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ORIGINAL PAPER

Rapid detection of *Leishmania infantum* infection in dogs: a comparative study using fast agglutination screening test (FAST) and direct agglutination test (DAT) in Iran

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Abstract Canine visceral leishmaniasis (CVL) is not only a veterinary problem but has also a serious public health importance. Rapid detection of CVL is highly important for control of human visceral leishmaniasis in Iran. This study was aimed to compare the fast agglutination screening test (FAST) with direct agglutination test (DAT) as a standard serological test for the detection of anti-*Leishmania* antibodies on dog serum samples. DAT and FAST antigens were prepared in the School of Public Health, Tehran University of Medical Science. Altogether, 73 serum samples from *Leishmania infantum* infection dogs and 74 sera from healthy controls were collected from human VL/CVL endemic and non-endemic areas of Iran, respectively. All the sera were evaluated with both FAST and DAT techniques. A sensitivity of 98.60% (95% CI, 98.57–98.62) and specificity of 78.70% (95 CI%, 69.20–88.20) were found at a 1:160 -(cut-off) titer when DAT confirmed cases were compared with healthy control. A good degree of agreement was observed between FAST and DAT (86.8%) by kappa analysis ($p<0.01$). In conclusion, this study showed that FAST is very practical and simple diagnostic tool for the sero-diagnosis of CVL in endemic areas of Iran.

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Introduction

Leishmaniasis is endemic in almost 88 countries with 350 million people at risk, 12 million patients, and 2 million new cases per year (Desjeux 2001). Visceral leishmaniasis (kala-azar) is the most important parasitic disease in some areas of Ardabil Province in the northwest and Fars Province in the south of the Islamic Republic of Iran (Nadim et al. 1978; Mohebali et al. 2005, 2006). In other provinces of the country, the disease has been reported in sporadic form (Nadim et al. 1978). Domestic dogs (*Canis familiaris*) are principal reservoir hosts of zoonotic visceral leishmaniasis (Ashford et al. 1998) and may play an important role in long-term maintenance of human VL (Bettini and Gradoni 1986).Therefore, one of the most important approaches to reduce the incidence of human VL (kala-azar) is to cull infected dogs (Ashford et al. 1998). Some diagnostic methods, like microscopical examination of Giemsa-stained smears or cultivation of bone marrow aspirates, are highly specific but not sensitive procedure (Piarroux et al. 1994).Others like the polymerase chain reaction are not practical for processing large numbers of samples, and their use should be restricted to the diagnosis of active cases of disease (Quinnell et al. 2001).Serology is probably the most widely used method for determination of *Leishmania* prevalence in canines because it is rather easy to perform and provides valuable information in a relatively short time (Scalone et al. 2002).Current diagnostic methods used for *Leishmania* mass-screening surveys including enzyme-linked immunosorbent assay and immunofluorescence antibody test have various sensitivity or specificity caused by required technological expertise and specialized laboratory equipment (Harith et al. 1989; Scalone et al. 2002). Direct agglutination test (DAT) is a relatively simple test with high sensitivity and specificity (Harith et al. 1989;

Table 1 DAT and FAST results in 147 serum samples collected of dogs from endemic and non-endemic areas of Iran

DAT		FAST	
Titer	(*n*)	+ (*n*)	-(*n*)
<80	46	5	41
80	15	6	9
160[a]	13	9	4
320	10	10	0
640	21	20	1
1,280	15	15	0
2,560	1	1	0
5,120	4	4	0
10,240	16	16	0
≥20,480	6	6	0
Total	147	92	55

[a] DAT cut-off >1:160

Edrissian et al. 1996; Mohebali et al. 2005).A drawback of the DAT is the relative long incubation time of 18 h and the need for serial dilutions of blood or serum. Therefore, fast agglutination screening test (FAST) was developed for the rapid detection (<3 h) of anti-*Leishmania* antibodies in serum samples (Schallig et al. 2002). In this study, we report the sensitivity and specificity of FAST compared to DAT as standard method for the detection of anti-*Leishmania* antibodies in canine visceral leishmaniasis (CVL) by two batches of DAT and FAST antigens, which were prepared in our laboratory at the School of Public Health, Tehran University of Medical Sciences.

Materials and methods

Sampling

Two groups of dog sera were used: dog sera with suspected clinically including lymphadenopathy, hair shedding, dermatitis, abdominal distention, and DAT positive (n=73) from Ardabil and East Azerbaijan Provinces, nortwest of Iran where *Leishmania infantum* are endemic (Moshfe et al. 2008) and sera from healthy dogs without any clinical and symptoms and DAT negative (n=74) from non-endemic areas (Nadim et al. 1978).

Blood samples (2–5 ml) were taken from all of the dogs (147 dogs). Samples were taken by venepuncture and put into 10 ml polypropylene tubes and processed 4–10 h after collection. The blood was centrifuged at 800×g for 5–10 min, and sera were separated and stored at −20°C.

Direct agglutination test

The *L. infantum* antigens for this study were prepared in the Protozoology unit of the School of Public Health, Tehran University of Medical Sciences. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of *L. infantum* LON-49 in RPMI1640 plus 10% fetal bovine serum, trypsinization of the parasites, staining with Coomassie brilliant blue, and fixing with formaldehyde 2% (Harith et al. 1989; Edrissian et al. 1996; Mohebali et al. 2006).The dog serum samples were tested by DAT according to the methods described by Harith et al. (1989).

To study the optimal DAT cut-off level, a receiver–operator characteristics curve was constructed according to the method Fletcher et al. (1982). Negative control wells (antigen only; on each plate) and known negative and positive controls were tested in each plate daily. The positive standard control serum was prepared from dogs with *L.infantum* infection from the endemic areas confirmed by microscopy, culture, and animal inoculation with 1:20,480 titers. Two individuals read the tests independently.

Therefore, we considered anti-*Leishmania* antibodies titers at >1:160 as *Leishmania* infection in this investigation.

Fast agglutination screening test

For FAST, antigen was prepared essentially as for DAT mentioned before, but the final density of promastigotes for the FAST antigen was calculated and arranged 2×10^8/ml. Twenty microlitres of 1:80 diluted serum in 0.9% saline and 1.56% β mercapto ethanol was incubated in a V-shaped microtitre plate for 1 h at 37°C; then, 20 µl of antigen was added. In this study, after 3 h incubation at room temperature, the results were read. The interpretation of the agglutination is the same as for the DAT with compact blue dots are scored as negative and large diffuse blue 'mats' as positive.

Statistical analysis

The sensitivity and specificity of the DAT and FAST in the present study were calculated as follows: sensitivity=TP/(TP+FN)×100% and specificity=TN/(TN+FP)×100% where TN represents true negative, TP true positive, FN false negative, and FP false positive. The sensitivity of the two tests, FAST and DAT, was assessed with sera from confirmed CVL from endemic areas (n=73). Sera of healthy controls from non-endemic areas (n=74) were used

Table 2 Comparison between FAST and DAT results

FAST	DAT		
	+ (*n*)	− (*n*)	Total
+ (*n*)	72	20	92
− (*n*)	1	54	55
Total	73	74	147

Concordance=86.8% by kappa analysis (p<0.01)

to determine the specificity of FAST. The degree of agreement between FAST and DAT was determined by calculating kappa (κ) values with 95% confidence intervals using SPSS version 13.5. Kappa values express the agreement beyond change, and a κ value of 0.21–0.60 represents a fair to moderate agreement, a κ value of 0.60–0.80 represents a substantial agreement, and a $\kappa > 0.80$ represents almost perfect agreement (Altman 2001). The calculation of the degree of agreement between DAT and FAST was based on all serum samples.

Results

The results of the serological analysis with DAT and FAST are presented in Tables 1 and 2, respectively. Seventy-four dogs were found sero-negative, and 73 were sero-positive with DAT using a cutoff titer of 1:160. FAST found 55 dogs to be sero-negative and 92 sero-positive. One of the DAT-positive samples (with tires of 1:640) showed FAST negative result (Table 1). The sensitivity of the FAST in the present study was calculated to be 98.6% and its specificity 78.7 % (11 false positive results). A good degree of agreement was observed between FAST and DAT (86.8 %) by kappa analysis ($p<0.01$; Table 2).

Discussion

Accurate and rapid diagnosis of CVL is of great importance in order to start early detection and to prevent transmission cycle of *Leishmania* parasite. Detection of anti-*Leishmania*-circulating antibodies using rapid and early serodiagnostic techniques of canine leishmaniasis is necessary (Bettini and Gradoni 1986). Currently, diagnostic tests that can be performed during short periods are needed for large-scale screening of *Leishmania* infection in dog populations. In general, the most satisfactory technique is DAT.

DAT has been proven to be a very important sero-diagnostic tool combining high validity and easy to performance (Harith et al. 1989; Edrissian et al. 1996; Mohebali et al. 2006). Otherwise, FAST can perform with only one serum dilution and making the test very suitable for the screening of VL in large populations of humans and dogs (Cardoso et al. 2004).

In the present study, only one of the FAST-negative serum sample was DAT-positive; therefore, the sensitivity of the FAST is calculated 98.63%. FAST specificity is lower because 20 of the DAT negative sera was FAST positive; therefore, the specificity of FAST is calculated 78.72%. These results credit FAST as a qualitative preliminary screening test considering high sensitivity rate and DAT as a quantitative confirmatory test especially in areas of high sero-prevalence. The same results were achieved by Cardoso et al. in Portugal in 2004 (Cardoso et al. 2004), but in this study, we used from the batches of DAT and FAST antigens that were prepared in our lab from Iran .With FAST, the final data often could be available in the same day after 3 h because it is not necessary to re-test those sero-negative samples with DAT. We only re-tested FAST-positive sera by DAT for confirmation, and results were available the next day. Therefore, by combining the two tests, large numbers of samples can be easily screened at the short time.

In conclusion, we can conclude that DAT and FAST are as very suitable techniques for the sero-diagnosis of CVL in different parts of Iran. Both tests are easy to perform, as well as being specific and sensitive. The FAST is very practical under field or rural conditions because neither specialized equipment for transportation is required nor cold chain is necessary for keeping of antigen. In addition, the FAST requires only one serum dilution and the results can be read within 3 h. The FAST can be used to screen large populations of suspected dogs in CVL endemic areas.

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