Serologic Analysis of Dogs, Horses, and Cottontail Rabbits for Antibodies to an Antigenic Flagellar Epitope of *Borrelia burgdorferi*

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Enzyme-linked immunosorbent assays (ELISA) and immunoblots using either whole-cell lysates of *Borrelia burgdorferi* or an antigenic region of flagellin (41-G) as the antigen were performed, and the abilities of the two assays to detect antibodies to this spirochete in dog, cottontail rabbit, and horse sera were compared. Assays using whole-cell *B. burgdorferi* lysates as the antigen were more sensitive for detecting antibodies. ELISA with 41-G as the antigen were specific for *Borrelia* antibodies but were not as sensitive as the assays with whole-cell lysates coated to the solid phase. Use of recombinant full-length flagellin, rather than 41-G, as the antigen in immunoblots increased the sensitivity of each assay. However, antibodies to other bacterial antigens cross-react with whole flagellin and may account for false-positive results. Antibodies to *B. burgdorferi* outer surface protein A or B were usually undetected when the sera were tested by immunoblotting methods. *Borrelia* lysates or the 41-G antigen may be used in ELISA or immunoblots to document host exposure to this spirochete. The use of 41-G as the antigen may increase the specificity of an assay or help confirm the serologic diagnosis of Lyme borreliosis in dogs, horses, and cottontail rabbits.

Lyme borreliosis is the most common tick-associated disease in the United States (30). The causative agent, *Borrelia burgdorferi*, is mostly transmitted by the bites of ticks in the *Ixodes ricinus* complex and can infect humans and various domesticated animals including dogs and horses (3, 4, 11, 13, 16, 19, 30). The clinical manifestations of disease in both humans and animals may be absent or difficult to discern (15, 23, 30). Signs of disease in dogs, horses, and other animals may include lameness, carditis, or a flu-like illness, but an accurate description of the stages of Lyme borreliosis in wildlife and domesticated animals is still being defined (12, 16, 17, 19, 21, 22, 31).

Serologic testing for antibodies to *B. burgdorferi* may be used to help document infection by this bacterium in wildlife, domesticated animals, and humans (5, 6, 9, 12, 14, 17, 19-23). The current serologic tests, however, are confounded by a lack of sensitivity in early infection and by false-positive results caused by the reactivity of antibodies produced during other infectious or inflammatory diseases to *B. burgdorferi* antigens (1, 18, 20, 25). Much of this work has been elucidated in human Lyme borreliosis, but the principles apply to veterinary diseases as well. Antibodies to the *B. burgdorferi* flagellin develop early during infection and may, therefore, be a good indicator of exposure to this spirochete (2, 5, 9, 10, 14, 30). Flagellin, however, shares epitopes with other infectious agents, such as treponemes and leptospires, and antibodies to flagellin may account for the false-positive reactivity (19, 20).

To develop more-specific reagents for antigens in diagnostic assays, we have identified immunogenic epitopes of flagellin. We have shown that an epitope of flagellin, containing amino acids 197 to 241 and designated 41-G, reacts

with antibodies in human sera (2, 9, 24). Others have also shown that antigenic epitopes in a similar region of flagellin are recognized by sera from patients (27, 28). Serologic tests, including both enzyme-linked immunosorbent assays (ELISA) and immunoblots using 41-G as the antigen appear to be more specific in documenting exposure to *B. burgdorferi* in humans than assays with whole-cell lysates (2, 9, 24). Indeed, Gassmann and coworkers have reported that certain epitopes of flagellin are reactive with animal sera as well (10). The usefulness of whole-cell lysates or 41-G as antigens in assays in diagnosing veterinary Lyme borreliosis, however, needs further study. The purpose of this study was to compare the abilities of ELISA and immunoblots with whole-cell *B. burgdorferi* lysates and an antigenic region of flagellin (41-G) from this spirochete as the antigens to help diagnose Lyme borreliosis in dogs, horses, and cottontail rabbits (*Sylvilagus floridanus*).

MATERIALS AND METHODS

Collection of serum samples. Blood samples were obtained from horses living in communities with the tick vector *Ixodes scapularis*, formerly known as *Ixodes dammini* (26), in Connecticut, Massachusetts, and New York State (19). Serum samples were also received from veterinarians who drew blood from privately owned dogs of various breeds in southern Connecticut, representing rural, suburban, and urban communities where Lyme borreliosis is prevalent (21, 23). Cottontail rabbits were captured in wooden box traps in New York State and Connecticut. Blood samples were taken from the rabbits, and serum samples were stored at -60°C until antibody analyses could be conducted (22). The sources of positive and negative serum controls for all animal groups have been reported elsewhere (17, 19, 22). The sera used included sera from a New Zealand White rabbit inoculated

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with *B. burgdorferi*, dogs that had antibodies to specific proteins (OspA and p39) of *B. burgdorferi*, and horses that had been exposed to *B. burgdorferi*-infected *I. dammini* and had limb or joint disorders.

Antigen preparation. Washed, whole-cell lysates of *B. burgdorferi* (strain 2591), the prototypic strain in our ELISA, were used as previously reported (24). Details on the amplification of the 41-kDa flagellin and outer surface protein A and B (OspA and OspB) genes by the polymerase chain reaction have been described elsewhere (2, 7–10). All recombinant antigens were expressed and purified as glutathione transferase fusion proteins. On the basis of previous serologic analyses, an antigenic region of the *B. burgdorferi* flagellin that contained amino acids 197 to 241 was designated 41-G. This antigen was strongly reactive with human sera (2, 9, 24). We chose the 41-G antigen for testing animal sera because of the encouraging results in serologic analyses of human sera in different laboratories (9, 24). Furthermore, 41-G has limited sequence similarity to the flagellin of treponemes, such as *Treponema pallidum*, and other bacteria that can cause immune responses in hosts and indirectly contribute to false-positive reactivity in diagnostic tests for Lyme borreliosis (9, 18, 24).

ELISA. To quantitate the concentration of total immunoglobulins to whole-cell lysates of *B. burgdorferi* or the recombinant 41-G antigen, an ELISA was used. Preliminary analyses of positive-control sera revealed that antigen concentrations of 3 and 5 µg of protein per ml were suitable for whole-cell lysate and the 41-G antigen, respectively. Dog, horse, and cottontail rabbit sera were diluted ≥1:160 and screened against both types of antigen in parallel tests on the same immunoplates. Polyvalent horseradish peroxidase-labeled antisera and substrate (2,2'-azino-di-3-ethyl-benzthiazoline sulfonate) were used in all assays. Details on sources and working dilutions of antigens, control sera, and other reagents, methods of determining optical density values for positive results, and procedures for performing ELISA with various mammalian species have been published (19, 22–24). Each plate contained positive- and negative-control sera, affinity-purified glutathione transferase, and phosphate-buffered saline (PBS) to ensure standardization before routine use. Glutathione transferase was included because the 41-G antigen was purified as a glutathione transferase fusion protein.

Tests on specificity were conducted to further evaluate the serum samples and antigens used in ELISA. Reference rabbit antisera to *B. burgdorferi* (homologous antibody titer of ≥1:10,240) or to *Borrelia hermsii* were reanalyzed as previously described (22) against whole-cell lysates of *B. burgdorferi* and screened against recombinant 41-G in parallel ELISAs. In addition, these antisera were tested against homologous and heterologous whole-cell antigens by indirect fluorescent-antibody staining methods to verify reagent activity. Fluorescein-labeled goat anti-rabbit total immunoglobulins were obtained commercially (Grand Island Biological Company, Grand Island, N.Y.) and were diluted in PBS to 1:50. The following antigens were tested: *B. hermsii* (strain HS1 serotype C), *B. burgdorferi* (strain 2591), *T. pallidum*, *Leptospira interrogans* serovar pomona (strain MLS) and *L. interrogans* serovar grippotyphosa (strain SC4397). The sources and methods of preparing antigens and antisera have been reported elsewhere (22).

Immunoblots. For the immunoblots, 1.5 µg of *B. burgdorferi* (strain 297) and 500 ng of a recombinant antigen (41-G, OspA, OspB, or flagellin) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 12% poly-

TABLE 1. Reactivities of dog, horse, and cottontail rabbit sera to *B. burgdorferi* whole-cell lysates and purified flagellin fragment (41-G) in ELISA

Study group	No. of serum samples tested	No. of serum samples positive		Geometric mean antibody titer ^a	
		WCL ^b	41-G ^c	WCL	41-G
Dogs	40	28	12	987	123
Horses	31	21	6	856	64
Cottontail rabbits	51	26	9	257	69

^a A value of 40, the average titer for negative sera, was used for each negative sample to compute geometric means.

^b WCL, whole-cell lysate of *B. burgdorferi*.

^c Recombinant protein.

acrylamide gel. Strain 297 of *B. burgdorferi* is the prototypic strain (used in our previous immunoblot assays) and is similar to strain 2591. Proteins were transferred to nitrocellulose sheets, and the nitrocellulose sheets were probed with sera, diluted 1:100 in PBS with 5% bovine serum albumin (BSA) for 1 h. The strips were washed three times with PBS and incubated with horseradish peroxidase-labeled goat antisera against rabbit, dog, or horse immunoglobulin, either immunoglobulin G (IgG) or IgM (Kirkegaard and Perry, Gaithersburg, Md.), diluted 1:3,000 in PBS with 5% BSA. After being washed in PBS, the sheets were developed using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, Ill.) and exposed to radioactive film.

RESULTS

Serum samples from dogs, horses, and cottontail rabbits, collected in regions of Connecticut and New York State where Lyme borreliosis is endemic, were tested for reactivity to *B. burgdorferi* lysates and 41-G by ELISA (Table 1). For the groups of sera that reacted positively in ELISA with whole-cell lysates, antibody titers ranged from 1:160 to 1:40,960. A total of 40 dog serum samples were evaluated. Of the 28 serum samples containing antibodies to whole-cell lysates, 12 (42.9%) reacted with 41-G. Six (28.6%) of 21 horse serum samples reactive in ELISA with whole-cell lysates were also positive in assays with 41-G antigen. Similarly, 9 (34.6%) of the 26 rabbit serum samples that had detectable antibodies to whole-cell antigen reacted positively in ELISA with 41-G. Additional tests were performed on serum samples from dogs ($n = 12$), horses ($n = 10$), and cottontail rabbits ($n = 15$) which were negative in ELISA with whole-cell lysates. One dog serum sample (titer, 1:1,280) and one rabbit serum sample (titer, 1:160) reacted with the 41-G antigen. The remaining specimens were negative when the recombinant antigen was used. In each group of animal sera, the geometric mean antibody titer was higher for sera tested with *B. burgdorferi* lysates than for sera tested with the 41-G antigen.

To test the specificities of whole-cell *B. burgdorferi* and 41-G antigens in ELISA, sera from New Zealand White rabbits inoculated with *Borrelia*, *Treponema*, or *Leptospira* spirochetes were tested. Rabbit anti-*B. hermsii* and rabbit anti-*B. burgdorferi* sera reacted with both the whole-cell lysate and the recombinant 41-G antigen of *B. burgdorferi* in ELISA. Antisera to *B. hermsii* had a titer of 1:2,560 to both types of *B. burgdorferi* antigen. Antisera to *B. burgdorferi* had titers of 1:40,960 and 1:10,240 to the homologous whole-

TABLE 2. Reactivities of serum samples to *B. burgdorferi* lysates, 41-G, and flagellin probed with dog, horse, or cottontail rabbit sera in immunoblots

Animal and no. of serum samples tested	Reactivity of sera in IgG immunoblots to antigen ^a :			Animal and no. of serum samples tested	Reactivity of sera in IgM immunoblots to antigen:		
	WCL	41-G	Flagellin		WCL	41-G	Flagellin
Dogs				Dogs			
5				6			
2	+			1			+
2	+		+	1		+	+
5	+	+	+	6	+	+	+
Horses				Horses			
1				12			
5	+		+	1	+		+
6	+	+	+				
1		+	+				
Cottontail rabbits				Cottontail rabbits			
6	+			2			
2	+			3	+		
2	+		+	7	+		+
2	+	+	+				

^a Whole-cell lysates (WCL) of *B. burgdorferi*, recombinant 41-G glutathione transferase fusion protein, and recombinant flagellin glutathione transferase fusion protein were used as antigens.

cell lysate and 41-G antigen, respectively. Rabbit antisera against *T. pallidum* and *L. interrogans* serovars pomona and grippityphosa showed little or no reactivity in ELISA regardless of the *B. burgdorferi* antigen used. By indirect fluorescent-antibody staining, homologous antibody titers of $\geq 1:16,384$ were recorded when whole-cell antigens were tested. Heterologous reactions for *Borrelia* species and *T. pallidum* had high titers ($>1:4,096$). However, as in ELISA, there was no cross-reactivity between the *Leptospira* antigens and antisera and those of *Borrelia* and *Treponema* species.

The animal sera, when quantities were sufficient, were used to probe *B. burgdorferi* lysates and recombinant antigens of this spirochete, including OspA, OspB, flagellin, and 41-G, in immunoblot assays. The results are summarized in Table 2. Representative immunoblots are shown in Fig. 1. Fourteen dog serum samples were evaluated in IgG immunoblot assays. Nine serum samples reacted with *B. burgdorferi* lysates, seven serum samples were positive to flagellin, and five serum samples reacted with 41-G. Since no dog sera showed reactivity with OspA or OspB, these data are, therefore, not individually included in Table 2. All of the recombinant *B. burgdorferi* antigens were expressed as glutathione transferase fusion proteins. Thus, glutathione transferase was included as a control antigen. As in ELISA, none of the serum samples reacted with glutathione transferase. In IgM immunoblots, 6 of 14 dog serum samples had antibodies to *B. burgdorferi* lysates, 8 serum samples had antibodies to flagellin, and 7 had antibodies to 41-G. In analyses of 13 horse serum samples, 11 samples had antibodies to *B. burgdorferi* lysates in IgG immunoblots. Twelve serum samples reacted with flagellin, while 7 serum samples reacted with 41-G. In IgM analyses, one serum sample reacted with whole-cell lysates and flagellin. There was no reactivity with 41-G. In IgG immunoblots of 12 cottontail rabbit serum samples, all specimens reacted with whole-cell lysates, 4 reacted with flagellin, and 2 reacted with 41-G. In IgM immunoblots, 10 cottontail rabbit serum samples reacted with *B. burgdorferi* lysates, while 7 reacted with flagellin.

DISCUSSION

Immunoplates coated with whole-cell lysates of *B. burgdorferi* can detect antibodies to this spirochete in dogs, horses, and rabbits. Cross-reactivity, however, between *B. burgdorferi* antigens and antibodies elicited during infection of animals with other bacteria, such as treponemes, or inflammatory disease, may account for false-positive results (1, 18, 25). Cross-reactivity has been documented in human disease and may confound the interpretation of serologic data for domestic animals and wildlife as well.

We performed serologic assays using 41-G, an antigenic region of flagellin, to determine whether this recombinant protein would be more specific and sensitive than whole-cell antigens for detecting antibodies to *B. burgdorferi*. 41-G has been shown to be a useful reagent in confirming human *B. burgdorferi* infection by serologic assays (9, 24). If the human and other mammalian immune responses are similar, as indicated previously, then 41-G would be an appropriate diagnostic antigen (10). On the basis of our analyses of animal sera and specificity tests, some animal sera that were reactive with whole-cell *B. burgdorferi* lysates in ELISA or immunoblots were also positive when 41-G was used. The 41-G antigen appears to be highly specific for *Borrelia* infection. Antibodies to OspA and OspB, however, were virtually undetected in the sera. This result agrees with our previous data, showing that the OspA and OspB humoral response does not occur until late in human infection (9). Although 41-G might be highly specific for *Borrelia* infection, the sensitivity of an ELISA using this antigen is not yet optimal.

Several possibilities exist for the current low sensitivity of the assay using 41-G. Immunoglobulins in sera of animals that react with the *B. burgdorferi* lysate may be cross-reactive antibodies elicited during other infectious or inflammatory diseases—as occurs in human disease. Some of the sera reactive to whole-cell lysates in our study could represent false-positive results. In this case, a specific assay, including 41-G antigen, might reduce the number of false-positive reactions, as previously reported for sera from

FIG. 1. Immunoblots of whole-cell lysates or recombinant antigens of *B. burgdorferi* probed with dog or horse sera. Lanes 1, whole-cell lysates of *B. burgdorferi* antigen; lanes 2, recombinant OspA glutathione transferase fusion protein; lanes 3, recombinant OspB glutathione transferase fusion protein; lanes 4, recombinant 41-G glutathione transferase fusion protein; lanes 5, recombinant flagellin glutathione transferase fusion protein; lanes 6, glutathione transferase. The immunoblots were probed with positive dog sera with a titer of 1:5,120 (A) or 1:1,280 (B) or with horse sera with a titer of 1:640 (C).

humans (24). Cross-reactivity could account for the greater number of serum samples positive to flagellin. Another possibility is that the ELISA using 41-G may need to be modified to enhance sensitivity. We have previously shown that using a biotin-streptavidin amplification system in conjunction with adsorption procedures can increase the sensitivity of a 41-G-based antibody detection system (24). An alternative explanation would be that although 41-G is frequently recognized by antibodies in humans, the animal antibody response may not be as strongly directed against this epitope. Therefore, other flagellin epitopes may be more important for animal diagnostic assays (27, 28). In addition,

other recombinant *B. burgdorferi* antigens, such as p39, an antigen recognized during human infection, may prove useful in laboratory diagnosis of Lyme borreliosis in animals (29).

The use of recombinant *B. burgdorferi* antigens for the laboratory diagnosis of veterinary Lyme borreliosis must be further explored to be clinically useful. Whole-cell lysates of this spirochete appear to be effective in detecting antibodies in dogs, horses, and rabbits. However, the potential for false-positive reactions may confound test data, as has occurred with serologic results for humans. The 41-G antigen, in particular, may be useful as a diagnostic reagent in ELISA because the specificity of this antigen is greater than that of whole-cell antigens.

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