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Leishmaniasis in Turkey: Visceral and cutaneous leishmaniasis caused by *Leishmania donovani* in Turkey

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ABSTRACT

In Turkey, the main causative agents are *Leishmania tropica* (*L. tropica*) and *Leishmania infantum* (*L. infantum*) for cutaneous leishmaniasis (CL) and *L. infantum* for visceral leishmaniasis (VL). In this study, we investigated leishmaniasis cases caused by *L. donovani* and established animal models for understanding its tropism in *in vivo* conditions. Clinical samples (lesion aspirations and bone marrow) obtained from CL/VL patients were investigated using parasitological (smear/NNN) and DNA-based techniques. For species identification, a real time ITS1-PCR was performed using isolates and results were confirmed by hsp70 PCR-N/sequencing and *cpb* gene PCR/sequencing in order to reveal *Leishmania donovani* and *Leishmania infantum* discrimination. Clinical materials from CL and VL patients were also inoculated into two experimental groups (Group CL and Group VL) of Balb/C mice intraperitoneally for creating clinical picture of Turkish *L. donovani* strains. After 45 days, the samples from visible sores of the skin were taken, and spleens and livers were removed. Measurements of the internal organs were done and touch preparations were prepared for checking the presence of amastigotes. The strains were isolated from all patients and amastigotes were seen in all smears of the patients, and then isolates were immediately stored in liquid nitrogen. In real time ITS1-PCR, the melting temperatures of all samples were out of range of *L. infantum, L. tropica and L. major*. Sequencing of hsp70 PCR-N showed that all isolates highly identical to previously submitted *L. donovani* sequences in GenBank, and *cpb* gene sequencing showed five isolates had longer *cpbF* allele, whereas one isolate contained a mixed sequence of both *cpbF* and *cpbE*. All mice in both experimental groups became infected. Compared to controls, the length and width of both liver and spleen were significantly elevated ($p < 0.001$) in both groups of mice. However, the weight of the liver increased significantly in all mice whereas the weight of spleen increased only in VL group. Amastigotes were also seen in all touch preparations prepared from skin sores, spleen and liver. *L. donovani* strain was isolated from autocutaneous a VL patient first time in Turkey. Animal models using clinical samples were successfully established and important clinical differences of the isolated strains were observed.

1. Introduction

Leishmaniasis is a group of diseases transmitted by infected female sand flies (*Phlebotomus* spp. in the Old World, and *Lutzomyia* spp. in the New World) and caused by the intracellular flagellate protozoa; *Leishmania* spp. Clinical manifestations of leishmaniasis vary from a self-limiting cutaneous infection (cutaneous leishmaniasis – CL) to a life-threatening visceral disease (visceral leishmaniasis – VL). It is currently endemic in 102 countries, areas or territories worldwide and 2 million new cases are recorded annually, three fourths of which are reported as CL. In 2015, WHO selected 14 and 12 countries as high-burden (case number > 2500) countries for VL and CL, respectively. Turkey is selected as one of the high-burden countries for CL (WHO, 2010, 2016). Situated on the crossroads between three continents under a subtropical climate, Turkey has been under the threat of leishmaniasis as well as other vector borne diseases. A total of 14.587 CL and 207 VL

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cases have been officially reported in Turkey between 2005 and 2014 (Gürel et al., 2012). Causative agents of VL and CL cases are known as *Leishmania infantum* and both *L. tropica* and *L. infantum*, respectively. Cutaneous leishmaniasis has been endemic in southeastern Anatolia for centuries; in last decades, new cases have sporadically been reported from some provinces in central and western Anatolia, which indicates the spreading of endemic foci in the country (Ozbel et al., 2011). It is well known that leishmaniasis is a significant public health concern in Turkey's southern and eastern neighbors (Karimkhani et al., 2016), where warfare has been a big problem for daily life in the last decade. Local health authorities in Turkey are currently taking some public health control measurements covering communicable diseases for more than 3 million immigrants coming from Syria, the southern neighboring country where both CL and VL are endemic.

Visceral leishmaniasis cases are predominantly seen in Aegean and Mediterranean Regions of Turkey, and the main causative agent is *Leishmania infantum* MON-1, as in other Mediterranean countries. *L. donovani,* which is another causative agent of VL in Asian and African endemic countries, was not documented in Turkey as a causative agent of VL until the year 2014. *L. donovani* was first diagnosed from 19 Turkish VL patients living in southeastern and east Mediterranean parts of Turkey using a species-specific real time PCR targeting *Leishmania* kDNA minicircle very recently (Koltas et al., 2014).

In the present study, we aimed to present six autocutaneous cases of leishmaniasis (five CL and one VL) from Turkish patients that all are infected with *Leishmania donovani,* and establish experimental CL and VL animal models using clinical materials of the patients.

2. Materials and methods

2.1. Patients, sampling and strain isolation

The *Leishmania* isolates included in the study were obtained from the patients initially admitted to the related Clinics of the hospitals in different provinces, where they were suspected clinically as CL or VL, and then referred to either Microbiology or Parasitology Laboratories of their hospitals for sample collection and certain diagnosis. Clinical samples obtained from the patients were inoculated into the NNN media and also injected to Balb/C mice according to the routine protocol in Parasitology Lab Celal Bayar University (the samples from VL patients and CL patients having specific/different clinic picture were injected into at least five mice). After application of molecular techniques mentioned below, isolates identified as *L. donovani* were included in the study. Four CL patients were from provinces located in southeastern Anatolia while one from northern part of Cyprus. Visceral leishmaniasis patient was diagnosed in Manisa province located in western Anatolia (Fig. 1). Demographic information of all patients and history of lesions were recorded (Table 1).

The lesion samples from CL patients were obtained using needle aspiration method. The surrounding intact skin of the patients was cleaned with 70% ethanol, and then saline solution (0.2–0.5 ml) was injected in the margin between the lesion and intact skin, and aspiration fluid was taken. The bone marrow sample from the VL patient was obtained using a fine-needle aspiration under the sterile conditions in pediatric clinic. The samples from CL and VL patients were first inoculated into enriched NNN medium. Two or more slides of smears of each patient's material were prepared to be use for parasitological examination. The culture tubes were kept at 24 °C and checked regularly for the presence of promastigotes every other day for 1 month. After initial reproduction of the promastigotes, they were transferred to RPMI-1640 medium containing 10% of fetal calf serum (FCS), 200 U/ml penicillin and 0.2 mg/ml streptomycin for mass production. When the medium volume in the flasks reached 10 ml, promastigotes were collected by centrifugation and used for trials after washing five times. All isolates were also cryopreserved in liquid nitrogen at −196 °C (Özbilgin et al., 2016).

One of the smears was stained with Giemsa stain after fixation with methyl alcohol and examined under the light microscope at x1000 magnification for the presence of *Leishmania* amastigotes, while the others were kept at −20 °C for further assessments.

2.2. Genotyping of Leishmania isolates

Each isolate were first cloned by limiting dilution method as follows: the log phase promastigotes were diluted serially from 1000 to 1 in a 96 well plate using RPMI-1640 (with 10% FCS) and the wells with a single promastigote were marked and incubated at 25 °C for growing (Garin et al., 2002). DNA isolation was performed from cloned promastigotes and clinical samples using High Pure PCR Template Preparation Kit (Roche, Germany) according to kit protocol. All DNA samples were kept at −20 °C until assessed by PCR.

2.3. Real time ITS-1 PCR

The real time ITS1-PCR method was performed using Old World species-specific primers and probes. All procedure was done as described in Ozensoy Toz et al. (2013). Melting curves were analyzed using channel 2 and 3. Four WHO reference strains were used as 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).

2.4. Heat shock protein 70 PCR

The species identification of the *Leishmania* isolates was performed using Heat Shock Protein 70 (Hsp-70) PCR-N, as reported previously (Van der Auwera et al., 2013). The mixture volume of PCR-N was 50 µl in total, containing 10X Taq buffer; 5X Tune Up solution (Nanohelix™); 10 µM of each primer; 10 mM dNTP mix; 1.25 U Helixamp™ Taq polymerase and 20 ng of DNA sample. Negative no-template control was included, along with positive controls consisting of DNAs from reference isolates of *L. infantum* (MHOM/TN/1980/IPT1), *L. tropica* (MHOM/A2/1974/SAF-K27), *L. major* (MHOM/TMSU/1973/5ASKH) and *L. donovani* (MHOM/IN/1980/DD8). PCR products were visualized after gel electrophoresis and positive samples (~593 bp) were sequenced commercially after ExoSap clean up step. Obtained sequence data were analyzed using Geneious R8 and compared with previously submitted sequences using Basic local alignment tool (BLAST®). The sequences were submitted to GenBank.

2.5. Real time cysteine protease b (cpb) gene PCR

A real-time PCR and sequencing of the *cpb* gene, which discriminates differences in *cpb* genes between *L. infantum* and *L. donovani* was also performed as described previously (Hide and Banuls, 2006). Briefly, a real-time PCR was performed using newly designed primers (NV_Leish-F GAACAGCAGTAAACTCGTTCCCGG, NV_Leish-R-new2 CTCAGGATGCGGTGCAGCA) and Cy5/BBQ labelled probe (NV_Leish-P TCATGTCTTACCAGAGCGGCGTGC) for detection on a LightCycler® 480 (Roche Diagnostics) instrument as described by the manufacturer.

2.6. Establishment of in vivo models

Using amastigotes from clinical specimens of a CL and a VL patient, animal models were generated to determine whether the parasites from CL and VL patients have different tropism. Biopsy material, taken in Dermatology clinic, obtained from CL patient (case 5) and bone marrow sample obtained from VL patient were initially suspended in sterile saline solution and both of them were injected intraperitoneally to two groups (Group CL and Group VL) of mice, each having five Balb/C mice. The control group was also established in same number of mice and only sterile saline were injected intraperitoneally at the same time with