

Original Article**Zoonotic cutaneous leishmaniasis in Shiraz, Southern Iran:****A molecular, isoenzyme and morphologic approach**

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**Abstract**

**BACKGROUND:** Cutaneous leishmaniasis (CL) with diverse clinical manifestations is prevalent and remains a major public health problem in Iran and its incidence has been doubled over the last decade. The present study is about the potential role of rodents in the epidemiology of CL in Kharameh district in Shiraz, Southern Iran.

**METHODS:** From April 2004 to April 2005, a total of sixteen rodents were collected in live traps from the endemic area of CL in Kharameh district in Shiraz. Evans medium was used for culture. Specific polymerase chain reaction and isoenzyme electrophoresis methods were performed to characterize the parasite.

**RESULTS:** The rodent species were *Tatera indica*. Three samples from *Tatera indica* were found positive (2 males and 1 female in Kafdehak and Sejel-Abad villages) for *L. major*. Macrophages in the bone marrow of femoral bone were infected with the amastigote form of the parasite.

**CONCLUSIONS:** It seems that *T. indica* is the reservoir host for CL in Kharameh (a district in Shiraz, Southern Iran). It was shown that the bone marrow of the rodents is the tissue of choice for light and ultrastructural studies of *L. major*.

**KEY WORDS:** Southern Iran, cutaneous leishmaniasis, isoenzyme, PCR, morphology.

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**L**eishmaniasis is distributed in tropical and subtropical areas and it is estimated that more than 15 million people are infected by this disease, with 400,000 new cases per year. More than one third of the world's population live in endemic areas and are at risk of infection. Leishmaniasis mainly occurs in the underdeveloped countries. Over twenty five countries and territories in the American continents and 75 countries and territories in Africa, Mediterranean region, the Middle East, the Southern part of Russia and Asia are

endemic foci of infection<sup>1</sup>. Zoonotic cutaneous leishmaniasis (ZCL) is an important health problem in Iran and a great economic burden on the health resources<sup>2</sup>. It is essentially a disease of rodents caused by *Leishmania major*, which is transmitted to humans mainly by *Phlebotomus Papatasi* sand flies<sup>3</sup>. In the west and south west of Iran, *Tatera indica* is primary and *Nesokia indica* or *M. libycus erythrourus* are the secondary reservoir hosts of the disease<sup>4, 5</sup>. In the South East of Iran (Baluchistan), *Meriones hurrianae* and *T.*

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indica are known as the main and the secondary reservoirs of this disease, respectively <sup>6</sup>. In south of Iran, *M. libycus* was confirmed as the main reservoir host of ZCL in Arsanjan <sup>7</sup> and Marvdasht <sup>8</sup>. The anthroponotic form of CL was described in Southern Iran by Nadim et al <sup>3</sup>. The endemic state of the CL in Kharameh district in Shiraz, its public health importance and the potential role of rodents in the epidemiology of CL in this area, were evaluated to help the authorities control this disease.

## Methods

### Study area

Fars Province is located in south of Iran and it contains 8% of Iran's area. It is mountainous with an average elevation of 5000 feet above the sea level and its climate is quite dusty and dry, with warm summers, mild winters, and a great deal of sunshine throughout the year <sup>9</sup>. Kharameh district is located in the northeast of Shiraz.

### Rodent trapping

The rodents were captured according to the methodology, standardized for the capture of small mammals <sup>10</sup>. They were caught in wire traps (35 cm × 12 cm × 12 cm), and baited with bread and toasted peanuts. From April 2004 to April 2005, during 96 consecutive nights in all the rural areas of Kharameh district in Shiraz, all the traps were set in different locations in agricultural plantations surrounding the houses. The traps were checked every morning. The identification of the specimens was based on the specific taxonomic criteria <sup>11</sup>, set by Department of Biology of Shiraz University. The captures were conducted under the permission of Fars Environment Protection Organization and the euthanasia was performed under the Ethics Code of Iran's Veterinary Organization.

### Preparation of smears and culture

Smears were provided from the ear, tail and the foot pads of the rodents and from any patient skin lesions <sup>12</sup> that were stained with Giemsa. They were examined under a light mi-

croscope for the amastigote form. Accordance to Iran's Veterinary Organization Ethics, the trapped animals were euthanized under chloroform anesthesia and were examined for any signs of cutaneous lesions and then they were necropsied. The sampling and the smear from skin of ear, tail and foot pads of the rodents were provided and inoculated into the Evans medium. Afterwards, they were incubated in 25°C for 1-4 weeks with weekly subcultures <sup>13</sup>. Samples were examined regularly to monitor the growth and the presence of contaminations. The contaminated cultures were discarded. Mass cultivation of the organism was carried out on RPMI medium containing 15% fetal calf serum (Gibco). The cultures were harvested at the end of the logarithmic phase of the growth and the number of the organisms was adjusted to 1-1.5×10<sup>7</sup>/ml. The cultures were centrifuged at 2000 g for 20 min at 4°C. The supernatant was discarded and the pellet of promastigotes was washed three times by resuspension and recentrifugation in cold-proline balanced salt solution.

### DNA Extraction

The precipitate of the cultivated sample was diluted by double distilled water (1:10) and the DNA of each cultivated sample was extracted by adding proteinase K to 5 ml of each sample. Then, lysis buffer was added (50 ml of Tris-HCl, pH=7.6; 1 mM of EDTA, pH=8.0; 1% Tween 20, 8.5 ml of proteinase K solution, 19 mg/ml) and was incubated at 37°C for 24 hours. Then, the lysate was extracted twice with phenol/chloroform/isoamyl alcohol before the DNA was precipitated with absolute ethanol; and it was re-suspended in 100 µL of double distilled water and stored at 4°C.

### PCR

The set of primers LINR4 (forward: 5'-GGGGTTGGTGTAATAATAGGG-3'), and LIN17 (reverse: 5'-TTTGAACGGGATTTCTG-3') were used for PCR <sup>14</sup>. A reaction mixture containing 1.5 mM of MgCl<sub>2</sub>, 200 µM (each) of deoxynucleoside triphosphate, 2.5 µL of 10 X taq polymerase buffer, 1.5 unit of taq DNA polymerase and 40 pmol of each primer were used in a total reaction volume of 25 µL including 5 µL of

the DNA sample. The mixture was amplified in a programmable thermocycler (Thence Cambridge, UK) for 5 min at 94°C (1 cycle) followed by 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 min followed by a final elongation at 72°C for 5 min (1 cycle) and kept at 4°C.

#### Agarose-gel Electrophoresis

A 10-µL sample of the final PCR product was subjected to electrophoresis in 1.5% agarose gel. Five µL of loading buffer was added to the product before electrophoresis and it was visualized under UV light with ethidium bromide. The WHO reference strains of *L. tropica* (MHOM/SU/71/K27), *L. major* (MHOM/SU/73/5ASKH) and *L. infantum* (MHOM/TN/80/IPT1) were obtained for parasite characterization from Pasteur Institute in Tehran.

#### Enzyme extraction

The following method was used for enzyme extraction from the pelleted organisms: an equal volume of hypotonic aqueous solution of enzyme stabilizers was added to the washed pellet of promastigotes, (1 mM ε-amino-n-caproic acid, 1 mM dithiothreitol and 1 mM EDTA, Sigma) and it was mixed thoroughly. Freezing was carried out at the vapor phase of liquid nitrogen and thawing was done at 25-30 °C for five times. The extract was centrifuged at 30000 g for 30 min at 4 °C. The supernatant was prepared in 10 µl beads in liquid nitrogen and was stored at -70 °C<sup>15</sup>.

#### Enzyme electrophoresis

Analysis was performed using discontinuous polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed using 3% of stacking gel, 7.5% of separating gel, a stacking buffer composed of Tris/HCl (pH=6.7), a resolving buffer of Tris/HCl (pH=8.9) and a tank buffer of Tris/HCl (pH=8.3), which ran under a constant current of 2 mA/well for 150 minutes. Each stain was tested for the activity of the 5 enzymatic system; namely, malate dehydrogenase (MDH) E. C. 1.1.1.37, phosphoglucosmutase (PGM) E.2.7.51, glucose-phosphate isomerase (GPI) E.C.5.3.1.9, nucleoside hydrolase I (NH1) E.C.3.2.2.1, and nucleoside hy-

drolase II (NH2) E.C.3.2.2.1. Electrophoretic band developing conditions were used for each enzyme system as described earlier<sup>13, 16-19</sup>.

#### Histopathological study

To demonstrate the presence of parasites in the tissues, the animals were euthanized and autopsied according to Iran's Veterinary Organization Ethics. Their foot pad, tail and ear tissues, lymph nodes, spleen, liver and femoral bones were removed and were fixed in 10% buffer formalin. After 24 hours of fixation, bones were decalcified in 7% nitric acid. In the foot pad, the tissue sample was provided from the plantar aspect of the foot and it was sliced several times, perpendicular to the epidermal plane. After fixation, the tissues were embedded in paraffin, and sections of 5 µm thickness were stained with hematoxylin and eosin (H & E) and then, they were studied under a light microscope.

#### Electron microscopy

For an electron microscopic study, a small fragment of fresh tissues of liver, spleen, popliteal lymph nodes, the plantar aspect of foot skin and femoral bones of each animal were fixed in 3% glutaraldehyde and 0.2 M cacodylate, at 4°C. They were postfixed in 1% osmium tetroxide and were dehydrated through ascending series of ethanol and were embedded in agar-100 resin. Semithin sections (1 µ thickness) were cut by ultramicrotome and stained with toluidine blue and they were used for light microscopic orientation. Ultrathin sections were cut from the selected areas that mounted on copper grids and were double stained with uranyl acetate and lead citrate. Then, they were examined under the transmission electron microscope and were screened for the presence of amastigotes<sup>20</sup>.

## Results

#### Live Trapping

Sixteen rodents were captured and they were only from one species, *Tatera indica*.

#### Identification of Parasites

Three animal isolates exhibited exponential growth in Evans medium. Using PCR, *L. major*

was isolated and identified as the causative agent of CL from all the three *T. indica*. Among the trapped rodents, one *T. indica* was male and one was female in Kafdehak village in Kharameh district in Shiraz. They were also positive for *L. major*. *L. major* was isolated from Sejel-Abad village in the area of another male of *T. indica*. The provided impression smears from the positive PCR samples revealed an amastigote form of the parasite. Figure 1 shows agarose gel electrophoresis of PCR products by the use of primers specific for the *L. major* complex from the rodents. The electrophoretic isoenzyme patterns using the five enzymes were shown in figure 2 and all electrophoretic patterns demonstrated similarity to *L. major*. The six samples had common patterns for *L. major* that were identical to the WHO reference strain.

#### **Histological features**

Histological studies revealed numerous clusters of amastigotes in the bone marrow of femoral bone which composed of foamy macrophages (with intracellular *Leishmania* amastigotes). No amastigote was visible in the sections of the lymph node, spleen, liver and skin.

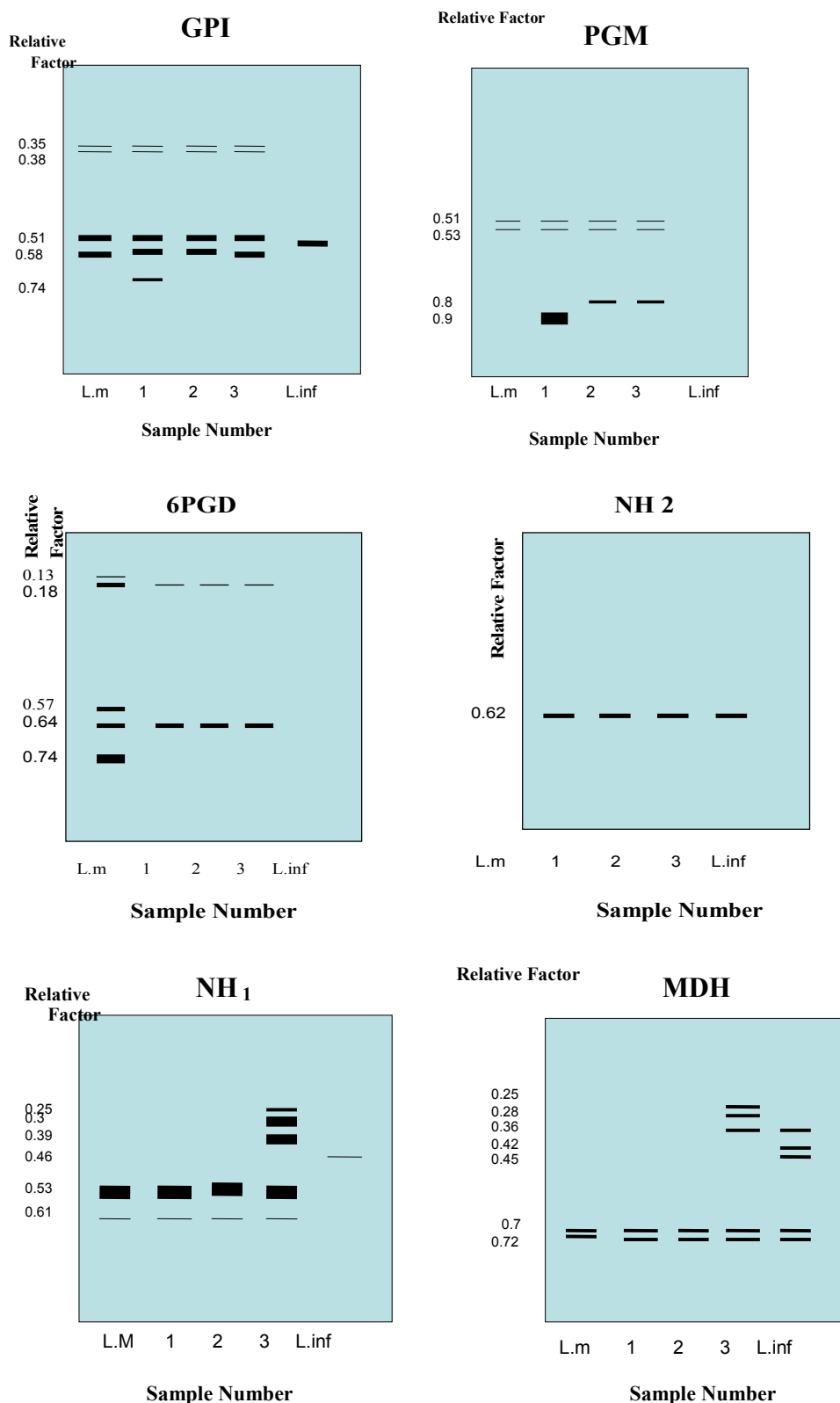
#### **Electron microscopy features**

Figure 3 shows a semi-thin plastic section of the bone marrow of 1  $\mu\text{m}$  thickness stained with toluidine blue  $\times 1000$  indicating amastigotes in the cytoplasm of macrophages. Figure 4 ( $\times 12100$ ) demonstrates electron micrograph of the bone marrow of femoral bone which indicates amastigotes within macrophages of the tissue.

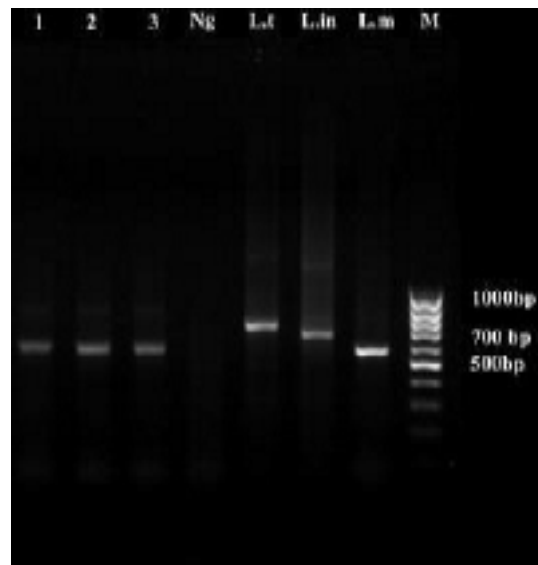
#### **Discussion**

By the application of PCR method, *L. major* was isolated as the causative agent of CL in Kharameh district in Shiraz and *T. indica* acted as the reservoir of the disease. In Southern Iran, Rassi et al 7 showed *L. major* infection in 6.8% of *Meriones libycus* in Arsanjan town as the principal reservoir of CL. Moemenbellah-Fard et al 8 isolated *L. major* infection in *Meriones libycus* in Marvdasht city in South-

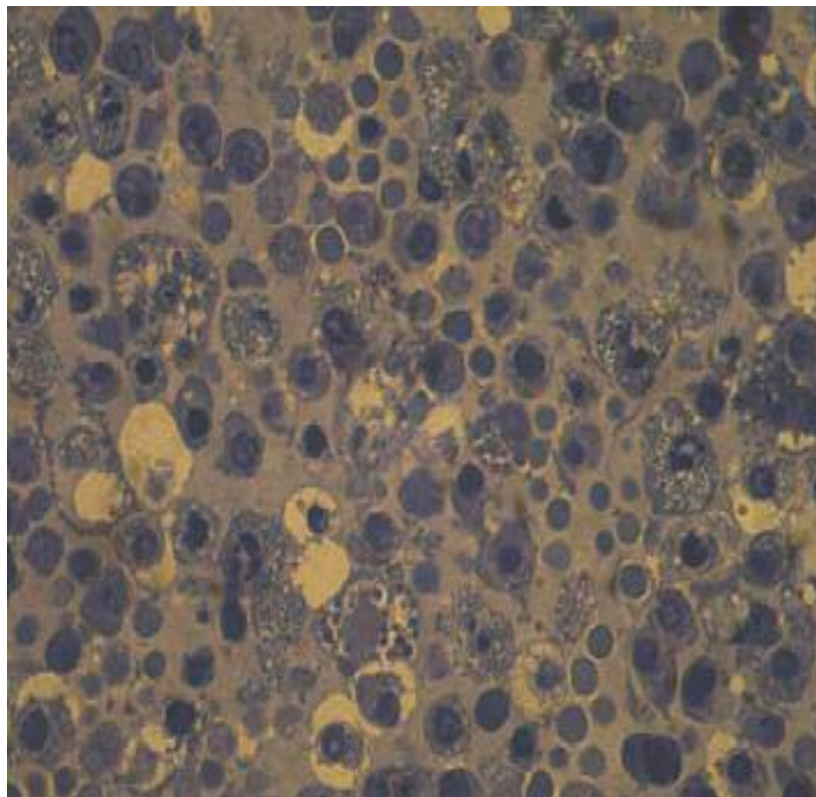
ern Iran. By the application of isoenzyme electrophoresis method, the enzymes MDH, NH1, NH2, PGM, GPI could differentiate *L. major* and *L. tropica* from *L. infantum*. The enzymes MDH, NH1, NH2, and GPI were found to be more efficient in characterizing these organisms. Our results are in accordance with the findings of Mebrahtu 21, Awadalla 22 and Hatam et al 15. The electrophoretic mobility of the isoenzyme bands in this system was consistent with Kreutzer 23 and Ebert 24 findings. Le-Blancq et al 17 and Mebrahtu 21 have also recommended MDH for the differentiation of *L. major* from *L. tropica*. Al-Tagi 25 and Mebrahtu 21 found NH and GPI to be more discriminative for the differentiation of *L. major* and *L. tropica*. In histological studies, the femoral bone of the rodents, if infected with the amastigote form of *Leishmania*, is the tissue which could clearly demonstrate the macrophages. Similar results were reported in golden hamster 26, 27. The histopathologic features reflect the host immune status in susceptible animals 28, 29. The histologic features are probably related to the presence of a mixed mononuclear cell reaction 30. Different tissue responses are observed in different animals infected by *Leishmania* parasites 31 and they are correlated with the resistance and the susceptibility of the animals 29, 30, 32. We demonstrated a diffuse infiltration of macrophages containing parasite antigens (parasitized macrophages). Promastigotes are phagocytosed by macrophages following the promastigotes of *Leishmania* opsonization by complement. Then, promastigotes will change into amastigotes (a form of a parasite that replicates in the phagolysosomal compartments of the host macrophages). Inhibition of phagosome maturation may constitute a strategy to provide an environment propitious for the promastigote-to-amastigote differentiation. Numerous round, 1- to 30-micron bodies within vacuoles in the macrophage cytoplasm reveal the fine structure of *Leishmania* amastigotes 21. We also indicated that the femoral bone marrow of rodents is the tissue of choice demonstrating the macrophages which contain amastigote form of *L. major* by light and electron microscopes.



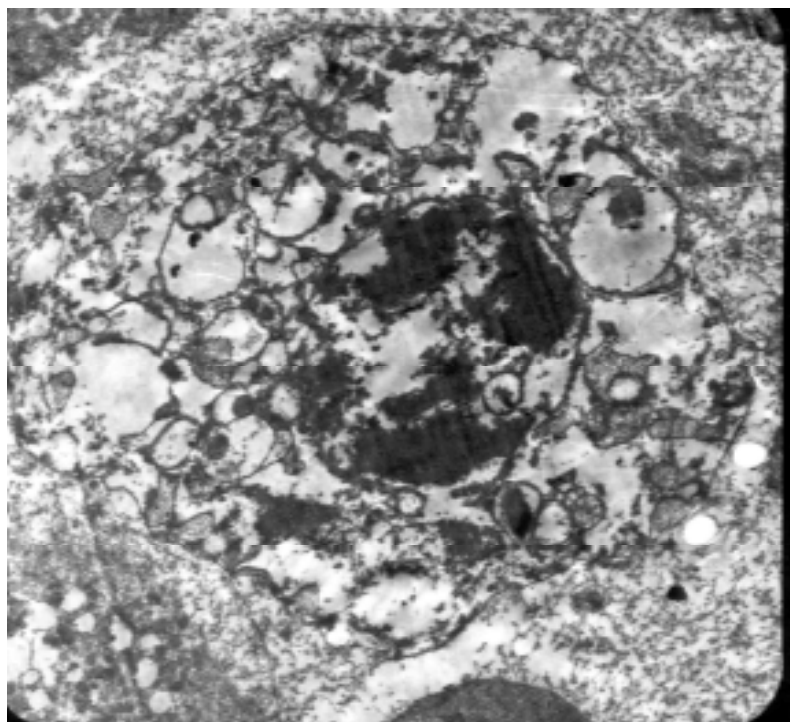
**Figure 1.** Gel electrophoresis obtained with soluble extracts of *Leishmania* promastigotes of six enzymatic systems (1 = Sejel-Abad village, Kharameh district, male; 2 = Kafdehk village, Kharameh district, male; 3 = Kafdehk village, Kharameh district, female; L.inf = leishmania infantum, Lm = leishmania major).



**Figure 2.** Gel electrophoresis of PCR products of *Leishmania* parasites isolated from trapped *T. indica* using the primers LINR4 and LIN17 (1 = Sejel-Abad village, Khrameh district, male; 2 = Kafdehak village, Khrameh district, male; 3 = Kafdehak village, Khrameh district, female); (L.t = *leishmania tropica*, L.in = *leishmania infantum*, L.m = *leishmania major*, M = Marker).



**Figure 3.** Bone marrow section of femoral bone of rodents infected to *Leishmania* (numerous leishman bodies are visible in macrophages, toluidine blue  $\times 1000$ ).



**Figure 4.** Electron microscopy of bone marrow of rodents infected to *L. major* (numerous leishman bodies in macrophages, Uranium acetate, Lead citrate  $\times 12100$ ).

In our study, the reservoir host of *L. major* was different from those reported in Southern Iran. This may be due to the difference in plantation, weather, sunshine duration, humidity and type of soil in the area. Our result is the first report of *L. major* in Kharameh district in Shiraz in *T. indica*. Rodent control is a challenge to the health authorities involved in the control of CL in southern parts of Iran. Socio-economic changes, rapid urbanization, building constructions in farms near the colonies of rodents, the storage of waste materials, new agricultural projects, and the existence of animal shelters among the old mud houses, may help increase the number of wild rodents and sand flies. The above factors provide a very efficient cycle for the transmission of this disease (ZCL). The migration of refugees from Afghanistan has also provided suitable conditions for the further spread of the disease <sup>1</sup>.

Our results indicated that *Tatera indica* can be a key epidemiological parameter to be brought into consideration when planning preventive measures.

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