The PCR-based detection of *Leishmania major* in *Mus musculus* and other rodents caught in southern Iran: a guide to sample selection

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In recent years, rural cutaneous leishmaniasis (CL) has become one of the most challenging public-health problems in several countries. Rodents play an important role as the ‘reservoir’ hosts of the parasite (*Leishmania major*) that causes the vast majority of the CL found in rural Iran. The present study deals with the role of rodents in the epidemiology of CL in Fars province, in the south of Iran, where the incidence of the disease has doubled over the last decade. Samples, of skin, foot pads, ears, livers and spleens, were collected from 89 rodents (three *Meriones persicus*, one *Me. libycus*, 64 *Tatera indica* and 21 *Mus musculus*), that had been caught in an endemic area, killed, and fixed in formalin [5% (w/v) formaldehyde]. When each sample was checked for *L. major* DNA by PCR, at least one sample from each of three (100%) of the *Me. persicus*, 35 (54.7%) of the *T. indica* and nine (42.9%) of the *Mu. musculus* was found to contain *L. major* DNA. Only 60% of the ear samples, 56% of the footpad samples, 38% of the liver samples and 38% of the spleen samples from the PCR-positive rodents were, however, found PCR-positive. Four of the rodents (two *T. indica* and two *Mu. musculus*) were each found to have PCR-positive ear and footpad samples but PCR-negative liver and spleen samples. Only four of the rodents (all

T. indica) had PCR-positive spleen and/or liver samples but PCR-negative ear and footpad samples. It is therefore recommended that, for the PCR-based detection of *L. major* in Iranian rodents, priority is given to the collection and investigation of both ear and footpad samples.

Zoonotic cutaneous leishmaniasis (CL) caused by *Leishmania major* is now endemic in 14 of the 29 provinces of Iran, with many rural foci in the north, east and south of the country (Yaghoobi-Ershadi *et al.*, 2005). Several species of rodents have been identified as ‘reservoir’ hosts for the causative parasite in Iran, including *Meriones libycus*, *Me. hurrianae*, *Nosokia indica*, *Rhombomys opimus*, *Tatera indica* and *Rattus norvegicus*. (Seyedi-Rashti and Nadim, 1967; Nadim and Faghih, 1968; Seyedi-Rashti and Salehzadeh, 1990; Javadian *et al.*, 1998; Motazedian *et al.*, 2010). Unfortunately, because *L. major* may cause no or only minor skin lesions in such hosts, it is not always easy to detect the infections in wild rodents (WHO, 1990; Moemenbellah-Fard *et al.*, 2003). Most infected rodents have been identified by the collection of skin scrapings from ears or feet and the culture of these samples (Edrissian *et al.*, 1982). PCR has, however, already been found useful for the identification of possible reservoir hosts, without the need for culture and parasite isolation (Alexander *et al.*, 1998; Oliveira *et al.*, 2005; Brandão-Filho and Shaw, 2006).

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The main aim of the present study was to use a PCR-based assay and ear, footpad, liver and spleen samples to explore the prevalences of leishmanial infection among rodents that had been trapped in various parts of Fars province, in southern Iran.

ANIMALS AND METHODS

Study Areas

Fars province lies in southern Iran, at 27°2'–31°42'N, 50°42'–55°36'E, and covers about 1,300,000 km² or 8.1% of Iran. Its mean altitude is 1540 m above sea level and its total population is about 4 million. Rodents were trapped in various parts of the province, including Shiraz, Kazerun, Firooz Abad, Fassa, Sarvestan, Kherem, Zarghan, Marvdasht, Sepidan, Arsanjan, Estahban, Kohmeh Sorkhi, Nobandgan, Kavar, Khafr, Lar, Abade, Bavanat, Khoram Bid, Niriz and Moshkan.

Sampling

Rodents were caught alive, in wire traps, between April 2004 and April 2005 (Mehrabani *et al.*, 2007). The traps were set inside and outside homes, in agricultural plantations close to houses and in both rural and urban settings. The trapped animals were identified by staff of Shiraz University's Department of Biology, using the relevant taxonomic criteria (Eisenberg and Redford, 1999), before being humanely killed (using methods approved by the Iranian Veterinary Organization), checked for skin lesions and then stored in formalin [5% (w/v) formaldehyde] until they could be processed further.

The Fars Environmental Protection Organization gave a permit for the trapping.

DNA Extraction and PCR

Small samples (each of about 30–40 mm³) of both ears, both rear footpads, the liver and the spleen were collected from each fixed animal. Each sample was homogenized in a 1.5-ml microcentrifuge tube, using a glass

rod, and then suspended in 200 µl lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% (v/v) Tween 20 and 15 µl of a solution that contained 19 mg proteinase K/ml]. Each sample was then incubated at 37°C for 48 h, homogenized again with a clean glass rod, and then incubated at 100°C for 15 min. After each resultant lysate was allowed to cool, the DNA in the lysate was recovered by phenol: chloroform: isoamyl-alcohol extraction (Pourmohammadi *et al.*, 2008). Precipitated DNA was resuspended in 50 µl double-distilled water, and stored at –20°C until it could be tested for leishmanial kinetoplast DNA in a PCR-based assay (Pourmohammadi *et al.*, 2008).

Data Analysis

For the rodent species collected in sufficient numbers (i.e. *T. indica*), χ^2 tests, conducted using the SPSS software package (SPSS Inc, Chicago, IL), were used to see which single type of sample (i.e. footpad, ear, liver or spleen), when investigated by PCR, gave a good indication of whether a rodent would be found PCR-positive (by the examination of any sample). A *P*-value of <0.05 was considered indicative of a statistically significant agreement.

RESULTS

Overall, 89 rodents (three *Me. persicus*, 64 *T. indica*, 21 *Mus musculus*, and one *Me. libycus*) were trapped and checked for leishmanial infection. Although no lesions were seen, 47 of the rodents — three (100%) of the *Me. persicus*, 35 (54.7%) of the *T. indica*, nine (42.9%) of the *Mu. musculus* and none of the *Me. libycus* — were found PCR-positive for leishmanial (all *L. major*) DNA.

With *T. indica*, the types of samples most frequently found PCR-positive were the ears (45.3%) and liver (29.7%) (Table 1). With *Mu. musculus*, the footpads (38.5%) and ears (30.7%) were the samples most frequently found PCR-positive (with all of the liver and spleen samples found PCR-negative;

TABLE 1. *Results of the PCR-based detection of Leishmania major DNA in samples from rodents caught in southern Iran*

	<i>Meriones persicus</i>	<i>Tatera indica</i>	<i>Mus musculus</i>	<i>Meriones libycus</i>
No. of rodents trapped	3	64	21	1
NO. AND (%) WITH PCR-POSITIVE:				
Right rear foot	3 (100)	10 (15.6)	3 (14.3)	0 (0)
Left rear foot	3 (100)	6 (9.4)	2 (9.5)	0 (0)
Right ear	3 (100)	16 (25.0)	1 (4.8)	0 (0)
Left ear	3 (100)	13 (20.3)	3 (14.3)	0 (0)
Liver	2 (67)	19 (29.7)	0 (0.0)	0 (0)
Spleen	1 (33)	18 (28.1)	0 (0.0)	0 (0)
Any sample	3 (100)	35 (54.7)	9 (42.9)	0 (0)

Table 1). In contrast, all of the samples from the three *Me. persicus* gave a positive result in the PCR (Table 1).

With *T. indica*, the PCR-based examination of either just footpad samples ($P<0.002$) or just ear samples ($P<0.001$) gave a good indication of whether the rodent would be found PCR-positive (i.e. by the examination of all samples). The PCR-positive *T. indica* were trapped in the Shiraz focus and rural areas in Kavar, Firooz Abad, Fassa, Sarvestan, Khrameh, Sepidan, Kohmereh Sorkhi and Nobandegan. All the *T. indica* trapped in Khafr and Abadeh were found PCR-negative. The nine PCR-positive *Mu. musculus* came from Kavar, Sepidan, Estahban, Moshkan and Niriz.

Four of the rodents (two *T. indica* and two *Mu. musculus*) were each found to have PCR-positive ear and footpad samples but PCR-negative liver and spleen samples (Table 2). Only four of the rodents (all *T. indica*) had PCR-positive spleen and/or liver samples but PCR-negative ear and footpad samples (Table 2).

DISCUSSION

In the detection of leishmanial infection in rodents, the investigation of biopsy/necropsy samples by PCR is more sensitive than the investigation of similar samples as smears or

TABLE 2. *The combinations of organs/tissues, from rodents caught in southern Iran, found PCR-positive for Leishmania major DNA*

	<i>Meriones persicus</i>	<i>Tatera indica</i>	<i>Mus musculus</i>	<i>Meriones libycus</i>
No. of rodents trapped	3	64	21	1
NO. AND (%) WITH PCR-POSITIVE:				
Foot/feet only	0 (0)	3 (3.1)	4 (19.0)	0 (0)
Ear(s) only	0 (0)	4 (6.3)	3 (14.3)	0 (0)
Liver only	0 (0)	2 (3.1)	0 (0)	0 (0)
Spleen only	0 (0)	4 (6.3)	0 (0)	0 (0)
Foot/feet and ear(s) only	0 (0)	2 (3.1)	2 (9.5)	0 (0)
Foot/feet and liver only	0 (0)	0 (0)	0 (0)	0 (0)
Foot/feet and spleen only	0 (0)	0 (0)	0 (0)	0 (0)
Ear(s) and liver only	0 (0)	4 (6.3)	0 (0)	0 (0)
Ear(s) and spleen only	0 (0)	1 (1.6)	0 (0)	0 (0)
Spleen and liver only	0 (0)	4 (6.3)	0 (0)	0 (0)
Ear(s), foot/feet and liver only	2 (67)	2 (3.1)	0 (0)	0 (0)
Ear(s), spleen and liver only	0 (0)	6 (9.4)	0 (0)	0 (0)
Ear(s), foot/feet and spleen only	1 (33)	3 (4.7)	0 (0)	0 (0)
Ear(s), foot/feet, spleen and liver	0 (0)	1 (4.8)	0 (0)	0 (0)

by culture (Nicolas *et al.*, 2002; Oliveira *et al.*, 2005; Parvizi *et al.*, 2008). Even though the samples investigated in the present study had been formalin-fixed, the overall prevalence of *L. major* detected (52.8%) was markedly higher than the value (16.2%) estimated, using microscopy and culture, for rodents caught elsewhere in southern Iran (Motazedian *et al.*, 2006; Mehrabani *et al.*, 2007). The results of rodent trapping in Fars (present study) indicate that *T. indica* is the most common rodent in the province, and the results of the subsequent PCR indicated that, at the time of the trapping, about one in every two (54.7%) *T. indica* in the province was infected with *L. major*. In earlier studies, *T. indica* infected with *L. major* have been detected in southern or south-eastern Iran (Javadian *et al.*, 1998; Mohebbali *et al.*, 2004; Mehrabani *et al.*, 2007) as well as the east and west of the country (Rassi *et al.*, 2001).

Meriones libycus is considered to be the main 'reservoir' host in the foci of zoonotic CL in northern and central Iran (Yaghoobi-Ershadi *et al.*, 2004; Pourmohammadi *et al.*, 2008). In the present study, this species and *Me. persicus* appeared to be rare in Fars province, although all three specimens of *Me. persicus* that were caught were found PCR-positive for *L. major* DNA, perhaps indicating that, despite their relative rarity, *Meriones* spp. are also important reservoir hosts for ZCL in southern Iran (Rassi *et al.*, 2006).

The present report appears to be the first to describe natural infection of Iranian *Mu. musculus* with *L. major*. Such infection may be epidemiologically significant because *Mu. musculus* is so widespread in and around housing, especially in urban areas, and probably occurs in every Iranian city and village. Laboratory mice bred from wild *Mu. musculus* are important experimental hosts of *L. major* (Mauricio *et al.*, 2001). After Aebischer *et al.* (1993) infected C57BL/6 mice with *L. major*, they were able to find the parasites in the lymph glands, livers, spleens and bone marrow of

the rodents. Nicolas *et al.* (2002) infected BALB/C mice with *L. major* and, 6–12 months later, found necropsy samples (of ears, lymph glands, livers, spleens, bone marrow, blood, foot pads and tail skin) to be PCR-positive for the parasite's DNA. As *Phlebotomus papatasi* can be infected by feeding on laboratory mice experimentally infected with *L. major* (Kimblin *et al.*, 2008), naturally infected mice may play an important role as reservoirs for human infection. The presence of *Mu. musculus* infected with *L. major* may explain why, in recent years, ZCL (which is usually considered a rural disease) has been increasingly detected in some urban areas of Iran (Razmjou *et al.*, 2009). In the present study, none of the livers and spleens of the *Mu. musculus* caught was found PCR-positive, perhaps indicating that *L. major* does not spread as much in *Mu. musculus* of the wild type as it tends to do in laboratory mice.

Taken together, the present results indicate that, for the PCR-based detection (or, presumably, the culture-based isolation) of *L. major* in Iranian rodents, priority is given to the collection and investigation of both ear and footpad samples.

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