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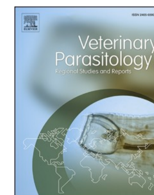

Short Communication

Performance of a point-of-care test for the detection of anti-*Leishmania infantum* antibodies is associated with immunofluorescent antibody titer and clinical stage of leishmaniosis in dogs from endemic regions

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ABSTRACT

Canine leishmaniosis (CanL) is caused by the protozoal parasite *Leishmania infantum*, which is transmitted by sand flies in warm climates across the world. Because dogs are considered a primary domestic reservoir for the parasite that causes leishmaniosis in humans, it is important from a One Health perspective that CanL be properly managed. In endemic regions, CanL is a common differential diagnosis in sick dogs because the clinical signs and clinicopathological disorders of the disease are non-specific, variable, and may overlap those of other common conditions. Diagnosis is based on the presence of compatible clinical signs, laboratory abnormalities, and confirmation by serological and parasitological evidence of infection. Here, we describe the performance of a point-of-care (POC) immunoassay that uses recombinant antigens to detect canine anti- *L. infantum* antibodies in a convenience sample set from a diagnostic laboratory, a group of canine patients with clinical staging, and in apparently healthy dogs from endemic areas. An immunofluorescence antibody test (IFAT) was used as the semiquantitative reference method. In the convenience sample set with high IFAT titers (≥ 1:800), the POC immunoassay demonstrated perfect agreement with IFAT (100%; 90/90). Using samples from dogs staged as either LeishVet Stage 2 or 3 or LeishVet Stage 1, positive agreement of the POC immunoassay with the IFAT was 98.8% (82/83) and 83.8% (31/37), respectively. The negative agreement with IFAT was 98.9% (272/275) in apparently healthy dogs from endemic areas of Greece and Italy. Since the performance of the POC immunoassay was associated with IFAT titer and clinical stage of CanL, the test may help veterinarians when determining if CanL is likely responsible for a patient's clinical picture or when evaluating an apparently healthy patient prior to vaccination.

1. Introduction

Canine leishmaniosis (CanL) is the clinical manifestation of the infection with the protozoal parasite *Leishmania infantum*. Clinical signs and clinicopathological disorders are non-specific, variable, and in endemic areas, may overlap those of other common conditions of dogs; subclinical infections with or without seroconversion in healthy dogs are common, but the clinical picture can also be severe. *Leishmania infantum* is transmitted primarily by phlebotomine sand flies and causes disease in humans, dogs, cats, and many other domestic and wild species (Molina et al., 2012). CanL is endemic in >70 countries in Europe, Africa, Asia, South and Central America, and is associated with travel in neighboring regions (Baneth et al., 2008; Miró et al., 2022). Sand fly distribution, and consequently, CanL-endemic regions, are expected to expand with climate change, including into western and central Europe (Chalghaf et al., 2018). A range of mammalian species can serve as reservoir hosts, facilitating this expanding geographic distribution (Molina et al., 2012; Müller et al., 2022). Because dogs are considered the primary domestic

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reservoir species in endemic regions, accurate diagnosis of CanL is crucial for limiting both zoonotic risk and the impact of disease in dogs.

Diagnosis of CanL is based on clinical signs, clinicopathological abnormalities, serology, and cytology, histopathology and/or molecular diagnosis by different PCR protocols (Paltrinieri et al., 2010; Roura et al., 2021; Solano-Gallego et al., 2011). Testing may be performed to confirm disease in a patient with clinical signs consistent with CanL, to evaluate apparently healthy dogs prior to vaccination, upon importation, or for routine medical checks, or to monitor seropositive animals (Solano-Gallego et al., 2011). Serological diagnosis may include qualitative or quantitative methods, where quantitative methods enable clinical staging and the monitoring of clinically healthy dogs. High titers from quantitative serology in the presence of compatible clinical signs and/or clinicopathological abnormalities are considered diagnostic, while low or negative titers in the face of high suspicion for CanL prompt the recommendation for diagnostic confirmation using parasitological methods (cytology, histopathology) and/or PCR (bone marrow, lymph node, spleen, skin, conjunctival swabs blood or urine) (Paltrinieri et al., 2010; Roura et al., 2021; Solano-Gallego et al., 2011). With this clinical and diagnostic information, dogs with CanL can be assigned to different clinical stages according to the LeishVet Guidelines (Solano-Gallego et al., 2011).

Common serological methods to detect antibodies against L. *infantum* include immunofluorescent antibody tests (IFAT), enzyme-linked immunosorbent assays (ELISA), and rapid serological tests, which use ELISA or immunochromatographic technology. The reported sensitivity and specificity for each method varies based on the population tested, selection of reference method, capture antigen(s) and clinical decision thresholds (Persichetti et al., 2017; Villanueva-Saz et al., 2022). Some serological tests for CanL use preparations of the parasite itself to detect antibodies in patient samples; these include whole cell antigen ELISAs and IFAT (Mancianti and Meciani, 1988). Most often, the promastigote stage of the parasites, which replicates in the sand fly host, is grown in culture. The promastigotes are then processed such that most of the antigens present in the whole organism are available to capture antibodies for detection in the serological test. A broader representation of parasite antigens may theoretically increase assay sensitivity by allowing detection of the patient's polyclonal humoral immune response (Almeida et al., 2005). On the other hand, using the whole parasite may decrease assay specificity because some of the antigens may be similar to those of other organisms, resulting in detection of cross-reactive antibodies (Fraga et al., 2016; Vrhovec Globokar et al., 2017).

An alternative to using parasite-derived antigens for detecting parasite-specific antibodies is the use of recombinant proteins. Immunodominant proteins, which elicit a robust immune response in most patients, are usually selected as targets, and several proteins from the amastigote stage of L. *infantum* have been reported for this use (Badaró et al., 1996; Bhatia et al., 1999; Burns et al., 1993). Recombinant protein-based ELISAs that are more sensitive and specific than whole cell antigen ELISAs for CanL diagnosis in dogs have been reported (Porrozzi et al., 2007).

Finally, serological methods can be qualitative or (semi-)quantitative. Qualitative serology, suitable for point-of-care (POC) applications, plays an important role in the initial diagnosis and screening of canine patients. However, the sensitivity of existing qualitative tests has underperformed compared to quantitative serological tests performed at a reference or academic laboratory (Solano-Gallego et al., 2014). Meanwhile, quantitative serology enables the clinician to stage the disease, monitor disease progression and, perhaps, to determine prognosis. To meet the expectations of practicing veterinarians, a qualitative, POC serological test should be nearly as sensitive as quantitative serology, maintain high specificity, and be easy to perform in the veterinary clinic. The purpose of the study reported here was to describe the diagnostic accuracy of a new point-of-care serological test that uses recombinant antigens for the detection of antibodies against L. *infantum* in dogs.

2. Methods

2.1. Canine samples

All samples for POC immunoassay testing were stored frozen on location at −20 °C until shipped on dry ice to Westbrook, ME, USA, and stored frozen at −20 °C until the time of testing. A total of 280 IFAT positive (titer >1:50) and 374 IFAT negative (titer < or = 1:50) serum samples were used in this study. These samples originated from either a commercial laboratory (*n* = 189), university teaching hospital (*n* = 190), or remnant serum samples from a vector-borne disease seroprevalence study (*n* = 275) (Table 1).

2.1.1. Commercial laboratory samples

Ninety serum samples selected based on high *Leishmania* IFAT titers (1:800 or greater) were obtained from commercial diagnostic laboratories. Samples were submitted by general practice veterinarians in Spain (*n* = 40, 44%), Italy (n = 40, 44%), Germany (*n* = 9, 10%), and Switzerland (n = 1, 1%) for *Leishmania* IFAT testing (IDEXX Reference Laboratory, Kornwestheim, Germany). An additional 99 serum samples were collected following general chemistry testing from the same commercial diagnostic laboratory in the United States (IDEXX Laboratories, Inc.), where L. *infantum* infection is considered non-endemic with only limited occurrence by breed (Rosypal et al., 2003). At the completion of all veterinarian-requested diagnostic testing, samples with at least 0.7 mL of remaining volume were retained for this study. No clinical information was available for these samples.

2.1.2. Samples from Uuniversity Complutense of Madrid

Remnant serum samples from a convenience set of canine patients that visited the Veterinary Teaching Hospital at University Complutense of Madrid (HCV-UCM), an area endemic for CanL (Gálvez et al., 2020), between December 2017 and September 2022 were obtained for the study (*n* = 190). At the time of clinical evaluation, serum samples were tested by IFAT (Pet Parasite Lab, Veterinary Faculty, UCM). Samples from two groups of patients were included; 1) dogs diagnosed with CanL and staged according to LeishVet CanL staging guidelines (Solano-Gallego et al., 2011) and 2) dogs that were being examined for routine medical checks, showed no clinical signs or clinicopathological abnormalities, but had non-negative IFAT titers (>1:50). Collection of patient samples at HCV-UCM was carried out in accordance with the international guidelines for the Care and Use of Experimental Animals and Spanish Legislation (RD 53/2013).

Table 1
Performance of the point-of-care (POC) immunoassay, reported as positive and negative agreement (95% CI) with the immunofluorescent assay test (IFAT).

	Sample Set	Positive Percent Agreement	Negative Percent Agreement
IFAT Positive (>1:50)	Diagnostic Laboratory High Titer (*n* = 90)	100 (96.0–100)	NA
	HCV-UCM LeishVet Stage 2 or 3 (*n* = 83)	98.8 (93.5–100)	NA
	HCV-UCM LeishVet Stage 1 (*n* = 37)	83.8 (68–93.8)	NA
	HCV-UCM Clinically Healthy Seropositive (*n* = 70)	51.4 (39.2–63.6)	NA
IFAT Negative (Negative or 1:50)	Apparently Healthy from Italy and Greece (*n* = 275)	NA	98.9 (96.8–99.8)
	Diagnostic Laboratory USA (*n* = 99)	NA	100 (96.3–100)

(NA - not applicable.)

2.1.3. Samples from Italy and Greece

We used banked serum samples from canine patients recruited for a seroprevalence study in Italy and Greece conducted in 2022 (Morelli et al., 2023). Remnant serum was retained following completion of diagnostic testing as part of a routine clinical visit ($n = 275$). Dogs were enrolled in that study during routine visits to their veterinarian if 1) their owners agreed, 2) they were old enough to have experienced at least one sand fly vector season, and 3) they displayed no clinical signs compatible with leishmaniosis, ehrlichiosis, anaplasmosis or heartworm disease. Specifically, they must have had fewer than five of a list of 75 clinical signs associated with any of these vector-borne diseases.

2.2. Diagnostic testing

2.2.1. Immunofluorescent antibody test (IFAT)

Because quantitative serology, including IFAT, is recommended for serodiagnosis and monitoring of CanL (Paltrinieri et al., 2010; Solano-Gallego et al., 2011), we compared the results of our POC immunoassay to IFAT results. The IFAT was performed at UCM as reported previously (Mancianti and Meciani, 1988; Müller et al., 2022) using sera obtained from dogs visiting the Veterinary Teaching Hospital. 1:50 titers are considered negative, 1:100 titers are considered borderline, and ≥ 1:200 titers are positive.

Serum samples obtained from the commercial reference laboratory, as well as those from the previous seroprevalence study in Italy and Greece, were tested by the commercially available IFAT method (Mega Screen® FLUOLEISH, Diagnostik MegaCor GmbH, Hörbranz, Austria) at IDEXX Laboratories in Kornwestheim, Germany or Westbrook, ME (Vrhovec Globokar et al., 2017). For this study, 1:50 was considered negative. Titers of 1:100 are classified as low/borderline and titers of ≥1:200 as positive.

2.2.2. Point-of-care (POC) immunoassay

Immunoassay reagents were developed for the detection of L. *infantum* specific antibodies using recombinant proteins from the immunodominant family of kinesin proteins (Badaró et al., 1996; Bhatia et al., 1999; Burns et al., 1993; Porrozzi et al., 2007). These reagents replaced the *Borrelia burgdorferi*-specific reagents in an existing POC immunoassay[1] to create a new commercial POC assay, without changing the assays for *Anaplasma*, *Ehrlichia* and Heartworm.[2] This POC immunoassay has four multiplexed assays that are determined to be positive by the visual observation of blue color at the corresponding position ("spot") on the device. Validation of the *Anaplasma*, *Ehrlichia,* and Heartworm assays was recently published (Beall et al., 2022). The POC test device uses ELISA technology with bidirectional flow of the patient sample, wash, and substrate followed by enzymatic amplification to generate visible blue spots when the target is detected (antigen for *Dirofilaria immitis* or anti- *Ehrlichia* spp., *Anaplasma* spp., or L. *infantum* antibody). Prior to POC immunoassay testing, all serum samples were blinded and randomized. Test devices were subject to three reads by three different professional laboratory technicians and consensus (2/3 or 3/3 visual operators) was required to make a positive or negative determination.

2.3. Statistics

Positive percent agreement was calculated by dividing the number of positive samples on the POC immunoassay by the number of samples that were positive on IFAT (titer >1:50). Negative percent agreement was calculated by dividing the number of samples that were negative with the POC immunoassay by the number of samples that were negative on IFAT (negative or 1:50 titer). 95% confidence intervals were calculated using the Clopper Pearson method in GraphPad Prism.

3. Results

All 90 canine samples from the diagnostic laboratory with anti-*Leishmania* IFAT titers of 1:800 or greater tested positive with the POC immunoassay resulting in 100% positive agreement (95% CI: 96.0–100.0%) (Table 1). The distribution of IFAT titers for these samples is depicted in Fig. 1. The 99 diagnostic laboratory samples from the non-endemic region had negative IFAT results (≤ 1:50) and all tested negative on the POC immunoassay for a negative agreement of 100% (95% CI: 96.3–100.0%) (Table 1).

All but one of the 83 dogs evaluated by the infectious diseases clinical unit (G. Miró in charge) at the HCV-UCM in Spain and staged according to the LeishVet Guidelines (Solano-Gallego et al., 2011) as having mild-to-moderate clinical disease (LeishVet stage 2 or 3) tested positive on the POC immunoassay resulting in a positive agreement with IFAT of 98.8% (95% CI: 93.5–100.0%) (Table 1). Twenty-three of these Stage 2 or 3 dogs had additional samples collected at follow-up visits (one dog had five total samples, three dogs had four samples, nine dogs had three samples and ten dogs had two samples). When these additional samples were included in the testing, the POC immunoassay was positive in 122/125 samples (97.6% [95% CI: 93.1–99.5%]). The three samples that were negative on the POC immunoassay had IFAT titers of 1:100 ($n = 1$) and 1:400 ($n = 2$). The distribution of IFAT titers from all 125 samples is shown in Fig. 1.

A total of 37 dogs were diagnosed at HCV-UCM with CanL and staged as LeishVet Stage 1. These dogs had positive IFAT titers (≥1:100, see Fig. 2A) with very subtle clinical signs. Thirty-one of 37 samples were positive on the POC ELISA for a positive agreement with IFAT of 83.8% (95% CI: 68.0–93.8%) (Table 1). We also tested 70 samples from clinically healthy dogs that were evaluated for routine medical checks at HCV-UCM, all of which had IFAT titers ≥1:100. The POC ELISA was positive in 36/70 dogs (51.4% [95% CI: 39.2–63.6%]) (Table 1). The distribution of IFAT titers for these clinically healthy seroreactive (CHS) dogs is shown in Fig. 2B. When all four IFAT positive populations were considered, the POC test provided a positive result for 261/271 (96.3%) of samples with an IFAT titer of 1:200 or greater, and 100% of the samples with IFAT titers of 1:800 or greater ($n = 168$).

Finally, all 275 apparently healthy dogs from endemic regions of Italy and Greece tested negative on IFAT (≤ 1:50) and 272 tested negative on the POC immunoassay for a negative agreement with IFAT of 98.9% (95% CI: 96.8–99.8%) (Table 1).

4. Discussion

In this study, we describe the performance of a POC immunoassay for the detection of antibodies against L. *infantum* using five regionally relevant sets of canine sera. The results of the POC immunoassay were directly associated with IFAT titers and clinical CanL staging; positive percent agreement with IFAT was high in those sample sets with high IFAT titers and advanced clinical disease. In addition, all but one dog at LeishVet stage 2 or 3 CanL had antibodies detected by the POC immunoassay which is consistent with previous reports that higher titers are often associated with clinical disease (Conde et al., 2022; Reis et al., 2006; Solano-Gallego et al., 2001). High negative percent agreement with IFAT was also observed in apparently healthy dogs from endemic regions, demonstrating the specificity of the recombinant antigen targets in the POC immunoassay. Together, these findings provide valuable insights to veterinarians regarding the expected performance of the POC immunoassay in their patient population compared to a well-established quantitative reference method (Mancianti and Meciani, 1988; Müller et al., 2022; Vrhovec Globokar et al., 2017).

The agreement between the two different *Leishmania* antibody detection tests, one using recombinant antigen targets and the other using whole cell organisms, was not unexpected. The POC immunoassay

[1] SNAP® 4Dx® Plus (Product Code 5P23.01), IDEXX Laboratories, Inc.

[2] SNAP® Leish 4Dx® (Product Code 5P50.U0), IDEXX Laboratories, Inc.

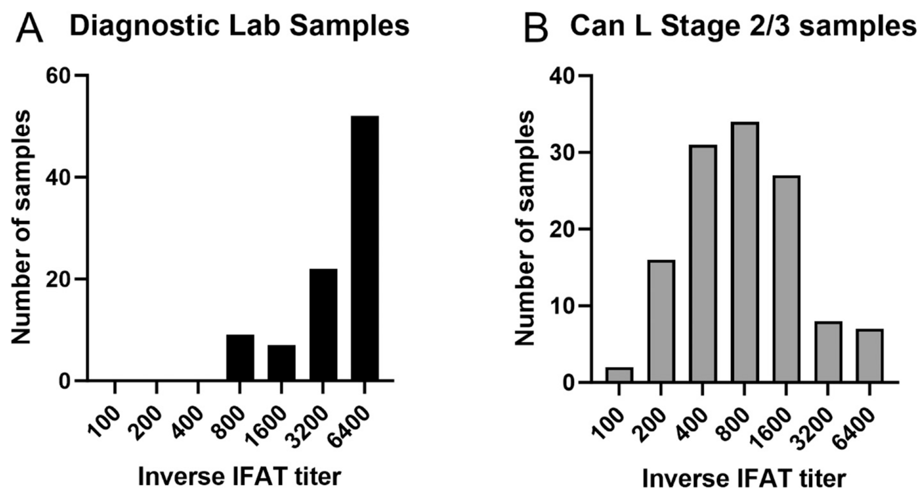

Fig. 1. Distribution of IFAT titers from A) the diagnostic laboratory samples with high IFAT titers and B) the HCV-UCM LeishVet Stage 2 or 3 patient samples from all visits.

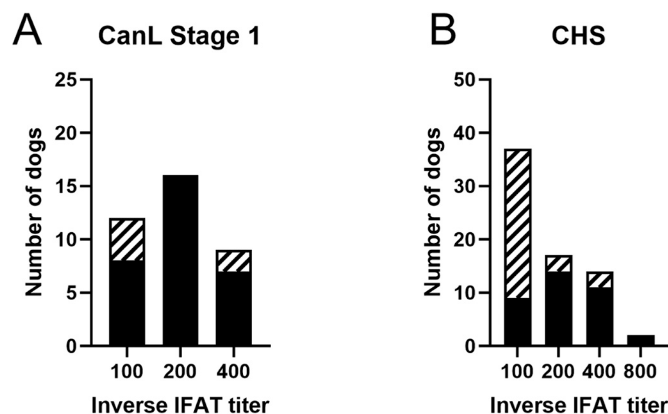

Fig. 2. Distribution of IFAT titers for A) LeishVet Stage 1 dogs and B) clinically healthy seroreactive (CHS) dogs. Samples that were positive on the POC ELISA are shown in filled bars and samples that were negative on the POC ELISA are shown in hatched bars.

used in our study contains recombinant kinesin proteins to detect *Leishmania* antibodies and performs comparably to what has been reported in the literature. Previous canine serological studies have demonstrated the ability of recombinant antigens, e.g. rK39, to reliably detect *Leishmania* antibodies in sick patients with clinical signs and parasitological confirmed infections (sensitivity of 96.7–100%) (Maia and Campino, 2008; Mettler et al., 2005; Porrozzi et al., 2007). However, in clinically healthy infected dogs, the sensitivity of recombinant antigens, whether used in either ELISA or rapid immunochromatographic tests, tends to be lower, ranging from 52.9 to 64.7%. Improvements in sensitivity have been achieved by combining recombinant targets derived from multiple parasite proteins previously used in the serodiagnosis of CanL (Costa et al., 2012; Maia and Campino, 2008; Porrozzi et al., 2007). Recombinant antigen targets have demonstrated better specificity than some soluble preparations of parasite antigens by avoiding detection of cross-reactive antibodies from other parasitic infections (Ferreira et al., 2007; Mettler et al., 2005; Porrozzi et al., 2007).

Our study had some limitations. The study used banked samples from previous patient visits or prior studies for testing on the POC immunoassay. As testing by both methods did not occur simultaneously, slight differences in antibody concentrations may have occurred due to an additional freeze-thaw cycle. Some of the convenience sample sets were sera to be discarded following routine reference laboratory testing and no clinical information was available for the patient to confirm a CanL diagnosis or stage. The convenience samples that were obtained for the study did not have parasitological confirmation of infection and therefore it was not possible to provide sensitivity and specificity estimates for the POC ELISA but required an evaluation of percent agreement with the IFAT. To address some of these limitations, a second study was performed with prospective sample collections over >1 year to assess the outcome of clinically healthy seropositive dogs tested on the POC immunoassay (Hernandez, et al. Submitted.). Additionally, future studies could compare the POC immunoassay to an established ELISA method performed in a single, diagnostic reference laboratory to serve as an additional gold standard, particularly for a sample set with confirmatory testing by parasitological methods and/or PCR. This could help to further elucidate the performance of the POC immunoassay in the CanL Stage 1 and CHS groups where IFAT titers tend to be low.

5. Conclusions

We evaluated a POC immunoassay for use in dogs living in CanL endemic regions of Europe. The recombinant antigen targets used in the test device detect antibodies against L. *infantum* and the performance demonstrated strong positive and negative agreement with IFAT, particularly when IFAT titers were over 1:200 or dogs were diagnosed with CanL of LeishVet stage 2 or 3. Providing a rapid POC immunoassay to veterinarians that agrees with a reference lab method and is able to detect seropositive dogs with no clinical signs or biochemical alterations will help with early diagnosis and better clinical management of the patient, as well as reduce the spread of a vector-borne disease of One Health concern (Miró and López-Vélez, 2018).

Ethical statement

Ethical review and approval were waived for the Italian and Greek samples, as all the study activities were performed in the frame of routine medical checks already needed by each animal involved in the present study and coordinated by local veterinarians. No procedures were performed on the animal solely for the purposes of the study.

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CRediT authorship contribution statement

Kristen Davenport: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Joe Liu:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Juliana Sarquis:** Writing – review & editing, Investigation. **Melissa Beall:** Writing – original draft, Supervision, Project administration, Data curation, Conceptualization. **Ana Montoya:** Writing – review & editing, Investigation. **Jan Drexel:** Writing – review & editing, Investigation. **Tori Denis:** Writing – review & editing, Investigation. **Ryan Toste:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Donato Traversa:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Guadalupe Miró:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

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