Lyme disease (LD) is a tick-borne infection caused by the bacterial pathogen *Borrelia burgdorferi*. Current diagnostic tests mostly use borrelial lysates or select antigens to detect serum antibodies against *B. burgdorferi*. These immunoassays are not entirely effective, especially for detection of early infection. We have recently characterized an *in vivo*-induced antigen, BBK07, as a serodiagnostic marker for LD. We now report that in a line blot assay, recombinant BBK07 protein-based detection is 90% sensitive and nearly 100% specific against *B. burgdorferi* infection in humans. Using an overlapping peptide library of 23 peptides encompassing full-length BBK07, we identified the immunodominant epitopes of BBK07 during human infection. We show that a select combination of amino-terminal peptides significantly enhanced BBK07-based diagnostic accuracy compared to that with the full-length protein. Although in enzyme-linked immunosorbent assay (ELISA) studies BBK07 peptides had overall lower sensitivity than established serodiagnostic peptides, such as the VlsE peptide C6 and OspC peptide pepC10, for the detection of early human LD, a subset of serum samples that failed to recognize either VlsE or OspC peptides were preferentially reactive to BBK07 peptides. These results highlight the fact that BBK07 peptides could be useful to complement the efficacy of VlsE and OspC peptide-based serodiagnostic assays. Finally, using a panel of canine sera, we show that BBK07 peptide is also effective for LD diagnosis in infected dogs. Together, our data show that peptides from the *B. burgdorferi* surface protein BBK07 are highly specific and sensitive serodiagnostic markers, and we suggest their future use in LD diagnostic assays.
for IgM antibodies during the early infection (4). The synthetic peptide C6, isolated from a conserved region of the variable membrane protein VlsE, is a target for host IgG and has been shown to be a sensitive and specific serodiagnostic marker (4, 22). While purified antigens show great promise, no recombinant or synthetic antigen has demonstrated sufficient sensitivity to replace the current two-tier approach (7, 14, 16, 20, 24, 26, 27, 35). Some of the highest sensitivities reported thus far have been obtained by the use of several antigens in combination to enhance diagnostic accuracy (4, 8, 19, 31, 32). However, there remains a need for improvement in sensitivity, especially for detection during the earliest stages of disease (1). The addition of new immunogenic epitopes could allow these tests to eventually supplant the two-tier approach, improving both the efficacy and cost of LD testing.

The *B. burgdorferi* lipoprotein BBK07 was identified as an immunodominant antigen in a study by Barbour et al. (6). We have recently shown that BBK07 is an *in vivo*-induced surface antigen which is selectively expressed during mammalian infection and is a promising serodiagnostic marker for LD (9). We demonstrated that an amino-terminal fragment of BBK07 could be used as a component of an effective serodiagnostic marker to detect human LD. In our current studies we further assessed the sensitivity and specificities of BBK07-based diagnosis using serum samples from North American and European patients with diagnosed LD and several other conditions, including syphilis and autoimmune diseases. We also assessed the serodiagnostic abilities of BBK07 using a full-length protein and an overlapping peptide library, identifying the most immunogenic epitopes of BBK07. We demonstrate that serum protein and an overlapping peptide library, identifying the most sensitive and specific antigen which is selectively expressed during mammalian infection and is a promising serodiagnostic marker for LD (9).

BBK07 peptides are able to detect both canine and human LD, even during the early stages of the disease. IgG and IgM ELISAs further show that a cohort of human LD sera failed to recognize VlsE-derived C6 or OspC-derived pepC10 peptide but reacted exclusively with BBK07 peptides, attesting to their potential use in enhancing the diagnostic sensitivity for early LD.

**MATERIALS AND METHODS**

**Bacteria and mice.** *Borrelia burgdorferi* isolates B31 A3 (13) and 297 (28) were used in the current study. *Borrelia burgdorferi* clinical isolates B408, B491, B500, B515, BL203, and BL206 were isolated from human patients with diagnosed LD within 2 weeks and 13 years after the onset of symptoms. Serum samples from North American and European patients with diagnosed LD and several other conditions, including syphilis and autoimmune diseases. We also assessed the serodiagnostic potentials of BBK07 using a fragment of BBK07 protein and an overlapping peptide library.

**Peptide synthesis.** A total of 23 overlapping peptides (P1 to P23) encompassing the mature BBK07 protein were commercially synthesized (Sigma). The peptides were 14 amino acids in length, with an overlap of 4 amino acids on the amino- and carboxyl-terminal sides. The C6 peptide (CGMKGWFOALK) (22) and the pepC10 peptide (PVVAESPKKP) (25) were also commercially synthesized (GenScript). All peptides were labeled with biotin at the amino terminus and were dissolved in dimethyl sulfoxide, aliquoted, and stored at −20°C until use.

**Serum.** Human serum samples used in the line blot assay were provided by Genzyme Virotech (Germany). The samples included sera collected from a total of 100 LD patients and 70 control individuals, including healthy donors and those with conditions of pregnancy, primary Epstein Barr virus infection, related spirochete infection (syphilis), and inflammatory complications (autoimmune diseases or rheumatoid factor). For ELISA studies, the healthy control sera included 25 samples from healthy human individuals who tested negative for LD, which were provided by the Centers for Disease Control and Prevention (Atlanta, GA) and by Marylou Breitentein (Yale University). These control sera were used to define the cutoff value in each assay (mean plus three standard deviations [SD]). Additional infected-serum panels were provided by the CDC.

**Expression of BBK07.** Full-length BBK07 consisted of 33 serum samples collected from human patients diagnosed with LD between 2 weeks and 13 years after the onset of symptoms. Serum panel 2 consisted of 60 samples collected from 20 culture-positive patients who presented to medical doctors with erythema migrans. Serum was collected at the first doctor visit after the onset of disease (day 0) and at subsequent visits 10 and 20 days thereafter. Groups of five mice were infected with *B. burgdorferi* clinical isolates B408, B491, B500, B515, BL203, and BL206 at 104 cells/mouse, as detailed previously (37). Two weeks after inoculation, serum samples from individual animals were collected and stored separately. Serum from a representative mouse infected with a specific isolate was used for the immunoblot assay. Canine serum samples were provided by the Symbiotics Corporation and consisted of control and infected samples, as defined by immunoblotting analysis using *B. burgdorferi* lysates.

**ELISA and Immunoblotting.** For ELISA, antigens were diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6, and applied to MaxiSorp microtiter plates (Nunc). Recombinant BBK07, BBK07N, and *B. burgdorferi* lysate were applied at 100 ng per well and synthetic peptides (P1 to P23) (see Table 2) at 500 ng per well. BBK07 peptides and the C6 peptide were applied directly on ELISA plates at 500 ng per well. Although the BBK07 and C6 peptides were biotinylated at the N terminus, the use of streptavidin-coated plates did not influence the sensitivity of the assay (data not shown). However, in experiments using the pepC10 peptide, the plates were first coated with 400 ng per well of streptavidin (MP Biomedicals) before the addition of the peptide, in order to enhance sensitivity. The mean optical density (OD) value for the control sera plus three SD was considered the cutoff value to determine positivity in each sample. The plates were blocked using 1% bovine serum albumin (BSA) in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) and were washed extensively with PBS-T between all steps. Human and canine sera were diluted in 1:200 or 1:10, respectively, in 1% BSA in PBS-T. Immunoblotting were performed using nitrocellulose membranes blocked with 5% skim milk in PBS-T, using murine serum dilutions from 1:200 to 1:2,000. Secondary antibodies against IgG conjugated to horseradish peroxidase were used with the following dilutions: goat anti-mouse IgG, 1:10,000; goat anti-human IgG, 1:5,000; goat anti-human IgM, 1:20,000; and goat anti-dog antibody, 1:2,000 (KPL). All steps were carried out either for 1 h at 25°C or overnight at 4°C. ELISA results were quantified using SureBlue tetramethylbenzidine (TMB) substrate (KPL). Before the optical density was read in a microplate spectrophotometer, the ELISA reactions were terminated with TMB stop solution (KPL), except for in the canine ELISA experiments, where the reactions were stopped using 1% sodium dodecyl sulfate solution.

For the line blot assay, recombinant protein was transferred to nitrocellulose membranes by a microdispensing method as described previously (14). Strips were incubated with human sera (dilution, 1:100), and the binding of specific antibodies was detected by using alkaline phosphatase-conjugated goat anti-human IgG serum (1:100; Genzyme Virotech, Germany). Immunoreactive bands were visualized with the addition of tetramethylbenzidine substrate. Cutoffs were based on a standardized band intensity scale (version V1.083093; Genzyme Virotech GmbH) of 0 to 5, with 5 as the most intense and 0 as no band visible. Line blots were considered positive if the band intensity was > 2.

**Statistical analyses.** Statistical analyses were performed using MedCalc (MedCalc Software, Mariakerke, Belgium). The diagnostic performance of each antigen was compared pairwise using the area under the curve (AUC) from re-
TABLE 1. Assessment of serodiagnostic potential of BBK07N in a line blot assay

| Serum panel* | No. positive/total (% positive) in BBK07N line blot assay |
|--------------|----------------------------------------------------------|
| U.S. sera, Lyme disease IgG positive | 18/20 (90) |
| German sera, Early Lyme disease | 1/20 (5) |
| Neuroborreliosis | 1/20 (5) |
| Lyme arthritis | 2/20 (10) |
| ACA | 4/20 (20) |

Control sera
- Healthy blood donors | 0/20 (0) |
- EBV | 0/10 (0) |
- Syphilis infections | 0/10 (0) |
- Pregnant women | 0/10 (0) |
- Autoimmune diseases | 1/10 (10) |
- Rheumatoid factor | 0/10 (0) |

* Human serum samples were analyzed by a line blot assay with various amounts of the recombinant fragment BBK07N. ACA, acrodermatitis chronica atrophicans; EBV, primary Epstein-Barr virus infection.

RESULTS

BBK07 is highly sensitive and specific against B. burgdorferi infection in North American and European patients. We have recently shown that an amino-terminal fragment of BBK07 (BBK07N) could be used for detection of human LD in North America (9). To test if BBK07 immunoreactivity was conserved in European patients, recombinant BBK07N was tested using a line blot assay which is widely used for serodiagnosis of LB in Europe (17). To assess the specificity of BBK07-based diagnosis, a set of German and U.S. patients with diagnosed LD infection, as well as sera from healthy individuals, pregnant women, and patients with other conditions, including autoimmune diseases and syphilis, were compared in a line blot assay, and the results were scored using a standardized band intensity scale. While BBK07 was highly sensitive (90%) in detecting B. burgdorferi infection in serum from the United States, the German patients with LB or other diseases or healthy controls showed minor reactivity (Table 1). The observed lower sensitivity of BBK07 reactivity in European LB patient sera likely results from the infection with other B. burgdorferi sensu lato species. Overall, these data underscore the potential sensitivity and specificity of BBK07-based diagnosis of B. burgdorferi infection in U.S. patients.

A BBK07-specific immune response is elicited during murine infection with several clinical isolates of B. burgdorferi sensu stricto. In order to further characterize the diagnostic potential of BBK07, we measured its conservation and immunogenicity in multiple clinical strains which were isolated from human patients from North America (37). These isolates have been previously characterized by restriction fragment length polymorphism analysis. Isolates B491, B515, BL203, and BL206 have a genotype associated with invasive disease, while B408 and B500 are likely to cause less invasive infections (37). The B. burgdorferi clinical isolates were grown in BSK medium and injected into mice, and serum was collected at 2 or 3 weeks following infection. Infected murine sera were used to probe equal amounts of recombinant BBK07N or FlaB protein by immunoblot analysis (Fig. 1). A BBK07-specific antibody response was detectable in mice infected with the B. burgdorferi B31 laboratory isolate A3, as well as all clinical isolates. Although we lack human data, these murine studies raise the possibility that BBK07-based serodiagnosis may be able to detect LD infection with multiple clinical isolates of B. burgdorferi sensu stricto.

Identification of immunogenic epitopes of BBK07. We next mapped the immunodominant epitopes of BBK07 using human sera. To accomplish this, a library consisting of 23 overlapping peptides was synthesized, covering the full-length mature BBK07 (Table 2). Each peptide was tested for ELISA reactivity in serum samples from 25 healthy donors and 33 patients diagnosed with LD from serum panel 1. Given the

TABLE 2. BBK07 peptide library amino acid sequences

| Peptide name | Amino acid sequence |
|--------------|--------------------|
| P1 | CKWHVDNPIDEATA |
| P2 | EATAEKSKALTSDV |
| P3 | TSVDQVLDEISEAT |
| P4 | SEATGSLSEKLIK |
| P5 | ITKLTPEELENKA |
| P6 | NLAKEAOQDSEKSK |
| P7 | EKSSKEIEikedNTK |
| P8 | KNTKEKSNIEVKT |
| P9 | VKDTPRRLIKIKNS |
| P10 | IKNSESQKSDVFQ |
| P11 | VFQOLINIGNATY |
| P12 | NATYAAKSNLKGL |
| P13 | KNGLKMVKLLD |
| P14 | DELLKISVSSNGDK |
| P15 | NGDKSTOKYNELK |
| P16 | VLDTPQPNFKNAENS |
| P17 | AENSTFKVPLENS |
| P18 | ENGSKEAKCKICT |
| P19 | CIKLMLTN VyTFK |
| P20 | TYFGVSTELKDK |
| P21 | KDKDVKDPXKILAA |
| P22 | ILALSEAIAAN |
| P23 | KIENAAAMAIHLCFN |
number of peptides and the limited supply of serum, all peptides were screened once with each serum sample, and the most immunogenic peptides were repeated in three independent experiments to validate the results. The cutoff value was set at the mean plus three SD for the healthy controls for each antigen. The results indicated that amino-terminal peptides P1 (CKWHVDNPIDEATA), P5 (ITKLTPEELENLAK), and P7 (EKSSKEIEDQKNTK) harbored the most immunodominant epitopes recognized by the infected sera (Fig. 2A). Samples were considered positive if they exceeded the cutoff value in at least two out of three experiments.

A combination of BBK07 peptides provides greater diagnostic accuracy than recombinant BBK07. We have demonstrated that a combination of BBK07 peptides (Fig. 2) and a recombinant amino-terminal fragment of BBK07 (9) are effective in LD diagnosis. To assess whether the presence of conformational epitopes could improve the sensitivity of BBK07-based serodiagnosis, we produced recombinant full-length BBK07 in insect cells using baculovirus expression system. The full-length protein was tested against serum panel 1 as detailed above, and its reactivity was compared to those of the recombinant amino-terminal fragment BBK07N and the BBK07 peptide combination. B. burgdorferi lysate was also included as a positive control. Three independent measurements were performed, which produced similar results (data not shown), and results of a representative experiment are shown (Fig. 3). While we expected that the full-length BBK07 (which distinguished 13 out of 33 patient sera from control sera [39%]) should provide more epitopes for antibody recognition, the amino-terminal fragment BBK07N (14 out of 33 [42%]) showed no significant reduction in diagnostic accuracy, as assessed by statistical analysis (P = 0.181). However, the peptide combination (17 out of 33 [52%]) displayed a diagnostic accuracy superior to that of either BBK07N or full-length BBK07. Statistical analyses further indicated that there was a significant improvement in test performance for the peptide combination compared to the full-length protein (P = 0.048) but not for the peptide combination compared to the recombinant fragment BBK07N (P = 0.252).

BBK07 peptide-based diagnosis is effective during early infection. We next assessed if BBK07-based diagnosis is effective in early LD infection. Since the peptide combination displayed...
the highest BBK07-based serodiagnostic sensitivity, this was used in the study. Serum samples (serum panel 2) were collected from patients displaying erythema migrans on their first visit to the physician. Biopsy specimens from all patients were later culture positive for *B. burgdorferi*, and serum from each patient was collected again at 10 and 20 days after the first visit (day 0) to the doctor. Note that the serum is first collected from the patients upon their first visit to a physician and not at the time of initial exposure. Therefore, while it is not known how long after infection the serum samples were collected, the appearance of erythema migrans on the first visit to a physician makes it likely to be within the first few weeks of *B. burgdorferi* infection (36). The control sera from serum panel 1 were used to define the cutoff for each antigen as detailed above, and each antigen was used to test serum panel 2 in three independent measurements (Tables 3 and 4). The BBK07 peptide

### TABLE 3. Individual patient IgM reactivity against BBK07 (P1P5P7) and OspC (pepC10) for peptide-based serodiagnosis of early LD

| Patient | P1P5P7 | pepC10 |
|---------|--------|--------|
|         | Initial visit (0/20, 0%) | Day 10 (4/20, 20%) | Day 20 (2/20, 10%) | Initial visit (3/20, 15%) | Day 10 (10/20, 50%) | Day 20 (9/20, 45%) |
| 1       |        | +      | +      |        | +      | +      |
| 2       |        | +      | -      |        | +      | -      |
| 3       |        | -      | -      |        | +      | +      |
| 4       |        | -      | -      |        | +      | +      |
| 5       |        | -      | -      |        | +      | +      |
| 6       |        | -      | -      |        | +      | +      |
| 7       |        | -      | -      |        | +      | +      |
| 8       |        | -      | -      |        | +      | +      |
| 9       |        | -      | -      |        | +      | +      |
| 10      |        | -      | -      |        | +      | +      |
| 11      |        | -      | -      |        | +      | +      |
| 12      |        | -      | -      |        | +      | +      |
| 13      |        | -      | -      |        | +      | +      |
| 14      |        | -      | -      |        | +      | +      |
| 15      |        | -      | -      |        | +      | +      |
| 16      |        | -      | -      |        | +      | +      |
| 17      |        | -      | -      |        | +      | +      |
| 18      |        | +      | -      |        | -      | -      |
| 19      |        | -      | -      |        | -      | -      |
| 20      |        | -      | -      |        | +      | +      |

**a** Boldface indicates samples negative for pepC10 but positive for P1P5P7.

### TABLE 4. Individual patient IgG reactivity against BBK07 (P1P5P7) and VlsE (C6) for peptide-based serodiagnosis of early LD

| Patient | P1P5P7 | C6 |
|---------|--------|----|
|         | Initial visit (3/20, 15%) | Day 10 (11/20, 55%) | Day 20 (15/20, 75%) |
| 1       |        | +  | +  |
| 2       |        | +  | +  |
| 3       |        | +  | +  |
| 4       |        | +  | +  |
| 5       |        | +  | +  |
| 6       |        | +  | +  |
| 7       |        | +  | +  |
| 8       |        | +  | +  |
| 9       |        | +  | +  |
| 10      |        | +  | +  |
| 11      |        | +  | +  |
| 12      |        | +  | +  |
| 13      |        | +  | +  |
| 14      |        | +  | +  |
| 15      |        | +  | +  |
| 16      |        | +  | +  |
| 17      |        | +  | +  |
| 18      |        | +  | +  |
| 19      |        | +  | +  |
| 20      |        | +  | +  |

**a** Boldface indicates samples negative for C6 but positive for P1P5P7.
BBK07 peptides as diagnostic tools for Lyme disease

We evaluated the potential of BBK07 as a serodiagnostic marker for LD (9). To further evaluate the potential of BBK07 as a serodiagnostic marker, we assessed its sensitivity and specificity using diverse sets of serum collected from the United States and Germany, where samples were chosen from patients with diagnosed LD and several relevant and cross-reactive diseases and from healthy controls. When mice were infected with seven different clinical isolates of *B. burgdorferi* originally cultured from human patients, each prompted a detectable antibody response to BBK07 suggesting wide conservation of BBK07 immunogenicity among clinical isolates. Using a library of 23 overlapping peptides and a human LD serum panel, we further identified the most immunodominant epitopes of BBK07 and showed that the combination of three amino-terminal peptides is a significantly more effective serodiagnostic marker than the full-length antigen. We also showed that this peptide combination can be used as a potential component of a serodiagnostic marker in early stages of the disease, effectively diagnosing some patients who lack reactivity to OspC- or VlsE-derived peptides, such as pepC10 or C6, respectively. Finally, we showed that these results may be applicable to other, nonhuman species at high risk of *B. burgdorferi* infection, as peptides from BBK07 were able to effectively detect LD in canine serum samples. As immunoreactivity could potentially vary across mammalian species, the effectiveness of a BBK07 peptide to diagnose canine LD in our preliminary study also testifies to the future use of BBK07-based diagnosis in companion animals. It has been suggested that dogs may serve as sentinels for LD, as they can be at a higher risk of tick exposure (23). Taken together, our data identified the most immunodominant epitopes of BBK07 and highlighted the usefulness of a BBK07 peptide combination as a potential component of a highly sensitive and specific test for human and canine LD.

The effectiveness of some *B. burgdorferi* antigens, including VlsE (18), OspC (12), and BmpA (33), has been reduced by sequence variation in the bacterial population. Our data indicate that BBK07 immunoreactivity is detectable across the *B. burgdorferi* isolates present in North America but not in European LD patients. Although the BBK07 gene is highly conserved in *B. burgdorferi* sensu stricto isolates in United States, the linear plasmid carrying the BBK07 gene or an ortholog...
thereof is absent in major B. burgdorferi sensu lato strains prevalent in Europe. Therefore, while BBK07 diagnosis is unlikely to be effective in Europe, reactivity to BBK07 or similar antigens absent in other B. burgdorferi sensu lato species could aid physicians or researchers in differentiating between individuals infected with B. burgdorferi or the European strains (6, 38).

Our previous work indicated that the amino-terminal half of BBK07 was immunogenic in humans and accessible to protein degradation on the surface of B. burgdorferi cells (9). This region is likely to be the major target of the host immune response, as our primary screen of the peptide library also showed that the most immunodominant peptides of BBK07 were located on the amino terminus of the protein. Two of the most effective BBK07 peptide antigens, P5 (amino acids 56 through 69) and P7 (amino acids 76 through 89), are situated only six amino acids apart on the amino-terminal region of BBK07. Their close proximity suggests that a longer peptide, encompassing P5, P6, and P7, could effectively harness the linear epitopes of each on a single molecule. In addition, the longer peptide could form limited conformational epitopes present in this region of the protein, potentially increasing sensitivity further. Moreover, BBK07 peptide diagnosis could further be enhanced by improvements in antibody detection techniques. In our study, we utilized a simple ELISA, absorbing the peptides directly on the plastic surface in a high-pH buffer. The sensitivity of the test is then dependent on the ability of the plate to bind and present the peptide. In our assay, each peptide was biotinylated on the amino terminus, but streptavidin-coated plates did not increase the sensitivity of the test. Chemical conjugation to solid surfaces or fusions with carrier proteins have been used in the past to improve peptide binding and display and could be applied to further enhance sensitivity of BBK07 peptide-based diagnosis (15). A recent study demonstrated enhanced sensitivity of LD detection using a luciferase immunoprecipitation system (8). This technique is performed entirely in the liquid phase and has a broad dynamic detection range. The highest sensitivity was achieved using a synthetic peptide hybrid containing epitopes from the B. burgdorferi proteins OspC and VlsE. Our data show that BBK07 peptides were able to detect an antibody response in several patients whose sera did not react with the VlsE peptide C6 or the OspC peptide. The mechanism by which C6- or OspC-negative individuals might respond to BBK07 peptides currently remains unknown. However, this could potentially result from the differential expression of spirochete surface antigens in certain humans or variation of individual immune responses against particular peptides, among other possibilities.

In conclusion, our data show that select peptides from BBK07 are more effective antigens than the full-length BBK07 protein, have the ability to detect both early and late human infections, and can detect a variety of B. burgdorferi infectious isolates. These factors, combined with their unique ability to detect B. burgdorferi in patients not reactive to C6 or pepC10 peptides, suggest that epitopes from BBK07 could be used as components of a more efficient sensitive and specific serodiagnostic test for LD.

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