

Investigation of asymptomatic visceral leishmaniasis cases using western blot in an endemic area in Turkey

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SUMMARY

In Turkey, *Leishmania infantum* is responsible for human visceral leishmaniasis (VL), which is seen mainly in the Aegean, Mediterranean, and Central Anatolia Regions. This study aimed to determine asymptomatic infections in an endemic area of VL in Turkey using the western blot technique.

A total of 82 persons including children and adults were chosen randomly in Denizli province which is one of the endemic sites for VL. Serum samples were collected and screened using indirect immunofluorescent test (IFAT), enzyme-linked immunosorbent assay (ELISA) and western blot (WB). One year later, 35 of the 82 persons were sampled and screened serologically for the second time.

Seven out of 82 samples were found to be positive by western blot analysis with the presence of 14 and/or 18 kDa bands. Two of these seven sera were also positive by IFAT, but only one of these two was positive by ELISA. Only one person showing seropositivity with all three tests had clinical symptoms and was diagnosed as VL with the presence of amastigotes in bone marrow aspirate. Because six people, including the one found to be seropositive in all two tests, had no clinical symptoms, they were accepted as asymptomatic carriers. The ratio of asymptomatic infection was calculated as 7.41% (6/81) in the region. In the second sampling, the western blot revealed antibodies against the same antigens in all seven subjects.

Our findings showed that the presence of antibodies against 14 and 18 kDa antigens are important for the diagnosis of symptomatic and asymptomatic infections. Western blot was found to be effective in the detection of asymptomatic persons in the epidemiological studies in endemic areas.

KEY WORDS: Leishmaniasis, Diagnosis, Asymptomatic, Western blot, Turkey

Received November 29, 2006

Accepted December 2, 2006

INTRODUCTION

Visceral leishmaniasis (VL) is a zoonotic parasitic infection transmitted by the bite of an infected female sandfly. It is the most severe form of

leishmaniasis and is usually fatal if left untreated (WHO, 2000; Desjeux, 2004). VL cases are observed mainly in children in Turkey as expected in the Mediterranean basin, but adult cases have also been observed in recent years. *Leishmania infantum* (*L. infantum*) MON-1 (the strains were identified in Montpellier, France and ISS, Italy, unpublished data) is responsible for human VL, which is seen mainly in the Aegean, Mediterranean, and Central Anatolia regions and dogs are accepted as main reservoir of the disease in Turkey (Ozbel *et al.*, 1995; Ozensoy *et al.*, 1998; Ok *et al.*, 2002).

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Chronically infected asymptomatic persons may serve as a reservoir of parasites and may be important in the transmission of VL (Costa *et al.*, 2002). The majority of infected individuals have a subclinical infection that may remain completely asymptomatic or have an oligosymptomatic form of the disease. Subjects with oligosymptomatic infection may develop clinical VL months after seroconversion or may self heal their infections one or two years after the seroconversion (Badaro *et al.*, 1986).

The most common methods for diagnosis of VL are classical parasitological methods such as direct microscopic examination and in vitro culture of bone marrow and/or splenic aspirates (Sundar and Rai, 2002). Several serological tests varying in terms of sensitivity and specificity are the most practical way of diagnosing VL infections (Bucheton *et al.*, 2003). Western blot (WB) has been used for diagnostic and epidemiological purposes, and antibodies against the 14, 16 kDa and/or 18 kDa antigens are important for the diagnosis of asymptomatic infections (Mary *et al.*, 1992; Marty *et al.*, 1994 and 1995; Bucheton *et al.*, 2003)

This study aimed to determine whether asymptomatic VL infections are present in an endemic area in Turkey using the WB technique.

MATERIALS AND METHODS

Study area and serum samples

Denizli province, located in southwestern Turkey, was chosen as the study area. A total of 16 patients (including six adults) from Denizli province were serologically diagnosed and parasitologically confirmed as VL in the Department of Parasitology of Ege University Medical Faculty between 1993 and 2000.

The sera were obtained from 38 children (aged between 1 and 13) and 44 adults (aged between 14 and 72) living in the rural area of Denizli province in June 2001. The people were chosen randomly and were examined physically for clinical signs of VL. Routine blood tests (hemogram, liver enzymes, etc.) were also analyzed. Serum samples were screened using indirect immunofluorescent test (IFAT), enzyme linked immunosorbent assay (ELISA) and WB techniques. Positive and negative control sera were

also used for all serological experiments. All three serological tests were performed three times. The area was visited one year later in June 2002 and 35 out of 82 people could be sampled for the second time.

Antigen

Local *Leishmania infantum*, MON-1 promastigotes were obtained by mass culturing in RPMI-1640 containing 10% foetal calf serum, washed eight times in sterile phosphate buffer solution after harvesting and used as the antigen for the serological tests.

For IFAT, promastigotes were resuspended at a concentration of 2×10^6 ml⁻¹ in saline and 10 microliters of this suspension were transferred per spot onto multispot IFAT slides. After air-drying, the slides were stored at -20°C until further use.

For ELISA and WB, after harvesting, the promastigotes were lysed using 1% sodium dodecyl sulfate (SDS) and supernatant was used after centrifugation. Antigen concentration was determined by checkerboard ELISA using positive and negative control sera.

Serological tests

IFAT: The IFAT was performed using standard procedures (de Korte *et al.*, 1990). Two-fold serial dilutions (1:16 to 1:16,384) of the sera in PBS were dispensed onto the antigen-coated wells. Following incubation at 37°C for 30 min, slides were washed and stained with FITC-labeled anti-human IgG conjugate (BioMerieux 75692) and examined under a fluorescence microscope. Titers $\geq 1:128$ were scored as positive.

ELISA: ELISA was performed as described elsewhere (Ozensoy *et al.*, 1998), except for the whole lysate promastigote antigen, using 1/100 single serum dilution, anti-human immunoglobulin G conjugated with alkaline phosphatase (dilution, 1/10,000; Sigma). The optical density was measured at 405 nm and subjects were considered positive when the OD was >0.300 which represents the mean plus 3 SD absorbencies obtained in sera from individuals accepting without exposure to *Leishmania*.

WB: SDS-polyacrylamide and gel electrophoresis (SDS-PAGE) were performed according to the method described by Marty *et al.* with modifications. (Marty *et al.*, 1994) A 12% polyacrylamide

gel was used as separating gel with Trisglycine buffer (pH 8,3) and Tris-Cl buffer (pH 8.8). A 4% stacking gel (pH 6.8) was used. Molecular weight markers (BioRad) were used in a separate tract. A total of 600 µg of antigens prepared in a solution were warmed up at 100°C for 3 min, the antigens were then applied to the surface of the gel. After electrophoresis for approximately 2.5 h at 100 V, the antigen was electrotransferred from the gel to a nitrocellulose membrane (Schleicher&Schuell) using transfer buffer (Trisglycine pH 8.3; 20% methanol). After the transfer, the membrane was blocked with TBS containing 0,05% Tween 20 for 15 minutes on a shaker and was later cut into strips 3 mm wide.

The strips were placed on a shaker and incubated for 1.5 h with 5 microliters of the serum to be tested, which was diluted in 1000 microliters TBS-Tw20 casein buffer. The strips were then

washed in TBS-Tw20, and were incubated in a 1:5000 dilution of anti-human IgG alkaline phosphatase conjugate (Sigma) in TBS-Tw20 for 1 h. After washing, strips were incubated with the nitroblue tetrazolium substrate (NBT/BCIP tablets-Boehringer Mannheim), which generated an insoluble dark gray product. All incubations were performed at room temperature on a rotator shaker. Each gel included a set of seven prestained molecular size standards (BioRad).

RESULTS

The seroprevalence of VL in Denizli province was detected 8.53% in WB, 2.45% in IFAT and 1.21% in ELISA. Although one (No. 2 in the Figure 1) serum sample was found to be seropositive by all three tests and one (No. 3 in the Figure 1)

TABLE 1 - Some characteristics and serological results of the 7 seropositive persons (†the order is same as in the figure)

							<i>Bands (kDa)</i>									
<i>Patients†</i>	<i>Age</i>	<i>Sex</i>	<i>Sampling</i>	<i>IFAT</i>	<i>ELISA</i>	<i>WB</i>	14	18	20	24	33	37	39	42	45	47+
1	14	M	1st 2nd	64 16	± Neg	+ +	+		+				+			+
2	30	F	1st 2nd	512 512	+ +	+ +	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
3	17	M	1st 2nd	128 64	± Neg	+ +	+	+	+			+		+	+	+
4	30	M	1st 2nd	Neg 16	Neg Neg	+ +	+	+			+	+				+
5	63	F	1st 2nd	Neg Neg	± Neg	+ +	+	+								
6	70	F	1st 2nd	16 Neg	Neg Neg	+ +	+	+								
7	34	F	1st 2nd	16 Neg	Neg Neg	+ +	+	+								

Intensity of bands

++++
+++
++
+
None

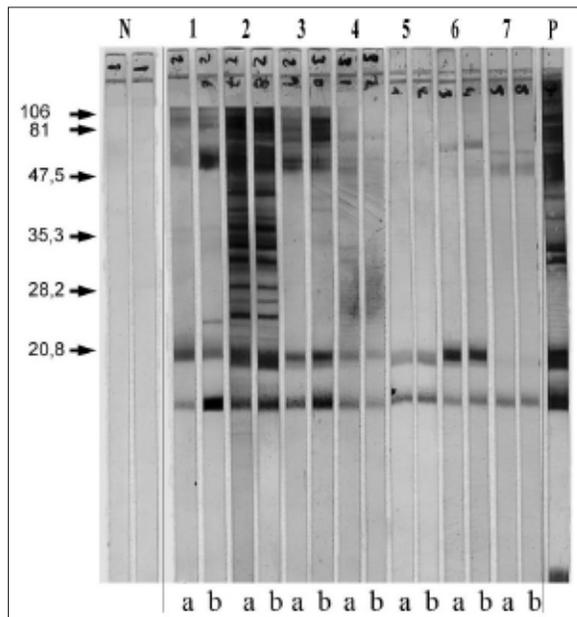


FIGURE 1 - Western blot analysis of sera from 7 seropositive samples. The first (a) and second (b) lines represent first and second samplings respectively. (Number 2 is the proven VL case) (N: Negative, P: Positive).

serum sample was found to be seropositive with IFAT and WB, WB results showed 5 more seropositives out of 82 serum samples (Table 1). Two seropositive sera (Nos. 2 and 3 in the Figure 1) reacted with many antigens, giving multiple bands (14-94 kDa) while another 5 sera reacted strongly with only 14 and/or 18 kDa antigens. In the second sampling, the WB revealed antibodies against the same antigens in all 7 samples with variations in intensity of certain bands (Figure). The ages of the 7 seropositive persons by WB were between 14 and 72, and three of them were female (Table 1).

Only one (No. 2 in the Figure) out of 7 seropositive samples in all three tests, showed clinical symptoms like hepatosplenomegaly and weakness. Pancytopenia was detected in laboratory analyses. Following these results, the patient was transferred to the University Hospital. Parasitological examination was performed using bone-marrow aspirate samples and amastigotes were shown in Giemsa-stained smears. She was diagnosed as VL and successfully treated with antimonials. There was no relapse during two years after therapy. Another

six people including one (No. 3 in the Figure 1) who was found to be seropositive by IFAT and WB had no clinical symptoms and routine blood tests were also normal.

DISCUSSION

There is a lack of gold standard techniques to detect VL infections. Serological tests are successful in the detection of VL but the low humoral immune response is a characteristic of asymptomatic subjects. However, WB is a sensitive tool to identify specific antibodies even when classical serological tests (IFAT, ELISA) are negative (Riera *et al.*, 2004). Similarly asymptomatic VL cases in a child population were not detected in different endemic sites in Turkey with using IFAT and ELISA in different field studies (Ozensoy *et al.*, 1998; Ertabaklar *et al.*, 2005).

In the present study, although Mediterranean type VL were usually observed in children, blood samples were also collected from adults because of previous detection of adult VL cases from Denizli province. One person showed seropositivity in all tests (IFAT, ELISA, and WB), had clinical symptoms diagnosed as VL and was treated with antimonials. One person who showed positivity only in IFAT (in cut-off titer, 1/128) and WB did not have any clinical symptoms for two years and was interpreted as asymptomatic VL infection. Another five people showed seropositivity only in WB, had no clinical symptoms with normal blood tests and were also accepted as asymptomatic carriers. The asymptomatic carrier ratio was calculated as 7.41% (6/81) in the region and most of them (5/6) were adults. According to these results, WB provided a significant increase in the sensitivity for detection of asymptomatic infection than IFAT and ELISA as previously mentioned (Mary *et al.*, 1992) but no other methods like parasite DNA-based PCR could not be performed for the detection of asymptomatic cases. A number of authors found PCR as the most sensitive method and WB as the most sensitive serological method in detecting asymptomatic carriers (Le Fichoux *et al.*, 1999; Riera *et al.*, 2004; Costa *et al.*, 2002).

The seroprevalence of VL in Denizli province was detected as 8.53% in WB, 2.45% in IFAT and

1.21% in ELISA. These results were consistent with the findings of other studies in blood donors from Barcelona (Cardeñosa *et al.*, 1995), Monaco (Le Fichoux *et al.*, 1999) and Eivissa, Balearic Islands (Spain) (Riera *et al.*, 2004). Other surveys, performed on healthy subjects from endemic areas in the south of France detected seropositivity to specific *L. infantum* fractions by WB in 13/20 and 19/50 individuals tested, while all samples were negative by ELISA and IFAT (Mary *et al.*, 1992; Marty *et al.*, 1994). In the present study, the *Leishmania* specific antibodies were revealed by WB in 7 sera samples out of 82, showing the characteristic 14 and/or 18 kDa bands in two sampling periods. Our results were consistent with previous findings. Marty *et al.* considered that the presence of a group of four bands (18, 21, 23 and 31 kDa) was sufficient to define clinical leishmaniasis in an endemic area (Marty *et al.*, 1995). However, in another study the authors pointed out the importance of 14, 16 and 18 kDa bands in the diagnosis of clinical leishmaniasis cases in addition to the group of 12-120 kDa bands. (Mary *et al.*, 1992; Marty *et al.*, 1994 and 1995; Bucheton *et al.*, 2003) In the present study, 5 sera giving positivity only in WB reacted with 20, 48, 55, 65 kDa antigens while 2 sera showing positivity also with other serological tests as ELISA and/or IFAT reacted with numerous antigens including 94 kDa previously described by Rolland *et al.* as the immunological marker of the *L. infantum* infection (Rolland Burger *et al.*, 1995). In conclusion, our findings also showed that the presence of antibodies against 14 and 18 kDa antigens are important for the diagnosis of symptomatic and asymptomatic infections and western blot can be a valuable tool in the detection of asymptomatic carriers in epidemiological studies in VL endemic areas. This study was also the first study to disclose asymptomatic VL cases in Turkey.

ACKNOWLEDGMENTS

This work was supported by Ege University Scientific Research Foundation (Project no: 2000/TIP/016). We thank the Director of the Infectious Diseases Department, Ismail Sancak, and the Branch of Ministry of Health in Denizli, Turkey.

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