BBK07, a Dominant In Vivo Antigen of *Borrelia burgdorferi*, Is a Potential Marker for Serodiagnosis of Lyme Disease

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One of the recently identified *Borrelia burgdorferi* immunogens, BBK07, is characterized for its expression in the spirochete infection cycle and evaluated for its potential use as a serodiagnostic marker for Lyme disease. We show that the BBK07 gene is expressed at extremely low levels in vitro and in ticks but is dramatically induced by spirochetes once introduced into the host and is highly expressed throughout mammalian infection. In contrast, the expression of BBK12, a paralog of BBK07 with 87% amino acid identity, although expressed in vitro, remained undetectable in vivo throughout murine infection and in ticks. BBK07 is localized in the outer membrane, and the amino-terminal domain of the antigen is exposed on the microbial surface. A truncated BBK07 protein representing the amino-terminal domain is able to effectively detect antibodies to *B. burgdorferi*, both in experimentally infected mice and in humans. Further characterization of the immunodominant antigens of *B. burgdorferi*, such as BBK07, could contribute to the development of novel serodiagnostic markers for detection of Lyme disease.

Since the identification of *Borrelia burgdorferi* as the causative agent of Lyme disease (LD) over 25 years ago, the number of reported cases of LD has increased steadily (4, 49). In some U.S. counties, the incidence is more than 500 cases per 100,000 individuals, and more than 20,000 cases in the United States are diagnosed each year (4). Difficulties in diagnosis have long complicated the treatment of LD, as the bite of an infected tick may go unnoticed by the patient, and the clinical manifestations of LD can significantly vary among diagnosed patients (47). Common symptoms, such as fever, malaise, and arthritis, can resemble those caused by other conditions, further complicating diagnosis. Antibiotic therapy is highly effective, especially if administered in the early stages of LD; however, serious complications can result from false diagnoses and inappropriate treatment (9, 17, 40, 50, 51). There is no commercially available vaccine for human LD, so the development of accurate, sensitive laboratory diagnostics is an important goal of LD research.

While many laboratory methods have been used to assess *B. burgdorferi* infection, direct detection of the bacterium is difficult, due to the low pathogen load in clinical samples (2, 24). Likewise, the extremely slow growth of *B. burgdorferi*, the high cost, and the labor-intensive procedure needed to culture this bacterium have limited the effectiveness of culture as a diagnostic tool (34, 46). PCR detection is possible (44), but not widely used for diagnosis, due primarily to low sensitivity in tissues, such as cerebrospinal fluid and blood (2). Instead, the primary means used to detect *B. burgdorferi* exposure is serodiagnosis (2). Immunodetection has been performed using whole-cell antigens, as well as recombinant proteins or peptide fragments (2). Whole-cell lysate provides a wide variety of antigens for detection, but is difficult to standardize due to variations in protein expression by culture growth phase (42). False-positive results are also an issue, as antibodies against other bacteria can cross-react with conserved *B. burgdorferi* proteins (5, 13, 21, 29).

To reduce cross-reactivity, several recombinant *B. burgdorferi* antigens and various fragments thereof have been evaluated as serodiagnostic markers for LD, including OspC (35), BmpA (45), VlsE (27), BBK32 (22), L25 (33), P37 (31), and DbpA (20). OspC is exposed on the *B. burgdorferi* surface, is produced during early infection, and is highly immunogenic (1, 13, 16, 35). A peptide fragment termed pepC10, containing a conserved immunogenic epitope, has been developed for serodiagnosis (32). BmpA, another surface-exposed protein, has also been studied for use in diagnosis (10, 45). Though immunogenic, significant protein sequence heterogeneity exists among *B. burgdorferi* isolates, constituting several serotypes, which limit the effectiveness of both OspC (14) and BmpA as serodiagnostic markers (43). VlsE is a dominant surface-exposed antigen of *B. burgdorferi*, a lipoprotein that undergoes antigenic variation by genetic recombination with silent vls cassettes (53). Expressed throughout late infection, VlsE and C6, a conserved peptide fragment of VlsE, have been evaluated as serodiagnostic markers for LD (15, 27, 28). These studies suggest that while the use of recombinant proteins can reduce cross-reactivity, thereby enhancing specificity, the use of only select antigens can reduce the sensitivity of the diagnostic test (30). A promising sensitivity in such tests was reported by Bacon et al. (3). Using kinetic enzyme-linked immunosorbent assay (ELISA), the combined detection of immunoglobulin M (IgM) against pepC10 and IgG against C6 provided 78% sensitivity in all tested samples. While assays using only recombinant antigens show promise, the identification and inclusion of more immunodominant antigens could improve the sensitivity of these tests.
In an effort to more completely catalogue antigens produced during infection, a recent study by Barbour et al. used synthetic protein arrays to test the immunogenicity of the majority of *B. burgdorferi* open reading frames (6). Though most open reading frames were not measurably immunogenic, they identified several novel antigens, including BBK07 and BBK12, putative lipoproteins from the linear plasmid lp36. These proteins are extremely similar in sequence, though BBK07 is slightly larger than BBK12 (250 and 232 amino acids, respectively) (18). The genes are members of paralogous family 59, and their products are 87% identical in their overlapping amino acid sequences. While both BBK07 and BBK12 were identified as immunogens and potential antigenic markers, a detailed characterization of their expression and the resulting immune response was not explored. We sought to characterize the expression, surface localization, and immune response against BBK07 to further evaluate its inclusion as a diagnostic marker to improve the accuracy and sensitivity of LD serodiagnosis.

**MATERIALS AND METHODS**

**Bacteria, ticks, and mice.** Isolate A3, a clonal derivative of *Borrelia burgdorferi* B31 M1 and a generous gift from Patricia Rosas, was used throughout the study. Bacteria were grown in BSK-II media at 34°C. The *Ixodes scapularis* ticks used in this study were maintained in the laboratory as described previously (36). C57BL/6J mice were purchased from the National Cancer Institute. All animal procedures were performed in compliance with the guidelines and with the approval of the Institutional Animal Care and Use Committee. Unless otherwise stated, a single intradermal needle inoculation of 10⁵ *B. burgdorferi* cells was used to infect each mouse. For generation of immunized serum, each mouse was injected with 100 μg of *B. burgdorferi* sonicate (five mice/group) intradermally. As injections with lysed spirochetes were performed without an adjuvant, all booster injections were performed at weekly intervals for 9 weeks. Polyclonal antibodies against truncated BBK07 protein representing the amino-terminal region of the mature protein (BBK07/N) were obtained by injecting mice intradermally with recombinant protein (10 μg/animal) emulsified in complete Freund's adjuvant once, and twice in incomplete Freund's adjuvant (Sigma) at 10-day intervals. Serum samples were collected and pooled 10 days after final injection (12).

**Purification of recombinant proteins.** The recombinant protein fragment, BBK07/N, containing the amino-terminal part of the protein and excluding the signal peptide, amino acids 18 to 142, was fused to an N-terminal six-histidine tag for purification on the pET30a/NT-His Champion vector (Invitrogen). The follow-owing oligonucleotide primers were used to construct the expression vector: forward primer (5′-AAT CTA GAA TGG GATG TAG ACA TTC CCA TTG 3′ [XbaI site italicized]) and reverse primer (5′-CCG GCA ATT ACA TCT GTA CAT TCT T 3′ [SamHI site italicized]). Purification was performed using commercial cell lysis buffer (FastBreak; Promega) and MagneHis TCT TTA GTC CAT TCT TTA GTC CAT TCT T 3′ (Sigma). Other murine sera were diluted to 1:5,000 in 1% bovine serum albumin in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]). Thirty-five serum samples from humans with a clinical history of LL, collected from the CDC Lyme patient serum panel, were used in the ELISA. The infected serum samples were collected from patients with clinical symptoms associated with either early or disseminated phases of LD. The intervals of serum sample collection from patients ranged from 2 weeks to 13 years following onset of disease. Five serum samples from normal individuals residing in areas where LD is not endemic were collected from the CDC, while additional serum samples from 20 individuals that tested negative for *B. burgdorferi* infection were provided by Marylou Breitentein at Yale University. The 25 control serum samples were used to define the cutoff value for each antigen (the mean plus 2 standard deviations) (39). Human serum samples were diluted to 1:1,000 in 1% bovine serum albumin in TBS-T. Secondary antibodies against IgG, conjugated to horseradish peroxidase, were used with the following dilutions: goat anti-mouse, 1:10,000; goat anti-human, 1:5,000 (KPL). All steps were carried out either for 1 hour at 25°C or overnight at 4°C. Immunoblots were developed on HyBlot CL film (Denville) using the ECL detection reagent (GE Healthcare). ELISA results were quantified using SureBlue TMB microwell peroxidase substrate and TMB stop solution (KPL).

**Statistics.** Results are expressed as the mean ± 2 standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by the two-tailed Student t test.

**RESULTS**

The BBK07 gene, but not the paralogous BBK12 gene, is selectively expressed in the mammal during the infection cycle of *B. burgdorferi*. The paralogous gene products BBK07 and BBK12 have recently been identified as potential immunogens of *B. burgdorferi* (6). The genes are highly homologous, with 87% amino acid identity in their overlapping sequences (18). Due to the nearly identical sequences of BBK07 and BBK12, it is unclear if the host immune response is directed against either or both genes. To ascertain their individual expression patterns, we developed two sets of oligonucleotide primer pairs targeting variable regions of each gene, which specifically amplified either the BBK07 or BBK12 gene with low cross-reactivity, as confirmed by the DNA sequencing of the corresponding amplicons (data not shown). These primers were then used to determine the relative expression levels of each gene in cultured spirochetes or infected host tissue by qRT-PCR analysis. While both genes were transcribed at relatively low levels in vitro, only the BBK07 gene was detectable in vivo, as shown in infected mouse dermis 1 week after inoculation (Fig. 1A). Strikingly, the transcriptional level of the BBK07 gene is more than 100-fold higher in the infected host tissue than in vitro.

Because of the relatively high transcriptional level of the BBK07 gene in the infected host tissue, we then studied the expression of the BBK07 gene in the *B. burgdorferi* life cycle,
covering the first 4 weeks of murine infection. Total RNA was isolated from experimentally infected tick and mouse tissues to generate cDNA representative of important stages in the *B. burgdorferi* life cycle: transmission from infected ticks, murine infection, acquisition by naïve ticks, and persistence through the tick molt. While the BBK07 gene was consistently expressed in multiple murine tissues during the first 4 weeks of murine infection, but only BBK07 transcripts were present in infected murine skin samples 1 week after inoculation. (B) BBK07 gene expression pattern in vivo. The transcriptional profile of the BBK07 gene throughout representative stages of the life cycle of *B. burgdorferi* was measured by qRT-PCR. Mice (three mice per time point) and ticks (three ticks per time point) were infected with *B. burgdorferi*. Total RNA was isolated from infected tissue samples representing the complete life cycle of *B. burgdorferi*: fed nymphs, infected murine tissue samples collected at weekly intervals through 4 weeks after infection, feeding larvae, and unfed nymphs. The BBK07 gene was expressed in all murine tissue samples tested but was undetectable in tick samples. All qRT-PCR results represent the mean and SEM of four qPCR measurements from two independent infection experiments.

**FIG. 1.** The BBK07 gene is selectively expressed during infection. (A) BBK07 and BBK12 gene expression by *B. burgdorferi*. qRT-PCR was performed using gene-specific primers to determine if the BBK07 gene, the BBK12 gene, or both are transcribed by *B. burgdorferi* in vivo and in vitro, normalized to flaB. Both gene transcripts were detectable at relatively low levels in vitro, but only BBK07 transcripts were present in infected murine skin samples 1 week after inoculation. (B) BBK07 gene expression pattern in vivo. The transcriptional profile of the BBK07 gene throughout representative stages of the life cycle of *B. burgdorferi* was measured by qRT-PCR. Mice (three mice per time point) and ticks (three ticks per time point) were infected with *B. burgdorferi*. Total RNA was isolated from infected tissue samples representing the complete life cycle of *B. burgdorferi*: fed nymphs, infected murine tissue samples collected at weekly intervals through 4 weeks after infection, feeding larvae, and unfed nymphs. The BBK07 gene was expressed in all murine tissue samples tested but was undetectable in tick samples. All qRT-PCR results represent the mean and SEM of four qPCR measurements from two independent infection experiments.

**FIG. 2.** The amino-terminal part of BBK07 is surface exposed and immunogenic. (A) The upper panel represents the schematic of BBK07 showing the location of the recombinant amino-terminal BBK07 protein fragment used in the current study. A protein fragment lacking the putative signal peptide (gray box) starting from the amino terminus to half of the protein length was purified and termed BBK07N. Generated mouse anti-BBK07N sera recognizes the recombinant truncated protein and native BBK07 (bottom left panel), while recombinant BBK07N detects BBK07-specific antibody in infected mouse serum 2 weeks after infection (bottom right panel). (B) Surface exposure of BBK07. Viable *B. burgdorferi* cells were incubated with (+) or without (−) proteinase K and subjected to immunoblotting by the use of antiserum against BBK07N, FlaB, and OspA. FlaB and OspA were used as subsurface and surface controls, respectively. While the levels of FlaB did not significantly decrease, both OspA and BBK07 showed significant degradation in the presence of proteinase K.
BBK07N also evoked a robust immune response, and BBK07N antiserum recognized both purified BBK07N and native BBK07 from *B. burgdorferi* lysate (Fig. 2A, lower panel).

To test the surface exposure of BBK07, a proteinase K accessibility assay was performed (12). Intact *B. burgdorferi* was incubated with and without proteinase K and probed with FlaB, OspA, or BBK07N antiserum. FlaB, a known subsurface protein, was not degraded, but both the surface protein OspA and BBK07 were significantly degraded, suggesting that the amino-terminal region of BBK07 is surface exposed (Fig. 2B).

**BBK07-specific immune response is pronounced during active borrelial infection but absent in hosts immunized with lysed pathogens.** Because qRT-PCR analysis indicated a dramatic induction of the BBK07 gene in vivo during early infection, we next assessed kinetics of BBK07 antibody development in the host over the first 9 weeks of *B. burgdorferi* infection. As qRT-PCR analysis indicated minor expression of the BBK07 gene in vitro, we also assessed, in parallel, BBK07 antibody development in mice immunized with sonicated spirochetes, in order to test whether BBK07 could differentiate infected hosts from ones vaccinated with killed pathogens. To accomplish this, groups of mice (five animals/group) were needle inoculated with a single *B. burgdorferi* inoculum (10⁵ cells/mice). In parallel, another group of mice (five animals/group) were injected with sonicated *B. burgdorferi* (100 μg/mice) at 7-day intervals for a total of 9 weeks. Serum samples were collected and pooled weekly. Equal amounts of *B. burgdorferi* lysate or BBK07N were used to detect specific antibodies present in each serum sample by ELISA (Fig. 3A). As a negative control, antibody development against the *B. burgdorferi* antigen Lp6.6, which is abundant in vitro but known to be downregulated during murine infection, was also measured (26). As expected, antibodies against *B. burgdorferi* lysate, but not against Lp6.6, were detected in infected mice (7, 26). BBK07 provoked a robust antibody development that was detectable after 1 week and remained elevated throughout the infection. In contrast, the mice immunized weekly with lysed spirochetes produced a low BBK07 antibody response that did not increase over the course of the experiment, while Lp6.6 provoked a robust antibody response (Fig. 3B). In order to confirm that the immunogenicity of BBK07 is not confined to needle-borne artificial murine infection, groups of naive mice were infected by tick bite. Serum samples were collected after 4 weeks of infection and tested by ELISA (Fig. 3C). The serum samples contained a similar response against both lysate and BBK07, indicating that the antibody response against BBK07 does not depend on the route of infection.

**Evaluation of BBK07N as a diagnostic marker for *B. burgdorferi* infection in murine hosts.** The robust and specific immune response provoked by BBK07 led us to investigate a possible diagnostic use of BBK07N. Using mouse serum samples collected 2 weeks after infection, we compared the immunogenicity of BBK07N to several other immunogenic *B. burgdorferi* antigens, such as VlsE, OspC, BmpA, and BbCRASP-2. As controls, *B. burgdorferi* lysate and Lp6.6 were also included in the assay. To measure the relative immunogenicity levels of each antigen, equal amounts of proteins and lysate were used in an ELISA, probed with the infected mouse serum (Fig. 4). Due to the high antibody titers detected by BBK07N and VlsE, which quickly reached the upper detection limit of the assay, the reaction was stopped shortly (1 min) after the addition of chromogenic substrate. As expected, naive serum samples had low reactivity to all antigens. Among all antigens tested, BBK07N reflected the most robust immune response, proving to be more sensitive than several of the antigens currently used in LD diagnosis.

**Detection of BBK07N-specific antibody response in human patients.** To further investigate a diagnostic use of BBK07N, human serum samples from patients diagnosed with LD and healthy human serum samples were used in an ELISA. Wells were coated with recombinant BBK07N, BmpA, OspC, or *B. burgdorferi* lysate and probed with human serum followed by the detection antibody. We did not have enough recombinant VlsE in our possession, and therefore, VlsE was excluded from the assay. The panel of control serum samples was used to
define the cutoff value for each antigen, representing the 95th percentile absorbance value. Samples with an absorbance higher than the cutoff value were considered positive. *B. burgdorferi* lysate displayed the highest sensitivity of the antigens tested but had the highest cutoff value due to low specificity. Due to its higher specificity, the recombinant antigen BBK07N (12 out of 35 total samples [34%]) was of comparable diagnostic accuracy to that of *B. burgdorferi* lystate (15 out of 35 [43%]) when detecting an antibody response in the infected sera (Fig. 5). In contrast, lower sensitivities were observed using BmpA (7 out of 35 [20%]) and OspC (3 out of 35 [9%]). These data suggest that BBK07N could be developed into a diagnostic tool for evaluating human LD patients.

**DISCUSSION**

The identification and characterization of in vivo antigens of *B. burgdorferi* is central to the improvement of current laboratory diagnostics for LD. A previous study identified BBK07 and BBK12 as novel immunogenic antigens of *B. burgdorferi* (6). We further extend the observation and establish that the BBK07 gene, but not the highly similar paralogous member BBK12, was expressed at relatively high levels in vivo. We show that a recombinant protein representing the amino-terminal region of BBK07 was able to provoke a specific antibody response against the native protein, providing antibodies that were then used to demonstrate the surface exposure of the amino-terminal region of BBK07. The recombinant protein could, accordingly, detect a specific antibody response to active infection with *B. burgdorferi*. As BBK07 had negligible expression in vitro, we show that this antigen could be useful in discriminating antibody development during active infection versus hosts vaccinated with killed pathogen preparations. The detected antibody response during infection was more robust than that detected by several currently used serodiagnostic antigens (2, 3). Finally, using a human serum panel with diagnosed LD, we show that BBK07 is a possible marker for the laboratory diagnosis of LD.

Because of the low numbers of *B. burgdorferi* cells present during disease, diagnosis of LD has principally relied on immunological methods (2). Serodiagnosis is more sensitive than is direct detection, but current serodiagnosis methodologies have been responsible for incidences of under- and overdiagnoses (9, 17, 40, 50, 51). False negatives can result from tests with low sensitivity, and test sensitivity can be improved by increasing the number of immunoreactive antigens tested. Because *B. burgdorferi* can be grown in vitro, many tests include whole *B. burgdorferi* cells or lysate, which provides an extensive set of antigens (2). However, the sensitivity gained by using cultured cells comes with a price, as some antigens of *B. burgdorferi* are conserved among other bacterial pathogens (13, 29). Antibodies against conserved antigens, such as flagellin and bacterial heat shock proteins, are naturally present in many uninfected individuals, increasing the chance of a false-positive result (5, 13, 21, 29). Perhaps most importantly, the use of *B. burgdorferi* cells makes standardization of the tests more difficult and, as a result, the outcome of tests more subjective. For example, *B. burgdorferi* antigen expression can vary by growth phase, and extended periods of in vitro culture can cause the loss of plasmids, some of which contain important antigens (41, 42). While in vitro-grown *B. burgdorferi* cells may increase
sensitivity by providing a wide array of antigens to detect an immune response, the decreased diagnostic specificity and standardization limit its effectiveness in serodiagnosis.

The use of recombinant proteins in diagnosis can eliminate cross-reactive epitopes and can ease standardization by reducing batch-to-batch variation. This increase in specificity need not come at the cost of sensitivity if the immunodominant antigens of B. burgdorferi are identified and characterized. Our data completely support a previous study showing that BBK07 is highly immunogenic during LD (6). However, plasmid lp36, which contained the BBK07 locus, could be lost during in vitro growth (23), and our data showing extremely low in vitro expression of BBK07 suggest that BBK07 is underrepresented in tests using in vitro-grown B. burgdorferi (6). The inclusion of BBK07 as a diagnostic marker could increase serodiagnostic sensitivity in human patients while maintaining the high specificity afforded by recombinant antigen tests. The low in vitro expression of the BBK07 gene and undetectable immune response against sonicated borrelial cells suggest additional use expression of the BBK07 gene and undetectable immune re-
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