Current Research in Parasitology & Vector-Borne Diseases 10 (2026) 100418

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.sciencedirect.com/journal/current-research-in-parasitology-and-vector-borne-diseases

A newly developed rapid K39-based LeishmaCHECK Ab ELISA for high-throughput serological screening of canine leishmaniasis

Irene Ferrero [a,*], Paolo Poletti [a], Enrica Giachino [a], Joel Filipe [b], Alessia Libera Gazzonis [b], Carolina Allievi [b], Paola Dall'Ara [b]

[a] Agrolabo S.p.A., Scarmagno, 10010, Italy
[b] Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi, 26900, Italy

ARTICLE INFO

Keywords:
Canine leishmaniasis
CanL
Leishmania infantum
LeishmaCHECK Ab ELISA
ELISA
IFAT

ABSTRACT

Canine leishmaniasis (CanL) is a zoonotic vector-borne disease caused by *Leishmania infantum*, with dogs acting as the main reservoir for human infection. Reliable serological assessment is essential for disease surveillance and control. This study describes the development and validation of LeishmaCHECK Ab ELISA, a new rapid qualitative and semi-quantitative assay for the detection of anti-*L. infantum* antibodies in canine serum. Cut-off values were established using multiple statistical approaches, including Youden's index and receiver operating characteristic (ROC) curve analysis. The assay was evaluated on 552 canine samples (313 positives, 206 negatives, 33 borderline), using IFAT as the reference serological method. LeishmaCHECK Ab ELISA showed excellent agreement with IFAT (Cohen's kappa of 0.93), achieving a relative sensitivity of 95.2% and a relative specificity of up to 99.5%. Comparative analyses also demonstrated superior diagnostic performance compared with other commercially available ELISA tests. These findings indicate that LeishmaCHECK Ab ELISA represents a reliable alternative tool for the serological screening of canine leishmaniasis.

1. Introduction

Leishmania spp. are protozoan organisms belonging to the family Trypanosomatidae (order Kinetoplastida) transmitted through the bites of phlebotomine sand flies. They are responsible for leishmaniases, a group of zoonotic diseases characterized by heterogeneous epidemiological and clinical manifestations, ranging from self-limiting cutaneous lesions to severe, potentially fatal visceral forms. Leishmaniasis remains a significant public health concern in the Mediterranean basin, the Americas, Western and Southwestern Asia, as well as in East and North Africa. *Leishmania* spp. can naturally infect a broad spectrum of domestic and wild mammalian species, which may act as parasite reservoirs. While wildlife hosts are generally considered not to play a major direct role in human transmission, canids, particularly the domestic dog (*Canis familiaris*), represent the most important reservoir, playing a key role in maintaining and transmitting the infection to humans (Martín-Sánchez et al., 2007; Malta et al., 2010; Carrillo et al., 2013; Ribeiro et al., 2018). *Leishmania infantum* is the principal etiological agent of canine leishmaniasis (CanL), a chronic multisystemic disease (Alvar et al., 2004; Dantas-Torres, 2007; Morales-Yuste et al., 2022). During its life cycle, *Leishmania* protozoans exist in two distinct morphological forms: promastigotes, which develop in the gut of the insect vector, and amastigotes, which proliferate within host monocytes and macrophages. In the midgut of an infected sand fly, the motile, flagellated promastigotes multiply and, during a subsequent blood meal, are inoculated into the host's dermis, where they are phagocytized by dermal macrophages or mononuclear phagocytic cells. Here, promastigotes lose their flagellum and differentiate into round-shaped amastigotes, which replicate intracellularly and progressively disseminate by infecting other cells, spreading to multiple tissues (Paltrinieri et al., 2010). In a later blood meal, sand flies may ingest infected macrophages containing amastigotes, thereby transmitting *Leishmania* parasites to new susceptible hosts, including dogs or humans.

In dogs, *Leishmania* infection may develop over weeks to several months (mean incubation period of approximately five months), during which the parasite disseminates systemically, leading to a broad spectrum of clinical outcomes ranging from subclinical or asymptomatic infection to disease (WOAH, 2021). Clinical manifestations vary in severity from cutaneous leishmaniasis (CL), characterized by skin lesions, to disseminated visceral leishmaniasis (VL), the most severe and

* Corresponding author.
E-mail address: irene.ferrero@agrolabo.it (I. Ferrero).

https://doi.org/10.1016/j.crpvbd.2026.100418
Received 28 April 2026; Received in revised form 21 July 2026; Accepted 22 July 2026
Available online 22 July 2026
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progressive form, which is almost fatal if left untreated (Castagnaro et al., 2007; Silva-Moreira et al., 2025). The infection can involve multiple tissues, causing cutaneous and visceral ulcerative lesions, ocular abnormalities, chronic weight loss, muscle atrophy, and nephropathy, which may lead to renal failure. In this case, an excessive antibody response can induce glomerulonephritis, usually associated with the deposition of immune complexes in the kidneys.

Control of canine leishmaniasis remains challenging. Current diagnostic approaches rely on a combination of parasitological, serological and molecular methods (Reimão et al., 2020). Routine laboratory investigations include a complete blood count, serum biochemical analysis, serum protein electrophoresis and urine examination. Although cytological and histopathological examinations may demonstrate the presence of *Leishmania* parasites within macrophages or affected tissues, these techniques show limited sensitivity and do not permit species-level identification. Parasitological methods remain the most specific diagnostic assays, as they involve the direct isolation and *in vitro* culture of promastigotes. Molecular techniques, particularly polymerase chain reaction (PCR)-based assays, enable the detection of *Leishmania* DNA and currently represent highly sensitive and specific tools for the assessment of canine leishmaniasis (Castagnaro et al., 2007; Solano-Gallego et al., 2011; Morales-Yuste et al., 2022). Among serological assays available as support for CanL diagnosis, such as indirect hemagglutination assay (IHA) and Western blot, the indirect immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are the most widely used in routine practice (Reimão et al., 2020). Among these, IFAT is regarded the reference serological method by the World Organization for Animal Health (WOAH, 2021) for estimating the prevalence of CanL (Vulpiani et al., 2011; Travi et al., 2018; Morales-Yuste et al., 2022; Davenport et al., 2024; Mahachi et al., 2024).

The sensitivity and specificity of ELISA vary considerably depending on the antigen employed (Elmahallawy et al., 2014). Numerous *Leishmania*-derived antigens have been characterized and applied in ELISA and rapid immunochromatographic tests, including crude soluble antigen (CSA) and several recombinant proteins such as rK39, rK9, rK26, rK28, rKRP42, rKE16, rA2, and rKDDR (Bhatia et al., 1999; Boarino et al., 2005; Mohapatra et al., 2010; Farash et al., 2017, 2022; Reimão et al., 2020), as well as the *Leishmania*-activated C-kinase antigen (LACK) (Ramiro et al., 2003; Kamoun-Essghaier et al., 2005; Fernández et al., 2018). Although these antigens show variable diagnostic performance in patients affected with VL, the rK39 antigen remains the most widely used for both human and canine VL serodiagnosis (Burns et al., 1993; Braz et al., 2002; Scalone et al., 2002; Rosati et al., 2003; Mohapatra et al., 2010; Maia et al., 2012; Reiter-Owona et al., 2016; Reimão et al., 2020).

This study aimed to develop the LeishmaCHECK Ab, a new qualitative and semi-quantitative indirect ELISA test designed for the identification of *L. infantum* antibodies in dog serum/plasma samples.

2. Materials and methods

2.1. Specimen collection

To establish the new ELISA assay and evaluate its analytical reliability, canine serum and plasma samples from animals presenting with diverse clinical conditions were collected from veterinary clinics located in northern and central Italy, as well as in Greece (Attica region). All samples were tested for anti-*Leishmania* antibodies using the reference serological method IFAT (Fluo *Leishmania* kit, Agrolabo S.p.A.) and classified as positive or negative prior to their use in assay development. A sample was considered IFAT-positive when reactive at a dilution of 1:80 or higher. Canine samples were derived from routine diagnostic submissions without pre-selection, and all laboratory analyses were performed under blinded conditions, as laboratory operators had no access to clinical records or previous diagnostic results. Some samples exhibited marked lipemia or hemolysis. Clinical information regarding disease outcome or ongoing pharmacological treatments was available for only a limited number of cases. The overall sample size was consistent with that reported in previously published ELISA validation studies (Zheng et al., 2003; Mei et al., 2012; Righi et al., 2022; Appeltrath et al., 2024).

2.2. ELISA plate setting

Nunc PolySorp and Nunc MaxiSorp microplates (Thermo Fisher Scientific, Waltham, MA, USA) were selected based on manufacturer recommendations (Ferrero et al., 2025a, 2025b, 2025c, 2025d) and on supporting evidence from the literature (https://www.genetex.com/upload/media/support/protocols/genetex_ELISA(indirect).pdf), to determine optimal assay performance. Overall, coating and blocking conditions adopted for the development of the new indirect ELISA assay were based on standardized procedures described in the literature (https://www.cusabio.com/protocols/ELISA_Protocol.pdf) and optimized according to internal protocols and technical expertise.

During assay development, plates were coated with different recombinant antigens derived from *L. infantum* promastigotes and amastigotes. Among these, the fusion protein K9-K26-K39 that contains immunodominant regions of *Leishmania* K9, K26 and K39 (MyBioSource, San Diego, CA, USA) or K39 antigen (Rekom Biotech, Granada, Spain) at 0.25 μg/ml (Kurkjian et al., 2005; Vaish et al., 2012; Mondal et al., 2019), 0.5 μg/ml (Zijlstra et al., 2001; Saliba et al., 2019) or 1 μg/ml (Silva et al., 2021) with or without LACK antigen (MyBioSource, San Diego, CA, USA). All antigens were coated in carbonate-bicarbonate (CB) buffer (pH 9.6) (Agrolabo S.p.A., Scarmagno, Italy) in accordance with published protocols (https://www.biomol.com/dateien/Bethyl–ELISA-Protocol.pdf) and incubated overnight at 2–8 °C. After a wash step, plates were blocked using two commercial blocking buffers (BB1 and BB2) (Surmodics, Eden Prairie, MN, USA) and allowed to dry at room temperature (RT, 20–25 °C) for one day before being stored at 2–8 °C.

In the finalized ELISA protocol, Nunc MaxiSorp microplates were prepared and coated with 0.5 μg/ml of recombinant *L. infantum* K39 antigen (Rekom Biotech). In the LeishmaCHECK Ab ELISA, a positive control (PC), consisting of anti-histidine (His) tag antibody labelled with horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO, USA), a negative control (NC, sample diluent) and samples, diluted 1:100 in sample diluent composed of phosphate-buffered saline (PBS) and bovine serum albumin (BSA) (Agrolabo S.p.A., Scarmagno, Italy), were dispensed in 100 μl into each well and incubated for 30 min at RT. During this incubation, specific anti-*L. infantum* antibodies present in positive samples bound to the immobilized antigen, forming antigen-antibody complexes. Following an initial washing step to remove unbound material, 100 μl of HRP-conjugated anti-dog IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:80,000 was added to each well and incubated for 15 min at RT. After a second wash cycle, 100 μl of the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Surmodics) was added and incubated for 10 min at RT in the dark, allowing color development. The enzymatic reaction was stopped by the addition of 100 μl of stop solution (0.2 M sulfuric acid; Agrolabo S.p.A.), and the optical density (OD) was measured at 450 nm by using an ELISA microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA). The assay run was considered valid when the OD value of the NC was below 0.1 and that of PC exceeded 0.6, within the limits described in the literature (Yuan et al., 2023; Appeltrath et al., 2024).

2.3. Validation of LeishmaCHECK Ab ELISA

The diagnostic performance of LeishmaCHECK Ab ELISA was assessed relative to IFAT (Fluo *Leishmania* kit, Agrolabo S.p.A.), the reference serological assay based on slides coated with whole *Leishmania* parasites. A total of 552 samples were tested by IFAT, using a titre of

1:80 as the diagnostic cut-off.

Two analytical approaches were applied to the borderline samples to evaluate the impact of these specimens. First, borderline samples were excluded to compare only clearly positive and negative animals. Secondly, they were classified as negative, following previous studies that considered dogs seropositive only at IFAT titres $\geq$ 1:160, as a titre of 1:80 may represent non-specific reactivity or antibody levels insufficient to reliably indicate infection (Oliva et al., 2014; Gizzarelli et al., 2020; Maurelli et al., 2020; Morales-Yuste et al., 2022; Mahachi et al., 2024; Santoro and Vellusi, 2015).

Briefly, IFAT was performed by incubating 20 µl of samples diluted 1:80 and appropriate controls on antigen-coated substrate slides in a humid chamber for 30 min at 37 °C. Following a washing step, 20 µl of fluorescein isothiocyanate (FITC)-labelled anti-dog IgG antibody conjugate was added to each well, and the slides were incubated again for 30 min at 37 °C in the dark. After washing and application of the mounting medium, coverslips were placed and slides were examined under a fluorescence microscope equipped with a FITC filter. A sample was considered negative when *Leishmania* parasites appeared greyish-red, whereas positive samples were characterized by a distinct yellow-green fluorescence localized on the parasite membrane and flagellar region.

Positive samples were further subjected to two-fold serial dilutions up to 1:640 to determine their antibody titre defined as the highest dilution yielding a positive fluorescence signal.

ELISA false-negative results were assigned to samples with IFAT titres above 1:80, in line with previous reports (Oliva et al., 2014; Anfossi et al., 2018; Stensvold et al., 2019; Gizzarelli et al., 2020; Maurelli et al., 2020; Morales-Yuste et al., 2022; Mahachi et al., 2024; Santoro and Vellusi, 2015).

Regarding controls, for PC, a minimum OD of 0.6 was set for test validity (Yuan et al., 2023; Appeltrath et al., 2024), consistent with prior ELISA studies (Raditic et al., 2011; Valero et al., 2012; Sirikajornpan et al., 2021). The sample diluent was used as NC, considering the 0.3 OD as threshold (Yuan et al., 2023; Appeltrath et al., 2024) as described in both research and commercial ELISA assays using PBS-based solutions (Hagler and Naranjo, 1994; Niedrig et al., 1999; Peng et al., 2013; EuroMAbNet, 2023; https://www.immunoreagents.com/protocols/view/2).

2.4. Determination of ELISA cut-off

Multiple approaches were employed to determine the LeishmaCHECK Ab ELISA cut-off. In preliminary experiments, the cut-off was calculated as the mean OD of negative samples plus three times the standard deviation (Lardeux et al., 2016; Khatri et al., 2020; Chen et al., 2021). Samples with OD values within ±10% of the cut-off were classified as borderline, following the approach used in other commercial ELISA assays (ABIN997000, DRG Instruments GmbH; Antibodies online, 2023; DRG Instruments, 2023). Cut-off was established by analyzing 132 samples (66 positives and 66 negatives) previously used for ELISA validation. According to various methodologies, optimal cut-off value was defined as the minimum difference between sensitivity and specificity (Mazzu-Nascimento et al., 2017) or as a value between the maximum OD of negative samples and the minimum OD of positive samples (Lardeux et al., 2016). Furthermore, receiver operating characteristic (ROC) curve analysis and Youden's index (*J*) ranging from 0 (ineffective test) to 1 (perfectly effective test), were used to identify the cut-off that maximized the distinction between true- and false positives. Finally, considering the amounts of true positives, false positives, false negatives and true negatives obtained from the LeishmaCHECK Ab ELISA against IFAT as reference assay, all diagnostic parameters including the accuracy, relative sensitivity and specificity, positive and negative predictive values (PPV and NPV, respectively) were determined at the selected cut-off value. The 95% confidence intervals (95% CI) were computed for sensitivity, specificity, PPV and NPV.

2.5. Evaluation of reproducibility and precision

The accuracy of the LeishmaCHECK Ab ELISA was evaluated by assessing variability within a single or different assay runs (intra- and inter-assay precision). Tests were conducted on different days under comparable conditions following the WOAH guidelines (WOAH, 2023). To account for potential differences in manual technique, assays were performed by two independent operators. Positive and negative samples were analysed in eight replicates across three separate batches, yielding a total of 24 measurements per sample, in accordance with International Council for Harmonisation (ICH) Q2(R1) recommendations, which require a minimum of nine determinations (at least three replicates under three distinct conditions) (FDA, 2021). For each sample, the mean, minimum and maximum OD values, standard deviation (SD), and percentage coefficient of variation (%CV) were calculated.

2.6. Comparison with commercial tests

The performance of the LeishmaCHECK Ab ELISA was evaluated in comparison with two commercially available ELISA assays commonly reported in the literature. In one study, 370 canine serum/plasma samples (152 IFAT-positive, 186 IFAT-negative and 32 IFAT-borderline) were tested using Leiscan ELISA or CIVTEST® Canis *Leishmania* (HIPRA, Amer, Spain) (Rodríguez-Cortès et al., 2013; Solano-Gallego et al., 2014). In a second study, 84 samples (42 IFAT-positive, 40 IFAT-negative and 2 IFAT-borderline) were assessed using INgezim *Leishmania* ELISA (Gold Standard Diagnostics, Madrid, Spain) (Risueño et al., 2012; Ledesma et al., 2017; Páez et al., 2025). All assays were performed according to the manufacturer's instructions.

2.7. Evaluation of serological cross-reactivity

Serum samples previously confirmed positive for *Ehrlichia canis* antibodies by both IFAT (Fluo *Ehrlichia* kit, Agrolabo S.p.A.) and ELISA (EhrlichiaCHECK Ab ELISA, Agrolabo S.p.A.), were tested for anti-*Leishmania* antibodies by LeishmaCHECK Ab ELISA to assess potential serological cross-reactivity between the two pathogens.

2.8. Determination of ELISA kit expiry

In the absence of specific regulations for stability testing of veterinary diagnostic kits, the ASTM F1980-21 Standard Guide for Accelerated Aging of Sterile Medical Device Packages (ASTM International, 2021), which uses the Arrhenius equation to estimate device degradation under accelerated conditions, was followed. Accelerated stability was conducted at 37 °C for 6 weeks, corresponding to 18 months of real-time stability at 2–8 °C, as calculated using online tools (https://keystonecompliance.com/aat-calculator/; https://pkgcompliance.com/resource/accelerated-aging-calculator/). For both accelerated and real-time stability studies, initial testing was performed at baseline (T_0), followed by weekly assays during the first six weeks. Thereafter, kits stored at 2–8 °C were tested every three months up to 18 months. For each sample and control, the percentage of remaining activity (%RA) was calculated as the ratio of the OD at a given time point to that at T_0, defining 70% as the minimum acceptable activity (Lexmond et al., 2011).

2.9. Statistical analysis

During comparison studies statistical analyses were performed using a t-test, with *P*-values < 0.05 considered statistically significant. Cohen's *d* was calculated to quantify the strength of the relationship (or effect size) with thresholds of 0.2, 0.5, and 0.8 representing small, moderate and large effects, respectively. Additionally, Cohen's kappa (κ) coefficient was used to assess the degree of agreement beyond chance. Moreover, a simple linear regression model was used to explain the

variation between the observations and the predicted values.

3. Results

3.1. Preliminary assays on Nunc PolySorp and MaxiSorp microplates

Preliminary assays were performed on Nunc PolySorp and Nunc MaxiSorp plates under different coating conditions and conjugate dilutions (1:30,000 and 1:80,000). Across all tested conditions, the NC and IFAT-negative sera consistently showed low OD values, whereas the PC and IFAT-positive sera displayed clear reactivity. No nonspecific signal was detected in uncoated wells, and the conjugate alone produced OD values comparable to the NC, confirming minimal background reactivity (Supplementary Table S1).

On PolySorp plates, assay performance was influenced by both antigen composition and blocking buffer. Among IFAT-negative samples, statistically significantly lower OD values were observed with K9-K26-K39 than with K9-K26-K39 plus LACK when BB1 was used, whereas no differences were detected with BB2. Furthermore, BB2 significantly reduced nonspecific reactivity compared with BB1 when K9-K26-K39 plus LACK was employed, indicating more effective blocking (Supplementary Table S2). No significant differences were observed between K39 alone, K39 plus LACK, and K9-K39-K26, suggesting that the addition of LACK did not improve assay performance when K39 was used as the coating antigen (Supplementary Table S2). The PC produced strong signals (OD > 1.5) only with K9-K39-K26 plus LACK or K39 coated with 0.5 μg/ml, whereas lower K39 coating concentrations generated suboptimal OD values (Supplementary Table S1).

Subsequent optimization focused on K39-coated MaxiSorp plates blocked with BB2. Antigen concentrations of 0.5 and 1 μg/ml and conjugate dilutions of 1:30,000 and 1:80,000 provided comparable discrimination between positive and negative samples without detectable nonspecific reactivity. However, the 1:80,000 conjugate dilution yielded improved signal intensity in positive sera. Evaluation on a larger panel of 101 sera (77 IFAT-negative and 24 IFAT-positive) showed that MaxiSorp plates produced significantly higher OD values in positive samples (mean OD ± SD of 2.130 ± 1.063) than PolySorp plates (mean OD ± SD of 1.450 ± 0.780; Cohen's $d = 0.303$) and eliminated the two false-negative results observed with PolySorp (Supplementary Table S2). Consequently, relative sensitivity and specificity compared with IFAT reached 100% on MaxiSorp plates (Cohen's kappa of 1.000, excellent agreement), whereas PolySorp plates achieved 91.7% sensitivity and 100% specificity (Cohen's kappa of 0.944, excellent agreement) (Table 1). The NC formed by sample diluent and tested with 1:80,000 conjugate dilution showed OD values in the range of negative samples. Regarding the PC, a dilution of 1:300,000 provided the strong reactivity (OD values roughly 2.7) with minimal background, while lower dilutions frequently generated saturated or overflow signals (Supplementary Table S1). Comparable results were obtained with K39 concentrations of 0.5 and 1 μg/ml under different incubation conditions. Although shorter incubation times reduced signal saturation, the 30/15/10 min protocol for sample/conjugate/TMB was selected because it enhanced positive-sample reactivity without increasing background (Supplementary Table S3).

Based on these findings, the final ELISA format was established using MaxiSorp plates coated with K39 as the sole antigen, BB2 blocking buffer, a conjugate dilution of 1:80,000 and incubation times of 30/15/10 min, as these conditions provided the best balance between signal intensity and low background reactivity.

3.2. Cut-off determination

The cut-off of the LeishmaCHECK Ab ELISA was determined using 132 samples (66 IFAT-positive and 66 IFAT-negative; Supplementary Table S4), considering IFAT as the reference test. According to previous studies, an appropriate cut-off may lie between the maximum OD of negative samples (Max OD–) and the minimum OD of positive samples (Min OD+) (Lardeux et al., 2016). In this dataset, Max OD− was 0.450 and Min OD+ was 0.318. Moreover, considering sensitivity and specificity curves (Mazzu-Nascimento et al., 2017), OD values between 0.360 and 0.450 corresponded to their intersection, and both parameters reached 98.5% (Supplementary Table S4). In addition, according to the ROC curve analysis, the optimal cut-off was OD 0.460, representing the best balance between sensitivity and specificity (Fig. 1) thus confirming the previous analysis. Similarly, the maximum Youden's index ($J = 0.985$) corresponded to OD values between 0.460 and 0.570 (Fig. 2).

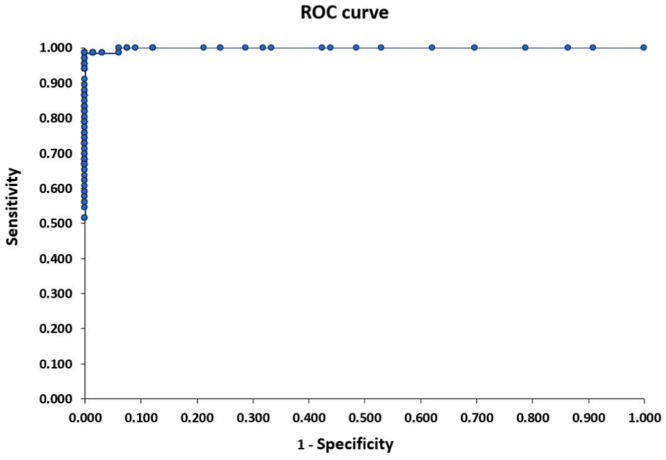

Fig. 1. Cut-off determination by ROC curve.

Table 1
Comparison between LeishmaCHECK Ab ELISA and IFAT.

LeishmaCHECK Ab ELISA		IFAT – Reference test			Relative sensitivity (%)	Relative specificity (%)	Cohen's κ
		Positive	Negative	Total			
Nunc PolySorp	Positive	22[a]	0[b]	22	91.7	100	0.944
	Negative	2[c]	77[d]	79			
	Total	24	77	101			
Nunc MaxiSorp	Positive	24[a]	0[b]	24	100	100	1.000
	Negative	0[c]	77[d]	77			
	Total	24	77	101			

Notes: Nunc PolySorp or Nunc MaxiSorp plates were coated with 0.5 μg/ml K39 and blocked with BB2. Incubation times were 30 min for samples, 15 min for conjugate and 10 min for TMB. Conjugate dilutions: 1:30,000 (PolySorp) and 1:80,000 (MaxiSorp). Total samples: 101 (77 IFAT-negatives, 24 IFAT-positives).
Abbreviation: IFAT, Fluo *Leishmania* kit (Agrolabo S.p.A.).
[a] True positives.
[b] False positives.
[c] False negatives.
[d] True negatives.

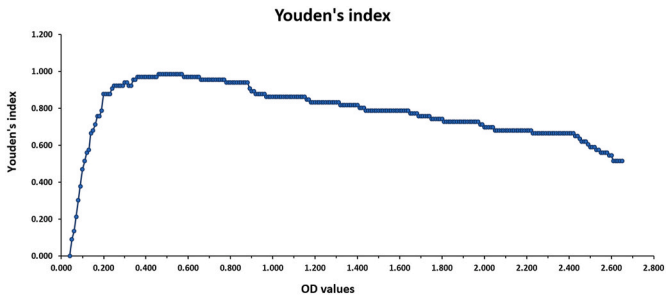

Fig. 2. Cut-off determination by Youden's index.

Based on these results, the cut-off value of LeishmaCHECK Ab ELISA was set at OD 0.460. An uncertainty interval of ±10% around this value was defined as a "doubtful zone".

3.3. LeishmaCHECK Ab ELISA validation

The diagnostic performance of LeishmaCHECK Ab ELISA was evaluated relative to IFAT. IFAT-negative samples showed no specific fluorescence or only weak red-greyish background staining, whereas positive samples exhibited the characteristic bright apple-green fluorescence of *Leishmania* parasites (Fig. 3).

Among the 552 samples analysed by IFAT, 313 were positive, 206 were negative and 33 were borderline (titres 1:80). To evaluate the impact of borderline samples, two analytical approaches were applied. In the first, they were excluded to compare only clearly positive and negative animals, while in the second, they were classified as negative, as described previously. No interference was observed with highly lipemic or haemolytic sera tested. In both cases, LeishmaCHECK Ab ELISA identified 15 false negatives and 1 or 3 false positives (Table 2). Among 15 false negatives, four had IFAT titre of 1:80–160, two 1:160–320, three 1:320–640, five above 1:640 and one had unknown IFAT titre (Supplementary Table S5). Compared to IFAT, LeishmaCHECK Ab ELISA achieved a relative sensitivity of 95.2% and a specificity of 98.7–99.5%. Agreement between ELISA and IFAT was excellent, with Cohen's kappa coefficients from 0.934 to 0.936. Overall diagnostic accuracy was 96.7–96.9%, while the PPV and NPV were in the range 99.0–99.7% and 93.2–94.0%, respectively. In summary, although there was a slight shift in the values, in all cases the overall diagnostic performance of the ELISA remains highly robust.

3.4. Reproducibility assessment

Reproducibility assay was conducted on controls, two positive (IFAT titres of 1:80–1:160 and > 1:640) and two negative samples (Supplementary Table S6). For ELISA assays, the acceptable limits for the coefficient of variation (%CV) are generally below 10% for intra-assay precision and between 15% and 20% for inter-assay one. Nevertheless, a 20% CV is commonly considered the upper acceptable threshold for both assay types (Lexmond et al., 2011; Conrad, 2023). The LeishmaCHECK Ab ELISA demonstrated good accuracy, with both intra-assay and inter-assay %CV values falling within the recommended limits (Table 3).

3.5. Comparative study with commercial Leiscan ELISA

A total of 370 canine serum/plasma samples (152 IFAT-positive, 186 IFAT-negative and 32 IFAT-borderline) were tested using the Leiscan ELISA kit and compared to IFAT and LeishmaCHECK Ab ELISA. As previously, the impact of IFAT-borderline samples (titer 1:80) was assessed both by excluding and including them in the evaluation.

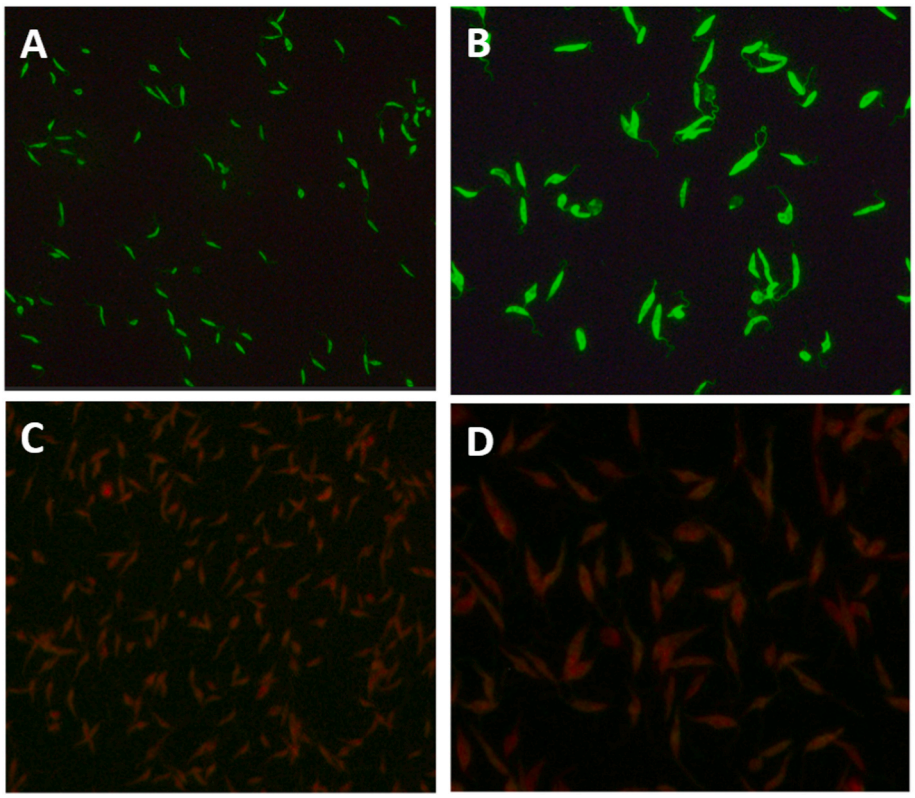

Fig. 3. Results obtained with IFAT. Example of positive (**A**, **B**) and negative (**C**, **D**) results in IFAT (Fluo *Leishmania* kit, Agrolabo), observed at 100× (**A**, **C**) and 400× magnification (**B**, **D**).

Table 2
Validation results of LeishmaCHECK Ab ELISA relative to IFAT assay: impact of different approaches to borderline IFAT samples (1:80).

Approach	LeishmaCHECK Ab ELISA	IFAT			RSe (95% CI) (%)	RSp (95% CI) (%)	PPV (95% CI) (%)	NPV (95% CI) (%)	Ac (%)	Cohen's κ
		Positive	Negative	Total						
# 1	Positive	298[a]	1[b]	299	95.2 (92.1–97.2)	99.5 (96.9–100)	99.7 (97.9–100)	93.2 (88.8–96.0)	96.9	0.936
	Negative	15[c]	205[d]	220						
	Total	313	206	519						
# 2	Positive	298[a]	3[b]	301	95.2 (92.1–97.2)	98.7 (96.1–99.7)	99.0 (96.9–99.7)	94.0 (90.1–96.5)	96.7	0.934
	Negative	15[c]	236[d]	251						
	Total	313	239	552						

Notes: In approach #1, borderline IFAT samples were excluded from the analysis, whereas in approach #2 they were included among negatives. A Cohen's κ value > 0.9 indicates excellent agreement.
Abbreviation: IFAT, Fluo *Leishmania* kit (Agrolabo S.p.A.); RSe, relative sensitivity; RSp, relative specificity; Ac, accuracy.
[a] True positives.
[b] False positives.
[c] False negatives.
[d] True negatives.

Table 3
Reproducibility assay and intra- and inter-assay coefficient of variation. ELISA tests were performed in 8 replicates on three plate batches.

Batch		PC	NC	NEG 1	NEG 2	POS 1	POS 2
Batch #1	Mean OD ($n = 8$)	2.821	0.047	0.056	0.071	3.053	0.664
	SD	0.029	0.003	0.003	0.003	0.049	0.013
	Intra-assay %CV	1.030	5.454	4.708	4.850	1.610	1.939
Batch #2	Mean OD ($n = 8$)	2.820	0.046	0.058	0.069	3.036	0.674
	SD	0.026	0.002	0.004	0.004	0.049	0.012
	Intra-assay %CV	0.926	4.791	6.812	6.065	1.601	1.819
Batch #3	Mean OD ($n = 8$)	2.828	0.047	0.055	0.068	2.983	0.673
	SD	0.033	0.002	0.002	0.004	0.047	0.013
	Intra-assay %CV	1.169	4.762	3.316	6.152	1.570	1.992
	Inter-assay %CV	1.042	5.002	4.946	5.689	1.594	1.917

Abbreviations: PC, positive control; NC, negative control; POS, positive samples (POS 1, IFAT titre >1:640; POS 2, IFAT titre 1:160); NEG, negative samples (NEG 1 and NEG 2, IFAT-negative); *n*, number of tests; SD, standard deviation; %CV, coefficient of variation in %.

3.5.1. Performance of Leiscan and LeishmaCHECK Ab ELISA excluding IFAT-borderline samples

The diagnostic performance of Leiscan and LeishmaCHECK Ab ELISA was initially assessed on 338 samples after excluding IFAT-borderline specimens. Compared with IFAT, both ELISA-based assays demonstrated excellent diagnostic performance, with only a limited number of false-positive and false-negative results (Table 4; Supplementary Table S7). Leiscan achieved a sensitivity of 94.1%, specificity of 98.9% and overall accuracy of 96.7%. LeishmaCHECK Ab ELISA showed slightly superior performance and an excellent agreement relative to IFAT (Cohen's kappa of 0.970), reaching a sensitivity of 97.4%, specificity of 99.5% and accuracy of 98.5%. Both assays also exhibited high PPV (> 98%) and NPV (> 95%), confirming their reliability in use. When Leiscan was evaluated directly against LeishmaCHECK Ab ELISA, sensitivity and specificity were 95.3% and 98.4%, respectively, further supporting the high concordance between the assays (Cohen's kappa of 0.939) (Table 4).

3.5.2. Performance of Leiscan and LeishmaCHECK Ab ELISA including IFAT-borderline samples

To evaluate assay performance under more challenging conditions, a secondary analysis was conducted including the 32 IFAT-borderline samples among negatives, increasing the study population from 338 to 370 specimens. The inclusion of borderline samples did not affect assay sensitivity but resulted in the expected reduction in specificity and predictive values due to the increased complexity associated with classifying low-titer or ambiguous samples.

Using IFAT as a reference, Leiscan maintained an unchanged sensitivity of 94.1%, whereas specificity decreased slightly from 98.9% to 96.8%. Consequently, the PPV declined from 98.6% to 95.3%. Similarly, compared to IFAT, LeishmaCHECK Ab ELISA retained a stable relative sensitivity of 97.4%, while specificity showed only a marginal reduction from 99.5% to 99.1%. Despite these changes, the overall accuracy remained high at 98.4% with excellent inter-test agreement (Cohen's kappa of 0.966) (Table 4).

In addition, agreement between Leiscan and LeishmaCHECK Ab ELISA remained excellent, with a Cohen's kappa coefficient of 0.922.

Notably, the inclusion of IFAT-borderline samples was associated with an increase in the number of false-positive results for both analyses, rising from 2–3 cases when borderline samples were excluded to 7 cases when included (Table 4; Supplementary Table S7). Nevertheless, the excellent agreement between Leiscan and LeishmaCHECK Ab ELISA confirmed the robustness and consistency of both assays even in the presence of diagnostically challenging samples.

Since the Leiscan ELISA kit provides an estimated IFAT-equivalent antibody titer for each sample, the relationship between the OD values obtained with the LeishmaCHECK Ab ELISA and the IFAT titres predicted by Leiscan was evaluated. After excluding samples with discordant ELISA results, 357 samples were analysed. A strong linear correlation was found between the mean OD values measured by the LeishmaCHECK Ab ELISA and the IFAT titres predicted by Leiscan ELISA, with a coefficient of determination (R^2) of 0.979 (Table 5).

3.6. Comparative study with commercial INgezim Leishmania ELISA

The performance of LeishmaCHECK Ab ELISA was also evaluated by comparing results with IFAT and the commercial INgezim *Leishmania* ELISA on a subset of 84 canine samples, comprising 42 IFAT-positive, 40 IFAT-negative, and 2 IFAT-borderline specimens (titre 1:80). In the primary analysis, IFAT-borderline samples were excluded, resulting in a study population of 82 samples, while in the second, they were included among negatives.

Table 4
Comparison of the results of Leiscan ELISA (HIPRA) against IFAT and LeishmaCHECK Ab ELISA under different approaches to borderline IFAT samples (1:80).

Approach		IFAT			RSe (%)	RSp (%)	Ac (%)	PPV (%)	NPV (%)	Cohen's κ	LeishmaCHECK Ab ELISA			RSe (%)	RSp (%)	Ac (%)	PPV (%)	NPV (%)	Cohen's κ
		Pos	Neg	Total							Pos	Neg	Total						
# 1	Leiscan	Pos	143[a]	2[b]	145	98.9	96.7	98.6	95.3	0.934	142[a]	3[b]	145	95.3	98.4	97.0	97.9	96.4	0.939
		Neg	9[c]	184[d]	193						7[c]	186[d]	193						
		Total	152	186	338						149	189	338						
	LeishmaCHECK Ab ELISA	Pos	148[a]	1[b]	149	97.4	99.5	98.5	99.3	97.9	0.970								
		Neg	4[c]	185[d]	189														
		Total	152	186	338														
# 2	Leiscan	Pos	143[a]	7[b]	150	94.1	96.8	95.7	95.3	95.9	0.911	143[a]	7[b]	150	95.3	96.8	96.2	95.3	96.8	0.922
		Neg	9[c]	211[d]	220							7[c]	213[d]	220					
		Total	152	218	370							150	220	370					
	LeishmaCHECK Ab ELISA	Pos	148[a]	2[b]	150	97.4	99.1	98.4	98.7	98.2	0.966								
		Neg	4[c]	216[d]	220														
		Total	152	218	370														

Notes: In approach #1, borderline IFAT samples were excluded from the analysis, whereas in approach #2 they were included among negatives. A Cohen's κ value > 0.9 indicates excellent agreement.
Abbreviations: IFAT, Fluo *Leishmania* kit (Agrolabo S.p.A.); Pos, positive; Neg, negative; RSe, relative sensitivity; RSp, relative specificity; Ac, accuracy.
[a] True positives.
[b] False positives.
[c] False negatives.
[d] True negatives.

3.6.1. *Performance of the INgezim Leishmania assay excluding IFAT-borderline samples*

Using IFAT as the reference method, the INgezim *Leishmania* assay demonstrated lower sensitivity than LeishmaCHECK Ab ELISA (66.7 *vs* 76.2%). Specifically, INgezim *Leishmania* assay failed to identify 14 IFAT-positive samples, whereas LeishmaCHECK Ab ELISA missed 10 IFAT-positive cases (Table 6; Supplementary Table S7). Despite its lower sensitivity, INgezim *Leishmania* assay maintained a high specificity of 92.5% with only a moderate agreement with IFAT (Cohen's kappa of 0.588) (Table 6). In the same cohort and against IFAT, LeishmaCHECK Ab ELISA showed higher specificity (100%) than INgezim *Leishmania* assay, correctly identifying all IFAT-negative samples and generating no false-positive results. Consequently, the assay achieved a relative specificity and PPV of 100%. However, despite the good agreement with IFAT (Cohen's kappa of 0.757), sensitivity was limited to 76.2% due to the presence of 10 false-negative samples, common to INgezim *Leishmania* assay (Table 6; Supplementary Table S7).

When compared directly with LeishmaCHECK Ab ELISA, the INgezim *Leishmania* assay achieved a sensitivity of 87.5% and a specificity of 94.0% with very good agreement between the two ELISA-based methods (Cohen's kappa of 0.819) (Table 6).

3.6.2. *Performance of the INgezim Leishmania assay including IFAT-borderline samples*

A secondary analysis was performed by including the two IFAT-borderline samples among negatives, increasing the sample size from 82 to 84 specimens. Interestingly, compared to IFAT, the INgezim *Leishmania* assay relative sensitivity remained unchanged at 66.7%, while specificity increased marginally from 92.5% to 92.9%. The agreement with IFAT remained within the moderate range (Cohen's kappa of 0.595). Despite the stability of these parameters, the assay continued to display substantially lower sensitivity and a higher false-negative rate than LeishmaCHECK Ab ELISA (14 *vs* 10 cases) (Table 6; Supplementary Table S7).

In contrast, LeishmaCHECK Ab ELISA maintained identical sensitivity and specificity values relative to IFAT (76.2% and 100%, respectively), with a Cohen's kappa of 0.762, confirming good agreement. Notably, the 10 false-negative samples identified by LeishmaCHECK Ab ELISA were part of those generated by the INgezim *Leishmania* assay (Supplementary Table S7).

Finally, the direct comparison between INgezim *Leishmania* assay and LeishmaCHECK Ab ELISA remained essentially unchanged after inclusion of the IFAT-borderline samples, further confirming the robustness of the observed agreement between the two assays (Table 6).

3.7. *Evaluation of serological cross-reactivity*

Co-infections involving ehrlichiosis, babesiosis, and leishmaniasis are relatively frequent, particularly in tropical, subtropical, and temperate regions, where hematophagous vectors are widely distributed (Krawczak et al., 2015). In addition, previous studies have reported serological cross-reactivity between antibodies against *Ehrlichia canis* and *Babesia canis* and *Leishmania* spp. in commonly used diagnostic assays (Mancianti et al., 1996; Mettler et al., 2005; Ferreira et al., 2007). Thus, given the availability of *E. canis*-positive samples, the potential serological cross-reactivity between anti-*Ehrlichia canis* and *L. infantum* antibodies was evaluated. A total of 46 canine samples, previously confirmed positive for *E. canis* antibodies by both IFAT (Fluo Ehrlichia kit, Agrolabo S.p.A.) and ELISA (EhrlichiaCHECK Ab ELISA, Agrolabo S.p.A.), were subsequently tested using the LeishmaCHECK Ab ELISA. As all samples yielded negative results, no evidence of cross-reactivity was observed between anti-*E. canis* antibodies and the *Leishmania* K39 antigen (Supplementary Table S8).

In the accelerated stability study, the PC, NC and five samples (two negative and three positive) were evaluated. All controls and samples showed percentage of remaining activity (%RA) values above 70% at

Table 5
Correlation between mean OD values in LeishmaCHECK Ab ELISA compared to IFAT titers predicted by Leiscan ELISA kit. Data analysis was performed on 357 samples (177 IFAT-negatives and 180 IFAT-positives).

	Negative	1:80	1:80–1:160	1:160–1:320	1:320–1:640	> 1:640
No. of samples	177	37	23	25	42	53
Mean OD values	0.134	0.291	1.178	1.815	2.223	2.720
R-squared	0.979					

each time point (Supplementary Table S9). These results indicate that kit reagents remain stable for up to 6 weeks at 37 °C, supporting an estimated shelf life of 18 months at 2–8 °C, pending confirmation through long-term stability studies.

4. Discussion

Serological detection of antibodies against *L. infantum* is crucial for confirming infection, assessing clinical disease and monitoring public health interventions. Ideally, the diagnostic sensitivity of a serological assay should be evaluated using animals with parasitologically confirmed infection, whereas specificity should be assessed in dogs whose uninfected status has been verified through longitudinal follow-up and consistently negative test results (Medeiros et al., 2021). However, such well-defined reference populations are often difficult to obtain under field conditions, and the absence of a perfect reference standard remains a common challenge in studies evaluating diagnostic performance. Among the available serological techniques, IFAT and ELISA are the most widely used serological methods for clinical evaluation and research on canine *L. infantum* infection. Although IFAT, based on whole promastigotes, is generally considered the reference serological method (WOAH, 2021; Travi et al., 2018; Mahachi et al., 2024), it has several limitations, including the need for specialized equipment and experienced personnel for result interpretation. Furthermore, IFAT may exhibit cross-reactivity with other trypanosomatids, particularly *Trypanosoma cruzi* and potentially with other *Leishmania* species (Troncarelli et al., 2009). Consequently, relying exclusively on IFAT as the reference method may introduce bias when assessing the diagnostic performance of novel assays. The performance of the K39-based LeishmaCHECK Ab ELISA was evaluated not only in comparison with IFAT but also against other commercially available ELISA assays. This approach provided an additional and more robust assessment of assay performance, reducing dependence on a single reference test and allowing a broader evaluation of agreement among currently available serological methods.

The classification of samples with borderline IFAT titres (1:80) represents a critical issue in seroepidemiological studies of CanL, as it can influence estimates of seroprevalence and diagnostic performance. To assess the impact of these samples, two approaches were evaluated. First, borderline samples were excluded to maximize epidemiological homogeneity between clearly seropositive and seronegative dogs. Secondly, they were classified as negative, consistent with previous studies that considered dogs seropositive only at IFAT titres $\geq$ 1:160 (Oliva et al., 2014; Gizzarelli et al., 2020; Maurelli et al., 2020; Morales-Yuste et al., 2022; Mahachi et al., 2024; Santoro and Vellusi, 2015). Under this assumption, a titre of 1:80 may indicate non-specific reactivity, early exposure to *Leishmania*, or antibody levels insufficient for a reliable assessment of infection status. In the absence of compatible clinical signs or additional confirmatory evidence, such dogs should not necessarily be considered infected. Regardless of the analytical approach adopted, LeishmaCHECK Ab ELISA based on K39 antigen achieved a high relative sensitivity (95.2%) and specificity up to 99.5% together with excellent agreement with IFAT (Cohen's kappa of 0.93). These findings indicate a strong ability to detect anti-*Leishmania* antibodies while minimizing false-positive results, a particularly valuable characteristic in endemic areas where confirmatory testing is often required.

The performance of LeishmaCHECK Ab ELISA was evaluated against two commercially available ELISA assays, INgezim *Leishmania* (GSD) and Leiscan (HIPRA). Both assays are based on crude immunodominant *L. infantum* antigens. However, they differ in their detection systems. In INgezim *Leishmania* assay, an HRP-conjugated monoclonal anti-canine IgG antibody is employed, whereas in Leiscan, HRP-labelled protein A is used for IgG detection (Páez et al., 2025). Despite these methodological differences, all ELISA assays produced both false-positive and false-negative results. Notably, the LeishmaCHECK Ab ELISA generated the lowest proportion of false-positive reactions. Direct comparison among LeishmaCHECK Ab ELISA, Leiscan, and IFAT further confirmed this observation.

The present study demonstrated that both LeishmaCHECK Ab ELISA and Leiscan are highly reliable serological tools for the detection of anti-*Leishmania* antibodies, exhibiting diagnostic performances comparable to IFAT. When IFAT borderline samples (titre 1:80) were excluded, the minimal discrepancies observed between the two ELISA assays and the excellent agreement values (Cohen's kappa > 0.93) indicated that they may be used interchangeably or complementarily in clinical screening protocols.

Consistent with previous findings (Santoro and Vellusi, 2015), both Leiscan and LeishmaCHECK Ab ELISA failed to identify a limited number of positive samples with IFAT titres of $\leq$ 1:160, 1:320, and $\geq$ 1:640, resulting in false-negative classifications. Conversely, inclusion of IFAT borderline samples led to a slight reduction in specificity for both ELISA platforms, attributable to a modest increase in false-positive results. Nevertheless, both assays maintained excellent performance compared with IFAT, with sensitivities exceeding 94%, overall accuracies above 95%, and excellent inter-test agreement (Cohen's kappa > 0.91). These results highlight the robustness and reliability of both ELISA systems, even when applied to clinically ambiguous cases or samples characterized by low antibody titres.

In CanL, the humoral response is typically associated with high antibody titres, whereas low antibody levels are characteristic of subclinical infections or dogs that have been exposed but remain uninfected (Travi et al., 2018). Elevated anti-K39 antibody levels may indicate an active VL or disseminated infection (Reiter-Owona et al., 2016). The detection of false positives and false negatives complicates the establishment of a precise correlation between IFAT antibody titres and ELISA results. Despite this issue, a strong correlation between LeishmaCHECK Ab ELISA results and Leiscan-predicted IFAT titres (coefficient of determination R^2 of 0.979) was determined, supporting the use of LeishmaCHECK Ab ELISA also as a reliable semi-quantitative assay for the serological screening of canine leishmaniosis.

Unlike Leiscan, the INgezim *Leishmania* kit exhibited more pronounced performance variability depending on the reference method used for comparison. When IFAT borderline samples were excluded, INgezim *Leishmania* assay showed substantially lower diagnostic performance, with a sensitivity of 66.7% and an overall accuracy of 79.3%, achieving only moderate agreement with IFAT (Cohen's kappa = 0.588). In contrast, under the same conditions, the LeishmaCHECK Ab ELISA demonstrated superior diagnostic performance, reaching a sensitivity of 76.2% and a specificity of 100%, together with good concordance with IFAT. These findings indicate that, although the INgezim *Leishmania* kit maintains acceptable specificity, its higher proportion of false-negative results limits its sensitivity as a screening tool when compared with the LeishmaCHECK Ab ELISA. The inclusion of

Table 6
Comparison of the results of INgezim *Leishmania* ELISA against IFAT and LeishmaCHECK Ab ELISA under different approaches to borderline IFAT samples (1:80).

Approach			IFAT			RSe (%)	RSp (%)	Ac (%)	PPV (%)	NPV (%)	Cohen's κ	LeishmaCHECK Ab ELISA			RSe (%)	RSp (%)	Ac (%)	PPV (%)	NPV (%)	Cohen's κ
			Pos	Neg	Total							Pos	Neg	Total						
# 1	INgezim *Leishmania*	Pos	28[a]	3[b]	31	66.7	92.5	79.3	90.3	72.5	0.588	28[a]	3[b]	31	87.5	94.0	91.5	90.3	92.2	0.819
		Neg	14[c]	37[d]	51							4[c]	47[d]	51						
		Total	42	40	82							32	50	82						
	LeishmaCHECK Ab ELISA	Pos	32[a]	0[b]	32	76.2	100	87.8	100	80.0	0.757									
		Neg	10[c]	40[d]	50															
		Total	42	40	82															
# 2	INgezim *Leishmania*	Pos	28[a]	3[b]	31	66.7	92.9	79.8	90.3	73.5	0.595	28[a]	3[b]	31	87.5	94.2	91.7	90.3	92.5	0.822
		Neg	14[c]	39[d]	53							4[c]	49[d]	53						
		Total	42	41	84							32	52	84						
	LeishmaCHECK Ab ELISA	Pos	32[a]	0[b]	32	76.2	100	88.1	100	80.8	0.762									
		Neg	10[c]	42[d]	52															
		Total	42	42	84															

Notes: In approach #1, borderline IFAT samples were excluded from the analysis, whereas in approach #2 they were included among negatives. Cohen's κ values of 0.4–0.6 indicate moderate agreement, values of 0.6–0.8 indicate good agreement, and values > 0.9 indicate excellent agreement.
Abbreviations: IFAT, Fluo *Leishmania* kit (Agrolabo S.p.A.); GSD, Gold Standard Diagnostics; Pos, positive; Neg, negative; RSe, relative sensitivity; RSp, relative specificity; Ac, accuracy.
[a] True positives.
[b] False positives.
[c] False negatives.
[d] True negatives.

IFAT borderline samples did not adversely affect the diagnostic performance of either assay. On the contrary, both ELISA platforms displayed remarkable stability across performance metrics. Nevertheless, when compared with IFAT, the INgezim *Leishmania* assay continued to show lower sensitivity than LeishmaCHECK Ab ELISA (66.7 *vs* 76.2%) and a higher number of false-negative results (14 *vs* 10 cases). Of the 14 false-negative samples identified by the INgezim *Leishmania* assay, four were also classified as negative by Leiscan, whereas ten were likewise negative using the LeishmaCHECK Ab ELISA. Notably, four of these ten samples were obtained from dogs undergoing anti-*Leishmania* treatment, a factor that may have influenced antibody detection. Overall, these findings confirm that, while both assays exhibit high specificity, the LeishmaCHECK Ab ELISA provides a more sensitive and robust diagnostic approach, regardless of whether IFAT borderline samples are included in the analysis.

The superior diagnostic performance observed for LeishmaCHECK Ab ELISA may be partially explained by the characteristics of the recombinant antigens selected for assay development. ELISA-based immunoassay performance largely depends on antigen quality. In this study, we used the recombinant fusion protein K9-K26-K39 together with LACK and K39, individually or in combination. LACK is a conserved *Leishmania* protein and a component of the NeoLeish® vaccine, representing an immunological target against *L. infantum* (Alonso et al., 2023). Kinesin-derived antigens K9, K26, and K39 have been widely used for VL serodiagnosis in humans and dogs (Boarino et al., 2005) and are involved in essential cellular processes in *Leishmania* (Gerald et al., 2007). Among these, K26 is an amastigote-upregulated hydrophilic protein containing 11 repeats of a 14-amino acid motif (Bhatia et al., 1999; Mohapatra et al., 2010; Travi et al., 2018). K39 is also predominantly expressed in amastigotes during VL (Rosati et al., 2003; Alvar et al., 2004; Silva et al., 2021) and includes a conserved 39-amino acid tandem repeat shared within the *L. donovani* complex (Burns et al., 1993; Badaro et al., 1996; Gerald et al., 2007; Dhom-Lemos et al., 2019). These repetitive regions enhance antibody recognition and induce strong humoral responses (Dhom-Lemos et al., 2019; Silva et al., 2021). In addition, K39 showed high sensitivity and specificity in ELISA-based serodiagnosis of human and canine VL (Burns et al., 1993; Braz et al., 2002; Scalone et al., 2002; Rosati et al., 2003; Mohapatra et al., 2010; Maia et al., 2012; Reimão et al., 2020), particularly in symptomatic cases (Badaro et al., 1996; Bhatia et al., 1999; Coelho et al., 2012). However, its sensitivity decreases in asymptomatic infections, as also observed for IFAT (Mettler et al., 2005). Based on its overall superior diagnostic performance, K39 was ultimately selected as the antigen of choice for the present study.

Compared to IFAT, the LeishmaCHECK Ab ELISA produced only one false-positive result. While some studies have reported reactivity of antibodies against *Ehrlichia canis* and *Babesia canis* with *Leishmania* spp. in common serological tests (Mancianti et al., 1996; Mettler et al., 2005; Ferreira et al., 2007), others found no cross-reactivity in IFAT, ELISA or rK39-based rapid tests in co-infections with these tick-borne pathogens (Krawczak et al., 2015). Consistently, our analysis of *E. canis*-positive samples showed no cross-reactivity with the *Leishmania* K39 antigen. Moreover, the LeishmaCHECK Ab ELISA does not differentiate between infections caused by *L. infantum* and the closely related *L. donovani*, both members of the *L. donovani* complex. However, *L. donovani* predominantly infects humans, whereas *L. infantum* is more pathogenic in dogs and is the primary agent of CanL (Konno et al., 2022).

False-negative ELISA results were defined as samples with IFAT titres > 1:80, consistent with previous studies (Anfossi et al., 2018; Stensvold et al., 2019; Mahachi et al., 2024). Among the positive samples included in this study, the LeishmaCHECK Ab ELISA yielded 15 false-negative results, with IFAT titres ranging from 1:80–1:160 to > 1:640. Given their limited frequency, these discordant findings are unlikely to reflect a systematic limitation of the assay. Rather, individual variability in antibody specificity or binding affinity may contribute to this discrepancy. Notably, four samples (no. 17, no. 72, no. 124, and no. 132)

yielded false-negative results not only with LeishmaCHECK Ab ELISA but also with the Leiscan and INgezim *Leishmania* assays.

Several explanations may account for false-negative serological results. The diagnostic performance of serological assays is influenced by the infecting *Leishmania* species, host immune response and assay sensitivity (Romero et al., 2005; Reiter-Owona et al., 2016). In cross-sectional studies such as the present one, some infected animals may not yet have undergone seroconversion at the time of sampling. Consequently, false-negative results may occur during the early stages of infection, when antibody levels remain below the detection threshold of serological assays. In the absence of parasitological confirmation and longitudinal follow-up, the true infection status of individual dogs cannot be established with complete certainty (Paltrinieri et al., 2010).

In addition, antibody levels may decline following successful treatment. Studies in both dogs and humans with VL have documented reductions in anti-*Leishmania* antibodies, including anti-K39 antibodies, after clinically effective therapy (Kumar et al., 2001; Reiter-Owona et al., 2016; Solano-Gallego et al., 2016). Clinical information was available for six of the fifteen false-negative dogs. Four animals (no. 191, no. 193, no. 195, and no. 196) received anti-*Leishmania* treatment, one dog (no. 194) was vaccinated, and one (no. 197) was asymptomatic. Importantly, all six samples were also negative with the INgezim *Leishmania* ELISA despite IFAT titres ranging from 1:80–1:160 to > 1:640. These findings support the hypothesis that reduced antibody detectability, rather than assay failure, may explain at least some of the discordant results.

The lower sensitivity of serological assays in asymptomatic infections should also be considered. ELISAs based on total, soluble or recombinant *Leishmania* antigens frequently fail to identify a proportion of asymptomatic infected dogs (Carvalho et al., 2002; Porrozzi et al., 2007; Rosenzweig et al., 2008; Coelho et al., 2012). Although K39-based ELISAs generally achieve high sensitivity in dogs with established clinical disease, they may yield negative results in asymptomatic animals with parasitologically confirmed infection. Similar observations have been reported in humans, where anti-K39 antibodies are most strongly associated with active VL or with subclinical infections that subsequently progress to overt disease (Badaro et al., 1996; Alvar et al., 2004).

A further consideration relates to the clinical form of leishmaniasis. Serological testing is generally less sensitive in CL than in VL, as antibody titres are typically lower due to the predominance of a cell-mediated (Th1) immune response over a humoral (Th2) response (del Giudice et al., 1998; Lombardo et al., 2014; Reiter-Owona et al., 2016; Villanueva-Saz et al., 2025). Consistent with this, most CL samples may result negative by K39-based ELISA despite detectable IFAT titres (Reiter-Owona et al., 2016).

Finally, a prozone (Hook) effect cannot be completely excluded. In VL patients with strong humoral responses, excess antibodies may interfere with antigen-antibody complex formation, leading to falsely negative serological results (Butch, 2000; Madariaga et al., 2006). Although prozone-like phenomena have been described in several immunoassays (Mathai et al., 2020; Mina et al., 2024), this mechanism was not specifically investigated in the present study. Therefore, its contribution to the observed false-negative results remains speculative and warrants further evaluation through serial dilution testing of discordant samples. In any case, when clinical signs are indicative of disease, negative results should be retested or confirmed using an alternative diagnostic assay. Therefore, serology should be interpreted in combination with clinical and molecular findings.

Moreover, LeishmaCHECK Ab ELISA accuracy was supported by reproducibility analyses, which showed intra- and inter-assay coefficients of variation within acceptable ranges (Lexmond et al., 2011; Conrad, 2023).

The present study was designed as a cross-sectional evaluation of assay performance in a heterogeneous population of dogs representative of routine diagnostic submissions, thereby reflecting real-world clinical conditions. Although this protocol does not allow for a longitudinal assessment of infectious status, and the lack of parasitological confirmation or comprehensive clinical data limits the definitive classification of the infection, the LeishmaCHECK Ab ELISA proved to be a useful tool for serological screening of anti-*Leishmania* antibodies in dogs. Additionally, most samples were analysed under blinded conditions, as recommended to minimize bias in diagnostic evaluations (Ribeiro et al., 2019), with clinical information available for only a limited subset of samples.

However, as samples were derived from routine clinical submissions rather than randomized selection, future prospective studies incorporating randomized sample selection, longitudinal follow-up, and fully blinded testing procedures would provide further evidence regarding the diagnostic accuracy, reproducibility, and field applicability of the LeishmaCHECK Ab ELISA. Although no nonspecific binding was observed, potential interference from drugs or endogenous compounds in inflamed blood cannot be completely excluded and warrants further investigation.

Regarding ELISA kit controls, because positive biological controls are difficult to standardize and may introduce batch-to-batch variability, only commercially available non-infectious reagents were included in the kit. Since the recombinant antigens carried a His-tag and no commercial antibodies against the immobilized molecules were available, an anti-His HRP antibody was selected as a positive control, as previously described (Zentgraf et al., 1995; Lindner et al., 1997; Bornhorst and Falke, 2000; Debeljak et al., 2006; He et al., 2022). Our results confirmed the suitability of using both the PC, consisting of the anti-His HRP antibody, and the NC as sample diluent.

The ELISA kit must be stored at a temperature between 2 °C and 8 °C to preserve its stability from the time of manufacture until use. Accelerated stability testing, typically carried out at 37 °C, is the most appropriate approach for simulating product degradation by increasing the rate of chemical and physical reactions in biological reagents. This method allows the shelf life of the product to be established more quickly (Cannell et al., 1985; Campa et al., 2021). During transport or storage, various unforeseen events may occur, such as breaks in the cold chain, which can negatively affect product quality and performance. Stability studies can be performed at defined temperatures that replicate the environmental conditions of the regions or countries where the product is to be distributed (Mechkovski, 1994; Bajaj et al., 2012). Unlike pharmaceutical preparations and veterinary medicinal products, where established stability testing guidelines are available (González-González et al., 2022; EMA, 2024), there is no dedicated legislative or regulatory framework governing the estimation of shelf life for veterinary diagnostic kits, such as ELISA or immunochromatographic assays. In this study, the Standard Guide ASTM F1980-21 (ASTM International, 2021), which applies the Arrhenius equation to predict medical device degradation under accelerated conditions, was followed to estimate the corresponding real-time shelf life. Our results showed that the kit remains stable for six weeks at 37 °C and can be stored for up to 18 months at 2–8 °C, evidence supported by in-house experience in developing ELISA tests (Ferrero et al., 2025a, 2025b, 2025c, 2025d). The accelerated stability assessment is adequate to support the commercialisation of the developed product and indicates that it remains suitable for use even if the cold chain is interrupted for up to six weeks.

5. Conclusions

The control of leishmaniasis remains challenging in both human and canine populations. The diagnosis of CanL should always integrate clinical history and presenting signs. Within this framework, the LeishmaCHECK Ab ELISA represents a rapid and reliable screening tool, providing accurate qualitative and semi-quantitative results in about one hour, without the need for thermostatic equipment. Positive results are highly suggestive of active infection and should be interpreted in conjunction with clinical and laboratory findings. However, false-

negative outcomes may occur in asymptomatic or subclinically infected animals, in cases of CL or due to the prozone effect. Moreover, pharmacological treatment may reduce anti-K39 antibody levels, potentially affecting test sensitivity. The stable performance also in samples with ambiguous serological status or low antibody titers highlights the diagnostic robustness, while its potential utility for post-treatment monitoring warrants further investigation. Collectively, these findings support the use of LeishmaCHECK Ab ELISA as a practical, robust and reliable alternative to IFAT for routine serological screening of canine leishmaniosis.

Ethical approval

Canine serum or plasma samples were collected with the owners' consent during routine veterinary check-ups and were voluntarily donated for research purposes. The procedures carried out do not qualify as animal experimentation and, therefore, are not governed by Italian Legislative Decree 26/2014 or Directive 2019/63/EU of the European Parliament.

CRediT authorship contribution statement

Irene Ferrero: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing. **Paolo Poletti:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft. **Enrica Giachino:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft. **Alessia Libera Gazzonis:** Validation, Investigation, Visualization, Supervision. **Carolina Allievi:** Validation, Investigation, Visualization, Supervision. **Paola Dall'Ara:** Writing - review & editing, Visualization, Supervision. **Joel Filipe:** Writing - review & editing, Visualization, Supervision.

Statement on the use of AI-assisted technologies

ChatGPT (OpenAI) was used solely to assist in the creation of the graphical abstract for this manuscript, in accordance with the journal's guidelines that permit the use of AI tools to support the creation of explanatory images. No AI-assisted technologies were used in the writing, analysis or interpretation of the manuscript.

Funding

This research received no external funding.

Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Irene Ferrero, Paolo Poletti and Enrica Giachino have conducted and funded the studies and are employees of Agrolabo S.p.A. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to express their gratitude to the veterinary practices and clinics in Italy (Veterinary Center of Lungo Dora Colletta, Turin; Veterinary Center Dr Vassia of Romano Canavese, Turin; Veterinary Center "Amici per sempre", Rome) and in Greece (Attica region) for their cooperation in the specimen collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2026.100418.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary file. Raw data are available from the corresponding author upon request.

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