

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

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## SUMMARY

A cross-sectional seroepidemiological survey of leishmaniasis was carried out among children and adults from four villages and one district of Denizli province located in the Southern Aegean Region of Turkey where 14 human visceral leishmaniasis (HVL) cases including 4 adults were reported between 1993 and 2000.

Blood samples were taken from 329 children, 217 adults and 140 dogs and a physical examination was also done. Indirect fluorescent antibody test and enzyme linked immunosorbent assay were performed for all sera.

All 329 sera collected from children were found to be negative while 2 (0.09%) out of 217 adult sera were found to be seropositive. One seropositive adult patient was confirmed parasitologically as HVL after bone marrow aspiration and treated with AmBisome<sup>®</sup> while the other was followed only serologically because of the absence of symptoms. The overall canine leishmaniasis seroprevalence was found to be 20.7%.

Sand flies were collected using CDC light traps in three out of five study sites and midguts of females were checked for promastigotes after dissection/identification. Eight *Phlebotomus* species were found in the region. *Phlebotomus neglectus* and *P. papatasi* were determined as dominant species with the ratio of 43.52% and 37.35%, respectively. No promastigotes were found in the midgut specimens. In addition, the results showed the presence of vector sand fly species, as well as a high seroprevalence of anti-*Leishmania* antibodies among dogs from rural and a suburban area of Denizli province with a large proportion of asymptomatic seropositive dogs.

**KEY WORDS:** Leishmaniasis, Visceral, Human, Canine, Sand flies, Western 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 leishmania-

sis (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).

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Dogs are the main reservoir of *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 Toz *et al.*, 2005).

Several sand fly species, including *P. ariasi*, *P. perniciosus*, *P. neglectus* and *P. perfiliewi* were reported as proven vectors of *L. infantum* in the Mediterranean Basin (Killick-Kendrick, 1999). Previous studies on sand flies in Turkey have demonstrated the presence of 20 *Phlebotomus* species (or subspecies recently raised to species) belonging to subgenera *Adlerius*, *Larrousius*, *Paraphlebotomus* and *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 *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<sup>2</sup> 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 ~+5°C in January and the mean maximum temperature is ~+28°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 (Gumusgay) 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

*Human:* Blood samples were obtained from 329

TABLE 1 - Some characteristics of the five study sites in Denizli province, including the number of samples of human and dogs.

Village/District	Latitude/ Longitude (DMS)	Altitude (m)	Population	Number of houses	Dogs sampled	Children sampled	Adults sampled
Asagidagdere	37° 48' 39N/29° 23' 50E	621	643	125	41	80	169
Dereciftlik	37° 48' 26N/29° 19' 34E	572	766	152	28	46	49
Ortatepe	37° 22' 60N/28° 46' 60E	639	240	70	13	52	0
Demirciler	37° 26' 21N/28° 50' 43E	1067	620	125	40	56	0
Gumusgay	37° 46' 27N/29° 5' 15E	428	7775	850	18	95	0
Total	-	-	10.044	1.322	140	329	217



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 titers 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 oligosymptomatic while 24.13% (7/29) of them were asymptomatic. Lymphadenopathy was the most frequent clinical sign followed by hair loss, conjunctivitis,

TABLE 2 - The serological and parasitological findings of dogs obtained from five study sites.

Village/district	Dogs sampled		Seropositive Dogs			Borderline Dogs		Smear positivity	
	n	M/F	n (%)	M/F	IFAT titer ranges	n (%)	M/F	Positive	M/F
Asagidagdere	41	25/16	9 (21.95)	6/3	128-8000	9 (21.95)	5/4	3/6	1/2
Dereciftlik	28	23/5	7 (25.00)	5/2	128-8000	4 (14.28)	3/1	0/6	0
Ortatepe	13	11/2	4 (30.76)	3/1	128-8000	0	0	nd	nd
Demirciler	40	37/3	7 (17.50)	7/0	128-4096	10 (25.00)	9/1	0/1	0
Gumusçay	18	14/4	2 (11.11)	2/0	1024-4096	0	0	1/1	1/0
Total	140	110/30	29 (20.71)	23/6	-	23 (16.42)	17/6	4/14	2/2

n: Number of dogs; M: Male; F: Female

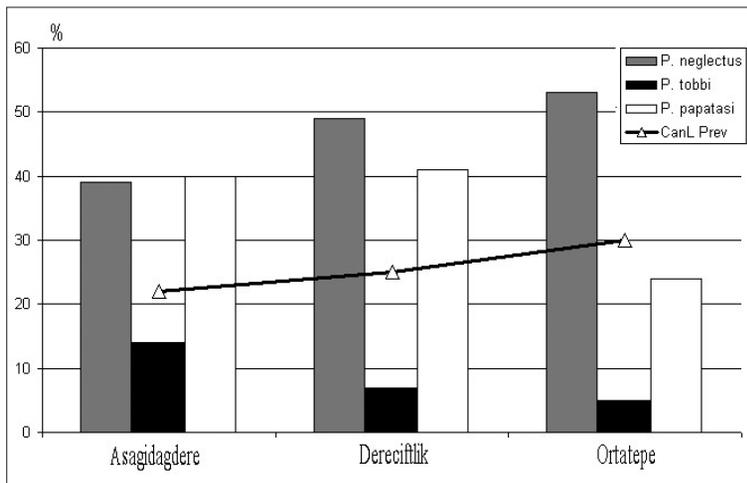


FIGURE 2 - The distribution of three dominant sand fly species in three villages and comparison with CanL seroprevalence.

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. simicci* 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 anarchic 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 also 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. papatasi* (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 *Larrousius* 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 Croatia (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 coast 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, Ivovic *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. similis*, belonging to the subgenus *Paraphlebotomus*, are closely related species, but the molecular analyses indicated that *P. similis* 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. similis* are present, but *P. similis* 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 the study 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.

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