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EPIDEMIOLOGY / *EPIDÉMIOLOGIE*

Dynamics of *Leishmania* infection rates in *Rhombomys opimus* (Rodentia: Gerbillinae) population of an endemic focus of zoonotic cutaneous leishmaniasis in Iran

Dynamique des taux d'infection à *Leishmania* chez les populations de *Rhombomys opimus* (Rodentia : Gerbillinae) dans un foyer endémique de leishmaniose cutanée zoonotique en Iran

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Abstract Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* is a great public health problem in the Old World. *Leishmania major* is widely distributed in populations of rodents in arid and savannah regions. In this study, seasonal variation of natural infection with *Leishmania* parasites in *Rhombomys opimus* (Rodentia: Gerbillinae) population of an endemic focus of ZCL in Iran was monitored. The study was conducted from October 2007 to October 2008 in the central part of the country. Nested polymerase chain reaction (PCR) assay was used for the detection and identification of *Leishmania* parasites, and the results were confirmed by PCR–restriction fragment length polymorphism (RFLP). The results showed that *Leishmania* infection rate was 55.8% (29 out of 52 gerbils) using nested PCR. The highest and lowest *Leishmania* infection rates were observed in fall and summer, respectively. Gerbils that were found to be infected only with *L. major* were 5.8%, and that with *Leishmania turanica* were 23.1%. A mixed natural infection was seen in the rodents with *L. major* and *L. turanica* (21.2%), with *L. major* and *L. gerbilli* (1.9%), and with all the three species (3.9%). *Leishmania major* infection alone was seen in fall and winter whereas mixed infection of *L. major* and *L. turanica* was observed in all seasons except in summer. *Leishmania turanica* infection was observed throughout the year. It is concluded that L. *major*, *L. gerbilli*, and *L. turanica* circulate in the population of *R. opimus* in central part of Iran. *Leishmania major* infection is 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 should be reconsidered.

Keywords *Rhombomys opimus* · *Leishmania major* · *L. gerbilli* · *L. turanica* · Seasonal variation · Zoonotic cutaneous leishmaniasis · Nested PCR · Badrood · Iran · Middle South Asia

Résumé La leishmaniose cutanée zoonotique (LCZ) due à *Leishmania major* constitue un problème de santé publique dans l'ancien monde. *L. major* est une espèce largement distribuée dans les populations de rongeurs vivant dans les régions arides et de savane. Dans la présente étude, la

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variation saisonnière de l'infection naturelle par les parasites du genre *Leishmania* chez la Grande Gerbille *Rhombomys opimus* (Rodentia: Gerbillinae) au sein d'une population d'un foyer endémique de LCZ en Iran a été contrôlée. L'étude a été menée d'octobre 2007 à octobre 2008 dans la partie centrale de l'Iran. Une PCR nichée a été utilisée pour la détection et l'identification du parasite *Leishmania*, et les résultats ont été confirmés par PCR-RFLP. Les résultats ont montré que le taux d'infection par *Leishmania* était de 55,8 % (29 sur 52 animaux) en utilisant la PCR nichée. Les taux d'infection par *Leishmania* les plus forts et les plus faibles ont été observés, respectivement, en automne et en été ; 5,8 % des gerbilles étaient infectées par *L. major* isolément et 23,1% par *L. turanica* isolément. Une infection mixte naturelle a été observée chez les rongeurs associant *L. major* et *L. turanica* (21,2 %), ou associant *L. major* et *L. gerbilli* (1,9 %), ou associant les trois espèces (3,9 %). L'infection due à *L. major* isolément a été observée en automne et en hiver. Une infection mixte associant *L. major* et *L. turanica* a été observée en toutes saisons, sauf en été. L'infection à *L. turanica* a été observée tout au long de l'année. En conclusion, les trois espèces *L. major*, *L. gerbilli* et *L. turanica* circulent dans la population de *R. opimus* dans la partie centrale de l'Iran. *L. major* est habituellement accompagnée par *L. turanica* chez les gerbilles naturellement infectées avec un plus fort taux en automne. Le rôle de *L. turanica* dans l'épidémiologie et la transmission de la LCZ devrait être reconsidéré.

Mots clés *Rhombomys opimus* · *Leishmania major* · *L. gerbilli* · *L. turanica* · Variation saisonnière · Leishmaniose cutanée zoonotique · PCR niche · Badrood · Iran · Moyen-Orient

Introduction

Zoonotic cutaneous leishmaniasis (ZCL), a neglected tropical disease, is a public health problem with a clear and disturbing increase in the number of cases in some areas of the world [2,6]. *Leishmania major* is widely distributed in various populations of rodents in arid and savannah regions [6]. The disease is endemic in many rural districts of Iran, in 17 provinces out of 30. Rodents belonging to subfamily Gerbillinae are the main reservoir hosts of ZCL in Iran and other countries where ZCL is endemic [3,15,18]. Gerbils are the most abundant mammals reported from natural ecosystems of Old World deserts [3]. Many rodent species act as reservoir hosts 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; *Meriones hurrianae* (Indian Desert Jird) in India and Iran; *Psammomys obesus* (Fat Sand Rat) and *Meriones crassus* in northern Africa and Middle East; and *Tatera* spp. in subsaharan Africa and Iran [6]. *R. opimus* (Cricetidae: Gerbillinae) is the principal *L. major* reservoir host in the vast territory of the Turan lowland (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* were reported from more than 200 places. The number of naturally infected great gerbils showed to be greater than any other mammals (other rodents, insectivores, and carnivores) [3].

All the proven vectors of ZCL belong to the subgenus *Phlebotomus*, i.e. *Phlebotomus papatasi*, the principal vector, and related species: *Phlebotomus salehi* and *Phlebotomus dubosqi*. Well-described stable ZCL systems are associated with *L. major*, *Psammomys obesus*, and *Ph. papatasi* in North Africa and Middle East and with *R. opimus* and *Ph. papatasi* in central Asia, Afghanistan, and Iran [6,19]. The distribution and the role of rodents as reservoir hosts of ZCL are geographically specific in Iran. *Rhombomys opimus* is the main reservoir of ZCL in Central and North East (N.E.) 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 rodent is *Tatera indica*, the Indian Jird (Cricetidae: Gerbillinae). In Baluchistan of Iran (border of Pakistan), *M. hurrianae* (Cricetidae: Gerbillinae) acts as a reservoir host [7,8,12,13,18,22]. One of the major problems for the control and understanding of this neglected disease is the lack of information about the dynamics of *Leishmania* parasites in rodent reservoir populations. In this study, seasonal variation of *Leishmania* species infection in *Rhombomys opimus* (Rodentia: Gerbillinae) population of an endemic focus in Central Iran was monitored.

Materials and methods

Study area

The investigation was conducted over a period of 12 months from October 2007 to October 2008 in Badrood rural district (33° 42′ N, 52° 2′ E), 25 km from the city of Natanz, Natanz county, Esfahan Province, central Iran where ZCL is endemic. Badrood district is located at an altitude of 1056 m, in the foothills of Karkas Mountains (altitude 3898 m) with a desert climate, hot summer and cold winter.

Rodent collection

Active colonies of gerbils in the district were identified and the rodents were caught using approximately 40 Sherman traps baited with cucumber, in different seasons. In spring

to fall, the traps were placed at the gerbil holes in the afternoon and were collected in the morning on the following day. In winter, Sherman traps were placed after sunrise and were collected at noon on the same day. 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.

The rodents were identified using morphological characters [5] and only great gerbils, *R. opimus*, were used for the study.

Direct examination test

In the laboratory, the rodents were anesthetized using intramuscular ketamine hydrochloride (60 mg/kg) and xylazine (5 mg/kg). Regardless of obvious lesions, impression smears were prepared from the ear lobes of the animals [4], stained by Giemsa, and directly examined carefully under a light microscope at high magnification (1000×) to search for *Leishmania* parasites. After preparing direct smears, ear lobe samples were removed while the rodents were anesthetized. 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. Animal experiments were approved by the Ethics 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; 100 mM 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 1 h and subjected to phenol–chloroform extraction (phenol–chloroform followed by chloroform). The DNA was precipitated with an equal volume of isopropanol and 1/10 volume of 3 M 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* in the specimens. The sequence of the 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 identity of all species identified by nested PCR was confirmed based on species-specific pattern of PCR-RFLP using the restriction digestion with MnlI. Digestion was performed by adding 5 U (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 by ethidium bromide staining.

Statistical analysis

The chi-square and Fisher's exact tests using SPSS 11.5 software were used and *P* value of less than 0.05 was considered significant.

Results

A total of 52 *R. opimus* (22 male and 30 female) were captured and examined by two diagnostic techniques, direct examination and nested PCR. Twelve out of fifty-two specimens (23.1%) were positive by microscopic examination and 29 (55.8%) by the nested PCR. In 17 samples in which the amastigote was not seen by direct examination, the nested PCR showed a positive result, and every positive smear was positive by nested PCR. Out of 29 positive samples by nested PCR, 3 (10.3%) were *L. major*, 12 (41.4%) were *L. turanica*, 11 (37.9%) were mixed infection of *L. major* and *L. turanica*, 1 (3.5%) was *L. major* and *L. gerbilli*, and 2 (6.9%) were mixed infection of all three species. The *Leishmania* infection rates of male and female gerbils were 63.6% and 50%, respectively, which was not statistically different. The highest (92.9%) and lowest (20%) infection rates of *Leishmania* were observed in fall and summer, respectively (Fig. 1). Statistically significant difference was observed in infection rates of *Leishmania* among different seasons ($P = 0.001$). Around 90% of the infected gerbils showed no cutaneous leishmaniasis lesion on their ear lobes.

Three out of fifty-two (5.8%) of the gerbils were found to be infected only with *L. major* and twelve (23.1%) with *L. turanica*. No *L. gerbilli* infection alone was seen in the gerbils. A mixed natural infection was seen in the rodents with *L. major* and *L. turanica* (21.2%), with *L. major* and *L. gerbilli* (1.9%), and with all three species (3.9%). Mixed infection of *L. gerbilli* and *L. turanica* was not seen in this study. Pure *L. major* infection was only observed in fall and winter whereas mixed infection of *L. major* and *L. turanica* was shown in all seasons except summer. Infection with *L. turanica* was seen throughout the year. The highest infection rate of *Leishmania* was seen with *L. turanica* (50%) and

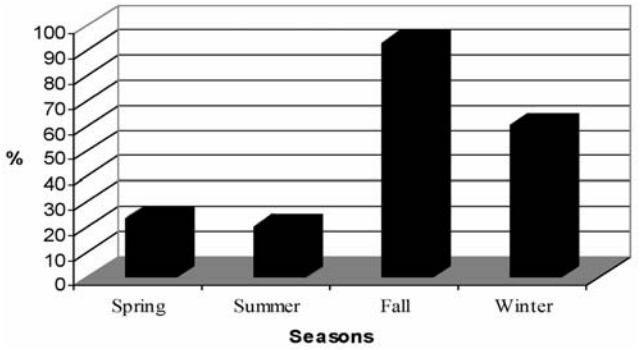

Fig. 1 Seasonal variation of infection rates of *Leishmania* of Great Gerbil population, Badrood rural district, Esfahan Province, Iran, October 2007–October 2008 / *Variation saisonnière des taux d'infection par* Leishmania *chez les populations de Grande Gerbille, dans les districts ruraux de Badroo, province d'Esfahan, Iran, oct. 2007 – oct. 2008*

mixed infection of *L. major* and *L. turanica* (35.7%), which was observed in fall (Table 1).

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. To our knowledge, seasonal variation of mixed natural infections of *R. opimus* with *L. major*, *L. turanica*, and *L. gerbilli* have not been reported earlier. Therefore, this is the first time that the dynamics of such mixed infections have been investigated in wild *R. opimus*, the Great Gerbil, in Iran or elsewhere. In spring, at the beginning of active season of sand flies [17], the highest *Leishmania* infection rate in *R. opimus* populations was due to *L. turanica*. The infection rate of *L. major* was less at this time but higher in fall (Table 1). In Turkmenia and Uzbekistan, epizootics among *R. opimus* populations always developed with *L. turanica* at the beginning of transmission season. The infection rate of *L. major* was extremely less in June and increased in late August and September [16]. In the district of Borkhar, an hyperendemic focus of the disease in Esfahan Province, in approximately 135 km from Badrood district, the highest infection rates of *Leishmania* in great gerbils were reported from August to December [22].

For vast territories of central Asia, mixed infections of wild rodents with *L. major* (pathogenic to humans) and *L. turanica* (nonpathogenic to humans) are typical. Each parasite has its own range of pathogenicity and virulence [15]. This animal proved to be susceptible to *L. major*, *L. turanica*, and *L. gerbilli*. Infection with *L. major* alone rarely occurred in *R. opimus*. *Leishmania turanica* promotes the persistence of *L. major* infection in the Great Gerbil [16].

Tableau 1 *Leishmania* species infection rates of *Rhombomys opimus* population in different seasons, Badrood rural district, Esfahan Province, Iran, Oct. 2007–Oct. 2008 / *Taux d'infection par les espèces de* Leishmania *chez les populations de* Rhombomys opimus *selon les saisons, dans les districts ruraux de Badrood*

Season	Species											
	L. major		*L. gerbilli*		*L. turanica*		*L. major* and *L. gerbilli*		*L. major* and *L. turanica*		*L. major*, *L. gerbilli* and *L. turanica*	
	No. of positive samples/no. of examined samples	Positive (%)	No. of positive samples/no. of examined samples	Positive (%)	No. of positive samples/no. of examined samples	Positive (%)	No. of positive samples/No. of examined samples	Positive (%)	No. of positive samples/No. of examined samples	Positive (%)	No. of positive samples/no. of examined samples	Positive (%)
Spring	0/13	0	0/13	0	2/13	15.4	0/13	0	1/13	7.7	0/13	0
Summer	0/5	0	0/5	0	1/5	20	0/5	0	0/5	0	0/5	0
Fall	1/14	7.1	0/14	0	7/14	50	0/14	0	5/14	35.7	0/14	0
Winter	2/20	10	0/20	0	2/20	10	1/20	5	5/20	25	2/20	10
Total	3/52	5.8	0/52	0	12/52	23.1	1/52	1.9	11/52	21.2	2/52	3.9

In an experimental *L. major* infection, the duration of the disease was 7 months; in *L. turanica* infection, it was 15 months; and in infection by *L. gerbilli*, it was 18 months. However, in coinfections of *L. major* and *L. turanica*, the disease duration was extended up to 39 months. Ulceration and visceralization never happened in great gerbils [14]. Great gerbils remained as ZCL reservoir hosts for life and seemed to be potential sources of *Leishmania* transmission until death [15]. *Leishmania turanica* proved to be the dominant species in *R. opimus* population located in hypoendemic, as well as in mesoendemic and hyperendemic foci of ZCL in Turkmenistan and Uzbekistan [16].

Rhombomys opimus is the main reservoir host of ZCL in Central and N.E. Iran and in some other countries [6,16,19,22]. In most of the earlier studies conducted in Iran, only *L. major* was isolated from great gerbils and characterized using isoenzyme or DNA-based molecular techniques [9,10,21]. In a study, which was conducted in the same area, only *L. major* was reported [18]. However, there are rare reports of *L. turanica* infection in *R. opimus* [20]. As most identification methods were based on the culture of *Leishmania* parasites, usually only one species of *Leishmania* was detected and identified in each study [9,18,19]. Regarding the sand fly vectors, *L. major* is also isolated and characterized from *Ph. papatasi, Ph. caucasicus*, as well as human lesions, in Iran [1,19,21,23]. Recently naturally infection of sand flies with *L. major*, *L. turanica* and *L. gerbilli* has been reported from Iran [11]. The three species were identified in naturally infected gerbils from Turkmenistan, Uzbekistan, and Kazakhstan [15,16]. The gerbils infected by *L. major* are important in the transmission cycle of ZCL [6,15,19]. The distribution of *L. major* as the causative agent of ZCL in central Asia has been found to coincide with *R. opimus* [15].

Conclusion

It is concluded that *L. major*, *L. gerbilli*, and *L. turanica* circulate in the population of *R. opimus* in central part of Iran. *Leishmania turanica* was the dominant species in the populations of the great gerbils. Infection with *L. major* alone in the population of the gerbil is rare. Infection of *Leishmania major* is 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 should be reconsidered carefully.

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