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Leishmaniasis in Turkey: molecular characterization of *Leishmania* from human and canine clinical samples

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Summary Human leishmaniasis, both visceral and cutaneous, and canine leishmaniasis have been reported in Turkey for centuries. However, the advent of new diagnostic tools during the last 30 years has led to the recognition that leishmaniasis is an important public health problem throughout the country. In most disease foci both canine and human leishmaniases exist together and identification of parasite species causing these diseases is a pre-requisite for understanding disease epidemiology. A total of 109 samples obtained from human and canine leishmaniasis cases were examined using internal transcribed spacer 1 PCR followed by restriction fragment length polymorphism analysis. Our results indicate that two species, *Leishmania tropica* and *Leishmania infantum*, are primarily responsible for cutaneous and visceral leishmaniasis, respectively, in Turkey. However, a new focus of human cutaneous leishmaniasis caused by *L. infantum* in Hatay region is described. This finding further stresses the importance of *Leishmania* species molecular characterization in prescribing appropriate therapy and understanding the disease's transmission in different endemic foci.

keywords *Leishmania infantum, Leishmania tropica,* cutaneous leishmaniasis, visceral leishmaniasis, canine leishmaniasis

Introduction

Leishmaniasis is a spectrum of diseases caused by multiple species in more than 88 countries worldwide. The main forms of human disease are cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL) (Desjeux 2004; WHO 2007). As the symptoms of leishmaniasis can vary and are frequently confused with other agents, diagnostic confirmation of the parasite is mandatory. Traditionally, direct detection of parasites is done by microscopic examination of clinical specimens or by cultivation. However, all *Leishmania* are morphologically similar and species identification is not possible using either of these techniques. Therefore, the ability to distinguish between *Leishmania* species is crucial in determining disease prognoses, as well as prescribing appropriate therapeutic regimens since some species are more refractory to treatment than others (Blum & Hatz 2009). Furthermore, knowledge of *Leishmania* species' geographical distribution, reservoir hosts and disease epidemiology is important in developing appropriate control measures (Schönian *et al.* 2003).

Human VL and canine leishmaniasis (CanL), caused by *Leishmania infantum*, are endemic throughout the Marmara, Ege, Black Sea and Mediterranean regions of western Turkey (Figure 1). The incidence of CanL in these regions is much higher than human cases, with infection rates in some dog populations approaching 30% (Ozensoy *et al.* 1998; Ertabaklar *et al.* 2005), similar to several VL foci around the Mediterranean Basin (Gradoni 1995; Papadopoulou *et al.* 2005). In other regions of Turkey these diseases occur only sporadically. Anthroponotic CL caused by *Leishmania tropica* is highly endemic in the Ege, Mediterranean and Southeastern Anatolia regions (Ok *et al.* 2002; Ertabaklar *et al.* 2005). In the eastern Mediterranean region *L. infantum*, in addition to *L. tropica*, has also been reported to cause human CL (Serin *et al.* 2005; Svobodova *et al.* 2009). In addition, CL due to *Leishmania major* is endemic in countries bordering Turkey to the south: Syria, Iraq and Iran (Khiami *et al.* 1991; CDC 2004; Talari *et al.* 2006). Species identification of the agent in Turkey is

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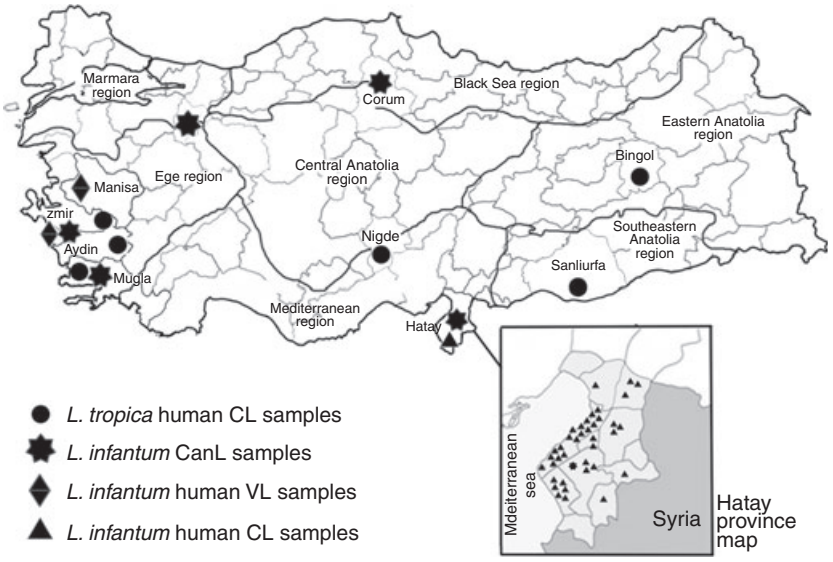

Figure 1 Map of Turkey indicating the origin of the samples analysed in this study by ITS1 PCR and RFLP. CL, cutaneous leishmaniasis and HVL, human visceral leishmaniasis and CanL, canine leishmaniasis.

crucial, since the country comprises seven geographical regions with environmental and ecological differences.

A universal PCR method targeting the internal transcribed spacer 1 (ITS1) region between the SSU and 5.8S rRNA genes was described for the direct diagnosis of different clinical manifestations of leishmaniasis and parasite identification. This method is applicable where several parasite species are aetiologically relevant. It is highly specific and sensitive able to detect approximately 0.2 parasites per sample (Schönian *et al.* 2003). Most of the medically important *Leishmania* species are then readily distinguished by DNA sequence or restriction enzyme analysis of the PCR product (Schönian *et al.* 2000, 2001, 2003; Davila & Momen 2002). ITS1 PCR restriction fragment length polymorphism (RFLP) can be used for direct species identification in patient tissues, blood or other samples without prior parasite culturing, microscopic analysis or other technique. In the present study, we analysed different specimens by ITS1 PCR RFLP collected from patients with human VL and CL, as well as CanL, in different regions of Turkey. We further show that species identification is extremely important in cases of CL since both agents *L. infantum* and *L. tropica* are present in the same regions.

Materials and methods

Samples

Specimens (n = 109) received from various localities in Turkey between the years 2006 and 2008 (Table 1 and supplemental data) were used in this study and included culture promastigotes (n = 52), tissue smears on slides (n = 40), peripheral blood (n = 16) and buffy coat (n = 1). Slides and blood samples collected in EDTA tubes were stored at −20 °C prior to DNA extraction. Diagnostic assessment by the methods employed in this study are part of the standard clinical procedures at the Ege University Medical School, Department of Parasitology, Turkey, and all patients gave informed consent.

DNA extraction

DNA extractions were performed using a commercial kit on 200 μl of cultured promastigotes, blood samples or tissue material scraped from slides and diluted with physiological saline according to the manufacture's protocols (High Pure PCR Template Preparation Kit, Roche®).

ITS1 PCR RFLP analysis

The rRNA ITS1 region was amplified using primers LITSR and L5.8S as described (Schönian *et al.* 2001, 2003; Bensoussan *et al.* 2006; supplemental data), except that we used 0.6 nM primers and PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem, Israel) in 25 μl total reaction. Leishmanial DNA's (20 ng) isolated from the following reference strains *L. major* (MHOM/P-S/1998/ISL389), *L. tropica* (ISER/IL/1998/LRC-L747), *Leishmania donovani* (MHOM/IN/1980/DD8) and *L. infantum* (MCAN/IL/1997/LRC-L720) were provided by the WHO Reference Centre, Jerusalem, Israel. These DNAs were used as a positive controls and leishmanial species DNA markers. Reaction buffers without

leishmanial DNA were also included as negative controls in each PCR analysis. Five μl of purified DNA was added to the PCR mixture. Amplicons were analysed on 1.5% agarose gels by electrophoresis at 100 V in Tris–acetate–EDTA buffer (0.04 M Tris–acetate and 1 mM EDTA, pH 8.0) and visualized by UV light after being stained with ethidium bromide. A reaction was considered positive when a band of correct size (300–350 bp) was observed. PCR products (17 μl) were digested with *Bsu*RI (MBI Fermentas), an isoschizomer of *Hae*III, according to the manufacturer's instructions, and the restriction fragments were analysed by gel electrophoresis at 120 V in 1 × Tris–acetate–EDTA buffer on 2.5% agarose gels (FMC BioProducts, Rockland, ME, USA). The fragments were visualized by UV light and the sizes of the restriction products are compared with DNA of reference *L. infantum*, *L. major*, *L. tropica* and *L. donovani* strains (Schönian *et al.* 2003).

Sequence analysis

DNA sequence analysis in both directions of the ITS1 PCR products from two human CL patients (SM2 and SM4) and one CanL sample (CanL-C55) from the Hatay province were performed by a commercial company (RefGen, Ankara, Turkey, http://www.refgen.com) and examined by BLAST using the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These ITS1 sequences were compared by multi-alignment (ClustalW2, http://www.ebi.ac.uk/Tools/clustalw2/index.html) with DNA sequences from three reference strains, two *L. tropica* (Accession No. EU683618: MHOM/PS/2000/GOKS23, LRC-L784 and Accession No. EU683617: ISER/IL/1998/LRC-L758) and one *L. infantum* (Accession No. AJ000289: MHOM/TN/1980/IPT1). ITS1 DNA sequences from *L. tropica* Turkish patient samples included for comparison are given in the results section.

Results

Samples (n = 109) from seven regions in Turkey were analysed by ITS1 PCR and RFLP (Table 1 and supplemental data). All 109 specimens tested positive by ITS1 PCR and gave one 300–350 bp amplicon of expected size when examined by gel electrophoresis (data not shown). Control reactions without DNA were negative.

The PCR products were further characterized by digestion with the restriction enzyme *Bsu*RI. The RFLP patterns obtained for all human VL and CanL specimens were identical to that seen with a *L. infantum* reference strain (Figure 2). However, RFLP patterns for samples analysed from CL patients showed that two *Leishmania* species,

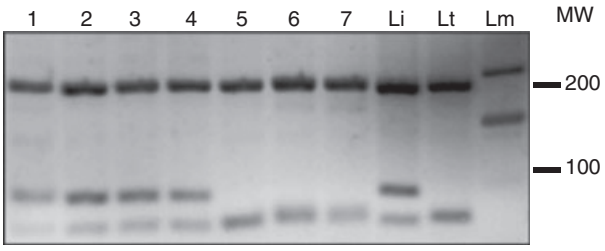

Figure 2 Restriction fragment length polymorphism (RFLP) analysis of amplicons obtained from Turkish human and dog samples following ITS1 region polymerase chain reaction (PCR). The PCR product was digested with the restriction enzyme *Bsu*RI, an isoschizomer of *Hae*III, and separated by electrophoresis on 2.5% agarose gels. *Leishmania* reference strains: Lm, *L. major* (MHOM/PS/1998/ISL389); Lt, *L. tropica* (ISER/IL/1998/LRC-L747) and Li, *L. infantum* (MCAN/IL/1997/LRC-L720). Lanes 1–7, clinical samples. *L. infantum*: lane 1, CL from Hatay (MHOM/TR/2006/SM 02); lane 2, CanL from Hatay (MCAN/TR/2007/EP134, C55); lane 3, CanL from Mugla (MCAN/TR/2005/EP126); lanes 4, Human VL from Aydin (MHOM/TR/2001/EP59); lane 5, CL from Aydin (MHOM/TR/2005/EP122); lane 6, CL from Sanliurfa (MHOM/TR/2004/EP95); lane 7, CL from Nigde (MHOM/TR/1996/EP20). MW, molecular weight markers (bp).

L. tropica or *L. infantum,* were responsible for CL disease in Turkey (Table 1, Figure 2 and data not shown).

Human CL samples from Eastern and Southeastern Anatolia regions were typed exclusively as *L. tropica*. Likewise, samples from CL patients in Ege, Central Anatolia and Marmara regions were exclusively *L. tropica,* even though human VL and CanL caused by *L. infantum* was present in the same areas. Surprisingly, in Hatay province, Eastern Mediterranean region, samples collected from human CL patients, as well as a dog with VL, were both *L. infantum* (Figure 2).

Sequence analysis

DNA sequence analysis of the ITS1 PCR products of two human CL patients (SM2 and SM4) and one CanL sample (CanL-C55) from Hatay province was carried out in order to confirm the RFLP identification. Overlapping regions from all three sequences were identical over 143 bp (Figure 3). Blast analysis also showed 100% identity over 143 bp with 27 additional *L. infantum* ITS1 DNA sequences deposited in the Genebank (data not shown).

Multiple alignment of the ITS1 DNA sequences from Hatay province, human CL and CanL samples, with reference *L. tropica* and *L. infantum* strains from Israel/Palestine, Turkey and Tunisia, confirms the RFLP analysis. DNA sequences for both Hatay CL strains showed

Table 1 Summary of samples analysed by ITS1 polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP)

Region†	Locality	Clinical‡	PCR-RFLP	Type of specimen				Total
				Culture	Smear	Blood	Buffy coat	
CA	Nigde	HCL	*L. tropica*	2	–	–	–	2
	Corum	CanL	*L. infantum*	1	–	–	–	1
EA	Bingol	HCL	*L. tropica*	1	–	–	–	1
Ege	Aydin	HCL	*L. tropica*	11	–	–	–	11
	Aydin	HVL	*L. infantum*	1	–	–	–	1
	Aydin	CanL	*L. infantum*	7	–	–	–	7
	Izmir	HCL	*L. tropica*	1	–	–	–	1
	Manisa	HVL	*L. infantum*	1	–	–	–	1
	Mugla	HCL	*L. tropica*	1	–	–	–	1
	Mugla	CanL	*L. infantum*	3	–	16	1	20
EM	Hatay	HCL	*L. infantum*	–	40	–	–	40
	Hatay	CanL	*L. infantum*	1	–	–	–	1
M	Bilecik	CanL	*L. infantum*	1	–	–	–	1
SE	Sanliurfa	HCL	*L. tropica*	21	–	–	–	21
	Total			52	40	16	1	109

†CA, Central Anatolia region; EA, Eastern Anatolia region; Ege, Ege region; EM, East Mediterranean region; M, Marmara region; SE, Southeastern region.
‡HCL, human cutaneous leishmaniasis; HVL, human visceral leishmaniasis; CanL, canine leishmaniasis.

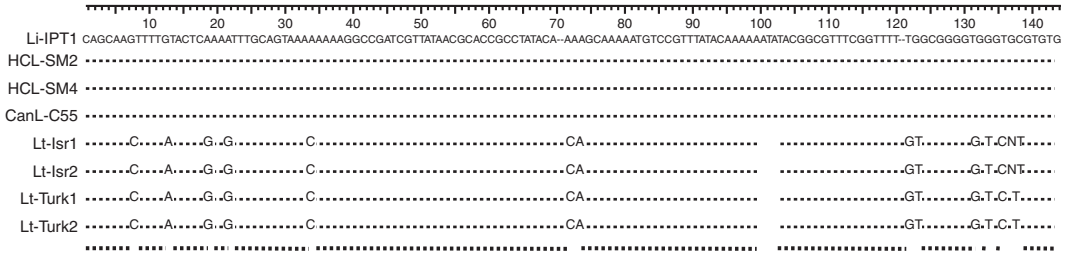

Figure 3 Multiple alignment of the ITS1 DNA sequences from patients with cutaneous leishmaniasis (CL) in Hatay province, and reference *L. tropica* and *L. infantum* strains. The 143 bp DNA sequence from the ITS1 region was aligned using Clustal W2 (ClustalW2, EMBL-EBI, http://www.ebi.ac.uk/Tools/clustalw2/index.html). Reference strains: *L. infantum*, Li-IPT1 (Accession No. AJ000289; MHOM/TN/1980/IPT1); *L. tropica*, Lt-Isr1 (Accession No. EU683618; MHOM/PS/2000/GOKS23, LRC-L784), Lt-Isr2 (Accession No. EU683617; ISER/IL/1998/LRC-L758), Lt-Turk1 (Accession No. FJ940894, MHOM/TR/2004/EP94), Lt-Turk2 (Accession No. FJ940895, MHOM/TR/2007/EP141). Hatay region: human CL samples, HCL-SM2 (MHOM/TR/2006/SM 02, Accession No. FJ94092) and HCL-SM4 (MHOM/TR/2005/SM 04, Accession No. FJ940893); CanL dog isolate, CanL-C55 (MCAN/TR/2007/EP134, Accession No. FJ940891).

100% identity to *L. infantum* strain IPT1, and several significant differences, including nucleotide insertions, deletions and replacements as indicated in Figure 3, from *L. tropica*. The *L. tropica* strains both in and outside Turkey demonstrated >99% identity over the 143 bp ITS1 region used for the multiple alignment (Figure 3).

Discussion

ITS1 PCR followed by RFLP proved useful for identifying *Leishmania* species found in Turkey. This technique worked well regardless of the clinical manifestation, or type of clinical sample examined. It appears that only two species, *L. infantum* and *L. tropica*, are responsible for most endemic leishmaniasis cases in Turkey.

In this study, ITS1 PCR RFLP showed that *L. infantum* is the causative agent of human VL and CanL in the Eastern Mediterranean, Ege, Central Anatolia and Marmara regions of Turkey, in agreement with the earlier studies using different methods (Ozensoy *et al.* 1998; Akman *et al.* 2000). These *L. infantum* strains were characterized as belonging to zymodemes MON-1 and MON-98 (S. Ozensoy Toz and Y. Ozbel, unpublished data).

According to the Turkish Ministry of Health (http://ww.saglik.gov.tr) both visceral and CL cases are found in Hatay, Mugla and Aydın provinces. Human CL caused by *L. tropica* is an ongoing problem in Southeastern Anatolia region, where epidemic outbreaks were described in the late 1990s (Akman *et al.* 2000). Sporadic cases of human CL caused by *L. tropica* have also been reported from the Black Sea, Central and Eastern Anatolia regions. *Leishmania tropica* zymodemes MON-200, MON-303 and MON-304 were found among strains isolated in Western Turkey (Izmir, Mugla and Aydin); MON-55 was seen only in central Anatolia (two strains from Nigde); while MON-304 was also found in southeastern Anatolian region (18 strains from Sanliurfa, S. Ozensoy Toz and Y. Ozbel, unpublished data).

Until recently, it was assumed that human CL in Turkey is caused exclusively by *L. tropica,* while *L. infantum* is responsible for human VL and CanL, however new molecular and epidemiological studies suggest that the situation is more complex. In 2005 three cases of CL caused by *L. infantum* were reported from the Adana region (Serin *et al.* 2005), and Svobodova *et al.* (2009) identified *L. infantum* as the parasite responsible for human CL in a focus located in northern Cukurova, Adana region approximately 130–200 km northwest of Hatay province. Our results show that both *L. tropica* and *L. infantum* cause human CL in Turkey. *Leishmania tropica* was responsible for this disease in all the regions examined, except Hatay province where the disease is caused by *L. infantum*.

This is the first time that *L. infantum* has been described as the causative agent of human CL in this region. CL has been endemic for decades in Hatay province, southern Turkey where the annual disease incidence was reported to vary from 5.5 to 16.2/100 000 during the years 1998–2005, respectively. The majority of CL patients (70.2%) are young, <20 years old, with the highest percentage (23.6%) seen in the 11–15 years group (Akcali *et al.* 2007). DNA sequencing of the ITS1 region from two of the human CL patient samples further supports the PCR results identifying these parasites as *L. infantum*.

L. infantum strains from both human CL and infected *Phlebotomus tobbi* sand flies in Cukurova region were examined by multilocus sequence typing (MLST) and found to be close to the *L. infantum* MON-188, rather than *L. infantum* MON-1 or MON-24. The latter two zymodemes are frequently associated with human VL (and CanL) or human CL, respectively (Svobodova *et al.* 2009). Human CL caused by *L. infantum* has also been described in adjacent regions of Lebanon and Syria where *L. infantum* MON-24 was isolated from both CanL and human CL cases (Knio *et al.* 2000). However, *L. infantum* MON-1 normally associated with human VL and CanL has also been reported to cause human CL in other areas around the Mediterranean Basin, such as Sidi Bourousi, Tunisia where this zymodeme was identified in 40% of the human CL cases (Belhadj *et al.* 2003).

The *L. infantum* strain (MHOM/TR/2000/OG-VL), isolated from a human VL patient in Hatay province, was shown by MLST to be distinct from parasites belonging to the MON-1 zymodeme, as well as parasites causing CL in the Cukurova foci (Svobodova *et al.* 2009). Interestingly, a *L. infantum* strain cultured from a dog with CanL (CanL-C55) in Hatay province, the first strain isolated in this region, was typed by multilocus enzyme electrophoresis (MLEE) as *L. infantum* MON-1 (S. Ozensoy Toz and Y. Ozbel, unpublished results), suggesting that at least two parasites genotypes are circulating in this region.

At this time strains from human CL patients in Hatay are unavailable; isolation and characterization of strains from CL and VL patients, and additional dogs with CanL, are necessary in order to understand local epidemiology. Identification of *L. infantum* genotypes causing human CL or VL in Hatay province will play an important role in determining whether zoonotic or anthroponotic transmission cycles are present, and have ramifications on subsequent public health measures designed to control this disease. To date treatment, topical (pentavalent antimony or cryotherapy) rather than systematic, of CL patients in Turkey infected with *L. tropica* and *L. infantum*, is identical. An open question remains as to whether immune suppression of cured CL patients where *L. infantum* was the causative agent can potentially result in relapses and the development of the visceral disease. This suggests that careful consideration should be given in choosing treatment regimes and in subsequent follow up CL caused by this species.

In summary, ITS1 PCR followed by RFLP can be used as a reliable method for diagnosis and identification of the *Leishmania* species found in Turkey. *Leishmania infantum* and *L. tropica* are present together in several endemic regions and identification of the parasite species is important not only for the appropriate therapy, but also in understanding disease epidemiology and parasite behaviour in the different endemic regions of Turkey.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Multiple alignment of the Internal Spacer Region 1 (ITS1) region of the rRNA operon for three *Leishmania* species: (1) *L. infantum* (2) *L. tropica* and (3) *L. major*. The primers used for amplification by ITS1 PCR, LITSR (forward primer - 5′-CTGGATCATTTTCC GATG-3′) and L5.8S (reverse primer - 5′-TGATACCACT TATCGCACTT-3′), sites where restriction enzyme *Hae*III cuts each amplicon and the sizes of the fragments generated are indicated.

Table S1. *Leishmania* strains used in this study.

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