Asymptomatic human carriers of *Leishmania infantum*: possible reservoirs for Mediterranean visceral leishmaniasis in southern Iran

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Over the last decade, the incidence of visceral leishmaniasis (VL) has increased in many districts of the province of Fars, in southern Iran. Recent epidemiological reports indicate that asymptomatic human infections with *Leishmania infantum* (the causative agent of VL throughout the Mediterranean basin) occur more frequently in Iran than was previously believed.

Between 2004 and 2006, blood samples were collected from 802 apparently healthy subjects from communities, in the north–west and south–east of Fars province, where VL cases had been recorded. Each of these samples was tested for anti-*Leishmania* antibodies, in direct agglutination tests (DAT), and for *L. infantum* kinetoplast DNA, in PCR-based assays. Of the 426 subjects from north–western Fars, eight (1.9%) were found seropositive and 68 (16.0%) PCR-positive. The corresponding values for the 376 subjects from south–eastern Fars were lower, with five (1.3%) seropositive and 32 (8.5%) PCR-positive. Of the 100 PCR-positive subjects, 18 (18.0%) each lived in a household in which there had been a case of VL, and six (6.0%) had had VL themselves (in each case, more than a year before the blood sampling for the present study). Although 21 of the PCR-positives have now been followed-up for at least 18 months, none has developed symptomatic VL. Since positivity in the PCR-based assay probably indicated the presence of *L. infantum* amastigotes in the peripheral blood of 12.5% of the subjects, it is clear that asymptomatic human carriers of *L. infantum* are quite common in the study areas and probably act as reservoirs in the transmission of the parasite, to humans and to dogs, by sandflies.

Human visceral leishmaniasis (VL) caused by *Leishmania infantum* is endemic in the countries that lie around the Mediterranean basin. Over the last 15 years, the incidence of Mediterranean VL has increased, largely because of *Leishmania*–HIV co-infection (Ashford, 2000; WHO, 2000). In contrast to VL caused by *L. donovani*, the transmission cycle of *L. infantum* is thought to be zoonotic, with canids — often domestic dogs (*Canis familiaris*) — acting as the principal hosts (Quinnell et al., 1997). In humans, *L. infantum* causes a wide spectrum of clinical manifestations, from asymptomatic or oligosymptomatic infection to acute or chronic disease (D’Oliveira et al., 1997).

In Iran, the main endemic areas for VL are Ardabil and East Azarbaijan, in the north–west, and the provinces of Fars and Bushehr, in the south (Mohebali et al., 2005). In the last few decades, epidemiological reports from Iran (Mohebali et al., 2005) and Brazil (Badaró et al., 1986) have shown that...
asymptomatic infection with *L. infantum* (=*L. chagasi*) occurs more frequently than was previously believed, and that a considerable number of infected individuals only develop a mild, oligosymptomatic disease that resolves without treatment. Costa et al. (2002) suggested that those with asymptomatic infection can act as reservoirs for VL.

The unpublished records of the provincial health service in the Iranian province of Fars indicate a worrying recent increase in the incidence of VL in many districts (unpubl. obs.). The aim of the present study was to explore the prevalence of asymptomatic human infection with *L. infantum* in the north–east and south–west of the province, by using direct agglutination tests (DAT) and PCR-based assays to check blood samples from asymptomatic subjects for evidence of recent or current infection.

**SUBJECTS AND METHODS**

**Study Area**

The study was conducted over a 2-year period (2004–2006) in the north–west (Nourabad-Mamasani district) and south–east (Darab district) of Fars province. VL is endemic in Fars, especially among the nomadic tribes (Asgari et al., 2006; Fakhar et al., 2006). Nourabad-Mamasani (52°7′E, 30°42′N) has an annual rainfall of 400–600 mm and lies at 800–2000 m above sea level whereas Darab (55°27′E, 28°57′N) is relatively dry (annual rainfall=200–300 mm) and less mountainous (400–1000 m above sea level). The mean annual temperature in both areas is similar, at about 21–22°C (Anon., 2003). The 802 subjects for the present study were selected at random from the apparently healthy adults and children living in villages and nomadic communities where cases of human VL had previously been identified.

**Sample Collection and Testing**

A 2-ml sample of venous blood was collected from each subject, into an EDTA-coated tube, and then centrifuged at 1000 × *g* for 5 min so that the plasma and buffy coat could be separated off and stored, separately, in 1.5-ml tubes at -20°C, until they could be tested (see below).

The plasma samples were tested in DAT (Harith et al., 1989), with Iranian *L. infantum* of the LON-49 zymodeme as the antigen. A sample giving a titre of 1:3200 or higher was considered seropositive.

The DNA in each buffy coat (of about 100 μl) was extracted using phenol:chloroform and ethanol precipitation (Motazedian et al., 2002). It was re-suspended in 100 μl sterile distilled water and stored at 4°C until it could be tested, in a PCR, for a sequence from the kinetoplast DNA (kDNA) of *Leishmania*. The primers used — RV1 (5′-CTT TTC TGG TCC CGC GGG TAG G-3′) and RV2 (5′-CCA CCT GGC CTA TTT TAC ACC A-3′) — amplify a 145-bp sequence from the LT1 fragment of the parasites’ kDNA minicircles (Ravel et al., 1995; Le Fichoux et al., 1999). Each 50-μl reaction mixture contained 20 μM of each deoxynucleoside triphosphate (dNTP), 3 mM MgCl₂, 1.5 U Taq polymerase (Sina Gene, Tehran), 50 pmol of each primer, and 5 μl DNA, in PCR buffer (Boehringer Mannheim, Mannheim, Germany). Each reaction mixture was overlaid with mineral oil before being transferred to a CG1-96 thermocycler (Corbett Research, Sydney, Australia) set to give 5 min at 94 °C followed by 35 cycles, each of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, and then a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in a 1.5%-agarose gel, stained with ethidium bromide, visualized under ultra-violet trans-illumination, and sized by comparison with a 100-bp ‘ladder’. Each sample found PCR-positive for leishmanial DNA was then investigated using the PCR described by Geramizadeh et al. (2006), which is based on the species-specific primers LIN4 and LIN17, to confirm that the DNA detected was that of *L. infantum*. 
RESULTS

Subjects
Of the 802 subjects (404 males and 398 females) investigated, 426 came from the north–west of Fars province and the other 376 from the south–east. Most (601) came from settled villages but the rest were from nomadic tribes. Overall, 39%, 44% and 17% were aged <5, 5–10 and >10 years, respectively. Ten (1.2%) of the subjects had suffered from VL, all at least 1 year before their blood was sampled for the present study.

Serological Screening
Eight (1.9%) of the subjects from the north–west of Fars and five (1.3%) of those from the south–east were found seropositive (see Table). Although a male subject was more likely to be found seropositive than a female one, the difference was not statistically significant (2.0% v. 1.3%; P=0.57). All 13 seropositive cases were children aged <10 years and eight of them came from nomadic tribes.

PCR Screening
Overall, 100 (12.5%) of the subjects — 16.0% of those from the north–west of Fars and 8.5% of those from the south–east (see Table) — were found PCR-positive in the Leishmania-specific PCR based on the RV1/RV2 primer set. All 100 were subsequently found positive for L. infantum DNA, using the PCR described by Geramizadeh et al. (2006), indicating that they probably had L. infantum amastigotes in their peripheral blood. Although a male subject was more likely to be found PCR-positive than a female subject, the difference was not statistically significant (13.9% v. 11.1%; P=0.33). Seventy-six (76%) of the PCR-positives were aged 5–10 or >10 years, 18 (18%) each belonged to the family of a known VL case, and six (6%) had had VL themselves (in each case, more than a year before the blood sampling for the present study). Only four (4%) of the PCR-positive subjects were also found seropositive. None of the PCR-positive cases complained of any illness at the time their blood samples were collected, all 100 then appearing entirely asymptomatic. Although 21 of the PCR-positive cases have now been followed-up for at least 18 months, none has yet shown any symptoms of VL. When the 21 were re-tested 18 months after the present study, only one remained PCR-positive for L. infantum DNA.

DISCUSSION

Thirteen (1.6%) of the 802 subjects investigated in the present study were found seropositive in a DAT based on L. infantum antigens. DAT is a simple, cost-effective and field-applicable method that is often recommended for the field diagnosis of VL in endemic regions and frequently used for that purpose in Iran (Mikaeili et al., 2007). Most epidemiological studies on VL are still performed using DAT or another immunological method, either for the detection of Leishmania-specific antibodies or for the detection of delayed-type hypersensitivity (DTH) to leishmanial antigens. Serological methods are simple, relatively non-invasive procedures for detecting active leishmaniasis, when large amounts of specific antibodies are present. They are not so useful, however, in the detection of subclinical infections because most such tests have low sensitivity when the titres of

<table>
<thead>
<tr>
<th>Area</th>
<th>Investigated</th>
<th>DAT-positive</th>
<th>PCR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>North–west</td>
<td>426</td>
<td>8 (1.9)</td>
<td>68 (16.0)</td>
</tr>
<tr>
<td>South–east</td>
<td>376</td>
<td>5 (1.3)</td>
<td>32 (8.5)</td>
</tr>
<tr>
<td>Both</td>
<td>802</td>
<td>13 (1.6)</td>
<td>100 (12.5)</td>
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Leishmania-specific antibodies are low (Badaro et al., 1983; Mary et al., 1992). In epidemiological studies, the extent of leishmanial infection is traditionally determined by quantitative serological tests, such as immunofluorescent antibody tests (IFAT), ELISA, DAT or DTH tests. Among the serological tests usually used, western blotting (WB) offers relatively high sensitivity when the titres of specific antibodies are low, and has been used successfully in epidemiological surveys in several endemic areas (Mary et al., 1992; Marty et al., 1994; Cardenosa et al., 1995; Le Fichoux et al., 1999). Unfortunately, positivity in antibody-detection or DTH tests does not necessarily indicate active infection, as it may be the result of exposure to Leishmania months or even years previously (Liew and O’Donnell, 1993).

An unfortunate problem in detecting cryptic L. infantum infections is the lack of an appropriate ‘gold standard’ technique (Kemp, 2000). De Gouveia Viana et al. (2008) showed that it was possible to use a combination of several diagnostic methods (IFAT, rk39 and L. chagasi ELISA, intradermal skin tests and PCR) to identify most asymptomatic L. chagasi carriers who present with modulated cytokine profiles. Although they found that PCR (29.7%) gave higher sensitivity than the other methods, they suggested that the recognition of a large proportion of asymptomatic carriers is facilitated when more than one diagnostic method is performed. Their low-level humoral immunoresponse usually means that those with asymptomatic infection have titres of Leishmania-specific antibodies that fall at or below the threshold for positivity in serodiagnostic tests. Such individuals do, however, often have sufficient amastigotes in their peripheral blood for leishmanial DNA to be detected using small samples and PCR.

PCR-based assays can be performed on any biological sample (skin, bone marrow, blood etc), with high sensitivity. Such assays have now been used several times to detect L. infantum DNA in the peripheral blood of symptomatic and asymptomatic subjects (Mathis and Deplazes, 1995; Le Fichoux et al., 1999; Lachaud et al., 2000; Otero et al., 2000; Cruz et al., 2002; Fisa et al., 2002). In the present study, the use of PCR revealed the kDNA of L. infantum in the blood of 100 (12.5%) of the asymptomatic subjects. In comparison, the results of the DAT revealed far fewer positives (because most asymptomatic subjects have low or undetectable levels of IgG antibodies to Leishmania) and, if the results of the PCR are assumed to have indicated all the current infections, most (nine) of the 13 DAT-positives that were detected did not have current infections. The insensitivity of DAT in the detection of asymptomatic or cryptic leishmanial infections has been reported before, for several members of the L. donovani complex, in both the Old and New Worlds (Le Fichoux et al., 1999; Costa et al., 2002; Riera et al., 2004).

It is unclear how long the asymptomatic human carriers of L. infantum carry amastigotes in their peripheral blood (and thus, presumably, remain infectious to any potential vector that feeds on them), and even whether such parasitaemia is continuous (over some unknown time) or periodic (Le Fichoux et al., 1999). In the present study, one subject appeared to remain parasitaemic but asymptomatic for at least 18 months (although the possibility that this subject was re-infected during follow-up cannot be excluded). In an endemic area of Spain, 50% of the asymptomatic blood donors found to be carrying L. infantum by Riera et al. (2004) were again found positive when re-tested 1 year later.

Unlike L. donovani, which is anthropozoonotic, L. infantum is generally anthropozoonotic, being mostly transmitted to humans from dogs, by sandflies. Deane and Deane (1962) showed that patients with active VL could infect the sandfly vector, however, and Costa et al. (2000, 2002) confirmed the possibility that healthy human carriers may act as reservoirs, since sandflies feeding on
such carriers can be infected in the process. Sandflies have certainly been infected by feeding on HIV-positive individuals co-infected with L. infantum, indicating that a new, natural, anthroponotic cycle may be developing as a result of the HIV pandemic (Molina et al., 1994). Asymptomatic carriers also pose a threat to blood banks in those areas were L. infantum is endemic, since the parasite can be transmitted by transfusion. Although asymptomatic carriers of L. infantum are probably less infective to sandflies than individuals with active VL, they clearly pose a problem to VL-control programmes, especially when, as in Fars province (present study), they are fairly common. Their relative contribution to the incidence of VL may be particularly high in endemic areas with low densities of dogs. In the communities of southern and south–western Iran, for example, there are relatively few dogs and only about 4%–6% of those present are seropositive for L. infantum (Mohebali et al., 2001; Asgari et al., 2006). In north–western Iran, in contrast, there are relatively large numbers of dogs (seven for every 100 humans) and >14% of those investigated have been found seropositive (Mohebali et al., 2005).

In conclusion, the results of the present, preliminary study indicate that many asymptomatic human carriers of L. infantum live in the endemic regions of southern Iran and potentially act as reservoirs of infection. Such carriers must be carefully considered by those planning VL-control programmes in the region. Large-scale studies of the problem posed by asymptomatic carriers in Iran are clearly warranted, and the investigation of other potential reservoirs, such as rodents and cats, must continue. All blood donors in the VL-endemic regions of Iran should be screened, in PCR-based assays, for Leishmania infection and/or the leucocytes from all samples of donated blood should be removed before the blood is transfused. The increasing numbers of immunocompromised and immunodeficient people living around the Mediterranean region may further exacerbate the problem posed by the anthroponotic transmission of L. infantum.

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