Diagnostic Techniques To Detect Cryptic Leishmaniasis in Dogs
Laura Iniesta,1 Salceda Fernández-Barredo,2 Béatrice Bulle,3 M. Teresa Gómez,2 Renaud Piarroux,2 Montserrat Gállego,1 José M. Alunda,2 and Montserrat Portús1*  

Laboratori de Parasitologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona,1 and Departament Patologia Animal I, Facultat de Veterinària, Universitat Complutense, Madrid, Spain, and Laboratoire Parasitologie-Mycologie, Faculté de Médecine et de Pharmacie, Université de Franche-Comté, Besançon, France2  

Received 26 November 2001/Returned for modification 7 March 2002/Accepted 17 May 2002

This study of several techniques for detecting cryptic leishmaniasis in dogs from areas in Spain where Leishmania infantum is highly endemic concludes that immunological techniques (enzyme-linked immunosorbent assay, immunofluorescence antibody test, Western blotting, delayed-type hypersensitivity reaction, and in vitro lymphocyte proliferation assay) do not clearly differentiate between noninfected and infected asymptomatic dogs and that culture and PCR are more reliable diagnostic tools.

Seroepidemiological studies of canine leishmaniasis have revealed a large number of asymptomatic seropositive animals (20, 24). Moreover, in areas where leishmaniasis is highly endemic, a high proportion of apparently healthy animals show low levels of anti-Leishmania antibodies. Serological follow-up of these animals has revealed that some of them are in the prepatent infection period, which will lead to increased antibody titers in subsequent blood extractions. Others have regressive forms of the disease, and their antibody levels will decrease in the following months or years; still others maintain low levels of antibodies without developing the disease for many years (1, 11, 12, 17). However, the total number of infected animals is unknown.

The detection of the extent of the infection, particularly among asymptomatic dogs, is of great importance for the control of leishmaniasis. Most epidemiological and control studies of canine leishmaniasis are performed by serological methods. Although such methods are traditionally considered to be more sensitive than parasitological techniques for the diagnosis of the disease, they underestimate the prevalence and incidence of the infection relative to those estimated by culture (10) and PCR (26). Indeed, experimentally infected dogs that develop the disease have an anti-Leishmania humoral immune response while those that remain asymptomatic present a cellular response (19). The application of highly sensitive techniques, such as PCR (3, 13) and Western blotting (WB) (1, 8, 11), as well as the optimization of culture (14), have improved the rate of detection of leishmaniasis. Here, we compare the results obtained by various diagnostic methods (direct examination, culture, PCR, enzyme-linked immunosorbent assay [ELISA], WB, delayed-type hypersensitivity reaction [DTH], and a lymphocyte proliferation assay [LPA]) for dogs from two areas in Spain where canine leishmaniasis is endemic, and we consider the results in the context of the clinical status of the animals.

The study was carried out with 72 animals, 38 of which were from the Priorat, an area in northeast Spain where leishmaniasis is highly endemic. The remaining 34 dogs were from an animal protection society in Madrid, Spain. Animals were selected for the study after extensive serological screening for anti-Leishmania-specific antibodies, and subjects were sometimes chosen for screening because of the presence of sick animals in the kennel. In other cases, we picked asymptomatic animals with specific antibodies or under other irregular circumstances. Thus, the dog samples were biased and cannot be considered representative of the whole dog population in the areas studied. Clinical symptoms and lesions consistent with canine leishmaniasis (skin abnormalities, onychogryposis, weight loss, epistaxis, apathy, ocular and other lesions, lymph node and spleen enlargement, etc.) were recorded for all dogs.

Blood was collected by cephalic or jugular venipuncture for complete blood count and biochemical analysis (serum proteins, the renal markers urea and creatinine, and the hepatic markers aspartate aminotransferase and alanine aminotransferase), for detection of anti-Leishmania antibodies, and for PCR and LPA. Popliteal lymph node aspirates were obtained for direct examination, culture, and PCR. Needle aspiration skin microbiopsy was also performed to obtain samples for PCR.

Anti-Leishmania antibodies were quantitatively detected by an immunofluorescence antibody test (IFAT) and an ELISA for immunoglobulin G2 (ELISA-IgG2) for dogs from Madrid and by ELISAs for protein A (ELISA-protein A) and for IgG2 for dogs from the Priorat. The IFAT was performed by standard methods (8) using a fluorescent conjugated anti-dog IgG (heavy plus light chains; Jackson ImmunoResearch, West Grove, Pa.) at a 1:50 dilution. ELISAs were performed as described elsewhere (22). Horseradish peroxidase-conjugated protein A (dilution, 1:30,000; Sigma, St. Louis, Mo.) and anti-dog IgG2 (dilutions, 1:2,000 to 1:5,000; Bethyl Laboratories, Montgomery, Tex.) were used as second antibodies. The reaction results were quantified in units relative to those of a...
positive serum sample that was used as a calibrator and arbitrarily set at 100 U.

WB analyses of sera from the Priorat area were performed at the Faculty of Pharmacy, Barcelona, Spain, as described elsewhere (1). Those sera that developed any of the polypeptide fractions of 14 or 16 kDa, previously reported as 12 and 14 kDa (1), were considered positive. WB analyses of sera from the Madrid area were performed at the Faculty of Veterinary Science, Madrid, as described previously (8). The sera that revealed the polypeptide fractions of 30, 42, 50, and 57 kDa were considered positive, as reported previously (8, 11). The correspondence between positive results detected by each method has been established (S. Méndez, M. J. Aisa, F. J. Fernández-Pérez, L. Iniesta, M. Portús, J. M. Alunda, and M. T. Gómez-Muñoz, submitted for publication).

DNA for PCR analysis was extracted with a PCR template preparation kit (Boehringer Mannheim, Mannheim, Germany), Leishmania-specific oligonucleotide primers A2 (5′-GGG AGAAGCTCTATTTGTG-3′) and B1 (5′-ACACTCAGTGCT GTAAAC-3′) were used to amplify a 650-bp fragment in the internal transcribed spacer region of genomic DNA (4a).

The DTH was performed by inoculation into the skin of the groin of 0.1 ml of Leishmania infantum antigen (3 × 10^6 pro-mastigotes/ml), which was kindly supplied by the Instituto de Salud Carlos III, Majadahonda, Spain. Reactions were read after 48 h and measured by the ballpoint pen method. The LPA was performed with peripheral blood mononuclear cells as described elsewhere (19).

Crude results are shown in Table 1. Each laboratory (Madrid and Barcelona) applied its current serological tests in addition to the ELISA-IgG2, which was performed in both laboratories with the same conjugates, the same quantification system, and the same calibrator serum. The IgG2 results correlated highly with the ELISA-protein A results (Spearman’s rank correlation coefficient, 0.950; P = 0.000) and the IFAT results (Spearman’s rank correlation coefficient, 0.627; P = 0.000). Therefore, to simplify this discussion, only results from the ELISA-IgG2 were included in the statistical analysis and comparison of techniques. In contrast, no correlation was observed between the techniques for detecting the cellular immunorespons, DTH, and LPA (Spearman’s rank correlation coefficient, 0.052; P = 0.75), and so the results obtained in each determination were analyzed independently. Attempts to link DTH and LPA findings for use in detecting human leishmaniasis have led to contradictory results. DTH and LPA results correlated in L. major-infected children of Jericho (2) and Tunisia (23). Nevertheless, the lymphoproliferative response to the L. major antigen by peripheral blood mononuclear cells from Sudanese individuals with a positive leishmaniasis skin test and no history of cutaneous leishmaniasis was similar to the response observed in Danish people with no reported exposure to Leishmania parasites (16).

The dogs that were positive by direct examination, culture, PCR, or at least two immunological methods were considered probably infected. Among them, those with external signs of leishmanial disease were considered to be symptomatic and those without external signs of disease, despite the detection of biochemical and hematological (analytical) disorders, were considered to be asymptomatic or to have cryptic infections.

The specific IgG2 levels detected by ELISA were directly related to the pathophysiological disorders detected during the physical and analytical examinations (Fig. 1). All symptomatic dogs (10 of 10) had high antibody titers as determined by the ELISA-IgG2, while only 35 of 51 animals with cryptic infections were positive by this technique.

The positive and negative results obtained with the techniques were compared two by two by the McNemar and Pearson tests in the whole dog sample studied, in animals considered to be infected (symptomatic and asymptomatic animals) and in those found to have cryptic infections (Table 2). Culture and PCR provided very similar results; they gave the same proportion of positives (McNemar test, P = 0.629) and a high degree of association (Pearson test, P < 0.001). The culture and PCR results differed for 17 of the 72 dogs studied (10 animals positive by PCR and negative by culture and 7 animals positive by culture and negative by PCR). In 11 of these animals, immunological techniques confirmed the presence of parasites. In three cases PCR was the only positive test, and in two cases only the culture was positive, as confirmed by new samples and repeated analyses.

The humoral immunoresponse, detected as L. infantum-specific IgG2 expression, was also associated with PCR and culture results when the whole dog population was considered (Pearson test, P ≤ 0.001) but was independent of those results when only the animals with cryptic infections were considered. The results obtained by immunological techniques were independent when compared two by two, even when these techniques were considered to have the same target as those of antibody detection (ELISA-IgG2 and WB) or cellular immunoresponse (DTH and LPA) techniques.

The lack of a “gold standard” for diagnosis of asymptomatic infections caused by L. infantum is a drawback to epidemiological studies of the disease. Parasitological techniques like direct microscopic examination and culture offer the only reliable evidence of the presence of parasites in a sample. However, direct examination lacks sensitivity when the parasite number is small, and the growth capacity in vitro varies from one Leishmania strain to another. Other analytical techniques, such as PCR and immunological methods, lack some specificity and sensitivity.

There is evidence of Leishmania persistence inside the host after recovery from the disease (4, 9, 22), and a positive response to Leishmania antigens detected by DTH and LPA has been associated with asymptomatic infection (19, 21). However, cellular immunoresponse or low antibody levels may be due to immunological memory rather than to the presence of the parasite in the host, especially in areas of endemicity. Moreover, positive lymphoproliferative responses to Leishmania antigen have been observed in nonexposed humans (15).

Whether a dog can be considered infected or uninfected is the key determinant of parameters such as sensitivity, specificity, and predictive values for diagnostic techniques. If we consider that only those animals that are positive by direct examination or culture are parasitized, the sensitivities and specificities of other methods such as PCR and immunological techniques are affected. Therefore, these parameters were not determined in the present study. The arbitrary definition of a cutoff in the ELISA and the lack of a clear separation between positive and negative results...
| Dog  |
|------|
| Signe | Analytical |
| Direct ex/culture |
| PCR  |
| ELISA-protein A  | ELISA-IgG  | IFAT-IgG  | WB  | DTH  | LPA  |
|------|------------|-----------|----------|------|------|------|
| B1   | +          | +         | +        | +    | 337  | 293  | +    | 0    |
| B2   | –          | +         | +        | –    | 31   | 21   | –    | 48   |
| B3   | –          | –         | –        | –    | 40   | 40   | –    | 80   |
| B4   | +          | –         | +        | +    | 177  | 177  | +    | 132  |
| B5   | –          | +         | –        | –    | 58   | 51   | +    | 462  |
| B6   | +          | +         | –        | +    | 174  | 159  | +    | 100  |
| B7   | +          | +         | +        | +    | 247  | 231  | +    | 80   |
| B8   | –          | –         | –        | +    | 17   | 11   | –    | 0    |
| B9   | –          | +         | –        | +    | 13   | 7    | –    | 0    |
| B10  | +          | +         | +        | +    | 252  | 229  | +    | 0    |
| B11  | –          | +         | +        | –    | 209  | 184  | +    | 30   |
| B12  | –          | –         | –        | –    | 33   | 37   | +    | 0    |
| B13  | –          | –         | –        | –    | 8    | 15   | –    | 0    |
| B14  | –          | –         | –        | –    | 11   | 13   | –    | 0    |
| B15  | –          | +         | –        | +    | 30   | 30   | –    | 117  |
| B16  | –          | –         | –        | –    | 27   | 27   | –    | 132  |
| B17  | –          | –         | –        | –    | 2    | 18   | –    | 132  |
| B18  | –          | +         | –        | +    | 12   | 12   | –    | 100  |
| B19  | +          | +         | +        | +    | 118  | 123  | +    | 0    |
| B20  | –          | +         | +        | +    | 16   | 24   | +    | 100  |
| B21  | –          | +         | –        | +    | 24   | 34   | +    | 144  |
| B22  | –          | –         | +        | +    | 69   | 59   | +    | 64   |
| B23  | +          | –         | +        | +    | 42   | 59   | +    | 594  |
| B24  | –          | –         | +        | +    | 20   | 27   | +    | 675  |
| B25  | +          | +         | +        | +    | 40   | 42   | –    | 144  |
| B26  | –          | –         | –        | +    | 75   | 72   | –    | 30   |
| B27  | –          | –         | –        | +    | 29   | 36   | –    | 360  |
| B28  | –          | –         | –        | –    | 90   | 85   | +    | 0    |
| B29  | –          | +         | –        | +    | 24   | 27   | –    | 120  |
| B30  | –          | +         | +        | +    | 69   | 78   | +    | 415  |
| B31  | –          | –         | –        | +    | 12   | 10   | +    | 110  |
| B32  | –          | –         | –        | –    | 13   | 16   | +    | 150  |
| B33  | +          | +         | +        | +    | 258  | 175  | +    | 400  |
| B34  | –          | +         | +        | +    | 348  | 169  | +    | 0    |
| B35  | –          | +         | +        | –    | 11   | 4    | –    | 0    |
| B36  | –          | +         | +        | –    | 12   | 6    | –    | 0    |
| B37  | –          | –         | –        | –    | 18   | 16   | +    | 0    |
| B38  | +          | +         | –        | –    | 375  | 173  | +    | 0    |
| M2   | –          | –         | –        | –    | 56   | 100  | +    | 75   |
| M4   | –          | +         | –        | –    | 28   | 100  | –    | 0    |
| M5   | –          | –         | –        | –    | 29   | 200  | +    | 0    |
| M7   | –          | –         | –        | –    | 23   | 100  | 0    | 4.93 |
| M10  | –          | –         | –        | –    | 10   | 100  | +    | 0    |
| M11  | –          | –         | –        | –    | 16   | 100  | 0    | 9.01 |
| M12  | –          | –         | –        | –    | 16   | 200  | +    | 0    |
| M16  | –          | –         | –        | –    | 17   | 50   | +    | 100  |
| M19  | –          | –         | –        | –    | 21   | 100  | –    | 0    |
| M20  | –          | –         | –        | –    | 14   | 100  | –    | 0    |
| M21  | –          | –         | –        | +    | 107  | 200  | +    | 0    |
| M23  | –          | –         | –        | –    | 12   | 100  | +    | 80   |
| M24  | –          | +         | –        | –    | 16   | 50   | –    | 460  |
| M27  | –          | –         | –        | –    | 14   | 100  | +    | 0    |
| M29  | +          | +         | +        | +    | 101  | 400  | +    | 0    |
| M30  | –          | +         | –        | –    | 44   | 200  | –    | 0    |
| M35  | –          | –         | –        | –    | 4    | 100  | +    | 0    |
| M36  | –          | –         | –        | –    | 18   | 100  | +    | 0    |
| M37  | +          | +         | –        | –    | 63   | 200  | +    | 0    |
| M46  | –          | +         | –        | –    | 36   | 100  | +    | 0    |
| M52  | –          | +         | –        | –    | 19   | 50   | –    | 0    |
| M57  | –          | +         | +        | –    | 64   | 400  | +    | 0    |
| M59  | –          | +         | –        | –    | 23   | 50   | +    | 0    |
| M65  | –          | +         | +        | +    | 38   | 400  | +    | 0    |
| M71  | –          | +         | +        | +    | 16   | 50   | +    | 0    |
| M72  | –          | +         | –        | –    | 25   | 50   | +    | 0    |
| M78  | –          | –         | –        | –    | 14   | 100  | +    | 0    |
| M79  | –          | –         | –        | –    | 10   | 100  | +    | 100  |

Continued on following page
entail a lack of specificity when this cutoff is established to identify bands. This may explain the independent results reporting the results cannot be avoided when very weak bands are detected. However, bands are difficult to identify, since several antigen fractions have similar molecular weights and subjectivity in reading the results cannot be avoided when very weak bands are detected. This may explain the independent results

(Pearson test, \( P > 0.05 \)) provided by the ELISA-IgG2 and WB. Moreover, since the antigen treatments in the ELISA and WB differed strongly, each technique identified a different set of epitopes, and this explains the lack of association in animal cohorts with a predominance of individuals with low humoral immunoresponses. In conclusion, visceral leishmaniasis in sick dogs from areas of endemcity can be easily diagnosed. It is characterized by a high humoral immunoresponse that is measurable by conventional serological techniques (e.g., IFAT and ELISA) and a high parasite burden that is detectable by parasitological and PCR methods. However, when we deal with asymptomatic animals, particularly in epidemiological studies, immunological techniques do not discriminate between infected and noninfected animals. Antibody detection alone and DTH or LPA, which are used to estimate the infected dog population (5, 6, 7, 25), are not suitable tools.

### TABLE 1—Continued

| Dog  | Clinical findings | Test results |
|------|-------------------|--------------|
| M80  | +                 | 74, 400, 400, 0, 1.8 |
| M84  | +                 | 48, 100, 400, 0, 4.17 |
| M85  | +                 | 107, 400, 400, 0, 0.71 |
| M95  | +                 | 11, 50, 50, 0, 2.22 |
| M101 | +                 | 124, 400, 400, 0, 9.12 |
| M106 | +                 | 16, 50, 50, 0, 1.19 |

a Designations indicate dogs from Barcelona (B) and Madrid (M).

b Clinical signs of leishmaniasis.

c Analytical (biochemical and hematological) disorders.

d Results of the direct examination and/or culture.

e PCR was considered positive when one or more samples (blood, skin, and/or lymph node aspirate) were positive.

f Positivity was established at values of \( \geq 24 \) U.

g Positive was established at values of \( \geq 200 \).

h WB results were considered positive when they showed any of the polypeptide fractions of 14 or 16 kDa (Barcelona) or of 30, 42, 50, and 57 kDa (Madrid).

i DTH induration area (square millimeters). Positivity was established at values of \( \geq 25 \) mm².

j LPA (stimulation index). Positivity was established at values of \( \geq 2.5 \).

### TABLE 2. Statistical analysis of results (positive/negative) obtained by various diagnostic techniques in three dog cohorts: (i) the whole dog population studied, (ii) dogs considered to be infected with *L. infantum*, and (iii) asymptomatic dogs with a cryptic *Leishmania* infection

| Technique(s) | Whole dog sample | Infected dogs | Dogs with cryptic infections |
|--------------|------------------|---------------|-----------------------------|
|              | naïve  | PP  | CC  | PMcN | naïve  | PP  | CC  | PMcN | naïve  | PP  | CC  | PMcN |
| Culture-PCR  | 66     | 0.000  | 0.432  | 0.629  | 56     | 0.003  | 0.369  | 0.629  | 47     | 0.017  | 0.329  | 0.607  |
| Culture-IgG2 | 72     | 0.000  | 0.389  | 0.000  | 61     | 0.017  | 0.293  | 0.000  | 51     | 0.065  | 0.251  | 0.001  |
| Culture-DTH  | 72     | 0.245  | 0.140  | 0.001  | 58     | 0.489  | 0.091  | 0.004  | 49     | 0.743  | 0.047  | 0.002  |
| Culture-LPA  | 40     | 0.583  | 0.087  | 0.052  | 34     | 0.397  | 0.144  | 0.15  | 31     | 0.675  | 0.075  | 0.031  |
| PCR-IgG2     | 66     | 0.001  | 0.380  | 0.027  | 56     | 0.052  | 0.252  | 0.027  | 47     | 0.183  | 0.191  | 0.041  |
| PCR-WB       | 62     | 0.304  | 0.129  | 0.008  | 54     | 0.636  | 0.064  | 0.043  | 45     | 0.885  | 0.035  | 0.021  |
| PCR-DTH      | 66     | 0.086  | 0.205  | 1      | 56     | 0.280  | 0.143  | 0.839  | 47     | 0.491  | 0.100  | 1      |
| PCR-LPA      | 35     | 0.070  | 0.291  | 0.210  | 29     | 0.020  | 0.396  | 0.383  | 27     | 0.058  | 0.343  | 0.167  |
| IgG2-WB      | 67     | 0.085  | 0.206  | 0.541  | 58     | 0.297  | 0.136  | 1      | 49     | 0.466  | 0.104  | 1      |
| IgG2-DTH     | 72     | 0.154  | 0.166  | 0.007  | 61     | 0.526  | 0.081  | 0.001  | 51     | 0.583  | 0.077  | 0.015  |
| IgG2-LPA     | 40     | 0.491  | 0.108  | 0.832  | 34     | 0.171  | 0.228  | 0.503  | 31     | 0.332  | 0.172  | 1      |
| WB-DTH       | 67     | 0.628  | 0.059  | 0.008  | 58     | 0.745  | 0.011  | 0.011  | 49     | 0.860  | 0.024  | 0.029  |
| WB-LPA       | 35     | 0.927  | 0.016  | 0.049  | 31     | 0.889  | 0.025  | 0.118  | 29     | 0.653  | 0.083  | 0.180  |
| LPA-DTH      | 40     | 0.855  | 0.029  | 0.027  | 34     | 0.656  | 0.076  | 0.064  | 31     | 0.675  | 0.031  | 0.075  |

a n, number of dogs.

b PP, Pearson test \( P \) value.

c CC, Pearson contingency coefficient.

d PMcN, McNemar test \( P \) value.
and parasitological methods and PCR offer more accurate results.

This study was supported by the EU, project FAIR CT98-4104. Financial support was also obtained from the Spanish and Catalan governments, projects PB94-0865 and 1999SGR00072.

We thank the veterinarians and dog owners for their collaboration in sampling and R. Rycroft for correcting the English version of the manuscript.

REFERENCES

1. Aisa, M. J., S. Castillejo, M. Gállego, R. Fisa, M. C. Riera, M. de Colmenares, S. Torras, X. Roura, J. Sentis, and M. Portús. 1998. Diagnostic potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. Am. J. Trop. Med. Hyg. 58:154–159.

2. Alvarado, R., C. Enk, K. Jaber, L. Schnurr, and S. Frankenburg. 1989. Delayed-type hypersensitivity and lymphocyte proliferation in response to Leishmania major infection in a group of children in Jericho. Trans. R. Soc. Trop. Med. Hyg. 83:189–192.

3. Ashford, D. A., M. Bozza, M. Freire, J. C. Miranda, I. Sherlock, C. Eulalio, U. Lopes, O. Fernandes, W. Degrave, R. H. Barker, Jr., R. Badaro, and J. R. David. 1995. Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. Am. J. Trop. Med. Hyg. 53:251–255.

4. Berrahal, F., C. Mary, M. Roze, A. Berenger, K. Escoffier, D. Lamouroux, and S. Dunan. 1996. Canine leishmaniasis: identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. Am. J. Trop. Med. Hyg. 55:273–277.

5. Cabral, M., J. O’Grady, and J. Alexander. 1992. Demonstration of Leishmania specific cell mediated and humoral immunity in asymptomatic dogs. Parasite Immunol. 14:531–539.

6. Cabral, M., J. E. O’Grady, S. Gomes, J. C. Sousa, H. Thompson, and J. Alexander. 1998. The immunology of canine leishmaniasis: strong evidence for a developing disease spectrum from asymptomatic dogs. Vet. Parasitol. 76:173–180.

7. Cardoso, L., F. Neto, J. C. Sousa, M. Rodrigues, and M. Cabral. 1998. Use of a leishmanin skin test in the detection of canine Leishmania-specific cellular immunity. Vet. Parasitol. 79:213–220.

8. Carrera, L., M. L. Fermin, M. Tesouro, P. García, E. Rollán, J. L. González, S. Méndez, M. Cuquerella, and J. M. Aludna. 1996. Antibody response in dogs experimentally infected with Leishmania infantum: infection course and antigen markers. Exp. Parasitol. 82:139–146.

9. Cavaliero, T., P. Arnold, A. Mathis, T. Glaus, R. Hofmann-Lehmann, and P. Deplazes. 1999. Clinical, serological, and parasitological follow-up after long-term allopurinol therapy of dogs naturally infected with Leishmania infantum. J. Vet. Intern. Med. 13:330–334.

10. Dye, C., E. Vidor, and J. Dereure. 1993. Serological diagnosis of leishmaniasis: on detecting as well as disease. Epidemiol. Infect. 103:67–656.

11. Fernández-Pérez, F. J., S. Méndez, C. de la Fuente, M. Cuquerella, M. T. Gómez, and J. M. Aludna. 1999. Value of Western blotting in the clinical follow-up of canine leishmaniasis. J. Vet. Diag. Investig. 11:170–173.

12. Fisa, R., M. Gállego, M. Portús, and J. Gállego. 1991. Evolución de la leishmaniasis canina en zona endémica a través de su seguimiento serológico. Cienc. Vet. 4:69–76.

13. Fisa, R., C. Riera, M. Gállego, J. Manubens, and M. Portús. 2001. Nested PCR for diagnosis of canine leishmaniasis in peripheral blood, lymph node and bone marrow aspirates. Vet. Parasitol. 99:105–111.

14. Howard, M. K., M. M. Pharoah, F. Ashall, and M. A. Miles. 1991. Human urine stimulates growth of Leishmania in vitro. Trans. R. Soc. Trop. Med. Hyg. 85:477–479.

15. Kemp, M., M. B. Hansen, and T. G. Theander. 1992. Recognition of Leishmania antigens by T lymphocytes from nonexposed individuals. Infect. Immun. 60:2246–2251.

16. Kemp, M., A. S. Hey, J. A. L. Kurtzhals, C. B. V. Christensen, A. Gaafar, M. D. Mustafa, A. A. Y. Kordofani, A. Ismail, A. Karazmi, and T. G. Theander. 1994. Dichotomy of the human T cell response to Leishmania antigens. I. Th1-like response to Leishmania major promastigote antigens in individuals recovered from cutaneous leishmaniasis. Clin. Exp. Immunol. 96:410–415.

17. Lannote, G., J. A. Rioux, J. Perières, and Y. Vollhardt. 1979. Ecologie des leishmaniasis dans le sud de la France. 10. Les formes évolutives de la leishmaniose viscérale canine. Elaboration d’une typologie bio-clinique à finalité épidémiologique. Ann. Parasitol. Hum. Comp. 54:277–295.

18. Mary, C., D. Lamouroux, S. Dunan, and M. Quilici. 1992. Western blot analysis of antibodies to Leishmania infantum antigens: potential of the 14-kilodalton and 16-kilodalton antigens for diagnosis and epidemiologic purposes. Am. J. Trop. Med. Hyg. 45:561–575.

19. Pinelli, E., R. Killick-Kendrick, J. Wagenaar, W. Bernadina, G. del Real, and J. Ruitenbergen. 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with Leishmania infantum. Infect. Immun. 62:229–235.

20. Portús, M., R. Fisa, T. Serra, M. Gállego, and M. Mora. 1987. Estudios seroepidemiológicos sobre la leishmaniasis canina en Cataluña. Med. Vet. 4:359–375.

21. Rahuel, A., H. Sahibi, N. Guessous-Idrissi, S. Lasri, A. Nataami, M. Riyad, and B. Berrag. 1999. Immune response against Leishmania infantum antigens in dogs naturally and experimentally infected with Leishmania infantum. Vet. Parasitol. 81:171–184.

22. Saad, A., H. Louzir, A. Ben Salah, M. Mokni, A. Ben Osman, and K. Dellagi. 1999. Leishmanin skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic Leishmania major infection in Tunisia. Clin. Exp. Immunol. 116:127–132.

23. Sideris, V., G. Papadopoulou, E. Dotsika, and E. Karagouni. 1999. Asymptomatic canine leishmaniasis in Greater Athens area, Greece. Eur. J. Epidemiol. 15:271–276.

24. Solano-Gallego, L., J. Llull, G. Ramos, C. Riera, M. Arboix, J. Alberola, and L. Ferrer. 2000. The ibizian hound presents a predominantly cellular immune response against natural Leishmania infection. Vet. Parasitol. 90:37–45.

25. Solano-Gallego, L., P. Morell, M. Arboix, J. Alberola, and L. Ferrer. 2001. Prevalence of Leishmania infantum infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. J. Clin. Microbiol. 39:560–563.