Comparison of a rapid immunochromatographic test kit with Immunofluorescence Antibody Test (IFAT) for assaying *Leishmania infantum* antibodies in dogs

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Introduction and aim of the study: Canine leishmaniasis (CanL) due to *Leishmania infantum* infection is a life-threatening zoonotic disease. The availability of reliable and rapid in-clinic serological tests could enable an immediate diagnosis in clinically suspected cases. Aim of this study is to compare the rapid immunochromatographic Theratest Leishmania® kit (Tt) with a quantitative reference method, Immunofluorescence Antibody Test (IFAT), for the detection of *L. infantum* antibodies in naturally infected dogs.
Material and Methods: Serum samples were obtained from 40 dogs, including 10 healthy control dogs and 30 dogs with confirmed CanL; the samples were evaluated with both tests. IgG-IFAT titres ≥ 1: 80 were considered positive. The sensitivity, specificity, and positive and negative predictive values were calculated; the concordance between the 2 tests was assessed with the Kappa (K) statistic test with a 95% CI.
Results: IFAT detected anti-*Leishmania infantum* IgG antibodies in 30/40 samples, with titres ranging between 1:80 and 1:5120. Using the qualitative Theratest Leishmania® kit, 31/40 samples tested positive. Compared to IFAT, the sensitivity and specificity of Tt were of 100% and 95%, respectively. The Kappa coefficient of 0.935 demonstrated a very good concordance between the two tests.
Discussion: The immunochromatographic test reliably identified canine sera with anti-*Leishmania infantum* IgG antibody titres ≥ 1:80. Tt requires neither special preparation of the serum nor specialized equipment; it can be used as a field test as it is easy to use and provides rapid results.

Key words: field test, canine leishmaniasis, IgG, IFAT, immunochromatography.

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INTRODUCTION

Canine leishmaniasis (CanL), caused by the protozoan Lei*shmania infantum*, is a potentially fatal zoonotic disease. In infected dogs, the development of the disease is influenced by several factors, including the type of immune response present, the breed, age, concomitant disorders and the level of exposure to the parasite. The clinical symptoms, which are extremely polymorphic, may be generalized (fever, weight loss, lymphomegaly, splenomegaly) or involve a single organ or apparatus (skin, kidney, ocular forms)[1]. The course of the disorder can be self-limiting, severe or fatal[2]. Canine leishmaniasis is endemic in many tropical and subtropical areas of the world. In the Mediterranean basin the average prevalence is of 22%[3,4,5,6] and it has also been reported in some areas of northern Europe[7,8,9]. **In Italy, the disease is present almost throughout the entire country, with a greater prevalence in the coastal regions of central and southern Italy**[5,10-13]. The transmission of *L. infantum* mainly occurs through a vector, the female phlebotomine sandfly, which transmits the parasite when consuming its blood meal; the dog is considered the main reservoir of the parasite[1,13]. In endemic areas the prevalence of infected dogs is much higher than the number of sick subjects[10] and the diagnostic suspicion of CanL is made difficult by the clinical presentation, which varies according to the immune status of the affected subject[1]. Although the clinical diagnosis of CanL can be confirmed by direct methods such as cytological examination and polymerase chain reaction (PCR) on biological tissues, the detection of antibodies is of considerable diagnostic value not only to confirm the disease in suspected clinical cases but also for the detection of asymptomatic infected dogs, which act as reservoirs for the parasite[14].

In Italy, canine leishmaniasis is present almost throughout the entire country, with a greater prevalence in the coastal regions of central and southern Italy.

Rapid, in-clinic tests allow an easy and rapid confirmation of the diagnostic suspicion of CanL, allowing the rapid management of clinical cases and facilitating epidemiological surveillance of the territory.

Quantitative serological tests are an important component in the diagnostic algorithm of canine leishmaniasis. The most widely used tests are Indirect Immunofluorescence Test (IFAT) and Enzyme Immunoassays (ELISA), which are used for both epidemiological studies and clinical diagnostics[15]. Elevated antibody titres are usually associated with a high parasitic load and evident clinical signs and confirm the diagnosis of the disease[15-18]. A limitation of these tests lies in the need for specialized laboratories, experienced staff and dedicated equipment.

Given the importance of this disease for the health of dogs and humans, the possibility of being able to easily and rapidly confirm the suspicion of CanL facilitates the rapid management of clinical cases and also reduces the risk of complications due to delayed therapeutic intervention[19]. To this purpose, in recent years rapid in-clinic tests have been developed in order to facilitate the diagnostic work-up of suspected clinical cases and to facilitate epidemiological surveillance[20-22]. Aim of this study is to evaluate the accuracy of a new immunochromatographic rapid test, using IFAT as the reference methodology.

Samples containing different IFAT antibody titres against *L. infantum* were selected in order to have samples reflecting the CanL cases commonly observed in clinical practice.

MATERIALS AND METHODS

For the evaluation of the immunochromatographic rapid test (Theratest Leishmania®, Bioforlife, Milan, Italy) samples containing different IFAT antibody titres against *L. infantum* were first selected in order to have samples reflecting the CanL cases commonly observed in clinical practice. The blood samples were taken from 40 dogs, age range 2-14, that were presented to the Vet Clinic of the Department of Veterinary Medicine of the University of Milan. Thirty of the 40 dogs originated from, or had travelled to, endemic areas for canine leishmaniasis and blood samples were taken as an integral part of their diagnostic work-up or during follow-up visits; when the samples were taken, 25/30 dogs showed symptoms compatible with CanL, reflecting the different stages of the disease[1], while 5/30 were asymptomatic. In the 30 dogs, the presence of *L. infantum* infection was confirmed with real-time PCR or cytology on samples of lymph nodes, spleen or marrow and IFAT positivity (titre ³ 1:80) for the detection of anti-*Leishmania infantum* antibodies. The control group consisted of 10 clinically healthy dog blood donors resident in a CanL-free area and which had never visited endemic areas. In these subjects the blood samples were collected during the periodic screening of donor dogs and were all negative at both PCR and IFAT for the detection of antigens and antibodies against *L. infantum*. In order to evaluate pos-

sible cross-reactions with antibodies to *Ehrlichia canis* an additional test was performed with the Theratest Leishmania® kit on the serum taken from an additional group of 10 dogs that were negative to PCR and to IFAT for the detection of antigens and antibodies against *L. infantum* but IFAT-positive for the detection of anti-*Ehrlichia canis* antibodies (titre ³ 1:80). In all the dogs the blood samples were taken from the cephalic vein of the forearm, after at least 8 hours of fasting; the blood was collected in tubes without anticoagulant and in tubes containing EDTA (Vacuette®, Preanalitica S.r.l., Caravaggio, Italy). The Tt was performed on samples of serum and of blood in EDTA within 24 hours of collection, while the serum aliquots for IFAT were frozen at -20°C until execution of the test.

Detection of antibodies against *Leishmania infantum* using IFAT

For the test to be considered valid, a red line, marked by the letter C (control), must always appear after 20 minutes of incubation at room temperature. The presence of the two lines (C and T) confirms test positivity, regardless of which of the two lines appears first and the intensity of their colour.

IFAT was performed as previously described[14], using a commercial kit (Leishmania-Spot IF®, BioMerieux Marcy L'Etoile, France) consisting of slides with fixed *L. infantum* promastigotes. The serum was diluted with a pH 7.2 buffered saline (PBS), deposited in serial dilutions in the wells present on the slide and incubated in a moist chamber for 30 minutes. The slides were then washed with PBS and incubated with murine anti-dog IgG antibodies conjugated with fluorescine (Sigma Aldrich, Munich, Germany) at 37°C for 30 minutes in a moist chamber. The slides were again washed with PBS, dried and examined under a fluorescence microscope (Zeiss Axiosop®, Germany) at 280 nm. As a negative and positive control, dog sera from a previously tested certainly healthy subject and from a subject diagnosed with CanL were included in each slide. The test was considered positive in the presence of membrane and/or cytoplasmic fluorescence at an antibody titre ≥ 1:80. IFAT for the detection of anti-*Ehrlichia canis* antibodies was performed using a commercial kit (Biopronix®, Agrolabo, Scamagno, Turin, Italy), as described above[15].

Detection of antibodies against *Leishmania infantum* using the Theratest Leishmania® kit

Theratest Leishmania® is a qualitative immunochromatographic diagnostic test for the detection of antibodies against *Leishmania infantum* in dog serum, plasma and whole blood. The test was performed following the manufacturer's instructions. Ten µl of the sample (serum or blood in EDTA) was pipetted into the sample pad hole and 2 drops of buffer provided in the package were immediately added.

The sample and the buffer migrated to the membrane area called the "results window", containing the adhering *L. infantum* antigen. After 20 minutes of incubation at room temperature, a red line, marked by the letter C (control), appeared in all the samples tested; this line must always be present for the test to be considered valid. In the positive samples, an additional red line appeared in the "results window", marked by the letter T (test). The presence of the two lines (C and T) confirms test positivity, regardless of which of the two lines appears first and the intensity of their colour. After 30 minutes from its execution the test can no longer be interpreted.

To establish the reproducibility of Tt, three IFAT-positive samples (titres 1:320; 1:2560; 1:5120) and one IFAT-negative sample were repeated 5 times, on the same day. In order to assess the impact of preservation on the interpretation of the sample, the same 3 positive samples were evaluated on fresh serum, frozen at -20°C for 7 days. The impact of haemolysis on Tt results was evaluated again on the same 3 serum samples, to which 1 g/L of haemoglobin was added. The different interpretation of the whole blood test was evaluated by performing duplicate tests on serum and blood in EDTA on 5 samples.

Statistical Analysis

Normality of the distribution of IFAT data was evaluated with the D'Agostino-Pearson test. For a general evaluation of the performance of the immunochromatographic method an assessment was made of the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), using the 2x2 table based on the concordance between Tt and IFAT results. In order to evaluate the concordance not expected by chance between IFAT and Tt results - for the detection of antibodies against *L. infantum* in canine serum -, the unweighted statistical K (k) was used, with a 95% CI referred to a 22% prevalence value, which is the average prevalence in the Mediterranean basin[1,9,18].

Based on the value of k, the concordance between the two tests was evaluated using the following guideline: 0: concordance not greater than expected by chance; 0.20 <: poor concordance; 0.21-0.40: weak concordance; 0.41-0.60: moderate concordance; 0.61-0.80: good concordance; 0.81-1.00: excellent concordance. The statis-

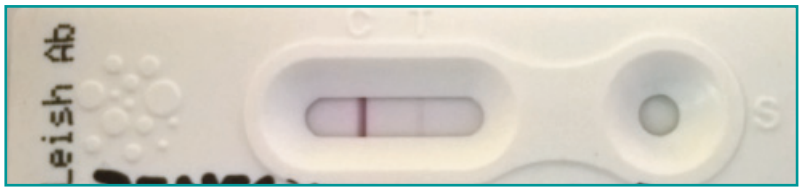

Figure 1 - Assessment of positivity for the detection of antibodies against *Leishmania infantum* using the immunochromatographic Theratest Leishmania® kit: IFAT-positive sample with titre 1:80 that tested positive to the immunochromatographic test with a very faint colour of the positive test line.

Using IFAT as reference, the immunochromatographic test showed a sensitivity of 100% (95% CI - 88.43%-100.00%), a specificity of 95% (95% CI - 75.13%-99.87%) and a K value of 0.935, indicating an excellent concordance between the two tests.

tical analysis was performed using the MedCalc program for Windows v. 9.2.1 (Mariakerke, Belgium). In all the analyses, a value of $P< 0.05$ was considered statistically significant.

RESULTS

With IFAT, 30/40 samples tested positive for the detection of antibodies against *L. infantum*, with an antibody titre ranging between 1:80 and 1:5120 (5-1:80; 6-1:160; 3-1:320; 6-1:640; 3-1:1280; 3-1:2560; 4-1:5120); with the immuno-chromatographic rapid test, 31/40 samples tested positive. Only one sample, taken from an asymptomatic subject, showed a discordant result, resulting negative to IFAT and positive to Tt. All Tt results were readable and interpretable; however, in 3 IFAT-positive samples with titres of 1:80 and 1:160, the red line of positivity in the "results window" appeared very faint and not as evident as the lines of the other samples, even with identical IFAT-positive titres (Figure 1). Using IFAT as reference, the immunochromatographic test presented a sensitivity of 100% (95% CI - 88.43%-100.00%) and a specificity of 95% (95% CI - 75.13%-99.87%), with a positive predictive value (PPV) of 84.94% (95% CI - 54.86-98.20) and a negative predictive value (NPV) of 100% (95% CI - 90.50-100.00). The value of the statistical kappa (k), calculated to assess the concordance beyond chance of the two methods in their capacity to detect antibodies against leishmania in the samples analysed, was of 0.935, indicating an excellent concordance between the two tests. None of the samples which tested positive for antibodies against *E. canis* and negative to IFAT for antibodies against *L. infantum* resulted positive with the immunochromatographic test. Tt showed a good reproducibility; the samples that were repeated 5 times always presented the same result (Figure 2), even if the sample with IFAT titre 1:2560 presented a marked variability in the intensity of the positive line (Figure 3). The results of

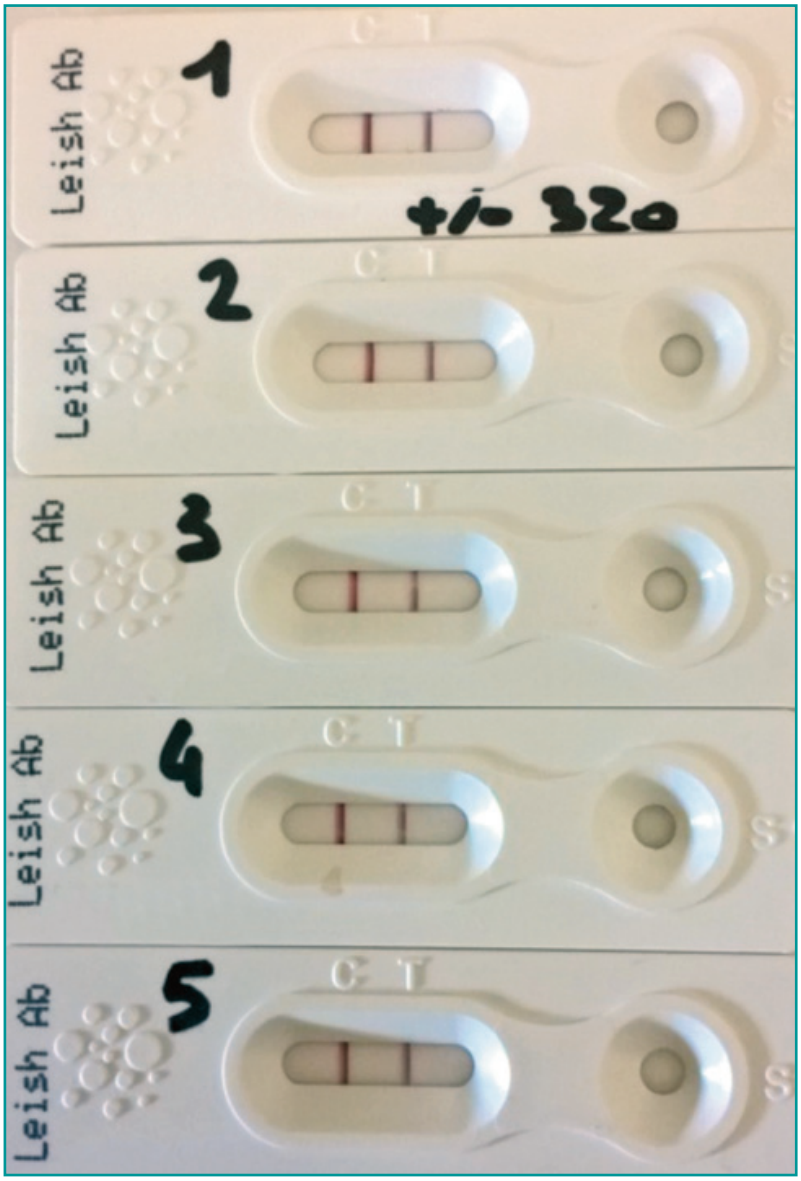

Figure 2 - Reproducibility test of the immunochromatographic Theratest Leishmania® kit for the detection of antibodies against *Leishmania infantum*: the same sample, tested 5 times on the same day, always showed the same positive result.

the immunochromatographic test remained unchanged also in the serum samples frozen at -20°C for 7 days. The haemolysis induced in 3 of the samples did not alter the result obtained. The interpretation of the results was also possible in whole blood samples, although the red line of the test appeared less distinct compared to the one in the serum samples (Figure 4).

DISCUSSION

The monitoring and epidemiological surveillance of canine leishmaniasis is of particular importance in both veterinary and human medicine, as the disease is a zoonosis with important public health implications and the dog is the main reservoir. The diagnostic approach to the disease involves the detection of anti-leishmania antibodies in the serum, associated with the identification of the parasite or of its genome in tissues. Serology is used to diagnose sick subjects, to screen blood-donor dogs, to identify clinically-healthy infected subjects living or coming from endemic areas or to carry out large-scale epidemiological studies[23,24,25]. Although no "gold standard" test with 100% sensitivity and 100% specificity is available for the diagnosis of CanL[18], quantitative tests such as IFAT and ELISA[19] are the most reliable tests for the detection of specific anti-leishmania antibodies (IgG). IFAT is considered the reference test for laboratory diagnostics[19] while ELISA is of variable sensitivity and specificity, depending on the different antigen used[23]. Apart from confirming the infection, quantitative methods allow to calculate the antibody titre (endpoint titration), the trend of which can be monitored during the course of the disease; the titre represents one of the parameters indicative of improvement or aggravation of the clinical picture[1,17,21]. Moreover, antibody titration allows the differentiation between diseased subjects, clinically symptomatic and with a high antibody titre, and infected ones, which generally present a low antibody titre[16]. Despite these undoubted advantages, these tests are not always easily performed in clinical practice as they require specialized laboratories and experienced staff for their execution and interpretation as well as long lead times. In clinical prac-

Theratest Leishmania® for the detection of anti-leishmania antibodies can be performed on serum or whole blood; it is easy to read, it does not require any special equipment or sample preparation and it can be stored at room temperature.

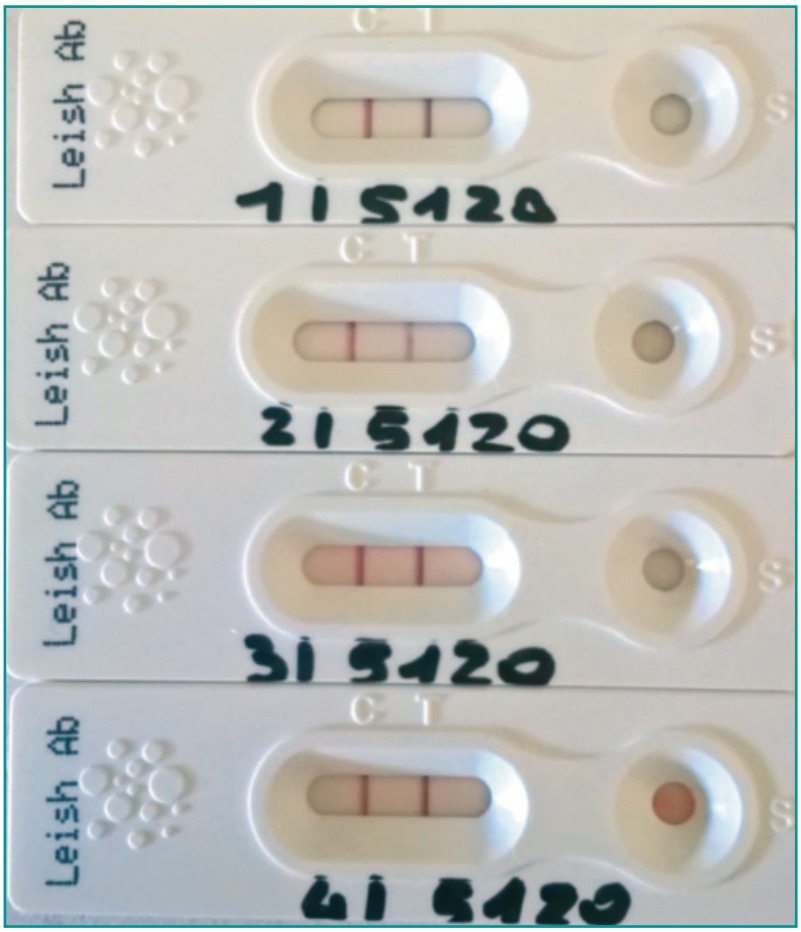

Figure 3 - Reproducibility test of the immunochromatographic Theratest Leishmania® kit for the detection of antibodies against *Leishmania infantum*: the reproducibility test on an IFAT-positive sample with titre 1:2560 showed a marked variability in the intensity of the positive test line.

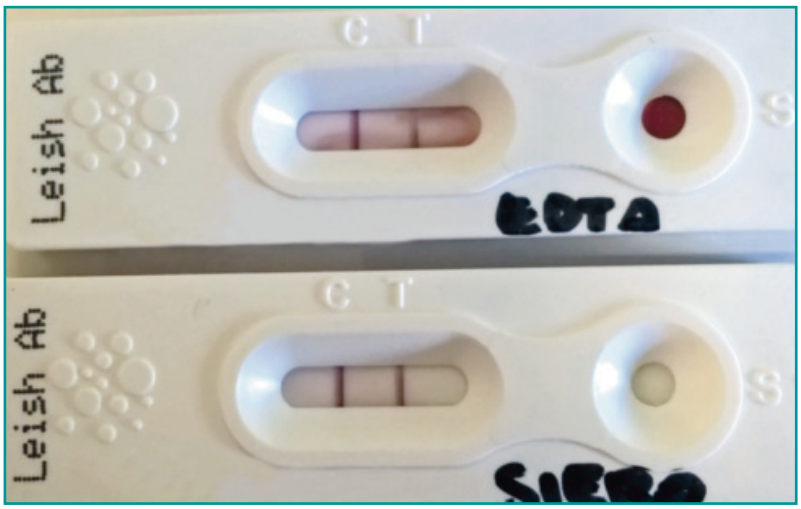

Figure 4 - Assessment of positivity for the detection of antibodies against *Leishmania infantum* using the immunochromatographic Theratest Leishmania® kit: test performed on the same IFAT-positive subject, on whole blood and serum. The reading of the result is possible with both samples.

tice, the availability of a rapid, easy-to-use test, which does not require any particular laboratory skills and can be performed in the clinic can facilitate and speed-up the diagnostic approach to the disease, as evidenced by the numerous rapid tests that have been marketed in recent years[20, 21, 22, 26]. Theratest Leishmania® for the detection of anti-leishmania antibodies can be performed on serum or whole blood; it is easy to read, it does not require any special equipment or sample preparation and it can be stored at room temperature. In less than 30 minutes the diagnostic suspicion can be confirmed, making the test ideal for in-clinic and field use. No cross-reaction with sera positive for *E. canis* has been observed, a co-infection which is not rare in the course of canine leishmaniasis. The statistical k value shows a very high concordance between the results obtained with the immunochromatographic test and IFAT. In samples with an IFAT antibody titre ≥ 1:80, the immunochromatographic test can detect anti-*Leishmania infantum* antibodies in dog sera with a specificity of 95% and a sensitivity of 100%, having never given false negative results. It should however be underlined that the occasional and random appearance of a very faint positive test line, with no correlation with the IFAT antibody titre, might lead to an incorrect interpretation, with a consequent false-negative result. For this reason, the authors emphasize the importance of a careful evaluation of the "results window", thus allowing to consider positive also samples with an extremely faint positive test line. In the presence of CanL the sensitivity of a test is critical to control the seroprevalence of the infection, while specificity is particularly important for the identification of sick patients[22]. In the presence of possible false positive results (PPV 84.94%) the test results should be considered in the light of an epidemiological and clinical-pathological context compatible with the presence of the disease. Positive results in subjects with clinical symptoms and laboratory alterations that do not reflect the typical picture of the disease should always induce a cautious interpretation of the results obtained.

Samples with an extremely faint positive test line should be considered positive.

The main limitation of the study is that it has not evaluated other possible cross-reactions of the immunochromatographic test with other parasitic diseases that may be present as co-infections in the course of CanL, such as babesiosis, rickettsiosis or anaplasmosis.

Test results should always be considered in the light of an epidemiological and clinical-pathological context compatible with the presence of the disease.

In conclusion, the immunochromatographic Theratest Leishmania® kit has shown an excellent diagnostic performance and has proved to be a valuable diagnostic aid in clinical practice.

The authors do however point out that for the proper management of leishmania patients any positive result obtained with a qualitative test must be then confirmed using a quantitative test that can confirm the diagnosis and provide the endpoint antibody titre.

KEY POINTS

- Canine leishmaniasis is a parasitic and zoonotic disease that is widespread in Italy and in the Mediterranean basin.
- The availability of an in-clinic diagnostic kit is a valid aid for the rapid confirmation of suspected clinical cases and for the evaluation of seroconversion in asymptomatic subjects.
- The immunochromatographic Theratest Leishmania® showed excellent concordance with Indirect Immunofluorescence Test (IFAT) (K=0.935).
- The immunochromatographic rapid test showed 100% sensitivity and 95% specificity in detecting antibodies against leishmania in the sera of dogs with canine leishmaniasis.
- In cases of suspected canine leishmaniasis the result of a qualitative test must always be confirmed with the detection of the antibody titre by means of a quantitative method.

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