



EVALUATION OF rK39 IMMUNOCHROMATOGRAPHIC TEST FOR THE DIAGNOSIS OF CANINE LEISHMANIASIS

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Summary

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Canine leishmaniasis (CanL) is a vector-borne zoonotic disease caused by the protozoan parasite *Leishmania infantum*, which is transmitted by the bite of a female sand fly. The aim of this study was to evaluate a human immunochromatographic test (IT-LEISH) for the detection of *Leishmania* infection in dogs by comparing this test with the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT). One hundred and seventy-six dogs chosen at random from the North-West region of Algeria were included in this study. All dogs were tested by three serological techniques namely IT-LEISH, IFAT and ELISA. The results of the present study showed that the sensitivity and specificity of IT-LEISH reached 94.4% and 87.2% compared with IFAT and 81.8% and 87% with ELISA respectively. The ROC curves and the area under the curve (AUC) allowed us to deduce that the results of the IT-LEISH test were good and excellent compared to ELISA and IFAT, respectively. In conclusion, the IT-LEISH test could be used in the diagnosis of CanL due to its reliability, fast and ease of use compared to other diagnostic methods.

Key words: canine leishmaniasis, diagnosis, IT-LEISH, IFAT, ELISA, sensitivity, specificity

INTRODUCTION

Canine leishmaniasis (CanL) is a vector-borne zoonosis that constitutes a serious public health problem (Elikaee *et al.*, 2019; Oliveira *et al.*, 2021; Ruiz-Postigo *et al.*, 2021; Akhzari *et al.*, 2024). It is endemic throughout the Mediterranean basin, and Algeria is among the most affected countries (Harrat *et al.*, 2003; Bachi, 2006; Vélez *et al.*, 2019). The disease is caused by a flagellated protozoan belonging to the genus *Leishmania*, mainly *Leishmania infantum* (Bachi, 2006; Zait *et al.*, 2012; Akhouni *et al.*, 2016; Moualek *et al.*, 2017), which is usually transmitted by the bite of an infected female sand fly (Moualek *et al.*, 2017; Tonelli *et al.*, 2021; Vilas-Boas *et al.*, 2024) and for which the dog constitutes the main reservoir of the disease (Zait *et al.*, 2012; Selim *et al.*, 2021; Garcia *et al.*, 2025).

CanL is a systemic disease that, when it occurs, presents a highly variable clinical picture (Gharbi *et al.*, 2015). Therefore, in addition to the clinical examination, its diagnosis requires various serological tests such as IFAT, ELISA, and immunochromatographic tests which are reliable, with a variety of antigens and easy to use (Saridomichelakis *et al.*, 2005; Elmahallawy *et al.*, 2014).

In foci of CanL, clinical disease occurs in less than 50% of the infected dogs and is characterised by a chronic evolution of viscerocutaneous signs (Oliva & Pagano, 2004) – a reason why the use of easy, fast, and efficient diagnostic tests is more than necessary. A recent study from Tiaret, northwestern Algeria, found a high prevalence of CanL in Atlas Shepherd dogs using various serological and molecular techniques (Bia *et al.*, 2022). Given the absence of immunochromatographic tests for dogs in Algeria, the

aim of the current study was to evaluate the performance of a human immunochromatographic test (IT-LEISH) for the diagnosis of leishmaniasis in dogs from different regions of western Algeria and to compare the results with those obtained by other diagnostic techniques.

MATERIALS AND METHODS

Ethics statement

This study was approved and validated by the scientific committee of the Institute of Veterinary Sciences, University Ibn Khaldoun, Tiaret, Algeria and registered under the number 205/VRPG/2017.

Animals

Our study involved a total of 176 dogs of both sexes; all dogs were adults older than one year, none of the dogs showed any signs related to canine leishmaniasis. The choice of dogs was random and the total number (n=176) was related to the availability of IT-LEISH kits. The investigation was carried out in different regions of the western part of Algeria (Tiaret (59 dogs), Mascara (59 dogs), Tisemsilt (58 dogs) from 2017 to 2020.

Blood samples preparation

One hundred and seventy-six peripheral bloods (4 mL) were collected in dry tubes, sera were separated and stored at -20°C until performance of serological test (IT-LEISH-ELISA-IFAT). The samples were sent to the WHO Collaborating Centre for Leishmaniasis, National Centre for Microbiology, Instituto de Salud Carlos III (Madrid), to perform two different paraclinical diagnostic techniques: indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and

to Laboratory of Parasitology and Mycology, Mustapha Tertiary Care Hospital, Algiers (Algeria), to perform the immunochromatographic test (IT-LEISH).

CanL special diagnostic tests

One hundred and seventy-six dogs underwent three serological tests (IT-LEISH, IFAT, ELISA).

Immunochromatographic test (IT-LEISH). The recombinant rK39 antigen immunochromatographic test on a strip (IT-LEISH) was performed with a commercial kit (Lot: 011140, Ref. 710124, Bio-Rad, France) following the manufacturer's instructions (IT LEISH. BIO-RAD, France).

Indirect Fluorescent Antibody Test (IFAT). The technique was performed according to Alvar *et al.* (2004) using *L. infantum* promastigotes as antigens (strain MHOM/FR/78/LEM 75). Stationary phase promastigote (MHOM/FR/78/LEM 75) were washed three times in PBS (PBS, pH 7.2) and 10 U1 of a 2×10^7 parasites/mL suspension are dispensed in 15-well immunofluorescence slides. Slides were then air dried for 1 h at 37 °C, fixed with cold acetone (−20 °C) for 5 min, and air-dried after one wash in PBS (−20 °C). Sera from dogs were assayed in serial two fold dilutions, from 1/40 to 1/640 then diluted sera (10 µL) were incubated in parasite-coated slides for 30 min at 37 °C into a wet chamber. After two washes in PBS-T20, 10 min each, the slides were air dried. The antibody fixation was revealed with 10 µL of FITC-conjugate diluted with PBS and Evans Bleu (0.01%) for counterstaining. The slides were then incubated for 30 min at 37 °C into a wet chamber in the dark. After three washes (10 min each), under shaking in the dark in PBS-T20, the slides were air dried, mounted with glycerol-PBS, pH=9.2 (9:1)

and a coverglass. Finally, the slides were examined using a Zeiss fluorescence microscope (40×).

Enzyme-linked Immunosorbent Assay (ELISA). The Maxisorp plates were sensitised with 100 µL of *Leishmania* antigen (1 µg of SLA per well) in temperon carbonate, during 1.30 h at 37 °C or 4 °C overnight. The contents of the plate was removed by vigorously tipping it over onto the wash. After washing 3 times without agitation with washing solution (PBS 1× + 0.05% Tween 20), and gently drying the remaining liquid on paper, the plates were blocked by adding 200 µL of blocking solution (1% casein in 1× PBS) at 37 °C for 1 hour. After washing 3 times with agitation for 3 min, 100 µL of plasma diluted 1/100 in washing solution was added for each well, incubated at 37 °C for 1 h and after washing 3 times, the peroxidase conjugate (HRP-mouse anti-dog Ig-), was added, the plate was covered with aluminum foil and incubated at 37 °C for 30 min. After washing 3 times, 100 µL o-phenylenediamine (OPD) substrate prepared 5 min before use was added, covered with aluminum foil. The plate was incubated in darkness (covered plate) with agitation at room temperature for 15 min. Then 50 µL stop solution (HCl 2N) was added and the plate was incubated in darkness (covered plate) with agitation at room temperature for 2 min. Finally, the plates were read at 490 nm.

Statistical analysis

The area under the curve (AUC) was calculated for ROC curves. The AUC corresponds to the probability that a positive event has a higher probability given by the model than a negative event. For an ideal model, AUC=1 and for a random model, AUC=0.5. A model is considered good when the AUC value is greater than 0.7, a

well-discriminating model must have an AUC between 0.87 and 0.9 whereas a model with an AUC greater than 0.9 is excellent (Hosmer& Lemeshow, 2000).

The sensitivity and specificity of the diagnostic tests were calculated using the following formulas:

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{number of false negatives}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{number of false positives}}$$

The positive predictive value (PPV) and the negative predictive value (NPV) were calculated as followed:

$$\text{PPV} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{number of false positives}}$$

$$\text{NPV} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{number of false negatives}}$$

Youden index = sensitivity+ specificity-1

All statistical analyses were performed

using XL-STAT software version 2016.02.28451, and 95% confidence intervals (CI) were established.

RESULTS

Table 1 summarises the the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), the 95% confidence interval of each approach. The best results were obtained with IT-LEISH/IFAT: 94.4% and 87.2% in terms of sensitivity and specificity respectively (Table 1); the PPV was equal to 45.9%, and the NPV was 99.3%. The IT-LEISH/ELISA decreased the estimated sensitivity to 81.8% and the specificity to 87% (Table 1), and the PPV and NPV were 47.4% and 97.1% respectively.

The results from IT-LEISH/IFAT and IT-LEISH/ELISA comparisons are presented in Tables 2 and 3. For IT-LEISH/IFAT, the AUC which is equal to 0.908 (P<0.05 (Fig. 1), the Youden index was 0.81 and according to Hosmer & Lemeshow (2000) the performance of the

Table 1. Sensitivity, specificity with 95% confidence intervals, PPV, and NPV for the diagnostic tests used in the study

Tests	Sensitivity [95%CI]	Specificity [95%CI]	PPV	NPV
IT-LEISH/IFAT	94.4% [72–100]	87.2% [80.9–91.6]	45.9%	99.3%
IT-LEISH/ELISA	81.8% [60.7–93.1]	87% [80.7–91.5]	47.4%	97.1%

Table 2. Results of IT-LEISH comparing to IFAT

IFAT \ IT-LEISH	Positive	Negative
Positive	17 (TP)	1 (FN)
Negative	20 (FP)	138 (TN)

TP: true positive, FP: false positive, TN: true negative, FN: false negative.

Table 3. Results of IT-LEISH comparing to ELISA

IT-LEISH \ ELISA	Positive	Negative
Positive	18 (TP)	4 (FN)
Negative	20 (FP)	134(TN)

TP: true positive, FP: false positive, TN: true negative, FN: false negative.

test was excellent. The results of IT-LEISH compared to ELISA were assessed as good in terms of AUC equal to 0.844, Youden index: 0.68 and $P < 0.05$ (Fig. 2).

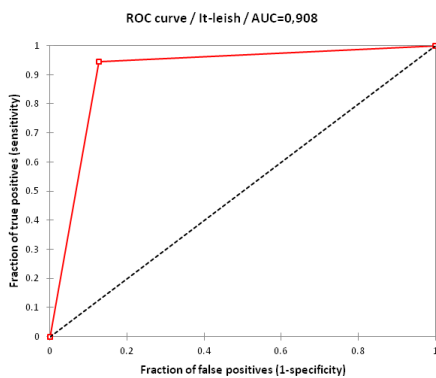


Fig. 1. Graphical representation of the ROC curve and the AUC of IT-LEISH compared to IFAT.

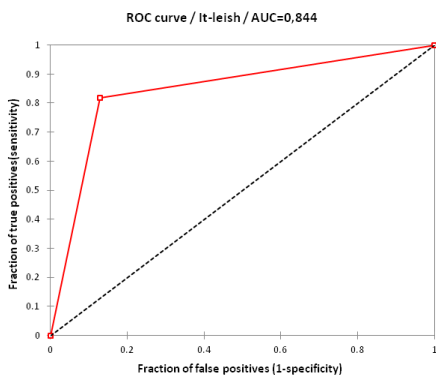


Fig. 2. Graphical representation of the ROC curve and the AUC of IT-LEISH compared to ELISA.

DISCUSSION

The rapid immunochromatographic test (IT-LEISH) was used to detect the presence of anti-*Leishmania spp.* antibodies in humans using a recombinant antigen (rK39). A variety of studies have validated the diagnostic performance of this rapid immunochromatographic test method, with sensitivity and specificity values between 90 and 100% (Maia *et al.*, 2012; Siqueira *et al.*, 2021).

In this study, the immunochromatographic test was used to detect antibodies against *Leishmania infantum* in dogs sampled from the northwest of Algeria. The efficiency of the recombinant rK39 antigen as a diagnostic marker for CanL has frequently been evaluated and proved in other studies (Lima *et al.*, 2022; Morales-Yuste *et al.*, 2022). According to our findings, IT-LEISH had a higher sensitivity and specificity (94.4% and 87.2%, respectively) compared to IFAT. Our results were better than those obtained by Reithinger *et al.* (2002), which demonstrated that the sensitivity and the specificity of rK39 dipstick varied from 72% to 77% and 61% to 75% respectively when used to diagnose visceral CanL. However, Otranto *et al.* (2005) described a better performance of the rK39 dipstick test compared to IFAT, and showing a respective sensitivity and a specificity of 97.06% and 100%. In a comparative study focused on evaluating the performance of a com-

mercially available rK39 dipstick test and ELISA using crude antigen, a sensitivity of 83% was obtained for the rK39 test, which is closer to our results, with a specificity of 100% (Lemos *et al.*, 2008). Concerning the ROC curve and the area under the curve (AUC) the results of the IT-LEISH test were good and excellent compared to ELISA and IFAT respectively (Hosmer & Lemeshow, 2000).

Despite the high sensitivity and specificity of IT-LEISH, the PPV (positive predictive value) is very low, only 45–47%, which means that almost half of the positive results may be false positive, which may be due to the clinical status of dogs (asymptomatic), limited ability of qualitative serological techniques to distinguish between past and acute infections, as well as the possibility of cross-reactions with other infectious agents. Therefore, it is proposed that the use of this test should be combined with other tests such as IFAT or ELISA, especially in asymptomatic dogs (Noli *et al.*, 2014).

The mismatch between the previous results and the divergence in performance may be attributed to the use of different approaches to estimate the true negative and true positive dogs, the principles of the techniques, the type of antigen and conjugate used, and modifications to the standard experimental protocol (incubation time, serum dilutions or type of microtitre plates used). On the other hand, the correct interpretation of IFAT results is subjective and depends on the operator's expertise, whereas the interpretation of the dipstick test is unequivocal (Mettler *et al.*, 2005; Barbiéri, 2006; de Lima *et al.*, 2010; Regina-Silva *et al.*, 2014; Solano-Gallego *et al.*, 2014).

In Brazil, a group of researchers evaluated the sensitivity of a rapid diagnostic test (RDT) based on serological detection

of the rK39 antigen in a cohort of naturally infected dogs (Quinnell *et al.*, 2013). The authors stated that the diagnostic performance of rK39 RDTs is reasonable for confirmation of *Leishmania* infection in suspected clinical cases, but the sensitivity to detect infected dogs is too low for large-scale epidemiological surveys and operational control programmes.

CONCLUSIONS

The IT-LEISH has proven its effectiveness in the diagnosis of CanL. The results show that the rK39 antigen is as an useful immunogenic antigen in the diagnosis of leishmaniasis in humans and even dogs, and because it is more available and easy to use, we recommend its use in veterinary settings to confirm tentative diagnosis of leishmaniasis in dogs.

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