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Evaluation of Serological Tests for the Diagnosis of Visceral Leishmaniasis*

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Aim: Visceral leishmaniasis (VL) is a zoonosis that affects both animals and man. VL is seen sporadically in the Aegean, Mediterranean, Black Sea and central Anatolian regions of Turkey. The aim of this study was to evaluate serological tests for the diagnosis of VL.

Materials and Methods: The performance of two agglutination tests based on freeze-dried whole promastigote Leishmania antigen, i.e. Fast Agglutination Screening Test (FAST) and Direct Agglutination Test (DAT), an Indirect Immunofluorescent Antibody Test (IFAT), and an Enzyme-Linked Immunosorbent Assay (ELISA) using soluble antigen were compared to parasitological diagnosis in the serum specimens of 59 patients with the clinical suspicion of VL presenting at a tertiary care center in Ankara.

Results: Twenty-four patients had a parasitologically proven VL infection and 35 VL suspects had a negative parasitological work-up. DAT and IFAT were positive in all parasitologically positive patients whereas FAST and ELISA were positive in 23 (95.8%) out of 24 of the cases. Out of 35 clinically suspected but parasitologically negative VL cases, four showed a positive reaction in DAT, five in FAST and IFA and six in ELISA. The agreement between the tests was excellent (agreement: 90-96.6%; κ value: 0.82-0.93).

Conclusions: The present study shows that the FAST is a rapid and cost-effective screening test. DAT, IFAT and ELISA tests are all very sensitive tests, but on grounds of simplicity and low cost, the DAT is considered the most suitable test for the sero-diagnosis of VL in our region.

Key Words: Parasitic diseases, protozoon infections, leishmaniasis, visceral, kala azar, Leishmania donovani, L. donovani promastigote antigen, serology, visceral leishmaniasis, diagnosis, serology, agglutination, IFA, ELISA

Visseral Leishmaniasis Tanısında Serolojik Testlerin Değerlendirilmesi

Amaç: Visseral leishmaniasis (VL) hayvan ve insanları etkiliyen bir zoonozdur. VL ülkemizde Ege, Akdeniz, Karadeniz ve İç Anadolu bölgesinde sporadik olarak görülmektedir. Bu çalışmada, VL tanısında parazitolojik tekniklerle birlikte serolojik testlerin değerlendirilmesi amaçlanmıştır.

Yöntem ve Gereç: Ankara'da üçüncü basamak sağlık hizmeti veren hastanelerde VL ön tanısı almış 59 olgudan alınan serum örneklerinde kuru-dondurulmuş tüm promastigot antijenine dayanan hızlı aglütinasyon tarama testi (FAST) ve Direkt aglütinasyon testi (DAT), IFAT ve çözünebilir antijen içeren ELISA yöntemlerinin etkinlikleri direkt parazitolojik tanı yöntemleri ile karşılaştırılmıştır.

Bulgular: Elli dokuz örneğin 24'ünde parazitolojik olarak VL tanısı konulmuş ve 35 örnek parazitolojik incelemede negatif olarak değerlendirilmiştir. DAT ve IFAT parazitolojik olarak tanımlanmış tüm örneklerde pozitif olarak bulunmuştur. FAST ve ELISA ise 23 (% 95.8) örnekte pozitif sonuç vermiştir. Parazitolojik teknikler ile negative olarak değerlendirilen 35 örneğin dördü DAT, beşi FAST ve IFA ve altısı ELISA yöntemiyle pozitif olarak bulunmuştur. İstatistiksel olarak testler arasındaki yüksek derecede uyumluluk (uyumluluk: % 90-96.6; κ değeri: 0.82-0.93) saptanmıştır.

Sonuç: Bu çalışma FAST'ın hızlı ve maliyet etkin bir tarama testi olduğunu göstermiştir. Tanı testlerinden DAT, İFAT ve ELISA yeterli duyarlılığa sahip olduğu ancak kolay uygulanabilirlik ve maliyet açından DA testinin bizim bölgemizde VL serolojik tanısını için en uygun test olarak değerlendirilmiştir.

Anahtar Sözcükler: Parazitik hastalıklar, Protozoon infeksiyonları, Leishmaniasis, Visseral, Kala-Azar, Leishmania donovani, L.donovani promastigot antijeni, seroloji, Visseral Leishmaniasis, Tanı, Seroloji, Aglütinasyon, İFA, ELISA

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Introduction

Leishmaniasis is infection with a species of flagellate protozoa belonging to the genus *Leishmania* that results in a group of globally widespread parasitic diseases. The disease is transmitted to humans by the bite of the infected female sand fly of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) (1). The disease predominantly manifests either in a cutaneous form, which causes skin ulcers on the exposed parts of the body, or in a visceral form that is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, and/or pancytopenia (1-3).

Visceral leishmaniasis (VL) is an endemic disease in the Aegean and Mediterranean basin, including Turkey, among humans and dogs. VL in this region is caused by *Leishmania infantum* and presents itself as the Mediterranean type of kala azar, which mainly affects children. Dogs are considered the main reservoir of the parasite and are primarily responsible for the persistence of VL in most urban areas (4).

In Turkey, epidemiology of leishmaniasis has not been extensively investigated and little detailed information is known about the distribution and prevalence of the disease, particularly VL. VL is mainly observed as endemic or sporadic in some areas of western and southern Turkey along the Aegean and Mediterranean coasts. In other parts of the country, the disease occurs sporadically (5). Recent studies have identified an emerging focus of human and canine disease near major population centers in the Central Anatolia region (6). Human VL type in Turkey is consistent with Mediterranean type of VL with a chronically debilitating disease that is predominantly seen in children younger than 11 years (7-9).

Since human VL is the most severe clinical manifestation of the disease that, if left untreated, has a mortality rate of almost 100%, accurate and rapid diagnosis is crucial. Clinical diagnosis of VL is supported by laboratory diagnosis mainly based on demonstration of parasites in tissue aspirates or cultures of biopsy specimens or serology (1-3,10).

In Turkey, diagnosis of VL generally relies on microscopic examination, mostly based on bone marrow aspirate, and culture of clinical samples in Novy, Nicolle, and MacNeal (NNN) medium. In addition, specific serological tests such as indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and rK39 antigen based tests (ELISA and dipstick) have been applied in the diagnosis of VL in both human and canine VL with satisfactory results in some centers (6,11-13). However, the application of IFAT and ELISA, which need an equipped laboratory with qualified technicians, is not feasible in remote VL endemic areas that lack sufficient infrastructure. The direct agglutination test (DAT), based on freeze-dried *L. donovani* promastigote antigen, may provide an alternative sero-diagnostic test, as it requires little equipment, no electricity or cold storage of the reagents and can be easily performed after appropriate training under harsh field conditions (14,15). However, there is only one sero-epidemiological study carried out in Turkey using DAT available in the literature (11). Therefore, the present study aimed at the evaluation of several sero-diagnostic tests, including the agglutination tests, DAT and fast agglutination screening test (FAST), in comparison to direct parasitological examination for the diagnosis of human VL in central Turkey.

Materials and Methods

Patients and Samples

A total of 59 bone marrow aspirations, peripheral blood smears and serum samples were obtained from patients (n=59; 42 males, 17 females) who were those referred to a tertiary care center on the basis of clinical suspicion of VL with signs of prolonged fever, splenomegaly and/or hepatomegaly and anemia/pancytopenia. Pancytopenia was defined as hemoglobin level <11 g/dl, white blood cell count <4 x 10^9/L, and a platelet count <100 x 10^9/L. A questionnaire with clinical and epidemiological data was completed for each patient at enrollment.

Diagnostic Procedures

All diagnostic procedures were carried out at Refik Saydam National Hygiene Center (RSNHC), Department of Communicable Diseases Research, in Ankara, Turkey.

The following methods were employed:

Parasitology

Standard parasitological examination was performed by microscopic observation of Giemsa-stained smears and followed by culture on NNN media. Cultures were incubated at 24°C and sub-cultured weekly for 4 weeks.

Serology – Agglutination Tests

The presence of anti-*Leishmania* antibodies in the serum samples was determined by FAST, DAT, IFAT and ELISA as described below.

DAT and FAST, based on freeze-dried antigen prepared from *L. donovani* 1S promastigotes, were provided from Royal Tropical Institute, Amsterdam, the Netherlands. FAST was performed as described by Schoone et al. (16). In brief, 20 mL 1:100 diluted serum in physiological saline (NaCl 0.85%), containing 0.78% b-mercaptoethanol, was mixed with equal volume antigen in microtiter plates with V-shaped wells (Greiner, Germany). The plate was carefully shaken and covered with a lid. After 3 h incubation at ambient temperature, the agglutination results were considered as negative (compact blue dot) or positive (large diffuse blue mat). Appropriate positive and negative controls were always included on each plate. DAT was performed as described previously (17). Briefly, two-fold serial dilutions of sera, ranging from 1:100 to 1:51200, were prepared in saline and 0.78% b-mercaptoethanol in V-shaped microtiter plates. DAT antigen solution (50 µL) was added to each well containing 50 µL diluted serum and the plate was covered with a lid. The negative control wells contained physiologic saline and antigen only. The results were read after 18 h of incubation at ambient temperature. The agglutination reaction was visually assessed against a white background. The end point titer was determined in reference to a clear sharp-edged blue spot (compact blue dot) observed in the control wells. Positive and negative controls were run for each test. Samples with a titer 1:1,600 were considered positive.

Serology – IFAT/ELISA

IFAT was carried out according to the procedure of De Korte et al. (18) using cultured promastigotes of a local *L. infantum* (MON-1) strain as antigen. Briefly, promastigotes were cultivated in RPMI-1640 containing 10% FCS and subsequently washed by centrifugation and resuspended at a concentration of $2x10^6$ parasites/ml. The prepared antigen suspension was dispensed onto multi-spot IFAT slides. Slides were air-dried and stored at -20 ^{o}C until use. Two-fold serial dilutions of serum in PBS from 1:16 to 1:256 were transferred onto the antigen-coated slides and incubated in a moist chamber for 30 min at 37 °C. After two washes in PBS, slides were stained with FITC labelled anti-human immunoglobulin G conjugate (Sigma F-4512, Germany) and incubated for 30 min at 37 °C. After removal of unbound conjugate by two washes, the air-dried slides were examined under a fluorescence microscope. Serum dilutions producing fluorescence on at least two-thirds of the promastigotes per view were considered to be positive. An antibody titer of IgG ≥1:128 was considered as an indication of *Leishmania* infection.

Finally, the presence of antibodies against *L. infantum* was also determined by a commercially available Leishmania IgM+IgG ELISA (Vircell SL Granada, Spain) according to the manufacturer's instructions.

Statistical Analysis

The data were processed with SPSS for Windows version 10.0.5 (SPSS, Inc., Chicago, IL). Statistical analysis of test results was done in terms of sensitivity, the degree of agreement between tests and Kappa (κ) values.

Sensitivity was estimated by the classic validation method. Parasitology was considered as the reference test in the 2 x 2 contingency table: a patient with a positive bone marrow aspirate was considered a VL case.

The degree of agreement between FAST, DAT, IFAT and ELISA was determined by calculating Kappa (κ) values with 95% confidence intervals using Epi-info version 6. Kappa values express the agreement beyond change, and a κ value of 0.21–0.60 represents a fair to moderate agreement, 0.60–0.80 a substantial agreement and >0.80 almost perfect agreement beyond change (19). The calculation of the degree of agreement between the tests and the κ values for FAST-DAT, DAT-IFAT, DAT-ELISA and IFAT-ELISA were based on the results obtained with the confirmed and suspected VL serum samples.

Results

Parasitology and Patient Characteristics

Parasitological diagnostic procedures revealed the presence of *Leishmania* amastigotes in smears and/or promastigotes in culture samples in 24 patients (40.7%). The remaining 35 (59.3%) suspected VL cases had a negative parasitological work-up.

The median age of the 24 confirmed VL patients was 6.75 years (range: 6 months to 19 years), with 22 (91.7%) confirmed patients under 14 years. The male:female ratio was 1.67:1. The age distribution of confirmed VL cases is shown in Figure 1.

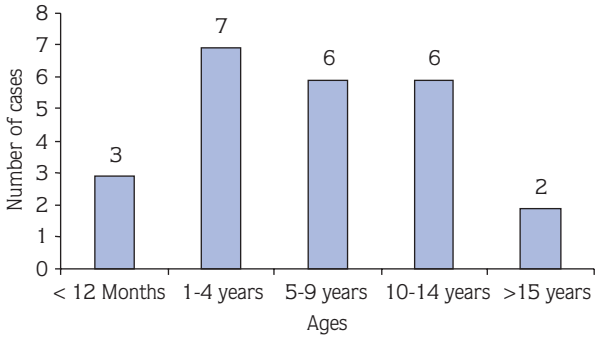

Figure 1. Age distribution of confirmed visceral leishmaniasis patients (n = 24) in the present study.

The most common clinical signs for the suspicion of VL were fever (87.5% of cases), splenomegaly (83.3%), pallor (75%) and anorexia (35%). The median duration of symptoms at first admission was eight weeks (range 1.5-52 weeks). Mean ± SD spleen and liver sizes were 6.0 ± 3.1 and 5.0 ± 4.0 cm below the costal margins, respectively. Anemia was present in 22 (91.6%) of the 24 parasitologically confirmed cases, leukopenia in 16 (75%), and thrombocytopenia in 13 (54.2%) VL patients. Pancytopenia was present in 10 (41.7%) of the 24 patients.

Serology

The results of serological testing of the suspected and parasitologically confirmed VL patients are presented in Table 1. Both DAT (agglutination titer ≥1:1600) as well as IFAT (positive fluorescence titer of ≥ 1:128) scored 100% of the parasitologically confirmed VL patients as positive. Twenty-three (95.8%) out of 24 confirmed VL cases were found positive by FAST and ELISA.

Anti-*L. infantum* antibody titers determined by DAT and IFAT are presented in Tables 2 and 3, respectively. Four out of 35 cases with a parasitologically negative bone marrow had a positive DAT result, with antibody titers ranging from 1:3200 to 1:51200. One suspected patient had a DAT titer below the cut-off value of the test. The IFAT was positive in five suspected, but parasitologically not confirmed, VL cases. Three IFAT-positive suspected patients only presented a low IFAT titer (1:64). Furthermore, it was observed that parasite-negative cases were found positive by means of FAST and ELISA in 5 and 6 cases, respectively.

Statistical Analysis

Calculation of the sensitivity of the assays revealed that the DAT and IFAT had a sensitivity of 100%, whereas FAST and ELISA both had a sensitivity of 95.8%.

Table 1. Results of serologic tests in VL patients.

Patient group	FAST (%)	DAT (%)	IFA (%)	ELISA (%)
Parasitology positive (n=24)	23 (95.8)	24 (100)	24 (100)	23 (95.8)
Parasitology negative (n=35)	5 (14.3)	4 (11.4)	5 (14.3)	6 (17.1)

Table 2. DAT results for anti-Leishmania antibodies in suspected and confirmed VL patients.

	Serial dilution series (Reciprocal)									
Patient group	800	1600	3200	6400	12800	25600	51200	102400	≥102400	Total (n)
Confirmed VL	(-)	(-)	(-)	(-)	(-)	5	4	2	13	24
Suspected VL	1	-	1	1	(-)	1	1	(-)	(-)	4

Table 3. Anti-Leishmania antibody titers determined by IFAT.

	IFA Titers						
Patient group	1:64	1:128	1:256	1:512	1:1024	1:2048	Total (n)
Confirmed VL	(-)	3	4	9	5	3	24
Suspected VL	3	1	1	(-)	-	-	5

The agreement between the two agglutination tests was 96.6%, with a calculated agreement beyond change (κ value) of 0.93 (See Table 4). The agreements (and κ values) between DAT-IFAT, DAT-ELISA, FAST-IFAT, FAST-ELISA and IFAT-ELISA were 94.9% (κ value: 0.90), 94.9% (0.90), 91.5% (0.83), 96.5% (0.93) and 90.0% (0.80), respectively (see Table 4).

Table 4. Comparison between DAT, FAST, IFAT and ELISA using serum samples from confirmed and suspected VL patients.

A. Comparison between DAT and FAST	FAST (+)	FAST (-)	Total
DAT (+)	27	1	28
DAT (-)	1	30	31
Total	28	31	59
B. Comparison between DAT and IFAT	IFA (+)	IFA (-)	Total
DAT (+)	27	1	28
DAT (-)	2	29	31
Total	29	30	59
C. Comparison between DAT and ELISA	ELISA (+)	ELISA (-)	Total
DAT (+)	27	1	28
DAT (-)	2	29	31
Total	29	30	59
D. Comparison between FAST and IFAT	IFA (+)	IFA (-)	Total
FAST (+)	26	2	28
FAST (-)	3	28	31
Total	29	30	59
E. Comparison between FAST and ELISA	ELISA (+)	ELISA (-)	Total
FAST (+)	27	2	29
FAST (-)	1	29	30
Total	28	31	59
F. Comparison between IFA and ELISA	ELISA (+)	ELISA (-)	Total
IFA (+)	26	3	29
IFA (-)	3	27	30
Total	29	30	59

Discussion

Unequivocal diagnosis of VL requires demonstration of parasites by Giemsa-stained smears or cultures of biopsy specimens from bone marrow, liver, enlarged lymph nodes, or spleen obtained by invasive procedures. Although demonstration of even a single amastigote upon microscopic examination of tissue smears or promastigotes in cultures is considered to be the gold standard, the sensitivities of these procedures, except in the case of splenic aspirate, are as low as 50-60%. Moreover, the procedures for obtaining tissue specimens have their own drawbacks, such as hemorrhage (1-3,10). In addition, identification of amastigotes requires considerable expertise and training and is subject to the ability of the observer. Parasitological techniques are also cumbersome, time-consuming and require expertise and costly equipment, severely restricting their use in routine clinical practice (1,10,14). These mentioned limitations of the direct diagnostic methods make serology the most useful diagnostic tool for laboratory diagnosis of VL (2, 3,10,14,20).

The comparison of the performances of agglutination tests (DAT and FAST) with IFAT and ELISA on serum samples of confirmed VL cases and suspects from Central Anatolia is reported. In the present study, the diagnosis of VL was confirmed with a positive parasitological procedure in 24 (40.7%) out of 59 VL suspects.

Serology based on the agglutination principle showed a sensitivity of 100% for DAT and 95.8% for FAST. Our findings corroborate well with observations from several other research groups, indicating high sensitivities (>92% - 100%) for these tests (15,20-26).

In our study, both IFAT and ELISA also showed good sensitivity values. These two tests are based on sonicated *L. infantum* antigen. The sensitivity of our ELISA assay is equivalent to that previously reported by several other research groups (27-29), who reported ELISA sensitivities varying between 97 to 100%. The sensitivity of IFAT in the present study is in agreement with the literature (24,30-32).

All serological tests employed found four to six additional patients as positive that were parasitologically negative. These patients were clinically VL suspected and had signs of the disease. The positive DAT, FAST, IFAT and ELISA may indicate a low parasitemia in these patients, which could not be detected by parasitological examination of their bone marrow aspirates. Perhaps more sensitive molecular methods, like polymerase chain reaction (PCR) and nucleic acid sequence based amplification (NASBA) are capable of detecting these low numbers of parasites (10,33).

Although the study population was rather small and the patients were recruited from an emerging focus in central Turkey, the serological results obtained indicate a high degree of agreement (agreement: 90-96.6%; κ value: 0.82-0.93) between the employed tests. Sophisticated serological tests such as IFAT and ELISA have not been extensively applied in the sero-diagnosis of VL in leishmaniasis endemic areas like Turkey, since both techniques require an equipped laboratory with qualified technicians. DAT and FAST are highly specific and sensitive tests as well as simpler and cheaper. They may provide a good alternative for the invasive and labor intensive methods that are required to perform a parasitological diagnosis, in particular in resource-poor environments. Based on our present experiences, and its slightly higher sensitivity, the DAT will be our method of choice for implementation of a serological test for the diagnosis of VL in our laboratory.

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