Evaluation of Anti-*Leishmania* Antibodies in Turkish Patients with Visceral Leishmaniasis using Western Blotting

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SUMMARY: This study examines Western Blotting (WB), which depends on the detection of the specific antibodies against the immunodominant antigens, with respect to its proficiency in the serodiagnosis and follow-up of patients with leishmaniasis in Turkey, in comparison to other serologic tests. A total of 56 patients, of whom 37 possibly had visceral leishmaniasis (VL) and whose bone marrow aspirates were available and nine with cutaneous leishmaniasis were studied using WB, ELISA and IFA. In order to determine the presence of cross-reactions, five patients with malaria and five with toxoplasmosis were also studied. WB was performed in sera diluted to 1/100, using antigens derived from two different *L. infantum* species. By using a combination of WB, ELISA and IFA, anti-*Leishmania* antibodies were detected in the sera of the thirty patients with VL. A number of bands with molecular weights varying from 14 kDa to 94 kDa were detected. In the present study, it appears that *L. infantum* specific antigens were recognized with 100% specificity and, with special reference to molecular weights, sensitivity varied between 15% for 14 kDa and 100% for 94 kDa antigens. There was no correlation between the number and intensity of bands in western blot analysis and IFAT and ELISA antibody titers, however, the sensitivity of the western blot analysis with ELISA and IFAT seem to be correlated. We concluded that WB is a reliable test for the diagnosis and follow-up of VL patients in Turkey.

Key words: Leishmaniasis, visceral, diagnosis, western blot

Visseral Leishmaniasisli Hastalarda Western Blot Yöntemi ile Anti-*Leishmania* Antikorlarının Değerlendirilmesi

ÖZET: Çalışmamızda, immunodominant antijenlere karşı oluşan özgül antikorların saptanması esasına dayanan, diğer testlerle kıyaslandığında Türkiye'deki Kala-Azar hastalarının tanısında ve takibinde yararlı olabilecek western blot (WB) yöntemi uygulanmıştır. Kala-Azar şüphesi olan 37 hasta serumu, çapraz reaksiyonlar açısından değerlendirmek üzere 9 şark çıbanlı hasta serumu, beş sıtma ve beş toxoplasmosisli hasta serumu olmak üzere toplam 56 hasta serumu WB, ELISA ve IFA yöntemleri ile çalışılmıştır. WB yönteminde, iki farklı *L. infantum* suşu antijen olarak kullanılmış ve serumlar 1/100 sulandırımda çalışılmıştır. Her üç yöntemle de Kala-Azar şüpheli 30 hastanın serumlarında anti-*Leishmania* antikorları saptanmıştır ve moleküler ağırlığı 14 kDa ile 94 kDa arasında değişen çok sayıda bant olduğu görülmüştür. Çalışmada kullanılan antijenlerle özgüllüğün %100 olduğu, ancak duyarlılığın %15 (14 kDa antijen için)ile %100 (94 kDa antijen için) arasında değiştiği görülmüştür. Bantların yoğunluğu ve sayısı ile IFAT ve ELISA'da elde edilen antikor titreleri ile doğrudan bir ilişki saptanmamıştır. Bununla birlikte, her üç testin de duyarlılığı arasında bir korelasyon olduğu görülmüştür. Sonuçta, WB yönteminin Kala-Azar hastalarının tanı ve takibinde kullanılabilecek bir test olduğu görüşüne varılmıştır.

Anahtar kelimeler: Leishmaniasis, Kala-Azar, tanı, western blot

GİRİŞ

Human visceral leishmaniasis (VL), caused by *L. donovani* and *L. infantum*, is characterized clinically by fever, hepato-splenomegaly, weight loss, anemia and leukopenia. The disease is endemic in tropics and subtropics, including the Aegean and Mediterranean regions of Turkey, with approximately 100,000 new cases each year. Since the disease causes life-threatening infection, early diagnosis is of great importance. However, a variety of diseases have to be considered in the differential diagnosis. Like bone marrow or splenic aspiration, which are invasive techniques requiring specialists to be performed and examined; the identification of the pathogen in infected tissue samples is needed for definitive diagnosis. Hence, serological tests have provided useful

Geliş tarihi/Submission date: 02 Mart/02 March 2004
Düzeltme tarihi/Revision date: 15 Nisan/15 April 2004
Kabul tarihi/Accepted date: 21 Nisan/21 April 2004
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alternatives, as more efficient diagnostic methods with high sensitivity and specificity. Immunoblots have been useful in studies of host serological responses during infection, and a few antigens with potential diagnostic value have been selected (2). This study examines the Western Blot (WB) technique, which depends on the detection of the specific antibodies against the immunodominant antigens, with respect to its competence in the serodiagnosis and follow-up of the patients with leishmaniasis in Turkey, in comparison with the other serologic tests.

MATERIAL AND METHODS

Human sera: A total of 56 patients, of which 37 patients suspected of visceral leishmaniasis (VL), whose bone marrow aspirates were also available, nine patients with cutaneous leishmaniasis, and for cross-reaction, five patients with malaria and another five patients with toxoplasmosis, were studied by WB, ELISA and IFAT. In five VL-infected patients, both the pre- and post-treatment sera were studied, in order to compare the post-treatment alterations in the number of the antigens recognized by VL sera and the intensity of the bands.

Antigen: The strains used in this study were: *L. infantum* (MHOM/TR/99/EP32) and *L. infantum* (MCAN/TR/99/EP33). Promastigotes were grown at 28 ^{o}C in RPMI 1640 medium (Biochrom-Cat No:F 1215) containing 10% heat inactivated fetal calf serum (FCS).

Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE): *L. infantum* promastigotes were lysed in a sample buffer consisting 125 nM Trisma base, 4% SDS, 20% glycerol, and 0.04% bromphenol blue (final concentrations). Samples were boiled for 5 minutes and immediately subjected to dual slab gel electrophoresis, using discontinuous buffer system of Laemmli 1970. SDS was performed on a 10% polyacrylamide gel (Figure 2).

Transfer and immunoblotting: Electrophoretic profiles of Leishmania components were transferred from gels to nitrocellulose membranes (7/8.4 cm, Bio-Rad 162-0145). Strips were blocked with 3% BSA, which was diluted in TBS (Tris-buffered saline). Then washed three times with TBS, and incubated 1 h at 37 ^{o}C with sera diluted 1:100 in TBS. The strips were washed again, and incubated with 1:1000 dilution of Anti-Human IgG goat alkaline phosphatase congugate (Sigma A3187) in TBS. After further washing enzymatic activity was revealed using nitroblue tetrazolium and bromo-chloro-indolyl phosphates (Sigma B-5655) chromogenic substrates. The reaction was stopped with distilled water when a background color began to appear.Strips were dried and stored in dark.

Immunoflourescence antibody test: Washed stationary-phase *L. infantum* promastigotes were dispensed in demarcated circles on 12 well glass slides. Sera samples diluted between 1:16 and 1:512 in PBS incubated 30 min at 37 ^{o}C and washed three times in PBS, and then, 1:300 diluted FITC-labeled anti-human IgG (Bio Meriux 75692) conjugate was added. The slides were then incubated 30 min at 37 ^{o}C, washed and examined under a fluorescence microscope (Olympus BH2-RFCA).

Enzyme-linked immunosorbent assay: Plates were coated overnight with 0.5 µg of antigen/well. Sera diluted 1:100 to 1:800 in casein buffer were added and the plates were incubated 1 h at 37 ^{o}C. Then The plates were washed 3 times with PBS-Tween 20 and incubated with a 1:10000 dilution of Anti-Human IgG goat alkaline phosphatase conjugate (Sigma A3187) for 1 h at 37 ^{o}C. After further washing immune complexes were revealed using DEAB (Diethanolamin, MERCK-803116). The Optical Density of wells was read at 405 nm.

RESULTS

In seven of the 37 patients clinically suspected of VL, no bands were detected by WB, whose ELISA, IFAT and bone marrow examinations were negative, and the diagnosis of VL was excluded. In the patients with malaria and toxoplasmosis, who were included for cross-reaction, WB was negative as well as ELISA and IFAT.

In nine patients with cutaneous leishmaniasis, sera of the seven patients (77%) gave one or more bands (ranged between 1 to 3) in 6 different molecular weights (Fig. 1). ELISA and IFAT tested sera of those patients gave low antibody titers, which were not correlated with the number of bands detected in WB.

The sera of the 30 patients with VL reacted with many antigens, giving multiple bands with molecular weights ranging from 14 to 94 kDa, having IFAT-antibody titers ranging from 1:128 to 1:4096 and ELISA-antibody titers ranging from 1:100 to 1:16,000.

In WB analysis, some bands were not present in all sera or were difficult to differentiate. We therefore selected four groups of bands, which were well differentiated and all present in all the 30 sera from VL-infected patients. These groups had molecular weights of 14-16; 21-31; 60-67 and 90-94 kDa. In those groups, L. infantum-specific antigens were recognized with 100% specificity and with special reference to molecular weights, sensitivity varied between 15% for 14 kDa and 100% for 94 kDa antigens (Fig. 1).

There was no correlation between the number and intensity of the bands detected in WB analysis and IFAT and ELISA-antibody titers, however, the direct comparison of the sensitivity of WB with ELISA and IFAT seemed correlated.

In the WB analysis of pre- and post-treatment sera of five patients treated with antimony, the number and the intensity of the bands from the post-treatment sera was reduced (Fig. 3).

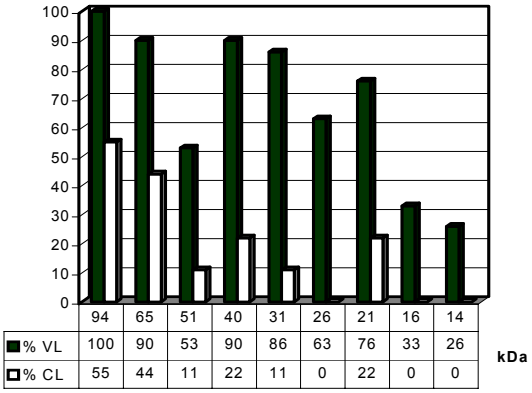


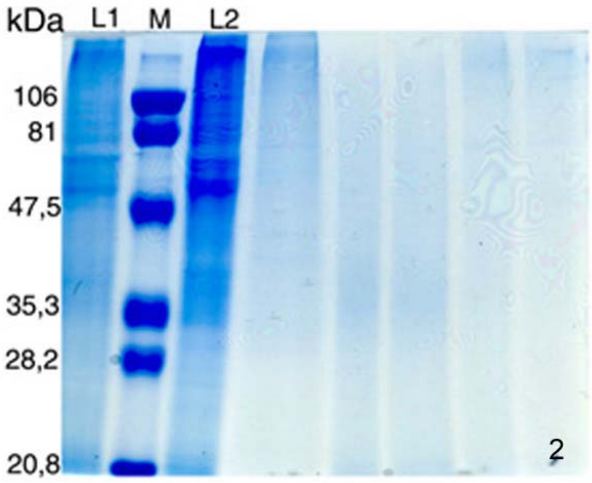
Figure 1. Percentages of the antigens recognized by patients with visceral and cutaneous leishmaniasis

sensitive (positive only in 26% and 33% of the patients with VL respectively).

Moreover, Marty et al. described specificity and sensitivity according to reactivity towards a group of four bands between 18 and 31 kDa and considered that, the presence of those bands was sufficient to define clinical leishmaniasis in an endemic area (1). In this study, we found that, all the patients with VL had reactivity towards the antigens between the 14 and 40 kDa, thus, WB technique proved useful in diagnosis with high sensitivity and specificity.

On the other hand, we found the 94-kDa antigens with the highest specificity (100 %), which have been described as the immunological markers of the L.infantum infection, previously by Rolland et al (3).

WB showed no cross-reactivity in sera obtained from Turkish patients with toxoplasmosis or malaria, which was also consistent with the previous data (4).



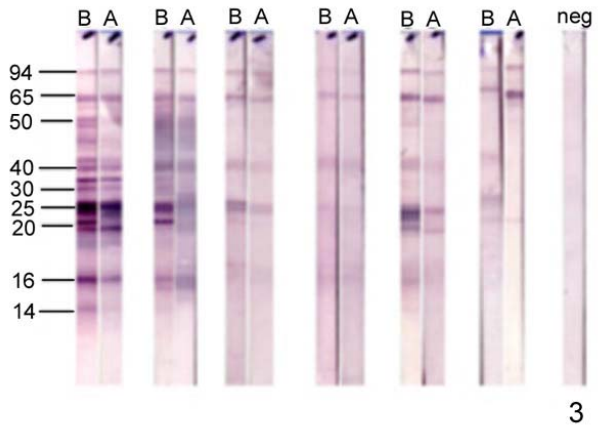


Figure 2. SDS-PAGE of the antigens using in western blotting
(**L1**: *L.infantum*- MHOM/TR/99/EP32; **L 2**: *L infantum*-MCAN/TR/99/EP33; **M**: Marker)

Figure 3. The bands obtained before and after treatment (**B**: Before therapy; **A**: After Therapy)

DISCUSSION

Numerous studies have investigated Leishmania antigen expression at the level of specific antibody recognition. Antigens with potential diagnostic value have been selected by their specific recognition by VL sera in WB assays (3).

Mary et al. described four groups of parasitic antigens available for diagnostic and epidemiological purposes, that antibodies against one or more of them have been found in all cases of leishmaniasis and confirmed that, the 14-16 kDa bands represented the most sensitive and specific antigens (2). Our findings were consistent with such grouping; however, the 14-16 kDa bands were found highly specific (not positive in any of the patients with cutaneous leishmaniasis), but not

As a result, we concluded that, WB is a reliable test for the diagnosis and follow-up of VL, which can be used as a routine diagnostic tool; however, it requires suitable laboratory environment and equipment.

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