Rapid detection of human *Leishmania infantum* infection: A comparative field study using the fast agglutination screening test and the direct agglutination test

Behnaz Akhoundi a, Mehdi Mohebali a,*, Leila Babakhan b, Gholam-Hossein Edrissian a, Mohammad-Bagher Eslami c, Hossein Keshavarz a, Hossein Malekafzali d

a Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, P.O. Box: 14155-6446, Tehran, Iran
b Department of Microbiology, Ghom University of Medical Sciences, Ghom, Iran
c Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
d Department of Biostatistics and Epidemiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Received 16 March 2010; received in revised form 4 September 2010; accepted 7 September 2010
Available online 12 October 2010

KEYWORDS
Leishmania infantum infection; Human; Fast agglutination screening test; Iran

Summary This study aimed to evaluate the performance of a fast agglutination screening test (FAST) for serodiagnosis of human *Leishmania infantum* infection in Iran. FAST is based on the direct agglutination test (DAT) but combines with a higher parasite concentration and is performed with only one serum dilution.

The validity of FAST for the detection of *L. infantum* infection in the field was compared with the direct agglutination test on 110 confirmed or patients suspected of infection with leishmaniasis, 177 healthy individuals and 41 patients with other infectious diseases who were from northwestern and southern parts of Iran. In this study, we found a 1:1600 cut-off point empirically by seeking the best correlation (90.8) between sera confirmed with visceral leishmaniasis and healthy control sera.

A sensitivity of 95.4% (95% CI, 91.4--99.4) and specificity of 88.5% (95% CI, 84.2--92.8) were found with 1:1600 as a cut-off titer when DAT-confirmed cases were compared with the control groups. A good degree of agreement was found between FAST and DAT (90.8%) by Kappa analysis. FAST requires 2 h for reading the results versus the 12--18 h needed for DAT.

* Corresponding author. Tel.: +98 2188951400; fax: +98 2188968258.
E-mail addresses: mohebali37@yahoo.com, mohebali@tums.ac.ir (M. Mohebali).
Introduction

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania*. Visceral leishmaniasis, also known as kala-azar in Asia, is the most severe form of the disease and if left untreated, the disease can have a fatality rate as high as 100% within two years (http://www.who.int/topics/leishmaniasis/en). Visceral leishmaniasis is caused most commonly by species pertaining to the *Leishmania donovani* complex: *L. (L.) donovani* in the Old World and *L. (L.) infantum* or *L. (L.) chagasi* in the Old World and the New World, respectively. Visceral leishmaniasis is common in less developed countries; with an estimated 500,000 new cases each year. Mediterranean visceral leishmaniasis is a potentially fatal protozoan infection that is endemic in some parts of Iran. Almost 68.4% of human visceral leishmaniasis cases in Iran have been detected in rural areas of the northwestern and southern regions, where health facilities are not well established and visceral leishmaniasis often co-exists with brucellosis, tuberculosis and other infectious diseases. Parasitological, serological and molecular methods are used for the diagnosis of human visceral leishmaniasis. Parasitological tests comprise commonly microscopic examination of bone marrow and spleen smears, but these methods are invasive. Although the IFAT and ELISA are two important serological methods for diagnosis of human visceral leishmaniasis, they require specific materials and equipment. Other immunodiagnostic methods, such as the latex agglutination test (KAtex) and recombinant antigens (rK39, rK26, rKe16), have limitations, such as low specificity in Sudanese subjects in the absence of clinical visceral leishmaniasis.

Molecular methods and serology based on recombinant antigens are very sensitive but have high variations of specificity and also require sophisticated equipments. Rapid and early detection of human *Leishmania infantum* infection by a fast, sensitive and non-invasive tool is highly necessary because it helps to initiate prompt treatment and subsequently to decrease the mortality rate of human visceral leishmaniasis.

Of the available serological tests for the diagnosis of visceral leishmaniasis, DAT is simple, highly specific and sensitive, cost-effective, reliable, safe and adaptable to micro-titer plates. Currently, DAT has been used for seroepidemiological studies of human visceral leishmaniasis in human and animal reservoirs in the northwestern and southern areas of Iran, where kala-azar is endemic. Although the DAT has high sensitivity and specificity, it has some limitations, particularly in the field; these include a need for long incubation time (12–18 h) and for serial dilutions of serum samples to read the final results. To circumvent these problems, a fast agglutination screening test (FAST) for rapid and early detection of anti-*Leishmania* antibodies was developed. The principal advantages of this method are its need for only one serum dilution and its short incubation time. In this study, we developed and modified FAST as a simple, practical, reliable and economical technique for epidemiological surveys and for rapid diagnosis of human *L. infantum* infection.

Materials and methods

Study area

The investigation was conducted over a period of 2 years (from 2007 to 2008) in the Meshkin-Shahr and Moghan districts in the northwest and the Shiraz suburbs in the south parts of Iran, where visceral leishmaniasis is endemic. Field laboratories were set up at the two locations for this purpose. No transmission of any other *Leishmania* spp. besides *L. infantum* was reported in humans in the studied areas.

Serum samples

Finger prick blood samples (~50 μL) were collected from confirmed and clinically suspected visceral leishmaniasis patients with fever, paleness, anemia, splenomegaly and hepatomegaly and control groups in the fields using micro-hematocrite tubes.

The blood was centrifuged at 3200 g for 5–10 min and the sera were separated. All of the serum samples were divided into four groups:

1. Sera that were clinically, parasitologically and serologically (DAT, IFA, rK39 and rKe16) positive (n = 77) and within a 2 years from clinically suspected and DAT positive (n = 103) 3. Sera from healthy controls without any clinical signs or symptoms that were DAT negative (n = 177) and 4. Sera from infectious diseases other than visceral leishmaniasis (n = 2,41), including cutaneous leishmaniasis (n = 12), hydatidosis (n = 5), malaria (n = 4), leprosy (n = 4), toxoplasmosis (n = 3), hepatitis B (n = 3), tuberculosis (n = 5), fascioliasis (n = 3), HIV+ (n = 1) and borreliosis (n = 1).

This study was approved by the Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran.

Preparation of FAST antigen

Antigen was prepared in the Protozoology Unit of the School of Public Health at Tehran University of Medical Sciences by modifying the methods of Harith and Schoone. In brief, promastigotes of *L. infantum* MCAN/IR/07/Mohebgh.(GenBank accession no. FJ555210) were cultivated in RPMI 1640 containing HEPES, L-glutamine, 20% FCS and
penicillin and streptomycin. They were harvested by centrifugation at 3200 g at 4 °C for 20 min and washed 3 times with cold Locke’s solution (pH 7.2–7.4). Then, digestion and treatment of surface proteins was performed with 0.4% trypsin. After fixation with 2% formaldehyde, the promastigotes were stained with 0.02% Coomassie brilliant blue (R-250) overnight at 4 °C and the final parasite density of the FAST antigen was adjusted to $1 \times 10^{6}$ promastigotes/ml in 1.2% formaldehyde-citrate saline.

**Analysis by FAST**

Twenty microliters of FAST antigen were added to an equal volume of 1:100 diluted sera in 0.9% normal saline and 0.78% 2-mercaptoethanol in V-shaped plate. After 2 h incubation at 30 °C in a refrigerated incubator, the results were read by two individuals separately. For evaluation of reproducibility of the FAST method, 19 serum samples were selected using a table of randomized numbers and were retested 3 times by FAST under the same conditions.

**Preparation of DAT antigen and performance of DAT test**

Direct agglutination antigen was prepared in the protozoology unit of the School of Public Health at Tehran University of Medical Sciences. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of Iranian strain of *L. infantum* (MCAN/IR/07/Moheb-gh. GenBank accession no. FJ555210) in RPMI 1640 plus 20% fetal bovine serum, trypsinization of the parasites, staining with Coomassie brilliant blue and fixing with 2% formaldehyde.

In this method, serum samples were diluted 1:10 to 1:102,400 in a V-shaped micro-titer plate into a dilution fluid containing 0.9% saline and 0.78% 2-mercaptoethanol. One equal volume (50 μL) of antigen suspension was added to each well. The results were read after 12–18 h incubation in a wet room at room temperature. The titer was defined as the highest dilution at which agglutination was still visible in comparison with positive and negative controls. Compact blue dots were scored as negative and large diffuse blue mats as positive.

The visceral leishmaniasis-positive control serum was taken from pooled serum consisting of 5 separate sera that were confirmed by parasitological and serological methods, such as dipstick rk39, IFA ($\geq 1:320$) and DAT (1:102,400). Negative control sera were prepared from individuals without measurable titers of anti-*Leishmania* antibodies from areas non-endemic for visceral leishmaniasis.

**Hemolytic degrees of the collected serum samples**

Based on hemolytic degree, the collected sera were divided into five groups, consisting of negative (−), weak (+), moderate (++) and strong (+++) and very strong (++++)). All of the sera were tested by FAST and DAT separately.

**Parasitological study**

Bone marrow aspirations were performed on 7 DAT-positive patients ($\geq 1:1600$) who had clinical signs and symptoms of visceral leishmaniasis. The prepared smears were fixed with methanol, stained with Giemsa and examined microscopically for the presence of amastigotes.

**Statistical analysis**

For the correlation finding between sensitivity and specificity of DAT and FAST with different potential cut-off points, we used from a receiver-operator characteristic (ROC) curve based on Altman’s method using SPSS 13.5. The true-positive rate (on the vertical axis) against the false-positive rate (on the horizontal axis) showed for each anti-*Leishmania* antibodies titers from 1:400 to 102,400 on the constructed ROC curve. All data were compared using the chi-square test ($X^2$) with a confidence level of 95% SPSS 13.5. A probability value ($P < 0.05$) was considered statistically significant. The agreement between the two methods was assessed using Epi info version 6.

The sensitivity and specificity of the DAT and FAST were calculated with the following formula: Sensitivity = TP/ (TP + FN)*100% and specificity = TN/(TN + FP)*100% where TP represents true positive, TN true negative, FN false negative and FP false positive. Data from the repeatability experiment were used to estimate the kappa value for the DAT and the FAST method. A kappa value of 1 represents perfect repeatability.

**Results**

Table 1 shows the results of FAST (1:100 dilution) and DAT (cut-off titer at 1:1600) on 328 human serum samples which were collected from patients with confirmed visceral leishmaniasis and clinically suspected visceral leishmaniasis, healthy controls and patients suffering from other infectious diseases. We could perform bone marrow aspiration on only seven patients with clinical manifestations including fever (of at least two weeks), anemia and hepatosplenomegaly. These patients showed different titers of anti-*Leishmania* antibodies (from 1:1600 to 1:102,400) by DAT, and amastigote forms of *Leishmania* sp. were also seen in their bone marrow aspiration materials. It should be mentioned that FAST was positive for all seven of the patients who were positive by parasitology and DAT.

Also, 218 (66.5%) serum samples were found negative and 110 (33.5%) were positive with DAT using a cut-off titer of 1:1600, while 198 (60.4%) sera were found negative and 130 (39.6%) were positive by FAST (Table 2). Based on DAT-positive results at titers of 1:1600 and higher, five of the DAT-positive sera (2 sera at 1:1600, 1 serum at 1:12,800, 1 serum at 1:25,600 and 1 serum at 1:102,400 titers) were found to be FAST-negative, while 25 of them had false positive results.

Table 3 shows the calculation of sensitivity and specificity rates of FAST for the complete series of confirmed and control sera and the agreement between the two tests that were analyzed separately. The best degree of agreement (90.8%) was observed between DAT (cut-off point = 1:1600) and FAST by kappa analysis ($p < 0.01$). The 1:1600 cut-off
point corresponded best to distinct when VL-confirmed cases were compared with the control groups. A sensitivity of 95.4% (95% CI, 91.4–99.4) and specificity of 88.5% (95% CI, 84.2–92.8) were found at the 1:1600 cut-off titer. Forty four out of 110 DAT-positive (40.0%) and 55 (42.3%) of 130 FAST-positive sera were showed different degrees of hemolysis. No statistical correlation was found between the FAST and DAT results with hemolytic sera (P = 0.718).

The reproducibility of the FAST method was determined to be 97% by re-testing three times on a few of the positive and negative serum samples.

Discussion

Rapid detection, early case-finding and treatment are essential components for reducing visceral leishmaniasis mortality and morbidity rates in endemic areas. As the clinical manifestations of visceral leishmaniasis have low specificity, confirmatory tests are required to decide which patients should be treated. These tests not only must be highly sensitive, but they also need to be specific because the current drugs used to treat visceral leishmaniasis are quite toxic. The most important limitation for the evaluation of serological tests is the imperfect standard for diagnosing visceral leishmaniasis and the absence of an appropriate gold standard. A definite diagnosis of visceral leishmaniasis depends upon the demonstration of the amastigote form of *Leishmania* parasites in bone marrow aspirates or in biopsy materials from the spleen, lymph nodes and liver. This procedure is invasive, and thus is acceptable only in the case of clinical suspicion of the disease. It has varying sensitivity. Parasitological confirmation might be the best standard for diagnosing visceral leishmaniasis, but not for diagnosing infection with the *L. donovani* complex in a community at risk.

In Iran, DAT is performed routinely for the diagnosis and for seroepidemiological studies of visceral leishmaniasis because it is simple and highly sensitive. Neither parasitological methods nor response to treatment is acceptable as a gold standard for the detection of *L. infantum* infection. Reciprocal titers of 1:1600 and 1:3200 serum dilutions were subsequently used for the detection of *L. infantum* infection in Iran. We used a cut-off titer of 1:1600 for detection of *L. infantum* in humans because the highest sensitivity and specificity rates and desirable concordance were observed at this titer. Nevertheless, the choice of a cut-off value cannot be based only on sensitivity and specificity because the predictive values of the test also depend on the epidemiological context and on the prevalence of infection.

When the prevalence rate is approximately 7.0% in visceral leishmaniasis endemic areas, the positive predictive value (PPV) will be 39%. If the prevalence rate is somewhat lower in non-endemic areas (2.0%), only 15% of FAST-positive individuals will actually have visceral leishmaniasis.

Of course, the negative predictive value (NPV) is more important throughout the whole range of prevalence rates. When the prevalence rate is approximately 7.0%, in visceral leishmaniasis endemic areas of Iran, the NPV will be calculated to be 99.6%, and when the prevalence rate is 2.0% in non-endemic areas, the NPV will be 99.9%. A few studies have been conducted to establish the sensitivity and specificity rates of FAST for diagnosis of the clinical form of the disease, both in visceral leishmaniasis endemic and non-visceral leishmaniasis endemic locations. A sensitivity of 91.1–100% and a specificity of 70.5–95.7% were reported for the diagnosis of humans with visceral leishmaniasis in a few studies. Interestingly, in our study, all seven of the parasitologically positive patients (confirmed visceral leishmaniasis) were DAT (≥ 1:1600) and FAST-positive. It is difficult to compare the results of different studies on the performance of FAST because of differences in reference tests (spleen or bone marrow aspirates, IFA test or DAT), the different populations studied and the various endemic/non-endemic locations of endemic areas.

### Table 1. Results of FAST (1:100 dilutions) and DAT (Cut-off value = 1:1600) of 328 human serum samples.

<table>
<thead>
<tr>
<th>Individual category</th>
<th>No.</th>
<th>Average of age groups (Years)</th>
<th>FAST + Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Visceral leishmaniasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— Confirmed (Clinical+, Microscopy+ and DAT+)</td>
<td>7</td>
<td>2.9</td>
<td>7</td>
</tr>
<tr>
<td>— Clinically suspected (Clinical+ and DAT-)</td>
<td>103</td>
<td>14.8</td>
<td>98</td>
</tr>
<tr>
<td>II Non-Visceral leishmanias</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— Healthy individuals (Clinical− and DAT−)</td>
<td>177</td>
<td>34.6</td>
<td>22</td>
</tr>
<tr>
<td>— Other infectious diseases (DAT−)</td>
<td>41</td>
<td>25.1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>328</td>
<td>19.3</td>
<td>130</td>
</tr>
</tbody>
</table>

### Table 2. Comparison between DAT (cut-off = 1600) and FAST results for human serum.

<table>
<thead>
<tr>
<th>Test</th>
<th>FAST+</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>DAT+</td>
<td>105</td>
<td>95.4</td>
<td>5</td>
<td>4.6</td>
<td>110</td>
</tr>
<tr>
<td>DAT−</td>
<td>25</td>
<td>11.5</td>
<td>193</td>
<td>88.5</td>
<td>218</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>39.6</td>
<td>198</td>
<td>60.4</td>
<td>328</td>
</tr>
</tbody>
</table>
leishmaniasis. However, studies of rapid detection of human *Leishmania infantum* infection and asymptomatic or sub-clinical forms of the infection in the field are limited.

In our study, five of the FAST-negative serum sample was DAT-positive; therefore, the sensitivity of the FAST is calculated 95.4%. FAST specificity is lower because 25 of the DAT negative sera was FAST positive; therefore, the specificity of FAST is calculated 88.5% with high negative predictive value (99.6%). The biggest limitation of FAST seems to be false positive reactions caused by higher parasite concentrations (1 x 10^9 promastigotes/ml) and by low serum dilutions; this leads to low specificity.

In conclusion, FAST can be used as a qualitative screening test to detect of large population of suspected individuals in VL endemic areas. With FAST, the final data often can be available in the same day after 2 h because it is not necessary to re-test those sero-negative samples with DAT. The advantages of short incubation period FAST can be used as qualitative screening test in the VL endemic area. FAST positives samples could be tested further with DAT for additional confirmation. Therefore, large numbers of serum samples can be easily screened with combination of the two tests at the short time.

Further research on test validation with larger populations and dried blood samples on appropriate filter papers in endemic and non-endemic areas of visceral leishmaniasis is recommended.

**Conflict of interest**

None.

**Acknowledgements**

This investigation was financially supported by National Institute of Health Research (NIHR), Tehran University of Medical Sciences (Project no: 241/1441). The authors thank Mr. Z. Zarei, Mrs. S. Charehder, Dr. H. Hajjaran, Mrs. N. Mirsamadi, Mrs. S. Molaei, Dr. Gh. R. Khanbaba, Dr. S. Shojaei, Mrs. A. Motevalli Haghi, Mr. S.E. Skandari, Mrs. A. Mir Amin Mohammad, Miss M. Roohnavaz, Dr. M.B. Rokni, Mrs. F. Mikaeli, Mr. Q. Asgari, Mrs. A. Mohammadia and other colleagues from the District Health Centers in the visceral leishmaniasis endemic areas of Iran for helping with the preparation of serum samples. We declare that there is no conflict of interest.

**References**

14. Harith A, Kolk AHJ, Kager PA, Leeuwenburg J, Muigai R, Kigus, and other colleagues from the District Health Centers in the visceral leishmaniasis endemic areas of Iran for helping with the preparation of serum samples. We declare that there is no conflict of interest.

### Table 3

<table>
<thead>
<tr>
<th>DAT titer</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:400</td>
<td>89.3 (83.6–94.8)</td>
<td>89.4 (85.0–93.6)</td>
<td>89.3</td>
</tr>
<tr>
<td>1:800</td>
<td>91.4 (93.3–99.9)</td>
<td>89.1 (84.7–93.3)</td>
<td>89.9</td>
</tr>
<tr>
<td>1:1600</td>
<td>95.4 (91.4–99.4)</td>
<td>88.5 (84.2–92.8)</td>
<td>90.8</td>
</tr>
<tr>
<td>1:3200</td>
<td>97.1 (93.9–100)</td>
<td>87.4 (83.0–91.8)</td>
<td>90.5</td>
</tr>
<tr>
<td>1:6400</td>
<td>96.8 (93.2–100)</td>
<td>83.3 (78.5–88.1)</td>
<td>87.2</td>
</tr>
<tr>
<td>1:12800</td>
<td>96.4 (92.4–100)</td>
<td>80.2 (78.5–88.1)</td>
<td>84.4</td>
</tr>
<tr>
<td>1:25600</td>
<td>97.2 (93.4–100)</td>
<td>76.6 (71.3–81.7)</td>
<td>81.1</td>
</tr>
<tr>
<td>1:51200</td>
<td>98.3 (95.0–100)</td>
<td>73.2 (67.8–78.6)</td>
<td>77.7</td>
</tr>
<tr>
<td>1:102400</td>
<td>98.1 (94.4–100)</td>
<td>71.6 (66.2–77.0)</td>
<td>75.9</td>
</tr>
</tbody>
</table>

Values in parentheses are exact binomial 95% confidence limits.


