

Comparison of recombinant A2-ELISA with rKE16 dipstick and direct agglutination tests for diagnosis of visceral leishmaniasis in dogs in Northwestern Iran

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ABSTRACT

Introduction: Various methods are used for the diagnosis of visceral leishmaniasis (VL), such as microscopic examination, culture and inoculation of laboratory animals; however, serological assays are commonly used for the detection of antibodies in serum samples with a wide range of specificity and sensitivity. **Methods:** The purpose of this study was to compare three serological methods, including rA2-ELISA, the recombinant KE16 (rKE16) dipstick test and the direct agglutination test (DAT), for the detection of antibodies against VL antigens. The assays utilized 350 statistically based random serum samples from domestic dogs with clinical symptoms as well as samples from asymptomatic and healthy dogs from rural and urban areas of the Meshkinshahr district, northwestern Iran. **Results:** Samples were assessed, and the following positive rates were obtained: 11.5% by rKE16, 26.9% by DAT and 49.8% by ELISA. The sensitivity among symptomatic dogs was 32.4% with rKE16, 100% with DAT and 52.9% with ELISA. Conversely, rA2-ELISA was less specific for asymptomatic dogs, at 46.5%, compared with DAT, at 88.9%. **Conclusions:** This study recommends rA2-ELISA as a parallel assay combined with DAT to detect VL infection among dogs. Further evaluations should be performed to develop an inexpensive and reliable serologic test for the detection of *Leishmania infantum* among infected dogs.

Keywords: A2 protein. DAT. Iran. rA2-ELISA. rKE16. Visceral leishmaniasis.

INTRODUCTION

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and has a fatality rate of almost 100% if left untreated. The mortality rate for this disease can be reduced by early diagnosis and appropriate treatment⁽¹⁾. Epidemiological studies have shown that Mediterranean VL caused by *Leishmania (Leishmania) infantum* occurs in different parts of Iran, and domestic dogs are the principal source of infection⁽²⁾.

Different methods are used for the diagnosis of VL based on aspirates or biopsy specimens of visceral tissues (spleen, liver, bone marrow), which are subjected to microscopic examination and culture; less specificity is obtained for bone marrow while more specificity is observed for spleen samples⁽³⁾. However, due to the high risk of splenic aspiration, serological assays

such as the indirect fluorescent antibody (IFA) method, the indirect hemagglutination assay (IHA), complement fixation (CF), the direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) are used for the detection of antibodies in serum samples from humans and dogs, with each exhibiting different specificities and sensitivities⁽⁴⁾⁽⁵⁾⁽⁶⁾.

Although several antigens have been investigated, the most commonly used antigen is a crude soluble antigen (CSA). ELISA generally uses crude antigens with different concentrations of CSA, representing a range of sensitivity from 80 to 100%. Cross-reactions with other diseases including trypanosomiasis, tuberculosis and toxoplasmosis have been reported⁽⁷⁾⁽⁸⁾.

However, classic diagnostic methods are limited by low sensitivity, requiring repeated tissue sampling and a trained laboratory staff. Therefore, there is a need to develop a more rapid and simple assay for the diagnosis of VL⁽⁹⁾. Because the elimination of infected dogs is an important mean for controlling the transmission of VL, an ideal diagnostic test for VL in dogs would have to be inexpensive and simple, in addition to being specific and sensitive⁽⁹⁾.

Rapid tests, such as the immunochromatographic dipstick test using the recombinant K39 (rK39)⁽¹⁰⁾, rK26⁽¹¹⁾ and rKE16⁽¹²⁾ proteins of *L. infantum* as antigens, appear to be well-suited for point-of-care diagnoses in symptomatic cases of canine VL,

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but a lack of sensitivity for asymptomatic dogs remains a primary problem⁽¹³⁾. Validations of rapid tests for the detection of canine *L. infantum* infection have been reported. The results of a rapid test can be influenced by the type of *Leishmania* antigen utilized, the manufacturer's production process, geographical location and clinical signs. In a study carried out in VL endemic areas of Iran, the rK39 dipstick test (Cypress, Belgium) showed moderate sensitivity (70.9%) and specificity (84.9%) in domestic dogs⁽¹⁴⁾. Because VL is endemic in northwest Iran, with dogs as the principle reservoir, the focus of this study is the application of rA2-ELISA in comparison to the rKE16 dipstick and direct agglutination tests for VL diagnosis in non-stray dogs in the Meshkinshahr district, Ardabil province, northwestern Iran. This approach would provide a simple and suitable tool for sero-diagnosis of VL in symptomatic and asymptomatic dogs. Efforts should be made to develop sensitive and specific methods capable of detecting asymptomatic carriers for mass surveys.

METHODS

Study area

This investigation was conducted over a period of 2 years (from 2011 to 2012) in the Meshkinshahr district, Ardabil province, northwestern Iran. Dogs were selected by random sampling in regions where human VL had been previously identified. This geographical zone was selected as an important endemic area for VL in Iran, as shown in **Figure 1**. A field laboratory was set up at each location to provide a base at which the animals could be examined.

Sample collection

This study used a panel of 350 serum samples from both male and female domestic dogs of various breeds and ages in the Meshkinshahr district, northwestern Iran as an endemic area for canine VL⁽²⁾⁽¹⁵⁾. Ethical permission was obtained from the dog owners to test their animals. The dogs were clinically examined by an expert veterinarian and classified as symptomatic based on the presence of at least two clinical signs, including lymphadenopathy, alopecia, dermatitis, skin ulceration, keratoconjunctivitis, onychogryposis, lameness, epistaxis, anorexia and weight loss. Peripheral blood collection (2.5ml) for serology was performed at the cephalic vein; the blood was processed and stored at -20°C. Samples from asymptomatic dogs with no detectable clinical signs of disease were collected and assessed using the standard DAT. Positive and negative samples were considered to correspond to asymptomatic and healthy dogs, respectively.

Parasitological study

The dogs were externally examined for signs of *Leishmania* infection. Smears were prepared from all skin lesions for each dog. All of the prepared smears were fixed with methanol, stained with 10% Giemsa stain and microscopically examined for the presence of amastigotes.



FIGURE 1- Geographical location of study area.

Direct agglutination test

The *Leishmania 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 producing DAT antigen included the mass production of promastigotes of *L. infantum* LON-49 in RPMI1640 plus 10% fetal bovine serum (FBS), trypsinization of the parasites, staining with Coomassie brilliant blue, and fixing with 2% formaldehyde⁽¹⁵⁾⁽¹⁶⁾. The serum samples from the dogs were tested using DAT, and according to previous studies, a ratio of 1:320 was indicated as a cut-off point for the canine sera⁽¹⁷⁾⁽¹⁸⁾. Initially, for screening purposes, 1:80 and 1:320 dilutions were utilized. Samples with 1:80 titers were diluted further to give end-point titers of 1:20480. Negative control wells (antigen only, on each plate) and known negative and positive controls were tested in each plate daily. The titer was defined as the highest dilution at which agglutination was still visible, represented as a blue dot, compared with negative control wells, which were represented as clear blue dots. A positive standard control serum prepared from dogs with *L. infantum* infection from the endemic areas was confirmed by microscopy, culture and animal inoculation with 1:20480 titers. Two individuals read the tests independently. The cut-off was determined in a previous study by experimental infection. To study the optimal DAT cut-off level, a receiver-operator characteristics (ROC) curve was constructed. Moreover, specific *Leishmania* antibodies at titers of 1:320 and above were considered as positive in previous experiments. Therefore, we considered anti-*Leishmania* antibody titers at $\geq 1:320$ to correspond to *Leishmania* infection in this investigation⁽¹⁷⁾⁽¹⁸⁾.

rKE16 dipstick rapid test

The rKE16 dipstick test (One step Rapid Immunochromatographic test for Kala Azar) is based on the rKE16 antigen. The assay was carried out using an rKE16 dipstick kit (Crystal KA, Co.,

Surat, India). The stored sample was brought to room temperature before the procedure was initiated. For this purpose, a number of blister packs were torn out from the pouch while the remaining blister packs were stored in a light zip lock pouch. All reagents reached room temperature prior to the assay. Dipsticks from the blister packs were taken out and labeled with the sample's identification code. Four drops of reaction buffer and 20µl of sample were added to the test tube and mixed well, and the dipstick was placed vertically in the test tube containing the diluted sample until the sample liquid front reached the arrow mark. After 15 min of incubation, the dipstick was removed from the sample and the result was read. The presence of only one pinkish red band at the control (C-region) and the absence of a band at the test (T-region) indicated a negative sample that was non-reactive for leishmaniasis. The presence of two pinkish red bands, at both the C- and T-regions, indicated a positive test sample reactive for leishmaniasis.

Recombinant A2 antigen

Recombinant A2 (rA2) antigen was kindly provided by Prof. S. Rafati, from the Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran, Iran and was prepared according to the protocol described in Mizbani et al.⁽¹⁹⁾. Briefly, the A2 gene with 3'UTR sequences was amplified from the pKSNEOA2-1 vector⁽²⁰⁾ using hot-start polymerase chain reaction (PCR) (Hot-Star kit, Qiagen) with the following primers: (forward, 5'-TTGAAGCTTACCGAGCACAATGAAGATCC-3'; reverse, 5'-AACAAAGCTTAGCAGAGGAAGTCAGCAAGG-3') including HindIII restriction sites indicated as underlined. For amplification, deoxyribonucleic acid (DNA) was denatured at 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 61°C for 30s and 72°C for 90s, with a final cycle of 72°C for 10 min. The amplified fragment was cloned into the pDrive cloning vector (Qiagen). After sequence confirmation, the A2 fragment was subcloned into the HindIII site of vector pNEO-GFP.

Dot blot

The reaction intensity of the purified antigen was investigated by a dot blot assay, which follows the same process as an immune blot assay, except that the antigen was applied at concentrations of 1, 5, and 10µg/ml and was directly spotted onto the polyvinylidene difluoride (PVDF) membrane. Monoclonal C9 (kindly provided by Dr. Greg Matlashewski, Microbiology and Immunology Department, McGill University, Montreal, Quebec, Canada) was used as a primary antibody at a dilution of 1:50, and Horseradish peroxidase (HRP) anti-mouse was used as the secondary antibody at a dilution of 1:2,500 dilution. The reactions were observed by the addition of cloronaftol, diaminobenzidine and H₂O₂.

Enzyme-linked immunosorbent assay

Serum samples from the dogs were analyzed by standard micro-ELISA⁽²¹⁾ to detect antigen-specific antibodies. Micro titer immunoassay plates were coated with rA2 in 100µL of coating buffer for 18h at 4°C. A titration curve was obtained to determine the optimal protein concentration for the ELISA. Free binding

sites were blocked with a 1% bovine serum albumin (BSA) solution for 2h at 37°C. After three washes with PBS-Tween20 at 0.05%, the plates were incubated with 100µL of canine or human sera for 1h at 37°C. The serum samples were diluted 1:100 in phosphate buffered saline (PBS), and the plates were then washed three times with PBS and incubated with 1:20000 anti-canine immunoglobulin G (IgG) antibody (Sigma, USA) conjugated with horseradish peroxidase. The reaction was stopped by the addition of 100µL of a 1M solution of HCl per well. The optical density (OD) was measured at 450nm in an ELISA microplate spectrophotometer (Molecular Devices, BIO-TEK, USA). The lower limit of positivity (cut-off) was determined by the OD mean of 20 negative canine control sera plus two standard deviations.

Cross-reactivity

To define potential cross-reactions with rA2-ELISA for the diagnosis of VL, the recombinant A2 antigen was assessed against different serum samples from other prevalent zoonotic infections including Kala Azar, cutaneous leishmaniasis (CL) due to *Leishmania major*, *Echinococcus granulosus*, *Leptospirosis*, and *Toxoplasma gondii* and infections with specific human pathogens including tuberculosis, *Plasmodium falciparum* and *P. vivax*.

RESULTS

Direct agglutination test

Figure 2 shows that out of the 350 serum samples collected from domestic dogs in villages known as endemic foci of leishmaniasis, 91 (26.9%) samples were shown to be positive by DAT ($\geq 1:320$) while 259 (73.1%) samples were negative ($<1/320$).

rKE16 strip tests

The immunochromatographic strip test (ICT) was performed for all cases. Among the 350 evaluated samples, 40 (11.4%) cases were detected as positive and 310 (88.6%) cases were found to be negative using the ICT strip test (**Figure 2**).

ELISA

The ELISA procedure was optimized with regard to the antigen concentration and serum dilution. The optimal antigen concentration was 5µg/ml for the rA2 protein. A clear separation between the parasite-positive dogs and healthy (uninfected) controls was obtained by using a single serum dilution of 1:100. Of 350 canine serum samples, 174 (49.7%) cases were shown to be positive while 176 (50.3%) cases were negative. All of the dog samples were compared for the DAT, rKE16 dipstick and ELISA methods, which indicated sero-positivities of 26.9%, 11.4% and 49.8%, respectively (**Figure 2**).

Figure 3 shows that the highest rate of correlation between assays among sero-positive cases was obtained between DAT and ELISA (12.3%), followed by rKE16 and ELISA (8%), rKE16 and DAT (7.7%) and rKE16, DAT, and ELISA (5.4%).

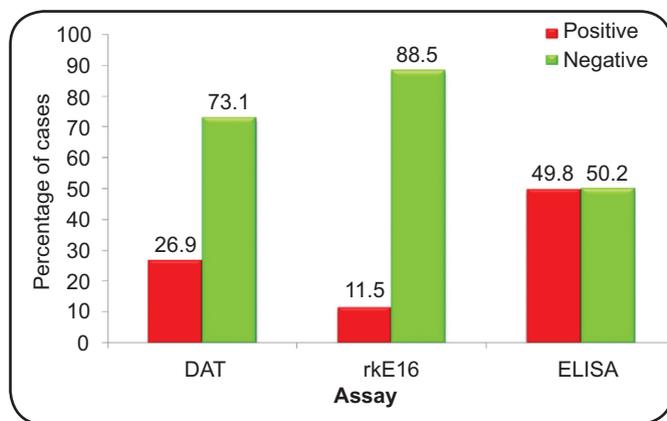


FIGURE 2- Comparison of the DAT, rKE16 dipstick and rA2-ELISA methods for the diagnosis of visceral leishmaniasis. DAT: direct agglutination test; rKE16: recombinant KE16; ELISA: enzyme-linked immunosorbent assay.

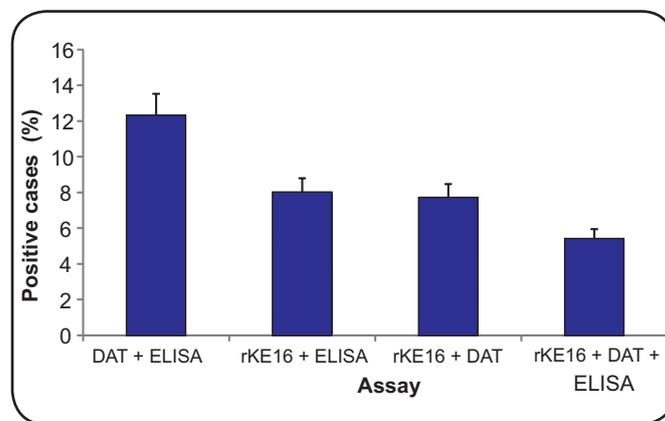


FIGURE 3 - Correlation between assays among sero-positive cases. DAT: direct agglutination test; ELISA: enzyme-linked immunosorbent assay; rKE16: recombinant KE16.

A comparison of all of the samples among the three groups, including healthy, asymptomatic and symptomatic dogs, was performed for the three assays: DAT, rKE16 dipstick and ELISA. The positive rates for the healthy dogs were 0% (DAT), 0% (rKE16), and 3.6% (ELISA); in asymptomatic dogs, the results were 11.1% (DAT), 7.1% (rKE16), and 53.5% (ELISA); and among symptomatic dogs, the results were 100% (DAT), 32.4% (rKE16), and 52.9% (ELISA) presented in **Table 1**.

Cross-reaction

No cross-reactivity was observed in rA2-ELISA for the diagnosis of VL with sera from other prevalent zoonotic infections, including Kala Azar, cutaneous leishmaniasis, hydatid cysts, leptospirosis and toxoplasmosis, or with human pathogens, including tuberculosis and malaria.

DISCUSSION

In addition to the authors' previous publications, which confirmed the presence of A2 antibodies among dogs infected with *L. infantum* in Meshkinshahr, Ardabil Province^{(22) (23)}, this complementary study was conducted to assess rA2-ELISA

in comparison to the rKE16 dipstick method and DAT for the diagnosis of VL. In the diagnosis of VL for healthy, asymptomatic and symptomatic dogs, the rKE16, DAT and ELISA methods accurately identified positive cases at rates of 11.5%, 26.9% and 49.8%, respectively. In terms of alignment techniques used in the diagnosis, the highest rate of positive cases was observed by both DAT and ELISA (12.3%). The sensitivity obtained for DAT was 100%, while the sensitivity of ELISA was 52.9%; thus, DAT obtained the highest sensitivity in symptomatic infected dogs. However, the rKE16 sensitivity was 32.4%, the lowest sensitivity among the three methods. The specificity obtained for rKE16 was 92.9%, while the specificities for DAT and ELISA were 88.9% and 46.5%, representing the negative cases among asymptomatic dogs. In addition, the highest rate of correlation between assays among sero-positive cases was observed between DAT and ELISA at 12.3%, while the lowest correlation was observed among rKE16, DAT and ELISA at 5.4%.

Contradictory results have been reported from Iran, Brazil, India, Ethiopia and Sudan, in which different assays have been applied for the detection of VL in dogs and humans. Akhoundi et al.⁽²⁴⁾ applied a DAT assay with only one serum dilution as a

TABLE 1- Comparison of DAT, rKE16 dipstick and ELISA assays among the three groups of healthy, asymptomatic and symptomatic dogs.

Assay	Result	Healthy	Asymptomatic	Symptomatic
DAT	Positive	0.0	11.1	100.0
	Negative	100.0	88.9	0.0
rKE16	Positive	0.0	7.1	32.4
	Negative	100.0	92.9	67.6
ELISA	Positive	3.6	53.5	52.9
	Negative	96.4	46.5	47.1

DAT: direct agglutination test; rKE16: recombinant KE16; ELISA: enzyme-linked immunosorbent assay.

simple, rapid, sensitive and non-invasive method that does not require much expertise for use in screening and sero-diagnosis of human *L. infantum* infection in Iran. Akhoundi et al.⁽²⁵⁾ also recommended a modified DAT antigen with high stability over a range of temperatures that can easily be transported in the field to VL endemic areas in Iran. In addition, de Assis et al.⁽²⁶⁾ reported that the DAT and the rK39 rapid test can be applied as useful assays to diagnose VL in Brazil. Sivakumar et al.⁽²⁷⁾ suggested that modified DATs and the ELISA-rK39 are useful tests for the diagnosis of VL and may replace the IFAT as a routine diagnostic test in Brazil. In contrast, a report from Ethiopia indicated that the Ld-rKDD8 antigen was less sensitive and less specific than the rKE16 antigen. In that study, the usefulness of the rK39 dipstick test, DAT and *Leishmania* skin test for detecting asymptomatic VL infection in children was reported⁽²⁸⁾. However, Cañavate et al.⁽²⁹⁾ confirmed two rK39 dipstick tests, the DAT and the indirect fluorescent antibody (IFA) test as suitable for the diagnosis of VL in Ethiopia. In Sudan, Abbas et al.⁽³⁰⁾ introduced rKLO8, a novel *L. donovani*-derived recombinant protein for VL detection exhibiting increased reactivity of Sudanese VL sera with the rKLO. These researchers confirmed this antigen as a potential candidate for the diagnosis of VL in Sudan. Recombinant A2 has also been investigated and indicated in several studies for diagnostic purposes in humans and dogs, by detecting anti-A2 antibodies using Western blot, ELISA and immunoprecipitation⁽⁹⁾⁽³¹⁾⁽³²⁾⁽³³⁾.

For diagnostic assays of *L. infantum* infection based on parasitology, PCR, real-time PCR, and DAT, sensitivities of 54%, 67.6%, 97.3%, and 100% VL were reported, respectively among symptomatic domestic dogs in Iran. For asymptomatic dogs, rates of 70.5%, 99.1%, and 100% were shown to be positive by PCR, real-time PCR, and DAT, respectively⁽³⁴⁾. In a study using rA2-ELISA, anti-A2 antibodies were detected in 14 out of 15 symptomatic dogs and in 10 out of 13 asymptomatic dogs in Brazil⁽³⁵⁾. This report from Brazil provided a comparison of ELISA using *L. infantum* rK26 and rK39 antigens and the *L. donovani* rA2 protein, as well as ELISA with crude soluble antigen (CSA), indicating a high sensitivity for rK26 and rK39 in the detection of symptomatic dogs (94% and 100%, respectively), followed by CSA (88%) and rA2 (70%). Conversely, rA2 was reported to be more sensitive for asymptomatic dogs (88%) than rK39 and rK26 (both 66%) and CSA (30%)⁽⁹⁾.

In conclusion, the findings of this study reveal that rA2-ELISA has a higher sensitivity for the diagnosis of VL in comparison to DAT and the rKE16 dipstick test; rA2-ELISA detected a higher rate of infection among dogs from Meshkinshahr, Ardebil province, Iran. The DAT has been used as a reference test in many sero-prevalence studies and is suitable for the sero-diagnosis of canine VL. Taken together, our findings suggest that the rA2 protein may be a valuable tool for the diagnosis of VL in the Old World, as suggested previously for the New World⁽³⁵⁾, particularly for the diagnosis of infection in dogs. Because A2 is a recombinant protein, it may also be useful in the development and improvement of other simpler and faster serological tests. Efforts should be made to

develop an inexpensive and reliable serological test based on epitope selection from such diagnostic markers for the sensitive detection of *L. infantum*-infected dogs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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