Comparison of Serological Methods (ELISA, DAT and IFA) for Diagnosis of Visceral Leishmaniasis Utilizing an Endemic Strain

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ABSTRACT

Background: The causative agent of visceral leishmaniasis (VL) in Iran is *Leishmania infantum (L. infantum)* (Mediterranean type) and its major reservoir host is the dog. **Objective:** To compare the serological methods including direct agglutination test (DAT), indirect immunofluorescent-antibody test (IFA) and enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of endemic strain of *L. infantum*. **Methods:** 61 blood samples from VL patients referred to Shiraz hospitals and 49 blood samples from control group were collected. Native strain of the parasite isolated from a VL patient from the region was cultured and characterized. Antigens from this *L. infantum* parasite were used in ELISA and IFA system. **Results:** Anti-*Leishmania* antibody was detected in 43 (70.5%), 49 (80.3%) and 51(83.6%) cases using DAT, IFA and ELISA, respectively. Based on these results, sensitivity and specificity of DAT was found to be 70.5% and 100%, respectively. Sensitivities of IFA and ELISA in diagnosis of VL were 80.3% and 83.6% and their specificity was 90.5%. **Conclusion:** Results of this study showed that DAT and ELISA have the highest specificity and sensitivity in diagnosis of VL. DAT is a simple, cost-effective and field applicable test. Thus, it can be recommended for early and accurate diagnosis of VL, especially in regions where malaria, brucellosis and tuberculosis are prevalent.

Keywords: Visceral Leishmaniasis, ELISA, IFA, DAT

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Iran.J.Immunol. VOL.4 NO.2 June 2007

116

INTRODUCTION

Visceral leishmaniasis (VL) or Kala-azar is the most severe form of leishmaniasis caused by *L. donovani* in the Indian subcontinent, Asia and Africa in adult and children and by *L. infantum* or *L. chagasi* in the Mediterranean region, Southwest and central Asia and South America primarily in young children (1). Domestic dogs are the principal reservoir hosts of the Mediterranean type of visceral leishmaniasis. More than 65 countries are currently affected by VL, and approximately 500,000 new human cases occur annually. Visceral leishmaniasis accounts for 75,000 deaths per year (1, 2). The clinical signs of VL in humans include prolonged fever, hepatosplenomegaly, substantial weight loss, progressive anemia and even death (3). The most highly endemic areas of Iran are Fars and Bushehr provinces in the south, the districts of Ardabil province in Northwest and Qom province in the center of Iran (4). The diagnosis of VL is complicated because commonly occurring diseases such as malaria, typhoid and tuberculosis have clinical features similar to VL (5). The laboratory diagnosis of VL is based on parasitological examination, molecular methods and serological techniques (6). Despite enormous efforts directed towards the development of a suitable test for the serological diagnosis of VL, the parasitological diagnosis of VL which relies on detection of parasite in bone marrow or spleen aspirate still remains the "gold standard" method. Serological methods as alternative to parasitological diagnosis are based on detection of antibodies or antigens in samples such as serum, plasma and urine (7). Several serological tests including indirect fluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) have provided good efficacy in diagnosis of VL (8). The sensitivity and specificity of such diagnostic methods depends on the type, source and purity of antigens employed, as some of *Leishmania* antigens have common cross-reactive epitopes shared with other microorganisms (9). The direct agglutination test (DAT) has proved to be a very important serodiagnostic tool combining high levels of intrinsic validity and ease of performance (10). ELISA is used successfully as an effective screening test for VL if the appropriate antigen and optimal conditions for specific antibody reaction are defined (11). Here we report the results of a study on the diagnosis of visceral leishmaniasis comparing the efficacy of DAT, IFA and ELISA for the diagnosis of VL.

MATERIALS AND METHODS

Blood Sampling. Blood samples were collected from sixty-one confirmed VL patients referred to Shiraz hospitals. Control samples (n=53) were obtained from healthy individuals (n=10) and patients with malaria (n=5), tuberculosis (n=4), toxoplasmosis (n=5), brucellosis (n=2), leukemia (n=2), hydatid cyst (n=6) and patients with fever of unknown origin (FUO) (n=19). Collected samples were tested by DAT, IFA and ELISA.

Direct Agglutination Test (DAT). The *L. infantum* Lon-49 (Iranian strain) antigen for this study was prepared in the parasitology department of the School of Health at Tehran University of Medical Sciences. DAT was performed as described by Harith et al (8). Briefly, samples were diluted in physiological saline (0.9% NaCl) containing 0.78% ß-mercaptoethanol and 0.2% gelatin. Two-fold dilution series of the sera were made in a V-shaped microtitre plate, starting at a dilution of 1:10 (step 1) and going up

to a maximum serum dilution of 1:6400 (step 7). Fifty microliters of DAT antigen (concentration of 5×10^{7} parasites per ml) was added to each well containing 50 µl of diluted serum. After two minutes of gentle shaking on a level surface, the plate was covered with a lid and checked after 18 hours. Antibody titer of 1:3200 and above was considered as positive.

Indirect Immunofluorescent Assay (IFA). Indirect immunofluorescent antibody test was performed by using formalin washed promastigotes of *L. infantum,* isolated from a 3 years old VL patient from the region. The serum samples were diluted 1:128 in PBS (0.1 M phosphate, 0.33 M NaCl, pH 7.2) for preliminary screening and the positive samples were serially diluted up to 1:1024 to obtain the real titer of IgG antibody. Ten microliter of each diluted serum was placed in the well of the slides and incubated in a humid chamber at 37°C for 30 minutes. Slides were washed in PBS (two times, each 7 minutes), dried and incubated for 30 minutes at 37°C with fluorescent conjugated rabbit anti-human IgG (Golafshan), diluted 1:100 and Evans blue solution, diluted 1:10000. Slides were washed and air dried. Finally, the samples were observed under immunofluorescent microscope and the titers of 1:128 and above were considered as positive.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA was performed as described by Hommel et al (12). Briefly, flat-bottomed 96-well microtiter plates were coated with 5 µg/mL (100 µL per well) of crude antigens of a native strain of the *L. infantum* isolated from a VL patient from the region. Parasite antigen was incubated overnight at 4°C. The wells were washed three times with 0.5% Tween 20 in PBS (PBST; pH=7.2) washing buffer. After blocking with 3% skimmed milk for one hour at room temperature, 100 µL of serum (1:100 dilution in PBST) was added, and the wells were incubated for 1.5 hours. After five washes with PBST, the plates were incubated with peroxidase-conjugated anti-human IgG (Sigma) (1:4,000 dilution in PBST) for one hour. The plates were then washed five times with PBST. 100 µL per well of substrate was then added and the plates were 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

Among VL patients, 55 cases (90.2%) belonged to children under 10 years of age and 6 cases (9.8%) to adults. 26 (42.6%) out of 61 cases were females and 35 cases (57.4%) were males who frequently lived in nomadic areas.

Serum samples of 61 VL and 53 non-VL patients along with samples from healthy people were tested with DAT. The assay showed a sensitivity of 70.5% (95%CI=57.2%-81.1%) and a specificity of 100% (95%CI=91.6%-100%) (Table1). Positive predictive value (PPV) and negative predictive value (NPV) of the test were 100% (95%CI=89.8%-100%) and 74.6% (95%CI=62.7%-83.9%) respectively. Using IFA a sensitivity of 80.3% (95%CI=68.7%-88.4%) and a specificity of 90.5% (95%CI=79.8%-96%) was found (Table 2). PPV and NPV of the test were 90.7% (95%CI=78.9%-96.5%) and 80% (95%CI=67.3%-88.8%), respectively. When serum samples were tested with ELISA, considering the cut off point of 0.267, the test showed a sensitivity of 83.6% (95%CI=72.4%-91.4%) and a specificity of 90.5% (95%CI=79.6%-96.7%) (Table 3). PPV and NPV of the test were 91.1% (95%CI=79.6%-96.7%) and 82.7% (95%CI=70.1%-91%), respectively. Statistical analysis of the data showed a fair agreement (kappa=0.279) between ELISA and DAT

whereas a better agreement (kappa=0.336) was found between ELISA and IFA. The best agreement was observed between DAT and IFA (kappa=0.389).

Table 1. Sensitivity and specificity of the Direct Agglutination Test(DAT) for diagnosis of VL

| Agglutination score | Sensitivity (%) | Specificity (%) | | | | | | | | |
	VL	H.P	M	T.B	T	B	ALL	H	FUO	Total
+*	43	0	0	0	0	0	0	0	0	0
-	18	10	5	4	5	2	2	6	19	53
Total	70.5%	100%	100%	100%	100%	100%	100%	100%	100%	100%

VL=visceral leishmaniasis; H.P=healthy people; M=malaria; T.B= tuberculosis; T= toxoplasmosis; B= brucellosis; ALL= leukemia; H=hydatid cyst; FUO= fever of unknown origin
* Titer of 1:3200 and above was considered as positive.

Table 2. Sensitivity and specificity of the Indirect Fluorescent Antibody (IFA) test for diagnosis of VL

| Score | Sensitivity (%) | Specificity (%) | | | | | | | | |
	VL	H.P	M	T.B	T	B	ALL	H	FUO	Total
+*	49	0	0	0	1	0	0	0	4	5
-	12	10	5	4	4	2	2	6	15	48
Total	80.3%	100%	100%	100%	80%	100%	100%	100%	78.9%	90.5%

VL=visceral leishmaniasis; H.P=healthy people; M=malaria; T.B= tuberculosis; T= toxoplasmosis; B= brucellosis; ALL= leukemia; H=hydatid cyst; FUO= fever of unknown origin
* Titer of 1:128 and above was considered as positive.

Table 3. Sensitivity and specificity of the Enzyme-Linked Immunosorbent Assay (ELISA) for diagnosis of VL

| Score | Sensitivity (%) | Specificity (%) | | | | | | | | |
	VL	H.P	M	T.B	T	B	ALL	H	FUO	Total
+*	51	0	2	1	0	0	0	0	2	5
-	10	10	3	3	5	2	2	6	17	53
Total	83.6%	100%	60%	75%	100%	100%	100%	100%	89.4%	90.5%

VL=visceral leishmaniasis; H.P=healthy people; M=malaria; T.B= tuberculosis; T= toxoplasmosis; B= brucellosis; ALL= leukemia; H=hydatid cyst; FUO= fever of unknown origin
* The cut off point (0.267) was set at mean ± 2SD.

DISCUSSION

Various noninvasive tests, with different specificities and sensitivities, are available for the diagnosis of leishmaniasis. Parasitological diagnosis which is an invasive method using splenic aspiration or bone marrow biopsy still remains as a gold standard (13). Among the different serodiagnostic methods, the direct agglutination test (DAT) seems to be a simple and suitable serodiagnostic test for VL. Joshi et al (1999) assessed the DAT for diagnosis of VL in Nepal. The specificity and sensitivity of the test in their study was reported as 99.2% and 100%, respectively (9). The direct agglutination test (DAT) based on freeze-dried (FD) *Leishmania donovani* antigen was used in eastern Sudan. The results showed that DAT is a suitable test for diagnosis of kala-azar in remote areas as it is sensitive (96.8%), specific (96.2%) and stable (14). Moreover DAT has been used for seroepidemiological study of VL in Iran by Edrissian et al (15). In a

Iran.J.Immunol. VOL.4 NO.2 June 2007

119

recent extensive study conducted by Mohebali et al, DAT has been used for diagnosis and seroepidemiological study of VL in different regions of Iran (16). In our study, sensitivity and specificity of DAT was found to be 70.3% and 100%, respectively, which is comparable with the previous studies.

IFA test was evaluated by Mukerji et al (1996) in a kala-azar endemic area of Bihar, India. The test was found to be highly sensitive (99%) and specific (99%) (17).

Chaudray et al (1992) employed the IFA test, using *L. donovani* promastigote and amastigote antigens, and showed a very high level of antibody in clinically and parasitologically established cases of kala-azar. None of the subjects from non-endemic area suffering with other parasitic diseases, such as malaria, filaria, amoebiasis, leprosy or tuberculosis gave a positive response to any of the antigens (18). In another study, Kohanteb and Ardehali (2005) evaluated the IFA for diagnosis of VL and showed false-positive results with malaria, tuberculosis, brucellosis and typhoid fever (19).

In our study, the sensitivity of IFA was 80.3%. However, 5 out of 53 patients with other diseases were positive by this method. Comparing these results with those obtained by DAT, it seems that DAT has a better performance in detecting anti-Leishmania antibodies in VL subjects.

In the last few years, an increasing number of *Leishmania* antigens have been employed in ELISA systems for diagnosis of VL. Some of which are considered to be species specific proteins such as gp46, lipophosphoglycan-associated protein KMP11, kinesin, heat shock protein, actin and tubulin (20). Kaul et al (2000) evaluated a 200 KDa antigen fraction from *L.donovani* axenic amastigotes and also *L.donovani* soluble antigen in an ELISA system for the detection of antibody in VL patients. A positive antibody response in confirmed VL cases was seen in 96.6% of cases, when using the 200 KDa fraction and 100% when the amastigote soluble antigens were used (21). In our study ELISA was the most sensitive method for diagnosis of VL.

Considering the other aforementioned assays, it appears that DAT has the highest specificity and ELISA the highest sensitivity in diagnosis of VL in our study. As kala-azar in Iran is prevalent in rural and remote areas such as nomadic regions where facilities for IFA and ELISA are not available, and since DAT is a simple, cost-effective and field applicable method, DAT can be recommended for serodiagnostic and seroepidemiological studies of VL especially where malaria, brucellosis and tuberculosis are prevalent.

ACKNOWNEDGEMENT

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