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Original Article

Development of an Indirect Fluorescent Antibody (IFA) Assay for the Detection of *Leishmania* RNA Virus 2 (LRV2) in *Leishmania* Parasites

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Abstract

Background: Detection of *Leishmania* RNA virus (LRV) in Old World *Leishmania* species and their possible role in the disease prognosis requires sensitive and specific methods, preferably independent of the viral genome. We aimed to develop an indirect immunofluorescence antibody (IFA) assay to detect LRV in the Old World *Leishmania* parasites.

Methods: Clinical samples were collected from 86 cutaneous leishmaniasis (CL) patients in different endemic areas of CL in Iran, during 2017-2019. For antibody preparation, the viruses were obtained from sediment of an LRV-infected *L. major* culture using freeze and thaw cycles followed by gradient cesium chloride centrifugation. The purified viruses were used to immunize a male 3-4 months rabbit. Various dilutions of the LRV-immunized rabbit's serum and a conjugated antibody were deployed to detect LRV in 48 isolates by IFA assay.

Results: LRV virus was detected in four of the 48 CL cases using IFA method. Amplification of a partial fragment of RNA-dependent RNA polymerase (*RdRp*) gene from the isolates confirmed the IFA results. In phylogeny, the generated *RdRp* sequences from four isolates were grouped with the other Old World LRVs, but separate from *L. aethiops* LRVs, which appear as a highly supported distinct clade.

Conclusion: Further optimization of this approach to detect the LRV directly in lesion scrapings can make it a more reliable tool for field studies and disclosing the virus's possible role in disseminating and unusual clinical features.



Introduction

Leishmaniasis is one of the most significant vector-borne parasitic diseases worldwide (1). Across 98 countries, more than 350 million people are at risk, and an estimated 12 million patients suffer from leishmaniasis (2). However, some 5 to 10 times as much as the reported cases remain asymptomatic and go unnoticed for hitherto unknown reasons (3). The disease manifests a broad range of clinical features ranging in severity from limited self-healing cutaneous lesions (CL) to disseminated mucocutaneous (DCL) infections and potentially fatal visceral leishmaniasis (VL) (4). The clinical features of the disease vary significantly with the species and strains of the parasites. However, the human host's genetic structure (e.g., genetic polymorphisms in TNF, IL-6, and HLA genes) and HIV coinfections contribute to leishmaniasis exacerbation (5).

In South America, *Leishmania* RNA virus (LRV) of the Totiviridae family play a crucial role in the mucosal and disseminated infections caused by *L. (Viannia) braziliensis* (6) and *L. (Viannia) guyanensis* (7). In addition, the LRVs also infect the Old World species, i.e., *L. aetiopica* (8), *L. major* (9), *L. infantum* (10), and *L. tropica* (11, 12).

Based on a tremendous difference in genome composition, the LRVs in the New World and Old World are grouped into LRV1 and LRV2 types with several subtypes in both types (13, 14).

In the New World, The LRV's underlying role in the enhanced pathogenicity and metastasis of the *Leishmania* infections is well documented (13, 15) and is associated with a high virus burden in parasites (8, 16). The innate immune system senses the LRVs in the early hours of infection. Some parasites die and release viral dsRNA that binds to Toll-like receptor 3 (TLR3) upon entering macrophages. This event triggers the subsequent IFN-type I-driven inflammatory cascade, intensifying the

disease (8, 17). In addition, the combined human and mouse data showed that LRV triggers TLR3 and TRIF to induce IFN-type I production (18). In human infections, disseminated cutaneous forms were accompanied by LRV infection of the parasites (8).

Iran is one of the significant endemic foci of CL in western Asia (19). CL appears in two main forms: zoonotic cutaneous leishmaniasis (ZCL) and anthroponotic cutaneous leishmaniasis (ACL) in the country (20). The emergence of disseminated and complicated forms of CL, mostly ZCL in the endemic areas, has diverted attention to the possible role of the LRV as a contributing factor. Previously, PCR amplification of the *RdRp* gene revealed LRV in *L. major* and *L. infantum* isolates in Iran (10). However, this method is time-consuming, costly, and relies on extracting a high-quality RNA virus followed by cDNA synthesis; and requires equipment like thermocyclers, electrophoresis, and gel documentation devices, which are not affordable in unprivileged areas. Besides, variations in the virus genome and mismatches in the primers and the target gene might lead to amplification failure (7).

The development of inexpensive and rapid tools with high sensitivity for detecting LRVs in *Leishmania* strains will assist studies on the possible role of LRVs in leishmaniasis prognosis. This study reports developing an IFA assay to detect LRV in *Leishmania* parasites of the Old World. This tool exploits the interaction of polyclonal antibodies with the epitopes in virus antigens that appear on the surface of the LRV- infected parasite.

Materials and Methods

Clinical samples and parasite culture

Samples were obtained from the 86 CL patients who were referred to leishmaniasis Laboratory, Department of Parasitology and

Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran during 2017-2019. Scrapings were obtained by a sterile lancet from the margin of the lesions. Smears were prepared, stained with 10% Giemsa and examined for the presence of amastigotes by microscopy under 1000X magnification. Moreover, lesion aspirates from patients were inoculated into RPMI-1640 medium (Invitrogen, USA) supplemented with 10%-20% fetal bovine serum and 0.1% pen-strep (21). The cultures were incubated at 24°C in a shaker incubator and monitored daily for promastigotes under an inverted microscope with 400X magnification.

Ethical Approval

We obtained written consent from all participants or their legal guardians, and the Ethics committee of Tehran University of Medical Sciences approved the study (code No.: IR.TUMS.SPH.REC.1397.283).

Molecular characterization of isolates

According to the manufacturer's instructions, DNA from the promastigotes of cultured isolates was extracted using a commercial kit (Bioneer, Korea). A partial sequence of ITS1 locus was amplified using the primers designed by others, followed by digestion with the HAEIII enzyme as described previously (21).

RNA extraction and cDNA construction

RNA was extracted from 10⁶ cultured promastigotes in the late logarithmic phase using a High pure RNA isolation kit (Roche, Germany) according to the manufacturer's instruction. The extracted RNAs were resuspended in 50 µL of nuclease-free water. The quantity of the extracted RNA, ng/µL, was measured by a Nanodrop instrument (ND-1000, Thermo scientific Fisher, US). The cDNA was synthesized from an RNA template with a combination of oligo (dT) 18 and random hexamer primers using the cDNA Synthesis Kit

(Roche, Germany) according to the manufacturer's instructions.

RdRp gene amplification and BLAST analysis

A 315-bp fragment from the RNA-dependent RNA polymerase (*RdRp*) gene was amplified using a semi-nested PCR assay (10). The resulting amplicons from the second amplification round of four specimens were sequenced in both directions using the same primers used for amplification in an ABI Prism™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) by a commercial Company (Macrogen, Seoul, South Korea). The generated sequences were BLASTed against similar sequences available in the GenBank database, and the similarities were obtained.

GenBank submission

The LRV sequences generated in this study were deposited in the GenBank database under the accession numbers MN13357-58 and MT268281-82.

Phylogenetic analysis of LRVs

A phylogenetic tree was constructed using the two generated ~280-bp sequences and other LRV sequences of the New World and Old World *Leishmania* species available in the GenBank database. The pairwise nucleotide identity of the sequences was performed using BioEdit, version 7.0.5, and examined using MEGA X (22). We selected the maximum likelihood (ML) method to draw a phylogenetic tree using Kimura 2-parameter models in MEGA X (22) with *Trichomonas vaginalis* virus (TVV) as the out-group. Bootstrap resampling analysis with 1,000 replications was used to assess branch confidence in clades in each tree.

Virus extraction

Totally, 500 ml of an LRV-infected *L. major* culture containing 2-3×10⁶ promastigotes/ml in the stationary phase (5-6 days after last pas-

sages) was centrifuged at 800 g for 15 min at 4 °C. The aqueous phase was discarded, and the sediment with the remainder of medium culture (15 ml) was alternately frozen and thawed at liquid nitrogen (-196 °C) and 37 °C, respectively. The lysate was centrifuged at 800 g for 10 min, and the supernatant, supposedly containing the virus, was centrifuged at 42000 g for 2.5 h at 4 °C. The resulting pellet was suspended in 1 ml of PBS and kept at -20°C until used (23, 24).

LRV purification

A decreasing gradient cesium chloride concentrations (1.4, 1.35, 1.3, 1.25, 1.2, 1.15, and 1.1 µg/ml in 0.1 Mol Tris buffer, pH=7.2) was added gently into an 8 ml tube. One ml of the PBS virus suspension was smoothly added to the tube and centrifuged at 71000 g for 3 h, 4 °C. The resulting phase was dialyzed against double distilled water (DDW) overnight and then stored at -70 °C until used (23).

Visualizing the LRV

For detecting the virus-like particles, we used negative staining as described elsewhere (25). Amounts of 5-10 µl from each phase were spotted on a carbon-coated copper grid and left for 3-5 min. Then, the grid was drained with filter paper and negatively stained with 2% uranyl acetate. The samples were examined under a transitional electron microscope (Zeiss LEO 906, Germany) at 100kV, with a 35970X magnification. Spherical virus-like particles measuring ≈50 nm in diameter were observed in the phase that matched the previous observations (26) (Fig. 1-A).

The semi-nested PCR method confirmed the presence of LRV2 in this layer.

This layer was used for the *in vivo* production of the polyclonal antibodies in a laboratory animal.

Rabbit inoculation with LRV2

The purified virus (0.5 ml) was mixed with 0.5 ml of Freund's complete adjuvant (F5881-Sigma, Germany) It was inoculated subcutaneously into the back of the neck of a male 3-4 months New Zealand Rabbit. Three boosting shots of one-week intervals, and a fourth with a 10-day interval, were inoculated with the virus preparation and Freund's incomplete adjuvant (F5506-Sigma, Germany). Ten days after the last shot, a blood sample was obtained from the animal's heart. The serum was separated by centrifugation, aliquoted, and stored alongside the animal's preimmunization serum at -20°C.

Indirect Fluorescent Antibody assay

Antigen preparation

In 15 ml falcon tubes, 5ml from individual 48 isolates and control cultures were mixed with an equal amount of 2% formalin and kept at room temperatures (RT) for 30 min. The suspensions were centrifuged at 800 g for 20 min, 4 °C, and the resulting pellets were washed three times with PBS (pH 7.2) and resuspended in PBS. 10 µl the suspensions containing 10⁶ parasites/ml were spotted on slides, allowed to dry for 2 h, at RT, and then kept at -20°C until used (27). LRV-negative and LRV+positive isolates previously checked by nested PCR amplification of the *RdRP* sequence were included in assays as controls (10). All the isolates were tested in duplicate.

IFA assay

Various dilutions of the LRV- immunized rabbit serum (1/10, 1/20, 1/40, and 1/80) in PBS were added to the spots on the slides, kept for 30 min at room temperature, and then washed three times with PBS (pH 7.2). Dilutions of a conjugated antibody in PBS (1/100, 1/150, and 1/200) were added to the spots, followed by incubation at RT for 30 min. The slides were washed as before and examined under an IFA microscope (Zeiss, Germany) with 400X magnification.

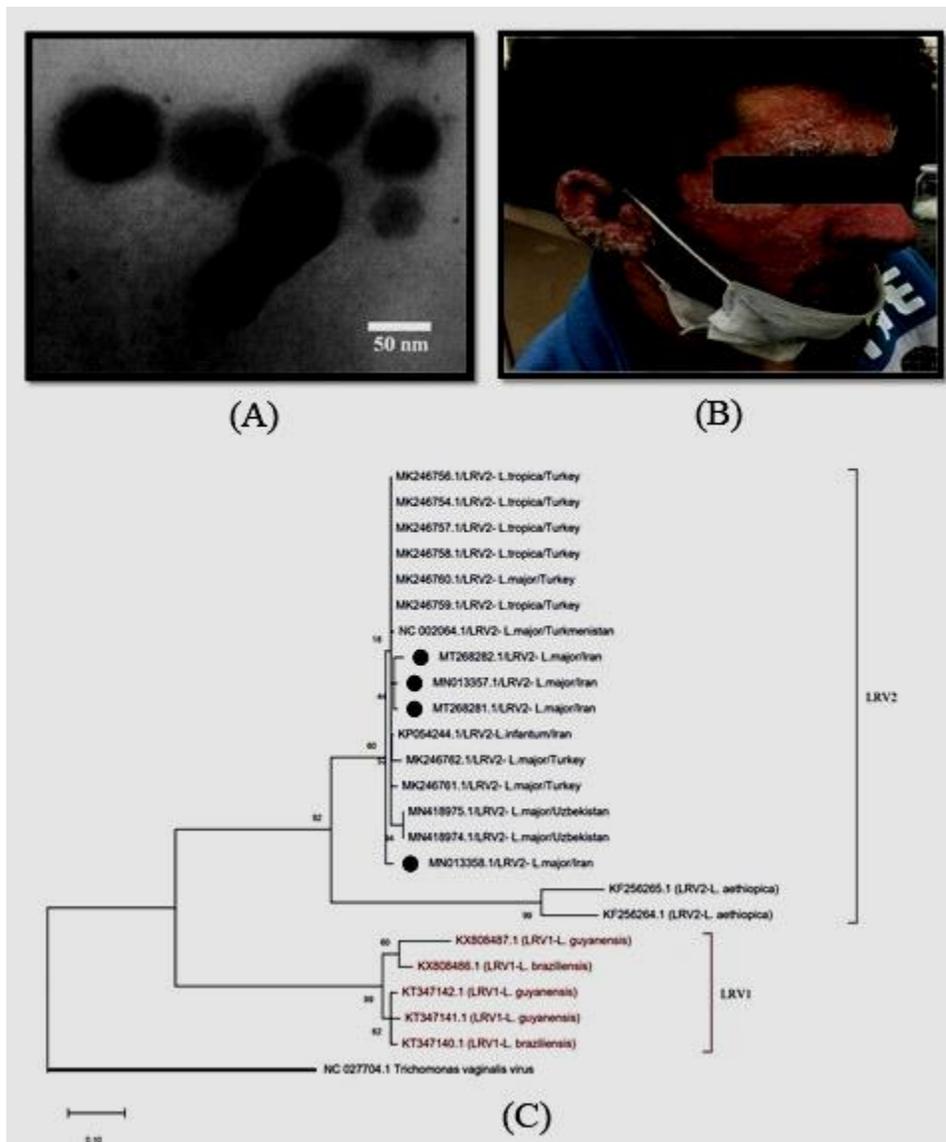


Fig. 1: (A): Virus-like particles measuring $\approx 30\text{-}50$ nm visualized by transmission electron microscopy, magnification 35970X. (B): Disseminated cutaneous leishmaniasis caused by an LRV-infected *L. major* isolate. (C): The phylogenetic analysis of *RdRp* sequences. The black bullets indicate the sequences generated in this study. The scale bar indicates 0.1-estimated nucleotide substitutions per site, and numbers on the nodes refer to bootstrap probability (BP) values

Results

Leishmania species

Of the 86 clinical samples that became positive by microscopy, 48 yielded a culture. PCR amplification of ITS1 sequence followed by RFLP identified 30 *L. major* and 18 *L. tropica* isolates.

Semi-nested PCR and BLAST analysis of LRV

PCR amplification of the *RdRp* gene in 48 samples revealed LRV in four *L. major* isolates, while all 18 *L. tropica* isolates were negative for the virus. The generated sequences exhibited the highest identity (95%-98%) with the LRV2 sequences from Iran (Acc. No KP054244), Turkey (Acc. No MK246754), and the com-

plete genome LRV2 isolates from Uzbekistan (Acc. No MN418975) and Turkmenistan (Acc. No U32108).

LRV Phylogeny

The phylogenetic tree revealed two deeply divergent groups representing the Old World and New World LRVs. The generated LRV sequences were grouped with the rest of the Old World LRVs. The *L. aethiopica* LRVs appeared as a highly supported distinct clade (PB:99) yet shared a common ancestor with the Old World LRVs (Fig. 1-C).

IFA assay

In the IFA assay, positive controls (Fig. 2-A) and 4 *L. major* isolates (Fig. 2-C, and D) emitted a brilliant green luminescence under immunofluorescence microscopy, consistent with the semi-nested PCR result. None of the

44 PCR negative isolates and negative control emitted a fluorescence glowing (Fig. 2-B). The optimum results were obtained with 1/20 and 1/40 dilutions of the LRV-immunized rabbit serum and 1/100, 1/150 dilutions of the conjugated antibody.

The positive LRV samples belonged to the ZCL foci of Iran in the northeast (Golestan province, n=2), the center (Semnan province, n=1), and the west of the country (Ilam province, n=1).

Out of the four CL patients infected with LRV-infected *L. major*, one had disseminated cutaneous leishmaniasis (Fig. A-B) and was quite resistant to complete treatment three courses with meglumine antimoniate (Glucantime®) and Liposomal Amphotericin B (AmBisome®); the other three patients had typical leishmaniasis lesions.

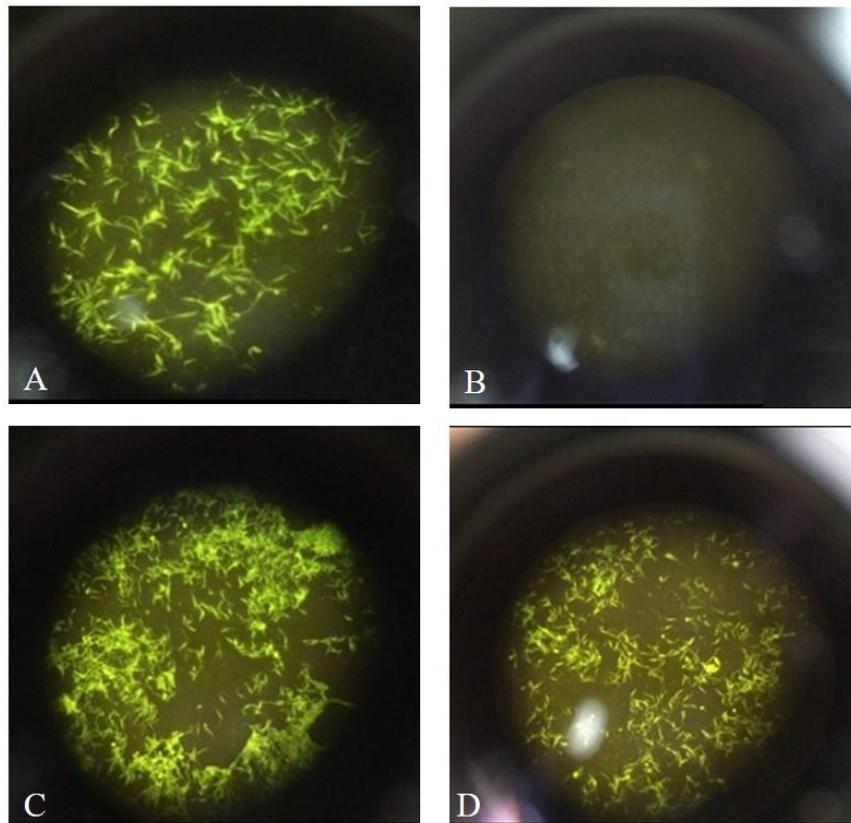


Fig. 2: Detection of LRV-infected promastigotes by IFA assay. A, LRV2-infected isolate (Positive control); B, negative control; C and D positive LRV2 isolates

Discussion

CL poses a severe health problem in the Middle East and Asia countries, including Iran. According to the World Health Organization, Iran had the highest disease prevalence in West Asia (28). In the country, the annual incidence of CL was estimated to be 30.9 per 100,000 in the Iranian people (29).

The recent emergence of unusual clinical signs and the disseminated lesions in some ZCL endemic regions of Iran diverted the attention to LRV's role as one of the possible contributing (30, 31).

Diagnosis of the virus in *Leishmania* parasites relies primarily on detecting the virus's genetic material. RT-PCR is highly sensitive and can successfully detect the LRV in the New World and the Old World species (8, 10, 12). However, it requires a high-quality RNA, followed by cDNA preparation and PCR amplification of the LRV sequence. This approach is costly and time-consuming and is limited to well-equipped laboratories.

Hence, straightforward and reliable screening methods to detect LRV in numerous parasite isolates under field conditions can assist studies on the possible role of the virus in the disease prognosis.

In the present study, we developed an IFA assay to detect LRV in the Old World *Leishmania* species using antibodies raised against the whole virus particle. Among 48 *Leishmania* isolates, our IFA detected LRV in four *L. major* isolates that also became positive by the semi-nested RT-PCR. Previously, Zangger et al., using polyclonal antibodies raised against capsid polypeptide of the *L. guyanensis*, detected LRV1 in the New World *Leishmania* species by both Western blot and immunofluorescence microscopy (8). However, due to LRV sequence diversity in the capsid polypeptide gene, the antibodies failed to detect the virus in a similar strain (8).

As shown in Fig. 2, the LRVs particles seem to be bound to the *Leishmania* parasite's surface. They most likely are those viruses that have exploited the *Leishmania* exosomal pathway to reach the extracellular milieu or released from dead parasites in the supernatant and then invaded other parasites. Recent studies have shown the intracellular packaging of the LRV within exosomes; actually, the virus seems to hijack the *Leishmania* exosomal pathway and finds its way to the extracellular environment (32).

Further investigation involving other LRV-infected *Leishmania* species, particularly *L. aethiops* isolates that harbor a divergent LRV, might approve this assay utility over a broader geographical area.

In our study, one of the four patients with LRV-infected *L. major* had disseminated lesions with no response to meglumine antimoniate (Glucantime®) and Liposomal Amphotericin B (AmBisome®) treatment courses. Without an LRV quantitative analysis, we could not link these features with the virus's burden in *Leishmania* parasites.

Conclusion

The culture of isolates was a prerequisite for the IFA assay. Further optimization of this IFA to circumvent the culture and directly detect LRV in lesion scrapings promise one-step closer to a screening method under field condition. We offer this IFA method for LRV detection in *Leishmania* parasites in endemic areas to determine the LRV prevalence. This technique can help us find a possible relationship between LRV infection of the parasite and clinical signs or treatment failure.

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Patent

IFA technique was patented by Dr. Hajjaran H, Dr. Ataei-Pirkooch A, and Dr. Mohebbali M. The Patent was filed in April 2019 and issued in January 2020 under the number: 100042 in Real Estate Registration Organization of Iran, Tehran. (<http://www.ssaa.ir/>). International classification is G01N 33/58:C07K 19/00; G01N 33/569.

Conflict of interest

The authors declare that there is no conflict of interests.

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