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Characterization of a *Borrelia miyamotoi* membrane antigen (BmaA) for serodiagnosis of *Borrelia miyamotoi* disease

Emma K. Harris[a,1], **Kevin S. Brandt**[a], **Taylor J. Van Gundy**[a], **Irina Goodrich**[a], **Gary P. Wormser**[b], **Brittany A. Armstrong**[c], **Robert D. Gilmore**[a,*]

[a]Bacterial Diseases Branch, Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA

[b]Department of Medicine, Division of Infectious Disease, New York Medical College, Valhalla, NY, USA

[c]Department of Pediatrics, Department of Molecular Virology and Microbiology, National School of Tropical Medicine, Baylor College of Medicine, Houston, Texas, USA

Abstract

Borrelia miyamotoi is a tick-borne pathogen that causes *Borrelia miyamotoi* disease (BMD), an emerging infectious disease of increasing public health significance. *B. miyamotoi* is transmitted by the same tick vector (*Ixodes* spp.) as *B. burgdorferi* sensu lato (s.l.), the causative agent of Lyme disease, therefore laboratory assays to differentiate BMD from Lyme disease are needed to avoid misdiagnoses and for disease confirmation. We previously performed a global immunoproteomic analysis of the murine host antibody response against *B. miyamotoi* infection to discover antigens that could serologically distinguish the two infections. An initial assessment identified a putative lipoprotein antigen, here termed BmaA, as a promising candidate to augment current research-based serological assays. In this study, we show that BmaA is an outer surface-associated protein by its susceptibility to protease digestion. Synthesis of BmaA in culture was independent of temperature at either 23 °C or 34 °C. The BmaA gene is present in two identical loci harbored on separate plasmids in North American strains LB-2001 and CT13-2396. *bmaA*-

*Corresponding author at: Division of Vector Borne Diseases, Centers for Disease Control and Prevention, 3150 Rampart Rd, CSU Foothills Campus, Fort Collins, Colorado, 80521, USA. rbg9@cdc.gov (R.D. Gilmore).

[1]Current address: Department of Microbiology, Immunology, and Pathology, Arthropod-borne and Infectious disease Laboratory, Colorado State University, Fort Collins, CO, USA.

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"The findings and conclusions in this report are those of the author (s) and do not necessarily represent the views of [the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry]."

CRediT authorship contribution statement

Emma K. Harris: Data curation, Formal analysis, Investigation, Visualization, Methodology, Writing - original draft. **Kevin S. Brandt**: Investigation, Methodology, Writing - review & editing. **Taylor J. Van Gundy**: Investigation, Methodology, Validation, Writing - review & editing. **Irina Goodrich**: Investigation, Methodology, Writing - review & editing. **Gary P. Wormser**: Resources, Investigation, Writing - review & editing. **Brittany A. Armstrong**: Investigation, Methodology, Writing - review & editing. **Robert D. Gilmore**: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101476.

like sequences are present in other *B. miyamotoi* strains and relapsing fever borrelia as multicopy genes and as paralogous or orthologous gene families. IgM and IgG antibodies in pooled serum from BMD patients reacted with native BmaA fractionated by 2-dimensional gel electrophoresis and identified by mass spectrometry. IgG against recombinant BmaA was detected in 4 of 5 BMD patient serum samples as compared with 1 of 23 serum samples collected from patients with various stages of Lyme disease. Human anti-*B. turicatae* serum did not seroreact with recombinant BmaA suggesting a role as a species-specific diagnostic antigen. These results demonstrated that BmaA elicits a human host antibody response during *B. miyamotoi* infection but not in a tested group of *B. burgdorferi*-infected Lyme disease patients, thereby providing a potentially useful addition for developing BMD serodiagnostic tests.

Keywords

Borrelia miyamotoi ; *Borrelia miyamotoi* disease; BmaA; Relapsing fever borrelia

1. Introduction

Borrelia miyamotoi disease (BMD) is caused by the tick-borne relapsing fever (TBRF) spirochete, *Borrelia miyamotoi*. First identified in Japan and thought to be a non-human pathogen (Fukunaga et al., 1995), human cases of *B. miyamotoi* infection were reported in Russia in 2011 with subsequent reports of BMD in Japan, United States, and the Netherlands (Chowdri et al., 2013; Gugliotta et al., 2013; Hoornstra et al., 2018; Krause et al., 2018, 2014; Molloy et al., 2015; Platonov et al., 2011; Sato et al., 2014). Afflicted individuals experience a febrile illness with chills, myalgia, arthralgia, and headache, and possibly fever relapses (Krause et al., 2015; Molloy et al., 2015; Platonov et al., 2011). Although *B. miyamotoi* is genetically grouped with TBRF borrelia that are transmitted by *Ornithodoros* ticks, *B. miyamotoi* is transmitted by *Ixodes* ticks, the same vector as *B. burgdorferi* sensu lato (s.l.) the causative agent of Lyme disease. The discovery of *B. miyamotoi* as a human pathogen in the same regions of endemicity as Lyme disease complicates the diagnosis and confirmation of BMD (Krause et al., 2015; Molloy et al., 2015; Platonov et al., 2011).

Laboratory diagnosis of BMD is dependent upon microscopic detection of spirochetes on blood smears, PCR amplification of *B. miyamotoi* DNA from patient blood samples, or serologic testing (Krause et al., 2015). A sensitive and specific serologic assay for BMD is needed to diagnose patients with late stages of disease, for confirmation of past infections, and to differentiate BMD from Lyme disease. Glycerophodiester phosphodiesterase (GlpQ) and its encoding gene, *glpQ*, are the standard targets for serology and PCR respectively as *glpQ* is present in relapsing fever (RF) borrelia, but not in *B. burgdorferi* (s.l.) thereby providing a target to distinguish RF infections from Lyme disease (Schwan et al., 1996). Studies have indicated that antibody detection for GlpQ alone is insensitive in the acute stage of BMD suggesting a limitation for diagnostic use (Jahfari et al., 2017; Molloy et al., 2015). A comprehensive study by Koetsveld et al. with 182 BMD PCR-positive serum samples (longitudinally collected from 50 patients with acute disease) demonstrated that combining GlpQ with *B. miyamotoi* antigens Vsp-1 and Vlps 15/16, 18, and 5 produced

a sensitive and specific assay (Koetsveld et al., 2018). However, antibodies against *B. miyamotoi* cross-react with the *B. burgdorferi* Vmp-like sequence, expressed (VlsE) C6 antigen (Koetsveld et al., 2019; Molloy et al., 2018).

Using an immunoproteomic approach, we previously reported that murine hosts infected with *B. miyamotoi* produced antibodies (IgM and IgG) against a 37-kDa membrane-associated protein identified as a putative lipoprotein, herein named Borrelia membrane antigen A (BmaA) (Harris et al., 2019). We demonstrated that serum antibodies from mice infected with *B. burgdorferi*, and a limited number of sera from human Lyme disease patients, were not reactive against recombinant (r) BmaA. In this study, we further characterize BmaA and expand the number of human BMD and Lyme disease patient samples to demonstrate the potential utility of this protein as a serodiagnostic tool.

2. Materials and methods

2.1. Borrelia strains

B. miyamotoi strain LB-2001 (provided by Joppe Hovius, Center for Experimental and Molecular Medicine, Amsterdam, the Netherlands), was originally isolated from *I. scapularis* in the Northeast United States and was maintained by passage in SCID mice (Scoles et al., 2001). *B. miyamotoi* LB-2001 low passage (less than 6) was cultivated in Modified Kelly-Pettenkofer Medium (MKP-F) (Wagemakers et al., 2014) at 34 °C in capped tubes and harvested at 4×10^7 spirochetes/mL. *B. burgdorferi* B31-A3 (Elias et al., 2002) and *B. turicatae* strain Oz-1 (Cadavid et al., 1994; Carlyon and Marconi, 1998) were propagated in complete BSK-II at 34 °C in capped tubes and harvested at 1.7×10^8 spirochetes/mL. All *Borrelia* spp. were enumerated by darkfield microscopy with a Cellometer counting chamber (Electron Microscopy Sciences, Hatfield, PA). Bacterial pellets for whole cell lysates were collected by centrifugation at 5000 × *g* for 10 min, followed by two washes with sterile 1 X PBS and storage at −80° C until protein isolation.

2.2. B. miyamotoi lysate preparation, recombinant protein production, and generation of anti-BmaA antiserum

Membrane-associated *B. miyamotoi* proteins for 2-DE were isolated as previously described (Harris et al., 2019). Briefly, spirochetes were pelleted at 4000 x g for 15 min at 4° C, followed by two washes in 1 X PBS. Spirochetes were resuspended in breaking buffer (20 mM Tris-HCl, pH 8.0) supplemented with 30 μg DNase I, 30 μg RNase A (Thermo Scientific, Rockford, IL), and one tablet of Complete Protease Inhibitor Cocktail (PIC) (Roche Applied Sciences, Indianapolis, IN). Spirochetes were lysed on ice using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT). Membrane-associated proteins were isolated by ultracentrifugation at 100,000 x g for 1 h at 4° C. Resultant pellets were resuspended in breaking buffer and incubated overnight at 4° C. Proteins were dialyzed in 10 mM ammonium bicarbonate.

B. miyamotoi recombinant BmaA was generated as previously described (Harris et al., 2019). *bmaA* was amplified from *B. miyamotoi* LB-2001 genomic DNA, and cloned into pETite N-His vector using the Expresso T7 cloning system (Lucigen, Middleton, WI)

and transformed into *E. coli* BL21 (DE3) for recombinant protein production under non-denaturing conditions according to manufacturer's instructions using the Ni-NTA Fast Start His tag affinity purification kit (Qiagen, Valencia, CA). Eluted protein was dialyzed in 1 X PBS overnight at 4° C.

Protein whole cell lysates for *B. miyamotoi*, *B. burgdorferi*, and *B. turicatae* were prepared by resuspending frozen pellets in IP Lysis Buffer (Pierce, Thermo Scientific, Rockford, IL) with 1X Roche protease inhibitor cocktail according to the manufacturer's instructions. Protein concentrations for all preparations were performed by BCA (Thermo, Rockford, IL).

Anti-rBmaA serum was generated in mice by a primary inoculation with 10 ug of rBmaA purified to remove endotoxin (Brandt et al., 2014) with Imject adjuvant followed by two booster inoculations of approximately 10 ug each, given 3 weeks apart. Mice were bled 8–14 days following the final boost, and an enzyme-linked immunosorbent assay (ELISA) was performed on the serum samples against recombinant protein to assess the antibody titers. Serum was stored at −20 °C. The experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee at the Division of Vector-Borne Diseases, CDC, Fort Collins, CO.

2.3. Protein sequence alignments

B. miyamotoi translated gene sequences were selected for amino acid alignments based on GenBank submissions that had the highest nucleotide identity to strain LB-2001 BmaA. TBRF and louse-borne RF borrelia protein sequences were selected based on a BLAST protein search against the *B. miyamotoi* LB-2001 protein sequence.

B. miyamotoi strain LB-2001 (GenBank ALN43426.1) alignment of translated sequences for amino acid identities was performed with *B. hermsii* (YOR) [GenBank AHH03916.1], *B. turicatae* (91E315) [GenBank ASJ27688.1], *B. parkeri* (GenBank WP_025407570), *B. coriaceae* (GenBank WP_051428636.1), *B. duttoni* (CR2A) [GenBank ETZ17857.1], *B. crocidurae* (DOU) [GenBank AHH07470.1), and *B. recurrentis* (A1) [GenBank ACH95210.1], along with the *B. miyamotoi* strains CT13-2396 (GenBank AOW96305), Izh-4 (GenBank ATQ21615.1), Yekat (GenBank ATQ15518.1), NL-IR-1 (GenBank QFP48637.1) and Fr64b (GenBank AHH06011.1). A *B. burgdorferi bmaA*-like sequence was found in the database search (GenBank NP_212978.1) corresponding to the annotated gene *bb0844*. Alignment analysis was performed by DNASTAR Lasergene 15 MegAlign Pro software using the ClustalW algorithm.

2.4. Temperature shift protein expression in culture

B. miyamotoi cultures (10 mL) were started in MKP-F media from a passage 3 frozen (−80 °C) glycerol stock and incubated at 23 °C in capped tubes for 23 days post-inoculation reaching a density of 1×10^6 bacteria/mL whereby a 3 mL cell pellet was collected, and washed 3X in Hanks buffered saline solution (HBSS) for SDS-PAGE analysis. The culture of the 23 °C cells was used to inoculate a fresh MKP-F culture (1:10 at 34 °C), with the remainder of the culture incubated at 34 °C for 3 days reaching a density of 1.2×10^6 bacteria/mL. A 3 mL cell pellet was collected and washed from each of the temperature-shifted cultures for SDS-PAGE analysis loading approximately 7.5×10^5 cells /

lane. Western blotting was performed by standard procedures using mouse anti-rBmaA (1:1000) followed by incubation with alkaline phosphatase conjugated goat anti-mouse IgG (1:5000) (KPL, Gaithersburg, MD).

2.5. Protease digestion of outer surface proteins

B. miyamotoi was grown to late log phase and 1 mL of culture was pelleted (approximately 5×10^7 organisms), washed 3X in sterile phosphate buffered saline (PBS) containing 5 mM $MgCl_2$, and resuspended in 100 μL PBS. Proteinase K was added to concentration of 400 μg/mL and the cell mixture was incubated for 90 min at room temperature whereby 50 μL was removed and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (5 μL of 10 mM stock) added to stop the reaction. The remainder of the *B. miyamotoi*/proteinase K mixture was continued to be incubated for an additional 90 min (180 min total) until addition of PMSF. Following the digestion step, the cells were pelleted and washed 2X PBS, resuspended in 2X SDS-PAGE loading buffer and fractionated by SDS-PAGE for immunoblotting according to standard procedures. A *B. burgdorferi* anti-FlaB monoclonal antibody cross-reactive against *B. miyamotoi* FlaB was used for the immunoblot. The protease digestion experiment was repeated as above.

2.6. Two-dimensional electrophoresis and immunoblotting

Two-dimensional electrophoresis (2-DE) followed by silver staining and immunoblotting was performed as previously described (Harris et al., 2019). 100 μg of solubilized, ampholyte-containing, membrane-associated *B. miyamotoi* protein was applied overnight at room temperature to 7 cm pH 4–7 or 6–11 Immobiline Dry Strips (GE Healthcare, Piscataway, NJ). Proteins were focused using a GE Multiphor II (GE Healthcare, Piscataway, NJ) with the following voltages: 50, 100, 150, 200, 250, and 300 V for 6 min, 500 V for 12 min, and 3000 V for 5 h. Strips were equilibrated at room temperature for 15 min in appropriate buffers. Size separation of proteins was achieved using 4–12 % Bis-Tris SDS-polyacrylamide gels (Life Technologies, Carlsbad, CA). Subsequently, gels were either silver stained (Pierce Silver Stain for Mass Spectrometry, Thermo Scientific, Rockland, IL) or transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting as described below for 1-dimension SDS-PAGE.

Proteins were transferred to PVDF membranes using the iBlot2 transfer apparatus (Invitrogen) and were blocked overnight in 3 % BSA in Tris-buffered saline–Tween 20 (TBS-T; 20 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.05 % Tween 20 [pH 7.4]). Human sera (pooled or individual) were diluted 1:200 in 1% BSA in TBS-T. Applied serum was incubated against membranes for two hours at room temperature and washed with TBS-T 3–5 times for five minutes each. Horseradish peroxidase (HRP)-labeled goat anti-human IgM (mu) F(ab')2 fragment or goat anti-human IgG (Fc) F(ab')2 fragment (Life Technologies, Carlsbad, CA) was diluted 1:10,000 in 1% BSA in TBS-T and incubated with membranes for one hour at room temperature followed by the wash step. Blots were developed by addition of Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and imaged on a ChemiDoc MP imaging system (BioRad).

Immunoblots using TBRF antisera were performed as follows. SDS-PAGE fractionated proteins were transferred to PVDF membranes and blocked overnight in Tropix Iblock (ThermoFisher). Membranes were incubated with primary antiserum (1:200 for human; 1:250 or 1:200 for mouse; 1:1000 for anti-rBmaA) for 1 h at room temperature. Following the wash step, goat anti-human IgA/G/M -HRP (Millipore, Billerica, MA), or Protein G-HRP (Invitrogen, Carlsbad, CA) (for mouse primary blots) were added at 1:4000 and incubated for 1 h at room temperature. Following the wash step, blots were developed by chemiluminescense with ECL reagent (Amersham, UK).

2.7. Line blotting

Nitrocellulose membranes (Millipore) were hydrated in TBS-T and placed in a Miniblotter45 (Immunetics, Boston, MA). Protein (10 μg) in TBS-T was loaded in each lane and incubated for 60 min with rocking at room temperature. The protein solution was aspirated by vacuum from the lanes followed by three washes in TBS-T. The membrane was removed from the manifold and blocked in T20 blocking buffer (Thermo-Fisher Scientific) at room temperature for 30 min. The membrane was rotated 90° and placed back in the manifold with serum samples diluted in T20 blocking buffer (1:200) added to the appropriate lane, followed by incubation at room temperature for 1 h on a rocking platform. After three washes in TBS-T, alkaline phosphatase-conjugated goat antihuman IgG antibodies (KPL, Gaithersburg, MD) diluted in blocking buffer (1:2000) were added to each lane and incubated with rocking at room temperature for 1 h. Following aspiration of the secondary anti-body solution, the membrane was removed from the apparatus, washed three times with TBS-T, and developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (Thermo-Fisher Scientific).

2.8. Mass spectrometry

Trypsin digestion and mass spectrometry to identify proteins were performed as previously reported (Harris et al., 2019). 2-DE-fractionated proteins were silver stained, excised from gels, and destained according to the manufacturer's instructions (Pierce Silver Stain for Mass Spectrometry, Thermo Scientific, Rockford, IL). Proteins were trypsin digested (83 ng/μl) overnight at 37° C. The addition of trifluoroacetic acid (TFA) terminated the reaction, after which peptides were extracted from gel slices using a solution of 0.1 % TFA/60 % acetonitrile (ACN) (v/v). Extracted peptide samples were dried via a Savant SPD1010 SpeedVac (Thermo Scientific, Rockford, IL). Peptides were submitted to the Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, Colorado for mass spectrometry analysis. Peptide sequences and corresponding protein identities were viewed in Scaffold (version 4.8.7). Identified proteins were accepted using the following criteria: > 90 % peptides and > 99 % protein probability threshold and at least two unique peptides per protein identity.

2.9. Clinical samples

BMD, Lyme disease, and healthy control patient serum samples were obtained from New York Medical College. All serum samples from BMD patients were collected in the convalescent phase. At time of presentation, these patients were confirmed as having BMD by PCR directed at *B. miyamotoi glpQ*. Lyme disease patient serum samples were selected

to represent early localized, early disseminated, and convalescent stages and were clinically diagnosed by presence of erythema migrans and confirmed by positive culture. Ten healthy control serum samples were collected from individuals located in either Lyme endemic (n = 5) or Lyme non-endemic (n = 5) regions of the United States, all of whom reported no known history Lyme disease. Serum samples from individuals infected with TBRF borrelia and control serum were obtained from Baylor College of Medicine. All patients provided informed consent. This study was approved and carried out under the auspices of the Institutional Review Boards of the Centers for Disease Control and Prevention, New York Medical College, and Baylor College of Medicine.

2.10. ELISA

Immulon 2HB 96-well plates were coated with 100 ng/well of purified *B. miyamotoi* recombinant BmaA diluted in carbonate bicarbonate coating buffer (90 mM $NaHCO_3$, 60 mM Na_2CO_3; pH 9.6) and incubated overnight at 4 ° C. The plate wells were subjected to five washes with Tris-buffered saline–Tween 20 [TBS-T; 20 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.05 % Tween 20 (pH 7.4)] using a BioTek 405 Select plate washer (BioTek, Winooski, VT), followed by addition of 300 μL blocking buffer (3 % fetal bovine serum in TBS-T) for 60 min at room temperature. Post-block, plates were washed, and sera in blocking buffer (1:100) was added to individual wells in duplicate and incubated for 45 min at room temperature. Plates were washed followed by addition of HRP-conjugated goat anti-human IgG Fc, F(ab')2 fragment (1:5000) (Life Technologies, Carlsbad, CA) and incubated for 45 min. Plates were washed and developed by addition of KPL SureBlue TMB Microwell Peroxidase substrate (100 μL) (Seracare, Milford, MA) for 10 min. Reaction was terminated with 1 N HCL and samples read at 450 nm using an ELx808IU Ultra microplate reader (Biotek, Winooski, VT). Cutoff values to determine positive samples were calculated by 3 standard deviations above the mean optical density for all healthy control samples. Patient samples were assayed in duplicate and replicated 2 times.

3. Results

3.1. BmaA gene and amino acid identities among relapsing fever borrelia

We initially described BmaA as a putative lipoprotein immunogen, part of a global immunoproteomic analysis of *B. miyamotoi* proteins (Harris et al., 2019). A GenBank database search found 2 identical *bmaA* copies in strain LB-2001 located on plasmids designated lpC and lpD (Barbour, 2016). The North American strain CT13-2396 also contained 2 identical copies of *bmaA* located on lp41 and lp30 with 100 % sequence identity to the LB-2001 genes (Kingry et al., 2017). Interestingly, *bmaA* is located on plasmids bearing *vsp* and *vlp* sequences that are the basis for antigenic variation that occurs in relapsing fever infections (Barbour, 2016). The GenBank database search revealed *bmaA*-like sequences located on other plasmids of the CT13-2396 strain, i.e. lp23, lp19, and lp20-2. The database search also revealed several *bmaA*-like genes in the *B. miyamotoi* Russian strains Izh and Yekat with locations on various plasmids (Kuleshov et al., 2018). The highest DNA sequence identity (88.7 %) was found in strain Izh-4 plasmid lp24 (Kuleshov et al., 2020). The database search also found *bmaA*-like genes to be harbored on separate plasmids on the Netherlands strains NL-IR-1 and −2 (Kuleshov et al., 2019)

and the Japanese strain FR64b. The presence of multi-copy *bmaA* genes, orthologous and paralogous sequences among and within strains respectively, is indicative of a gene family. Indeed, an annotation of P12 family has been accorded to homologs of the protein sequence in the GenBank database for several submissions.

The strain LB-2001 BmaA protein is composed of 311 amino acids with a calculated molecular mass of 35,442 daltons (Barbour, 2016). The amino terminus of the protein predicts a signal peptide and contains a motif consistent with a signal peptidase site II cleavage site, LLSC, commonly seen in other prokaryotic lipoproteins (Fig. 1A) (predicted by SignalP-5.0 [http://www.cbs.dtu.dk/services/SignalP/]).

Strain LB-2001 alignment of translated DNA sequences for amino acid identities was performed with *B. miyamotoi* strains and louse-borne and TBRF borrelia as described in Materials and Methods. The amino acid alignments are shown in Fig. 1A with percent amino acid identities in Fig. 1B.

3.2. Temperature independent expression in culture and surface localization of BmaA

We assessed BmaA synthesis under different temperature conditions during MKP-F culture growth. BmaA was detected by immunoblot in *B. miyamotoi* whole cell lysates cultivated at

23 °C and after the shift to 34 °C (Fig. 2A). BmaA was produced either by temperature shift to 34 °C from a subculture of 23 °C organisms to fresh media, or when incubating the original

23 °C culture at 34 °C. FlaB was constitutively expressed at both temperatures (Fig. 2B).

We previously identified BmaA by isolation of *B. miyamotoi* membrane proteins suggesting a surface localization (Harris et al., 2019). We subjected *B. miyamotoi* culture-grown cells to proteinase K digestion to assess BmaA cellular localization. Whole cell lysates incubated with proteinase K demonstrated protein degradation as observed by SDS-PAGE GelCode Blue staining compared with lysates incubated in the absence of the protease (Fig. 3A). Immunoblot analysis showed BmaA was diminished following 90 and 180 min of incubation with proteinase K indicating its external cellular location (Fig. 3B). The periplasmically located FlaB was protected from protease digestion serving as the subsurface control protein (Fig. 3C).

3.3. BMD human seroreactivity to B. miyamotoi proteins by 2-dimensional electrophoresis (2-DE) immunoblotting

Serum from 5 BMD-infected individuals (Table 1) were pooled and used to probe *B. miyamotoi* 2-DE fractionated proteins for IgM and IgG reactivity (Fig. 4). The purpose was two-fold; i) to confirm the *B. miyamotoi*-infected human antibody response against native BmaA; and ii) to illustrate the human BMD immunoproteomic pattern to compare against the murine profile previously performed (Harris et al., 2019). Nine immunogenic spots around 37 kDa were selected for identification by mass spectrometry. Several proteins were identified in the selected spots including BmaA (annotated as “putative lipoprotein” in Supplemental Table 1), thereby demonstrating that the protein was well-represented

among the antigenic profile of *B. miyamotoi* (Fig. 4A–F). The mass spectrometry results are provided in Supplemental Table 1.

Also observed were immunoreactive protein pattern differences detected by IgM in the 100, 26, and 15 kDa range that were not represented in the IgG immunoblot, and IgG-reactive proteins that were not represented in the IgM blots at 25 kDa (pH 6–11) (Fig. 4). The identification of these proteins and those unique to the human response vs the mouse response found previously (Harris et al., 2019) may represent antigens for BMD detection worthy of further study.

3.4. BMD patient serum seroreactivity against rBmaA by Western blot

We performed IgG Western blots against rBmaA with 5 individual serum samples from patients confirmed for BMD by PCR (Table 1). Four of 5 (80 %) BMD patients had an observable IgG response to rBmaA (Fig. 5, lane 1). Only BMD patient #1 failed to react with rBmaA.

BMD patient IgG seroreactivity against the current standard antigen, rGlpQ, was included as a comparative to the individual patients' response against rBmaA. Three patients showed variable reactivity (i.e. either weak or strong) to both antigens (BMD-2, −3, and −4), while patient BMD-1 was reactive to GlpQ only and patient BMD-5 was reactive to BmaA only. All BMD patients were reactive to the *B. miyamotoi* whole cell lysate.

Note that in each immunoblot there is a major immunoreactive band(s) at 37-kDa in the *B. miyamotoi* whole cell lysate lane for each patient serum sample. Because of the similar molecular masses of BmaA, GlpQ, and various Vmps, the immunodominant bands may be one or more of these co-migrating proteins.

3.5. BMD and Lyme disease patient IgG seroreactivity to rBmaA measured by ELISA

A baseline ELISA cutoff for positive rBmaA IgG reactivity was set at 3 standard deviations above the mean optical density (OD) for healthy patients from Lyme disease endemic and non-endemic regions (HC, Fig. 6A). IgG antibody reactivity to rBmaA was detected in 4 of 5 (80 %) BMD patients (Fig. 6A), in agreement with the Western blot analyses (Fig. 5A). The BMD patient with a negative ELISA value (BMD-1) was also negative by Western blot (Table 1, Fig. 5). Serum from this patient was collected 157 days after PCR confirmation of BMD. This result suggests that additional BMD serum samples collected at all stages of disease presentation will be required to determine whether detection of anti-BmaA antibodies is more sensitive in acute or convalescent stages of disease.

Twenty-three serum samples from culture-confirmed Lyme disease patients representing early localized, early disseminated, and convalescent stages were assayed to assess cross-reactivity against rBmaA. Twenty-two of the 23 Lyme disease patient samples were negative for rBmaA suggesting a high level of specificity for BMD cases vs Lyme disease (Fig. 6A). However, one sample had detectable antibodies to rBmaA. This patient (LD-6) had multiple erythema migrans, reported known tick exposure, and was confirmed as having Lyme disease based on positive skin culture and positive PCR. Testing of the serum was negative by IgG ELISA and Western blot. Interestingly, we found the IgG Western blot of

this patient's serum sample was strongly reactive to several *B. miyamotoi* and *B. turicatae* antigens with only a single reactive band seen in the *B. burgdorferi* lane (Fig. 6B). These observations suggest this patient may have also been infected with a TBRF borrelia as a coinfection or as a separate prior infection.

3.6. Lyme disease patient serum samples reactivity to rBmaA by Western blot

We performed IgG line immunoblotting with the 23 Lyme disease patient serum samples to reassess the ELISA results by a second assay. In agreement with the ELISA results, the Lyme disease patients demonstrated little or no observable reactivity to rBmaA above background (Fig. 7A) except patient sample LD-6 as seen in Fig. 6A–B. All Lyme patient samples were reactive to *B. burgdorferi* whole cell lysate except for LD-1. Several, but not all, Lyme patient samples were positive for recombinant OspC, a major protein used in Lyme disease serology, which was included in the line blot as a single antigen control for comparative purposes with rBmaA. Representative Western blots were performed on 8 samples and supported the serologic non-reactivity against rBmaA from *B. burgdorferi*-infected individuals that was observed by ELISA and line immunoblotting (Fig. 7B, lane 1). All 8 samples were positive for antibodies to antigens in both the *B. burgdorferi* and *B. miyamotoi* whole cell lysates (lanes 3 and 4 respectively), with some samples (LD-18, −20, −23) also exhibiting reactivity against rGlpQ (lane 2). This result illustrates the antigenic cross-reactivity between the two species and underscores the need for additional specific serodiagnostic antigens.

3.7. TBRF human and mouse serum samples seroreactivity to rBmaA

We performed IgG immunoblotting against rBmaA with human serum collected from 3 patients infected by *B. turicatae* at the same locale, plus two additional serum samples from persons who were present at the exposure site but did not become ill. None of the 5 samples exhibited a positive reactivity to rBmaA including the 3 persons who became ill and were diagnosed with relapsing fever (Fig. 8A, lane 3). However, the 3 TBRF patients' serum were positive for antibodies to antigens in the *B. turicatae* whole cell lysate (Fig. 8A, lane 1) with some cross reactivity observed to *B. miyamotoi* whole cell lysate (Fig. 8A, lane 2). Healthy patient sample 1 demonstrated cross reactivity to a band at 37 kDa (Fig. 8A, lanes 1 and 2) which was likely to be against FlaB.

We also tested mouse anti-*B. turicatae* antiserum and the serological profile was strongly positive for antibodies to antigens in the *B. turicatae* whole cell lysate but negative for rBmaA (Fig. 8B). As a control, mouse anti-*B. miyamotoi* was positive for antibodies to rBmaA (Fig. 8B). These early results suggest that *B. miyamotoi* BmaA may be a species-specific serological antigen for RF borrelia.

4. Discussion

Standardized, validated, and commercially available serodiagnostic laboratory tests for BMD are currently unavailable. Recent studies have documented limitations in BMD serodiagnostic assays used for research purposes, e.g. GlpQ lacks sensitivity for acute disease detection and BMD patients can have cross-reactive antibodies against the Lyme

disease C6 antigen and other borrelial antigens (Koetsveld et al., 2019; Krause et al., 2018; Molloy et al., 2015, 2018). We demonstrate that a *B. miyamotoi* surface lipoprotein, BmaA, is recognized by convalescent serum IgG antibodies from PCR-confirmed BMD patients by Western blot and ELISA, and specifically, BmaA did not react with 22 of 23 serum samples from a cohort of Lyme disease patients.

We showed that BmaA was surface exposed with a characteristic lipoprotein signal peptide and peptidase cleavage site. BmaA was synthesized during in vitro cultivation independent of temperature. This observation was unlike synthesis seen for some *B. burgdorferi* outer membrane lipoproteins that are induced by temperature changes mimicking the environmental shift during tick bloodmeal acquisition (Ojaimi et al., 2003; Revel et al., 2002; Schwan et al., 1995; Tokarz et al., 2004).

Two identical copies of the gene encoding BmaA are found on plasmids that also harbor *vlp* and *vsp* genes, and genes with sequences homologous to *bmaA* are found on at least three other plasmids according to sequence data available for strain CT13-2396. These findings point to a *bmaA* paralogous gene family in *B. miyamotoi*. Determination of functional roles for BmaA and other *B. miyamotoi* outer membrane lipoproteins in the tick-mammalian host infectious cycle awaits.

2-DE immunoblotting of *B. miyamotoi* membrane proteins with human BMD serum samples reflected a pattern of antigen recognition as was observed previously with mouse anti-*B. miyamotoi* serum (Harris et al., 2019), i.e. the immunodominant antigens were present mainly in the 37 kDa region where the Vmp antigens migrate (Barbour, 2016; Harris et al., 2019; Koetsveld et al., 2018; Wagemakers et al., 2016). BmaA was identified by mass spectrometry confirming that this antigen was immunoreactive with human anti-*B. miyamotoi* antibodies. 2-DE immunoblotting showed some immunoreactive profile differences between IgM and IgG suggesting such antigens may be useful targets for detection of recent vs more remote BMD infection. Additionally, the antigenic patterns seen previously between the mouse antibodies vs the human antibodies presented in this study offer clues for immunogens specific for human disease.

We tested 5 convalescent serum samples from patients diagnosed with BMD confirmed by PCR, with 4 of the 5 BMD patients seroreactive against rBmaA. The lone BmaA-negative BMD patient presented to the clinic for blood collection 157 days after the date of PCR testing whereas the BmaA-positive patients presented between 38–100 days. While it cannot be concluded from the small sample size, it would be interesting in future studies to determine the timing of development and resolution of the anti-BmaA response. Answering this question could aid in determining the role of BmaA (and other immunogenic proteins including GlpQ) in BMD pathogenesis and address comparisons between the mouse model and human BMD infections.

Except for one, the Lyme disease patient serum samples demonstrated no observable IgG seroreactivity against rBmaA by Western blot and ELISA. However, the lone BmaA-positive Lyme patient provided some interesting observations. The clinical report for this patient stated multiple erythema migrans, tick exposure, positive skin culture, and a positive

PCR, all signs of a *B. burgdorferi* (s.l.) infection. However, the patient was IgG ELISA and Western blot negative at the time of presentation. The negative Western blot was corroborated by the immunoblot we performed (with only one prominent *B. burgdorferi* band, Fig. 6B) while there were several strongly positive bands seen in *B. miyamotoi* and *B. turicatae* lysate lanes. This patient may have had a BMD coinfection or a prior BMD infection.

Immunoblot assays with anti-*B. turicatae* antibodies demonstrated no observable seroreactivity to the *B. miyamotoi* rBmaA suggesting that this antigen may be species-specific and therefore potentially useful to differentiate among infections caused by RF borrelia. We were not able to obtain anti-*B. hermsii* antiserum or antiserum against other RF borrelia for this study, but future work to fully assess specificity will need to include more anti-RF borrelia serum samples.

An obvious limitation of the current study was the low number of well-characterized BMD samples available for evaluation. Statistical assessment of the sensitivity and specificity for rBmaA as a serodiagnostic antigen will require the acquisition of a larger sample set of BMD and TBRF-infected patient serum.

In conclusion, we found that BMD patient serum was seroreactive with rBmaA indicating that this antigen may be useful for serological assessment for *B. miyamotoi* and potentially other TBRF borrelial infections. Our initial observations showing little to no antigenic cross-reactivity with anti-*B. burgdorferi* antibodies suggests BmaA may be an attractive candidate to add to the growing list of described TBRF borrelial antigens, GlpQ, BipA, BrpA, Bta112, Vmps (Schwan et al., 1996; Barbour, 2016; Embers et al., 2019; Lopez et al., 2009; Lopez et al., 2010; Lopez et al., 2013; Wagemakers et al., 2016), and others identified by global analyses (Barbour, 2016; Lopez et al., 2009; Tokarz et al., 2020) to improve laboratory serologic differentiation between patients with BMD vs Lyme disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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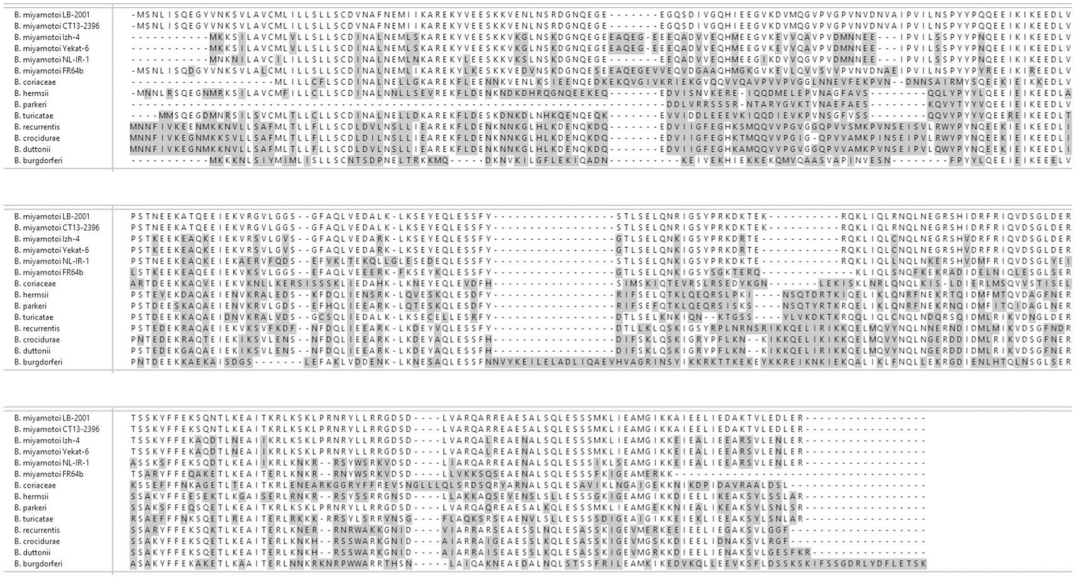
a



b

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		100.0	81.1	81.1	75.4	67.6	44.1	52.4	50.3	56.8	50.3	51.1	51.4	35.4	1
2	0.0		81.1	81.1	75.4	67.6	44.1	52.4	50.3	56.8	50.3	51.1	51.4	35.4	2
3	19.1	19.1		100.0	83.8	66.5	47.8	51.4	52.7	58.1	49.2	49.7	50.0	37.8	3
4	19.1	19.1	0.0		83.8	66.5	47.8	51.4	52.7	58.1	49.2	49.7	50.0	37.8	4
5	30.6	30.6	19.7	19.7		62.7	45.7	53.2	50.3	58.6	51.1	51.1	51.4	37.8	5
6	40.4	40.4	40.5	40.5	47.4		43.0	49.7	40.0	50.0	48.1	47.0	47.0	33.2	6
7	104.9	104.9	102.8	102.8	107.3	105.1		38.6	40.3	43.0	41.1	41.4	40.5	28.4	7
8	85.4	85.4	82.7	82.7	82.7	81.8	123.9		60.5	65.4	54.1	55.1	56.2	33.5	8
9	61.4	61.4	60.5	60.5	73.2	83.7	117.5	42.5		52.2	41.9	43.8	44.6	33.8	9
10	70.0	70.0	63.8	63.8	66.5	77.9	105.7	57.0	69.8		52.7	53.0	53.8	34.9	10
11	94.4	94.4	86.0	86.0	86.0	90.7	111.7	81.6	94.4	83.5		85.9	87.3	32.2	11
12	90.1	90.1	82.7	82.7	84.6	93.5	108.9	78.1	85.7	82.6	17.5		94.6	33.8	12
13	89.5	89.5	82.2	82.2	84.0	90.7	109.4	75.2	82.7	80.3	14.7	5.3		33.8	13
14	103.9	103.9	97.5	97.5	102.5	92.8	139.0	120.3	114.8	117.4	122.2	112.0	114.2		14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

B. miyamotoi LB-2001
B. miyamotoi CT13-2396
B. miyamotoi Izh-4
B. miyamotoi Yekat-6
B. miyamotoi NL-IR-1
B. miyamotoi FR64b
B. coriaceae
B. hermsii
B. parkeri
B. turicatae
B. recurrentis
B. crocidurae
B. duttonii
B. burgdorferi

Fig. 1.

A) Amino acid alignment of BmaA homologs from *B. miyamotoi* strains, RF borrelia, and *B. burgdorferi*. Amino acid differences from strain LB-2001 are shaded. The only sequence available in GenBank for *B. parkeri* listed a partial sequence missing the N-terminal sequences for this protein. B) Table showing percent amino acid identity and divergence among strains. Divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign. Percent identity compares sequences directly, without accounting for phylogenetic relationships. Note that divergence is not usually the inverse of percent identity (i.e., the sum of the percent identity and divergence values for a given pair is not usually 100).

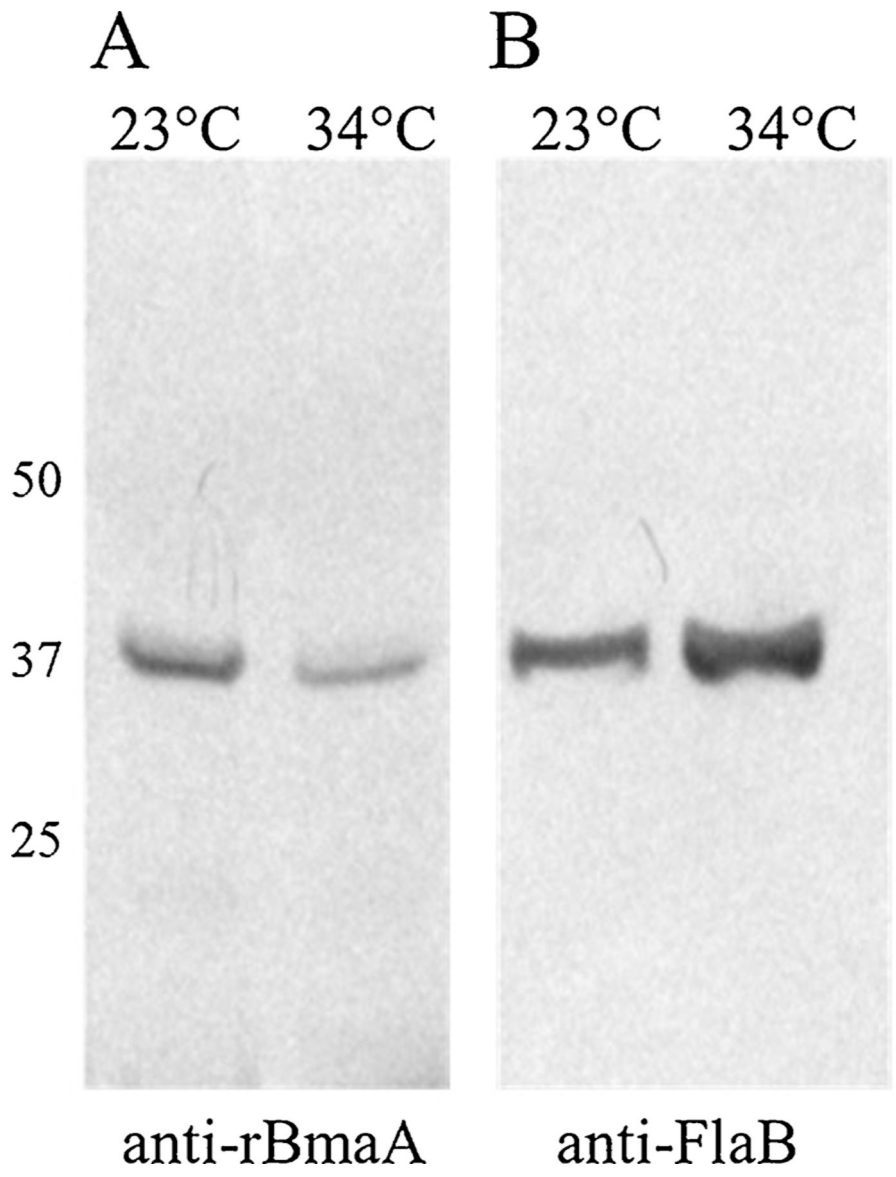


Fig. 2.

Immunoblots of *B. miyamotoi* cultures cultivated at 23 °C and shifted to 34 °C. Immunoblots probed with A) mouse anti-*B. miyamotoi* rBmaA serum; B) mouse anti-*B. burgdorferi* FlaB monoclonal antibody. Molecular weight markers in kilodaltons are on left of panel.

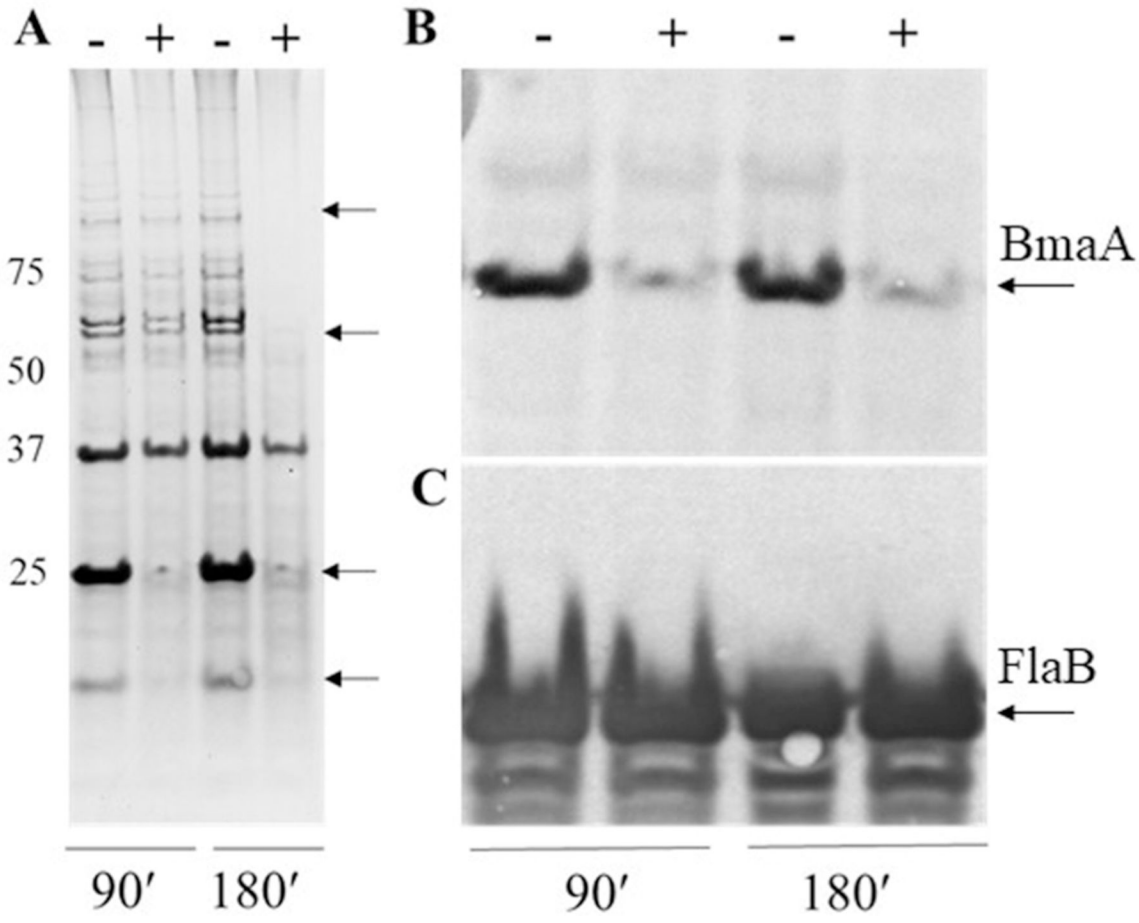


Fig. 3.

Surface localization of BmaA detected with (+) or without (−) addition of proteinase K at 90 and 180 min incubation. A) SDS-PAGE GelCode Blue staining of *B. miyamotoi* whole cell lysates. Arrows indicate areas of proteins affected by protease digestion. Molecular weight markers in kilodaltons are left of panel. B) Immunoblot of *B. miyamotoi* whole cell lysates probed with mouse anti-*B. miyamotoi* rBmaA sera. C) Immunoblot of *B. miyamotoi* whole cell lysates probed with mouse anti-*B. burgdorferi* FlaB monoclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

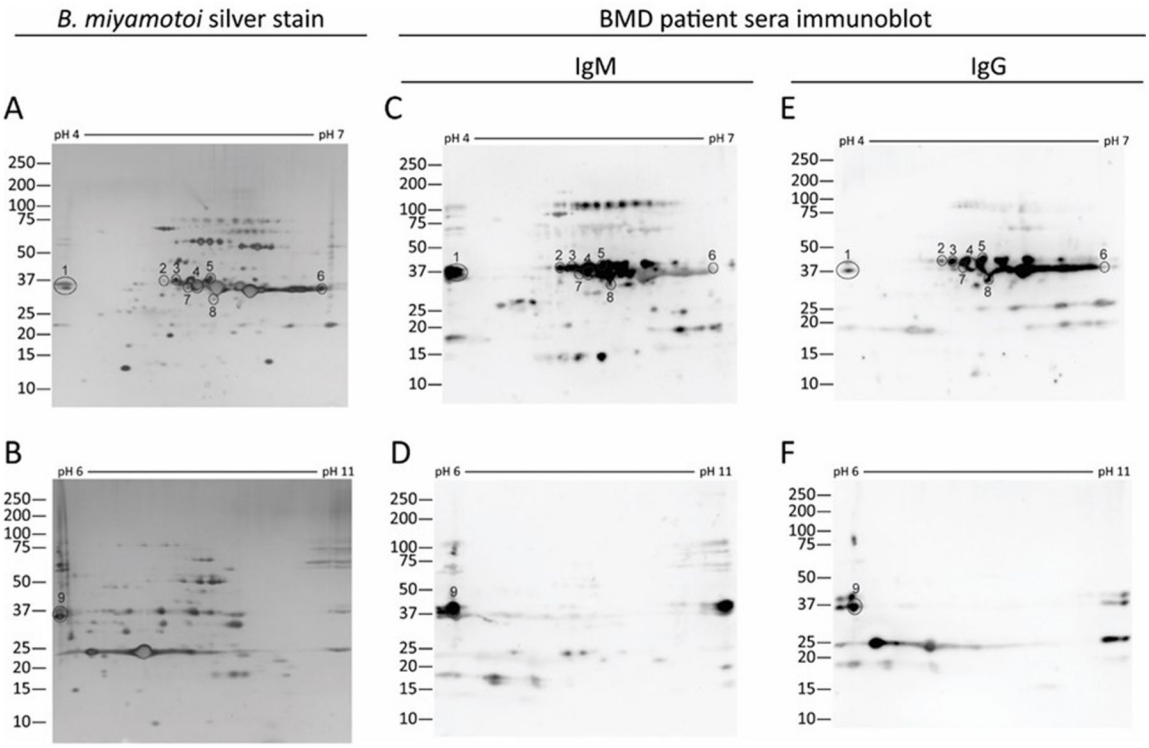


Fig. 4.

2-DE fractionation of *B. miyamotoi* membrane proteins by pH range 4–7 panels A,C,E and by 6–11 panels B, D, F. A and B) Fractionated proteins visualized by silver stain. C-F) Immunoblots probed with pooled serum from 5 BMD patients for IgM (panels C-D) and IgG (panels E-F). Proteins selected for mass spectrometry analysis are circled and numbered 1–9. Molecular weight markers are on left of panels.

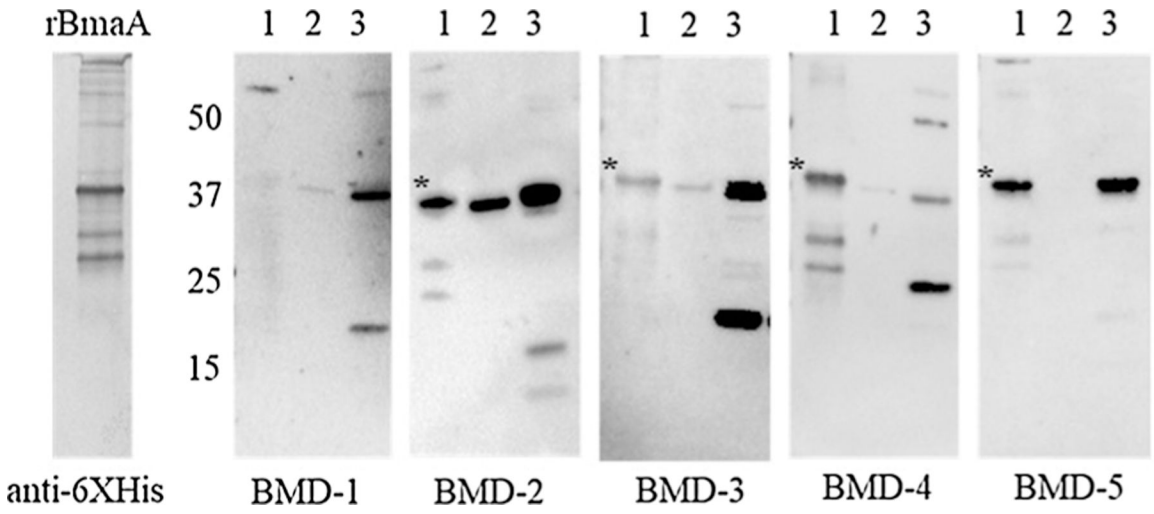


Fig. 5.

IgG immunoblots probed with individual BMD patient serum samples. Lanes: 1) rBmaA; 2) rGlpQ; 3) *B. miyamotoi* whole cell lysate. Asterisks denote rBmaA. Molecular weight markers in kilodaltons are on left. Left panel depicts rBmaA probed with anti-6X His-Tag antibody.

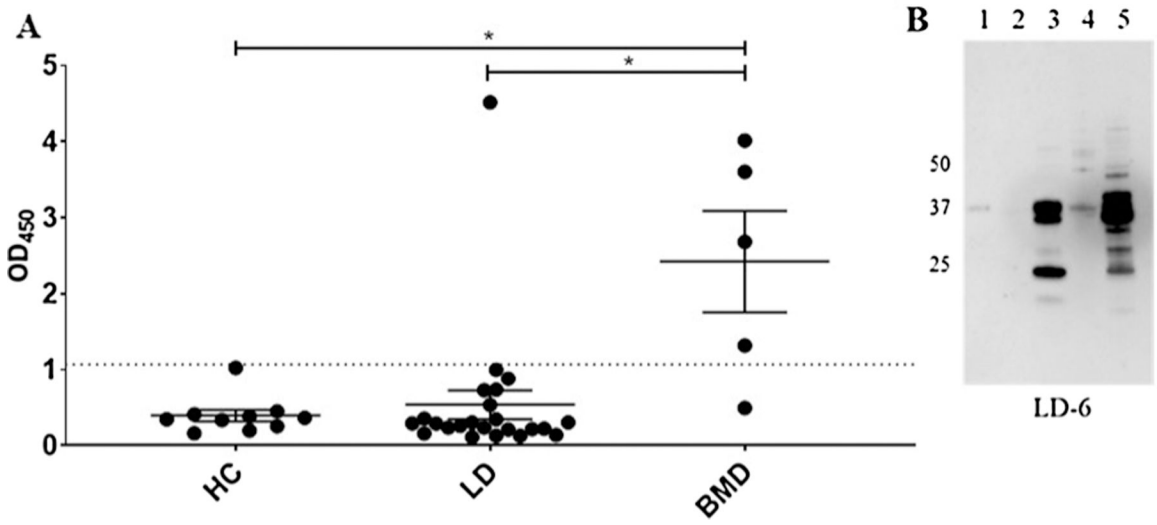


Fig. 6.

A) Patient serum sample IgG seroreactivity to rBmaA by ELISA. Healthy control individuals from endemic and non-endemic Lyme areas (HC); Lyme disease patients (LD); *Borrelia miyamotoi* disease patients (BMD). The dotted line indicates the cutoff value calculated by 3 standard deviations above the mean optical density for all healthy control samples. Each closed circle represents the average OD for each individual patient. Data are representative of two replicates within and across plates. Error bars indicate the standard error of the mean, while the horizontal bars represent mean values for each group. A P-value of ≤ 0.05 was considered significant and denoted by *. Statistical significance was determined using a one-way ANOVA followed by a Tukey's multiple comparisons test. B) Immunoblot of rBmaA-ELISA positive Lyme disease patient (LD-6), Lanes: 1) rBmaA; 2) rGlpQ; 3) *B. miyamotoi* whole cell lysate; 4) *B. burgdorferi* whole cell lysate; 5) *B. turicatae* whole cell lysate. Molecular weight markers are on left of panel.

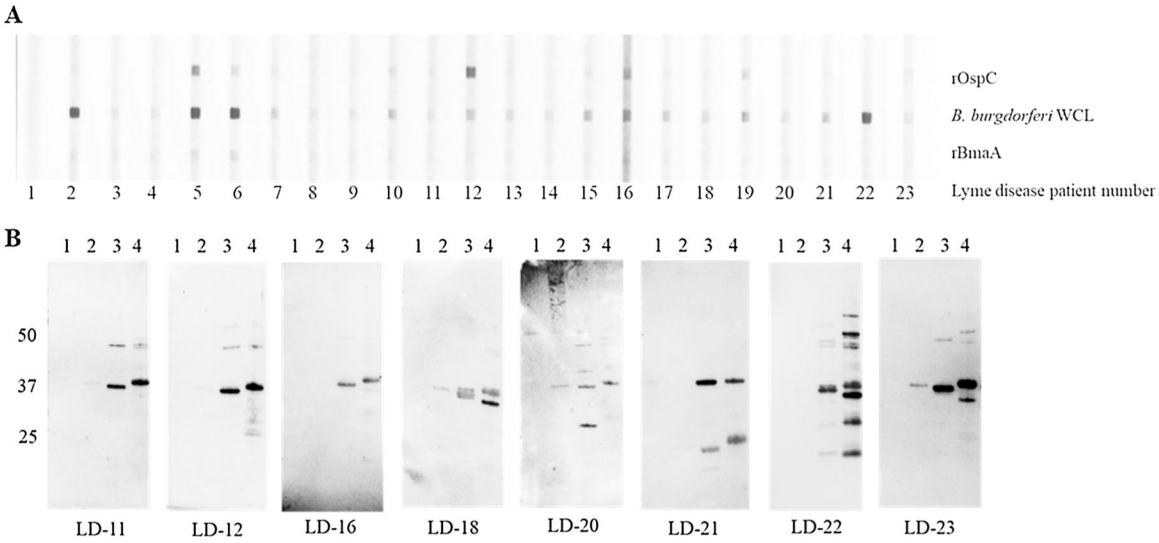


Fig. 7.

A) Line immunoblot of rBmaA, *B. burgdorferi* whole cell lysate, and rOspC probed with Lyme disease patient serum samples. B) Representative immunoblots probed with Lyme disease patient samples from line immunoblot and ELISA in Fig. 7A. Lanes: 1) rBmaA; 2) rGlpQ; 3) *B. miyamotoi* whole cell lysate; 4) *B. burgdorferi* whole cell lysate. Molecular weight markers in kilodaltons are on left.

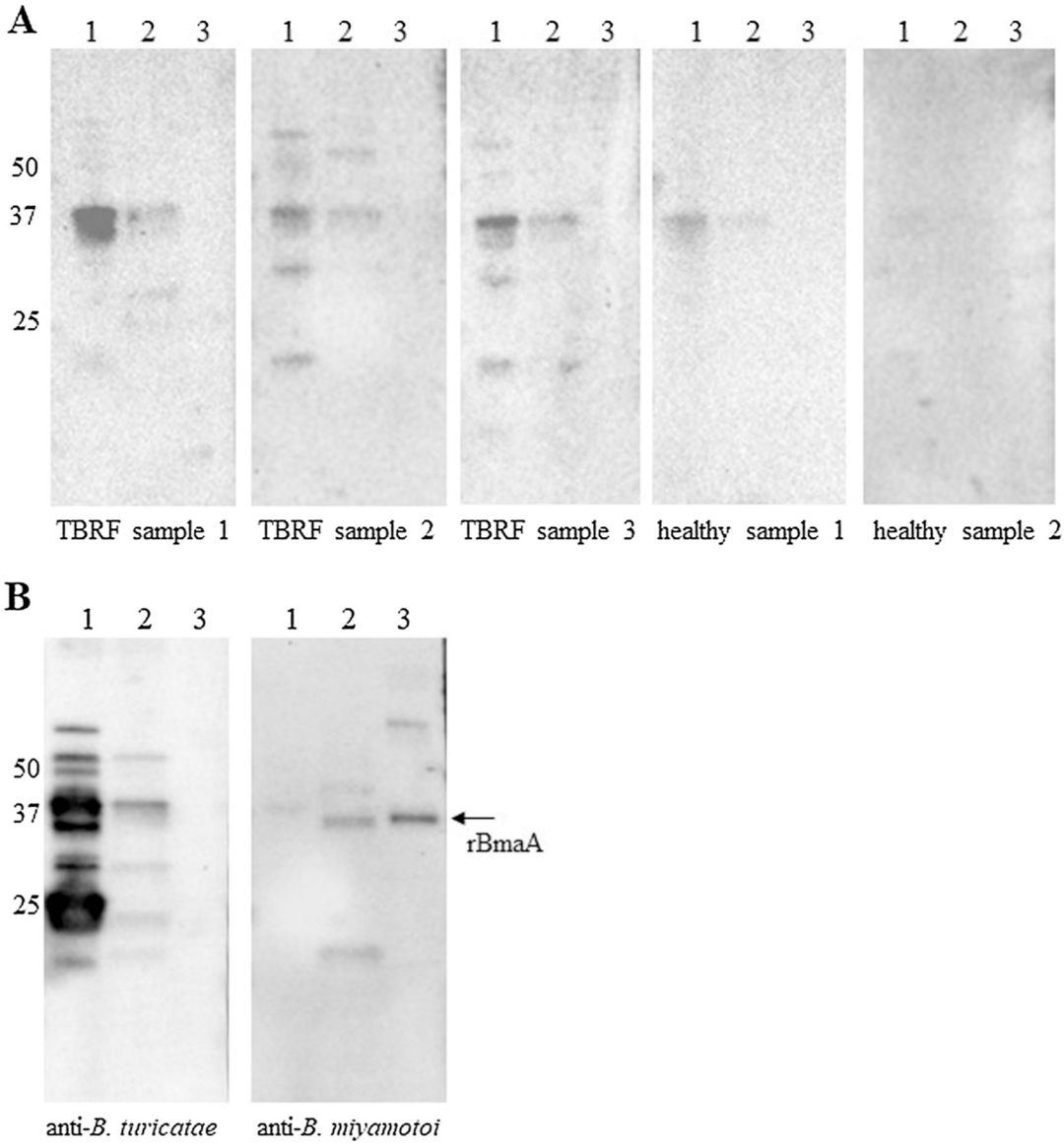


Fig. 8.

Western blots determining seroreactivity against rBmaA and lysates of *B. turicatae* and *B. miyamotoi*. Lanes: 1) *B. turicatae* whole cell lysate; 2) *B. miyamotoi* whole cell lysate; 3) rBmaA. A) Human serum samples collected from site of *B. turicatae* exposure. TBRF samples were from ill patients; healthy samples were from persons from the exposure site that did not become ill. B) Mouse antiserum generated against *B. turicatae*, *B. miyamotoi*, and rBmaA as labeled. Molecular weight markers in kilodaltons are on left of panels.

Table 1

Borrelia miyamotoi disease (BMD) patient data describing clinical symptomology.

Sample ID	Antibiotic administration	Days post-symptom onset	Confirmatory testing for BMD	Presence of EM	Previous history Lyme disease
1	Y	157	PCR	N	Unknown
2	Y	100	PCR	N	N
3	Y	57	PCR	N	Unknown
4	Y	13	PCR	N	N
5	Y	38	PCR	N	Y

Y = yes.

N = no.

Unknown indicates no previous diagnosis of either Lyme disease or tick-borne disease according to the patient's medical history or recollection.

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