Full Length Research Paper

Molecular and seroepidemiological study of *Leishmania infantum* infection among humans, dogs and wild canines from Azarshahr (new endemic focus), Iran

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An epidemiological survey of visceral leishmaniosis among domestic and wild canines as well people was carried out in the new endemic focus of Azarshahr (northwest of Iran) to assess the distribution of the disease and the possible association between infection in dogs and people during 2008-2009. At first using direct agglutination test (DAT), an examination of visceral leishmaniosis was carried out among 1500 Azarshahr district residents children in the age from 6 month to 10 years old. Then, one hundred and twenty domestic dogs, ten jackals, ten foxes and ten wolves from the district were examined for clinical and serological signs of canine leishmaniosis. The sera were evaluated by DAT then parasitological study was performed for all seropositive animals. Human sera samples, the overall seropositive rate of disease was 1.3% (19/1500) (95% CI, 0.7 to 1.8) by DAT (1:3200 and above). Data analysis revealed that serological positivity was statistically associated with age groups ($\chi^2 = 7.46, P = 0.023$). Of the 120 serum sampled collected from 120 domestic and stray dogs, 19.2% (23/120) (95% CI, 12.1-26.2) were positive by DAT (1:320 and above). No statistically significant difference was found between male (17.9%) and female (37.5%) seroprevalence ($P=0.172$). Also, no statistically significant difference was found between serological positivity and age groups ($P=0.107$). Serology and parasitology tests that were performed in 30 wild canines showed 10% of these animals were infected by *Leishmania infantum*. Amastigotes of Leishmania were observed in 14 seropositive dogs after dissection and parasitological examinations. A polymerase chain reaction (PCR) genotyping tool was developed and used to identification of species of the seropositive dog isolates. These isolates were identified as *L. infantum*. The present study provides, for the first time, information on the distribution of the visceral leishmaniosis in Azarshahr county of Iran, as a new endemic focus of disease.

Key words: Visceral leishmaniosis, Seroprevalence, Polymerase chain reaction, *Leishmania infantum*, Azarshahr.

INTRODUCTION

Infection with the protozoan parasite *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) can lead to three different forms of disease: cutaneous, mucocutaneous, and visceral leishmaniasis (VL). Visceral leishmaniasis, also called Kala-azar, is the most severe form of leishmaniasis caused by the *Leishmania donovani* complex species of *Leishmania* (*L. donovani, Leishmania donovani infantum,* and *Leishmania donovani chagasi*) (Farajnia et al; 2008). Visceral leishmaniasis, is one of the most important parasitic diseases worldwide and remains a challenge to public health in at least 65 countries (Fallah et al., 2011; Khanmohammadi et al., 2010; Moreno et al., 2002; Desjeux et al., 2004). This disease is a zoonosis caused by *Leishmania* (*Leishmania*) *infantum* (Kinetoplastida: Trypanosomatidae). This organism is an obligatory intracellular protozoon that is transmitted to a

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susceptible vertebrate host an infected female phlebotomine sand fly (Diptera: Psychodidae) is feeding (Fallah et al., 2011; Khanmohammadi et al., 2010). Domestic dogs (Canis familiaris) are principal reservoir hosts of Mediterranean type of visceral leishmaniasis. These parasites cause a wide spectrum of clinical manifestations in humans and it is estimated that the annual occurrence of human visceral leishmaniosis (HVL) cases worldwide is 500,000 (WHO, 2008). VL is dispersly endemic in several provinces of Iran including East Azerbaijan, Ardabil, Fars and Gom (Mohebali et al; 2005). In other Provinces of the Iran, the disease has been reported in sporadic form (Edrissian et al., 1988; Fallah et al., 2001). Although, dogs are the main reservoirs for human infection to VL (Mohebali et al., 2006), wild carnivores such as jackals (Canis aureus) and foxes (Vulpes vulpes) have been also found infected with Leishmania spp. These animals are assumed to be reservoirs for parasites, particularly in regions where sporadic cases of disease have been reported. (khanmohammadi et al., 201; Mohebali et al., 2006). The objectives of this study were to determine the sero-prevalence of human and canine visceral leishmaniosis in Azarshahr county of Iran as a new endemic focus of VL, and to identify the natural reservoir of human kala-azar in this area. Focus was particularly on dogs, with a view to determining infection rates among them and their role in transmission of the disease to humans.

MATERIALS AND METHODS

Study area

The present cross-sectional study was carried out in Azarshahr (latitude 37° 43' 17'' N, longitude 46° 2' 59'' E, altitude 1367 m), a municipality located on the south-east of Urmieh sea and have 45 km distance from Tabriz city (East Azerbaijan's capital). Azarshahr had a population of about 114918 inhabitants (Figure 1). The suspected vectors found in the focus include Phlebotomus kandelakii and Phlebotomus perfiliewi (Rassi et al., 2005).

Sample and data collection

This study was carried out over a period of 15 months during 2008 - 2009 on the 15 villages of Azarshahr. Blood sampling from 10% of children ≤10 years old was done. All study participants completed an epidemiological questionnaire and gave informed consent to participate in the study. Basic demographic data, including age, sex, address, and altitude of the house, were obtained, as well as potential risks factors for kala-azar. A 5 ml blood was taken and serum separated and stored at -20°C until serological analyses. Also, a total of 120 domestic and stray dogs (males and females, different ages) were selected by simple random sampling in the present study. Dogs were captured in fifteen areas of Azarshahr. Feral dogs are abundant in the study areas, living close to man and wandering around farm buildings and houses, where they are considered to be a nuisance by the local population. Obtained Dog’s age by dental formula and dental erosions should be considered. The minimum sample size required (n=120) was calculated considering the dog population. 3 to 5 ml blood samples from all dogs were collected. Additionally, samples were taken from 30 wild canines in this area: 10 jackals (C. aureus), 10 foxes (V. vulpes) and 10 wolves (Canis lupus). Samples were centrifuged at 800 g for 5 to10 min. Then, the obtained sera were frozen at -20°C until it could be tested for anti-Leishmania antibodies. After sample collection, a questionnaire was used to collect information regarding the age, gender, and district of origin of each dog. All of the sera (human and dog samples) were tested for kala-azar using the DAT, according to the methods described by Harith et al. (1989).

Serological test

Promastigotes of Leishmania were mass produced in RPMI
Parasitological study

The dogs were examined externally for signs of infection, and then dissected for the following investigations: Smears were prepared from any skin lesion detected, and from the livers and spleen of each dog. These were fixed with methanol, stained with Giemsa stain and examined microscopically for the presence of amastigotes. Biopsy specimens were collected aseptically from spleen and liver, then cultured into biphasic culture media (prepared from nutrient agar containing 10% whole rabbit blood overlaid with liver infusion tryptose broth (LIT) containing 100 U/ml penicillin G and 1 μg/ml streptomycin). The inoculation cultures were incubated at 21°C for up to 6 weeks and examined weekly for the presence of promastigotes.

PCR amplification

The promastigotes were isolated from the RPMI 1640 media and tested using PCR. To prepare template DNA from Leishmania cultures, the pellet was suspended in 10 to 20 fold of disruption buffer containing 100 mM Tris, 10 mM EDTA, pH 8.0, 2% SDS and 2% 2-mercaptoethanol which was added before use. The suspension was incubated at 65°C for at least 30 min and cooled to room temperature before precipitating with half volume of ice-cold 3.0 M KAc, pH 5.5. The supernatant was then precipitated by an equal volume of ice-cold isopropanol at ~15k/15 min/4°C. The pellet was rinsed with 70% ethanol and air-dried before re suspending in sterile water. To prepare template from biopsy samples of spleen, 50 to 500 mg of infected tissue was homogenized in a proper glass homogenizer or ground in a micro fuge tube by a heat-sealed blue tip. After adding 2 to 3 fold disruption buffers, DNA preparation was followed according to the above procedure. Specific primers were designed using a 778 bp partial sequence file of L. infantum minicircle DNA (gi|2558170|gb|AF027578.1|) through Blast search and analyzed by Oligo Tech version 1.0. These included homologous 5'-CCC AAA CTT TTC TGC TCC TTC G-3', positioned at 24-45 and complementary 5'-CCA CGA CGC ATC CAA TCC AA-3’, positioned at 360-341 flanking a 337-bp fragment. Reaction cocktail contained 1.0x PCR buffer, 2.0 mM MgCl2, 0.2 mM dNTPs, 0.5 mM each primers, 1-2 units of recombinant Taq DNA polymerase (Sina gen, Iran).

The final volume was adjusted to 20 or 25 μl per reaction including 2-5 μl of template. The reaction conditions included lid temperature of 105°C along with 4 min of initial denaturation at 95°C followed by 35 cycles of 95°C/30 s, 65°C/30 s and 72°C/1.0 min. The reactions were ended by additional extension at 72°C/10 min. PCR products were separated by horizontal electrophoresis in 1 to 1.5% agarose gel, along with a molecular weight marker. After staining the gel in ethidium bromide solution, the photograph was taken under UV illumination (Farajnia et al., 2004; Noyes et al., 1996).

Statistical analysis

Chi-squared ($\chi^2$) was used to compare seroprevalence rates (SPR) relative to gender and age. The differences were considered statistically significant when probability (P) value $\leq$0.05. The 95% confidence intervals (95% CI) of seroprevalence rates were calculated. Statistical analysis was performed using Epi Info software, version 6.

RESULTS

Out of the 1500 human sera examined from the fifteen localities, 19 (1.3%) (95% confidence interval: 0.7-1.8) sera showed a reaction equal to or greater than 1:320. All of the cases were children under 10 years. Data analysis revealed that serological positivity was statistically associated with age groups ($\chi^2$ = 7.46, P = 0.023). The highest proportion of positive cases was in children < 1 year old and the rate among children decreased with age. Seroprevalence rates in relation to data are shown in Table 1. About 1.3% of the seropositive individuals were males and 1.2% females, giving a male to female ratio of 1.08:1. One hundred and twenty dogs from the municipality of Azarshahr were examined. Anti-Leishmania specific antibodies, using the cut-off value of 1:320 and above were detected in male and female dogs. 112(93.3%) of the dogs were males and 8(6.6%) were females. The sero-prevalence values among male and female animals were 17.9 and 37.5%, respectively (Table 2). No significant difference was found in the seroprevalence rates between canine Leishmania infection and gender ($\chi^2$ = 1.86, P = 0.172).

Of the 120 samples, 23 tested positive to anti-Leishmania antibodies, confirming previous exposure to Leishmania parasites. Referring to animal age groups, the highest sero-prevalence (44.4%) was found in dogs greater than 8-year-old and the lowest values (15.3%) in dogs less than 3-year-old. No significant difference was found in the seroprevalence rates between canine Leishmania infection and age ($\chi^2$ = 4.46, P = 0.107). Six of the 23 sero-positive dogs showed at least one clinical sign including lymphadenopathy, hair shedding, dermal lesions, onychogriposis and cachexia which may be regarded as symptomatic form of the disease, while the other 17 dogs (73.9%) were asymptomatic.

The proportion of symptomatic infections in sero-positive dogs was 26% (6/23) and overall prevalence of CVL was 5% (6/120) (Table 3). Of 30 wild canines captured in Azarshahr County, 5 cases were positive to anti-Leishmania antibodies confirming previous exposure to Leishmania parasites. Results of parasitological and serological tests in the wild canines that were captured around the villages from endemic foci of HVL in Azarshahr county of Iran are shown in Table 3. Visceral leishmaniasis in a wolf from Azrashahr county of Iran was
Table 1. Seroprevalence rates of anti-Leishmania antibodies among humans from Azarshahr in relation to epidemiological data.

<table>
<thead>
<tr>
<th>Human's data</th>
<th>n</th>
<th>Direct agglutination test</th>
<th>Seroprevalence rates</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Suspected</td>
<td>Negative</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>≤1</td>
<td>176</td>
<td>6</td>
<td>2</td>
<td>168</td>
</tr>
<tr>
<td>2-5</td>
<td>790</td>
<td>7</td>
<td>16</td>
<td>767</td>
</tr>
<tr>
<td>6-10</td>
<td>534</td>
<td>6</td>
<td>14</td>
<td>514</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>757</td>
<td>10</td>
<td>16</td>
<td>731</td>
</tr>
<tr>
<td>Female</td>
<td>743</td>
<td>9</td>
<td>16</td>
<td>718</td>
</tr>
<tr>
<td>Total</td>
<td>1500</td>
<td>19</td>
<td>32</td>
<td>1449</td>
</tr>
</tbody>
</table>

a Titer of antibody ≥ 1:3200 b Titer of antibody between 1:800-1:1600 c Titer of antibody lower than 1:800 d 95% confidence intervals are in brackets.

Table 2. Sero-prevalence of canine Leishmania infection by gender and age groups in Azarshahr district of Iran.

<table>
<thead>
<tr>
<th>Dog’s data</th>
<th>n</th>
<th>Direct agglutination test</th>
<th>Sero-prevalence rates</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td>a</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>0-3</td>
<td>72</td>
<td>11</td>
<td>15.3 (6.9-23.5)</td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>39</td>
<td>8</td>
<td>20.5 (7.8-33.1)</td>
<td></td>
</tr>
<tr>
<td>≥ 8</td>
<td>9</td>
<td>4</td>
<td>44.4 (11.9-76.9)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>112</td>
<td>20</td>
<td>17.9 (10.7-24.9)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>3</td>
<td>37.5 (3.9-71.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>23</td>
<td>19.2 (12.1-26.2)</td>
<td></td>
</tr>
</tbody>
</table>

a Titer of antibody ≥ 1:320 b 95% confidence intervals are in brackets.

Table 3. Results of parasitological and serological tests in wild canines captured around the villages from endemic foci of HVL in Azarshahr county of Iran.

<table>
<thead>
<tr>
<th>Type of animal</th>
<th>No. of animal</th>
<th>Parasitological positive</th>
<th>Serological positive</th>
<th>Leishmania spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxes</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>L. infantum</td>
</tr>
<tr>
<td>Jackals</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>L. infantum</td>
</tr>
<tr>
<td>Wolves</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>L. infantum</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>5</td>
<td>5</td>
<td>L. infantum</td>
</tr>
</tbody>
</table>

The prevalence of Kala-azar in this area, Among the specific serological tests, direct agglutination test (DAT) was found to be more specific (72 to 100%), sensitive (92 to 100%), and practical particularly in endemic area of the world (Edrissian et al., 1996). The results of the DAT for detection of L. infantum infection in humans and dogs were excellent. Therefore, we used of the DAT for the determination of seroprevalence of human and canine Leishmania infection. In this study, serological surveys using DAT analysis showed 1.9% of the population in the study areas had anti-Leishmania antibodies in titres of ≥ 1:3200, all of them children under 10 years old. These were lower than those reported by Mohebali et al. (2001)
in Bushehr (3.4% n =1496). The peak number of cases was in children ≤ 1 year old and the seropositive rate decreased with increasing of age of the children. Prior studies in Iran have shown a seropositive rate of about 50% in the age group 1 to 2 years and 96% of seropositive cases in children up to 8 years old (Soleimanzadeh et al., 1993; Edrissian et al., 1999). About 1.3% of seropositive individuals were males and 1.2% females. No statistically different (P<0.05) was observed between them. Previous DAT serological survey of VL in Azarshahr by Mirsamadi et al. (2003) was showed that (1.9% n = 1252) of the collected specimens were anti-\textit{Leishmania} antibody positive with ≥ 1:3200 titers and 1.2% with 1:1600 titers were suspicious. The overall prevalence among dogs in our study (19.2%) was higher than that reported in other endemic areas of East Azerbaijan Province by Mohebali et al. (2005). Of the 120 serum sampled collected from stray and domestic dogs, 19.2% were anti-\textit{Leishmania} antibody positive. No statistical differences were found among canine \textit{Leishmania} infection with regard to gender in our study.

Similar results were found by Abranches et al. (1992) in Portugal, Sidersis et al. (1996); Bokai et al. (1998); Mohebali et al. (2005); Fakhar et al. (2004); Gavagni et al. (2002) and Moshef et al. (2008) in Iran. In the current study, we found canine \textit{Leishmania} infection mostly in younger dogs (≤3 years). There seems to be an increased sero-prevalence of the infection associated with animal age. Regarding to highly \textit{L. infantum} infection of domestic dogs, similarity between \textit{L. infantum} isolated from infected humans and domestic dogs in the Azarshahr county, domestic dogs seem to be the main reservoirs of infection in endemic foci of VL focusing in Azarshahr county of Iran. From a worldwide public health point of view zoonotic visceral leishmaniasis (ZVL) is one of the most important parasitic diseases emerging in recent years (WHO, 1990). On the basis of our results, this endemic focus of VL in East Azerbaijan province is similar to the endemic foci of Kalaybar and Ahar and Sarab in this province, Jahrum, Ghar and Firooz Abad in Fars province, Meshkin-shahr (Moshef et al., 2008; Mohebali et al., 2005; Fallah et al; 2011) in Ardabil province and other endemic areas of the Islamic Republic of Iran where the kala azar is of the Mediterranean type (WHO, 1990).

The human serological survey indicates the possible existence of sub clinical infection, which may thus serve as a reservoir of infection. In spite of the small sample size of the canine survey, the existence of a reservoir of infection was confirmed and suggests an alarming percentage higher than that of other some endemic foci, and far higher than that of the Mediterranean region. Kala-azar should be made a notifiable disease in Azarshahr and further field studies should be organized to define more clearly the foci of human kala-azar in Azar-shahr, and to trace and confirm possible reservoirs among canines and other wild animals, as well as to study vectors. The health authorities should take the following measures: one case finding, including a search for overt cases of kala-azar by spleen puncture and serology to detect recent infections which may or may not become overt; and control of the human reservoir, which is especially important in epidemic situations. Case finding and treatment form the major component of any control scheme. Also, the canine reservoir may be controlled by reduction of the stray dog population (Tesh, 1995).

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