*Iranian J Publ Health, Vol. 36, No.2, 2007, pp.1-6*

**Original Articles**

# Diagnosis of Canine Visceral Leishmaniasis by ELISA Using K39sub Recombinant Antigen

*M Taran [1], *M Mohebali [1], MH Modaresi [2], S Mamishi [3], M Mahmoudi [1], M Mojarad [2]*

[1]*Dept. of Medical Parasitology and Mycology, School of Public Health and Institute of Public Health Research, Medical Sciences/University of Tehran, Iran*
[2] *Dept. of Medical Genetics, Faculty of Medicine, Medical Sciences/University of Tehran, Iran*
[3] *Pediatric Medical Center, Faculty of Medicine, Medical Sciences/University of Tehran, Iran*

**(Received 15 Jan 2007; accepted 3 May 2007)**

## Abstract

**Background:** Surveillance of the canine reservoir is highly important to help control of visceral leishmaniasis in human. It is therefore imperative to improve and develop new tools reliable, easy to use, and cheap for the diagnosis of canine leishmaniasis. K39 sub recombinant antigen of *Leishmania infantum* was expressed in prokaryotic system and evaluated for sero-diagnosis of canine visceral leishmaniasis (CVL).

**Methods:** The gene fragment encoding a single 39-amino acid subunit of the kinesin-related protein k39 (k39sub) was amplified from DNA of Iranian strain of *L. infantum* (MCAN/IR/96/LON49) and cloned into a pMAL-p2 expression vector in frame with maltose-binding protein (MBP) fusion. The antigenic properties of *L. infantum* recombinant K39 subunit (39 amino acids) have been tested for the serological diagnosis of CVL by ELISA. K39sub ELISA for CVL was compared with a standard direct agglutination test (DAT) on 55 clinically infected dogs and 71 healthy controls from endemic areas of Ardabil and East Azerbaijan provinces, north-west of Iran.

**Results:** A sensitivity of 72.7% and specificity of 87.3% were found at a 1:320 cut off titer when DAT confirmed cases were compared with healthy control. A good concordance was found between k39sub ELISA and DAT (k= 81.0).

**Conclusion:** Given the antigenic properties shown by the k39sub, we think this protein carry immunodominant epitopes and are valuable for the sero- diagnosis of *L. infantum* infection in dogs.

**Keywords:** *K39antigen,* Leishmania infantum*, Agglutination test, Dogs*

## Introduction

Protozoan parasites of the genus *Leishmania* are widely distributed and transmitted by the bite of sandflies. In the vertebrate host, the infecting promastigotes differentiate into and replicate as amastigotes within macrophages (1). Depending on the species of *Leishmania*, infection can lead to a variety of diseases. Visceral leishmaniasis (VL), also known as kala-azar, is commonly caused by *L. infantum* (Mediterranean), *L. donovani* (India and Africa), or *L. chagasi* (South America) (2). Although humans are the sole reservoir hosts for some *Leishmania* species, in most cases other animals play a major role in the maintenance of infections (3). Domestic dogs (*Canis familiaris*) are principal reservoir hosts of zoonotic visceral leishmaniasis (ZVL) caused by *L. infantum*. Therefore, one of the most important approaches to reduce the incidence of human VL (Kala-azar) is to cull infected dogs (4).

Then the diagnosis of visceral leishmaniasis infection in dogs is important in veterinary practice and in surveillance of zoonotic visceral leishmaniasis (ZVL). The direct agglutination test (DAT) and immunofluorescence antibody test (IFAT) are routinely used for the detection of specific antibodies. DAT in spite of high sensitivity and specificity is very time consuming (5, 6). IFAT requires technological expertise and specialized laboratory equipment, and can be labor-intensive,

***Corresponding author:** Tel: +98 21 88951400, fax: +98 21 66462267, E-mail: mmohebali@hotmail.com

and is difficult to standardize and to interpret (7). Immunoenzymatic assays such as the enzyme-linked immunosorbent assay (ELISA) are easier to standardize and more practical as routine laboratory tools. The performance of ELISA test is greatly affected by the quality of the antigens used, however, and test specificity limitations are the main drawback when crude antigen preparations are used. Recombinant technology, together with the characterization of specific immunodominant antigens at the genetic level, allowed the development of a second generation of diagnostic immunoassays, and the recent validation of a recombinant k39-ELISA as a diagnostic marker for canine leishmaniasis represents a good example (8). The k39 antigen is a repetitive of 39 amino acid residues in visceral *Leishmania* isolates examined so far (9, 10) and is mainly expressed in the amastigote stage and elicits a strong immunoresponse in both asymptomatic and clinically infected dogs (7). The 39-amino acid subunit of k39 (k39sub) had not been evaluated as diagnostic marker for canine Leishmaniasis in Iran. For the first time, we determined sensitivity and specificity of k39sub-ELISA from Iranian strain of *L. infantum* compared to direct agglutination test for the serodiagnosis of canine visceral leishmaniasis.

## Materials and Methods

***DNA isolation, PCR, cloning, expression and purification of recombinant protein*** DNA was extracted from promastigotes of the *L. infantum* reference strain (MCAN/Ir/96/Lon 49) by conventional procedures (11). Primers were synthesized for the amplification of the 3'-ter-minal gene fragment encoding a single 39-amino-acid unit of k39 (k39sub) (GenBank accession number L07879; fw 3146 to 3169, rv 3248 to 3265). Oligonucleotides contained restriction sites for *Bam*HI (sense) and *Hind* III (antisense) to facilitate cloning and stop codon. Amplification of the target gene was carried out in a 50-µl reaction volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 10 pmol of each primer, 200 ng of the template, and 1 U of *PFU* DNA polymerase (Gibco). PCR was performed in 14 cycles of 95 °C for 30 s, 66.4-0.5 for 30 s, and 72 °C for 20 s and 20 cycles of 95 °C for 30 s, 59.4 for 30 s, and 72 °C for 20 s. Amplified products of the expected length were subcloned into pBluescript II SK (+) (Stratagene), miniprepared, digested with appropriate restriction enzymes, and cloned into pMAL-p2 expression vector (New England Biolabs, Inc) in frame with maltose-binding protein (MBP) fusion.

To express recombinant protein, early log-phase cultures of positive clones were induced for 5 h with 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) under agitation. Bacterial cells were recovered by centrifugation and lysed by conventional physicochemical methods. Recombinant fusion proteins were recovered in the soluble fraction and purified by affinity chromatography on amylase columns according to the supplier's method (New England Biolabs). The expected molecular mass was 47 kDa for MBP-K39sub. To avoid false-positive reactions to the carrier moiety, recombinant MBP was expressed and purified under the same conditions and used in the ELISA procedure as a negative antigen. The purity and yield of antigen was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Bradford methods (12).

***Sampling*** Blood samples (2–5 ml) were taken from 126 dogs from Ardabil and East Azerbaijan Provinces, Nort-west of Iran where *L. infantum* are endemic. 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. Dog age was determined by interviewing dog owners. The mean age of dogs was 35 mo (range 2-192 mo); 79 were male and 47 were female. No transmission of *Leishmania* spp. or *Trypanosoma cruzi* was reported in dogs in the studied areas.

***Enzyme linked immunosorbent assay*** The ELISA was performed according to Hommel et

al. (13). For ELISA, microtiter plates were coated overnight with 20µg/ml of MBP-k39sub and MBP in 100µl of 0.1M bicarbonate buffer, pH 9.0. Following overnight incubation at 4 °C and subsequent washes with PBS- 0.05% Tween 20 (phosphate-buffered saline- 0.05% Tween 20) excess protein binding sites were blocked at room temperature for 3 h with 250 µl /well of PBS containing 1% bovine serum albumin. After tree washes, 100 µl of 1:100 dog sera in serum diluent (PBS containing 0.1% bovine serum albumin and 0.05% Tween 20) was added to duplicate wells. After three washes, 100µl of anti-human conjugated with alkaline phosphatase (Sigma Co.) (with dilution 1/1000) was added to each well and incubated for 30 min. after three washes, 100µl p-nitro phenyl phosphate (Sigma Co.) in diethyl amine buffer was added to each well and incubated for 20 min , the reaction was stopped with 100µl of 3 N NaOH and The absorbance at 405 nm was measured. The ELISA reader (Anthos 2020) was set to subtract the reading of MBP as the negative antigen from that of MBP-k39sub. The cut off point was calculated by X+3SD in normal dog population. Optical Density (OD) reading of ≥0.23 was considered as positive.

***Direct agglutination test*** The *L. infantum* antigens for this study were prepared in the protozoology unit of the School of Public Health in 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% (6, 14). The dog serum samples were tested by DAT according to the methods described by Harith et al. (6, 15). Specific *Leishmania* antibodies at a 1:320 and above were considered as positive (6, 15).

***Parasitological study*** Twenty suspected dogs were examined externally for signs of *Leishmania* infection; afterwards, all suspected dogs were dissected for the observation of amastigote form of *L. infantum* in the liver and spleen of each dog. All the prepared smears were fixed with methanol, stained with Giemsa stain 10% and examined microscopically for the presence of amastigotes. Twelve out of 14 dissected dogs were parasitologically positive and the results were used for confirming K39 sub recombinant antigen.

***Negative and positive controls*** Three groups of dog sera were used. Dog sera with suspected clinically and DAT positive (n= 55), sera from healthy dogs and DAT negative (n= 71), and the positive standard control sera came from dogs with *L. infantum* infection confirmed by microscopy (n= 12).

***Data analysis*** Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) for each diagnostic test were calculated according to the method of Fleiss and Smith (16, 17).

## Results

MBP and MBP-k39sub were highly expressed, found in the soluble fraction, and successfully purified by affinity chromatography (Fig. 1).

The frequency distribution of DAT titers showed of all 126 serum samples were collected from the dogs and their comparison results with k39sub-ELISA were summarized in Table 1. Nine out of 71 negative control sera were positive by using the k39sub-ELISA.

Using a cut off value of 1:320 for DAT and that of 0.23 for k39sub- ELISA the concordance between two tests was 81.0 ($P< 0.01$). The most concordance rate between two tests was occurred in 1: 640 DAT cut off value.

The validity of DAT regarding the complete series of clinically suspected dogs and concordance between two tests were analyzed separately in Table 2.

Table 3 showed that positive (PPV) and negative (NPV) predictive values of k39sub-ELISA according to prevalence rates of canine visceral leishmaniasis in endemic foci of Iran. When the prevalence rate is 14%, the PPV will be 48.3%.

It should be mentioned that k39sub- ELISA test was positive in all 12 authopsied dogs that they were parasitologically and serologically (DAT) positive (≥1:320).

**Table 1:** Comparison of k39sub-ELISA and DAT in diagnosis of *L. infantum* infection in dogs

| DAT | k39sub-ELISA | | | | | |
|---|---|---|---|---|---|---|
| | Positive | | Negative | | Total | |
| | No. | % | No. | % | No. | % |
| Negative | 4 | 8.2 | 53 | 67.8 | 57 | 45.3 |
| 1:80 | 2 | 4.1 | 7 | 9 | 9 | 7.1 |
| 1:160 | 3 | 6.1 | 2 | 2.6 | 5 | 4 |
| 1:320 | 10 | 20.4 | 15 | 20.6 | 25 | 19.8 |
| 1:640 | 6 | 12.2 | 0 | 0 | 6 | 4.8 |
| 1:1280 | 2 | 4.1 | 0 | 0 | 2 | 1.6 |
| 1:2560 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1:5120 | 1 | 2 | 0 | 0 | 1 | 0.8 |
| 1:10240 | 9 | 18.4 | 0 | 0 | 9 | 7.1 |
| 1:20480 | 12 | 24.5 | 0 | 0 | 12 | 9.5 |
| Total | 49 | 100 | 77 | 100 | 126 | 100 |

**Table 2:** Sensitivity, specificity and concordance values of k39sub-ELISA compared with DAT according to the different cut off titers

| DAT titer | Sensitivity | Specificity | Concordance |
|---|---|---|---|
| 1:80 | 65.2± 8.3 | 93.0± 4.5 | 77.0± 7.3 |
| 1:160 | 71.7± 8.0 | 91.0± 5.0 | 81.8± 6.8 |
| 1:320 | 72.7± 7.8 | 87.3± 5.8 | 81.0± 6.9 |
| 1:640 | 93.8± 4.2 | 83.0± 6.6 | 85.7± 6.1 |
| 1:1280 | 96.0± 3.4 | 77.2± 7.3 | 81.0± 6.9 |
| 1:2560 | 95.6± 3.6 | 75.7± 7.5 | 79.4± 7.1 |
| 1:5120 | 100± 0 | 75.0± 7.6 | 79.4± 7.1 |
| 1:10240 | 100± 0 | 74.3± 7.6 | 78.6± 7.2 |
| 1:20480 | 100± 0 | 68.4± 8.1 | 71.4± 8.0 |

Values are binomial 95% confidence limits

**Table 3:** Positive predictive (PPV) and negative predictive values (NPV) for a 1: 320 direct agglutination test cut off titer (sensitivity= 72.7%, specificity= 87.3%), according to different prevalence rate in the studied endemic areas of Iran

| Prevalence rate (%) | PPV (%) | NPV (%) |
|---|---|---|
| 14 | 48.3 | 98.7 |
| 21 | 60.5 | 96.3 |
| 28 | 69.2 | 93.9 |

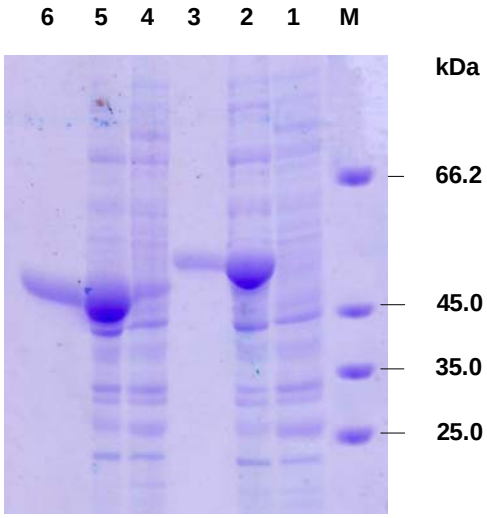


**Fig. 1:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis result showing expression and purification of recombinant antigens in *E. coli*. M, molecular-weight standard; lanes 1, 2, 4, 5, total noninduced and induced bacterial lysates expressing MBP and MBP-K39sub, respectively; lanes 3 and 6, affinity-purified, MBP and MBP-K39sub respectively.

## Discussion

The development of serodiagnostic tests for VL seems to be a show case of the multiple methodological pitfalls in test validation (16, 18). The most important question is the imperfect standard for diagnosing VL and the absence of such a reference test (gold standard) for infection with the parasite that causes VL. The definite diagnosis of VL depends upon the demonstration of leishmanial amastigotes in bone marrow or biopsy material (spleen, lymph nodes, liver). These procedures are invasive, and thus are acceptable only in case of clinical suspicion of the disease. Moreover, they have poor sensitivity (19). Parasitological confirmation might be the best standard for diagnosing VL, but not for diagnosing infection with *L. donovani* complex in a community at risk (20). According to previous studies (6, 14, 19) the performance of the DAT for detection of *L. infantum* infection in humans and dogs was excellent. Therefore, we have used DAT as the gold standard and compared the ELISA results with DAT. The serodi-

agnostic potential of k39 for VL, is shown previously with subjects from Brazil, Sudan, China and Pakistan (9, 5, 21).

Findings indicate not only the conservation of the K39 epitope among visceralizing species of *Leishmania* in different geographical regions, but also its high antigenicity (21).

Six reported studies used a k39-ELISA to detect *Leishmania* infection in dogs (7, 8, 22-25). The k39-ELISA was 100% sensitive in 90 para-sitologically confirmed, high-antibody-titer dogs in Brazil (22) and in 37 parasitologically confirmed dogs in Venezuela (negative controls not included in either study) (25). In a Turkish study, 18 of 494 dogs were positive by k39-ELISA; sensitivity and specificity were reported to be 93% and 100%, respectively (23). In a large epidemiological survey in Italy, k39-ELISA sensitivity and specificity were 97 and 99%, re-spectively (8). Finally, in a Moroccan study, the k39- ELISA was 100% sensitive in detecting 11 parasitologically confirmed, clinically symptomatic dogs, but failed to detect ZVL infection in 9 parasitologically confirmed, clinically asymptomatic dogs (25). The k39sub-ELISA was 95% sensitive and specific in 40 parasitologically confirmed dogs in Italy (7).

Our results demonstrated that k39sub antigen carries highly reactive and diagnostically relevant epitopes. The sensitivity of k39sub antigen is lower than that of k39 antigen in ELISA. This resulted in a complete subset of overlapping epitopes which might be lost if a single repetition were to be used. The k39sub ELISA showed the good agreement with the DAT results.

## Acknowledgements

This study was financially supported by the Institute of Public Health Research, Academic Pivot for Education and Research, Medical Sciences/ University of Tehran No: 240/ 5437. We wish to express our thanks to Mrs B Akhoundi, Mr Z zarei, Dr H Hajjaran, and Mrs S Charedar for laboratory and field help. We are also grateful for the sincere cooperation of the staff of Medical Genetics department of Medical Sciences/ Tehran University.

## References

1. Haldar JP, Ghose S, Saha KC, and Ghose AC (1983). Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. *Infect Immunol*, 42(2): 702–7.
2. Lee MB, Gilbert HM (1999). Current Approaches to Leishmaniasis. *Infect Med*, 16: 34-7.
3. Mauricio IL, Stothard JR, Miles MA (2000). The strange case of Leishmania chagasi. *Parasitol Today*, 16(5): 188-89.
4. Ashford DA, David JR, Freire M, David R, Sherlock I, Eulalio MC, et al. (1998). Studies on control of visceral leishmaniasis impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. *Am J Trop Med Hyg*, 59: 53–7.
5. Reithinger R, Quinnell RJ, Alexander B, Davies CR (2002). Rapid Detection of Leishmania infantum Infection in Dogs: Comparative Study Using an Immunochromatographic Dipstick Test, Enzyme-Linked Immunosorbent Assay, and PCR. *J Clin Microbiol*, 40: 2352-56.
6. Harith A, Salappendel RJ, Reiter I, Knapen F, Korte P, Huigen E, Kolk, RHG, (1989). Application of a direct agglutination test for detection of specific anti-*Leishmania* antibodies in the canine reservoir. *J Clin Microbiol*, 27: 2252–57.
7. Rosati S, Ortoffi M, Profiti M, Mannelli A, Mignone W, Bollo E, et al. ( 2003). Prokaryotic Expression and Antigenic Characterization of Three Recombinant Leishmania Antigens for Serological Diagnosis of Canine Leishmaniasis. *Clin Diag Lab Immunol*, 10: 1153-56.
8. Scalone A, De Luna R, Oliva G, Baldi L, Satta G, Vesco G, et al. (2002). Evaluation of the *Leishmania* recombinant K39

antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. *Vet Parasitol*, 104: 275-85.

9. Burns JM JR, , Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG (1993). Molecular characterization of a kinesin-related antigen of Leishmania chagasi which detects specific antibody in African and American visceral leishmaniasis patients. *Proc Nati Aca Sci*. *USA*, 90: 775-79.
10. Singh S, Gilman-Sachs A, Chang KP, Reed SG (1995). Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. *J Parasitol*, 81: 1000-3.
11. Requena JM, Lopez MC, Jimenez-Ruiz A, De la Torre JC, Alonso C (1988). A head-to-tail tandem organization of hsp70 genes in *Trypanosoma ctuzi*. *Nuc Aci Res*, 16: 1393-406.
12. Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann Biochem*, 72: 248-54.
13. Hommel M , Peters W, Ranque J, Quilici M, Lanotte G (1978). The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. *Ann Trop Med Parasitol*, 72: 213-18.
14. Edrissian GH, Hajjaran H, Mohebali M, Soleimanzaeh G, Bokaei S (1996). Application and evaluation of direct agglutination test in sero-diagnosis of visceral leishmaniasis in man and canine reservoirs in Iran. *Iranian J Med Sci*, 21: 119-24.
15. Mohebali M, Edrissian Gh.H, Nadim A, Hajjaran H, Akhoundi B, Hooshman B, et al. (2006). Application of Direct agglutination test (DAT) for the diagnosis and seroepidemiological studies of visceral leishmaniasis in Iran. *Iranian J Parasiolt*, 1: 15-25.
16. Smith PG, Richard HM (1992). *Methods for Field Trials of Interventions Against Tropical Diseases*. 2nd ed. Oxford Medical Publications.
17. Fleiss JL (1981). *Statistical Methods for Rates and Proportions*. 2nd ed. Wiley, New York .pp.212-36.
18. Valenstein P (1990). Evaluation diagnostic tests with imperfect standards. *Am J Clin Pathol*, 93: 252-58.
19. Saul J, Semiao S (1996). *Canine visceral leishmaniasis in Evora district. In: Protogal: A Seroepidemiological Study*. ACP/ Academic Press, Amsterdam. pp. 9-74.
20. Mohebali M, Taran M, Zarei Z (2004). Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick rk39 test and direct agglutination. *Vet Parasitol*, 121: 239-45.
21. Qu Qi, Zhong L, Masoom-Yasinzai M, Abdur-Rab M, Aksu HS, Reed SG, et al (1994). Serodiagnosis of Asian leishmaniasis with a recombinant antigen from the repetitive domain of a Leishmania kinesin. *Trans Roy Soc Trop Med Hyg*, 88: 543-45.
22. Badaró R, Benson D, Eulálio MC, Freire M, Cunha S, Netto EM, et al. ( 1996). rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *J Infec Dis*, 173: 758-61.
23. Ozensoy S, Ozbel Y, Turgay N, Alkan M Z, Gul K, Gilman-Sachs A, et al. (1998). Serodiagnosis and epidemiology of visceral leishmaniasis in Turkey. *Am J Trop Med Hyg*, 59:363-69.
24. Rhalem A, Sahibi H , Guessous-Idrissi N, Lasri S, Natami A, Riyad M, et al. (1999). Immune response against *Leishmania* antigens in dogs naturally and experimentally infected with *Leishmania infantum*. *Vet Parasitol*, 81:173-84.
25. Zerpa O, Ulrich M, Negrón E, Rodríguez N, Centeno M, Rodríguez V, et al. (2000). Canine visceral leishmaniasis on Margarita island (Nueva Esparta, Venezuela). *Trans Roy Soc Trop Med Hyg*, 5: 484-87.