Original Article

Direct Agglutination Test and Enzyme Linked Immunosorbent Assay with Urine Samples for the Diagnosis of Visceral Leishmaniasis

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Abstract

Background: Visceral leishmaniasis (VL) or Kala azar is an infectious disease caused by various species of *Leishmania* parasites. The aim of this study was to detect and compare the presence of anti-*Leishmania* antibodies in the urine of visceral leishmaniasis patients using ELISA and DAT methods.

Methods: A total of 30 urine samples were collected from VL patients referred to Shiraz (southeast of Iran) hospitals. Moreover 31 urine samples were collected from healthy individuals and patients with other diseases such as malaria, brucellosis, hydatidosis and cutaneous leishmaniasis. Collected samples were examined to detect anti-*Leishmania* antibodies in urine, using ELISA and DAT.

Results: Anti-*Leishmania* antibody was detected in urine of 18 out of 30 (60%) VL patients by DAT while ELISA detected anti-*Leishmania* antibodies in urine of 28 out of 30 (93.3%) of VL cases. Sensitivity and specificity of urine-based DAT was 60% and 83.9%, respectively while sensitivity and specificity of urine-based ELISA were 93.3% and 93.5%, correspondingly.

Conclusion: Urine-based DAT and ELISA have a reasonable specificity and sensitivity in diagnosis of VL. Accordingly, urine-based ELISA might be a suitable alternative for serum based assays for diagnosis of VL.

Keywords: *Visceral leishmaniasis, ELISA, DAT, Antibody, Urine*

Introduction

Visceral leishmaniasis (VL) or kala-azar, is an infectious disease caused by protozoan parasite *Leishmania donovani* complex. The causative agent of VL in Iran is *L. infantum*. The endemic areas in Iran include Fars and Bushehr in the South, Ardabil in the North West, East Azerbaijan and Qom Province in the central of Iran. However sporadic cases of VL have been reported from all provinces (1-5). VL is a potentially fatal disease that affects an estimated of 500, 000 people each year worldwide (6). Clinical manifestations of the disease in human include prolonged fever, hepatosplenomegaly, substantial weight loss, progressive anemia and even death (7).

Different methods have been developed for diagnosis of VL but parasitological diagnosis which relies on detection of parasite in bone marrow or spleen aspirate still remains the gold standard for diagnosis of VL. Several serological tests including indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) have been used for diagnosis of VL (8, 9). Detection of anti-*Leishmania* IgG antibodies in serum have been used for diagnosis of VL

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over the past decades. Recently detection of antibodies in urine has been the focus of several studies for diagnosis of infectious diseases (10-13).

Here we evaluated the significance of antibody detection in urine for diagnosis of VL in an ELISA and DAT system.

Materials and Methods

Urine samples

Thirty urine samples were collected from VL patients referred to Shiraz (southeast of Iran) hospitals. The subjects consisted of 12 (40%) females and 18 (60%) males. VL cases had been parasitologically (bone marrow aspiration, 11 cases) or serologically (IFA, 23 cases) confirmed. Moreover 31 urine samples were collected from healthy individuals and patients with other diseases such as malaria, brucellosis, hydatidosis, and cutaneous leishmaniasis. The control samples had no history, in the past, or sign, at the time of sampling, of VL. Collected samples were stored at -20 ºC until use.

DAT with urine samples

The urine samples were tested by DAT according to the methods described by Islam *et al.* (11). Fifty µl of urine sample, without dilution, was added to each well of V-shaped microtitre plate. The plate left at 37 °C for 10 min. The DAT antigen (50 µl) was then added to each well. After two minutes of gentle shaking on a level surface, the plate was left overnight at room temperature. The results were checked visually according to the agglutination size.

Preparation of antigen for ELISA

Promastigotes of *L. infantum* were cultured in RPMI supplemented with 10% fetal calf serum (FCS). Soluble antigens were extracted from parasites by washing the parasites (5×10^7 cell/ml) in PBS, three times, followed by three freeze/thawing cycles. The extract was then centrifuged at 1300 g for 15 min. The supernatant was removed and protein content was estimated by Bradford protein assay (14).

ELISA with urine samples

The ELISA with urine samples was performed as described earlier (15). Briefly, microtiter plate was coated with 5 µg/ml of *L. infantum* antigen in coating buffer and incubated overnight at 4 °C. After blocking with 3% skimmed milk for one hour at room temperature, 100 µl of urine samples were added to each well and incubated for 1.5 hour. After washing the wells with PBST, 100 µl of peroxidase-conjugated anti-human IgG (Sigma) (1:4000 dilution in PBST) was added to each well and incubated for 1 h. Then, 100 µL/well of substrate was added and the plate was incubated for one hour at room temperature. Finally the optical density was measured at 490 nm as a reference. The cut off point was set at mean+2SD.

Results

DAT with urine samples

Urine samples of 30 VL patients and 31 non-VL patients and healthy subjects were tested by DAT for detection of anti-*Leishmania* antibodies in urine samples. The assay showed a sensitivity of 60% (95% CI=40.8%-76.8%) and a specificity of 83.9% (95% CI= 65.5%-93.9%). False positive reaction was seen with two urine samples from cutaneous leishmaniasis patients, one urine sample from patients with brucellosis and two urine samples of healthy subjects. Table 1 shows the performance of DAT in detection of anti-*Leishmania* antibodies.

ELISA with urine samples

Among 30 VL patients studied, considering the cut off point of 0.087, the urine-based ELISA was positive in 28 patients and was negative in 2 samples. Based on these results, the sensitivity and specificity of the assay were 93.3% (95% CI= 76.5%-98.8%) and 93.5% (95% CI= 77.2%-98.9%), respectively. Table 1 shows the results of ELISA on urine samples.

Table 1: Results of DAT and ELISA on urine samples for diagnosis of visceral leishmaniasis

Urine samples	No. of Positive cases				No. of Negative cases			
	DAT		ELISA		DAT		ELISA	
	n	%	n	%	n	%	n	%
Visceral leishmaniasis	18	60	28	93.3	12	40	2	6.7
Healthy people	2	33.3	0	0	4	66.7	6	100
Cutaneous leishmaniasis	2	16.7	1	8.3	10	83.3	11	91.7
Malaria	0	0	0	0	5	100	5	100
Brucellosis	1	50	0	0	1	50	2	100
Hydatidosis	0	0	1	16.7	6	100	5	83.3

Discussion

The diagnosis of VL can be performed by detection of antibody using serological methods such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) (8, 9). Serum is the common sample for detection of antibodies for diagnosis of VL. Several serum antibody detection assays have been applied for diagnosis of VL. Edrissian *et al*. assessed ELISA and IFAT for the serodiagnosis of visceral leishmaniasis (16). In this study ELISA was a little more sensitive than IFAT, but IFAT seemed to be more specific in detecting leishmanial antibodies (16). Moreover DAT has been used for seroepidemiological study of VL in Iran (17). In a recent extensive study, DAT has been used for diagnosis and seroepidemiological study of VL in different regions of Iran (18). In this study DAT has been used for detection of anti-*Leishmania* antibody in serum of 12144 human samples, collected from four geographical districts of Iran. From 516 detected kala-azar cases, 50.6% were from Meshkin-shahr and Moghan districts in Ardabil Province while the rest (49.4%) were from other areas of Iran (18).

Validity of antibody detection assays, using serum, has not reached the satisfactory level because of false positive or negative reactions. Urine might be an alternative sample for detection of antigen or antibody which produced against microbial agents and released in urine. Antigen detection in urine of VL patients have been first reported by Kohanteb *et al.*, where they demonstrated soluble antigen in urine of 19, out of 21, VL cases (10). This has been followed by Sarkari *et al.*, where they used a latex agglutination test (Katex) and capture-ELISA for detection of antigen in urine of VL patients (19, 20). Specificity of Katex and capture-ELISA was found to be 100% in these studies.

The presence of anti-*Leishmania* antibodies in the urine of patients with visceral leishmaniasis has also been described (10, 11). Release of antibody in urine in VL patient might be partially related to renal damage caused by VL infection (21, 22). In a prospective study of 50 patients with visceral leishmaniasis, laboratory abnormalities suggestive of renal involvement were frequent. Proteinuria and/or microscopic hematuria or pyurias were observed in 51% of such cases. Renal involvement in visceral leishmaniasis was mild and seemed to revert with the cure of the leishmanial infection (21).

DAT and ELISA, using serum and urine samples have been used for diagnosis of VL (11,

15). The urine-based ELISA demonstrated the best sensitivity (93.3%) and specificity (97.3%) compared with other assays. In the present study, we assessed the detection of anti-*Leishmania* antibodies in the urine of patients with visceral leishmaniasis by ELISA and DAT. Results of this study showed that urine-based ELISA had a satisfactory specificity and sensitivity in diagnosis of VL. In a recent study we showed that DAT is a specific while ELISA is more sensitive assay in detecting of anti-*Leishmania* antibody in serum of VL patients (9). Comparing the findings of the present study with those obtained from our previously published study, it is noticeable that serum-based DAT has the highest specificity and urine based ELISA has the highest sensitivity in diagnosis of VL (9). Furthermore when the results of urine-based ELISA was compared with those of serum-based ELISA in our previous study, a high degree of agreement (86.7%) was observed (kappa= -0.071).
Taken together, findings of this study revealed that urine based ELISA is a sensitive and specific assay in detecting anti-*Leishmania* antibodies in VL patients. It was not necessary to concentrate urine before the assay. The urine-based ELISA seems to be a desirable method in diagnosis of VL because of noninvasive and easier collection of urine samples. Accordingly, we consider that the urine-based ELISA might be an alternative, to serum-based serological tests, for diagnosis of VL.

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