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The relevance of molecular diagnosis in a dog vaccinated against leishmaniasis

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Abstract

Background: Canine leishmaniasis (CanL) is an important problem in veterinary medicine. Since 2011, a vaccine against leishmaniasis is commercially available in Europe. This vaccination allows an approximate four-fold decrease of symptomatic active infection risk. At present, clinical observations on vaccinated dogs are limited, and in controlled experiments it has been reported that a certain number of vaccinated dogs can still be infected. In practice, these cases could be difficult to interpret based on conventional serological diagnostic tools. The current case report describes a complex diagnosis of CanL in a vaccinated dog and emphasizes the role of molecular techniques as a useful diagnostic approach.

Case presentation: An intact female 6-year-old boxer dog weighing 28 kg vaccinated against leishmaniasis was admitted to the Veterinary Clinic with mild alopecia, moderate dermatitis with abundant dandruff and a diffuse and marked lymphadenomegaly. While no relevant alterations were evidenced by hematological and biochemical analyses, the indirect fluorescent antibody test (IFAT) for *Leishmania infantum* was positive with a titer of 1:1280, and a qPCR analysis targeting *L. infantum* kinetoplast minicircle DNA suggested the diagnosis of CanL. In light of the qPCR results, the dog was subjected to anti-*Leishmania* therapy with miltefosine for 28 days and allopurinol for 6 months. The dog was monitored 4 times after initial diagnosis until day 294, and a progressive overall improvement and complete clinical remission was observed.

Conclusions: This case report evidences a further complexity in CanL diagnosis in vaccinated dogs, underlining the utility of a molecular approach in these particular complex cases, as previously verified in borderline cases or during disease relapse.

Keywords: Canine leishmaniasis, *leishmania infantum*, qPCR, vaccine

Introduction

Canine leishmaniasis (CanL), resulting from *Leishmania infantum* infection, is a serious parasitological disease in veterinary medicine [1]. CanL is endemic in the Mediterranean basin, Central and South America and parts of Asia [2]. CanL diagnosis has been a matter of discussion in the literature, not only because the clinical manifestation of CanL is highly variable with various atypical forms, but also because of the absence of a diagnostic gold standard [3]. Nevertheless, indirect fluorescent antibody test (IFAT) is considered an important reference method among serological techniques by the World Organization for Animal Health (OIE-Office International des Epizooties) [4].

Since 2011, a vaccine for CanL (LiESP/QA-21) is available in Europe. When administered to dogs exposed to natural *L. infantum* infection, the vaccine was shown to decrease the risk of progression to symptomatic active *L. infantum* infection by approximately four-fold [5]. Moreover, dogs developing the disease despite vaccination appeared less infectious to sand flies [6], suggesting an additional benefit of vaccination on an epidemiological scale.

However, the increasing presence of vaccinated dogs could also bring new challenges in CanL diagnosis. In fact, vaccinated dogs have been shown to develop IFAT positive titers for almost one year post first vaccination [7]. To date, clinical experience regarding the evaluation of the antibody titers in vaccinated dogs is limited, especially considering boosters.

The current case report describes a complex case of CanL

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in a vaccinated dog with positive IFAT titer and oligosymptomatic clinical aspect but normal hematochemical values, and emphasizes the role of molecular techniques as a useful diagnostic approach.

Case presentation

An intact female 6-year-old boxer dog weighing 28 kg was admitted to the Veterinary Clinic "Santa Teresa" (Fano, PU, Italy) due to mild alopecia and moderate dermatitis with abundant dandruff. The clinical examination also revealed a diffuse and marked lymphadenomegaly. The score of clinical signs, calculated as previously reported [8], resulted in a 3 (oligosymptomatic) on a 0-14 scale. The dog had been vaccinated and boosted against leishmaniasis with LiESP/QA-21 vaccine (CaniLeish®) (Virbac, Carros, France), accordingly to manufacturer's guidelines, 18 and 5 months before admission to the clinic, respectively. Prior to vaccination, the dog tested negative for anti-*Leishmania* antibodies by the SNAP *Leishmania* Test (IDEXX Laboratories Inc., Westbrook, Maine). The results of chemical, hematological and clinical parameters on the day of admission are shown in **Tables 1** and **2** (Day 0 columns). No alterations were evidenced with the exception of a slight increase in alanine aminotransferase and gamma glutamyltransferase values. Notably, the values of serum proteins, albumin and albumin/globulin ratio, often altered in CanL, were in the normal range, as well as erythrocyte cell count, hemoglobin and hematocrit. Lymph node fine needle aspiration was performed in order to obtain a cytological smear, as a lymphoproliferative disorder was suspected. The results of cytological examination evidenced a lymph node hyperplasia/reactivity, excluding a lymphoproliferative disorder. No parasites were evidenced at this stage. At least 100 oil immersion fields were examined, corresponding to a sensitivity of about 84% [9].

Table 1. Clinical chemistry values and score of clinical signs.

Parameter	Normal range	Day 0	Day 294
Score of clinical signs (scale 0-14)	0	3	0
Total proteins (g/dl)	5.2-8.2	6.5	6.4
Albumins (g/dl)	2.3-4.0	2.8	2.4
Globulins (g/dl)	2.5-4.5	3.7	4.0
Albumins/Globulins (ratio)	0.5-1.7	0.8	0.6
Alkaline phosphatase (U/L)	23-212	101	76
Alanine amino transferase (U/L)	10-100	110	35
Pancreatic amylase (U/L)	500-1500	1320	933
Blood urea nitrogen (mg/dl)	7.0-27.0	12	20
Calcium (mg/dl)	7.9-12.0	9.6	8.9
Total cholesterol (mg/dl)	110-320	208	296
Creatinine (mg/dl)	0.5-1.8	0.9	1.2
Gamma glutamil transferase (U/L)	0-7	9	0
Glucose (mg/dl)	74-143	83	102
Pancreatic lipase (U/L)	200-1800	1387	1974
Phosphorus (mg/dl)	2.5-6.8	4.7	4.6
Total bilirubin (mg/dl)	0-0.9	0.3	0.2

Table 2. Hematological values.

Parameter	Normal range	Day 0	Day 53	Day 294
White blood cells (x10^3/µl)	6.0-17.0	8.9	8.1	7.4
Red blood cells (x10^6/µl)	5.50-7.90	6.10	6.61	6.53
hemoglobin (g/dl)	12.0-20.5	17.0	18.7	14.3
Haematocrit (%)	37.0-55.0	45	48.0	49.4
Mean corpuscular volume (fl)	60-76	74	73.0	76
Red blood cell distribution width (%)	12.0-16.0	17.1	17.3	17.5
Platelets (x10^3/µl)	176-400	256	312	320
Plateletcrit (%)	0.120-0.390	0.270	0.322	0.323
Platelets distribution width (%)	10.0-27.0	13.1	14.2	14.6

The clinical aspect and the fact that the dog originated from an area endemic for leishmaniasis [10] suggested a possible *L. infantum* infection, despite the dog's vaccination. Therefore, IFAT and qPCR-based molecular analyses were performed as previously described [11]. Briefly, IFAT was performed on serum samples with an in house assay validated and provided by the Institute of Experimental Preventive Veterinary Medicine (Istituto Zooprofilattico Sperimentale) of Sicily [12]. IFAT titers ≥1:160 were considered positive in accordance with Gradoni et al. [10]. The qPCR targeting *L. infantum* kinetoplast minicircle DNA was performed on DNA extracted from exfoliative epithelial cells collected from the right and left conjunctiva (conjunctival swab), bone marrow, popliteal and prescapular lymph nodes. The conjunctival swab samples were processed as previously described [8]. Biopsied samples from lymph nodes and bone marrow were harvested in EDTA tubes for blood collection and stored at -20°C until processing. DNA extraction was performed with the DNeasy blood & tissue kit (Qiagen).

The IFAT titer was high (1:1280), and qPCR results were positive in the conjunctival swab, bone marrow and popliteal lymph node samples (a range from 0.69 to 8.45 parasites/5x10^4 cells, depending on the sample) (**Table 3**). These results suggested a *L. infantum* infection, despite the cytological sample was negative and the lymphadenomegaly was not accompanied by other hematological signs or dysproteinaemia, as reported in vaccinated but symptomatic dogs [13]. Therefore, the dog was subjected to therapy with miltefosine (2 mg/kg per os) for 28 days and allopurinol (15 mg/kg b.i.d. per os) for 6 months. The clinical evaluation and molecular analyses were re-per-

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Table 3. IFAT and qPCR results.

Parameter	Units	Day 0	Day 53	Day 108	Day 195	Day 294
IFAT	--	1:1280	n.a.	1:1280	1:640	1:640
CS* Left	Parasites/5x10^4 cells	0.69	9.06	neg§	neg§	neg§
CS* Right	Parasites/5x10^4 cells	neg§	1.17	neg§	neg§	neg§
Bone marrow	Parasites/5x10^4 cells	8.45	n.a. ‖	n.a. ‖	n.a. ‖	neg§
PopL†	Parasites/5x10^4 cells	4.55	neg§	neg§	neg§	n.a. ‖
PresL‡	Parasites/5x10^4 cells	n.a. ‖	neg§	n.a. ‖	neg§	0.85

*conjunctival swab; †popliteal lymph node; ‡prescapular lymph node; §negative; ‖not available

formed after 53, 108, 195 and 294 days (**Tables 1-3**), and a progressive overall improvement was observed. In particular, starting from the first follow-up, the alopecia disappeared, the dermatitis became mild and the lymph node dimensions diminished noticeably. The last follow-up evidenced a complete normalization of lymph node size and absence of other clinical signs. The score of clinical signs dropped to "2" on days 53-195 and "0" by day 294. The IFAT titer decreased to 1:640 on days 195 and 294. The qPCR results showed different trends in the tested samples (**Table 3**). At the first follow-up (day 53), while the parasite load in the popliteal lymph node turned negative, the parasite load increased in both conjunctival samples, most likely due to different parasite dissemination in the various tissues. At the second follow-up (day 108) the conjunctival samples also turned negative. The molecular analysis at the last follow-up (day 294) evidenced the absence of parasite DNA in all the matrices tested, with the exception of the prescapular lymph node, which showed a parasite load near the qPCR detection limit. Overall, the improvement of clinical signs and the decrease of IFAT titer and parasite load, following the anti-*Leishmania* therapy, demonstrate the compatibility of original clinical states with the *L. infantum* infection, despite the dog having been vaccinated.

Discussion

It has been shown in controlled experiments that a certain number of dogs vaccinated against leishmaniasis can still be infected, but the risk of progression to active infection is significantly reduced [**14**]. However, in the clinical practice, these cases could be difficult to interpret if conventional serological diagnostic tools (i.e., IFAT) are used. Furthermore, a positive IFAT could also be the result of parasite contact before vaccination, since the dogs can be routinely tested with a rapid serological test prior to vaccination.

Concerning this case report, the major difficulty was the lack of correlation between initial clinical hypotheses (leishmaniasis or lymphoproliferative disorder, due to the marked lymphadenomegaly) and the negative results of cytological analysis. Moreover, the dog did not show pathological values in hematological or biochemical parameters. This could be due to the fact that vaccination could have attenuated symptomatic severity (e.g., no weight loss was found) as previously reported in a randomized controlled trial [**13**]. Nevertheless, the high IFAT titer suggested a *L. infantum* infection, which was also confirmed by qPCR in different samples. The qPCR results evidenced a low parasite burden, which could explain the lack of parasite identification in cytological samples. If taken by themselves, the results of qPCR in the diagnosis of this case could have been elusive, since the dog came from an endemic area where many healthy dogs can be found PCR positive. However, considering the positivity of all samples tested and the clinical signs including lymphadenomegaly in absence of lymphoproliferative disorders, the qPCR analysis resulted helpful for the diagnosis of leishmaniasis. This diagnosis was then supported by the positive response to anti-*Leishmania* treatment.

The success of anti-*Leishmania* therapy was accompanied by a slow decrease of antibody titer, which was not surprising since it is known that the decrease of antibody titer could not be detected during the first 6 months of treatment [**15**]. Moreover, the fact that the prescapular lymph node sample at day 294 was found to be positive by qPCR was also unsurprising since it has been previously reported that qPCR can retrieve *Leishmania* DNA in lymph node aspirates for up to 12 months after therapy with miltefosine and allopurinol, even in dogs without relapse of clinical signs [**16**].

Conclusions

Since vaccination does not confer complete protection but can induce positive IFAT titers, the use of molecular methods could be helpful to limit drawbacks. Here we presented a complex diagnosis of leishmaniasis in which qPCR was part of the evaluation process. Because the dog came from an endemic area, qPCR results were considered together with the clinical signs and always in a context of a differential diagnosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	MC	LG	AD	EG	AM	MM
Research concept and design	✓	✓	--	--	--	--
Collection and/or assembly of data	✓	--	✓	✓	--	--
Data analysis and interpretation	✓	✓	✓	--	✓	--
Writing the article	✓	✓	--	--	--	--
Critical revision of the article	--	--	--	--	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓
Statistical analysis	--	--	--	--	--	--

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