Serological and entomological survey of zoonotic visceral leishmaniasis in Denizli Province, Aegean Region, Turkey

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INTRODUCTION

In Turkey, human visceral leishmaniasis (HVL) and canine leishmaniasis (CanL), caused by Leishmania (L.) infantum MON-1, are endemic along the Aegean and Mediterranean coasts and occur sporadically in other regions (Ozensoy et al., 1998). Anthroponotic cutaneous leishmaniasis (ACL) caused by L. tropica is still highly endemic in the South and Southeast regions. In Eastern Part of Mediterranean Region, L. infantum is also responsible for cutaneous leishmaniasis (CL) besides L. tropica (Serin et al., 2005). The number of epidemiological studies and retrospective case reports on childhood HVL from different provinces of Turkey has increased in recent years (Ozensoy et al., 1998, Ertabaklar et al., 2005, Saltoglu et al., 2004). However, there are few case reports on HVL in immunocompromised adults (Ozensoy et al., 1998, Saltoglu et al., 2004, Buyukasik et al., 1998, Kose et al., 2004 and 2005).
Dogs are the main reservoir of \textit{L. infantum} and previous studies carried out in some provinces located in western Turkey reported a seroprevalence ranging between 0.72\% and 33.30\% (Ozensoy et al., 1998, Ozbel et al., 2000, Ozensoy Töz et al., 2005).

Several sand fly species, including \textit{P. ariasi}, \textit{P. perniciosus}, \textit{P. neglectus} and \textit{P. perfiliewi} were reported as proven vectors of \textit{L. infantum} in the Mediterranean Basin (Killick-Kendrick, 1999). Previous studies on sand flies in Turkey have demonstrated the presence of 20 \textit{Phlebotomus} species (or subspecies recently raised to species) belonging to subgenera \textit{Adlerius}, \textit{Larroussi}, \textit{Paraphlebotomus} and \textit{Phlebotomus} (Houin et al., 1971, Yaman&Ozbel, 2004, Yaman&Dik, 2006). Nine of them are proven or could be probable vectors of the Old World leishmaniases (Killick-Kendrick, 1990).

According to the Turkish Ministry of Health official records, within a period of 5 years (2000-2004) a total of 127 HVL cases were reported in Turkey, 30.7\% (39/127) of which occurred in Aegean Region including Denizli province (available in http://www.saglik.gov.tr/extras/istatistikler/temel2004/index.htm). Between 1993 and 2000, 14 cases of HVL were diagnosed from Denizli province and 4 of them were adults without immunodeficiency. The adult cases had been diagnosed in 1999 and 2000. Because of previously reported adult HVL cases caused by \textit{Leishmania infantum} MON-1, we aimed to carry out a serological and entomological survey in Denizli province.

**MATERIALS AND METHODS**

**Study area**

The survey was carried out in Denizli province (latitude 37° 46’ 27N, longitude 29° 5’ 15E) located in the Southern Aegean Region of Turkey and it is a bridge between Aegean, Mediterranean and Central Anatolia Regions. The province is divided into 372 villages, belonging to 18 administrative districts covering in area of 11.868 km$^2$ and according to the 2000 census the total population of the province and villages was 850,029 and 432,346, respectively.

In Denizli province, the climate is between continental and Mediterranean, the mean minimum temperature is $\sim+5^\circ$C in January and the mean maximum temperature is $\sim+28^\circ$C in July. The average rainfall is 100 mm. People are mainly engaged in the production of tobacco, wheat, vegetables and cattle/sheep breeding. Some of the people are the forest workers. All villages have similar ecological properties like vegetation, soil type, etc. The houses and animal sheds are usually made of brick, mud, wood and/or stone. Most of the families own at least one dog. The four villages (Asagidagdere, Dereciftlik, Ortatepe, Demirciler) and one district (Gumuscay) where one HVL case was reported in each one were included in the study and (Table 1, Figure 1) the survey was carried out in two different periods, June 2001 and August 2002.

**Physical examination and sampling**

\textit{Human:} Blood samples were obtained from 329

<table>
<thead>
<tr>
<th>Village/District</th>
<th>Latitude/Longitude (DMS)</th>
<th>Altitude (m)</th>
<th>Population</th>
<th>Number of houses</th>
<th>Dogs sampled</th>
<th>Children sampled</th>
<th>Adults sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asagidagdere</td>
<td>37° 48’ 39N/29° 23’ 50E</td>
<td>621</td>
<td>643</td>
<td>125</td>
<td>41</td>
<td>80</td>
<td>169</td>
</tr>
<tr>
<td>Dereciftlik</td>
<td>37° 48’ 26N/29° 19’ 34E</td>
<td>572</td>
<td>766</td>
<td>152</td>
<td>28</td>
<td>46</td>
<td>49</td>
</tr>
<tr>
<td>Ortatepe</td>
<td>37° 22’ 60N/28° 46’ 60E</td>
<td>639</td>
<td>240</td>
<td>70</td>
<td>13</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>Demirciler</td>
<td>37° 26’ 21N/28° 50’ 43E</td>
<td>1067</td>
<td>620</td>
<td>125</td>
<td>40</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Gumuscay</td>
<td>37° 46’ 27N/29° 5’ 15E</td>
<td>428</td>
<td>7775</td>
<td>850</td>
<td>18</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>10.044</td>
<td>1.322</td>
<td>140</td>
<td>329</td>
<td>217</td>
</tr>
</tbody>
</table>
children, aged between 1 and 14 years old in five study sites (Table 1). In addition, a total of 217 adults were sampled from Asagidagdere and Dereciftlik villages only. The physical examination was carried out by authorized physicians and informed consent was obtained from all participants involved in the study.

Dogs: Blood was collected from 140 dogs (males and females, different ages and breeds, selected at random) and sera were obtained. All dogs sampled in the villages were owned and used primarily for hunting and/or guarding. Dogs were always kept outside the houses.

Popliteal lymph node aspirates were taken from 40 dogs (15 seropositive, 18 seronegative, 7 borderline) with enlarged lymph nodes. Smears were fixed in methanol for Giemsa staining. The aspirates were also inoculated in NNN culture to isolate the parasite. Parasitological examination was regarded as positive on the basis of smear and/or culture positivity.

The clinical status of each dog was carefully evaluated and clinical signs were separated into two groups as visceral (weight loss, enlarged lymph nodes, fever, epistaxis, fatigue, conjunctivitis) and cutaneous (dermatitis, hair loss, mouth and skin ulcers, onychogryphosis) signs (Ozensoy Toz et al., 2005) and a dog with only one or without any signs was intended as asymptomatic.

The instructions and policies of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) were applied in the study.

Serological tests

Antigens: The antigens for IFAT and ELISA were prepared using promastigotes from local *L. infantum* MON-1 stocks obtained by mass cultivation in RPMI-1640 containing 10% FCS.

IFAT: The IFAT was performed using standard procedures for human (de Korte et al., 1990) and dog sera (Abranches et al., 1991). Slides were stained with FITC-labeled anti-human IgG conjugate (BioMerieux 75692) for human sera and FITC labeled anti-dog IgG conjugate (Sigma, A9042) for dog sera. Titers ≥1:128 were scored as positive for both groups of sera while 1/64 titer was accepted as borderline in reference to relevant publications (Ozbel et al., 2000, Abranches et al., 1991).

Enzyme Lynked Immunosorbent Assay (ELISA): ELISA was performed as explained before (Ozensoy et al., 1998), except the whole lysate promastigote antigen, using 1/100 single serum dilution. The optical density was measured at 405 nm and the subjects were considered as positive when the OD was >0.300 which represents the mean plus 3xSD absorbencies obtained in sera from individuals accepting without exposure to *Leishmania*.

Sand fly collection

The sand fly collection was carried out in three villages (Asagidagdere, Dereciftlik and Ortatepe) at two different times, June 2001 and August 2002 using CDC miniature light traps. The male sand flies were immersed in 70% ethanol, and mounted in chloralhydrate medium for later identification. The midguts of 182 females were searched.
for live promastigotes in the field. After the examination, the cover slips were carefully removed and the slides were stained with Giemsa after drying for later checking the promastigotes under the high magnification (X1000). Each fly was identified to species, using the keys and descriptions of Theodor (1958), Perfil’ev (1968), Artemiev (1980), Lewis (1982) and Killick-Kendrick et al. (1991).

RESULTS

Human

Among 329 children, no specific clinical signs for HVL were found and there was no seropositive subject above cut-off titer. Five children were determined in 1/64 titer in IFAT. Among 217 adults, hepatomegaly was detected in one male and 6 villagers were determined in 1/64 titer in IFAT. However, two adults (one female and one male) from Asagidagdere village were found to be seropositive by IFAT and ELISA tests. rK39 rapid dipstick assay (InBios International, Inc., Seattle, USA) was also performed for only these two sera for serological confirmation. The seropositivity ratio among adults was detected as 0.09% (2/217) in the region.

The first serum sample was positive at 1/256 titer in IFAT and rK39 rapid test was strongly positive. She was 30 years old and had been followed up with a diagnosis of aplastic anemia in the state hospital for two years. Her hospital records pointed out pancytopenia, hepatosplenomegaly and weakness. The old bone marrow aspirates were examined retrospectively and she was diagnosed as visceral leishmaniasis by the presence of amastigotes. She was treated with AmBisome® and no relapse was reported after two years. The second serum sample was positive at 1/128 titer in IFAT and rK39 rapid test was weak positive. He was followed up physically and serologically for two years and did not show any clinical signs and the IFAT titers were decreased in a year. This might be evaluated as an asymptomatic case of HVL.

Dogs

The seroprevalence of CanL in five study sites ranged from 11.11% to 30.76%. The overall seroprevalence of CanL was 20.71% (29/140). Five out of 29 IFAT and/or smear positives were found to be negative by ELISA. The concordance between IFAT and ELISA was found to be 82.75%. All results obtained from dog sera were shown in Table 2 (75.8%) out of 29 dogs were seropositive; 13 (56.5%) out of 23 borderline dogs and 52 (59.09%) out of 88 seronegative dogs showed at least one clinical sign of CanL and 68.96% (20/29) of seropositive dogs were olygosymptomatic while 24.13% (7/29) of them were asymptomatic. Lymphadenopathy was the most frequent clinical sign followed by hair loss, conjunctivitis,

<table>
<thead>
<tr>
<th>Village/district</th>
<th>Dogs sampled</th>
<th>Seropositive Dogs</th>
<th>Borderline Dogs</th>
<th>Smear positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/ M/F</td>
<td>n (%)</td>
<td>IFAT titer ranges</td>
<td>n/ M/F</td>
</tr>
<tr>
<td>Asagidagdere</td>
<td>41/25/16</td>
<td>9 (21.95)</td>
<td>128-8000</td>
<td>9/21.95</td>
</tr>
<tr>
<td>Dereciftlik</td>
<td>28/23/5</td>
<td>7 (25.00)</td>
<td>128-8000</td>
<td>4/14.28</td>
</tr>
<tr>
<td>Ortatepe</td>
<td>13/11/2</td>
<td>4 (30.76)</td>
<td>128-8000</td>
<td>0/0</td>
</tr>
<tr>
<td>Demirciler</td>
<td>40/37/3</td>
<td>7 (17.50)</td>
<td>128-4096</td>
<td>10/25.00</td>
</tr>
<tr>
<td>Gumuscay</td>
<td>18/14/4</td>
<td>2 (11.11)</td>
<td>1024-4096</td>
<td>0/0</td>
</tr>
<tr>
<td>Total</td>
<td>140/110/30</td>
<td>29 (20.71)</td>
<td>-</td>
<td>23(16.42)</td>
</tr>
</tbody>
</table>

n: Number of dogs; M: Male; F: Female
onycogryphosis, skin lesions, weakness, scaling, epistaxis and fever, respectively. Epistaxis was only seen in seropositive dogs and evaluated as the most specific sign.

**Sand flies**
A total of 340 sand flies were collected from three villages in two different periods and eight species of *Phlebotomus* were identified as *P. neglectus*, *P. tobbi*, *P. papatasi*, *P. alexandri*, *P. sergenti*, *P. similis*, *P. simici* and *P. halepensis*. *P. neglectus* (43.52%) and *P. papatasi* (37.35%) were detected as dominant species in the province (Figure 2). During the study, more female specimens than male specimens were collected and the male/female ratio was found to be 1:1.15. No promastigotes were found after the dissection of 182 females.

The minimum and maximum temperatures were recorded as between 18°C and 32°C; 19 and 29°C; 19 and 32°C in the villages of Asagidagdere, Dereciftlik and Ortatepe, respectively. The humidity was noted between 45% and 60% during sand fly collection.

**DISCUSSION**

*L. infantum* is responsible for HVL and CanL, while *L. tropica* and *L. infantum* is responsible for CL in Turkey (Ozbel *et al.*, 2000, Serin *et al.*, 2005). So far, VL *Leishmania* strains isolated from patients or dogs in Turkey were identified as *L. infantum* MON-1 and MON-98 by isoenzyme analysis (Ozensoy Toz, unpublished data).

It is reported that Mediterranean HVL is not only a children’s disease and 12 adult HVL cases were observed between 1971 and 1980 in France and associated with anarcho fever, splenomegaly, anemia, etc. (Sebahoun *et al.*, 1980). Mediterranean HVL was accepted as infantile disease and adult VL cases were reported sporadically in the past, but HIV-VL co-infection is being seen more and more frequently in the Mediterranean Basin, especially in Spain, France and Italy (Desjeux, 2004).

Interestingly, in Italy, 12 (19%) out of 64 adult HIV-negative HVL cases had an underlying disease, and Liposomal amphotericin B is reported to be more convenient in adult cases than antimonials (Pagliano *et al.*, 2003). In Greece, 4 HIV-negative adult cases (between 25 and 40 years) out of 111 suspected cases from the native population and immigrants from South Albania were diagnosed as HVL and they were treated with antimonials successfully. In addition, 5 adult VL cases were determined in the screening of 1200 healthy people (Papadopoulou *et al.*, 2005).

So far, 9 adult HVL patients were reported between 1993 and 2005 from the Aegean (in Manisa province) (Ozensoy *et al.*, 1998, Kose *et al.*, 2004 and 2005) and Mediterranean Regions (Saltoglu *et al.*, 2004, Buyukasik *et al.*, 1998) of Turkey. In the present study, Denizli province was chosen as the study site because of the highest adult/children ratio (28.5%; 4/14) of HVL patients in 1993 and 2000. All 14 patients had been successfully treated with antimonials except two children who died after a period of antimonial ther-
apy with a secondary infection because of late diagnosis. During our study, another two adult patients were also diagnosed as HVL, one was acute and another was asymptomatic. Our results increased the ratio in the province from 28.5% to 42.8%. For determining the asymptomatic cases, an additional study was carried out using 82 sera by western blotting and 5 more asymptomatic carriers were detected in the study region (Sakru et al., 2007).

Seroepidemiological studies on human and canine leishmaniasis were previously carried out in different endemic regions in Turkey using IFAT, DAT and/or rK39 ELISA. The seroprevalence of CanL was reported between 0.72% and 33.30% in previous field studies in different regions (Ozensoy et al., 1998, Ozbel et al., 2000, Ertabaklar et al., 2005). The overall seroprevalence rate was determined as 20.71% in the present study while it changed from 11.11% to 30.76% in different locations. A pilot program for active surveillance among humans and dogs of VL in the endemic regions of Turkey like the present study area will be useful and reasonable like that performed in Italy (Gradoni et al., 1993).

The clinical signs of CanL were evaluated in depth at another endemic site in the Aegean region of Turkey and 23.7% of the dogs that had at least one sign of CanL were diagnosed as CanL by serological and/or parasitological methods. Epistaxis was found to be the least common but highly specific sign and was seen only in seropositive dogs (Ozensoy et al., 2005) as also recorded in the present study. According to our results, we can suggest that the grouping of clinical signs in the dog will be more helpful in the differential diagnosis.

Previous sand fly studies disclosed nine proven or probable vector species of the Old World leishmaniasis (Killick-Kendrick, 1990) in Turkey (Yaman&Ozbel, 2004). In three villages of the study area, sand fly collection was carried out for the first time and 8 Phlebotomus species were identified. Among them, P. neglectus, P. tobbi, P. alexandri which are proven or probable vectors of L. infantum have medical importance (Killick-Kendrick, 1999) for this province too. Among all Phlebotomus specimens collected, P. neglectus (43.52%) was the dominant species, followed by P. papatasii (37.35%) and P. tobbi (11.17%). These three most dominant species constituted 92.04% of the total sand flies collected throughout the study. Two Phlebotomus species, P. neglectus and P. tobbi belonging to Larroussius subgenus, constitute potential vectors of HVL and CanL in the study area.

The presence of P. neglectus in Northern Italy was shown first time in 1995 (Maroli et al., 1995). In addition to this paper, from a review of previous published papers, the author’s hypothesis was the western limit of P. neglectus maybe Northern Italy and the species had migrated from the East (Maroli et al., 2002). In the later studies in Albania (Velo et al., 2005) and Crotia (Bosni et al., 2006), P. neglectus was found as the most abundant species. The epidemiological studies on leishmaniasis carried out in Turkey showed that Turkey can play a role as a bridge for P. neglectus as well as other species from East to West. This hypothesis was supported by the detection of P. neglectus in the Eastern, Central Part (Ertabaklar et al., 2005) and Northwestern part of the Aegean cost of Turkey (Ozbel Y., unpublished data).

Our results and unpublished records showed that vector species and general Phlebotomus fauna of the Aegean Region of Turkey are very similar to the Greek Islands and mainland of Greece (Chaniotis et al., 1994, Iovic et al., 2007). In the present study, P. neglectus, a proven vector for L. infantum in Greece, was also found as the most abundant species (43.52%) for the first time in the present study region and in Anatolia. But because of promastigotes was not seen in dissected sand flies, we can only speculate about P. neglectus as a potential vector in Denizli province. The high seroprevalence of the dogs in all villages and the adult cases besides child patients were indicators of the potential high risk of the disease in the region.

Two species, P. sergenti and P. simillis, belonging to the subgenus Paraphlebotomus, are closely related species, but the molecular analyses indicated that P. simillis is closer to P. jacusieli than to P. sergenti. It was previously described that Turkey is the only country in which P. sergenti and P. simillis are present, but P. simillis should occur only west of Taurus and Antitaurus and P. sergenti only to the east (Depaquit et al., 2002). But both these species were found as sympatric in Central Anatolia where they were located in the west of Taurus and Antitaurus (Yaman&Dik, 2006). In the present study, we also noted that these two
species exist in our study area in the western part of Turkey. In conclusion, because of the detection of high ratio of CanL and the presence of a probable vector Phlebotomus species in thestudy area, we strongly advocate preventive measures and an extension of the study to the neighbouring provinces to have more epidemiological data on the disease in Southwestern Turkey. These studies will also yield more information on the role of Anatolia in the distribution of sand fly species from east to west.

ACKNOWLEDGEMENTS
We thank to Dr. Ismail Sancak (Director of Infectious Diseases of the Branch of Ministry of Health, Denizli) for helping us during the field work. This work received financial support from Ege University Funds (Project No. 2000 TIP 016).

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