Short communication

Evaluation of an ELISA rapid device for the serological diagnosis of Leishmania infantum infection in dog as compared with immunofluorescence assay and Western blot

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Abstract

In this study we compared a commercial enzyme linked immunosorbent assay (ELISA) rapid test (Snap CLATK Canine Leishmania Antibody Test Kit, IDEXX-Snap) with indirect immunofluorescence assay (IFA) and Western blot (WB) for the detection of Leishmania infantum antibodies in dogs. In total sera from 234 dogs were collected: 59 positives and 51 doubtful sera (IFA 1:40–1:80) from an L. infantum endemic area and 124 negative sera from a non-endemic area were tested. To evaluate the Snap CLATK’s performances on whole blood, blood in EDTA and sera from 37 dogs were tested in parallel with Snap CLATK.

Snap CLATK sensitivity and specificity compared to IFA were 91.1% and 99.2%, while compared to WB were 93.4% and 98.3%, respectively. When IFA doubtful sera (titers of 1:40 or 1:80) were tested Snap CLATK, using WB as reference, sensitivity and specificity were 90.9% and 100%, respectively. Moreover, a complete concordance was observed when Snap CLATK rapid assay was carried out on whole blood or sera from 37 dogs. The Snap CLATK has demonstrated simplicity and performance and can be considered a quick and reliable alternative for the diagnosis of L. infantum infection in dogs.

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1. Introduction

Visceral leishmaniosis is a disease of dogs and humans caused by Leishmania infantum and transmitted by sandflies of the Phlebotomus and Lutzomyia genera, respectively in the Old and New Worlds. The domestic dog (Canis familiaris) is the reservoir of this parasite which is endemic in the Middle East, in many tropical and subtropical areas of the world and in Mediterranean areas of Europe where the seroprevalences range from 1.7% to 48% (Ozbel et al., 1995; Gradoni, 1999).

Human visceral leishmaniosis (HVL) is one of the top 10 diseases of concern to the Office International des Epizooties (OIE) and is rapidly spreading, often in association with HIV infection, especially in sub-Saharan Africa and South America. In 1996 it was estimated that some 12 million people were infected and 350 millions were at risk of acquiring infection (http://www.who.int/docstore/water_sanitation_health/vectcontrol/ch07.htm).

Recently, canine leishmaniosis (CanL) has spread in continental areas of the USA (Enserink, 2000) and
continental regions of North Italy (Ferroglio et al., 2005) where CanL was previously non-endemic.

Accurate diagnosis of CanL is necessary for the management of clinical canine cases and, considering that dogs are the reservoir also for human infection, it is also important on a public health approach. CanL diagnosis is generally based on clinical signs and confirmed by serological or parasitological tests. Parasitological tests are usually invasive or labor-intensive and time-consuming, so the diagnosis is often based on serological tests which are easier and less expensive (Ferrer et al., 1995). Even if immunofluorescent assay (IFA) is the most used test for both epidemiological studies and clinical practice (Gradoni, 1999, 2002; OIE, 2000), a lot of enzyme linked immunosorbent assay (ELISA) and direct agglutination tests have been developed and are available for diagnostic laboratories (Scalone et al., 2002; Boarino et al., 2005). IFA response is considered unequivocal for serum titres <1:40 (negative) or ≥1:160 (positive), but it is ambiguous for titres of 1:80 and 1:40 which are evaluated as doubtful (Ferroglio et al., 2002). Recently Western blot (WB) has proved more sensitive than IFA and many authors have suggested its use in the diagnosis of CanL (Aisa et al., 2001; Reithinger et al., 2002; Mohebali et al., 2004). A rapid sensitive and specific diagnostic test would be useful as part of the diagnostic protocol for in-clinic diagnosis, but it is also extremely valuable in mass screening surveys and intervention campaigns in endemic areas. To control the infection it is really important to have a rapid in-clinic test to identify asymptomatic dogs which can be as infective to the vectors as symptomatic ones (Alvar et al., 1994). The rapid and cost-effective detection of infected dogs allows a quick implementation of control measures and can greatly reduce the risk of infection transmission.

Considering the above mentioned points, we deemed it interesting to compare a commercial rapid ELISA test (Snap™ Canine Leishmaniasis Antibody Test Kit, IDEXX-Snap™ CLATK, http://idexx.com) with IFA and WB for the serological diagnosis of CanL and to evaluate its sensitivity and specificity in whole blood and serum samples.

2. Materials and methods

Samples: blood samples were collected from January to April 2003 in 2 veterinary clinics in an endemic area (Liguria, Italy) and a clinic from a non-endemic area (High Aosta Valley, Italy) from the radial vein from 234 dogs: 59 positive sera (IFA ≥ 1:160) and 51 doubtful sera (IFA 1:40–1:80) from dogs living in an endemic area of L. infantum and 124 negative sera (IFA < 1:40) from dogs living in a non-endemic area. Blood was allowed to clot, centrifuged and the serum was separated, frozen and stored at −20°C until testing.

Fresh blood and sera were also collected from another 37 dogs, 24 dogs from non-endemic areas and 13 IFA and WB seropositive dogs, and tested within 1 h with the Snap™ CLATK, while sera aliquots were frozen and stored at −20°C until IFA and WB testing.

IFA was carried out according to the procedure reported in Mancianti and Meciani (1988). Because of limited volume of some aliquots only 175 serum samples have been tested in IFA.

WB was carried out according to Ferroglio et al. (2002). Briefly late-log phase promastigotes of L. infantum (IPT-1 Roma) were lysed by freezing and thawing cycles. Two hundred micrograms (1 mg/1 ml) of lysate were boiled for 5 min in a reducing sample buffer and run using a Bio-Rad Mini-PROTEAN II apparatus (Bio-Rad, Hercules, CA, USA) on a 12% polyacrylamide gel under denaturising condition (SDS-PAGE). Molecular weight markers (Biotinylated SDS-PAGE standards, Bio-Rad, Hercules, CA, USA) were used in a separate lane.

Fractionated proteins were electroblotted (350 mA, 1 h) onto nitrocellulose sheets that were saturated (3 h) with 5% Tris buffer saline (TBS)–bovine serum albumin (BSA). Dogs’ sera were diluted 1:10 in TBS and incubated overnight in a Multiscreen Apparatus (Bio-Rad, Hercules, CA, USA). After washing three times with 0.05% Tween 20-TBS, the secondary antibody (horseradish peroxidase labelled rabbit anti-dog IgG, 1:6000; Sigma) was incubated for 1 h. Nitrocellulose membrane was washed twice and anti-L. infantum antibodies were revealed on a Hyperfilm ECL (ECL System, Amersham, Uppsala, Sweden) using a chemiluminescent Western blot detection kit (ECL System, Amersham, Uppsala, Sweden). Samples were considered positive when at least two bands of 169, 115, 66 or 33 kDa were found (Ferroglio et al., 2002).

Because of lack of enough serum aliquots 183 serum samples have been tested in WB as previously described.

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Snap<sup>R</sup> CLATK: the Snap<sup>R</sup> CLATK Canine Leishmania Antibody Test Kit was carried out according to the manufacturer’s instructions. It is a rapid ELISA for detection of antibody to <i>L. infantum</i> in canine whole blood, serum or plasma. The antigen used in the Snap<sup>R</sup> CLATK is derived from <i>L. infantum</i> promastigotes prepared by sonic disruption, filtration, and diethylaminoethyl (DEAE) column purification.

This test uses IDEXX’s proprietary Snap<sup>R</sup> device format, which provides reversible chromatographic flow of the sample and automatic, sequential flow of wash and enzyme substrate. Color develops in spots on the flow matrix to provide the test results, which are visually read.

Statistical analysis: agreement (k) among the tests and the evaluation of their sensitivity and specificity were calculated using Win Episcope (ver. 2.0) program.

### 3. Results

The results of IFA, WB and Snap<sup>R</sup> CLATK comparison have been summarized in Tables 1–3. The first two tables show the comparative data between Snap<sup>R</sup> CLATK and IFA (Table 1) and between Snap<sup>R</sup> CLATK and WB (Table 2). In Table 3 the results of the comparison between Snap<sup>R</sup> CLATK and WB on IFA doubtful sera are reported.

Using IFA as the reference standard (Table 1), Snap<sup>R</sup> CLATK sensitivity and specificity were 91.1% (95% CI = 83.6–98.5%) and 99.2% (95% CI = 97.5–100.0%), respectively, with a high degree of agreement (k = 0.92). Compared to WB (Table 2), the Snap<sup>R</sup> CLATK test sensitivity and specificity were 93.4% (95% CI = 87.2–99.7%) and 98.4% (95% CI = 96.1–100.0%), respectively. Also in this case the agreement (k = 0.92) was very good.

When IFA doubtful sera (1:40 and 1:80 titers) were tested by WB and Snap<sup>R</sup> CLATK, Snap<sup>R</sup> CLATK sensitivity (100.0%) (95% CI = 73.9–100.0%), specificity (90.9%) (95% CI = 100.0–100.0%) and agreement (k = 0.94) were very good compared to WB results (Table 3). Finally, we did not observe differences (k = 1) when whole blood in EDTA and sera were tested within 1 h from blood collection with Snap<sup>R</sup> CLATK. The comparison testing was performed on 24 IFA and WB negative dogs from non-endemic areas and 13 IFA and WB seropositive dogs.

### 4. Discussion

Dog is the <i>L. infantum</i> main reservoir host, so monitoring and surveying in this species are very important in order to prevent spreading of the disease (Dye et al., 1993; Ozbel et al., 1995; Dye, 1996; Raj et al., 1999). Rapid and reliable indirect diagnostic assays are necessary to detect infections in dog, because of the large variability of clinical symptoms and the presence of asymptomatic but infective dogs (Dye et al., 1993; Alvar et al., 1994).

Until a few years ago the definitive diagnosis of CanL depended upon the demonstration of <i>Leishmania</i> spp. amastigotes in bone marrow or biopsy material (spleen, lymph nodes, liver). These procedures are invasive, and thus are acceptable only in case of clinical suspicion of the disease. In addition, this technique has poor sensitivity so it cannot be considered a gold standard for CanL diagnosis (Gradoni, 1999).

In the last decade many PCR protocols to detect <i>L. infantum</i> DNA has been developed for the direct diagnosis of CanL, and PCR has been shown to be a sensitive and highly specific technique for the detection of infection. However, PCR is neither routinely used in

Table 2

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<th>Snap&lt;sup&gt;R&lt;/sup&gt; CLATK</th>
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<tr>
<td></td>
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<td>Negative</td>
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<tr>
<td>Snap&lt;sup&gt;R&lt;/sup&gt; CLATK Positive</td>
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<td>2</td>
</tr>
<tr>
<td>Snap&lt;sup&gt;R&lt;/sup&gt; CLATK Negative</td>
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Table 3

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<th>WB (IFA 1:40–1:80)</th>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
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<td>0</td>
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<tr>
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clinical medicine nor in wide field surveys in many countries where the infection is endemic. Moreover, some researches suggest that PCR is less sensitive in detecting asymptomatic dogs (Ashford et al., 1995; Quinnell et al., 2001; Reithinger et al., 2002) or, conversely, too sensitive and therefore with a low positive predictive value in detecting disease (Lachaud et al., 2002).

Serological methods are usually used for both clinical and mass screening diagnosis of CanL. Although IFA represents the serological reference test its use is limited by the subjective interpretation of results often not repeatable among different laboratories (Gradoni, 1999) and by the different cut off used in each laboratory.

Recently, many authors have suggested the use of WB in the diagnosis of CanL (Aisa et al., 1998; Gradoni et al., 1999). This technique requires a good technical background and is limited to research labs and is not applicable to routine diagnosis.

During the last decades many rapid diagnostic tests have been developed in order to follow rapid diagnosis and interventions both in humans and dogs (Mancianti et al., 1996; Jelinek et al., 1999; Brandonisio et al., 2002; Edrissian et al., 2003; Mohebali et al., 2004; Zijlstra et al., 2001).

In comparison with other studies where immunochromatographic dipstick tests for *L. infantum* are compared to ELISA and PCR (Reithinger et al., 2002) and to DAT (Mohebali et al., 2004), the agreement between the rapid device and more sophisticated laboratory techniques (IFA and WB) seems to be higher with Snap<sup>®</sup> CLATK than the other rapid device. Sensitivity and specificity for rapid tests, including dipstick, range from 52.9% to 99.2% and from 61% to 87.9%, respectively (Reithinger et al., 2002; Mohebali et al., 2004; Schalling et al., 2004; Mettler et al., 2005). On the contrary, two trials demonstrate high sensitivity and specificity for two different dipstick tests (Otranto et al., 2004; Da Costa et al., 2003). These differences can be explained by the different technologies of the tests or by the different approach in estimating the reference populations.

The complete agreement between the Snap<sup>®</sup> CLATK results from sera and the whole blood samples is likely the result of a washing solution used in Snap<sup>®</sup> ELISA technology. This solution runs on the matrix leaving the device background clean even if the samples are whole blood in anticoagulant.

In conclusion, our results showed that Snap<sup>®</sup> CLATK, compared to the IFA and WB technologies, is a simple and rapid assay with high sensitivity and specificity for the diagnosis of *L. infantum* infection in dog. The possibility of carrying out the test on whole blood and the quick (5 min) response allows for a rapid and affordable diagnosis of CanL in a large number of samples both in clinics or directly on the field during surveillance surveys. Snap<sup>®</sup> CLATK cannot be used as the sole diagnostic tool and should be utilized with other quantitative serological tests (i.e. IFA), direct test (i.e. PCR) and clinical laboratory analysis.

The availability of a reliable rapid diagnostic test can greatly increase the diagnostic capabilities of veterinarians involved in field survey. The diagnosis of asymptomatic untreated dogs can significantly decrease the mean duration of infectiousness reducing the risk of disease transmission from dogs, thus allowing a better control of the infection in dogs and, indirectly, in humans as well.

**References**


