

## Molecular Epizootiology of Rodent Leishmaniasis in a Hyperendemic Area of Iran

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### Abstract

**Background:** Zoonotic cutaneous leishmaniasis (ZCL) is an expanding disease and public health problem in Iran. In the current study, natural *Leishmania* infection rate and seasonal fluctuation of the infection in *Rhombomys opimus* population of a hyperendemic focus of ZCL in Iran was investigated.

**Methods:** The study was conducted from October 2006 to October 2008 in Esfahan Province, central part of Iran. An extensive sampling of rodents using Sherman traps was done in different seasons. Nested PCR assay was used for detection and identification of *Leishmania* species and the results were confirmed using PCR-RFLP.

**Results:** *Leishmania* infection rate was 58.6% (34 of 58) using nested PCR. 44.8% of the gerbils were infected only with *L. turanica* and 1.7% with *L. gerbilli* alone. A mixed natural infection with *L. major* and *L. turanica* was seen in 12.1% of the rodents. *L. major* infection alone was not seen in *R. opimus* population in the study area. The highest and lowest *Leishmania* infection rates were observed in fall and spring respectively. *L. turanica* infection was observed throughout the year whereas mixed infections with *L. major* and *L. turanica* was not seen in spring.

**Conclusion:** It is concluded that in the study area, *L. major*, *L. gerbilli* and *L. turanica* circulate in the population of *R. opimus*. *Leishmania major* infection usually accompanied by *L. turanica* in naturally infected gerbils with the highest rate in fall. It is recommended that the role of *L. turanica* in the epidemiology and transmission of ZCL be revisited.

**Keywords:** *Rhombomya opimus*, *Leishmania mixed infection*, *Cutaneous leishmaniasis*, *Molecular epizootiology*, *Iran*

### Introduction

Cutaneous leishmaniasis due to *Leishmania major*, a neglected tropical disease, is a major public health problem in some areas of the old world (1). *L. major* is widely distributed in various populations of rodents in arid and savannah regions (1). The disease is endemic in many rural districts of Iran, in 17 out of the 30 provinces. Rodents belong to Gerbillinae subfamily are the main reservoir hosts for ZCL in Iran and other countries where ZCL due to *L. major* is endemic (2-4). Gerbils are the most abundant mammals

reported from natural ecosystems of old world deserts (2). Many rodent species act as reservoir host of ZCL. *Rhombomys opimus* (great gerbil) in central Asia, northern Afghanistan and Iran; *Meriones libycus* (Libyan jird) in the Arabian Peninsula, central Asia and Iran; *M. hurrianae* (Indian desert jird) in India and Iran; *Psammomys obesus* (fat sand rat) and *M. crassus* in northern Africa and Middle East; and *Tatera* spp. in subsaharan Africa and Iran (1). *R. opimus* (Cricetidae: Gerbillinae) is the main *L. major* reservoir host in the vast territory of the Turan low-

land (west and south Kazakhstan and central Asia with adjacent parts of Afghanistan and Iran), Mongolia, and apparently, in some provinces of China. In the Turan lowland, naturally infected *R. opimus* are reported from more than 200 regions. The number of naturally infected great gerbils showed to be greater than any other mammals (other rodents, insectivores, carnivores) (2).

All the proven vectors of ZCL belong to the subgenus *Phlebotomus* (*Phlebotomus*), i.e. *P. papatasi*, the main vector, and related species *P. salehi* and *P. dubosqi*. Well-described stable ZCL systems are associated with *L. major* and *P. obesus*/*P. papatasi* in North Africa and Middle East, and *R. opimus*/*P. papatasi* in central Asia, Afghanistan and Iran (1, 5). The distribution and the role of rodents as ZCL reservoir hosts are geographically specific in Iran. *R. opimus* is the main reservoir of ZCL in Central and North East Iran followed by *M. libycus* (Cricetidae; Gerbillinae), which is the primary reservoir of ZCL in some areas of the central and southern Iran. In the south and south west of the country including the Iran-Iraq border, the reservoir is *T. indica*, the Indian jird (Cricetidae: Gerbillinae). In Baluchistan of Iran (border of Pakistan), *M. hurrianae* (Cricetidae: Gerbillinae) acts as a reservoir host (4, 6-10). One of the major problems for control of this neglected disease is lack of information about the dynamics of *Leishmania* parasites infection rates in rodent populations as the reservoir hosts.

In the present study, an investigation was carried out on natural infection rates of *Leishmania* parasites and seasonal fluctuations of the infection in *Rhombomys opimus* (Rodentia: Gerbillinae) population in a hyperendemic focus of ZCL in Iran.

## Materials and Methods

### Study area

The investigation was conducted over a period of 24 mo from October 2006 to October 2008 in Borkhar and Sejzi rural districts, 15- 35 km, northeast and east of Esfahan City (32° 39' 35" N/51° 40' 17" E), Esfahan Province, central Iran,

respectively where ZCL is hyperendemic. The study areas are located at an altitude of around 1,550 m, with a desert climate, hot summer and cold winter. In 2007, the maximum and minimum mean monthly temperatures were 37.8 °C and -6.3 °C in July and January, respectively. The total annual rainfall in this year was 109.7 mm. The maximum mean monthly relative humidity was 83.3% in January and the minimum was 13.9% in September (Esfahan Metrological Organization). Wheat, barley, cotton, vines, beetroot, pistachio, alfalfa, Indian corn, clover and summer crops are cultivated in these areas (10).

### Collection of rodents

In the first year of the study, active colonies of gerbils in the district were identified and the rodents were caught using 20-45 Sherman traps baited with cucumber for detection and identification of *Leishmania* parasites. In the second year, the rodents were collected using around 40 Sherman traps baited with cucumber each season to determine the seasonal fluctuations of the parasites in the populations of *R. opimus*. In spring to fall, the traps were placed at the gerbil holes in the afternoon and collected in the morning of the following day. In winter, Sherman traps were placed after sunrise and collected at the same day afternoon. The trapped gerbils were transferred to the animal house facility at the Esfahan Training and Health Research Center, National Institute of Health, Esfahan, Iran, and maintained until use for parasitological and molecular testing.

Identification of the rodents was done using morphological characters (11) and only great gerbils, *R. opimus*, were included in the study.

### Direct examination test

In the laboratory, the rodents were anaesthetized using intramuscular Ketamine hydrochloride (60 mg/kg) and Xylazine (5 mg/kg). Regardless of having any obvious lesions, impression smears were prepared from the ear lobes of the animals (12), and stained using Giemsa and directly examined under a light microscope at

high magnification (1000x). After preparing direct smears, ear lobe samples were removed while the rodents were anaesthetized. The ear lobes were transferred to cold phosphate-buffered saline (pH=7.4) and thoroughly disrupted by grinding with a pestle and kept at -20 °C until use. The animals were nursed to complete recovery.

### **Ethical consideration**

Animal experiments were approved by the Ethical Committee of Tehran University of Medical Sciences, Tehran, Iran.

### **Nested PCR assay**

Genomic DNA was extracted and purified using a conventional phenol-chloroform protocol. Briefly, 200 µl of lysis buffer (100 mM Tris-HCl, pH=8; 10 mM EDTA, pH=8; 1% SDS; 100mM NaCl; 2% Triton X-100) with proteinase K (100 mg/ml) were added to 100 µl homogenized suspension of disrupted tissues and cells. The sample was incubated at 56 °C for one hour and subjected to a phenol-chloroform extraction (phenol-chloroform followed by chloroform). Extracted DNA was precipitated with an equal volume of isopropanol and 1/10 volume of 3M sodium acetate (pH= 5.2). The pellet was washed with 70% ethanol, air dried at room temperature and resuspended in 20 µl of sterile distilled water.

Fragment length polymorphism of the second internal transcribed spacer (ITS2) in the ribosomal RNA gene (rDNA) based on a nested PCR system was used for detection and species-identification of *Leishmania*. The sequence of the designed primers were as follows: Leish out F (5'-AAA CTC CTC TCT GGT GCT TGC-3') and Leish out R (5'-AAA CAA AGG TTG TCG GGG G-3') as the outer primers, and Leish in F (5'- AAT TCA ACT TCG CGT TGG CC-3') and Leish in R (5'-CCT CTC TTT TTT CTC TGT GC-3') as the inner primers. The PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. The results of nested PCR were confirmed based on species-specific pattern of PCR-RFLP using the restriction digestion with MnlI. Digestion was per-

formed by adding 5U (0.5 µl of the enzyme) and 1.5 µl of the relevant buffer to a 13 µl aliquot of the nested PCR product in a final volume of 15 µl. The mixture was incubated at 37 °C for 3 h and the products were separated using 2.5% agarose gel electrophoresis and visualized using ethidium bromide staining.

### **Statistical analysis**

The Fisher's exact test using SPSS 11.5 software was used and  $P < 0.05$  was considered as significant.

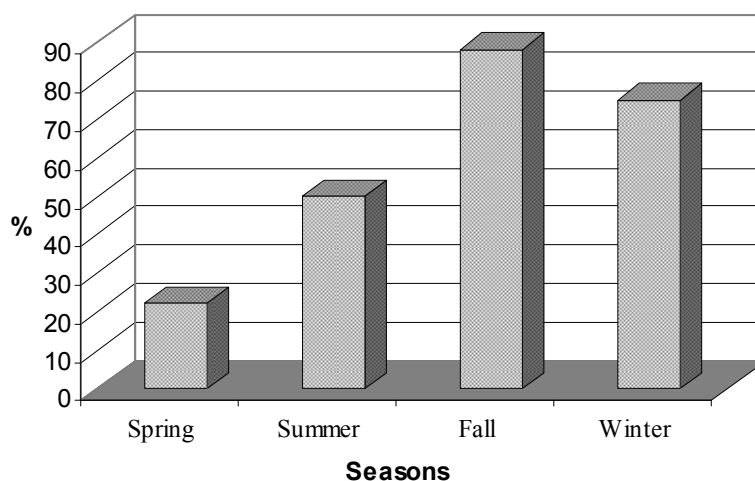
### **Results**

A total of 58 *R. opimus* (32.1% male and 67.9% female), were captured and examined by two diagnostic techniques, direct examination and nested PCR. Fourteen out of 58 specimens (24.1%) were positive by microscopic examination and 34 (58.6%) by the nested PCR. In 29 samples which the amastigote was not seen by through direct examination, the nested PCR showed positive results, and every positive smear was also found positive by nested PCR. Out of 34 nested PCR positive samples, 26 (76.5%) were identified as *L. turanica*, 1 (2.9%) was *L. gerbilli*, and 7 (20.6%) were mixed infection of *L. major* and *L. turanica*. *Leishmania* infection rate of male and female gerbils were 52.9% and 61.1% respectively which was not statistically different. Based on direct smear examination, none of the *Leishmania* positive gerbils showed cutaneous lesion.

Twenty six out of 58 (44.8%) of the gerbils were identified to be infected only with *L. turanica*, and 1 (1.7%) with *L. gerbilli*. Interestingly no gerbil was found to be infected with *L. major* alone. A mixed natural infection with *L. major* and *L. turanica* was seen in 12.1% of the rodents. Mixed infection of *L. major* and *L. gerbilli* and also *L. gerbilli* and *L. turanica*, were not seen in this study. The results of the seasonal variation of *Leishmania* infection which was performed only in the second year showed that pure *L. turanica* infection was seen throughout the year whereas mixed infection of *L. ma-*

major and *L. turanica* was seen in all seasons except spring. The highest *Leishmania* infection rate was observed with *L. turanica* (70.6%) in fall (Table 1). The highest (88.2%) and lowest

(22.2%) *Leishmania* infection rate was observed in fall and spring respectively (Fig. 1). Statistically significant difference was observed in *Leishmania* infection rate in different seasons ( $P=0.005$ ).



**Fig. 1:** Seasonal fluctuations of *Leishmania* infection rate of great gerbil population, Sejzi and Borkhar rural districts, Esfahan Province, Iran, Oct 2007- Oct. 2008

**Table 1:** *Leishmania* species infection rate of *Rhombomys opimus* population in different seasons, Sejzi and Borkhar rural districts, Esfahan Province, Iran, Oct 2007- Oct 2008

Species	<i>L. turanica</i>		<i>L. major</i> and <i>L. turanica</i>	
	No. of positive samples/no. of examined samples	% Positive	No. of positive samples/no. of examined samples	% Positive
Season				
Spring	2/9	22.2	0/9	0
Summer	3/8	37.5	1/8	12.5
Fall	12/17	70.6	3/17	17.7
Winter	4/8	50	2/8	25
Total	21/42	50	6/42	14.3

## Discussion

The results of the current study showed that *L. major*, *L. gerbilli* and *L. turanica* circulate in *R. opimus* populations in the study region. *L. turanica* was the dominant species detected in *R. opimus* population in the study areas in all seasons. Infection with *L. major* alone was not seen in *R. opimus* and *L. gerbilli* alone was rare. *L. major* infection was mostly accompanied by *L. turanica*. In spring, at the beginning of active season of sand flies (13), the only *Leishmania* infection in *R. opimus* population was due to *L. turanica*. (Table 1). In Turkmenia and Uzbeki-

stan, epizootics among *R. opimus* population always developed with *L. turanica* at the beginning of transmission season. *L. major* infection rate was extremely low in June and increased in late August and September (14). In the district of Borkhar, an hyperendemic focus of the disease in Esfahan Province, the highest *Leishmania* infection rate in great gerbils was previously reported from August to December (10).

For vast territories of central Asia, mixed infections of wild rodents with *L. major* (pathogenic to human) and *L. turanica* (non-pathogenic to human) are typical. Each parasite has own range

of pathogenicity and virulence (3). This animal proved to be susceptible to *L. major*, *L. turanica* and *L. gerbilli*. Infection with *L. major* alone rarely occurred in *R. opimus*. *L. turanica* promotes the persistence of *L. major* infection in the great gerbil (14). In an experimental *Leishmania* infection, the duration of *L. major* infection was 7 months, in *L. turanica* infection was 15 months, and *L. gerbilli* infection was 18 months. However, co-infection of *L. major* and *L. turanica* the duration of infection was extended up to 39 mo. Ulceration and visceralization never reported in great gerbils (15). Substantial part of great gerbils population live for more than one year (2) and remained as ZCL reservoir host for entire life and seem to be potential sources of *Leishmania* transmission until death (3). *L. turanica* proved to be the dominant species in *R. opimus* population located in hypoendemic, as well as meso- and hyperendemic foci of ZCL in Turkmenistan and Uzbekistan (14).

*Rhombomys opimus* is the main reservoir host of ZCL in Iran as well as some other countries (1, 10, 14, 15). In the previous studies, only *L. major* was isolated from great gerbils and characterized using isoenzyme or DNA-based molecular techniques in Iran (5, 10, 16, 17). However, there are rare reports of *L. turanica* infection in *R. opimus* (18). In most of the studies, identification of *Leishmania* species was done after isolation of *Leishmania* parasites from the culture media, which usually resulted in growth of only one species of *Leishmania* (4, 5, 16). Regarding the sand fly vectors, *L. major* is also isolated and characterized from *Phlebotomus papatasi*, *P. caucasicus*, as well as human lesions, in Iran (5, 19-21). Recently naturally infection of sand flies with *L. major*, *L. turanica* and *L. gerbilli* are reported from Iran (22). The three species were identified in naturally infected gerbils from Turkmenistan, Uzbekistan and Kazakhstan (3, 14). *L. major* infection of gerbils is crucial in the transmission cycle of ZCL (1, 3, 5). The distribution of *L. major* as the causative agent of ZCL in central Asia has been found to coincide with *R. opimus* (3).

It is concluded that *L. major*, *L. gerbilli* and *L. turanica* circulate in the population of *R. opimus* in central part of Iran. *L. turanica* was the dominant species in the population of great gerbils. Infection with *L. major* alone was not seen in the population of the gerbil. *Leishmania major* infection usually accompanied with *L. turanica* in naturally infected gerbils with the highest rate in fall. It is recommended that the role of *L. turanica* in the epidemiology and transmission of ZCL be revisited carefully.

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