

A comparative study of antigen and antibody detection in visceral leishmaniasis using serum and urine-based ELISA

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Abstract. An antigen-based ELISA system was evaluated for diagnosis of visceral leishmaniasis (VL). Urine samples from confirmed VL cases were tested by the system in comparison with urine samples from patients with non-VL infectious disease and patients with non-infectious diseases. Antigen was detected in urine of 21 out of 35 (60%) of VL cases. No cross reaction was found with samples from healthy individuals except in 3 samples from non-VL infectious diseases. Two samples from cutaneous leishmaniasis patient and one from patient with toxoplasmosis. The results obtained with the antigen-based ELISA were compared to those obtained with direct agglutination test (DAT), an antibody-based ELISA and indirect immunofluorescent antibody (IFA) revealed that the antigen-based ELISA is comparable in terms of specificity (91.2%; 95% CI=75.2-97.7%) but with a lower sensitivity (60%; 95% CI=42.2-75.6%). These results suggest that the antigen detection in urine by the noninvasive antigen-based ELISA system might offer a useful method for diagnosis of VL.

INTRODUCTION

Visceral leishmaniasis (VL) is a disease caused by protozoan parasites of the genus *Leishmania*, in particular *L. donovani* and *L. infantum*. VL is a serious public health problem in many tropical and subtropical regions of the world, including Iran (Mohebbali *et al.*, 2001, 2005). Parasitological diagnosis of VL, which is an invasive and not sensitive method, still remains as a routine method for diagnosis of VL (Berman, 2005).

A number of serological techniques have been developed for diagnosis of VL including enzyme linked immunosorbent assay (ELISA), dot ELISA and direct agglutination test (DAT). The sensitivity and specificity of such diagnostic methods depends on the type, source and purity of antigen employed. An alternative to antibody detection is antigen detection in urine which is a recent

approach in diagnosis of infection diseases.

A study in field of antigen detection in leishmaniasis carried out by Attar *et al* resulted in the demonstration of an antigen in the urine of VL patients by a latex agglutination test ("KAtex") (Attar *et al.*, 2001). The sensitivity and specificity of Katex was found to be 70-80% and 100%, respectively if the sample was boiled for 5 minutes before testing. Further work showed that the target antigen for this agglutination test is a low molecular weight (5-20kDa) carbohydrate antigen (Sarkari *et al.*, 2002). The current study was performed to use an ELISA system for detection of antigen in urine of VL patients.

MATERIALS AND METHODS

Parasites: *Leishmania* parasite (*L. infantum*) isolated from a VL patient was



used for rabbit immunization for production of anti *Leishmania* antibody.

Urine and serum samples: Serum (61) and urine (35) samples were collected from VL patients. Moreover 53 serum and 34 urine samples were obtained from healthy controls, patients with non-infectious diseases and patients with different microbial infections, but not visceral leishmaniasis, including toxoplasmosis, hydatidosis, brucellosis, tuberculosis, malaria and cutaneous leishmaniasis.

Production of anti-*Leishmania* antibody: Two rabbits were selected and the immunization schedule was started by IV injection of live promastigote (*L. infantum*) over a period of one month at an interval of 4-5 days. The inoculum size was doubled each time starting from 10^7 increasing to 3.2×10^8 cells. The rabbits were bled, five days after the last injection, were tested for anti *L. infantum* antibodies. Anti-*Leishmania* antibody was purified by affinity chromatography, using protein A Sepharose CL 4B (Sigma Chemical Co.) column. Purified IgG was labeled with Horseradish peroxidase.

Capture ELISA for the detection of the urinary antigen: Capture ELISA for detection of urinary antigen was carried out in flat-bottom 96-well microtiter plates (Dynatech). The plates were coated with 5 µg/ml of the purified IgG (100 µL/well) in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) followed by incubating at 4°C overnight. The plates were washed three times with PBS buffer containing 0.5% Tween 20, pH 7.4 (PBST) every 5 minutes. Urine samples (100 µL) from VL patients along with controls were added and the plate was incubated for 1.5 hours at room temperature followed by washing step as before. Horseradish peroxidase conjugated anti-*Leishmania* antibody (100 µL, in PBST) was added to the plates and incubated for one hour at room temperature. After being washed as before, the plates were incubated with chromogen/substrate (100 µL/well of 0.4 mg/ml OPD, 0.025% H_2O_2 in 0.1 M citrate buffer, pH 5) for 45 min at room temperature and the OD was measured at 490 nm.

Direct agglutination test (DAT): DAT was performed as described by Joshi *et al.* (1993). Briefly, sera samples were diluted in physiological saline (0.9% NaCl) containing 0.78% β-mercaptoethanol and 0.2% gelatin. Two-fold dilution series of the sera were prepared in a V-shaped microtitre plate, starting at a dilution of 1:10 and rising to a maximum serum dilution of 1:6400. Fifty microliter of DAT antigen (concentration of 5×10^7 *L. infantum* per ml) was added to each well already containing 50 µl of diluted serum. The plate wrapped with cling film, rotated gently by hand and incubated at 26°C overnight and then checked against a white background.

Enzyme-linked immunosorbent assay: ELISA was performed with *L. infantum* crude antigens to detect anti-*Leishmania* antibody in serum of VL patients.

RESULTS

Samples were taken from 61 VL cases. Most of patients (80.3%) had a positive IFA test while 38.5% of them had a positive parasitological test using bone marrow aspiration. Bone marrow aspiration was not performed in all cases.

Serum samples from VL patients along with samples from healthy controls and samples from no-VL patients were tested by DAT. Antibody was detected in 43 cases while no positive reaction was found in serum of healthy controls and non kala azar patients. A sensitivity of 70.5% (95% CI= 57.2-81.1) and specificity of 100% (95% CI=91.6-100) was calculated for this assay. A significant agreement (61.5%) was found between DAT and direct parasitological test. When the serum samples were tested by an ELISA system, using crude *L. infantum* antigen, a sensitivity of 83.6 (95% CI= 71.4-91.4) and specificity of 90.5% (95% CI= 78.6-96.5) was found for this assay. An agreement rate of 48% was found between ELISA and bone marrow examination.

The urine samples were tested for detection of leishmanial urinary antigen. The samples were 35 from VL cases and 34

samples from non VL patients and also healthy subjects. Antigen was detected in urine of 21 from 35 cases of VL patients. While none of healthy individuals gave a positive reaction in the assay, three samples from non VL patients, one from toxoplasmosis patient and 2 samples from cutaneous leishmaniasis patients had a false positive reaction with this system. Accordingly, a sensitivity of 60% (95% CI= 42.2-75.6) and specificity of 91.2% (95% CI= 75.2-97.7) was calculated for the antigen detection assay. Statistical analysis showed a significant agreement (Kappa=0.412) between this assay and direct parasitological examination. Also the system had a reasonable agreement with DAT and indirect ELISA (Kapp=.338 and 0.317 respectively), (54.3%), using serum sample. Table 1 shows the features of capture ELISA for detection of urinary antigen and table 2 shows the features of this assay in compare to other antibody detection assays in diagnosis of VL.

Table 1: Features of antigen-based capture ELISA for diagnosis of VL

Test result	Diseases							Total
	VL	HP	M	CL	T	B	H	
+	21	0	0	2	1	0	0	24
-	14	10	3	10	0	3	5	45
Total	35	10	3	12	1	3	5	69

VL: visceral leishmaniasis, HP: healthy person, M: malaria, CL: cutaneous leishmaniasis, T: toxoplasmosis, B: brucellosis, H: hydatidosis.

Table 2: Features of antigen-based capture ELISA in compare to antibody detection assays in diagnosis of VL

Assay	Antigen-based Capture ELISA	DAT	Indirect ELISA
Sensitivity (%)	60	70.5	83.6
Specificity (%)	91.2	100	90.6
PPV (%)	87.5	100	91.1
NPV (%)	68.9	74.6	82.7

PPV: Positive predictive value, NPV: Negative predictive value.

DISCUSSION

Serological methods for diagnosis of VL are mostly based on detection of antibodies in serum and a number of tests including indirect fluorescent antibody test (IFAT), ELISA and DAT have provided a relatively good efficacy in diagnosis of VL (Zijlstra *et al.*, 1998; Rajasekariah *et al.*, 2001; Mikaeili *et al.*, 2007). An alternative to antibody detection is antigen detection and a few assays have been improved for antigen detection in diagnosis of VL. The most studied -antigen detection assay is latex agglutination test (Katex) with a sensitivity ranged from 40-95% and specificity ranged from 90-100% (El-Safi *et al.*, 2003; Sunder *et al.*, 2005; Vilaplana *et al.*, 2004). The main drawback of this test is that the sample needs to be treated (heating at 100°C for 5 min) before testing.

Here we evaluated the usefulness of antigen-based ELISA for diagnosis of VL and compared the results with antibody detection assays. Our results showed that the *Leishmania* antigen can be detected in urine of more than 60% of VL patients by ELISA system. None of the healthy control was positive by the system while 3 samples from non-VL patients gave a positive reaction by the system. The introduced antigen detection assay was not able to detect the antigen in urine of a few of cases and this reduced the sensitivity of the assay which in turn hinder the applicability of the assay. However, when the results was compared with routine antibody detection assays (indirect ELISA, DAT and IFA), a high specificity was found for the introduced assay. ELISA uses urine specimens, rather than serum, which reduces the risk of infection to the person carrying out the test. Comparing our results with other studies which uses urine for diagnosis of VL, pre-treatment of urine sample (boiling), which is a drawback of other antigen detection assays, is not necessary for this ELISA system (El-Safi *et al.*, 2003; Sunder *et al.*, 2005; Vilaplana *et al.*, 2004). The urine based ELISA detect antigen which, as for many antigen detection assays, can be used for monitoring of the treatment in patients.

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