Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay

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Abstract

Canine infections with *Leishmania infantum* are important as a cause of serious disease in the dog and as a reservoir for human visceral leishmaniasis (VL). Accurate diagnosis of canine infections is essential to the veterinary community and for VL surveillance programs. A standardized ELISA using a purified recombinant antigen (rK39) specific to VL was compared to the immunofluorescent antibody test (IFAT) as the standard. The ELISA was developed, optimized and evaluated using sera from 6368 dogs. The standardized ELISA and IFAT results were highly concordant. The timing and pattern of ELISA and IFAT seroconversion in dogs followed prospectively after natural infections were very similar. Antibodies reacting with rK39 were more common in asymptomatic canine infections than reported for subclinical human VL. The rK39 ELISA is a relatively simple and rapid assay for assessing the infection status of dogs, and is an alternative to IFAT, especially when screening large numbers of samples. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania infantum*; Recombinant antigen; Dog; Diagnosis; IFAT; ELISA

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1. Introduction

Dogs are the domestic reservoir for *Leishmania infantum* (synonym: *L. chagasi*) (Maurício et al., 2000), the parasite causing zoonotic visceral leishmaniasis (VL) in both the Old and New Worlds (World Health Organization, 1990). Clinical forms of canine leishmaniasis, characterized by chronic evolution of viscerocutaneous signs, occur in less than 50% of infected dogs (Lanotte et al., 1979). On the other hand, both symptomatic and asymptomatic animals are infective to sandfly vectors (Gradoni et al., 1987; Molina et al., 1994). Furthermore, routine canine leishmaniasis diagnosis is important for monitoring *L. infantum* transmission in a given territory, as the dog may serve as a sentinel host for the assessment of leishmaniasis risk to human susceptible populations, such as HIV-infected individuals (Gradoni et al., 1996).

Given the frequent lack of signs in dogs and the difficulty of direct detection of the organism, rapid and accurate indirect diagnosis of canine infection represents an essential tool in VL surveillance programs. The principal serodiagnostic tests include immunofluorescent antibody test (IFAT), direct agglutination, enzyme-linked immunosorbent assay (ELISA), dot-ELISA, immunochromatography and immunoblot. Among them, IFAT appears to be recognized as the gold standard, being the most sensitive and specific test (see Gradoni (1999) for a review). These conventional tests employ crude antigen preparations, either as whole promastigotes or their soluble extracts, which limit standardization of the assays and reproducibility of results.

A recombinant (r) antigen suitable for VL diagnosis is K39, a repetitive immunodominant epitope in a kinesin-related protein that is highly conserved among viscerotropic *Leishmania* species (Burns et al., 1993). It has been demonstrated in a number of endemic sites that rK39 ELISA is sensitive and specific for serodiagnosis and prognosis of human VL (Qu et al., 1994; Singh et al., 1995; Badaro et al., 1996; Houghton et al., 1998; Zijlstra et al., 1998). Preliminary studies have shown that this test was suitable also for the serodiagnosis of canine leishmaniasis (Ozensoy et al., 1998; Zerpa et al., 2000). Here, we report on the development and standardization of a rK39 ELISA, which was validated with a large number of canine sera through a multi-center study involving five regions of Italy.

2. Materials and methods

2.1. Study design and dogs

Preliminary antigen evaluation and determination of test conditions using a prototype rK39 ELISA was performed with serum samples from the following dog groups: (i) 209 dogs from an endemic area in southern Italy, in which a well-established natural *L. infantum* infection was demonstrated by standard parasitological techniques (all were part of a population screened for drug clinical trials (Oliva et al., 1995, 1998) and displayed clinical signs of different degrees of severity); (ii) 81 clinically healthy dogs from the same endemic area and proven to be negative by standard parasitological techniques and IFAT; (iii) 90 normal dogs from the USA; (iv) 62 *Leishmania*-free dogs known to be infected with other parasites.
For standardization of test conditions using field samples, 415 sera were collected from a dog population of northern Sardinia as part of the local VL surveillance program.

To monitor the appearance and trend of anti-rK39 antibodies, four naive beagles (Stefano Morini Co., Reggio Emilia, Italy) were housed in a kennel for stray dogs in a highly endemic focus of disease and exposed to natural *Leishmania* transmission during one sand fly season (from June to September 1997). Serum samples were collected periodically over 13 months.

A standardized laboratory kit ELISA using rK39 (Heska Corporation, Fort Collins, CO; distributed in Italy by A.T.I. s.r.l., Ozzano Emilia, Bologna) was validated in a multi-center study using 5507 serum samples collected as part of the routine sampling of both owned and stray dogs. The samples were collected by veterinary practitioners of five Italian regions endemic for human and canine leishmaniasis caused by *L. infantum*, and sent for diagnosis to the local Institutes for Zooprophylaxis (IZs). The IZs are public institutes for animal health that act as diagnostic reference centers for canine leishmaniasis. Diagnostic evaluation was requested due to suspicion of clinical disease or as part of epidemiological surveys in the VL surveillance programs. Thus, this group of dogs included both clinically ill and healthy animals.

2.2. Standard Leishmania serology and parasitology

IFA T was used as reference serological test following standard protocols (Gradoni and Gramiccia, 2000). The antigen was prepared from promastigotes of the WHO reference strain of *L. infantum* (MHOM/TN/80/IPT1). A titer of 40 or greater was considered as positive at the conditions developed at the Department of Parasitology of Istituto Superiore di Sanità (Pozio et al., 1981). Material from bone marrow and popliteal or pre-scapular lymph node aspirates was smeared on slides and stained with Giemsa’s stain and/or cultured in Evan’s modified Tobie’s medium.

2.3. K39 antigen

The prototype ELISA was developed using a histidine tagged antigen (rK39他的). The pET-17b plasmid (Novagen Inc., Madison, WI) containing the insert for rK39他的 was obtained from Corixa Corporation, Seattle, Washington. The plasmid was transformed into BL21(DE3) *Escherichia coli* via standard methods (Sambrook et al., 1989). The protein rK39他的 consisted of amino acids 658–955 from GenBank accession L07879, with the addition of seven amino acids at the amino-terminus (MHHHHHNN) and 14 amino acids at the carboxy-terminus (RNSAEFAQKRPGHD). Purification from the cell lysate was performed by nickel-chelate chromatography using a Ni-NTA matrix (Qiagen Inc., Valencia, CA) and EDTA elution.

The laboratory kit ELISA was developed using a slightly different antigen (rK39他的), which was identical to that described for the prototype ELISA except that amino acids 2–7 were removed (HHHHNNH). The insert encoding rK39他的 was subcloned into the plasmid λPRcro/T2ori/RSET-B (PCT Publication no. WO 95/24198, 14 September 1995). The plasmid was transformed into HB101 *E. coli* via standard methods (Sambrook et al., 1989). Purification from the cell lysate was performed by anion-exchange chromatography using
a Q-Sepharose high performance column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and NaCl gradient elution.

2.4. ELISA (prototype and laboratory kit formats)

2.4.1. Prototype ELISA

Nunc PolySorp® 96-microwell plates (Nalgene Nunc International, Rochester, NY) were coated overnight at 4 °C with 25 ng per well of rK39His in 100 μl per well of 50 mM carbonate buffer, pH 9.6. The microwells were blocked with StabiCoat (Surmodics, Eden Prairie, MN) and dried. Dog serum was added to the microwells at a final concentration of 1/50 in 4% (v/v) fetal bovine serum/phosphate-buffered saline containing 0.05% (v/v) Tween-20 (FBS/PBS-T) and the plates were incubated 30 min at room temperature. The microwells were washed four times with PBS-T, followed by the addition of peroxidase labeled goat–anti-dog IgG (H+L) (KPL, Gaithersburg, MD) diluted to 100 ng/ml in FBS/PBS-T and incubated 30 min at room temperature. The microwells were washed four times as before, and the bound enzyme detected by incubating 10 min with the TMB Microwell Peroxidase Substrate System (KPL). The reaction was stopped with 2.5N H2SO4 and absorbance was read at 450 nm.

2.4.2. Laboratory kit ELISA

The protocol was identical to the prototype ELISA except the microwells were coated with 2.2 ng rK39acro per well and the peroxidase labeled goat–anti-dog IgG (H + L) was diluted to 22 ng/ml.

2.5. Statistical analysis

To compare ELISA and IFAT results, concordance between the tests was further analyzed using kappa statistics. Kappa is an index which indicates how much greater test concordance is than would be expected by chance (Fleiss, 1986). The kappa values range from 0 (chance agreement only) to 1.0 (perfect agreement). Values above 0.4 represent good concordance, and those above 0.75 represent excellent concordance.

3. Results

3.1. Sensitivity and specificity of the prototype ELISA in established L. infantum infection

The prototype ELISA was used to test sera from 209 dogs with parasitological evidence of leishmaniasis. In these animals, amastigote density in bone marrow and lymph nodes ranged from one parasite per 1000 microscopic fields to one parasite per microscopic field. IFAT was positive in all these dogs, with titers ranging from 80 to 5120. Sera from 81 uninfected Italian dogs and 90 normal US dogs were used as negative controls.

Of the confirmed infected dogs, 203/209 (97.1%) were positive at absorbance greater than 0.220. For the normal dogs, 1/81 (1.2%) of the Italian dogs and 0/90 of the US dogs
were positive at the absorbance greater than 0.220. Thus, the sensitivity of the assay was 97.1% while the specificity was 98.8%, if only the Italian dogs were included, or 99.4% if the US dogs were also included. There was no correlation between absorbance values recorded with the ELISA and either the IFAT titer or the severity of disease (data not shown).

Sera from 62 *Leishmania*-free dogs infected with *Ehrlichia canis* (13), *Babesia gibsoni* (9), *Trypanosoma cruzi* (2), *Dirofilaria repens* (4), *Dirofilaria immitis* (7), *Dipetalonema reconditum* (3), *Dipylidium caninum* (5), *Trichuris vulpis* (6) or *Ancylostoma caninum* (13) were also examined and found to have absorbance values less than 0.220 (data not shown).

3.2. Sensitivity and specificity of the prototype ELISA in field samples

Based on the absorbance mean and standard deviation for Italian negative dogs above, the cut-off value was set at $A_{450} = 0.400$. Using these test conditions, the prototype ELISA was further evaluated on a sample of 415 sera with known IFAT values collected from the northern Sardinia area during an epidemiologic survey. Parasitological and clinical features of the animals were not available. Of the 157 IFAT-positive sera with a titer of 40 or greater, 156 (99.4%) were also positive in the prototype ELISA. The single ELISA negative sample had a corresponding IFAT titer of 40. Among the 258 IFAT-negative sera, 1 (0.4%) was positive in the prototype ELISA (Fig. 1). Again, there was no correlation between absorbance values and IFAT titers (data not shown). The test sensitivity and specificity as compared to IFAT were 99.4 and 99.6%, respectively. The observed concordance between the two tests was 0.99 and the kappa value 0.89.

![Fig. 1. Distribution of absorbance ($A_{450}$) values obtained using the prototype rK39 ELISA with 415 canine serum samples collected in northern Sardinia and examined by IFAT at the threshold titer of 40. Values lower and greater than 0.400 are shown at different scales on the x-axis.](image-url)
Fig. 2. Appearance and trend of antileishmanial antibodies revealed by IFAT (●) and laboratory kit ELISA (▲) in four beagles exposed to natural Leishmania infection after June 1997 and found parasite positive in March 1998. In each of the four graphs, the dotted line is the threshold value for both tests, i.e. a titer of 40 for IFAT (expressed as natural log, left) and an absorbance value of 0.400 (A_{450}, right).
3.3. Appearance and trend of anti-rK39 antibodies using the standardized laboratory kit ELISA

Four naive beagle dogs were housed in a kennel from June 1997 through March 1998. By the end of March 1998 all animals showed evidence of an established *Leishmania* infection as demonstrated by positive microscopy and culture. Only one dog (dog 1, Fig. 2) developed clinical disease during the observation period. Serological assessments were made in parallel using the laboratory kit ELISA and IFAT. As shown in Fig. 2, both the appearance and trend of anti-leishmanial antibodies showed high concordance with the two tests (0.88; kappa: 0.85). In dog 1, high levels of antibodies were detected very early after *Leishmania* exposure, with an increasing trend up to the animal’s euthanasia. A similar trend, but with temporary seroconversion to negative revealed by both assays, was observed in dog 2. Both dogs 3 and 4 developed a late positive serologic response, about 10 months after placement at the endemic site. Interestingly, IFAT titers and ELISA absorbance values recorded prospectively in each dog demonstrated similar patterns and appeared to be correlated, while this was not evident in the cross-sectional determinations reported above.

3.4. Multi-center study for the validation of the standardized laboratory kit ELISA

This study was designed to evaluate the performance of the standardized laboratory kit ELISA at IZ departments that routinely run a high number of IFAT for canine leishmaniasis. The sera were examined by both methods for 1–2 months. To maximize the standardization of IFAT procedures, the collaborating IZs were provided with the same *L. infantum* strain for antigen preparation and batch of FITC-conjugated anti-dog immunoglobulins, as well as with a common protocol for the assay procedure. To facilitate the evaluation, only two serum dilutions (1:40 and 1:80) were examined by IFAT. If a sample had discordant results with the two tests, it was sent to the Department of Parasitology of Istituto Superiore di Sanità for blind re-evaluation.

Five IZs, hereafter referred to as Palermo, Sassari, Naples, Imperia and Padua, examined a total of 5507 sera. Of these sera, 275 (5.0%) had discordant results between the two tests, 145 of which were re-evaluated. In Table 1, which shows the laboratory kit ELISA results at the IFAT threshold titer of 40, data are presented after re-evaluation of discordant samples. The observed test concordance varied from 0.91 to 0.99 between the IZs, whereas the kappa value varied from 0.76 to 0.98.

Because several sera giving discordant results had an IFAT titer of only 40, and because the percentage of the positives represented by these sera varied greatly between the IZs (4.1% Sassari, 11.7% Palermo, 11.8% Padua, 18.5% Naples, 42.6% Imperia), the laboratory kit ELISA results were also compared with the IFAT threshold titer of 80 (Table 2). Using this criteria, both the observed test concordance (0.95–0.98) and the kappa values (0.84–0.95) were much more consistent between the IZs.

Information on the clinical condition was available in a subset of 1028 dogs. Of these, 628 dogs showed one or more signs referable to a leishmanial infection, including aspecific signs such as slight lymph adenopathy and/or loss of weight. In this group of dogs, 265 were IFAT-positive at the threshold titer of 80, and 259 were positive in the laboratory kit ELISA. Sensitivity and specificity of the ELISA as compared to IFAT for this group of dogs
Table 1
Laboratory kit ELISA results compared to an IFA T threshold titer of 40 with 5507 canine sera examined by five Italian Institutes for Zooprophylaxis (IZs)

<table>
<thead>
<tr>
<th>Result</th>
<th>IZ Palermo ((N = 1839))</th>
<th>IZ Sassari ((N = 1119))</th>
<th>IZ Naples ((N = 1675))</th>
<th>IZ Imperia ((N = 467))</th>
<th>IZ Padua ((N = 407))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
</tr>
<tr>
<td>Positive</td>
<td>733</td>
<td>743</td>
<td>344</td>
<td>330</td>
<td>365</td>
</tr>
<tr>
<td>Negative</td>
<td>1106</td>
<td>1096</td>
<td>775</td>
<td>789</td>
<td>1310</td>
</tr>
<tr>
<td>FP(^a)</td>
<td>18</td>
<td>3</td>
<td>19</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>FN(^b)</td>
<td>8</td>
<td>17</td>
<td>53</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>OC(^c)</td>
<td>0.99</td>
<td>0.98</td>
<td>0.96</td>
<td>0.91</td>
<td>0.99</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.98</td>
<td>0.95</td>
<td>0.88</td>
<td>0.76</td>
<td>0.96</td>
</tr>
</tbody>
</table>

\(^a\) rK39 ELISA false positive compared to IFAT.
\(^b\) rK39 ELISA false negative compared to IFAT.
\(^c\) Observed concordance between rK39 ELISA and IFAT.

Table 2
Laboratory kit ELISA results reported in Table 1 compared to an IFA T threshold titer of 80

<table>
<thead>
<tr>
<th>Result</th>
<th>IZ Palermo ((N = 1839))</th>
<th>IZ Sassari ((N = 1119))</th>
<th>IZ Naples ((N = 1675))</th>
<th>IZ Imperia ((N = 467))</th>
<th>IZ Padua ((N = 407))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
<td>ELISA IFAT</td>
</tr>
<tr>
<td>Positive</td>
<td>647</td>
<td>743</td>
<td>330</td>
<td>330</td>
<td>308</td>
</tr>
<tr>
<td>Negative</td>
<td>1192</td>
<td>1096</td>
<td>789</td>
<td>789</td>
<td>1367</td>
</tr>
<tr>
<td>FP(^a)</td>
<td>98</td>
<td>13</td>
<td>49</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>FN(^b)</td>
<td>2</td>
<td>13</td>
<td>26</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>OC(^c)</td>
<td>0.95</td>
<td>0.98</td>
<td>0.95</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.89</td>
<td>0.95</td>
<td>0.84</td>
<td>0.91</td>
<td>0.89</td>
</tr>
</tbody>
</table>

\(^a\) rK39 ELISA false positive compared to IFAT.
\(^b\) rK39 ELISA false negative compared to IFAT.
\(^c\) Observed concordance between rK39 ELISA and IFAT.

were 96.6 and 99.2%, respectively. Four hundred dogs were apparently in good health. In this group of asymptomatic animals, 53 were IFAT-positive at the threshold titer of 80, and 52 were positive at the laboratory kit ELISA. Sensitivity and specificity of the ELISA as compared to IFAT for this group of dogs were 92.4 and 99.1%, respectively.

4. Discussion

Among 380 dogs examined in the first part of the study, IFAT was positive at titers of 80 or greater in 100% of animals with parasitological evidence of leishmaniasis, whereas the assay was negative at the threshold titer of 40 in 100% of clinically healthy, Leishmania-free dogs. Therefore, the first set of experiments have confirmed the value of IFAT for the evaluation of the new serodiagnostic method, and justified its use as the sole reference test in subsequent investigations involving larger dog samples.
When used with sera from two large group of dogs, both the sensitivity and the specificity of the prototype ELISA were remarkably high as compared to IFAT (97.1–99.4 and 99.4–99.6%, respectively). Sensitivity was similar to, and specificity higher than those reported for conventional ELISA (Ashford et al., 1993; Mancianti et al., 1995; Sideris et al., 1996) or dot-ELISA (Mancianti et al., 1996) using parasite derived antigen. Furthermore, the distribution of absorbance values revealed a clear difference between negative and positive sera (see Fig. 1), while this is not usually seen with the conventional Leishmania ELISA. It is noteworthy, however, that in early canine infections both IFAT and rK39 ELISA failed to reveal specific antibodies for a variable period of time, as shown in three out four naive beagles exposed to natural Leishmania transmission. Due to serological latency, which appears to be common in dogs and may include both incubation and transient seroconversion-to-negative periods (Acedo-Sánchez et al., 1998), Dye et al. (1993) have estimated that even the most efficient serological method will probably miss more than 20% of newly established infections. As a consequence, serological techniques should be complemented by other methods currently in use when an accurate diagnosis is necessary (Campino et al., 2000), or suspected cases that are seronegative should be re-tested after a suitable period of time.

The laboratory kit ELISA detected anti-leishmanial antibodies in a large number of asymptomatic dogs. The high sensitivity of the assay in this group of dogs (92.4%), which was only slightly lower than in dogs showing signs referable to leishmanial infection (96.6%), indicates that antibodies reacting with rK39 are more common in asymptomatic canine infections than in subclinical human VL. Only 4% of sera from 130 subjects with early, stationary or self-healing subclinical VL (as detected by serology using crude Leishmania antigen) reacted with rK39, whereas 87% of sera from 15 subclinical cases progressing to clinical VL reacted with the antigen (Badaro et al., 1996). These different patterns suggest that the asymptomatic condition should be treated as a markedly different biological feature between canine and human subjects infected with L. infantum.

Data from the multi-center study on the laboratory kit ELISA validation showed some variation between collaborating institutes in performance of the IFAT, the rK39 ELISA, or both. It is unlikely that the dog populations examined by the IZs differed somehow in the biological response to Leishmania, however, it should be noted that the dogs tested at each IZ were different, and each IZ received these samples based on the different criteria for that area. The great variation in frequency of sera showing the low IFAT titer of 40 (from 4.1 to 42.6%) strongly suggests the greatest variation was due to IFAT interpretation. To overcome this, the IFAT threshold value was increased to 80 for all IZs, which may have resulted in the removal of some true positives from some locations and, therefore, a slight decrease of specificity. The “gray zone” for a positive interpretation appears to be a common problem for IFAT in many laboratories, which report threshold titers ranging from 20 to 160 (Jambou et al., 1986; Abranches et al., 1991; Dye et al., 1993; Berrahal et al., 1996; Sideris et al., 1996; Acedo–Sánchez et al., 1996, 1998).

In conclusion, the laboratory kit ELISA is a relatively simple and rapid assay for assessing the infection status of dogs. The use of a specific purified recombinant protein as the K39 antigen allows better standardization than culture derived parasite material, and the quantitative assessment removes the operator subjectivity that does exist with IFAT. The laboratory kit ELISA can be used as an alternative to IFAT, especially when screening large numbers of samples.
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References


