudice et al., 1998; Serin et al., 2005). Long-term research carried out in the Abruzzi region of Italy showed an intensive CL focus, with thousands of recorded cases caused by dermatotropic *L. infantum* (Corradetti, 1952; Gramiccia et al., 1987; Maroli et al., 1987). Several studies from different areas evaluating the rK39 dipstick test confirmed high sensitivity and specificity of this method for the visceral form of leishmaniasis (Chappuis et al., 2006). Our data obtained by DiaMed-IT LEISH dipstick test clearly demonstrated the non-visceral form of leishmaniasis in tested patients from both parts of the study area.

Molecular methods revealed that isolates from humans and sand flies were identical. Moreover, identical parasites were isolated from *P. tobbi* females in the Eastern part and Western parts of the study area. Our findings suggest that both parts of study area, despite being up to 65 km apart, represent one focus with the same circulating parasite. Phylogenetic analysis based on MLST showed that this *L. infantum* strain is unambiguously distinct from the MON-1 zymodeme as well as from a viscerotropic isolate of *L. infantum* from South-East Turkey. Among accessible isolates typed using the MLST method, our strain is close to MON-188, the *L. infantum* strain isolated by Gramiccia (Pratlong et al., 2003) in Sicily from an HIV-positive patient with visceral leishmaniasis.

Phlebotomus (Larrousius) tobbi was clearly shown to be a vector of CL in the Cukurova focus. Prevalence of infected females was almost 1.5%; out of 898 dissected *P. tobbi* females 13 were positive for *L. infantum* promastigotes. The relatively high *L. infantum*

prevalence in *P. tobbi* coincides with widespread human infection throughout the region.

We have found quantitative and qualitative differences in the phlebotomine fauna in both studied sites of the current focus. Although the sites are less than 65 km apart, *P. papatasi* and *P. perfiliewi* are absent in the Eastern part of the focus while *P. simici* is exclusively present here. General climatic conditions seem to be similar in both parts of the focus; however, local microclimatic differences probably occur. Differences in sand fly fauna composition in close *Leishmania* foci of CL including involvement of different vectors are known in other Mediterranean regions (Svobodová et al., 2006).

Despite the differences in phlebotomine fauna composition, *P. tobbi* is the most dominant species in both parts of the focus. Being a member of the *Larrousius* sub-genus, *P. tobbi* is a suspected vector of *L. infantum*. The only previous isolation of *L. infantum* from this species has been reported from Cyprus. However, human *L. infantum* is rare in Cyprus; only visceral cases are reported and the infected sand fly specimen was caught near an infected dog (Léger et al., 2000).

Blood-meal analysis assigned the close relationship of local *P. tobbi* populations with large ruminants, mainly with cattle. However, human blood was the second major blood source and 10% of females had fed on humans. In addition, human blood was detected in four females infected by *L. infantum*. We speculate that the population of *P. tobbi* in Çukurova region is more anthropophilic than populations from other areas. In Azerbaijan and Cyprus, *P. tobbi* were not attracted to humans (Gasanzade et al., 1990; Léger et al., 2000) and in Albania none of the blood-engorged *P. tobbi* females had fed on humans (Velo et al., 2005). The anthropophily of *P. tobbi* populations in the Cukurova region might be caused by local housing conditions, such as close cohabitation of humans with domestic animals and sleeping outdoors. Leishmaniasis caused by *L. infantum* is usually a zoonosis with dogs being reservoir hosts. Surprisingly, within our study we did not find any dog blood in engorged sand fly females, which corresponds with low numbers of dogs in both sites. Relatively high prevalence of CL in local people allows us to speculate about another transmission cycle and an anthroponotic cycle cannot be excluded.

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