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Full Length Research Paper

Comparative performance of direct agglutination test, indirect immunofluorescent antibody test, polymerase chain reaction and bone marrow aspiration method

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The objectives of this study were to compare the sensitivities and reliabilities of direct agglutination test (DAT), indirect immunofluorescent antibody test (IFAT), polymerase chain reaction (PCR) and bone marrow aspiration for the diagnosis of Mediterranean visceral leishmaniasis (MVL) using blood samples. Blood samples were collected from 67 confirmed MVL patients and 47 patients with other diseases such as malaria, tuberculosis, echinococcosis, toxoplasmosis and leukemia and; 10 healthy individuals as control group. MVL was more frequently diagnosed in children less than 2 years-old (44.8%). The highest sensitive diagnostic method was IFAT (85.1 %) while the least one was bone marrow aspiration (42.8%). The specificity of DAT (100%) was higher than IFAT (80.8%, CI = 66.3 to 90.4%). Comparison of the two serological methods revealed that DAT had a higher positive predictive value (100%) whereas IFAT had a higher negative predictive value (86.4%). The highest degree of agreement (agreement = 71.6%, Kappa = 0.13) was found between IFAT and DAT. Moreover, the minimum number of false negative results (1 out of 55 patients) was found when IFAT and PCR were applied simultaneously (agreement = 69.1%, Kappa = -0.09). Therefore, we suggest the application of IFAT and PCR methods in endemic areas of visceral leishmaniasis for early and accurate diagnosis and to avoid misdiagnosis of MVL.

Key words: Mediterranean visceral leishmaniasis, indirect immunofluorescent antibody test, direct agglutination test, polymerase chain reaction, bone marrow aspiration.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a systemic disease caused by *Leishmania donovani complex*. In the countries of the Mediterranean basin and Middle East, including Iran, VL is caused by *L. donovani infantum* (Mohebali et al., 2005; Fakhar et al., 2008). The annual occurrence of human visceral leishmaniasis (HVL) cases worldwide is estimated to be 500,000 and accounts for 75,000 deaths (WHO, 2000). Clinical signs and symptoms include prolonged fever, hepatosplenomegaly, substantial weight loss, progressive anemia, and even death (Caldas et al., 2006). Diagnosis of VL is complex because common diseases such as malaria, typhoid, and tuberculosis have clinical features similar to HVL which mentioned above (Singh, 2006). Moreover, some of VL cases have

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been misdiagnosed as autoimmune hepatitis, acute lymphoblastic leukemia, malignant lymphoma, and acute myeloid leukaemia (AML) (Kawakami et al., 1996; Jones et al., 2003; Asgari et al., 2007; Dalgic et al., 2005). Laboratory diagnosis of HVL is based on parasitological examination, molecular methods, and serological techniques (Sundar and Rai, 2002). Demonstration of parasite in spleen's smear is considered as the gold standard test for diagnosis of VL. Although this method is highly sensitive, it can be associated with the risk of serious hemorrhage. Culture, as another parasitological method can apply to improve sensitivity (Lightner et al., 1983).

Immunodiagnostic tests are based upon the detection of antibodies or antigens in serum, plasma, or urine samples. These methods are highly sensitive, noninvasive and more suitable in endemic areas. (Sundar and Rai, 2002). New methods such as fast agglutination screening test (FAST) and immunochromatographic strip test (based on rK39 antigen) have also been developed for use in field conditions (Hailu et al., 2006).

Recently, detection of parasite DNA in tissues (Gatti et al., 2004), serum (Fissore et al., 2004) and urine (Motazedian et al., 2008) via polymerase chain reaction (PCR) is used for early and more specific diagnosis. The PCR assay using peripheral blood as a relatively noninvasive and accessible clinical sample was shown to be highly efficient (Fakhar et al., 2008). Despite the development of these new techniques there are some problems in diagnosis of this disease especially in endemic area because of cross reactivity and sensitivity variation with these tests as well as misdiagnosing with other diseases (Kohanteb and Ardehali 2005; Hailu et al., 2006 Asgari et al., 2007). The objectives of this study were to compare the sensitivities and specificity of different current diagnostic methods for the diagnosis of Mediterranean visceral leishmaniasis (MVL) using blood samples in Fars Province as a main endemic focus in southern Iran, to evaluate and present the suitable test for the diagnosis of HVL.

MATERIALS AND METHODS

Blood sampling and testing

Blood samples were collected from 107 patients suspected of MVL referring to Shiraz hospitals. In addition, control samples (n = 57) were taken from healthy individual (n = 10), 47 patients with other diseases such as FUO (fever of unknown origin) (n = 24), malaria (n = 5), tuberculosis (n = 5), echinococcosis (n = 6), toxoplasmosis (n = 4), brucellosis (n = 2) and leukemia (n = 2).

Two ml of the blood samples were collected in EDTA coated tubes. Samples were centrifuged at 1000 g for 5 min and plasma and buffy coat were separated and liquated into 1.5 ml microtubes and stored at -20°C.

Plasma samples were tested by direct agglutination test (DAT) as described by (Harith et al., 1989). They were diluted 1:3200 (cut off) for preliminary screening and positive samples were serially diluted up to 1:12800 to obtain the real titer of IgG antibody.

The highest titer in which agglutination was still visible was reported.

Plasma samples were tested by indirect fluorescent antibody test (IFAT) as described by Walton et al. (1972). They were diluted 1:128 in PBS (0.1 M phosphate, 0.33 M NaCl, PH 7.2) for preliminary screening and positive samples were serially diluted up to 1:1024 to achieve the real titer of IgG antibody. The cut off IFAT assay for visceral leishmaniasis was considered 1:128 dilutions.

Bone marrow (B.M) aspiration was performed on 63 out of 67 MVL confirmed patients. B.M smears were fixed with methanol, stained with Giemsa and examined under a compound light microscope for the amastigotes.

For the DNA extraction, the buffy coat of 67 confirmed MVL patients and control group (n = 57) were collected and then lysis buffer was added (50 mM of Tris-HCl, pH = 7.6; 1 mM of EDTA, pH = 8.0; 1% Tween 20, 8.5 µl of proteinase K solution, 19 mg/ml) and incubated for 24 h at 37°C. The lysate was then ext racted twice with phenol/chloroform/isoamyl.

The DNA was precipitated with absolute ethanol and resuspended in 100 µL of double distilled water and stored at 4°C (Motazedian et al., 2002).

For the PCR screening, we adapted the PCR protocol previously described by Lachaud et al. (2000) for identification of *Leishmania* in the level of genus. This method uses the primers RV1 (5-CTTTTCTGGTCCCGCGGGTAGG-3) and RV2 (5-CCACCTGGCCTATTTTACACCA-3) to amplify a fragment of 145 bp present on the highly reiterated minicircles of KDNA. The procedure was the same as described by Fakhar et al.(2008).

The set of primers LIN-4 (forward: 5'-GGGGTTGGTGTAAAATAGGG-3'),LIN-17(reverse:5'-TTGAACGGGATTTCTG-3'), a major modification of the assay described by Aransay et al. (2000), was used for species- specific PCR in order to identify *Leishmania* species. The same protocol was used as described by Fakhar et al. (2008).

Finally, the results were analyzed by SPSS and EPI info softwares using Chi-Square test and a P-value < 0.05 was considered statistically significant.

RESULTS

A total of 107 patients suspected of VL, 67 were confirmed (clinically and response to therapy together with at least one positive diagnostic test) to be infected with MVL. Of 67 MVL patients, 55.2% (n = 37), 44.8% (n = 30) were males and females, respectively. The range of age groups was from 3 months to 33 years-old with the mean of 5.7 years-old. MVL was more frequently observed in children less than 2 year-old (44.8 %) and least common in 10 year-old and above group (7.5 %).

Only 31.3% (21/67) of 67 confirmed VL patients were found positive in all four diagnostic tests. In contrast, 4.5%, 40.3 and 23.9% of these patients were positive by one, two, and three methods, respectively.

Nine of 24 patients with fever of unknown origin (FUO) were false positive by IFAT; because they have been treated for leishmaniasis with no success, or the origin of fever was discovered later on, while all subjects infected with other diseases were negative by DAT and PCR, (Tables 1 and 3).

The most sensitive (85.1%) (95%, CI = 73.8 to 92.3%) diagnostic method was IFAT, (Table 2) and the least sensitive (42.8%) (95%, CI= 28.8 to 53%) was bone

Table 1. Sensitivity and specificity of the direct agglutination test (DAT) for diagnosis of HVL.

	Sensitivity (%)				Specificity (%)					
Score	VL	H	M	T.B	Toxo	Br	L	E	F.U.O	Total
Positive*	50	0	0	0	0	0	0	0	0	0
Negative	17	10	5	5	4	2	2	6	24	57
Total (%)	74.6	100	100	100	100	100	100	100	100	100

VL = visceral leishmaniasis; H= healthy people; M=malaria; T.B = tuberculosis; Toxo= toxoplasmosis; L = leukemia; E = echinococcosis; FUO = fever of unknown origin Br= brucellosis; *Titer of 1:3200 and above was considered as positive.

Table 2. Sensitivity and specificity of the indirect fluorescent antibody test (IFAT) for diagnosis of HVL.

	Sensitivity (%)				Specificity (%)					
Score	VL	H	M	T.B	Toxo	Br	L	E	F.U.O	Total
Positive*	57	0	0	0	0	0	0	0	9	9
Negative	10	10	5	5	4	2	2	6	15	48
Total %	85.1%	100	100	100	100	100	100	100	62.5	84.2

VL = visceral leishmaniasis; H= healthy people, M=malaria; T.B = tuberculosis; Toxo = toxoplasmosis; Br= brucellosis; L = leukemia; E = echinococcosis; FUO = fever of unknown origin. *Titer of 1:128 and above was considered as positive.

Table 3. Sensitivity and specificity of the polymerase chain reaction (PCR) for diagnosis of HVL.

	Sensitivity (%)				Specificity (%)					
Score	VL	H	M	T.B	Toxo	Br	L	E	F.U.O	Total
*Positive	55	0	0	0	0	0	0	0	0	0
Negative	12	10	5	5	4	2	2	6	24	57
Total %	82.1%	100	100	100	100	100	100	100	100	100

VL = visceral leishmaniasis; H= healthy people, M=malaria; T.B= tuberculosis; Toxo = toxoplasmosis; Br = brucellosis; L = leukemia; E = echinococcosis; FUO= fever of unknown origin.

marrow aspiration. The specificity (100%) (95%, CI=90.5 to 100%) of DAT was higher than IFAT (80.8%) (95%, CI=66.3 to 90.4%) (Table 1).

Comparing the serological tests revealed that DAT had the highest positive predictive value (100%), while IFAT had the highest negative predictive value (86.4%).

As a whole, the efficacy of IFAT (91.2%) was higher than DAT (85.1%). The highest degree of agreement (agreement = 71.6%, Kappa = 0.13) was found between IFAT and DAT, as 44 and 4 out of 67 MVL patients were positive and negative in both tests, respectively (Table 4). Moreover, the minimum number of missed cases was found when these two tests (IFAT and DAT) were performed together, compared to other combination of paired groups.

The fair degree of agreement (agreement = 69.1%, Kappa = 0.09) was observed between IFAT and PCR.

Bone marrow aspiration and PCR had the least degree of agreement (agreement = 49.2%, Kappa = 0.06) as only 23 and 8 out of 67 MVL patients were positive and negative in both tests respectively. The maximum number of false negative results (8 /63 patients) was found when using PCR and bone marrow aspiration, (Tables 1, 2 and 3).

DISCUSSION

We compared the efficacy of four diagnostic methods including DAT, IFAT, PCR, and bone marrow aspiration on 67 VL confirmed patients. Demonstration of *Leishman* bodies (amastigotes) in spleen, bone marrow, and lymph node aspirations is considered as the gold standard test for diagnosis of VL. (Sundar and Rai, 2002). Although splenic aspiration can be associated with the risk of serious hemorrhage and long searches may be required to demonstrate the parasite in bone marrow smears, as reduction of microscopic search time from 30 min to 1 min will reduce the sensitivity from 92 to 40.2% (da Silva et al., 2005). We believe that the low sensitivity (42.8%)

Table 4. The Sensitivity, specificity, PPV, NPV, validity and efficacy of different diagnostic tests for diagnosis of HVL.

Test	B.M smear	IFA	DAT	PCR
Sensitivity (%)	42.8	85.1	74.6	82.1
Specificity (%)	100	80.8	100	100
Positive Predictive Value (PPV) (%)	100	86.4	100	100
Negative Predictive Value (NPV) (%)	56.6	79.2	73.4	9.7
Validity (%)	71.4	82.9	87.3	91
Efficacy	64.9	91.2	85.1	89.5

of this method in our study could be the result of low parasitemia, diluted B.M through aspiration materials and possibly due to inadequate time spent by the pathologist to view microscopic fields. Moreover, bone marrow aspiration is a painful and the sensitivity ranges from 76 to 85% reported in most studies (Singh, 2006).

The direct agglutination test has been used in epidemiologic studies in several endemic areas and is used in large-scale screening of VL as a simple, valid test with high sensitivity and specificity (Mohebali et al., 2005). The sensitivity and specificity of this method varies in different studies between 90 to 100% and 72 to 100%, respectively (Sundar and Rai, 2002).

In our study, the sensitivity of DAT (74.6%) was low which might on one hand be due to DA antigen's fragility and sensitivity to vigorous shaking , and on the other hand, the inefficacy of immune system in production of antibody at the age of 6 months and below. (Caldas et al., 2006; Gatti et al., 2004). However, the specificity of the test (100%) in this study was higher than in other studies (Chappuis et al., 2006).

Several studies on IFAT have reported a sensitivity of 55 to 95%, and specificity of 70 to 89% (Sundar and Rai, 2002; Choudhry et al., 1992). According to researches in Iran, this test has low specificity and has cross-reaction with typhoid fever, tuberculosis, malaria, toxoplasmosis, and brucellosis (Kohanteb and Ardehali 2005). However, DAT has shown high specificity without cross-reaction with these diseases but it has less sensitivity (Edrissian, 1996; Mikaeili et al., 2007). Our findings are similar to our (Mikaeili et al., 2007) and other previous studies.

Recently, the use of Polymerase chain reaction with high sensitivity (70–100%) and specificity (100%) has become popular in different parts of the world (Sundar and Rai, 2002; Singh, 2006), and the sensitivity of 82.1% and specificity of 100% in our study is similar to previous findings. The PCR technique has several advantages including the ability to work with small amounts of target material, fast detection of *Leishmania* in symptomatic patients and asymptomatic carriers. Moreover, it was applied to detect Leishmania/HIV co-infected patients and the follow up of treatment (Maurya et al., 2005; Bossolascoet al., 2003). Six primer pairs were compared for detecting *L. infantum* DNA by Gao et al., (2006). The primer pairs RV1-RV2 (0.1 parasite/ml blood) were most sensitive and suitable in detecting the asymptomatic infection of *L. infantum*, and the prevalence of the asymptomatic infection is high in human population in the endemic area. In our study, we applied RV1- RV2 primers set as described above. The PCR on peripheral blood sample can be used for treatment response evaluation, and it is also very efficient for early detection of the disease especially in patients with cryptic infection, small children, and immunocompromised patients (Campino et al., 2000) and also subclinical and asymptomatic infections in endemic area (Fakhar et al., 2008).

The sensitivity of PCR on peripheral blood sample depends on a variety of factors including the type of primer (genomic DNA versus. kinetoplast DNA), parasite species, parasite concentration in peripheral blood, type of gel (agarose versus. polyacrylamide), and DNA extraction method. The sample used for extraction is also very important, as the use of buffy coat instead of whole blood, can increase the sensitivity up to 10 times (Lachaud et al., 2002). We used buffy coat for DNA extraction.

According to a study by Disch et al.,(2004) the clearance time of *Leishmania infantum* KDNA from blood was 37 days after the start of treatment. In 5 patients participating in our study, the parasite could not be detected after 14 days or in some cases 21 days of treatment with Amphotericin B, and the symptoms of the disease had also subsided.

There were also a 3-month-old, a 5-month-old, and 32-year-old patients who were only positive in PCR and all their other tests were negative. Consequently, PCR assay is helpful in detection of early stages of the disease, in patients whose antibody production has not been triggered yet due to infancy or immunosuppressant, or in patients with subclinical infections whose serological tests become negative or below the threshold for positivity.

In the present study, the minimum number of missed cases was found when IFAT and PCR tests were performed together. Consequently, we suggest the use of IFAT and PCR methods to avoid misdiagnosis of MVL with other diseases. Most of misdiagnosed patients were referred from non-endemic regions where the occurrence of the disease is not expected by the physician.

Moreover, atypical cells and different blast may be observed in bone marrow aspiration of VL patients. (Asgari et al., 2007).

Based on our study, the simultaneous use of IFAT and PCR is proper for decisive diagnosis of the disease. This is especially important in endemic areas of the disease where subclinical and asymptomatic forms of the disease in adulthood and childhood with cryptic infections are frequent.

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