Characterization of *Leishmania* infection in rodents from endemic areas of the Islamic Republic of Iran

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خصائص عدوى القوارض بالليشمانيات في المناطق الموطونة بها في جمهورية إيران الإسلامية

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الخلاصة: تمَّ استفراد أنواع الليشمانيات والتعرُّف على خصائصها وعلى النظائر الإنزيمية فيها، كما تم تحليل الجزيئات المأخوذة من القوارض التي أُمسك بها في أجزاء مختلفة من جمهورية إيران الإسلامية في ما بين عامَيْ 1991 و2000. أما في المناطق الموطونة بداء الليشمانيات الجلدي فقد وُجدت الطفيليات الآتية التي كشفت بالفحص المجهري المباشر من 18.6٪ من اللطاخات التي أُخذت من 566 نموذج: الليشمانيات الكبيرة وقد تم استفرادُها من 4 نماذج. المعيَّنات الأوبيمية والمريونيسة الليبية والتاتيريَّة الهندية والمريونية الهريانية. وقد استُفردت الليشمانية الطورانية لأول مرة في هذا البلد من المعيَّنات الأوبيمية. وفي المناطق الموطونة بداء الليشمانيات الحشوية لوحظت الطفيليات في كبد وطحال 13.7٪ من أصل 504 من القوارض. وقد كانت نتيجة الزرع إيجابية في حالتَيْن، واستُفردت المُشَيْقات من المريونية الفارسية، وأمكن من دراسة خصائصها التعرُّف على أنها من نوع الليشمانية الدونوفانية ذات الإنزيم LPN50 ومن المتوسطة الذهبية، وقد أمكن من دراسة خصائصها التعرُّف على أنها من الليشمانية الطِّفلية ذات الإنزيم LON49.

ABSTRACT Between 1991–2000, ***Leishmania*** species were isolated and characterized by isoenzyme and molecular analysis from rodents caught in various parts of the Islamic Republic of Iran. In areas endemic for cutaneous leishmaniasis, parasites were observed by direct microscopy in smears from 18.6% of 566 specimens. ***L. major*** was isolated from 4 species: ***Rhombomys opimus, Meriones libycus, Tatera indica*** and ***Mer. hurrianae***. ***L. turanica*** was isolated from ***R. opimus*** for the first time in this country. In endemic areas of visceral leishmaniasis, parasites were observed in liver and spleen from 13.7% of 504 rodents. Two species were positive on culture; promastigotes isolated from ***Mer. persicus*** were characterized as ***L. donovani*** zymodeme LON50 and from ***Mesocricetus auratus*** as ***L. infantum*** LON49.

Caractérisation de l'infection à *Leishmania* chez des rongeurs des zones endémiques de la République islamique d'Iran.

RÉSUMÉ Entre 1991 et 2000, des espèces de ***Leishmania*** ont été isolées et caractérisées par isoenzymes et analyse moléculaire chez des rongeurs capturés dans diverses parties de la République islamique d'Iran. Dans les zones d'endémie de la leishmaniose cutanée, des parasites ont été observés par microscopie directe dans des frottis provenant de 18,6 % des 566 échantillons. ***L. major*** a été isolé chez quatre espèces : ***Rhombomys opimus, Meriones libycus, Tatera indica*** et ***Mer. hurrianae. L. turanica*** a été isolé chez ***R. opimus*** pour la première fois dans ce pays. Dans les zones d'endémie de la leishmaniose viscérale, des parasites ont été observés dans le foie et la rate de 13,7 % des 504 rongeurs. Deux espèces ont donné des cultures positives ; les promastigotes isolés chez ***Mer. persicus*** ont été caractérisés comme zymodème LON50 de ***L. donovani*** et ceux isolés chez ***Mesocricetus auratus*** comme LON49 de ***L. infantum.***

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Introduction

Leishmaniasis is an important health problem in the Islamic Republic of Iran. There are several foci of zoonotic cutaneous leishmaniasis (CL) in the north, east and south of the country [*1–6*]. Zoonotic CL is essentially a disease of gerbils, transmitted by *Phlebotomus papatasi* and *P. cocausicus* and other species of sand fly that breed in gerbil burrows [*7*]. The human disease is secondary to the infection of gerbils and is seen only in places where the infected gerbils live [*8*]. Three different epidemiological types of zoonotic CL have been observed in this country and 4 species of rodents (Gerbillidae) are the principal animal reservoir hosts in all foci [*9*].

Visceral leishmaniasis (VL), or kala-azar, is also seen sporadically all over the Islamic Republic of Iran and is of the Mediterranean type. Wild and domestic carnivores are the main animal reservoirs [*10–12*], but rodents have been reported as reservoirs in the Meshkin-Shar district [*13*]. Sand flies of the genus *Phlebotomus* are the most likely vector of VL in the endemic areas [*10*].

The study of *Leishmania* infection in rodents in the Islamic Republic of Iran started in 1953 in the north-east of the country [*8*] but, while it was extended to other parts of the country, the isolation and characterization of the parasites has not been investigated in these areas. In this study, we report the isolation and characterization of *Leishmania* species infection from a number of species of rodents that were trapped alive in different parts of the Islamic Republic of Iran in the last decade.

Methods

Study area

The investigation was conducted over a period of 10 years from 1991 to 2000 in endemic foci of zoonotic CL and VL in the Islamic Republic of Iran (Figure 1).

Collection and examination of rodents

The study sites were determined by reports from local health authorities of outbreaks of human CL and VL infection. 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 CL or VL were 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: colour, body measurements, ears, tail, feet, teeth and cranium [*14,15*].

Isolation of parasites from the caught rodents

For detecting CL infection, 2 impression smears were taken from the ears of each rodent [*13,16*]. For detecting VL parasites, 2 impression smears from the spleen and liver of each rodent were prepared. The smears were fixed in methanol, stained by standard Giemsa methods and examined for parasites by light microscope at high magnification (× 1000).

The samples from infected rodents were cultured in Novy–MacNeal–Nicolle (NNN) culture and liver infusion broth tryptose (LIT) and RPMI 1640 medium (Gibco Life Technologies, New York, USA) containing 10% heat-inactivated fetal calf serum. The cultures were checked for promastigotes twice a week for a period of 6 weeks.

Leishmania species were characterized by random amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR)

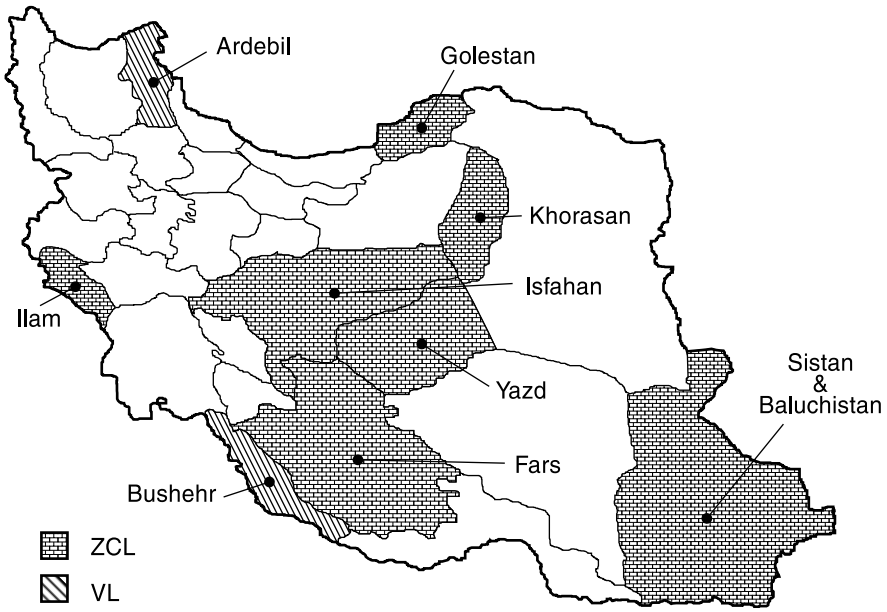

Figure 1 **Areas endemic for zoonotic cutaneous leishmaniasis (ZCL) and visceral leishmaniasis (VL) where rodents were collected for the study**

analysis [*17,18*] at the Medical Faculty, Shiraz University of Medical Sciences and the School of Pubic Health, Tehran University of Medical Sciences and by isoenzyme analysis at the London School of Hygiene and Tropical Medicine, United Kingdom, and the Faculty of Medicine, University of Montpellier, France.

Characterization of isolated parasites

For the RAPD–PCR analysis, DNA was extracted from the promastigotes, cultured at 20 °C in RPMI1640 medium (10 000 parasites per 10 mL) and washed with Locke's solution. The pellet was resuspended in 100 µL lysis buffer. The lysate was extracted once with equal volumes of 1:1 (v/v) phenol:chloroform and once with 24:1 (v/v) chloroform isoamylalcohol and precipitated by ethanol. The DNA was resuspended in the specified materials and amplification were done in a mixture containing 20 mmol/L $(NH_4)_2(SO_4)$, 75 mmol/L Tris-HCl, pH.9, 0.01% (w/v) Tween 20, 2 mmol/L $MgCl_2$, 200 µmol/L deoxynulcleotide triposphate, 1 mmol/L primer and 1 unit of Taq polymerase. Then 1 µL of DNA (20 ng/µL) was added by centrifugation through the mineral oil overlay and the reaction was carried out in a thermocycler (Genius, Techne Ltd, United Kingdom) programmed for 1 cycle of 2 min at 94 °C, followed by 30 cycles of 30 s at temperatures of 94 °C, 1 min at 36 °C and 2 min at 72 °C. Aliquots from each reaction (12 µL) were run on 1.5% agarose gel and visualized under ultraviolet light with ethidium

bromide. The primers used in this study were as follows:

- AB1-07 GGT GAC GCA G
- 327. ATA CGG CGT C
- 329. GCG AAC CTC C
- 333. GAA TGC GAC G
- 335. TGG ACC ACC C

For the isoenzyme characterization, after mass production of promastigotes, samples were cultured in monophasic media with 10% to 20% fetal calf serum, washed with phosphate-buffered saline at 4 °C with centrifugation at 2500–3000 × g for 20 min 3 times and freeze-thawed in liquid nitrogen several times, followed by electrophoresis on polyacrylamide gel. In this technique 12 enzymes were used: pyruvate kinase (PK), superoxide dismutase (SOD), phosphoglucomutase (PGM), peptidase D (PEPD), alanine aminotransferase (ALT), aspartate aminotransferase (AST), nucleoside hydrolase (NH), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate isomerase (GPI), esterase (ES), methanol dehydrogenase (MDH) and mannose-6-phosphate isomerase (MPI) [*19*].

Results

Areas endemic for cutaneous leishmaniasis

Altogether, 566 rodents (Gerbillidae) were trapped alive in several CL-endemic areas throughout the Islamic Republic of Iran from 1991 to 2000. *Leishmania* parasites were observed in cutaneous smears from 105 (18.6%) of the rodents by direct high magnification microscopy examination (Table 1).

L. major was isolated from *Rhombomys opimus*, *Meriones libycus*, *Tatera indica* and *Mer. hurrianae* and characterized by isoenzyme analysis and molecular procedures (RAPD–PCR). All of the *Leishmania* species and strains were similar to *Leishmania* species that had been isolated from human infection in the same areas. *L. turanica* was isolated from an infected *R. opimus* for the first time in this country.

R. opimus was the principal reservoir host of zoonotic CL in the north-eastern (Minoo Dasht) district where 85.2% of isolates tested positive (Table 1). It was also prominent in the central parts of the country (Badrood, Ardakan and Sabzevar districts). *Mer. libycus* was found in 35.1% of isolates in the south-west (Fars province) and 25.0% in the central area. *T. indica* was the main reservoir host in foci of the south-west (14.3%) and south (Dashti and Dashtestan districts) of the country (4.5% of isolates tested positive). In the south-east of the country (including southern parts of Baluchistan, Dashtyari, Konarak and Chabahar areas) the main animal reservoir was *Mer. hurrianae* (17.9% of isolates).

Areas endemic for visceral leishmaniasis

A further 504 rodents (Gerbillidae, Cricetidae) were caught during 1994 to 2000 in 2 areas endemic for VL: Meshkin-Shahr district (north-west) and Dashti and Dashtestan districts (south). *Leishmania* parasites were seen in livers and spleens of 69 (13.7%) of these rodents by microscopy (Table 2).

Leishmania spp. were isolated from 2 specimens of *Mer. persicus* and 1 specimen of *Mesocricetus auratus* in culture media (Table 2). Although parasites were observed in a few specimens of *Cricetulus migratorius*, none were positive on culture. Using isoenzyme techniques the promastigotes isolated from *Mer. persicus* were characterized as *L. donovani* zymodeme

Table 1 ***Leishmania*** **species isolates from rodents caught in areas of the Islamic Republic of Iran endemic for cutaneuos leishmaniasis (1991–2000)**

Location of capture/ rodent species	No. tested	Positive on microscopy No.	%	*Leishmania* species identified
North-east (Minoo Dasht district)				
Rhombomys opimus	27	23	85.2	***L. major***
Meriones libycus	1	0	0	–
South (Dashti and Dashtestan district)				
Tatera indica	133	6	4.5	***L. major***
Meriones crassus	48	0	0	–
Rattus rattus	3	0	0	–
Nesokia indica	3	0	0	–
Mus musculus	5	0	0	–
South-east (Baluchestan)				
Meriones hurrianae	28	5	17.9	***L. major***
Tatera indica	27	1	3.7	–
Rattus rattus	3	0	0	–
Rattus norvegicus	4	0	0	–
Mus musculus	5	0	0	–
Nesokia indica	2	0	0	–
Funambulus pennanti	1	0	0	–
West (Mehran district)				
Tatera indica	22	2	9.1	***L. major***
Nesokia indica	8	0	0	_
Central (Badrood district)				
Meriones libycus	36	9	25.0	***L. major***
Rhombomys opimus	25	8	32.0	***L. major***
South-west (Fars province)				
Meriones libycus	97	34	35.1	***L. major***
Tatera indica	21	3	14.3	–
Central (Ardakan district)				
Rhombomys opimus	26	3	11.5	***L. major***
Meriones libycus	19	3	15.8	–
Central (Sabzevar district)				
Rhombomys opimus	22	8	36.4	***L. major*** and ***L. turanica***
Total	566	105	18.6	

LON50 and those from *Mes. auratus* were identified as *L. infantum* LON49.

Discussion

Both CL and VL are endemic in the Islamic Republic of Iran. Mucosal leishmaniasis is usually an extension of the cutaneous form, except for 3 cases of lesions of the palate for which the causative organisms are unknown [*20*].

The cutaneous form of leishmaniasis is seen in 2 forms: anthroponotic and zoonotic. Anthroponotic CL is endemic in many large- and medium-size cities, as well as villages in the suburbs of these foci. The main reservoir host of CL is man, although the lesions have been observed on dogs in Tehran, Mashad, Shiraz and Kerman [*9*]. Zoonotic CL is endemic in many foci in the north, east and south of the country [*9*]. This is essentially a disease of gerbils, transmitted by sand flies that live and breed in the gerbil burrows. The human disease is secondary to the infection of gerbils and is seen only in places where the infected gerbils live.

Our results show that *R. opimus* (great gerbil) is the principal reservoir host of zoonotic CL in the central and north-east parts of the country. *Mer. libycus* (Libyan jird) was also found to be infected and can act as a secondary reservoir host in the absence of *R. opimus*. Of course, in some areas from the centre and south of the country, gerbils have become the primary reservoir of zoonotic CL due to ecological changes [*21*]. Other foci are in Turkemen-Sahara, Lotfabad and Sarakhs, that is the border with Turkemenistan Republic, Esfarayen in Khorasan, Bakran in Semnan, Abarkuh in Yazd, Neiriz and Estahban in Fars provinces. Natural *Leishmania* spp. infection of *R. opimus* is found in Abardej of Varamin near Tehran but far from human residences and *Leishmania* species have not yet been determined [*9,22*].

T. indica (Indian jird) is the main reservoir host of zoonotic CL in foci of the south-west and south of the country.

Table 2 ***Leishmania*** **species isolates from rodents caught in areas of the Islamic Republic of Iran endemic for visceral leishmaniasis (1994–2000)**

Location of capture/ rodent species	No. tested	Positive on microscopy		Positive on culture media		*Leishmania* species and zymodemes identified
		No.	%	No.	%	
Meshkin-Shahr						
Cricetulus migratorius	15	2	13.3	0	0	–
Mesocricetus auratus	2	1	50.0	1	50.0	***L. infantum*** LON49
Meriones persicus	394	66	16.8	2	0.5	***L. donovani*** LON50
Mus musculus	7	0	0	0	0	–
Allactaga spp.	1	0	0	0	0	–
Dashti and Dashtestan district						
Tatera indica	85	0	0	0	0	–
Total	504	69	13.7	3	0.6	

These areas include the Iran–Iraq borders from Sumar to the Gulf, all the provinces of Khuzestan and some parts of Ilam, Bushehr and Hormozegan [*1,11,23*].

In foci of the south-east of the country, the main animal reservoir is *Mer. hurrianae* (Indian desert jird). These areas include the southern parts of Baluchistan, Dashtyari, Konarak and Chabahar areas. This type of zoonotic CL is similar to the foci of the disease reported from Rajasthan in India [*4,7,9*].

The visceral form of leishmaniasis is seen in sporadic form all over the Islamic Republic of Iran and is endemic in Ardebil and east Azerbaijan provinces in the north-west, and in Fars and Bushehr in the south. Wild and domestic dogs are the main reservoir hosts of VL [*12*]. In this study, amastigotes were observed in 13.7% of the rodents on microscopic examination of the smears prepared from internal organs.

L. donovani LON-50 was isolated from 2 specimens of *Mer. persicus* (Persian jird). It seems to transmit from infected rodents to humans in these endemic areas. *L. infantum* LON-49 was isolated from 1 specimen of *Mes. auratus* (golden hamster). This species of *Leishmania* is zoonotic and had been previously isolated from humans [*10*] and dogs in the Meshkin-Shahr area [*12*], and also from dogs and foxes in the Dashti district of Bushehr province [*11*]. *L. infantum* had been isolated from *Rattus rattus* (black rat) in Italy and Iraq [*24*]. In one study, *Mer. persicus* was reported to be naturally infected with *Leishmania* spp. in east Azerbaijan, in the north-west of the Islamic Republic of Iran. In the smears prepared from the cutaneous lesion of this gerbil, considerable numbers of amastigotes were seen. However, microscopic examination of the smears prepared from the internal organs and blood of this rodent did not show any amastigotes [*25*]. In the other study that was carried out in the Semeskandeh area of Mazanderan province in the north of the Islamic Republic of Iran, *Leishmania* spp. infection was reported in internal organs of *R. rattus* but *Leishmania* parasites were not isolated from them (Gholami, personal communication).

In conclusion, this study has shown that rodents harbour *Leishmania* spp. infection and may therefore have a role in transmission of leishmaniasis to humans, particularly to children. Further ecological and biological studies of rodents and sand flies are necessary in endemic foci of zoonotic VL from the Islamic Republic of Iran until the exact role of the rodents as animal reservoirs is clarified completely.

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