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## ***Leishmania major* in *Tatera indica* in Fasa, Southern Iran: Microscopy, Culture, Isoenzyme, PCR and Morphologic Study**

<sup>1</sup>D. Mehrabani, <sup>2</sup>M.H. Motazedian, <sup>2</sup>G.R. Hatam, <sup>2</sup>Q. Asgari, <sup>3</sup>S.M. Owji and <sup>4</sup>A. Oryan

<sup>1</sup>Stem Cell and Transgenic Technology Research Center, Gastro-Entero-Hepatology Research Center, Nemazee Hospital, Iran

<sup>2</sup>Department of Parasitology and Mycology,

<sup>3</sup>Department of Pathology, Shiraz University of Medical Sciences, P.O. Box 71345-1744, Iran

<sup>4</sup>Shiraz University, Shiraz, Iran

*Corresponding Author: Davood Mehrabani, Gastro-Entero-Hepatology Research Center, Department of Pathology, Nemazee Hospital, Shiraz University of Medical Sciences, P.O. Box 71345-1744, Shiraz, Iran Tel: +98-711-6474263 Fax: +98-711-6474263*

### **ABSTRACT**

This study was undertaken to identify rodents infected with *Leishmania* in Fasa town, Fars Province, Southern Iran. From April 2004 to April 2005, a total of 17 rodents were collected in live traps in the area. Microscopical examination of stained tissue smears and the culture of tissue samples, PCR, isoenzyme electrophoresis and morphological review were used for detection of the parasite. The captured rodents were 10 *Rattus rattus* and 7 *Tatera indica*. One male *T. indica* was infected with *L. major* in Zahedshahr District of Fasa town. In histological and ultrastructural studies, numerous clusters of amastigotes were seen in the foamy macrophages of bone marrow of the femoral bone. We can conclude that *T. indica* is one of the rodents that may be a potential reservoir host of *L. major* in the region and femoral bone marrow is morphologically the tissue of choice to confirm the presence of macrophages containing the amastigote form of the parasite.

**Key words:** *L. major*, *T. indica*, isoenzyme, PCR, epidemiology, histology, ultrastructure, reservoir, rodent, Iran

### **INTRODUCTION**

Cutaneous Leishmaniasis (CL) is still one of the important health problems in several countries in the Mediterranean regions, Africa and the Middle East (WHO, 2008). In Iran, Zoonotic Cutaneous Leishmaniasis (ZCL) is considered to be focally endemic in several foci of the country (Nadim, 2000). In most ZCL endemic areas in Iran, the causative organism was identified as *L. major* and is of public health importance in 15 out of 32 provinces of the country (Yaghoubi-Ershadi *et al.*, 2005). Many rodents were reported as the reservoir hosts of the parasite in Iran, including *Meriones libycus*, *M. hurrianae*, *M. persicus*, *Nesoicia indica*, *Rhombomys opimus*, *Tatera indica* and *Gerbillus* (Seyed-Rashti and Nadim, 1967a; Nadim and Faghieh, 1968; Momenbellah-Fard *et al.*, 2003; Parvizi *et al.*, 2008). In Southern Iran, *L. major* was identified in *M. libycus* (Momenbellah-Fard *et al.*, 2003; Rassi *et al.*, 2001), *T. indica* (Asgari *et al.*, 2007) and *Gerbillus* (Mehrabani *et al.*, 2007). The increasing worry about the incidence of human CL in Southern Iran may be due to spread of the local human population into regions where *L. major* has been maintained in a rodent-sand fly-rodent cycle (Desjeux, 2001). Several attempts were also

made to correlate histopathologic changes with leishmaniasis (Bryceson, 1969; Destombes, 1960; Ridley *et al.*, 1980). This study was performed to identify rodents infected with *Leishmania* sp. in Fasa town, Southern Iran, using a combination of classical microscopy, culture, isoenzyme electrophoresis, PCR and morphology.

## MATERIALS AND METHODS

**Study area:** Fasa is situated in the South-East of Shiraz city, the capital of Fars Province in Southern Iran. Its geographical coordinates are 28° 56' 22" North and 53° 39' 0" East (Fig. 1). The province is dry, dusty and mountainous, with an altitude of 1500 m above the sea level. Its summer is warm and its Winter is mild, with much sun throughout the year (Iran Budget and Planning Organization, 2007).



Fig. 1: A sketch map of Iran, showing the location of Fasa town, Fars province, Southern Iran

**Trapping:** The rodents were caught alive between April 2004 and April 2005 as described by Mehrabani *et al.* (2007) in both urban and rural settings in Fasa town, Fars province, Southern Iran. The trapped animals were identified by staff of Department of Biology, Shiraz University, using the relevant taxonomic criteria (Eisenberg and Redford, 1999). A permit for the trapping was given by the Fars Environmental protection organization and all the caught rodents were handled and sacrificed using methods approved by the Iranian veterinary organization.

**Smears and cultures:** Rodents were sacrificed using chloroform and then carefully examined for any skin lesion. An impression of each tissue sample provided from the feet, tail, ears and any patent lesion (Edrissian *et al.*, 1982) was stained with Giemsa's stain and studied microscopically for the presence of amastigotes. Tissue samples were provided in an aseptical condition from the liver, spleen and the skin and cultured at 25°C in rabbit-blood agar (Evans, 1989). The cultures were checked regularly for parasites and considered negative if no promastigotes were observed within 2 months. The parasites from any positive cultures were recorded and cryopreserved in liquid nitrogen, pending their identification in an isoenzyme electrophoresis and PCR-based assays.

**Enzyme electrophoresis:** Enzyme extraction from the pelleted organisms was carried out as described by Asgari *et al.* (2007). Analysis was done using discontinuous polyacrylamide gel electrophoresis (PAGE) by a 3% of stacking gel, 7.5% of separating gel, a stacking buffer 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) run under a constant current of 2 mA/well for 150 min. Each stain was examined for the activity of 6 enzymatic systems including malate dehydrogenase (MDH) E.C.1.1.1.37, phosphoglucumutase (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, nucleoside hydrolase II (NH2) E.C.3.2.2.1 and glucose-6-phosphate dehydrogenase (G-6-PD) EC 1.1.1.49. Electrophoretic band developing conditions were used for each enzyme system as described earlier (Evans, 1989).

**PCR:** Total DNA was extracted from each promastigote culture as described before until it could be tested for leishmanial kinetoplast DNA (KDNA) in the PCR (Mehrabani *et al.*, 2007; Motazedian *et al.*, 2002; Aransay *et al.*, 2000). The primers used in the PCR were identical to their study.

Samples (10 µL) of the PCR products were each mixed with 5 µL loading buffer and then separated by electrophoresis in a 1.5% agarose gel. The bands were stained with ethidium bromide and visualized by ultra-violet transillumination. The reference strains of *L. major* (MHOM/TM/1973/5ASKH), *L. tropica* (MHOM/AZ/1974/SAF-K27) and *L. infantum* (MHOM/TN/1980/IPT1) which were provided from the Pasteur Institute in Tehran, Iran, were also run in the PCR.

**Histological study:** To show the presence of parasites, the rodents were euthanized based on Iranian Veterinary Organization Ethics Committee. The rodents were autopsied and the femoral bones were removed and fixed in 10% buffered formalin. After 24 h, the bones were decalcified in 7% nitric acid. The resultant slices were then dehydrated with graded alcohols, embedded in paraffin blocks and sections of 5 µm in thickness were prepared and stained with hematoxylin and eosin and studied using light microscopy.

**Ultrastructural study:** For electron microscopy, 1 mm from the femoral bones of each animal were fixed in 3% cold glutaraldehyde, buffered with 0.2 M sodium cacodylate and postfixed in 1% osmium tetroxide, dehydrated through ascending series of ethanol and embedded in agar-100 resin. Semithin sections (1  $\mu$ m in thickness) were cut by ultramicrotome and stained with toluidine blue and were examined under a light microscope to confirm the preparation orientation. Ultrathin sections were cut from selected areas, mounted on copper grids and double stained with uranyl acetate and lead citrate, then examined under a transmission electron microscope and screened for the presence of amastigotes (Rasmusson and Descoteaux, 2004).

## RESULTS AND DISCUSSION

Seventeen rodents were captured including 10 *Rhattus rattus* and 7 *T. indica* in agricultural plantations close to houses. One male *T. indica* was just found smear-positive for amastigotes and culture-positive for leishmanial infection in Zahedshahr district of Fasa town. The electrophoretic isoenzyme patterns were shown in Fig. 2a-f, demonstrating the similarity to *L. major* with RFs of 0.72 and 0.75. The result of the PCR represented one *T. indica* harboring *L. major* too (Fig. 3).

In histological study, several clusters of amastigotes were visible in the foamy macrophages of bone marrow in the femoral bone containing intracellular *Leishmania* amastigotes (Fig. 4). In

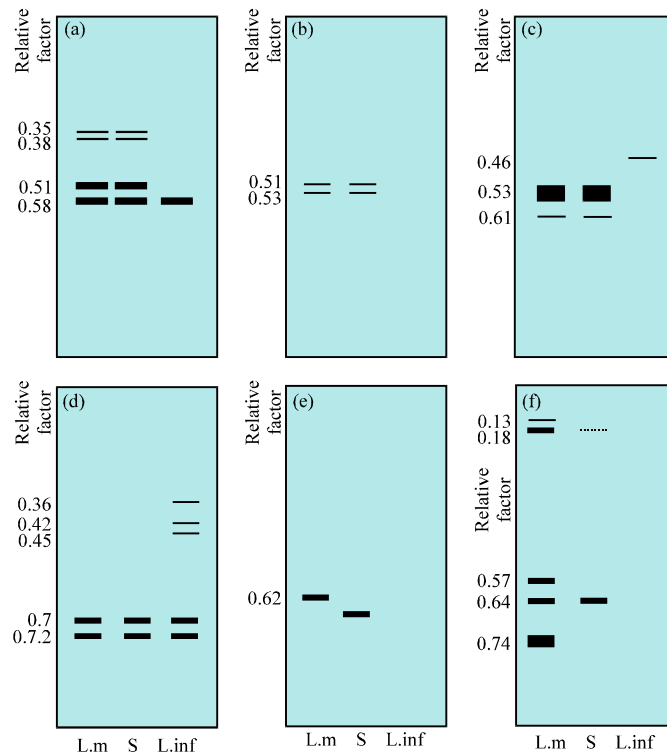


Fig. 2: Gel electrophoresis obtained with soluble extracts of *Leishmania* promastigotes of 6 enzymatic systems, (a) GPI, (b) PGM, (c) NHI, (d) MDH, (e) NH2 and (f) G6PD including malate dehydrogenase (MDH) E.C.1.1.1.37, phosphoglucomutase (PGM) E.2.7.51, glucose-phosphate isomerase (GPI) E.C.5.3.1.9, nucleoside hydrolase I (NH1) E.C.3.2.2.1, nucleoside hydrolase II (NH2) E.C.3.2.2.1 and glucose-6-phosphate dehydrogenase (G-6-PD) EC 1.1.1.49; s = tissue sample of *T. indica*, L.inf = *Leishmania infantum*, Lm = *Leishmania major*

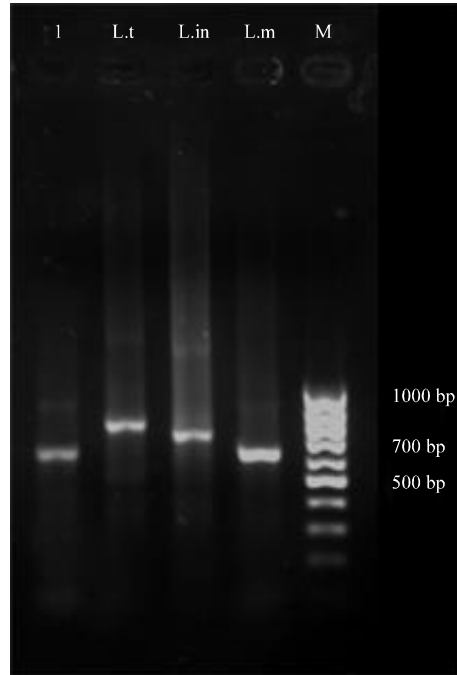


Fig. 3: The amplicons produced, in the PCR based on the LINR4 and LIN17 primers, from samples from a male *T. indica* caught in Fasa town. For reference, samples from *Leishmania tropica* MHOM/AZ/1974/SAF-K27 (L.t), *L. infantum* MHOM/TN/1980/IPT1 (L.in) and *L. major* MHOM/TM/1973/5ASKH (L.m) and molecular-weight markers (M) were also run

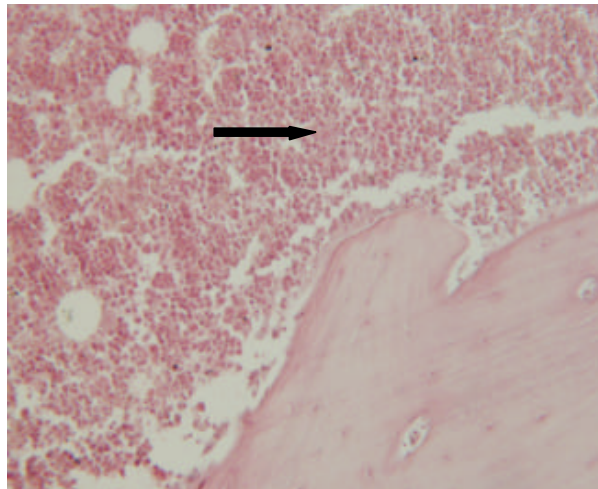


Fig. 4: Femoral bone marrow of *Tatera indica* infected with *L. major* showing parasitized macrophages (hematoxylin and eosin, 160x)

ultrastructural study, the amastigotes were typically noticed in the cytoplasm of the macrophages of the bone marrow in the femoral bone (Fig. 5).

The CL is the most common form of leishmaniosis accounts for nearly 50-70% of all new cases appear worldwide (WHO, 2008). This form of the disease has diverse clinical manifestations and

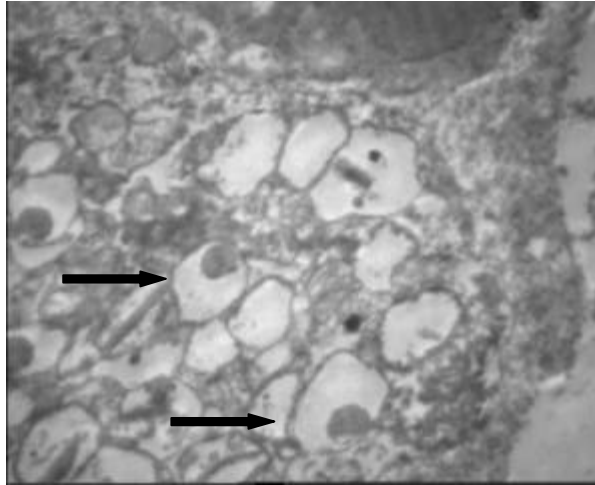


Fig. 5: Ultrastructural features of femoral bone marrow section of *Tatera indica* infected with *L. major* (several *Leishmania* bodies are noticed in macrophages; uranium acetate, lead citrate, 6000x)

is still prevalent and remains a major public health problem in Iran, while its incidence has been doubled over the last decade (Motazedian *et al.*, 2006).

In Iran, *T. indica* and *N. indica* were reported as the reservoir hosts in Western parts (Javadian *et al.*, 1989; Hajjarian *et al.*, 2009), *Rhombomis opimus*, *M. libycus* and *M. persicus* in the central parts (Parvizi *et al.*, 2008; Doroudgar *et al.*, 1995; Yaghoubi-Ershadi *et al.*, 2001), Northern areas (Pourmohammadi *et al.*, 2008), Khorasan Province (Yaghoubi-Ershadi *et al.*, 2003), Yazd Province (Yaghoubi-Ershadi *et al.*, 2004), Tehran Province (Yaghoubi-Ershadi *et al.*, 2004) and Southern Iran (Asgari *et al.*, 2007; Mehrabani *et al.*, 2007).

In Fars province, Southern Iran, Rassi *et al.* (2001, 2006, 2007) isolated *L. major* from *M. libycus* collected from Arsanjan, Neyriz and Marvdasht towns respectively, indicating that this species of rodent was probably the main reservoir host for the parasite causing human CL in the area. In another report, *M. libycus* was found to be harbouring *L. major* in Marvdasht (Momenbellah-Fard *et al.*, 2003).

Although, many rodent species (such as *M. libycus* and *Rh. opimus* in Northeastern, eastern and central parts; *T. indica*, *N. indica* and *M. libycus* in the Southwest and *M. hurrianae* in the Southeast of the country) were found naturally infected with *L. major* (Nadim, 2000), only one report of *L. major* in Iranian *Gerbillus* recorded in Eastern region, Southern Iran (Mehrabani *et al.*, 2007).

In the country, *T. indica* infected with *L. major* were reported from several regions (Seyed-Rashti and Nadim, 1967b; Asgari *et al.*, 2007; Mehrabani *et al.*, 2007; Javadian *et al.*, 1989; Seyed-Rashti *et al.*, 1967b). No other collected rodents were found infected with *L. major* in the present study.

In Fars Province, Southern Iran, *M. libycus* was reported to be infected with *L. major* in Neyriz and Arsanjan and *Gerbillus* sp. in Larestan region (Momenbellah-Fard *et al.*, 2003; Rassi *et al.*, 2001, 2006, 2007). Infection with *L. major* was reported in several rodents worldwide. Schlein *et al.* (1984) demonstrated amastigotes in *N. indica* in the lower Jordan Valley, a region where CL due to *L. major* is hyper-endemic and *Ps. obesus* is believed to be the main reservoir host (Le Blancq *et al.*, 1986).

Using isoenzyme electrophoresis, the enzymes MDH, PGM, GPI, NH1 and NH2 could discriminate *L. major* from *L. tropica* and *L. infantum*. The enzymes MDH, GPI, NH1 and NH2 were more efficient to characterize these parasites. Our findings are identical to several reports (Mebrahtu *et al.*, 1992; Awadalla *et al.*, 1987; Hosseini *et al.*, 2005). The electrophoretic mobility of the isoenzyme bands in the system was consistent with other reports (Kreutzer *et al.*, 1987; Ebert, 1987). Finding of some authors suggested MDH for differentiation between *L. major* and *L. tropica* too (Le Blancq *et al.*, 1986; Mebrahtu *et al.*, 1992), while others observed GPI and NH for differentiation between *L. major* and *L. tropica* (Mebrahtu *et al.*, 1992; Al-Tagi and Evans, 1978). Results of the present study, finding of *L. major* in *T. indica* was similar to the other results in the province (Asgari *et al.*, 2007; Mehrabani *et al.*, 2007). In our study, the electrophoretic isoenzyme patterns as shown in Fig. 2 using 6 enzymatic systems including MDH, PGM, GPI, NH1, NH2 and 6GPD demonstrated the similarity to *L. major* but with RFs of 0.72 and 0.75.

At histological level, the femoral bone of the infected rodents with the amastigote form of *Leishmania*, was shown to be the tissue of choice demonstrating the macrophages. Similar reports were presented in golden hamster (Ridley *et al.*, 1980; Kahl *et al.*, 1991). The histologic features may be related to a mixed mononuclear cell reaction (Barral-Netto *et al.*, 1987). The present study, also showed a diffuse infiltration of macrophages with parasite antigens (parasitized macrophages). Promastigotes are phagocytosed by macrophages after opsonization of the *Leishmania* promastigotes by the complement. Identically several round, 1-to 30-micron bodies within vacuoles in the macrophage cytoplasm were shown as fine structures of *Leishmania* amastigotes (Mebrahtu *et al.*, 1992).

We can conclude that changes in socioeconomic status, urban and rural construction developments, new agricultural projects, the storage of waste products nearby the town all may be a risk for presence of wild rodents in the area. The present study, showed that *T. indica* which is the first report in this area was the rodent infected with *L. major* and may play a potential reservoir host for *L. major* in the region. The present study demonstrated that the femoral bone marrow of rodents is the tissue of choice showing the macrophages with amastigote form of *L. major* by light and electron microscopy methods.

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