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Clinical manifestations and genetic variation of *Leishmania infantum* and *Leishmania tropica* in Southern Turkey

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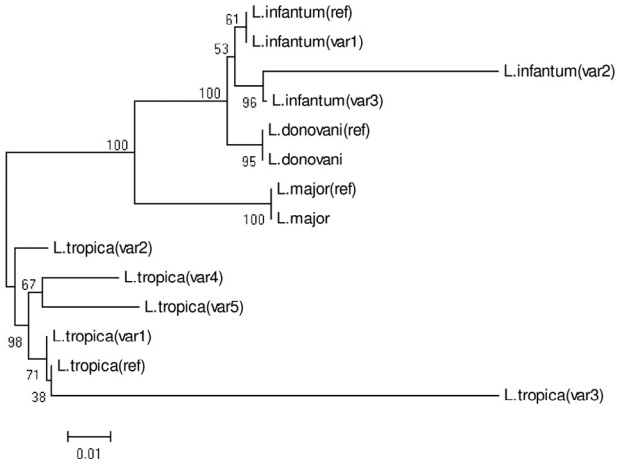
HIGHLIGHTS

- We isolated *Leishmania* strains from cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) cases.
- PCR-RFLP, melting curve real-time PCR, and DNA sequencing were used for discrimination of *Leishmania* species.
- *L. infantum* (variant 3) was identified from only CL cases.
- *L. tropica* (variant 2) was identified from only VL cases.
- The genetic variation might play a role in the causation of CL and VL cases.

GRAPHICAL ABSTRACT



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ABSTRACT

L. infantum was isolated from cutaneous leishmaniasis (CL) skin lesions in patients having no signs and symptoms of visceral leishmaniasis (VL). Similarly, *L. tropica* had previously been isolated from patients with VL in the absence of cutaneous lesions. It was not certain how visceralization occurred. Smears (207) and bone marrow samples (135) were taken from CL and VL-suspected patients, respectively. Microscopic examination, ITS1-PCR, RFLP and DNA sequencing for all samples were analyzed. The microscopic examination of smears was found to be 61.3% (127/207) in CL-suspected cases and bone marrow samples were found to be positive 8.8% (12/135) in VL-suspected cases. *L. tropica* 48.6% (72/148), *L. infantum* 35.8% (53/148), *L. major* 15.6% (23/148) in CL, and *L. infantum* 56.3% (18/32), *L. donovani* 31.2% (10/32), *L. tropica* 12.5% (4/32) in VL were found with PCR-RFLP. In addition, the DNA sequencing revealed a genetic variation in *L. infantum* (variants 1–3) and *L. tropica* (variants 1–5). We assume that the increased disease occurrence may have resulted from geographical expansion of disease, changing patterns of

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international travel, population migrations, non-immune people into endemic regions of infected people into non-endemic regions. In this study, *L. infantum* (variant 3) only in CL-patients and *L. tropica* (variant 2) only in VL-patients were identified. We hypothesize that genetic variation might play a role in the causation of CL and VL in southern Turkey and the genetic variants may differ according to the geographical location among *Leishmania* strains.

1. Introduction

Leishmania tropica (*L. tropica*) and *Leishmania major* (*L. major*) are believed to cause cutaneous leishmaniasis (CL); whereas *Leishmania infantum* (*L. infantum*) and *Leishmania donovani* (*L. donovani*) cause visceral leishmaniasis (VL) (World Health Organization, 2013). The introduction of new and fast methods for identification of *Leishmania* species enables researchers to use more precise approach to study epidemiology, treatment and clinical prognosis about leishmaniasis (El Tai et al., 2000).

Molecular methods based on polymerase chain reaction (PCR) have recently been developed in determination of genotypes and pathogenicity of *Leishmania* species (Asato et al., 2009). Furthermore, *L. infantum* has been described in CL-patients without VL history and *L. tropica* has been determined in VL-patients without CL history (Eroglu et al., 2011; Koltas et al., 2014; Serin et al., 2005; Toz et al., 2013). In addition, *L. major* and *L. donovani* are reported as causative agents of CL-patients and VL-patients, respectively, in southern Turkey (Akman et al., 2000; Koltas et al., 2014). However, the genetic variation of the parasites is one of the most important factors commonly discussed and the subject of controversy (Oryan et al., 2013). This genetic variation may result in different phenotypes that can be associated with the diversity of the clinical manifestations and geographical distribution (Mahnaz et al., 2011; Schönian et al., 2003). Therefore, typing has been conducted by PCR, based on methods which use polymorphic DNA targets with high discriminatory power (Oryan et al., 2013). For analysis of genetic diversity, several nuclear DNA markers such as the small subunit rRNA genes, the gp63 gene locus and the internal transcribed spacer 1 (ITS1) have previously been used (Oryan et al., 2013; Schönian et al., 2003).

The aim of the current study was to identify the *Leishmania* species causative agents of CL and VL by employing the ITS1 PCR-RFLP methods. Genetic variations among *Leishmania* species were established by DNA-sequencing. Furthermore, the relationship between genetic variation of *Leishmania* species and clinical manifestation of leishmaniasis were identified.

2. Materials and methods

2.1. Collection of samples

Smears from 207 CL-suspected patients were taken from the patients applied to Cukurova University, Faculty of Medicine, and Department of Dermatology. In addition, 135 bone marrow samples from VL-suspected patients were obtained from the patients applied to Cukurova University Faculty of Medicine Department of Pediatric Infectious Diseases. We started the study after the approval of Cukurova University Ethics Committee. All of the CL-suspected and VL-suspected patients were clinically examined, and a survey was administered to find out whether CL suspected patient had a previous VL infection.

2.2. Microscopic examination

All of the smears and peripheral bone marrow samples were fixed by dipping in absolute methanol, stained with Giemsa 10% stain, and then they were examined under light microscope with magnification 100×. All of the amastigote observed preparations were accepted to be positive, and those amastigotes not observed were negative.

2.3. Leishmania strains and DNA extraction

Various *Leishmania* strains from American Type Culture Collection (ATCC) were used as reference strains; *L. donovani* (ATCC 50212; WHOM/IN/80/DD8; Bihar, India, 1980), *L. infantum* (ATCC, 501340; WHOM/TN/80/IPT-1; Monastir, Tunisia, 1980), *L. major* (ATCC, PRA-385; WHOM/SN/74/SD; Senegal, West Africa, 1973) and *L. tropica* (ATCC, 50129; WHOM/SU/74/K27; Baku, Azerbaidjanskaya, 1974). The smears (5 mg) were scraped from each slide with a toothpick, resuspended in 20 µl of double-distilled water and transferred to 1.5 ml sterile microtubes. In addition, bone marrow samples (100 µl) were transferred to 1.5 ml sterile microtubes. DNA extraction was performed using Agencourt DNA blood kit (Beckman Coulter, Beverly, USA) according to the manufacturer's instructions for all samples.

2.4. ITS1 PCR-RFLP analysis

The ITS1 region was amplified using DNA extracted from the *Leishmania* and ITS1-specific primers LITSR and L5.8S (Schönian et al., 2003). The 50 µl PCR reaction mixtures consisted of PCR buffer 1× (75 mM KCl, pH 8.3 and 20 mM Tris–HCl, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Fermantas, Burlington, Canada)), 0.2 mM $dNTP_s$ (Fermantas, Burlington, Canada), 0.5 pmol of each primer and 5 µl of the DNA sample. After the initial denaturation (5 min at 94ºC), 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 54 °C, and elongation for 1 min at 72 °C were carried out, and the PCR was terminated by final extension at 72 °C for 10 min. The PCR products were analyzed in 1% agarose gel by electrophoresis at 100 V in 1X Tris–boric–EDTA buffer (0.04 mM Tris–boric and 1 mM EDTA, pH 8) and visualized by UV light after being stained with ethidium bromide. All of the samples approximately fragment length 350 bp with LITSR-L5.8S were accepted to be positive by PCR.

ITS1 PCR products (10 µl) were digested with the *Haemophilus aegyptius* (HaeIII) restriction enzyme (Promega, Wisconsin, USA) according to the manufacturer's instructions, and the restriction fragments were analyzed by 2% agarose gel electrophoresis. The HaeIII restriction enzyme digest 5′…GG/CC…3′ and 3′…CC/GG…5′ nucleotide. The restriction products were compared with DNA of reference *L. donovani*, *L. infantum*, *L. major*, *L. tropica* (Schönian et al., 2003).

2.5. DNA sequencing and phylogenetic analysis

The ITS1 PCR products were purified using a SentroPure DNA purification kit (Sentromer DNA, Istanbul, Turkey), and they were sequenced using the sense primer and the DNA sequencing kit Big Dye Terminator™ (Applied Biosystems, California, USA) according to the manufacturer's instructions. DNA sequencing was performed with an ABI Prism 310™ Genetic Analyzer with forward and reverse primers (Applied Biosystems, California, USA). The double stranded sequences were edited and aligned using the BioEdit program, v5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The results of BioEdit program were confirmed using Codoncode aligner 5.01 programs.