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# Study on the prevalence of visceral Leishmaniasis in rodent's of Azarshahr district (new focus), northwest of Iran

**Fallah*, E., Farshchian, M., Mazlomi, A., Majidi, J., Kusha, A., Mardi, A., Mahdipoorzareh, N.**

*Department of Parasitology and Immunology, Faculty of Medicine, Tabriz University of Medical Sciences,, Tabriz, Iran*

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**ABSTRACT**

To examine the seroprevalence of zoonotic visceral leishmaniasis (ZVL) among rodents in Azarshahr County, and to assess the possible association of infection among rodents in respect with transmission/prevalence of the disease among children, a survey was conducted during 2003-2004. Azarshahr County is an endemic region for leishmaniasis and this research is the first study in determining the host reservoirs in this county. In this survey, 265 rodents belonging to 7 genera/species were trapped alive. Anti-Leishmanial antibodies were detected through direct agglutination test (DAT), indirect fluorescent antibody tests (IFAT) and microscopic examination. Fourteen (5.3%) animals were shown to be seropositive, 27 (10.2%) provided relatively lower titers, and 224 (84.5%) turned out to be seronegative. Amastigotes of *Leishmania* were observed in four seropositive rodents including one *Meriones persicus*, two *Cricetalus migratorius* and one *Mesocicetus auratus* after dissection and parasitological examinations. Multiple analyses of PCR were used to reassure the identity of purified isolates and infected clinical samples. According to the results of this study, the isolates were identified as *Leishmania infantum* and those infected rodents are assumed to be potential host reserviors for visceral leishmaniasis in the region. This work is the first report of detecting *L. infantum* infection in *Cricetalus migratorius, Meriones persicus* and *Mesocricetus auratus* from Azarshahr of Iran.

***Keywords:*** Leishmaniasis, Kala-azar, epidemiology, host reservior, PCR

## INTRODUCTION

Visceral leishmaniasis (VL), or Kala-azar, is dispersly endemic in several provinces of Iran including East Azerbaijan, Ardabil, Fars, and Quam.

In other provinces of the country, the disease has been reported in sporadic form (Edrissian *et al* 1988, Fallah and Mohebali 2001, Mazlumi-Gavgani *et al* 2002). Azarshahr County has been reported to be endemic for VL (Mirsamadi *et al* 2003, Farshchian *et al* 2004). Although dogs are the main reservoirs for human infection to VL (Edrissian *et al* 1988),

* Author for correspondence. E-mail: fallahe@tbzmed.ac.ir

wild carnivores such as jackals and foxes have been also found infected with *Leishmania* spp. These animals are assumed to be reservoirs for parasites, particularly in regions where sporadic cases of disease have been reported. (Nadim *et al* 1978, Hamidi *et al* 1982, Mohebali *et al* 2005). Infections of *L. infantum* in rodents have been previously reported in multiple genera/species (Edrissian 1990, Mohebali *et al* 1998, Fernanda *et al* 2005).

The main objective of this investigation was to screen *leishmania* infection in rodents through serological and parasitological examination in Azarshahr County, an endemic part of East Azerbaijan. The second aim of this study was to assess the possible role of rodents in transmission of the disease to children and animals.

## MATERIALS AND METHODS

**Source of samples.** Rodents were trapped alive in various parts of Azarshahr County, located in East Azerbaijan. The active colonies of rodents were identified and the rodents were trapped alive in various parts of these areas. Specimens were collected from the colonies of gerbils located about 1–1.5 km around villages where VL was endemic. Around 20–30 live traps were used each week and rodents were caught in all seasons. The genus and species of the rodents were determined by external characteristics: color, body measurements, ears, tail, feet, teeth and cranium (Boitani 1980, Ziaei 1996).

Blood samples were collected from each animal in two heparinized capillary tubes before sacrificing them for further examinations. Initially, blood samples were tested by direct agglutination test (DAT) and indirect fluorescent antibody test (IFAT) according to the procedures described by Harith and coworkers (Harith *et al* 1988, 1989).

**Antigen preparation and source of isolate.** Briefly, promastigotes of *Leishmania* were mass-produced in RPMI 1640 (Sigma®) containing fetal bovine serum (FCS) followed by trypsinization of the parasites, staining with Coomassie blue and fixing by formaldehyde. The parasite source was *Leishmania donovani* strain I-S, where kindly provided by Dr. Harith to the Pastor Institute, Tehran. The live culture was maintained in Novy-MacNeal-Nicolle (NNN) medium enriched with liquid phase of liver infusion broth tryptose (LIT) and used for antigen preparation (Sadigursky & Brodeskeym 1986). *L. donovani* antigen prepared in our laboratory was used for testing rodents' serum samples through IFAT (Edrissian *et al* 1981).

**Sample preparation and testing.** The spleen and liver samples of seropositive rodents (≥1: 80 in DAT and IFAT) were cultured in NNN medium containing FCS and checked twice a week for six weeks. A total of 1512 smears were prepared from blood (as thick and thin smears), and spleen/liver samples (as impression smears). The smears prepared from all animals were stained with standard Giemsa, and examined microscopically for presence of *Leishmania* amastigotes. The promastigotes were isolated from the media as well as corresponding organ samples were tested using PCR.

**PCR analysis.** Template preparation used in PCR was described by Mahmoudpour (2000, 2002, and 2004). Briefly, to prepare template DNA from *Leishmania* cultures, the pellet was suspended in 10-20 fold of disruption buffer containing 100 mM Tris, 10 mM EDTA, pH 8.0, 2% SDS and 2% 2-mercaptoethanol which was added before use. The suspension was incubated at 65°C for at least 30 min and cooled to room temperature before precipitating with half volume of ice-cold 3.0 M KAc, pH 5.5. The supernatant was further precipitated by an equal volume of ice-cold iso-propanol at ~15k/15min/4 °C. The pellet was rinsed with 70% ethanol and air-dried before resuspending in sterile water. To prepare temple from biopsy samples of spleen, 50-500 mg of infected tissue was homogenized in a proper glass homogenizer or ground in a microfuge tube by a heat-sealed blue tip. After adding 2-3 fold disruption buffers, DNA preparation was followed according to

the above procedure.

Specific primers were designed using a 778 bp partial sequence file of *Leishmania infantum* minicircle DNA (gi|2598170|gb|AF027578.1|) through Blast search and analyzed by Oligo Tech version 1.0. These included homologous 5'-CCC AAA CTT TTC TGG TCC TTC G-3', positioned at 24-45 and complementary 5'-CCA CGA CGC ATC CAA TCC AA-3', positioned at 360-341 flanking a 337-bp fragment. Reaction cocktail contained 1.0x PCR buffer, 2.0 mM MgCl2, 0.2 mM dNTP's, 0.5 mM each primers, 1-2 units of recombinant *Taq* DNA polymerase (Cinnagen Inc., Iran). The final volume was adjusted to 20 or 25 µl per reaction including 2-5 µl of template.

The reaction conditions included lid temperature of 105 °C along with 4 minutes of initial denaturation at 95 °C followed by 35 cycles of 95 °C/30 sec, 65 °C/30 sec and 72°C/1.0 min. The reactions were ended by additional extension at 72°C/10min.

**Gel Electrophoresis.** Entire PCR products were loaded into 1-1.5% agarose gel and electrophoresed for 1-1.5 hr in 1% TAE buffer along with a molecular weight marker. After staining the gel in ethidium bromide solution, the photograph was taken under UV illumination.

## RESULTS

A total of 265 rodents belonging to 7 genera/species were trapped alive in various parts of Azarshahr County. These included 8 (3%) *Cricetulus migratorius* (grey hamster) and 117 (44.2%) *Mus musculus* where both species trapped in residential houses. Animals trapped outdoors in villages included 75 (28.3%) *Meriones persicus*, two (0.8%) *Mesocricetus auratus*, 60 (22.5%) *Rattus -*

**Table 1.** Number of rodents trapped alive in Azarshahr County, located in north western region of Iran, during 2003- 2004 as grouped by the genus, species and location of trapping.

| Ttrapping Location | Specie s | | | | | | |
|---|---|---|---|---|---|---|---|
| | M.M.* | M.P.* | R.N.* | C.M.* | M.A.* | S.A.* | H.I.* |
| Azarshahr-Pazikuh | 25 | 0 | 0 | 3 | 0 | 0 | 2 |
| Azarshahr-Kurdan | 17 | 0 | 0 | 0 | 0 | 0 | 0 |
| Azarshahr-Shahidbehshti | 16 | 0 | 0 | 0 | 0 | 0 | 0 |
| Azarshahr (river, sewage, stream) | 0 | 0 | 60 | 0 | 0 | 0 | 0 |
| Segayesh village | 23 | 27 | 0 | 5 | 2 | 1 | 0 |
| Gavahir village | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Jaraghil village | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Yengjeh village | 10 | 30 | 0 | 0 | 0 | 0 | 0 |
| Germizigol village | 2 | 11 | 0 | 0 | 0 | 0 | 0 |
| Amirdizaj village | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Almalx village | 3 | 7 | 0 | 0 | 0 | 0 | 0 |
| Total | 117 | 75 | 60 | 8 | 2 | 1 | 2 |
| Percentage % | 44.2 | 28.3 | 22.5 | 3 | 0.8 | 0.4 | 0.8 |

* *M.M.: Mus musculus., M.P.: Meriones persicus, R.N.: Rattus norvegicus, C.M.: Cricetalus migratorius (grey hamster), M.A.: Mesocricetus auratus (golden hamster), S.A.: Sciurus anomalus and H.I.: Hystrix indica*

**Table 2.** Parasitological and serological (DAT) screening of 265 rodents trapped in Azarshahr County, located in north western region of Iran, during 2003 - 2004.

| Species | Tested number | Positive Number on Microscopic Exam | Positive Number on Culture Medium | DAT, *Leishmania* antibody titers Number | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Neg | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 |
| *M.M.*[*] | 117 | 0 | 0 | 106 | 5 | 4 | 3 | 0 | 0 |
| *M.P.*[*] | 75 | 3 | 3 | 53 | 8 | 7 | 4 | 3 | 0 |
| *RN.*[*] | 60 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 |
| *C.M.*[*] | 8 | 3 | 3 | 3 | 0 | 2 | 2 | 0 | 1 |
| *M.A.*[*] | 2 | 2 | 2 | 53 | 0 | 1 | 0 | 1 | 0 |
| *S.A.*[*] | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| *H.I.*[*] | 1 | 0 | 0 | 54 | 0 | 0 | 0 | 0 | 0 |
| Total | 265 | 8 | 5 | 224 | 13 | 14 | 9 | 4 | 1 |

[*] *M.M.: Mus musculus., M.P.: Meriones persicus, R.N.: Rattus norvegicus, C.M.: Cricetalus migratorius (grey hamster), M.A.: Mesocricetus auratus (golden hamster), S.A.: Sciurus anomalus and H.I.: Hystrix indica*

**Table 3.** Parasitological and serological (IFAT) screening of 265 rodents trapped in Azarshahr County, located in north western region of Iran, during 2003 - 2004.

| Species | Tested number | Positive Number on Microscopic Exam | Positive Number on Culture Medium | IFAT, *Leishmania* antibody titers Number | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Neg | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 |
| *M.M.*[*] | 117 | 0 | 0 | 106 | 5 | 2 | 2 | 2 | 0 | 0 |
| *M.P.*[*] | 75 | 3 | 3 | 50 | 5 | 6 | 7 | 5 | 2 | 0 |
| *RN.*[*] | 60 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C.M.*[*] | 8 | 3 | 3 | 3 | 0 | 0 | 2 | 2 | 0 | 1 |
| *M.A.*[*] | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| *S.A.*[*] | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *H.I.*[*] | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 265 | 8 | 5 | 222 | 10 | 8 | 12 | 9 | 3 | 1 |

[*] *M.M.: Mus musculus., M.P.: Meriones persicus, R.N.: Rattus norvegicus, C.M.: Cricetalus migratorius (grey hamster), M.A.: Mesocricetus auratus (golden hamster), S.A.: Sciurus anomalus and H.I.: Hystrix indica*

*norvegicus*, one (0.4%) *Sciurus anomalus* and two (0.8%) *Hystrix indica*. Tables 1-3 describe the results of the parasitological and serological tests.

*Leishmania* spp. was isolated from spleens of two *Meriones persicus*, *Leishmania* spp. was isolated from spleens of two *Meriones persicus*, one *Mesocricetus auratus* and one *Cricetulus migratorius* in NNN plus FCS. The promastigotes isolated from these animals were identified as *Leishmania infantum* through PCR analysis.

Figure 1 represents the results of multiple PCR analyses conducted to reconfirm the identity of purified isolates of parasite and those of infected clinical samples.

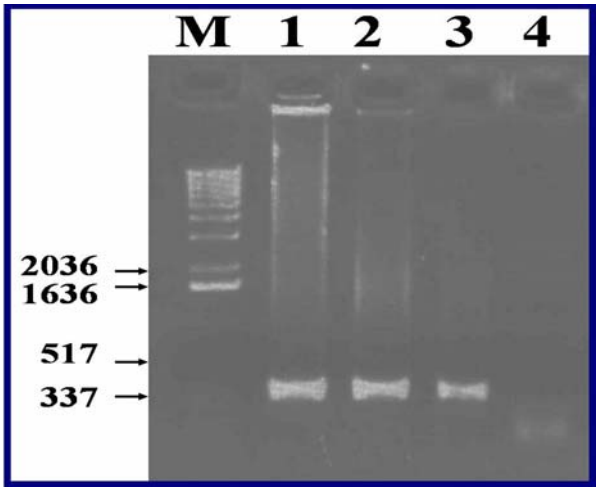


**Figure 1.** Agarose gel analysis demonstrating the results of PCR on 2 clinical samples 11 obtained from dog spleen (lane 2) and hamster spleen (lane 3). A pure culture of 12 *Leishmania infantum* MON 1 was used as positive control (lane 1) and a no template 13 reaction used as negative control (lane 4).

## DISCUSSION

*Meriones persicus* was reported to be naturally infected with *Leishmania* in East Azerbaijan, Iran. Considerable numbers of amastigotes were observed in the smears prepared from the cutaneous lesions of *Meriones persicus*, while no amastigote was seen in microscopic examination of the smears prepared from the internal organs and blood samples of this rodent (Edrissian *et al* 1975). According to data collected in this study, amastigotes were observed in 1.5% of the rodents after microscopic examination of the smears prepared from internal organs. As reported by Mohebali and coworkers from the Meshkinshahr County, *Leishmania* spp. was isolated from spleens of two *Meriones persicus* and one *Mes. auratus* as cultured in NNN+LIT. Amastigotes were observed in 16.5% of the rodents, and *L. donovani* LON-50 was isolated from two *Meriones. persicus.* Meanwhile, using isoenzyme analysis, promastigotes isolated from *Mes. auratus* were identified as *L. infantum* zymodem LON-49 (Mohebali *et al* 1998; Mohebali 1995).

*Rattus rattus* and *Thrichomys apereoides* were shown to be the most abundant rodent species in an endemic area of visceral leishmaniasis in Brazil. Meanwhile, DNA belonging to *L. braziliensis*, *L. mexicana* and *L. donovani* complexes was confirmed in several individuals of *R. ratus* (Fernanda *et al* 2005). As reported by Edrissian (1990), *L. infantum*, a zoonotic species, was isolated from humans in Meshkinshahr County. Parasitology and serology tests were performed in 30 wild canines provided that 10% of these animals were infected with *L. infantum* (Edrissian 1993). According to Mohebali and coworkers (1995, 1998 and 2005), the parasite was isolated from two *Meriones. persicus*, one *Mes. Auratus*, one *Mes. migratorius* and dogs in Meshkinshahr County and from dogs in Karaj vicinity located 40 km from west Tehran. Using molecular and biochemical procedures, 10 out of 11 *Leishmania* isolates obtained from dogs and wild canines were identified as *L. infantum* and one as *L. tropica*. In addition to humans and dogs, wild carnivores such as jackals and foxes have been reported to be infected with *Leishmania* in Iran (Hamidi 1982; Edrissian 1993). *L. infantum* was isolated from *Rattus rattus* in Italy and Iraq (Desjeux 1991). According to the PCR analysis shown on Figure 1, the parasites isolated

from rodents trapped in Azarshahr County produced similar results seen on samples obtained from a patient derived isolate and a biopsy sample of infected dog spleen.

To establish a stronger relation within these samples from different hosts, the sequence of corresponding k-DNA will be compared after cloning and sequencing. To explore the biological role of rodents as reservoirs of *L. infantum* and mechanisms of disease spread among different hosts, further studies are needed to trace the infection in insect vectors feeding on infected rodents.

Observing natural *Leishmania* infection in rodents trapped in a highly endemic area may provide an association of rodents, particularly those living in houses, with transmission and the spread of disease to the children. Further studies are needed to clarify the exact role of rodents as reservoirs of kala-azar in endemic areas.

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