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Case Report

Molecular Tracking of *Leishmania major* in an Archived *Rattus norvegicus* Spleen Sample in Iran: A Case Report

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Abstract

Rodents are the primary reservoir hosts for zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major*. Knowing reservoir hosts is crucial for leishmaniasis surveillance and control programs in endemic areas. In this study, we examined an archived spleen of *Rattus norvegicus* obtained during a pest control program in 2000 in Tehran, the capital of Iran. The sample was analyzed using polymerase chain reaction (PCR) and sequencing to determine the presence of Trypanosomatidae based on the internal transcribed spacer (*ITS*) 1 gene. Amplification and sequencing of the discriminative region of the *ITS1* gene followed by BLAST analysis showed the highest similarity with *L. major* isolates. Also, the phylogenetic analysis revealed that our sample was grouped with *L. major* isolates retrieved from the GenBank database. This finding might support the claim that *R. norvegicus* acts as a potential reservoir host for *L. major*. Further studies, including a survey on more rodent samples as well as studying sandflies in the area, might uncover the possible presence of such pathobiological conditions in ZCL transmission in urban and suburban settings.

Introduction

Cutaneous leishmaniasis (CL) is a vector-borne disease caused by several *Leishmania* species and transmitted by phlebotomine sandflies (1). In Iran, *L. major* and *L. tropica* are causative agents of zoonotic (ZCL) and anthroponotic CL (ACL), respectively (2). Meanwhile CL is prevalent in many rural areas across 19 out of 31 provinces in the country, mainly in Fars, Khuzestan, and Isfahan (3).

Rodents of the subfamily Gerbillinae are the main reservoir of ZCL in most endemic parts of Iran (4). Nonetheless, accidental infection of other rodent species by *L. major* could suggest the potential role of these species in the distribution of the zoonotic cycle of *L. major*. However, some reports indicated *Leishmania* infections in synanthropic species like *R. norvegicus* (Norway rat) and *R. rattus* (roof rat) (5). The rats in densely populated urban areas can cause extensive physical damage and spoil foodstuffs. The urban environment promotes close contact between rats and people(6), which increases the risk of zoonotic disease transmission.

The increased *R. norvegicus* populations in recent decades in urban settings like Tehran could expose humans more significantly to the risk of infectious diseases harbored by this rodent, underscoring the urgent need for further research and surveillance.

In the present study, we investigated the potential role of *R. norvegicus* as a reservoir of *Leishmania* spp. in Tehran by examining the formalin-preserved spleens of this animal.

Case presentation

The present analyzed spleen sample was accidentally found amongst the preserved materials from the pest rodents control program in 2000 by Tehran's municipality in highly populated localities in the south near the old bazaar (District 12).

The formalin-preserved samples were embedded in paraffin, and 4-5 µm thick sections were prepared using a Manual Rotary Microtome (Leica Biosystems, USA). Histopathological analysis was conducted by processing the tissue and performing standard hematoxylin-eosin (H&E) staining (7) and examined under light microscopy at 1000x magnification.

Total DNA was extracted from three randomly selected parts of the formalin-fixed spleens according to manufacture instructions provided by a commercial DNA-Formalin-Fixed Paraffin-Embedded (FFPE) Tissue kit (Sinaclone Technology and Services, Tehran, Iran).

Since the genus of Trypanosomatidae parasite was not detectable using microscopical examination, Universal primers targeting a highly homologous region of the ribosomal internal transcribed spacer (ITS) 1 for the *Leishmania*, *Trypanosoma,* and *Crithidia* species, were designed Using the Primer 3 software (https://primer3.ut.ee/), consisted of forward (5'-AGGAAGCAAAAGTCGTAAC-3') and reverse (5'- GAAGCCAAGTCATCCATC-3) to amplify a ~ 350-bp fragment of the *ITS1* gene. The PCR reaction was performed *in 25 µL* volume comprising 12.5 µL master mix (Ampliqon, Denmark), 10 pmol of each primer, 4 µL of DNA, and 6.5 µL distilled water. The amplification began with an initial denaturation at 94°C for 6 min, followed by 35 cycles of 94°C for 1 min, 48°C for 30s and 72°C for 1 min, with a final extension at 72°C for 5 min. *L. major* strain (MRHO/IR/75/E.R.) DNA and DNA-free samples were as positive and negative controls, respectively. The amplicon was sequenced in both directions and submitted to the GenBank database with accession number LC746245. In addition, kinetoplast DNA (kDNA) was used as another genetic marker to confirm the *Leishmania* genus, as described previously (8).

The phylogenetic tree was constructed using the maximum likelihood (ML) method with the Kimura 2-parameter model using MEGA soft-

ware version 11 (9) with bootstrap values estimated with 1000 replicates for sequences from the GenBank database belonging to *L. major* (n=20), *L. turanica* (n=4), *L. tropica* (n=3), and *L. aethiopica* (n=1), alongside a *Trypanosoma brucei* sequence (accession no. JN673390), as the outgroup.

The size of our merely archived spleen used in the present study was measured at 3.5 cm in length, 1 cm in width, and 1 cm in thickness. As shown in Fig. 1, the normal spleen in *R. norvegicus* had measurements of 2.2 cm in length, 0.5 cm in width, and 0.5 cm in diameter, which is in consistent with the literature (10). The archived spleen was 1.59, 2, and 2 times larger in length, width, and depth, respectively, compared to the normal spleen. No pathological and/ or parasitological signs were seen histosections.

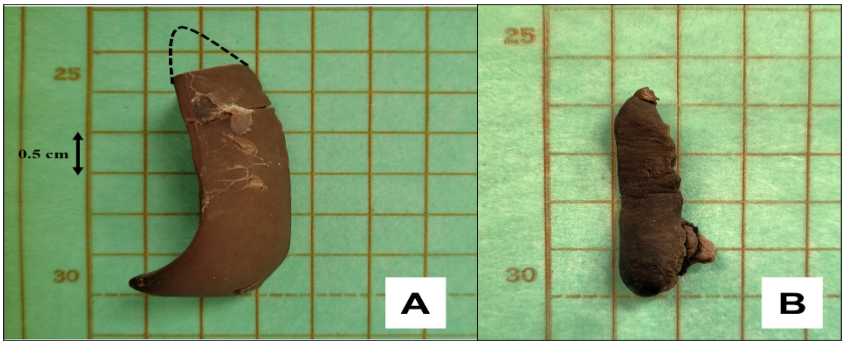

Fig. 1: The present studied spleen in *R. norvegicus* compared with a normal spleen. The enlarged *R. norvegicus* spleen with the missing part is shown with the dotted line (A) compared to a normal *R. norvegicus* spleen (B)

Amplification of the ITS1 fragment resolved a 350-bp amplicon by gel electrophoresis (Fig. 2). In BLAST analysis, the generated sequence exhibited 100% identity with *L. major*. The amplification of an identical fragment for the kDNA gene was 560-bp as reference sample.

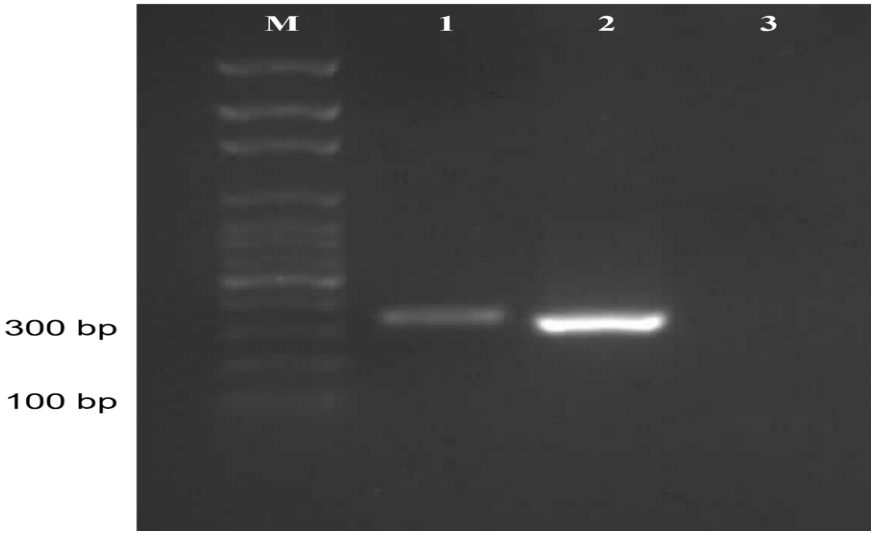

Fig. 2: Electrophoresis of the ITS1 amplicon on 1.5% agarose gel. M, 100-bp DNA Marker (Gene Ruler™, Fermentas); 1, standard strain of L. major (MRHO/IR/75/ER); 2, DNA from a *R.norvegius* spleen; 3, negative control

Our phylogenetic tree revealed two main clades comprising the *L. major* and *L. tropica* complexes. The sequence obtained in this study emerged alongside *L. major* isolates from Iran, Turkey, Uzbekistan, and Iraq (Fig. 3).

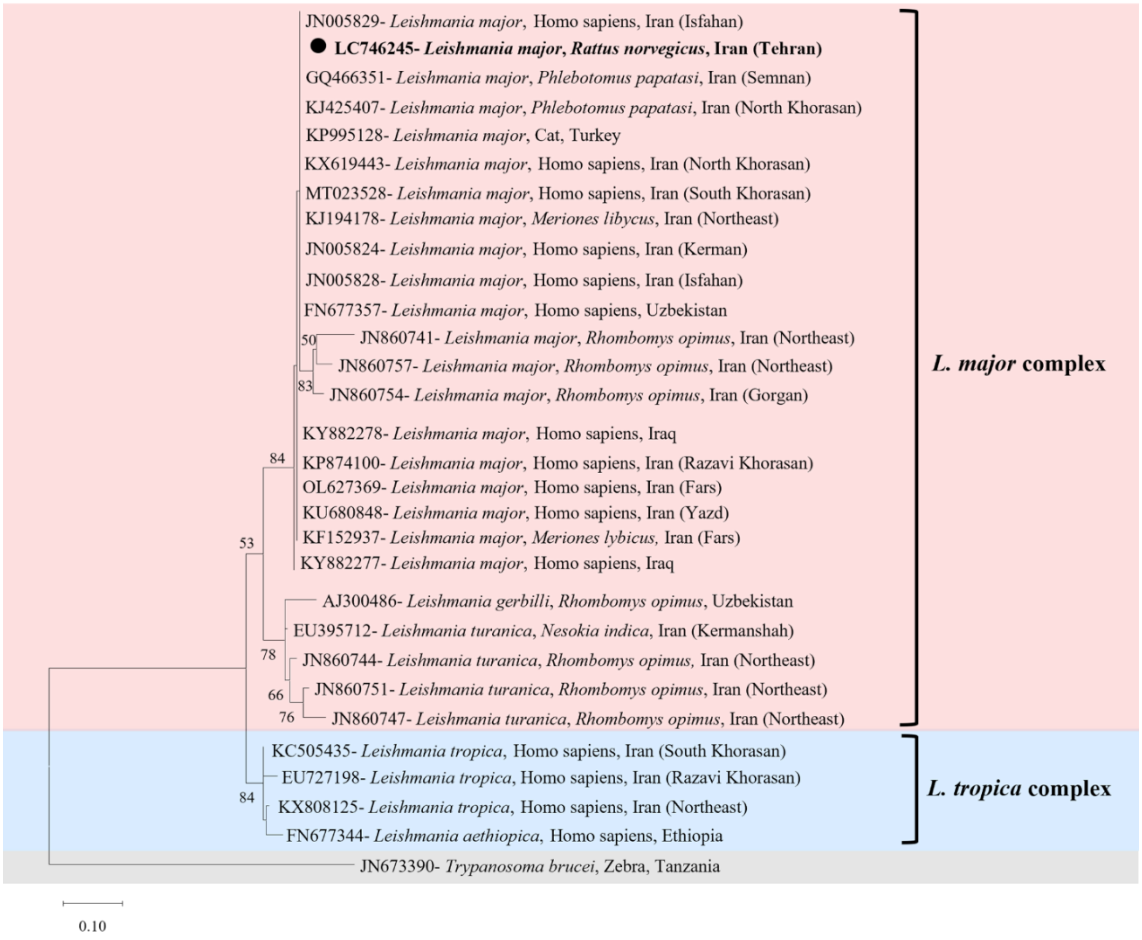

Fig. 3: The phylogenetic tree inferred based on 302-bp of ITS1 sequences of *Leishmania* spp. retrieved from the GenBank and the sequence from the present study (indicated by a black circle). The evolutionary history was derived using the maximum likelihood (ML) method and the Kimura 2-parameter model in MEGA11. Bootstrap values (>50%) are shown at the nodes based on 1000 replicates

Discussion

Several wild, domestic, and synanthropic mammals have been reported as hosts and reservoirs for *Leishmania* spp. *Rattus norvegicus,* the most common pest worldwide, is a reservoir for various zoonotic parasites. The infection of *R. norvegicus* with *L. major* was first reported by Morsy et al. (11) and Sami et al. (12) in Egypt. Given the potential role of *R. norvegicus* in transmitting *Leishmania* spp., the importance of looking back to uncover the possible same scenario using PCR on our archived spleen in Tehran was highlighted. Following positive molecular analysis results, the histopathological procedure did not illustrate any reliable signs of tissue changes or parasitological findings. Despite preserving the sample in formalin over the years, DNA extraction and amplification were successfully carried out. Sequences analysis of

ITS1 revealed the presence of *L. major* parasite in the spleen sample of *R. norvegicus*. Phylogenetic analysis indicated that our sequence was located with *L. major* complex alongside *L. major* isolates obtained from Iran, Turkey, Uzbekistan, and Iraq. However, seeing the current result has made it more tolerable to hear the previous reports indicating the presence of *L. major* in the rat stools, although based on the lifecycle of *L. major*, the identification of *Leishmania* species in the fecal specimens obtained from rodents has raised questions. In line with our results, in 2020, *L. major* was also reported in fecal samples of *R. norvegicus* of Tehran province by nested- PCR amplification of the kinetoplast DNA (kDNA) sequence (13).

Moreover, prior to abovementioned paper, *L. major* was also reported in 2010 in *R. norvegicus* skin samples in Fars province using PCR targeting kDNA (14). In both records, the target sequences were not registered in GenBank. Regardless of the lack of reliable histopathological results in these reports, to our knowledge, the present paper can be considered the first actual record of *L. major* in twenty-year-old archived samples from *R. norvegicus* in Iran.

Meanwhile, the recording of sporadic cases of ZCL due to *L. major* and *L. tropica* causing ACL in Tehran might be in favor of the potential role of *R. norvegicus* in leishmaniasis in certain spaces and times in this part of the country. Nevertheless, considering no available report of human cases in Tehran, studies on the existence of the infection transmission cycle in this geographical region are needed.

In sudden climatological and natural environmental changes as well as unexpected population movement, the explosion of these rodents that leads to closer contact between humans and the rats, the occurrence of such vector-borne parasitic infections may facilitated (15), which urban managing planners should seriously regard.

The facts described here recommend careful and regular monitoring to avoid a sudden confrontation with rats in human residing environments, parallel to investigating their possible role in cutaneous leishmaniasis. The present paper has also emphasized the need for more studies on different *Leishmania* species' lifecycles in neglected localities within the endemic territories to support a more successful national control program on leishmaniasis.

Conclusion

Our present finding in *R. norvegicus* illustrates the increased possible role of this urban pest in cutaneous leishmaniasis in Tehran despite no previous records. In this study, further research towards finding the reservoirs of rural cutaneous leishmaniasis is recommended, with specific attention to Muridae family members that have not yet been defined as reservoirs.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors have no affiliations with or involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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