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Comparison of Indirect Immunofluorescent-Antibody Assay, Enzyme-Linked Immunosorbent Assay, and Western Immunoblot for the Diagnosis of Lyme Disease in Dogs

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Enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent-antibody assay (IFA), and Western immunoblot were used to test serum samples from 128 dogs for the presence of antibody to *Borrelia burgdorferi*. Sera included 72 samples from dogs suspected of having Lyme disease, 32 samples from dogs residing in areas in which Lyme disease was not considered endemic, and 24 samples from dogs with clinical and serologic evidence of immune-mediated disease (n = 10), Rocky Mountain spotted fever (n = 5), or leptospirosis (n = 9). Results of Western immunoblotting were used as the standard against which performances of ELISA and IFA were measured. ELISA was significantly more sensitive than IFA (84.8 versus 66.7%), although both tests were equally specific (93.5%). Eight samples that were positive by Western immunoblot were simultaneously negative by ELISA and IFA. Of these eight, four were from dogs suspected of having immune-mediated disease, two were from dogs suspected of having leptospirosis, and two were from dogs suspected of having Lyme disease. These results may indicate that sera from dogs with immune-mediated disease, and to a lesser extent sera from those with leptospirosis, cross-react with *B. burgdorferi* antigens. Alternatively, Western immunoblot results may not truly reflect Lyme disease status, particularly in the case of dogs with immune-mediated diseases. At present, however, the use of Western immunoblotting as a diagnostic standard for dogs offers the best alternative to a clinical definition of disease.

Lyme disease (LD) was first described by investigators at Yale University in the early 1970s as a distinct constellation of symptoms in patients from the Lyme, Conn., area (18). Subsequent studies have shown that the disease is caused by infection with the spirochete *Borrelia burgdorferi* following exposure to infected ixodid ticks (2, 4, 17). With the advent of more careful surveillance and reporting techniques, LD has now received the dubious distinction of being the most common tick-transmitted zoonotic disease in the United States (5). Although early investigations were directed toward characterizing the disease in humans, it has been shown more recently that this organism can also infect both dogs and horses (9, 10, 13). The detection of exposure to *B. burgdorferi* in domestic animals is of interest to public health officials as well as to veterinarians. Dogs, in particular, inhabit the same environment as people. Because dogs have been shown to be at greater risk for exposure to the spirochete (7), their use as sentinel animals to detect areas in which LD is endemic has been proposed (11, 16).

Several methods are presently in use for detecting antibody to *B. burgdorferi* in people; these include antibody capture, Western immunoblot (WB), enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay (IFA) techniques (3, 6, 12, 15, 20). Antibody capture has recently replaced WB as the most sensitive test when measured against a clinical definition of disease (3). ELISA, however, has been used extensively in clinical and epidemiologic studies because of its relatively high sensitivity and specificity coupled with its ease of performance (6, 8, 12, 15, 20). For detection of *B. burgdorferi* antibody in dogs, no clinical standard against which to measure the performance of serologic tests currently exists. Since antibody capture techniques have not yet been adapted for use with dogs, we used WB results as a standard against which the performance of an ELISA developed in this laboratory and that of a standard IFA for the detection of *B. burgdorferi* antibody in dogs was evaluated.

MATERIALS AND METHODS

Case definition. For two reasons, it was not possible to develop a clinical definition of LD in dogs against which performance of diagnostic tests could be measured. Erythema migrans, an early and characteristic sign of LD in people, has not yet been reported in dogs (9, 10). Furthermore, the clinical signs of LD in dogs are nonspecific and easily confused with those of other diseases and conditions, such as immune-mediated disease, Rocky Mountain spotted fever (RMSF), and traumatic injury. Positive and negative canine sera in this study were thus identified on the basis of immunoblot test results. Serum samples from 128 dogs were tested by WB; positive sera were considered to have been taken from dogs exposed to *B. burgdorferi*, and negative sera were considered to have been taken from dogs not previously exposed to *B. burgdorferi*. The 128 samples included 72 serum samples from dogs suspected by clinicians of having LD, 32 serum samples from a random sample of dogs residing in areas in which LD is not endemic and presented to veterinarians for reasons unrelated to LD (e.g., flea control, spaying and neutering procedures, vaccinations, heartworm testing, and routine biochemical and hematologic tests), and 24 serum samples sent to the Tufts Veterinary Diagnostic Laboratory for diagnostic testing

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other than for LD. The latter group included dogs with presumptive diagnoses of immune-mediated disease (e.g., systemic lupus erythematosis or rheumatoid arthritis), RMSF, and leptospirosis. A total of 10 dogs with positive serologic results for antinuclear antibody or rheumatoid factor or both, clinical signs consistent with immune-mediated disease, and 9 of 10 dogs with unlikely exposure to *Ixodes dammini* constituted the immune-mediated arthritis group. Nine dogs with clinical signs consistent with acute onset of leptospirosis and a single antibody titer of ≥1:1,600 for leptospirosis and *Leptospira interrogans* serovar canicola, pomona, icterohaemorrhagiae, grippotyphosa, or hardjo constituted the leptospirosis group. Five dogs with clinical, epidemiologic, and serologic results interpreted by veterinary clinicians as being strongly suggestive of RMSF constituted the RMSF group.

Sera were stored at −20°C, and initial tests were performed within 7 days of arrival. Sera were subsequently stored at −20°C for up to 18 months, until further testing was performed.

Case and control sera were coded, intermixed, and tested by WB, IFA, and ELISA without prior knowledge of the clinical status of an animal or its response to specific therapy.

Antigen preparation. *B. burgdorferi* from an Ipswich, Mass., strain that had been passaged multiple times was donated by Andrew Spielman. Actively growing cultures of *B. burgdorferi* in modified Kelly medium (1) were centrifuged at 12,500 × *g* for 1 h at 4°C. The pellet of organisms was suspended in Hanks balanced salt solution without Ca^{2+} and Mg^{2+}. The organisms were centrifuged again and suspended in one-tenth the original volume of Hanks balanced salt solution.

IFA for antibody to *B. burgdorferi*. Immunofluorescence slides were prepared by diluting the *Borrelia* suspension 1:16 in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} and placing 5 μl on each of 12 wells of an IFA slide (Cel-line, Newfield, N.J.). Slides were air dried, fixed for 20 min in cold acetone at −20°C, and stored at −70°C until used. Serum samples were tested at twofold dilutions from 1:16 to 1:2,048 by a standard IFA technique (14). Polyvalent rabbit anti-canine gamma globulin fluorescein isothiocyanate conjugate (Antibodies, Inc., Davis, Calif.) was used at a predetermined optimum dilution. Previous work in this laboratory has established that a titer of ≥1:128 was consistent with clinical and epidemiologic signs of LD in dogs.

ELISA. Reagents for ELISA were obtained from Cambridge BioScience, Inc. (Worcester, Mass.). Serum samples and positive and negative controls were diluted to 1:100 in sample diluent, added to wells coated with antigens derived from *B. burgdorferi*, and incubated at 22°C for 1 h. Wells were washed with a solution of deionized water and 0.05% nonionic detergent, and horseradish peroxidase-labeled goat anti-canine immunoglobulin G (heavy and light chain specific) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well. Wells were incubated for 30 min at 22°C, and the wash procedure was repeated. Hydrogen peroxide and tetramethyl-benzidine were added to each well and allowed to react for 10 min. The reaction was then stopped with 1 N sulfuric acid, and the plate was read at 450 nm on a microwell reader (Biotek Instruments, Winooski, Vt.). A cutoff value of 0.170 absorbance units was used to distinguish between positive and negative samples. This value is 3 standard deviations above the mean optical density (OD) for serum samples from 34 dogs residing in areas in which LD was nonendemic and presented to veterinarians for routine reasons (e.g., flea allergy dermatitis, spayings and castrations, and vaccinations). Known high-positive (≥0.400), low-positive (≥0.170 and <0.400), and negative (<0.170) sera and an antigen-only blank control were included on each plate tested.

WB. The procedures used for immunoblotting were similar to those described by Towbin et al. (19). Briefly, the proteins in the concentrated antigen preparation were transferred overnight at 0.6 A from a reducing sodium dodecyl sulfate-polyacrylamide electrophoresis gel to nitrocellulose in 192 mM glycine–24 mM Tris base–20% methanol. Strips of the nitrocellulose were blocked with a detergent and then incubated for 2 h at room temperature with test sera at a 1:100 dilution. After the strips were washed, they were incubated for 1 h at room temperature with peroxidase-labeled goat anti-canine immunoglobulin G conjugate (Kirkegaard & Perry). The strips were washed again. Reacting bands were visualized with a solution of 0.05% 4-chloro-1-naphthol with 0.015% hydrogen peroxide. Based on previous test results for erythema migrans-positive human cases (unpublished observations), serum samples that exhibited reactivity at two or more bands were considered positive.

Statistical analysis. (i) Correlation between IFA and ELISA. The degree of correlation between ELISA absorbance values and the logarithms of IFA titers was assessed by a standard linear regression technique.

(ii) Intra-assay reproducibility of ELISA. Ten serum samples, 2 each demonstrating high, low, and no reactivity by ELISA and 4 demonstrating reactivity near the cutoff value between negative and positive OD values (0.170), were replicated nine times on a single ELISA plate. Replicates for a given serum sample were randomly assigned to well positions. Standard deviations were calculated for each sample.

(iii) Interassay reproducibility of ELISA. Nine serum samples, three each demonstrating high, low, and no reactivity by ELISA, were tested in 11 separate assays over a 2-month period. A repeated-measures analysis of variance was used to determine the contribution of time to total variation in OD measurements.

RESULTS

WB. A total of 66 samples tested positive and 62 samples tested negative by WB. Five of ten dogs (50%) with immune-mediated disease, two of nine dogs (22%) with leptospirosis, and one of five dogs (20%) with RMSF tested positive by WB.

Comparison of ELISA and WB. Of the 66 serum samples positive by WB, 56 (84.8%) tested positive by ELISA. Of the 10 false-negative samples, 5 (50%) were from dogs with immune-mediated disease, 3 (30%) were from dogs whose sera were submitted for LD testing, and 2 (20%) were from dogs with leptospirosis. OD measurements on 8 of the 10 dogs were within 1 standard deviation of the mean used to establish the cutoff point for seropositivity (0.049 ± 0.040). All five of the serum samples from dogs with immune-mediated disease fell into this category.

Of the 62 serum samples negative by WB, 58 (93.5%) tested negative by ELISA. Of the four false-positive samples, one was taken from a dog with mammary adenocarcinoma, one was from a dog with leptospirosis, and two were from dogs with clinical signs suggestive of LD. The OD values for these four dogs were all within the low-positive OD range.

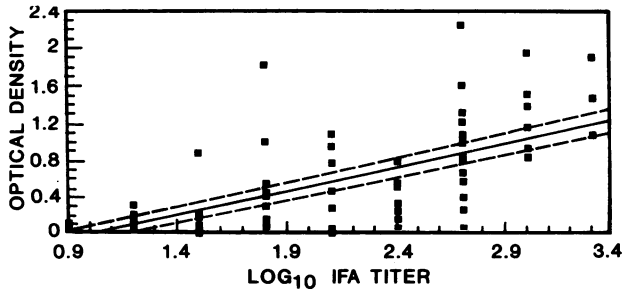

FIG. 1. Relationship between $\log_{10}$ antibody titer as measured by IFA and the OD at 450 nm as measured by ELISA for antibody to *B. burgdorferi* (n = 128). ——, Mean; ----, 95% confidence interval.

When the 10 serum samples from dogs with immune-mediated disease were excluded from the analysis, sensitivity of ELISA increased to 91.8% (56 of 61 samples) and specificity declined slightly, to 93.0% (53 of 57 samples).

Comparison of IFA and WB. Of the 66 serum samples positive by WB, 44 (66.7%) tested positive by IFA. Of the 22 false-negative samples, 14 (63.6%) were from dogs whose sera had been submitted for LD testing, 4 (18.2%) were from dogs with immune-mediated disease, 2 (9.1%) were from dogs with leptospirosis, 1 (4.5%) was from a dog with RMSF, and 1 (4.5%) was from a dog whose serum had been submitted for a hematologic and chemistry profile. None of the false-negative samples had a titer greater than 1:64, and 68% had titers of ≤1:32. Reactivities of three of four false-negative samples from dogs with immune-mediated disease measured less than 1:16.

Of the 62 serum samples negative by WB, 58 (93.5%) tested negative by IFA. Three of the four false-positive serum samples were from dogs whose sera had been submitted for LD testing; the other sample was from a dog whose serum had been submitted for a hematologic and clinical chemistry profile. Three of the four were positive at the cutoff value of 1:128, and one was positive at 1:256. None of the false-positive IFA samples were simultaneously positive by ELISA.

When the 10 serum samples from dogs with immune-mediated disease were excluded from the analysis, sensitivity of the IFA increased slightly, to 70.5% (43 of 61 samples), and specificity decreased slightly, to 93.0% (53 of 57 samples).

Comparison of ELISA and IFA. By simple linear regression of OD versus log IFA, r^2 was 57.7% ($P < 0.0001$) (Fig. 1). Overall agreement of ELISA and IFA was 75% (96 of 128 samples). ELISA demonstrated greater sensitivity than IFA, regardless of whether sera from dogs with immune-mediated diseases were or were not included in the analysis (84.8 versus 66.7%, $\chi^2 = 7.56$, and $P < 0.01$ and 94.8 versus 74.1%, $\chi^2 = 11.30$, and $P < 0.005$, respectively). Eight samples that were positive by WB were simultaneously negative by ELISA and by IFA. Of these eight, four were from dogs with immune-mediated disease, two were from dogs with leptospirosis, and two were from dogs suspected of having LD. With regard to specificity, there appears to be no difference between the two tests regardless of whether sera from dogs with immune-mediated diseases were or were not included (93.5 and 93.0%, respectively). None of the four samples that were negative by WB but positive by IFA were likewise positive by ELISA.

Intertest variation of ELISA. By repeated-measures analysis of variance, the effect of testing nine serum samples by ELISA on different days over a 2-month period was determined to be insignificant ($P = 0.21$). For the three high-positive samples, standard deviations ranged from 29 to 35% of each mean. For the three low-positive samples, standard deviations ranged from 22 to 28% of each mean. For the three negative samples, standard deviations ranged from 18 to 22% of each mean.

Intratest variation of ELISA. For the 10 samples tested by ELISA, standard deviations ranged from 9 to 44% of the mean values.

DISCUSSION

Without a more specific clinical marker of disease, canine LD cannot at the present time be clinically defined. Lacking such a clinical definition, other criteria must be relied on in order to document exposure. Previous research has demonstrated that the most sensitive and specific serologic test available for diagnosis of LD antibody is the antibody capture method (3). This test has not yet been adapted for detection of *B. burgdorferi* antibodies in dogs. Equally specific but slightly less sensitive is the WB, which had previously been the diagnostic standard for LD in people (8). At present, this test offers the best alternative to established clinical criteria for documenting *B. burgdorferi* infection in dogs.

When WB results were used as the standard against which the performances of other tests were evaluated, ELISA demonstrated significantly greater sensitivity than did IFA (84.8 versus 66.7%). Specificities of both tests were the same. There appears to be no pattern to the distribution of false-positive samples. None of the false-positive ELISA samples were from the same dogs as the false-positive IFA samples. The arithmetic mean OD of the four ELISA-positive samples was 0.240. This value is more than 1 standard deviation below the mean OD value for the remaining ELISA-positive samples (0.812 ± 0.499). The geometric mean titer for the four IFA-positive samples was 152. This value was well below the geometric mean titer for the remaining IFA-positive samples, which was 430. Relative to the distributions for all positive ELISA and IFA measurements, then, false-positive measurements fell at the lower end of the distribution.

With regard to the false-negative samples, however, a striking pattern was observed. For ELISA, 50% (5 of 10) and 20% (2 of 10) of the samples and for IFA, 18.2% (4 of 22) and 9.1% (2 of 22) of the samples were from dogs with presumptive immune-mediated disease and leptospirosis, respectively. No WB-positive dog with elevated leptospirosis titers had ELISA and IFA results that were simultaneously negative. However, of the five WB-positive dogs with presumptive immune-mediated disease, four were simultaneously negative by both ELISA and IFA. Furthermore, all five OD measurements on the samples positive by ELISA were well within 1 standard deviation of the mean established for LD-negative dogs. Similarly, all four titers on the samples positive by IFA were well below the cutoff value of 1:128 (three were nonreactive at <1:16; one was positive at 1:32).

Two possibilities could explain the discrepancy between WB test results and those of other serologic tests with dogs with presumptive immune-mediated disease. These dogs may have been seen in the early stages of LD, when clinical signs often resemble those of the immune-mediated arthritides. If this were the case, immunoblotting, because of its greater sensitivity, may have correctly identified these cases, whereas the presumably less sensitive ELISA and

IFA could not. This still does not explain the facts, however, that most of these dogs tested positive for antinuclear antibody or rheumatoid factor or both and that 9 of the 10 dogs were presented to veterinarians in areas in which LD is not highly endemic. An alternative explanation is that the increased levels of circulating antibody produced in immune-mediated disease cross-reacted with *B. burgdorferi* antigens. This would be more likely to occur with immunoblotting than with ELISA because immunoblotting makes use of whole spirochetal antigens, whereas the ELISA tested here uses spirochetal antigens that have been purified. Low inherent sensitivity may explain the lack of reactivity in IFA despite the use of whole organisms. At present, we cannot rule out either possibility. The latter explanation does, however, allow for the possibility that in the case of dogs with immune-mediated disease, WB results may not be the best indicator of the true LD status of an animal.

For these reasons, then, data excluding results with serum samples from dogs with immune-mediated disease were also examined. Under these circumstances, sensitivity of the ELISA increased to 91.8% and specificity declined slightly, to 93.0%. Sensitivity of the IFA likewise increased, to 70.5%, although specificity decreased slightly, to 93.0%.

The ELISA may be particularly useful in differentiating between cases of RMSF and cases of LD. RMSF cases often present with clinical signs nearly identical to those of LD. Furthermore, some areas in which LD is endemic, such as Cape Cod, Mass., are likewise areas in which RMSF is endemic, although the tick vectors of these diseases are not the same. In the acute stage of RMSF, it is not uncommon for antibody production to *Rickettsia rickettsii* to be low, necessitating the submission of a convalescent-stage serum sample several weeks later for demonstration of seroconversion. Dogs with LD, on the other hand, because they lack an early sign of disease, usually present to the veterinarian somewhat later in the course of this disease than do people, at a time when levels of antibody to *B. burgdorferi* are more likely to be elevated. Of the five cases of presumptive RMSF examined here, all five tested negative for LD by ELISA, IFA, and WB. There appears to be no cross-reactivity between these two diseases. Thus, if a single serum sample tested negative for both diseases, a presumptive diagnosis of RMSF would be more likely than one of LD. This distinction may be academic, since the recommended therapies for these two diseases are the same. On the other hand, given the current debate concerning duration of treatment in human LD cases, the distinction between these two diseases may be important should recommended duration of therapy for LD be extended.

Dogs are routinely vaccinated with *Leptospira* bacterins. Previous work has shown minor antigenic relatedness between *B. burgdorferi* and 20 *Leptospira* serovars (10). The authors of that study found no positive correlation between antibody titers to *Leptospira* serovars and *B. burgdorferi* for individual serum samples, although the presence of leptospiral antibody was distinctly different for serum samples with and without antibody to *B. burgdorferi*. They concluded that minor antigenic cross-reactivity or polyclonal B-lymphocyte stimulation secondary to *B. burgdorferi* infection could explain these observations. In the present study, nine dogs with clinical and serologic evidence of exposure to leptospiral antigens were tested. Two of the nine were positive for LD by WB. Another dog was positive for LD by ELISA. None of the nine were positive for LD by IFA. It is possible that the two WB-positive dogs were concurrently infected with *B. burgdorferi* and that WB, because of its inherently greater sensitivity, correctly identified these sera as positive. An alternate interpretation is that these results provide additional evidence of cross-reactivity between spirochetal antigens.

ELISA developed in this laboratory, then, appears to be more sensitive than the previously employed IFA for the detection of antibody to *B. burgdorferi*. Practicality likewise supports the use of ELISA in preference to IFA. WB will continue to be useful for sorting out equivocal test results or for whenever timely confirmation of LD status is required. Results of WB testing should be interpreted with caution, however, in the case of dogs suspected of having immune-mediated disease. Studies to better characterize the clinical course of LD in dogs are currently under way in this laboratory in the hope that an alternative to using WB as a diagnostic standard may be found.

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