

# First Report and In Silico Analysis of Leishmania virus (LRV2) identified in an autochthonous *Leishmania major* isolate in Turkey

Özgür Kurt<sup>1</sup>, Nesteren Mansur<sup>2</sup>, İbrzhim Çavuş<sup>3</sup>, Orhan Özcan<sup>4</sup>, M. Burak Batır<sup>5</sup>,  
Cumhur Gündüz<sup>6</sup>, Ugur Sezerman<sup>4</sup>, Ahmet. Özbilgin<sup>3</sup>

<sup>1</sup>Acibadem Mehmet Ali Aydınlar University School of Medicine Department of Medical Microbiology, Istanbul;

<sup>2</sup>Acibadem Mehmet Ali Aydınlar University Institute of Health Sciences Department of Biotechnology, Istanbul;

<sup>3</sup>Celal Bayar University School of Medicine Department of Medical Parasitology, Manisa;

<sup>4</sup>Acibadem Mehmet Ali Aydınlar University School of Medicine Dept. of Biostatistics and Bioinformatics, Istanbul;

<sup>5</sup>Celal Bayar University School of Science and Arts Department of Biology, Manisa;

<sup>6</sup>Ege University School of Medicine Department of Medical Biology, Izmir, Turkey

## SUMMARY

Leishmania virus (LRV) has previously been identified in different *Leishmania* species. Host-LRV interaction is associated with exacerbated clinical manifestations of cutaneous leishmaniasis (CL) and may cause poor therapeutic response. CL cases due to *L. major* with large skin lesions resistant to routine therapy were recently identified in Turkey. Here, we report the first autochthonous case of cutaneous leishmaniasis caused by LRV-positive *Leishmania major*, using conventional PCR targeting the viral capsid protein of LRV. The lesion of the case was 6 months old, relatively large (4 cm), and did not recover despite three consecutive intralesional applications of glucantime. Assessment of LRV's influence on prognosis and clinical outcomes of leishmaniasis, based on additional studies, is required.

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## INTRODUCTION

Leishmaniasis is a vector-borne disease transmitted by sand flies (*Phlebotomus* spp. in the Old World, and *Lutzomyia* spp. in the New World), and caused by *Leishmania* species that are intracellular, flagellated protozoan parasites (Culha *et al.*, 2014). It is endemic in 102 countries with an annual rate of 2 million new cases, three-fourths of which appear as cutaneous leishmaniasis (CL) (WHO, 2017). CL has long been endemic in Turkey, especially in southeastern Anatolia; however, its incidence has been on the rise in recent years due to a huge influx of immigrants, especially from Syria, and to an increase of infection foci throughout Anatolia (Özbilgin *et al.*, 2016). Its predominant causative agent in the Old World is *Leishmania tropica*, while CL cases due to *L. infantum*, *L. donovani* and *L. major* have also been reported (Ok *et al.*, 2002; Culha *et al.*, 2014; Özbilgin *et al.*, 2017).

CL cases due to *L. major*, which is also known as zoonotic cutaneous leishmaniasis (ZCL), are common in Northern Africa and Middle East (Elfari *et al.*, 2005; Pralong *et al.*, 2009). It is known to be prevalent in 15 of the 30 provinces of Iran, a neighbor of Turkey, where unusual clinical outcomes such as mucosal leishmaniasis and disseminated leishmaniasis have already been reported (Akhoundi *et al.*,

2013; Hajjaran *et al.*, 2013). *L. major* was previously reported in a retrospective study in Turkey (Koltas *et al.*, 2014); however, its first clinical isolation and successful cultivation in Turkey were done later, together with demonstration of its in vivo effects in a hamster model (Özbilgin *et al.*, 2016). A large spectrum of clinical manifestations of CL with autochthonous *L. major* isolates has been identified in Turkey, varying from a single, tiny skin lesion with good response to therapy to large, crusted skin lesions that lead to the amputation of the extremities in laboratory animals (Özbilgin *et al.*, 2016; Remadi *et al.*, 2016). This huge variation may be due to a complex association between the parasite and the host, where an endosymbiotic RNA virus present in *Leishmania* parasites may also play a role (Hartley *et al.*, 2012; Hajjaran *et al.*, 2016; Ives *et al.*, 2011; Brettman *et al.*, 2016). Leishmania virus (LRV) is a 40 nm viral particle, classified within the *Totiviridae* family. Described almost 30 years ago, LRV is now classified as LRV-1 (infecting New World parasites) and LRV-2 (infecting Old World parasites), according to some genomic differences. It consists of a non-segmented dsRNA that encodes a major capsid protein and a capsid RNA-dependent RNA polymerase (RDRP) fusion protein essential for replication (Scheffter *et al.*, 1995; Hartley *et al.*, 2012). LRV has been identified in many New and Old World leishmaniasis agents, such as *L. braziliensis*, *L. major* and *L. infantum* (Hartley *et al.*, 2012; Zangger *et al.*, 2013). Studies on LRV-Host interactions on animal models showed that LRV presence in *Leishmania* is associated with destructive hyper-inflammation leading to exacerbation and metastasis of disease (Ives *et al.*, 2011; Zangger *et al.*, 2014; Alves-Ferreira *et al.*, 2015; Hartley *et al.*, 2016). Indeed, various human studies have shown that LRV may influence the clinical prognosis in cutaneous

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Corresponding author:

Özgür Kurt

E-mail: Ozgur.Kurt@acibadem.edu.tr

leishmaniasis patients, and may increase the risk of mucocutaneous leishmaniasis development (Cantanhede *et al.*, 2015) and even treatment failure in patients (Bourreau *et al.*, 2015; Aduai *et al.*, 2016; Hajjarian *et al.*, 2016).

Presented here is the first case of CL in Turkey caused by LRV-positive *L. major*, with initial analysis of its phylogenetic analyses.

## MATERIALS AND METHODS

**Parasitological Diagnosis:** Samples obtained from her skin lesion were sent to Parasitology Laboratory of Manisa Celal Bayar University for further analysis. Here, Giemsa-stained smears were initially prepared from the lesion samples; they were then inoculated in NNN (Novy-McNeal-Nicolle) medium for culture. A PCR reaction that targeted the ITS region of *Leishmania spp.* was also conducted using both the skin lesion and culture material to identify the *Leishmania* species, as previously described (Töz *et al.*, 2009). To investigate the presence of LRV, *L. major* isolate was transferred to Acibadem Mehmet Ali Aydinlar University's Research Laboratory in Istanbul, where it was preserved in liquid nitrogen at -196°C until the day of the experiments. The isolate was then thawed and inoculated in RPMI medium (GIBCO®) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin (Penicillin, 10,000 units/ml - Streptomycin, 10 mg/ml, GMBiolab®, Taiwan) and 0.2% Gentamicin (Gentamicin, 50 mg/ml, GMBiolab®, Taiwan). Total RNA extraction was done using *L. major* promastigotes at stationary phase, with Total RNA Miniprep Purification Kit® (GMBiolab, Taiwan). The RNA amount was quantified with NanoDrop® (Implen, Germany). First-strand synthesis of complementary DNA (cDNA) was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers in a 20 µl mixture, containing 1µg of isolated RNA.

The genetic content of Leishmania virus (LRV) had initially been downloaded from NCBI® Database via "Geneious R10", with data retrieval agent. Specific detection primers that targeted the viral capsid antigen of LRV were initially designed, considering the presence of high mutation rates identified in RNA viruses (Elena and Sanjuán, 2005). The primers for LRV1 (F-AATTCAAACGAGATGCCTATGAG and R-GTGCTAGTGGTAGTTCTATACGA) and LRV2 (F-ATGCTGATAACTTGAAACAGGAG and R-CAT-

CATTGCCTGTAAGTGAGTAG), which could amplify specific sequences of 2576 and 954 base pairs (bp), respectively, were used in the analyses. A primer pair for KMP-11 (F-AGATGCAGGAACAGAACGCC and R-TGCTTGAA-GTGCTCCGAGTG) that amplified a 160-bp long sequence, was used as the Internal Control. Conventional PCR was applied using PCR Hot Start Master Mix II (GMBiolab®), 1µl of cDNA and 7.5 pmol from each primer, in a total of 25 µl reaction mixture. PCR conditions for LRV1 and LRV2 were as follows: Initial denaturation for 3 minutes (min) at 95°C, followed by 35 cycles of denaturation for 30 seconds (sec) at 95°C; annealing at 53°C for 40 sec for LRV1 and 30 sec for LRV2; extension at 72°C for 3 min for LRV1 and 2 min for LRV2; and a final extension for 7 min at 72°C. PCR products were visualized with agarose gel electrophoresis in a 2% gel stained with Sybr Gold® (Invitrogen®) and 100 bp DNA marker (GMBiolab®). The sequence analysis of the sample was done in GATC Biotech laboratories in Germany, and the results were compared with the data present on the NCBI database. The BLAST analyses were done in our laboratory using "Standalone BLAST Setup for Windows PC" (<https://www.ncbi.nlm.nih.gov/books/NBK52637>). This "PCR + Sequencing" method was previously used to detect LRV in *Leishmania* isolates in similar studies (Zangger *et al.*, 2013; Hajjarian *et al.*, 2016; Aduai *et al.*, 2016; Cantanhede *et al.*, 2018).

**Construction of a Phylogenetic Tree:** A phylogenetic tree was built using the capsid protein sequences of LRV, as described (Koonin and Dolja, 1993). The outlier reference was selected as *Trichomonas vaginalis* double stranded (ds) RNA virus, due to its similarities to LRV in terms of classification (Totiviridae) and host (human). The viral sequences were downloaded from the NCBI Nucleotide database, aligned with MUSCLE 3.8.425 (Edgar, 2004) by eight iteration parameters: kmer4\_6 (distance measure), neighbor-joining (Clustering method) pseudo (tree rooting method), CLUSTALW (sequence weighting scheme), hydrophobicity multiple was set to 1.2, anchor spacing was set as 32, diagonal minimum length was set as 24 and finally window size was set to 5. This alignment was used by PAUP4 to build taxonomic trees (Swofford, 2003). The Maximum Likelihood Method with GAMA distributed time reversal (GTR) was used as the rate variation parameter of 100 bootstrapped PAUP run. GTR was selected for the rate variation of the virus since our sample was a fast-evolving RNA virus. For the in-silico analyses in the study, LRV sequences were retrieved from NCBI genome database by Geneious R10®161.

## CASE REPORT

### Case presentation

A 15-year-old school girl from Manisa province located in western Anatolia was referred to Parasitology Laboratory with CL suspect due to her 6-month old, 4 cm long, ulcerated skin lesion near her left ear (Figure 1). She was initially given 20 mg/kg of intralesional glucantime, three times a week for 3 weeks. This treatment protocol was repeated two times more due to treatment failures.

### Parasitological Assessments

Initial parasitological assessments of patient samples indicated the presence of *Leishmania* amastigotes and promastigotes in Giemsa-stained smears and culture materi-



**Figure 1** - The skin lesion of the cutaneous leishmaniasis patient from Manisa province.

al, respectively. ITS-targeted PCR analysis indicated that the causative agent was *Leishmania major*. The second PCR analysis conducted using specific detection primers of LRV that targeted viral capsid antigen showed the presence of LRV in *L. major* isolate of the patient. Sequencing of viral DNA followed by bioinformatics interpretation showed that the viral isolate was linked to LRV2 in the phylogenetic tree. The viral sequence was then named as “LRV2-LmjManisa” and submitted to NCBI Nucleotide Database, together with all necessary parameters (Bioproject Submission No: SUB3183189). A phylogenetic tree was developed using the viral capsid protein (Figure 2), in which the Turkish isolate from Manisa province was found to be closely associated with NC002064 viral genome defined as the “Leishmania virus LRV2-1, a virus of an Old-World parasite strain” in NCBI database. ([https://www.ncbi.nlm.nih.gov/nuccore/NC\\_002064](https://www.ncbi.nlm.nih.gov/nuccore/NC_002064)).

## DISCUSSION

The first report on both the clinical isolation of *L. major* in Turkey as well as demonstration of its clinical effects in a mouse model was published recently (Özbilgin *et al.*, 2016). To our knowledge, this is the first report of LRV in autochthonous *L. major* isolates in Turkey. The presented CL patient infected with LRV-positive *L. major* had a larger lesion and did not respond to two previous courses of regular anti-leishmanial therapy. Bioinformatics assessments showed that the isolated virus was linked to the same root as other LRV-2 isolates in the phylogenetic tree (Figure 2) and was named LRV2-LmjManisa. It showed both high sequence similarity (after the bootstrap value of 100) and small nucleotide sequence variances compared to other LRV2 isolates in the database.

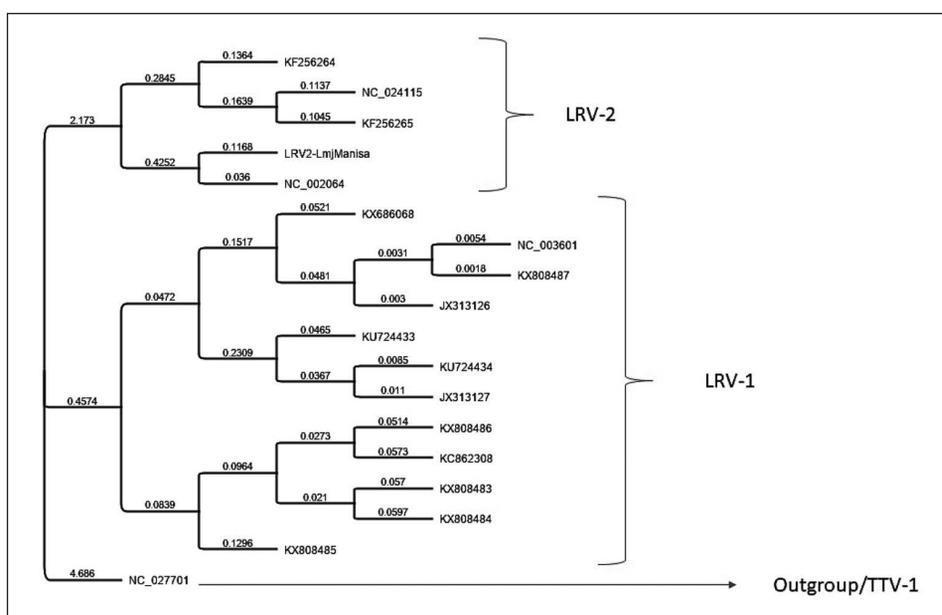
The first report of LRV in *Leishmania* species dates to 1988, when it was detected in *L. guyanensis* (Tarr *et al.*, 1988), followed by its identification in Old World agents, such as *L. major*, *L. aethiopica* and *L. infantum*. Studies on animal models using the isolates from both the New and Old World (*L. guyanensis* and *L. aethiopica*) showed that the nucleic acid of LRV acted as a potent immunogen

and caused a severe inflammation through interactions with Toll-like receptor 3 (TLR3), which were intra and extracellular receptors that initiated immune responses against pathogens (Ives *et al.*, 2011; Hartley *et al.*, 2012; Gomez-Arreaza *et al.*, 2017). Studies on animal models showed that activation of TLR3 influenced the production of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-alpha), which might lead to higher parasitic load and development of worse clinical outcomes or even metastatic lesions in humans, such as mucocutaneous leishmaniasis (MCL) (Ives *et al.*, 2011; Cantanhede *et al.*, 2015). LRV is also associated with treatment failures and symptomatic relapse in human cases, and treatment failure is not related to intrinsic pentavalent antimonial resistance in parasites, but probably to modulation of the host's immune system and metabolism (Bourreau *et al.*, 2015; Adai *et al.*, 2016). Recently, Castiglioni and colleagues demonstrated that immunization with LRV1 capsid was beneficial in alleviating the LRV-related pathology and lowering the parasitic burden in animal models (Castiglioni *et al.*, 2017). In addition, it was shown that anti-LRV treatment using agents like antiviral adenosine analogues may be beneficial to clinical outcomes in leishmaniasis patients (Kuhlmann *et al.*, 2017). Thus, investigation of LRV in clinical cases of leishmaniasis is useful to assess the prognosis of infection and lower the risk of treatment failures in patients.

Virus-specific GTR models of viral protein evolution have been shown to improve phylogenetic accuracy for retro-transcribing (RNA viruses) elements (Dimmic *et al.*, 2002; Dang *et al.*, 2010), as in our presented case. For the in-silico analyses in the study, LRV sequences were retrieved from NCBI genome database by Geneious R10<sup>®</sup>161. Since many LRV sequences in the database were produced by detection primers that targeted conserved regions of the genes having at most 100 amino acids, we could do the comparisons only with a limited number of sequences on NCBI Database, which had more than 300 amino acids.

In conclusion, this case report demonstrates the presence of LRV2-LmjManisa in an autochthonous *L. major* isolate

**Figure 2** - The phylogenetic tree of *Leishmania Virus-2* (LRV2) isolated from a patient in Manisa province of Turkey (LRV2-LmjManisa). *Trichomonas vaginalis* double-stranded RNA virus was selected as the outliner due to its similarities with LRV. The numbers on the phylogenetic tree represent substitution rates per site.



in Turkey for the first time. Regarding the emerging incidence of leishmaniasis in Turkey and its neighbors, we plan to assess the presence of LRV in *Leishmania* isolates from Turkey on a broader scale, not only within *L. major* but in all *Leishmania* isolates in Turkey, including the hybrid *Leishmania* isolates in Turkey. Hybrid *Leishmania* strains have recently been identified in Turkey (Özbilgin *et al.*, 2016), and since they may show non-typical clinical manifestations and remain unresponsive to routine diagnosis and treatment, they are regarded as potential public health threats. Assessment of LRV in hybrid *Leishmania* isolates will eventually provide not only an epidemiological marker to identify the original species of the hybrid isolates, but also an estimation for prognosis of the infection. We also plan in vitro and in vivo projects to compare the immune responses received from LRV (+) and LRV (-) *Leishmania* isolates. Viral genomics will be instrumental in future studies, to unveil alterations in the genomic context of the virus as well as host-parasite interactions.

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