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Diagnosis of *Leishmania infantum* using Direct Agglutination Test and rKE16 Dipstick Rapid Test in Domestic Dogs from Ardabil Province, Iran

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ABSTRACT

Leishmaniasis is a parasitic disease, caused by several *Leishmania* species. Clinical symptoms range from Cutaneous Lesions (CL) to severe Visceral Leishmaniasis (VL). The main cause of VL in Iran is *Leishmania infantum* and domestic dogs are the major reservoir host. Different methods are used for diagnosis of VL including the parasitology staining from tissue aspiration and determination of antibodies against VL. The aim of this study was to compare direct agglutination (DAT) and rKE16 dipstick rapid tests for VL in domestic dogs from Meshkin Shahr district, Ardabil Province, Northwestern Iran. Blood samples of 200 dogs from five different regions were collected and their sera tested with DAT and rKE16 methods. Collected data were compared for VL detection, antibody titers and their association with age, gender and location. The highest rate of infection was detected by DAT (24.5%) in compare to rKE16 (11.5%). Majority of dogs suffering from VL were 2-7 years old and more male infection (82%) was observed with the highest rate in Parikhan village. These findings showed that DAT has a higher sensitivity than rKE16 dipstick for dogs VL detection. More infection was observed among dogs from Parikhan village of Meshkin Shahr district, Ardabil province, Northwestern Iran.

Key words: DAT, Iran, *L. infantum*, Meshkin Shahr, rKE16, Visceral Leishmaniasis

INTRODUCTION

Visceral Leishmaniasis (VL) or Kala-azar is a parasitic infectious disease that is fatal if left untreated. Two *Leishmania* species are causal agents of VL including *Leishmania* (L) *infantum* and *L. donovani*. The first is a zoonosis and is endemic in countries around the Mediterranean basin and in Latin-America. The second is assumed to be an anthroponosis and is endemic in East-Africa and the Indian subcontinent (WHO., 2010). The main clinical symptoms of VL are as follows: Development of skin lesions, periocular alopecia, nasal and/or auricular ulcers, fever, weight loss, lymphadenopathy, epistaxis, onychogryphosis, splenomegaly, conjunctivitis, emaciation and signs of renal failure. Clinical diagnosis of VL is difficult due to the myriad clinical symptoms that may be exhibited by apparently healthy animals, oligosymptomatic animals and animals at advanced disease stages (Gontijo and Melo, 2004).

Up until the 1990's accurate VL diagnosis necessitated parasitological confirmation by microscopy or culture of the blood, bone-marrow, lymph nodes or spleen (Srividya *et al.*, 2012). Microscopic detection of parasites in clinical material from the spleen is still considered the reference standard; however, splenic aspirates are associated with risk of patient's life. The invasiveness and potentially fatal complications associated with splenic aspiration has spurred the development of non-invasive serological tests such as direct agglutination test (DAT) (El Harith *et al.*, 1988). Various diagnostic assays are available, but they vary in sensitivity as a function of the clinical condition, parasite density, biological material and the diagnostic methods used for analysis (Bisugo *et al.*, 2007). Different methods are used for the laboratory diagnosis of VL including the observation of the parasite in stained specimens and determination of specific antibodies against *Leishmania* spp. There are some publications from Iran, Brazil, India, Ethiopia and Sudan applied different assays for detection of VL in dogs and humans including DAT assay used for screening and serodiagnosis of human *L. infantum* infection in Iran (Akhoundi *et al.*, 2010, 2013), DAT, rK39-based rapid test and ELISA-rK39 for diagnosis of VL in Brazil (De Assis *et al.*, 2011; Pedras *et al.*, 2008), Ld-rKDD8 and rKE16 antigens for the diagnosis of VL in India (Sivakumar *et al.*, 2008), rK39 Dipstick tests, DAT, IFA test and Leishmanin skin test for diagnosis of VL in Ethiopia (Gadisa *et al.*, 2012; Canavate *et al.*, 2011), rKLO8 and rKLO as a candidate for diagnosis of VL in Sudan (Abass *et al.*, 2013). There are also comparisons of some assays for VL detection including rK39 and rKE16 in the Indian subcontinent, Brazil and East Africa (Cunningham *et al.*, 2012), immunochromatographic tests and DAT in Latin-America, East-Africa and Asia (Adams *et al.*, 2012) and rKE16 and rK39 in diagnosis of VL in India (Vaish *et al.*, 2012).

Regarding to similarity between *L. infantum* isolated from infected humans and high *L. infantum* infection of domestic dogs in several published studies; domestic dogs seem to be the main reservoirs of infection in endemic foci of VL focusing in northwest and central parts of Iran (Soleimanzadeh *et al.*, 1993; Mohebali *et al.*, 2001, 2005, 2006; Moshfe *et al.*, 2009; Mohebali *et al.*, 2011; Mohebali, 2013). In order to emphasise importance of VL in Iran and also to verify diagnosis assay, this study has been conducted aiming to investigate a comparative serological study on VL using direct agglutination test (DAT) and rKE16 dipstick rapid test in domestic dogs from Meshkin Shahr district, Ardabil province, northwestern Iran.

MATERIALS AND METHODS

Geographical distribution of study area: The investigation was conducted in northwest of Iran, where VL is endemic among dogs and human. Meshkin Shahr district in the central northern part of the Ardabil province located at an altitude of 1490 m above sea level. Forty-two percent of their populations settle in urban areas, 58% live in rural areas and small population has nomadic living. The study area has moderate mountainous weather. In this area there are a lot of dogs with a friendly relationship with the human population which used as guard and herd dogs and also the stray dog is found. In this region, the amount of animal manure can be seen in yards and alleys which is considered as the resting place of dogs.

Sample collection: A descriptive cross sectional study was carried out and 200 blood samples from five different regions of Meshkin Shahr district during 2011-2013 were collected from domestic dogs in villages where human VL had been reported at least 5 cases during 3 years ago. General information including age, gender, location and having symptoms from each dog were documented

by interviewing dog owners. Blood sample were collected into 10 mL polypropylene tubes and centrifuged at 8000 g for 5-10 min and then serum aliquots were stored at -20°C until examined. All of the serum samples were transferred leishmaniasis laboratory in the School of Public Health, Tehran University of Medical Sciences and were tested by DAT and rKE16 (Barati *et al*., 2013).

Direct Agglutination Test (DAT): The DAT antigen used in this study was prepared at the Protozoology Unit of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran and stored at 4°C until used. The method was applied as previously described (Mohebali *et al*., 2005; Barati *et al*., 2013; Akhoundi *et al*., 2012). Briefly, the principal phases of the procedure for preparing the DAT antigen were mass production of promastigotes of *L. infantum* (Iranian strain) in RPMI1640 medium supplemented with fet al bovine serum, trypsinization of the parasites, staining with Coomassie Brilliant blue and fixing with formaldehyde. Gelatin was diluted in DW and placed in water bath until the gelatin is completely dissolved; the solution must be cooled, then 2-mercaptoethanol was added to it. For DAT a v-shaped microplate was used; initially, serial dilutions of serum diluent solution were prepared starting at a dilution of 1:10 to a maximum dilution of 1:20480. Then the plates incubated at 37°C and finally kept at room temperature. Reconstituted antigen was added to microplate wells. In position of a positive test, the wells represented a cloudy condition, otherwise during a negative result, the point is deposited at the bottom of well. Concluding results of DAT, depended on the titration, 1:80 and less showed negative DAT result and titration of 1:160 was doubtful and the titration more than 1:320 represented a positive test.

rKE16 dipstick: The assay was carried out using rKE16 dipstick kit (Crystal KA, Co., Surat, India). The stored sample was brought to room temperature before starting of the procedure. The required number of blister pack was torn out from the pouch and stored remaining blister packs in lightly zip lock pouch. All reagents attained room temperature before starting the assay. Dipstick form blister pack was taken out and labeled with patient's identification code. Four drops of reaction buffer and 20 µL sample was added into the test tube, mixed well and dipstick placed vertically into the test tube containing diluted sample, until the sample liquid front reaches the arrow mark. After 15 min incubation, the dipstick was removed from the sample and result was read. Presence of only one pinkish red band at control C-region and no band at test T-region indicated that the sample is negative and is non-reactive for leishmaniasis. Presence of two pinkish red bands, at both C and T-regions indicated that the sample is positive and is reactive for leishmaniasis.

Statistical analysis: Statistical analysis was applied by Chi-Square and Kappa methods, using Open Epi and Graph Pad Prism Software (Graph Pad, San Diego, California, USA). Graphs were plotted by Microsoft Excel software.

RESULTS AND DISCUSSION

Positive and negative dog samples were detected by DAT and rKE16 methods and compared based on these laboratory assays. The results considered highest rate of positive samples were detected by DAT (49 samples, 24.5%) in compare to rKE16 method (23 samples, 11.5%). However, the most negative samples were recognized by rKE16 (177 samples, 88.5%) in compare to DAT (151 samples, 75.5%) (Fig. 1). According to antibody titers which were assessed and compared by
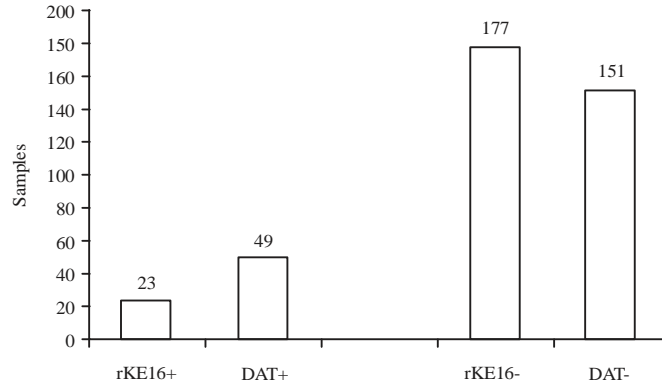
Fig. 1: Comparison of positive and negative samples based on laboratory methods used, positive and negative dog samples were detected by DAT and rKE16 methods and compared based on these laboratory assays, the results considered highest rate of positive samples were detected by DAT (24.5%) in compare to rKE16 method (11.5%). However, the most negative samples were recognized by rKE16 (88.5%) in compare to DAT (75.5%), statistical analysis was done by Kappa method and Graph Pad software (Kappa: 0.130, SE of Kappa: 0.038, p-value: 0.055)
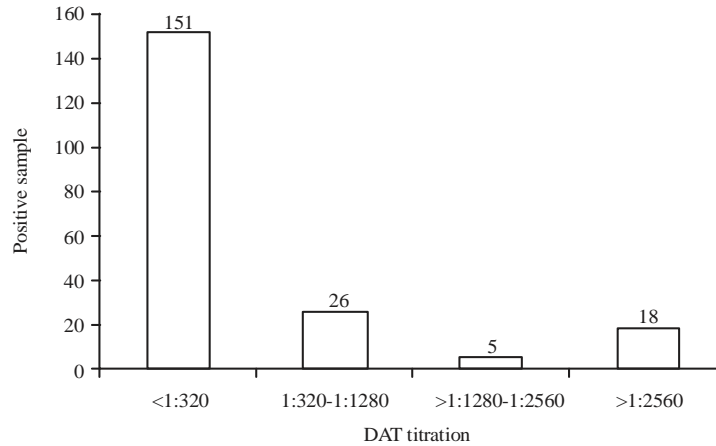

Fig. 2: Comparison of antibody titer by DAT in samples, antibody titer was assessed and compared using DAT. According this assay, the titers with less than 1:320 were considered as negative and equal or more than 1:320 titers marked as positive. The highest rate of antibody was among less than 1:320 titer. Statistical analysis was done by Chi Square method and OpenEpi software (Chi Square: 4.724, Degree of freedom: 2; p-value: 0.094)

DAT; the titers with less than 1:320 were considered as negative and equal or more than 1:320 titers marked as positive. The highest rate of antibody was among group with less than 1:320 titer (Fig. 2). Positive samples were confirmed by rKE16 and DAT methods and their association presented according to age groups of dogs. The majority of dogs suffering from VL, were among 2-7 years old, however the lowest rate of infection were shown in groups older than 7 years old which were confirmed by both assays (Fig. 3). Positive samples were assessed by both DAT and rKE16 methods and compared according to gender of dogs in Meshkin Shahr. Results
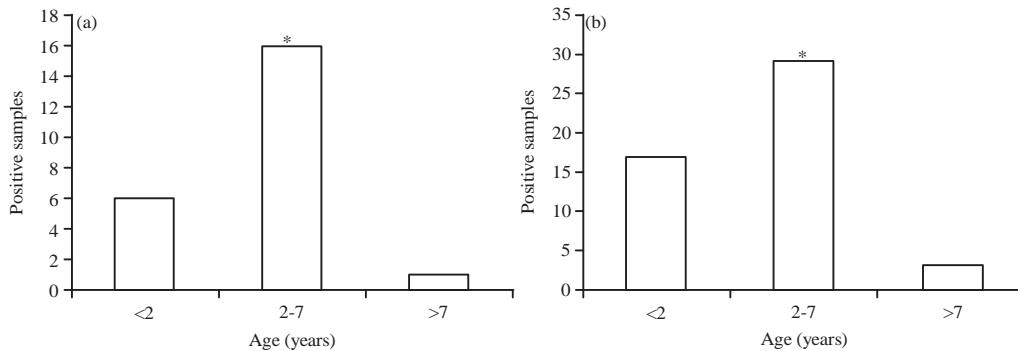
Fig. 3(a-b): Association of positive samples by rKE16 and DAT assays based on age groups, positive samples were confirmed by rKE16 and DAT methods and their association presented according to age groups of dogs. The majority of dogs suffering from Leishmania parasite were among 2-7 years old. However, the lowest rate of infection were shown in groups older than 7 years old which confirmed by both assays. Statistical analysis was done by Chi Square method and OpenEpi software, (a) rKE16: Chi Square: 4.724, Degree of freedom: 2, p-value: 0.094 and (b) DAT: Chi Square: 3.341, Degree of freedom: 2, p-value: 0.188

indicated majority of positive dogs were male (82%) in comparison with female infected dogs (18%) (Fig. 4). Positive samples were assessed by rKE16 and DAT methods and compared based on geographical locations. Considering both assays, the highest rate of VL in dogs were observed in Parikhan village and the lowest rate of infection detected at Jehadabad and Kojnagh villages (Fig. 5).

In this study, the sensitivity of DAT and rKE16 assays for detection of VL in dogs indicated more detection of positive samples by DAT (24.5%) in compare to rKE16 (11.5%). However, the most negative samples were recognized by rKE16 (88.5%) in compare to DAT (75.5%). Antibody titer was assessed and compared by DAT; the highest rate of antibody titer was among negative samples with less than 1:320. The majority of positive cases have 1:320-1:1280 titers. VL in dogs and their association with age group revealed a high rate of infection among 2-7 years old and the lowest rate of infection was shown in older dogs than 7 years old. Hunting and food obtaining among 2-7 years old dogs are common; they probably are more exposed to contamination than younger dogs. Since older dogs were also unable to obtain food, the infection rate is low. The data of this study also presented a relationship of VL infection to gender of dogs in Meshkin Shahr. Results indicated majority of positive dogs were male (82%) in comparison with female infected dogs (18%). This may due that female dogs do less travel for hunting during pregnancy and milk-feeding, therefore they expose to low risk of infection. The association of positive samples based on geographical locations revealed, more VL infection among dogs from Parikhan village and low rate of infection at Jehadabad and Kojnagh villages. This may be due to high population of dogs and close distance of Parikhan village to Meshkin Shahr city.

There are some publications from Iran, Brazil, India, Ethiopia and Sudan applied different assays for detection of VL in dogs and humans in support or in opposite of this study. A DAT assay was applied but combines with a higher parasite concentration and is performed with only one serum dilution as a simple, rapid, sensitive and non-invasive method which does not require a higher volume of antigens or much expertise and it can be used for screening and serodiagnosis of
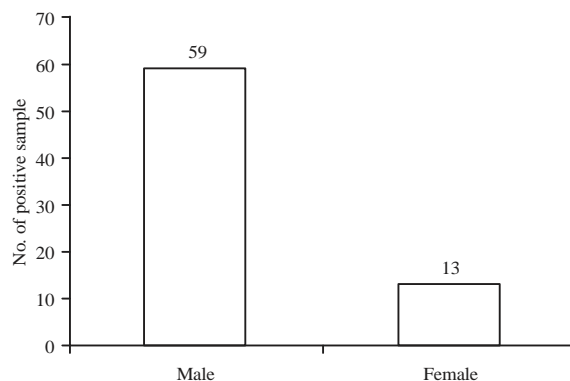
Fig. 4: Comparison of positive samples by both DAT and rK16 tests according to gender, positive samples were assessed by both DAT and rKE16 methods and compared according to gender of dogs in Meshkin Shahr. Results indicated majority of positive dogs were male (82%) in comparison with female infected dogs (18%). Statistical analysis was done by Chi Square method and OpenEpi software (Chi Square: 32.45, Degree of freedom: 1, p-value: <0.0000001)
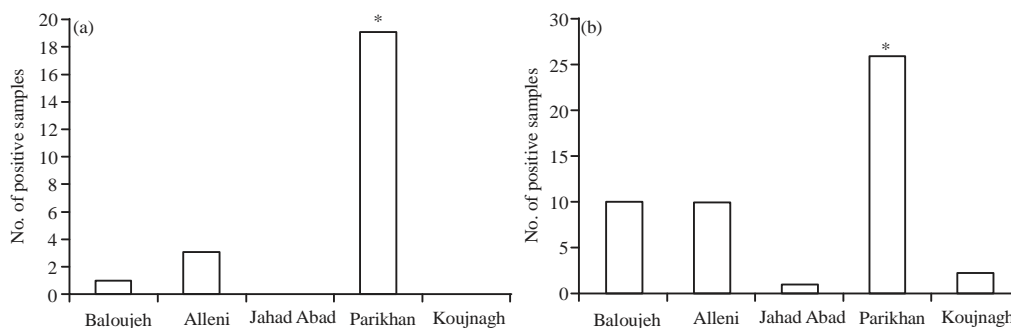

Fig. 5(a-b):Number of positive samples by (a) rKE16 and (b) DAT methods according to geographical locations of sampling, positive samples were assessed by rKE16 and DAT methods and compared based on geographical locations. Considering both assays, the highest rate of VL among dogs were observed in Parikhan village and the lowest rate of infection detected at Jehadabad and Kojnagh villages. Statistical analysis was done by Chi Square method and OpenEpi software, (a) rKE16: Chi Square: 7.857, Degree of freedom: 4, p-value: 0.096), (DAT; Chi Square: 10.08, Degree of freedom: 4, p-value: 0.039)]

human *L. infantum* infection in Iran (Akhoundi *et al.*, 2010). They also recommended a modified DAT antigen with high stability over a range of temperatures and easy transport in the field of areas with endemic VL in Iran (Akhoundi *et al.*, 2013). In addition, a publication showed that the DAT and the rK39-based rapid test can be applied as a useful assay to diagnose VL in Brazil (De Assis *et al.*, 2011). Other researchers suggested that modified DAT and ELISA-rK39 are useful tests for the diagnosis of VL and could replace IFAT as the routine diagnostic test in Brazil (Pedras *et al.*, 2008). Moreover in India, a study revealed that Ld-rKDD8 antigen was less sensitive

and less specific as compared to rKE16 antigen for the diagnosis of VL (Sivakumar *et al.*, 2008). Although, in an opposite data of a study in Amhara State, Ethiopia, the usefulness of the rK39-Immunochromatographic test, DAT and Leishmanin skin test for detecting asymptomatic *Leishmania* infection in children in a new VL focus were indicated (Gadisa *et al.*, 2012), however in a supporting study, an evaluation of two rK39 Dipstick tests, DAT and IFA test for diagnosis of VL in a new epidemic site in highland of Ethiopia was made and both rK39 tests performed as well as DAT and they were indicated as suitable assays for VL diagnosis in first-level health centers in this area of Ethiopia (Canavate *et al.*, 2011). In Sudan, a method was introduced as rKLO8, a novel *L. donovani*-derived recombinant immunodominant protein for sensitive detection of VL and it reported the increased reactivity of Sudanese VL sera with the rKLO and this antigen was confirmed as a potential candidate for diagnosis of VL in Sudan (Abass *et al.*, 2013).

There are some reporting comparisons of different assays for VL detection. A commercial VL containing bound rK39 or rKE16 antigen were evaluated using archived human sera from confirmed VL cases and endemic non-VL controls in the Indian subcontinent (ISC), Brazil and East Africa to assess sensitivity and specificity. Diagnostic accuracy of this test varies between the major endemic regions, suggests it is inadequate for excluding a VL diagnosis (Cunningham *et al.*, 2012). A comparative evaluation of immuno-chromatographic diagnostics for VL, DAT-antigen from a single batch and common protocol was sent to nine laboratories in Latin-America, East-Africa and Asia. Due to significant inter-laboratory disagreement on-site refresher training was provided to all technicians performing DAT. The most probable explanation for disagreement was subjective endpoint reading (Adams *et al.*, 2012). In another study, two formats of rKE16 and rK39 antigen-based rapid tests were compared for the diagnosis of VL. Sensitivities varied between 99.5 and 95.5%. In subjects with different diseases, the specificities were comparable for all rapid tests among endemic areas (Vaish *et al.*, 2012).

CONCLUSION

In conclusion, the findings of this study revealed that DAT method has a higher sensitivity for diagnosis of VL than rKE16 dipstick and more infection was observed among 2-7 years old male dogs from Parikhan village of Meshkin Shahr district, Ardabil province, Iran.

ACKNOWLEDGMENT

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