https://doi.org/10.48047/AFJBS.6.16.2024.780-797

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Diagnostic Performance of IFAT, Blood and Lymph Node Culture and Rapid Tests in Canine Leishmaniasis and Their Association with Clinical Signs

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Volume 6, Issue 16, Nov 2024

Received: 28 Oct 2024

Accepted: 22 Nov 2024

Published: 07 Dec 2024

doi: 10.48047/AFJBS.6.16.2024.780-797

Abstract

Canine leishmaniasis (CanL) is an important public health issue in the Mediterranean basin. This study aimed to enhance the diagnosis of CanL in Algeria and to assess its association with clinical manifestations. We evaluated the effectiveness of parasitological and immunological diagnostic methods in 109 dogs, both with and without clinical signs of Canl. The diagnostic methods assessed included the indirect Immunofluorescent antibody test (IFAT), Immunochromatographic rapid test (RIM), lymph node culture (LNC), and blood culture (BC).The sensitivity and specificity of each technique were analyzed. The results indicated that IFAT had the highest proportion of positivity (15.6%), followed by RIM (11%) BC (7.3%)and LNC (6.4%). RIM, BC,and LNC exhibited high specificity (97.8%,100%, and100%, respectively), though their sensitivity varied:58.8% for RIM, 41.18% for LNC, and 64.71% for BC, compared to IFAT (the Gold Standard). The association between tests and clinical signs revealed that IFAT was to detect seropositive in dogs presenting a single clinical sign, skin lesions (4.4%) and altered general condition (100%), whereas other tests were negative. All four tests were positive for dogs with multiple clinical signs,with IFAT showing the highest proportion of positivity,followed by blood culture, LNCand RIM.The study results demonstrate variability in test characteristics across different clinical signs.

Keywords: Canine leishmaniasis; IFAT; Blood culture; Lymph node culture; Rapid testing; Clinical signs.

INTRODUCTION

Leishmaniasis is a protozoal disease caused by intracellular agents of the genus *Leishmania spp* (CanL), transmitted by the bite of female sandflies (Miro et al., 2017). It is a group of zoonotic infectious parasitic diseases that affect both humans and animals, particularly domestic dogs, which are considered the main reservoir for humans (Knight et al., 2022). These serious emerging parasitosis have been reported in 88 countries, including 72 developing countries (Carvalho et al.,2017). In fact, CanL affects several million domestic dogs in countries bordering the Atlantic Ocean, mainly in Europe (22.09%) (Berriatua et al.,2021; Knihaa et al.,2023) and America (50%) (Marcondes and Day,2019; DantasTorres, 2024), but is also spreading to Asia (Ribeiro et al., 2018). Similarly, North Africa is considered a highly endemic area with a high prevalence of *L. infantum*: 35.7% in Algeria (Velez et al., 2019; Medkour et al.,2020), 58.3% in Tunisia (Bouattour et al., 2021), and 33.3% in Morocco (Idrissiet al.,2021). Clinical manifestations depend on the *Leishmania* species involved. Indeed, several species have been identified, many of which cause zoonoses (Morales-Yusteet al.,2022). At least 13 species of Leishmania infecting dogs have been reported worldwide, including the recent detection of *L. tarantolae* infection in dogs in Italy (Iatta et al., 2021). The main zoonotic species detected in infected humans are *Leishmania major*, *Leishmania guyanensis*, *Leishmania braziliensis*, and *Leishmania infantum*(Ascencio et al.,2020). The main clinical forms observed are cutaneous, mucocutaneous, and visceral (Alanazi et al., 2019). These forms can cause significant morbidity and mortality in domestic animals, leading to considerable economic losses associated with livestock farming and concern among pet owners (Alanazi et al., 2019). Diagnosis is primarily based on the presence of anti-*Leishmania* IgG antibodies in the serum of infected dogs (Knight et al., 2022). Commonly used serological tests for diagnosis include the indirect immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and rapid tests (rapid ELISA and immunochromatographic), with varying sensitivity and specificity depending on the antigens used (Ascencio et al.,2020; Knight et al., 2022). Due to the large antigenic variety of *Leishmania*, several serological tests are currently available. Studies evaluating the sensitivity and specificity of these tests are crucial for diagnosing this infection. However, few studies have been conducted to demonstrate the correlation between clinical signs and various diagnostic tests. To address this, we tested the efficacy of four diagnostic tests, including IFAT, blood culture, lymph node culture, and the qualitative immunochromatographic rapid test (RIM), and their correlation with clinical signs in dogs

suspected of having leishmaniasis at the Higher National Veterinary School (ENSV)- Algiers Algeria canine clinic.

MATERIALS AND METHODS

Animals

A total of 109 dogs of different breeds, ages and sexes were received at the Canine Clinic of ENSV- Algiers Algeria for various reasons during a six-month period (October 2023 to April 2024). The description and clinical examination of each dog were recorded (Table 1).

Blood Samples

For each dog, 5 mL of blood was collected from the radial vein into Sec and EDTA tubes for blood culture tests and serological screening, respectively. One drop of whole blood from each sample was used for the rapid test.

Diagnostic Tests

Indirect Immunofluorescent Antibody Test (IFAT)

This is a quantitative method whose threshold varies from laboratory to laboratory (generally 1/80 or 1/100) and is considered the "Gold Standard" (Boelaert et al., 1999). Therefore, we have used it as the reference test in this study. The test was conducted at the Pasteur Institute of Algeria, in the Parasitic and Genetic Eco-Epidemiology Department. A homologous canine antigen prepared by the National Reference Center for Leishmania, consisting of promastigote forms, was used. After a 1:20 dilution, 20 µL of each serum dilution was applied per well. The plates were incubated for 30 minutes at 37°C in a humid chamber and then washed in PBS buffer (PH 7.2). The prepared slides were observed under a fluorescence microscope. A positivity threshold of 1/80 was retained for the reaction. The indirect immunofluorescent antibody test (IFAT) is the reference technique.

Immunochromatographic-based qualitative rapid test (RIM)

The WITNESS Leishmania test (Zoetis, France) was performed according to the manufacturer's instructions using one drop (10 µL) of whole blood from each dog collected. The appearance of two lines indicates a positive response.

Blood Culture (BC)

This technique requires one to several weeks, and results are determined from four successive cultures. After centrifugation, the plasma is transferred into a separate tube. The white interlayer containing leukocytes is aseptically collected and inoculated onto NNN medium (Novy-MacNeal-Nicolle), which is a two-phase medium supplemented with penicillin

(100,000 IU/ml) and streptomycin (50 µg/ml). The tube is placed in an incubator at 24°C. Three days later, a subculture and a direct examination are performed, followed by direct examination between a slide and coverslip on day 7 to observe amastigote forms under the microscope. Readings are repeated on days 14, 21, and 27 before a negative result is confirmed.

General and clinical information Generally, a culture is considered negative after four successive negative sub-cultures.

Lymph Node Culture (LNC)

Puncture is performed on the superficial lymph nodes of the thigh (popliteal lymph node). The content of the syringe (aspirate) is then smeared onto a microscope slide, dried, and stained with May-Grunwald-Giemsa.

Statistical Analysis

about the dogs from which samples were obtained and the results of the diagnostic tests were presented in a Microsoft Excel table. Statistical analysis was performed using SPSS software (IBM, version 26) to calculate the prevalence of leishmaniasis and various parameters related to the sensitivity and specificity of the diagnostic tests. Sensitivity (Se) and specificity (Sp) of the tests were reported in comparison with the reference test (IFAT) for the dogs tested in the study. Sensitivity of the test is estimated by the proportion of true positives (TP) among patients, and specificity of the test is estimated by the proportion of true negatives (TN) among healthy patients:

$$\text{Sensitivity: (Se)} = TP / (TP + FN)$$

$$\text{Specificity:(Sp)} = TN / (TN + FP)$$

where FP and FN refer to false positives and false negatives, respectively.

The degree of agreement between the diagnostic tests and the IFAT test was estimated using the kappa index (κ). The significance level was set at $p < 0.05$. The Yule coefficient (Q) measures the strength of the association between each diagnostic test and the IFAT reference test. The Youden index (ranging from 0 to 1), which combines sensitivity and specificity, is equal to 1 for a perfect test:

$$\text{Youden Index} = (Se + Sp - 1)$$

RESULTS

Clinical Outcomes

Based on clinical signs, 21 (19.26%) dogs showed no clinical signs, while 88 (80.74%) dogs exhibited more than one clinical sign that raised suspicion of leishmaniasis. The clinical signs observed in the dogs are summarized in (Table 2). Skin lesions were the most common sign, observed in 72 dogs, including alopecia, pruritus, squamous hyperkeratosis, and ulcers. General condition alterations (such as despondency, weight loss, hyperthermia, and anorexia) and visceral lesions (lymphadenopathy, splenomegaly, and hepatomegaly) were noted in 30 and 29 dogs, respectively. Onychogryphosis was observed in 12 dogs, representing the lowest rate.

Prevalence of Leishmaniasis by the Four Tests

(Table 3) shows the prevalence of seropositive dogs for each test in the total population (n=109). The IFAT test identified 15.60% of dogs as positive, compared to 11.01% for the RIM, 7.34% for the BC and 6.42% for the LNC. This suggests a prevalence of canine leishmaniasis between 7% and 16%, indicating an underestimate of almost 9% of dogs with leishmaniasis compared to the gold standard test (IFAT).

Diagnostic Tests

The results of the four diagnostic tests applied to the 109 dogs are presented in (Table 4), with the IFAT considered the gold standard (Boelaert et al., 1999). Of the dogs tested, 90 (82.57%) were negative on all tests, while 6 dogs (5.50%) were positive on all tests. Additionally, 4 dogs (3.67%) were positive on all tests except one: the LNC test and RIM (2.75% and 0.92%, respectively). Two dogs (1.84%) were positive in only 2 out of 4 tests, namely IFAT+RIM and IFAT+BC (0.92% each). Finally, 7 dogs (6.42%) were positive in only 1 test, specifically IFAT and RIM (4.59% and 1.83%, respectively).

Sensitivity and Specificity

The results of sensitivity and specificity for the diagnostic tests (RIM, LNC, and blood culture) compared to the gold standard are presented in (Table 5). In terms of sensitivity, blood culture was the most sensitive test (64.71%) among the tests analyzed, indicating a higher probability of identifying seropositive animals, followed by RIM (58.82%). The lymph node culture test showed the highest specificity (100%) and a false positive rate of 0%. Lymph node culture and blood culture identified the highest number of true positives (100% and 9.8%, respectively) and the lowest rates of false negatives (6.12% and 7.22%, respectively). The specificity of the lymph node culture, blood culture, and RIM tests showed a statistically significant difference. The similarity between the test results revealed a high agreement between the lymph node culture test and blood culture, with lesser similarity with RIM. Evaluating the concordance of test results with the infection status of each sample

showed that the blood culture test had the highest kappa concordance index (0.756), while the lymph node culture test had the lowest index (0.542) (Table 5). Additionally, the blood culture was the only test with nearly perfect classification consistency.

Clinical Signs and Different Tests

A wide variety of clinical manifestations were observed due to different types of immune responses (Table 6). Regarding skin lesions, 44 dogs had only skin lesions. It was noted that three tests—blood culture, RIM, and LNC—detected the presence of *Leishmania*, unlike the IFAT test, which detected the parasite in dogs with skin lesions alone or with another sign, albeit at lower percentages (4.44% and 13.33%, respectively). The detection of anti-*Leishmania* antibodies by the IFAT test was positive in 2 dogs with an altered general condition alone, representing a prevalence of 8.33%. However, *Leishmania* was detected in 10 dogs with clinical signs associated with an altered general condition, with a prevalence of 41.66% by all four tests, including IFAT (60%), RIM (50%), blood culture (40%), and LNC (30%). Additionally, 12 dogs tested positive in all four tests (IFAT: 66.66%, RIM: 58.33%, blood culture: 41.66%, and LNC: 25%), presenting multiple clinical signs. For onychogryphosis and adenomegaly, none of the four tests detected *Leishmania*. For onychogryphosis, 10 dogs tested positive in all four tests for one or more signs associated with onychogryphosis, with the highest rate observed for the IFAT test, followed by RIM and blood culture. For adenomegaly, 24 dogs presented adenomegaly associated with one or more clinical signs. The IFAT, RIM, and blood culture tests showed an identical prevalence of 55.56%.

DISCUSSION

Canine leishmaniasis (CanL) is the most prevalent vector-borne disease in dogs in the Mediterranean region and poses a significant concern for human health (Ribeiro et al., 2018). Of the 109 dogs examined, 21 (19.2%) showed no clinical signs, while 88 (80.74%) exhibited multiple clinical signs suggestive of leishmaniasis, including skin lesions, lymphadenopathy, altered general condition, weight loss, splenomegaly, and hepatomegaly.

In this study, the Indirect Immunofluorescence Antibody Test (IFAT) is considered the preferred serological method for diagnosing CanL (De Carvalho et al., 2018), and according to Boelaert et al. (1999), IFAT is regarded as the gold standard. Using IFAT with a dilution of 1:80, our study found an overall seroprevalence of 15.6% (Table 3), which is higher than the 6% prevalence reported in Tunisia (Zoghlami,2014) but lower than the 18.7% prevalence reported by Boussaa et al. (2014)in Morocco. In some endemic regions of Algeria, the

prevalence can reach 37.5% (Dereure et al., 1999), with IFAT showing sensitivities and specificities close to 100% (Mettler et al., 2005; Souza Filho,2016).

The immunochromatographic-based qualitative rapid test (RIM) is straightforward to perform but less effective than IFAT. In our study, RIM showed a prevalence of 11%, with a sensitivity of 58.8% and a high specificity of 97.83%. These results are higher than those reported by Grimaldi et al. (2012) and Asfaram et al.(2018), who reported a prevalence of 47% using the rapid test (TR DPP Bio-Manguinhos), but lower than Mendonça et al, in 2017 and Laurenti et al. (2014), who reported sensitivities of 97.9% and 90.6%, respectively. Paltrinieri et al. (2016) confirmed that while membrane immunochromatography (M.I.C.) serology has good specificity, sensitivity varies between 30% and 70% depending on the kit. The main drawback of RIM is the significant rate of false-negative and false-positive results (7.22% and 16.67%, respectively), indicating that more sensitive methods should be used when clinical suspicion is high. Maia and Campino (2008) highlighted that rapid tests often have low specificity (ranging from 61% to 75%), leading to a high proportion of false positives. Rapid immunochromatographic tests are considered screening tests (De Carvalho et al., 2018).

For lymph node culture (LNC), the prevalence was 6.4% (Table 3). The sensitivity of cytologic diagnosis was 41.18%, while specificity was 100%. This test had a false-negative rate of 9.8% and a false-positive rate of 0% (Table 5). The low sensitivity may be due to a low parasite burden in the dogs or poor dissemination, meaning the absence of visible parasites does not exclude Leishmania infection (Guerra et al.,2019).

Blood culture (BC) had an overall prevalence of 7.3% (8/109) (Table 3). The sensitivity of leishmaniasis cultures on NNN medium was 64.71% in our study, similar to findings by Belhadj et al. (2005). It is the most specific test with a specificity of 100%. This test had a false-negative rate of 6.1% and a false-positive rate of 0% (Table 5). Despite its disadvantages, culture techniques, particularly NNN medium, remain widely used for leishmaniasis diagnosis (Limoncu et al., 1997).

Our statistical analysis revealed several observations. The most notable was the low sensitivity of LNC (41.18%) and its high false-negative rate (9.8%) compared with other tests (RIM and BC). The concordances (kappa values) for LNC, RIM, and BC tests were 0.542, 0.644, and 0.756, respectively. Based on our results, the best tests for identifying true positives are lymph node culture and blood culture, both with high specificity (100%), making them reliable confirmatory tests. Additionally, dogs with negative RIM results should be tested using other methods.

Canine leishmaniasis (CanL) is a systemic disease characterized by non-specific clinical signs (Campino and Maia, 2018; Pennisi et al., 2015). Our study observed a wide range of clinical manifestations due to different immune responses (Table 6). A significant association was noted between IFAT and the presence of symptoms. It is also worth noting that 45 dogs had only skin lesions at the first visit with no detectable systemic abnormalities, and 2 dogs had altered general conditions without other abnormalities. IFAT was able to detect the parasite in dogs with skin lesions only or altered general condition, with prevalences of 4.44% and 100%, respectively. These results support the notion that clinical forms of CanL, especially general condition, may indicate the presence of leishmaniasis. This percentage is lower than the 16.5% reported by Cabassu et al. (1988) for dogs with skin lesions only.Regarding other tests, parasite detection was observed in dogs with multiple clinical signs, with high prevalence for BC and RIM tests. According to Paltrinieri et al.(2016), the sensitivity of rapid kits (RIM) improves when dogs exhibit suggestive clinical signs. Dogs with more than three clinical signs generalized lymphadenomegaly, skin lesions, onychogryphosis, and altered general condition had a higher prevalence of 100% for IFAT, RIM, and BC tests.

CONCLUSIONS

The results of the present study showed a strong correspondence between the observedclinical signs of leishmaniasis and the IFAT results, mainly when the disease is manifested bya single clinical sign. In addition, BC appears to have better sensitivity and specificity thanother tests, making it a good alternative to IFAT for veterinary clinicians.

Funding source

We hereby declare that this study received no external funding. All aspects of the research were conducted without financial support.

Conflict of Interest

The authors declare that theyhave no conflict of interest.

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Table 1 : Characteristics of the dogs included in the study (n=109)

Parameters	**n (%)**
Breed	
German shepherd dog	57 (52,30%)
Mongrels	33 (30,28%)
Pitbul	13 (11,92%)
Retrievers	06 (5,50%)
Age (years)	
1-2	45 (41,29%)
3-7	49 (44,95%)
8-10	15 (13,76%)
Sex	
Females	51 (46,79%)
Males	58 (53,21%)

Table 2. Clinical signs associated with Canl.

Symptoms	**n**	**Clinical manifestations**
Alteredgeneral condition	30	Despondency,Weightloss,hyperthermiaanorexia,
Skin lesion	72	Alopecia, pruritus, squamosis, hyperkeratosis, ulcers
Viscerallesions	29	Lymphadenopathy, splenomegaly, hepathomegaly
Onychogryphosis	12	Longer claws.

Table 3: Prevalence of different method test (n=109).

	No positive	**Prevalence (%)**
IFAT	17	15.60
RIM	12	11.01
LNC	7	6.42
BC	8	7.34

IFAT: Immunofluorescence antibody test; RIM: Rapid Immunomigration, LNC :Lymph Node Culture, BC :Culture Blood ;

Table 4: Serological test results with different combination (n=109).

IFAT	RIM	LNC	BC	Number	%
–	–	–	–	90	82.57
–	+	–	–	2	1.83
+	–	–	–	5	4.59
+	–	–	+	1	0.92
+	–	+	+	1	0.92
+	+	–	–	1	0.92
+	+	–	+	3	2.75
+	+	+	+	6	5.50
			Total	109	100

IFAT: Immunofluorescence antibody test; RIM: Rapid Immunomigration, LNC :Lymph Node Culture, BC :Culture Blood ; +:Positive test result; -: Negative test results.

Table 5: Sensitivity and specificity of each test, given IFAT as a gold standard.

	RIM	**LNC**	**Blood culture**
Sensitivity (%)	58.82	41.18	64.71
Specificity (%)	97.83	100	100
Kappa	0.644	0.542	0.756
P(Kappa)	0.000	0.000	0.000
Positive predictive value (%)	83.33	100	100
Negativepredictive value (%)	92.78	90.2	93.88
False positive rate (%)	16.67	0	0
False negative rate (%)	7.22	9.8	6.12
Youden index (0-1)	0.57	0.41	0.65
Yule's Q coefficient (0-1)	0.97	1	1

RIM: Rapid Immunomigration,LNC :Lymph Node Culture, BC :Culture Blood

Table 6:Main clinical signs observed in dogs based on diagnostic tests (n=109).

	NOT (%)	**IFAT not (%)**	**LNC not (%)**	**BC not (%)**	**RIM not (%)**
No signs	21 (100.00)	0 (0)	0 (0)	0 (0)	0 (0)
Skin lesion					
Only	45 (66.18)	2 (04.44)	0 (00)	0 (0)	0 (0)
with 1 sign	15 (22.06)	2 (13.33)	0 (00)	0 (0)	0 (0)
with 2 signs	08 (11.76)	6 (75.00)	4 (50)	5 (62.5)	5 (62.5)
General condition					
Only	02 (08.33)	2 (100)	0 (0)	0 (0)	0 (0)
with 1 sign	10 (41.66)	6 (60)	3 (30)	4 (40)	5 (50)
with 2 signs	12 (50)	8 (66.66)	3 (25)	5 (41.66)	7 (58.33)
Onchyogryphosis					
Only	00 (00.00)	0 (0)	0 (0)	0 (0)	0 (0)
with 1 sign	04 (40.00)	3 (75)	1 (25)	2 (50)	2 (50)
with 2 signs	06 (60.00)	4 (66.67)	1 (16.67)	3 (50)	4 (66.67)
Adenomegaly					
Only	04 (14.29)	0 (0)	0 (0)	0 (0)	0 (0)
with 1 sign	15 (53.57)	4 (26.67)	4 (26.67)	4 (26.67)	3 (20)
with 2 signs	09 (32.14)	5 (55.56)	4 (44.44)	5 (55.56)	5 (55.56)
All signs	4 (100.00)	4 (100)	3 (75)	4 (100)	4 (100)

IFAT: Immunofluorescence antibody test; RIM: Rapid Immunomigration, LNC :Lymph Node Culture, BC :Culture Blood