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Borrelia miyamotoi strain LB-2001 retains plasmids and infectious phenotype throughout continuous culture passages as evaluated by multiplex PCR

Robert D. Gilmore[*],
Sierra Mikula,
Emma K. Harris[1],
Taylor J. Van Gundy,
Irina Goodrich,
Kevin S. Brandt

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

Abstract

Borrelia miyamotoi is a tick-borne spirochete of the relapsing fever borrelia group and an emerging pathogen of public health significance. The genomes of relapsing fever borreliae and Lyme disease borreliae consist of multiple linear and circular plasmids in addition to the chromosome. Previous work with *B. burgdorferi* sensu lato found diminished infectivity upon continuous in vitro culture passage that was attributable to plasmid loss. The effect of long-term culture passage on *B. miyamotoi* is not known. We generated a series of plasmid-specific primer sets and developed a multiplex PCR assay to detect the 14 known plasmids of *B. miyamotoi* North American strains LB-2001 and CT13–2396. We assessed the plasmid content of *B. miyamotoi* LB-2001 over 64 culture passages spanning 15 months and determined that strain LB-2001 retained all plasmids upon prolonged in vitro cultivation and remained infectious in mice. We also found that strain LB-2001 lacks plasmid lp20-1 which is present in strain CT13–2396. These results suggest that *B. miyamotoi* remains genetically stable when cultured and passaged in vitro.

Keywords

Borrelia miyamotoi ; Tick-borne relapsing fever; Multiplex PCR

1. Introduction

Borrelia miyamotoi, a tick-borne spirochete causing Borrelia miyamotoi disease (BMD), is an emerging pathogen of public health importance (Krause et al., 2015; Wagemakers et al., 2015). *Borrelia miyamotoi* is genetically related to the tick-borne relapsing fever

[*]Corresponding author. 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.

(TBRF) group of borreliae, but unlike other TBRF borreliae, it is transmitted by *Ixodes* spp. ticks which are also the vector for the Lyme disease spirochete, *B. burgdorferi* sensu lato (s.l.) (Krause et al., 2018; Platonov et al., 2011; Telford et al., 2015). Both Lyme disease borreliae and TBRF borreliae possess a genomic structure composed of an approximate megabase-sized chromosome and multiple linear and circular plasmids. The number of plasmids varies between borrelial species and often between strains. *Borreliaburgdorferi* s.l. can possess as many as 21 linear and circular plasmids ranging in size from 56 to 5 kilobases (Casjens et al., 2000). During the early days of *B. burgdorferi* studies, it was discovered that maintaining the organisms in culture by serial passages led to an eventual loss of a subset of plasmids (Barbour, 1988; Schwan et al., 1988). Although loss of these plasmids did not affect viability or growth in culture, the plasmids were found to harbor genes essential for borrelial infectivity in mice and ticks (Labandeira-Rey and Skare, 2001; Norris et al., 1995; Purser and Norris, 2000). Later studies revealed that *B. burgdorferi* s.l. could lose plasmids during other manipulations such as genetic transformations, reisolation from infected experimental animals, or frozen storage (Elias et al., 2002; Grimm et al., 2003). Therefore, assessing plasmid content of reisolated *B. burgdorferi* became essential in experiments involving genetic determinations of phenotypic changes. In contrast, a previous study reported no plasmid loss following prolonged culture passage and consequently no loss of infectivity in mice for the TBRF borreliae *B. hermsii* and *B. turicatae* (Lopez et al., 2008). The effect of multiple culture passages on plasmid stability and infectivity for *B. miyamotoi* is unknown. As investigations into the molecular pathogenesis of *B. miyamotoi* progress, an understanding of genomic plasmid integrity relating to infectivity for this pathogen is required.

In this study, we developed a plasmid-specific multiplex PCR assay and assessed the plasmid profile of *B. miyamotoi* strain LB-2001 over 64 culture passages throughout a 15 month period. We report finding no plasmid loss which correlated to an infectious phenotype following injection into mice.

2. Materials and methods

2.1. Strains and culture passaging

Borrelia miyamotoi strain LB-2001 passage 3 (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 maintained by passage in SCID mice (Scoles et al., 2001). *Borrelia miyamotoi* strains LB-2001 passage 4, CT13–2396, and FR64b (passages unknown) from frozen stock cultures were cultivated in Modified Kelly-Pettenkofer Medium (MKP-F) supplemented with 7% gelatin (Wagemakers et al., 2014) at 34 °C in capped tubes to create microaerophilic condition. Every 7 days, cultures at late log stage were counted by darkfield microscopy using a Cellometer counting chamber (Electron Microscopy Sciences, Hatfield, PA). A 2–4 ml culture aliquot was collected and pelleted in a microfuge tube, followed by a wash in 1X phosphate buffered saline (PBS), re-pelleted, and stored at −80 °C for future multiplex PCR analysis. The remaining culture was inoculated into fresh MKP-F at 1:10 dilution to generate the subsequent passage. The procedure was repeated weekly for a period of 64 passages.

2.2. Primer generation for strain LB-2001 plasmids

Primer sequences specific for individual plasmids were determined from the North American strain CT13–2396 deposited in GenBank (Kingry et al., 2017). Primer selection was performed from a download of each plasmid sequence retrieved from GenBank using PrimerQuest software (Integrated DNA Technologies, Coralville, IA) with a function of "Parameter Set: General PCR". We designed primer pairs to the 14 respective plasmids with the criteria that they did not share homology with other plasmids and possessed a Tm of at least 60 °C. Primers were determined to produce amplicons of staggered size for visualization as a ladder by size migration when analyzed by agarose gel electrophoresis or Bioanalyzer DNA chip (Agilent, Santa Clara, CA) according to manufacturer's directions. Primer sequences with amplicon sizes are listed in Table 1. Amplicons generated from each primer set were agarose gel purified and DNA sequenced to verify identity. The sequenced amplicon was compared to the strain CT13–2396 sequence and subjected to a BLAST search to ensure identity to only one plasmid.

2.3. Multiplex PCR

Multiplex PCR was performed in 3 groups to amplify segments of plasmids; Group LP1 (linear plasmid 1): lp23, −6, −41, −19, −30, and −72; Group LP2 (linear plasmid 2): lp20–1, −26, and −20–2; Group CP (circular plasmid): cp1, −2, −3, −4, and −5 (Table 1). A 5X master primer mix for each group was generated with a concentration of 0.5 μM for each primer. The multiplex reaction (20 μL) consisted of 5X primer master mix (4 μL), 5X NEB Multiplex PCR Master Mix (New England Biolabs, Ipswich, MA) (4 μL), DNA template (1 μL), and water (11 μL). Cycling Parameter 1 was 95 °C for 2 min (1 cycle), 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (35 cycles), 72 °C for 5 min (1 cycle). Cycling Parameter 2 was 95 °C for 2 min (1 cycle), 95 °C for 20 s, 60 °C for 1 min, 68 °C for 1 min (30 cycles), 68 °C for 5 min (1 cycle).

Borrelia miyamotoi templates were generated by purifying DNA from pellets of each passage stored at −80 °C using a DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's directions. DNA templates were also generated by resuspending a frozen 1 mL culture pellet in 50 μL of water and boiled in a water bath for 10 min followed by a brief microfuge spin to pellet cell debris. Following PCR, each multiplex group was analyzed on a Bioanalyzer DNA chip (Agilent) according to manufacturer's directions. Amplicons from individual PCR reactions were electrophoresed and observed on 4% Tris-acetate-EDTA (TAE) agarose gels.

2.4. Mouse inoculation and infectivity assays by culture, PCR, serology, and direct observation microscopy

Animal experiments were approved and conducted in accordance with guidelines and regulations as established by the Division of Vector-Borne Diseases Institutional Animal Care and Use Committee (IACUC) protocol number 19–012.

Six-week old outbred female CD-1 mice (Charles River, Raleigh, NC) were needle-inoculated subcutaneously with 1×10^5 *B. miyamotoi* strain LB-2001 passage 5 (n = 2) and passage 64 (n = 1). Blood (1–5 μl) was collected by tail-nick incision for spirochete culture

and direct microscopic observation at days 3, 7, 14 post-inoculation and ear biopsy samples were also taken at this time (Sinsky and Piesman, 1989). Higher volumes of blood were collected from the submandibular vein (cheek punch) for PCR analysis, culture, serology, and direct microscopic observation at days 21 and 30. Terminal blood collections were performed at day 42 post-inoculation whereby organs (heart, spleen, and bladder) were harvested for PCR and culture.

Approximately 2–5 μl of blood samplings were inoculated into 4 mL of MKP-F. Organs (heart, spleen, bladder) harvested at necropsy were divided with half cultured in MKP-F and half stored at −80 °C until DNA isolation was performed. Cultures were assessed for spirochetes by dark-field microscopy once/week up to 5 weeks post-inoculation.

DNA was isolated from approximately 1–5 μl of blood collected at days 3, 7, 14, 21, and 42 (50 μL) using the DNA Micro Kit (Qiagen) following manufacturer protocols. DNA was isolated from MKP-F cultures previously inoculated with blood. 4 mL of culture was pelleted at 14,000 × g for 10 min followed by washing in 1 mL sterile PBS. Pellets were resuspended in 200 μL TES buffer (50 mM Tris–HCl pH 8.0, 50 mM EDTA, 15 % sucrose), and SDS was added to 1% final concentration followed by addition 1.8 units of proteinase K (ThermoFisher Scientific, Rockland, IL) and incubated at 37 °C for 30 min. The sample was extracted with phenol/chloroform/isoamyl alcohol and precipitated by addition of 1/10 vol sodium acetate (pH 5.3) and 3 volumes ethanol. 70 % ethanol wash was followed by air drying the pellet and resuspension in 20 μL nuclease free water. DNA isolation from organs was performed by the phenol/chloroform method above with suspension in 400 μL TES and proteinase K digestion time increased to overnight at 55 °C with resuspension in 30 μL nuclease free water.

qPCR was performed utilizing Luna Universal Probe qPCR master mix (New England Biolabs, Ipswich, MA) with primers designed to amplify a fragment of *B. miyamotoi glpQ*; Forward- CACGACCCAGAAATTGACACA (0.5 u M), Reverse- GTGTGAAGTCAGTAGCGTAAT (0.5 u M), FAM -labeled probe- TCTTCCGTTTTCTCTAGCTCGATTGGG (0.2 u M). Cycling conditions were 95 °C 1 min (1 cycle), 95 °C 10 s, 60 °C 1 min (39 cycles) in 20 μL reaction volumes. Samples were assessed in triplicate with 3/3 reactions with a Ct < 39 required for positive sample designation. Amplicons generated from qPCR were visualized on 1% Tris-acetate-EDTA agarose gels.

For direct observation by microscopy, 1–5 μl of blood from tail-nick bleeds was resuspended into 50 μL of sterile PBS. Samples were stored overnight at −20° C, thawed on ice the next morning, and vortexed. Diluted blood (1 μL) was pipetted into a microscope slide reaction well with cover slip and visualized by dark-field microscopy (400X magnification). Borrelia were counted from 20 fields. Images were collected by Infinity Capture software (Version 6.3.0).

2.5. SDS-PAGE and immunoblotting

Borrelia miyamotoi (1×10^8) were pelleted, washed 3X in PBS or HBSS (Hanks Buffered Saline Solution), resuspended in 100 μL of 2X SDS-PAGE gel loading buffer and heated

to 99 °C for 10 min in a heat block. 10 μL (1×10^7 cells) per lane were electrophoresed on Criterion AnykD TGX gels (Bio-Rad, Hercules, CA). Gels were stained with GelCode Blue (ThermoFisher Scientific) or blotted to polyvinylidene difluoride (PVDF) membranes using the iBlot 2 system (ThermoFisher Scientific). Membranes were blocked for 45 min in SuperBlock (ThermoFisher Scientific) followed by incubation with primary mouse serum (1:500) 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]) for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG H + l secondary antibody (1:5000) in TBS-T was added and incubated for 45 min at room temperature followed by addition of colorimetric substrate nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (BCIP-NBT). All washes between incubations were in TBS-T 3X, 3 min each.

3. Results

3.1. Primer generation and PCR amplification of individual plasmid segments

The genomic sequence of strain LB-2001 is incomplete consisting only of the chromosome and a few plasmids. Therefore, we used the published genome sequence of *B. miyamotoi* strain CT13–2396 to derive sequences for plasmid-specific primers (Kingry et al., 2017). Like strain LB-2001, strain CT13–2396 is a North American strain isolated from *I. scapularis* from the same geographic region.

Each primer pair produced a distinct amplicon of the predicted size except for lp20–1 (Fig. 1). Multiple attempts to amplify lp20–1 using 4 additional sets of primers (Table 1), were unsuccessful (data not shown). However, all five lp20–1 primer sets generated amplicons from strain CT13–2396 (Fig. 2A–B). The data provides evidence suggesting that strain LB-2001 lacks lp20–1.

PCR for detection of the 14 plasmids was consolidated into 3 multiplex groups to streamline the process. The first and second group consisted of primers that amplify fragments of the nine linear plasmids (LP1 and LP2), with the third group for amplification of circular plasmid regions (CP; Table 1). A test using *B. miyamotoi* LB-2001 (passage 36) was performed with two separate PCR amplification parameters to assess optimal conditions considering the multiple primer pairs in a reaction. The first parameter was based on standard PCR amplification conditions (PCR parameter 1). The second parameter was from the multiplex PCR described by Bunikis et al. for *B. burgdorferi* which is routinely used in our laboratory (PCR parameter 2) (Bunikis et al., 2011). Both parameters successfully amplified all plasmids, however PCR parameter 2 performed better to amplify lp72 fragment (Fig. 3). We tested both amplification conditions for plasmid detection in multiple *B. miyamotoi* LB-2001 culture passages and found that PCR parameter 2 consistently produced a stronger lp72 amplicon (data not shown). We used PCR parameter 2 for all subsequent multiplex reactions.

We tested the multiplex primer pairs on genomic DNA isolated from the North American *B. miyamotoi* strain CT13–2396 and the Japanese strain FR64b. As expected, the primer sets successfully amplified the plasmid fragments from strain CT13–2396 (Fig. 4). However, amplicons were not detected from strain FR64b for lp30 (with smaller lp23) in LP1, lp20–2,

lp26, and lp20–1 in LP2, and cp3 and cp4 in CP (Fig. 4). This result was not unexpected because BLAST searches indicated that many of the primers used in the assay were not completely identity specific for FR64b.

3.2. Multiplex PCR of LB-2001 shows no loss of plasmids after multiple culture passages

Multiplex PCR reactions were performed on genomic DNA isolated from each of the *B. miyamotoi* LB-2001 culture passages (4–53 and 64) and were assessed by Bioanalyzer runs. We found complete plasmid profiles (except for lp20–1) in DNA from each of the culture passages (data not shown). The amplicon profile of passages 4 and 64 were identical (Fig. 5) demonstrating complete retention of plasmids during prolonged culture passage of *B. miyamotoi* strain LB-2001.

3.3. High passage B. miyamotoi strain LB-2001 retains infectivity in mice

Passages 4 and 64 of *B. miyamotoi* strain LB-2001 were needle inoculated separately into CD-1 outbred mice. Infectivity was measured by i) direct microscopic observation of blood; ii) culturing of blood and other tissues; iii) PCR of blood culture and tissue samples; and iv) serology.

Borrelia were observed by microscopy of blood collected from mice injected with either low or high passage *B. miyamotoi* usually at days 3 and 7 post-inoculation (Table 2, Fig. 6). Spirochetes were not numerous in 1–2 μl of blood, i.e. 0–1 borrelia / field in 20 fields indicating that mice were infected regardless of culture passage but did not have a high systemic density.

MKP-F cultures of blood or organ tissues collected from high- or low-passage inoculated mice did not yield visible spirochetes by dark field microscopy following 6–8 weeks incubation (Table 2). Since live borrelia were observed directly from blood smears in the mice, we surmised that live borrelia may be present in the cultures in low densities undetectable by the small volumes assessed by direct microscopy, therefore we concentrated the 4 mL MKP-F blood cultures by centrifugation and collected pellets for DNA detection by PCR. Blood culture pellets from both low- and high-passage inoculated mice were PCR positive (Table 2). DNA isolated from organs taken at necropsy 42 days post-inoculation were also PCR positive (Table 2). Cultured ear tissue in MKP-F became contaminated due to absence of antibiotics in the media so no determination could be made regarding spirochete localization in this tissue. *Borrelia miyamotoi* are sensitive to rifampicin that is used in culture media for ear biopsies for *B. burgdorferi*. It is difficult to completely sterilize ear tissue prior to culture inoculation, therefore whether *B. miyamotoi* can be cultured from ear tissue remains to be resolved.

We tested the mice for seroconversion as another confirmation for infection. All mice seroconverted against multiple antigens as observed by Western blot indicative of prior active infection (Table 2, Fig. 7).

We conclude from these results that high passage *B. miyamotoi* does not lose plasmids upon continuous passages in culture and thereby retains infectivity when needle-inoculated into mice.

4. Discussion

The discovery that *B. burgdorferi*, the causative agent of Lyme borreliosis, becomes non-infectious when inoculated into mice following continuous in vitro culture passaging of its infectious parental clonal strain became an important consideration for investigations of pathogenic mechanisms. Studies determined that upon long-term culture passage, *B. burgdorferi* s.l. did not retain the full complement of plasmids, specifically those harboring genes necessary for mammalian infectivity, although viability and growth in culture media was unaffected (Labandeira-Rey and Skare, 2001; Purser and Norris, 2000; Schwan et al., 1988). As investigations into the infectious processes of *B. miyamotoi* unfold, we undertook the current study to assess whether long-term culture passage of *B. miyamotoi* results in a similar loss of plasmids and consequently a non-infectious phenotype. As a component of this investigation, we developed a multiplex PCR assay to more easily assess plasmid content.

Borrelia miyamotoi strain LB-2001 was selected for this study because it is a North American tick isolate that has been used in previous investigations. It was initially described by Scoles et al. as a spirochete isolated from *I. scapularis* in northeastern United States that was not *B. burgdorferi* s.l. and was not culturable using existing growth media for borrelia (Scoles et al., 2001). Subsequent studies by the Hovius laboratory developed culture medium and conditions to grow strain LB-2001 in vitro, and they used this strain in their investigations into the antigenic properties of the surface proteins (Koetsveld et al., 2018; Wagemakers et al., 2016, 2014). We used this strain in our immunoproteomic investigation to uncover novel antigens for serodiagnostic purposes (Harris et al., 2019). The chromosome sequence and partial sequences of some plasmids have been reported for strain LB-2001 but a complete genomic representation and annotation has not been performed. However, the genomic sequence for the North American strain CT13–2396 was published and is publicly available (Kingry et al., 2017). Therefore, to generate primer pairs specific for plasmids of LB-2001, we speculated that since the North American strains were geographic relatives, CT13–2396 plasmid sequences could be used to amplify regions of LB-2001.

Primer pairs specific for each of the 14 plasmids (except lp20–1) successfully produced amplicons of staggered lengths that could be used in a multiplex PCR platform consisting of 3 reactions. We assessed 2 thermocycling protocols for amplification; one was a standard for general PCR, and one was reported for a *B. burgdorferi* multiplex that we have used successfully in our laboratory. Both cycling parameters worked well, although lp72 was better amplified with parameter 2, therefore determination of optimal conditions was not subjected to further empirical analyses.

We found that the multiplex primer set for lp20–1 did not produce an amplicon from strain LB-2001, nor did any of four additional lp20–1 primer pairs. Since the five sets of lp20–1 primer pairs were determined from the *B. miyamotoi* strain CT13–2396, they successfully amplified the respective portions of the lp20–1 plasmid in that strain. The findings of this experiment suggest that strain LB-2001 lacks lp20–1 thereby revealing a genotypic difference from strain CT13–2396. The putative absence of lp20–1 in LB-2001 did not influence growth in culture or the establishment of infection in mice by needle inoculation.

The GenBank entry for lp20-1 for strain CT13–2396 (CP017133) reports 14 open reading frames annotated as hypothetical proteins with no known functions in biological processes. Therefore, possible phenotypic effects of lp20–1-encoded gene products in the natural tick infectious cycle of *B. miyamotoi* and whether high passage LB-2001 is vector-competent remain to be determined. The presence or absence of this plasmid, together with the other plasmid primer sets will aid future studies assessing the differentiation of genotypes among other North American isolates.

BLAST analyses revealed that the North American plasmid primers were not always exact matches with *B. miyamotoi* strains from Russia, the Netherlands, or the Japanese strain FR64b thereby implying that this multiplex PCR would not be applicable for these strains. Indeed, we found that some, but not all, plasmid fragments were amplified from strain FR64b DNA, although not always of the sizes observed in the North American strains. Genomic sequencing of multiple Russian Izh and Yekat isolates report a differential number of plasmids ranging from 14 to 19 (Kuleshov et al., 2019, 2018; Kuleshov et al., 2020). Strains and isolates from geographic regions of the world will need specific primer pairs, but North American strains may be closely related to utilize the primer pairs described here. A genome assembly and annotation report of current strains with plasmids that have been sequenced is available at https://www.ncbi.nlm.nih.gov/genome/genomes/16651 and at https://www.ncbi.nlm.nih.gov/genome/plasmids/16651.

A limitation of our study is that the LB-2001 strain has not been cloned. The strain was originally propagated in mice and subsequently cultivated in liquid media and is the source of the spirochetes used here. We cannot confirm that the strain in these experiments represents a single population with regards to plasmid content. Our attempts to clone the strain by colony plating have so far been unsuccessful. Cloning by limiting dilution has also been problematic as we have observed that *B. miyamotoi* growth in culture results in little to no color change of the media in which to visually ascertain growth. We have also observed that initiating a culture with a low-density inoculum of this spirochete results in slow to no exponential growth, therefore a culture beginning with a single cell may not be possible or will at least prove difficult.

A second caveat was that the possibility of specific gene rearrangements on plasmids during the prolonged cultivations were not evaluated. Relapsing fever spirochetes have been shown to retain plasmids throughout passages and a previous investigation indicated genetic rearrangements or deletion within plasmids can occur (Lopez et al., 2008).

There is currently little information on experimental animal infections with this organism, particularly in outbred mouse strains. Therefore, we performed multiple analyses in mice to provide an accurate assessment of *B. miyamotoi* infection. We observed borrelia by microscopy from blood collected within a week post-inoculation albeit in low numbers. We also cultured the collected blood samples attempting to expand the population and confirm active infection. However, we found that inoculating a 4 mL culture tube with microliters of blood did not result in exponential growth that could be observed by microscopy of a 3 μL sample. Concentrating the culture by centrifugation and performing PCR on the pellet did provide a positive result for the presence of *B. miyamotoi* and confirmed the

direct microscopic blood examination. We had the same result when culturing organs (which contained blood within the tissue) when mice were sacrificed 42 days post-inoculation, i.e. there was no observable growth from culture, but PCR of organ tissue was positive. Our observations regarding culture of *B. miyamotoi* demonstrate that this spirochete is more fastidious than other RF borrelia or *B. burgdorferi*, and that larger inoculums may be required to obtain logarithmic growth in liquid media.

We were also unable to detect *B. miyamotoi* by PCR from DNA extracted from a small volume of mouse blood. This result may have been due to the low density of borrelia in the blood sample or the kit extraction method we initially utilized. When we extracted DNA by the phenol/chloroform method, we obtained PCR positive results. Lynn et al. described *B. miyamotoi* strain CT13–2396 detection by PCR from infected mouse blood from both inbred and outbred strains of mice using higher volumes of blood than was performed in our study (Lynn et al., 2019). That finding illustrates PCR detection of *B. miyamotoi* DNA from infected blood is achievable and will aid in future investigations into mechanisms of *B. miyamotoi* systemic growth and relapses.

In conclusion, we designed primer pairs to specifically amplify regions of each of the 14 plasmids for *B. miyamotoi* strain LB-2001 and developed a multiplex PCR assay whereby all plasmid reactions could be performed in 3 tubes. We used the multiplex PCR to examine genomic DNA isolated from successive culture passages of *B. miyamotoi* and determined that continuous culture passaging did not affect plasmid stability, and consequently infectivity. The findings differ from *B. burgdorferi* where plasmid loss in culture reflects a loss of infectivity, and coincide with studies on the TBRF borreliae, *B. hermsii* and *B. turicatae* (Lopez et al., 2008).

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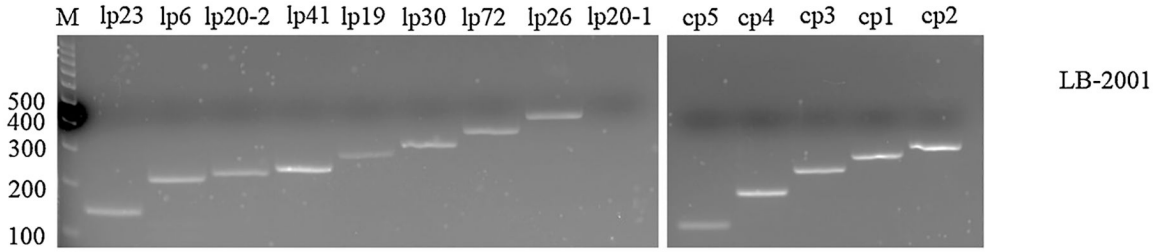

Fig. 1.

Amplicons generated from each plasmid (designated by lane) of *B. miyamotoi* strain LB-2001 run in individual reactions and electrophoresed on 4% TAE agarose gel. M; molecular weight markers with base pair sizes denoted on left.

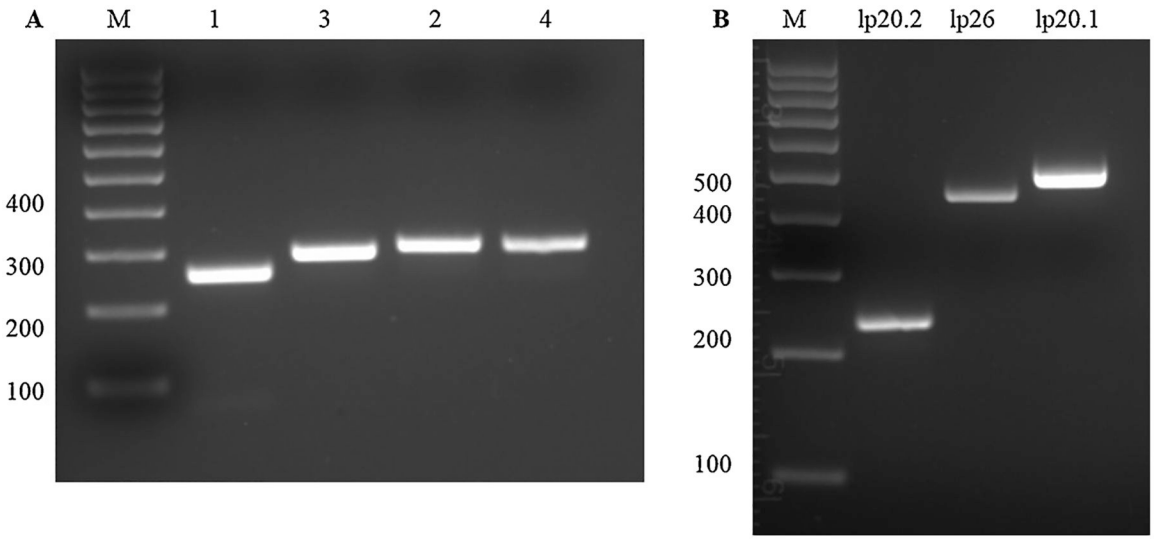

Fig. 2.

Borrelia miyamotoi strain CT13-2396 lp20-1 segment PCR amplification electrophoresed on 4% TAE agarose gels. A) Separate lp20-1-specific primer sets (designated 1–4, see Table 1); B) Multiplex LP2 primer set showing band identity of lp20-2, lp26, and lp20.1. M; molecular weight markers. Base pair designations on left of panels.

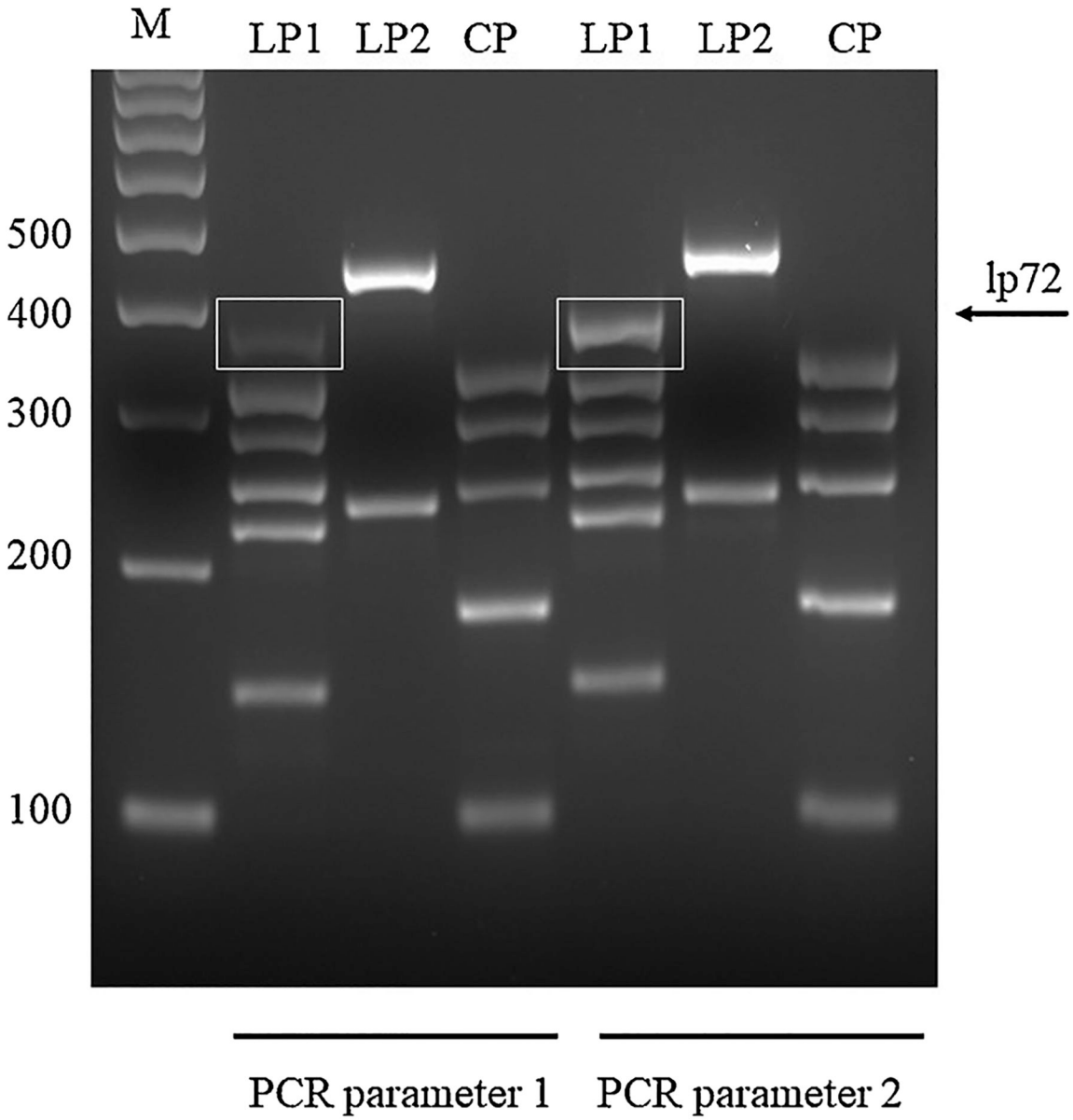

Fig. 3.

Comparison of PCR amplification efficiency using parameters 1 and 2 in the multiplex PCR assay with *B. miyamotoi* passage 36 DNA as template. lp72 amplicons are boxed. Amplicons were electrophoresed on 4% TAE agarose gels. M; molecular weight markers. Base pair designations are on left of panel. The three multiplex reactions are designated on top of panel.

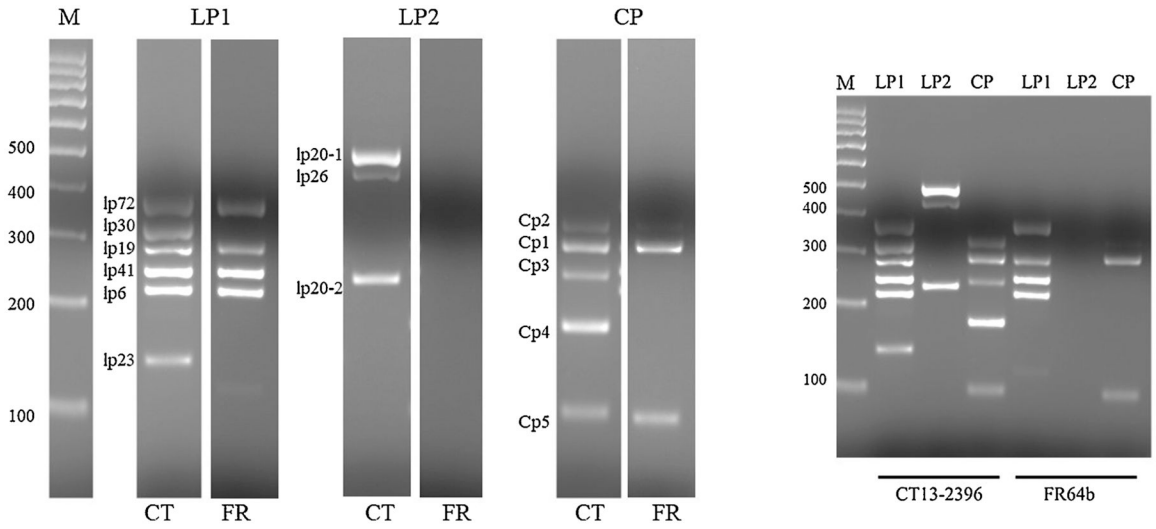

Fig. 4.

Plasmid multiplex PCR of *B. miyamotoi* strains CT13-2396 and FR64b. A) Strains aligned side-by-side for LP1, LP2, and CP. CT, strain CT13-2396; FR, strain FR64b. M, molecular weight markers with base pair sizes on left of panel. B) Agarose gel with multiplex PCR of both strains from which panel A was generated.

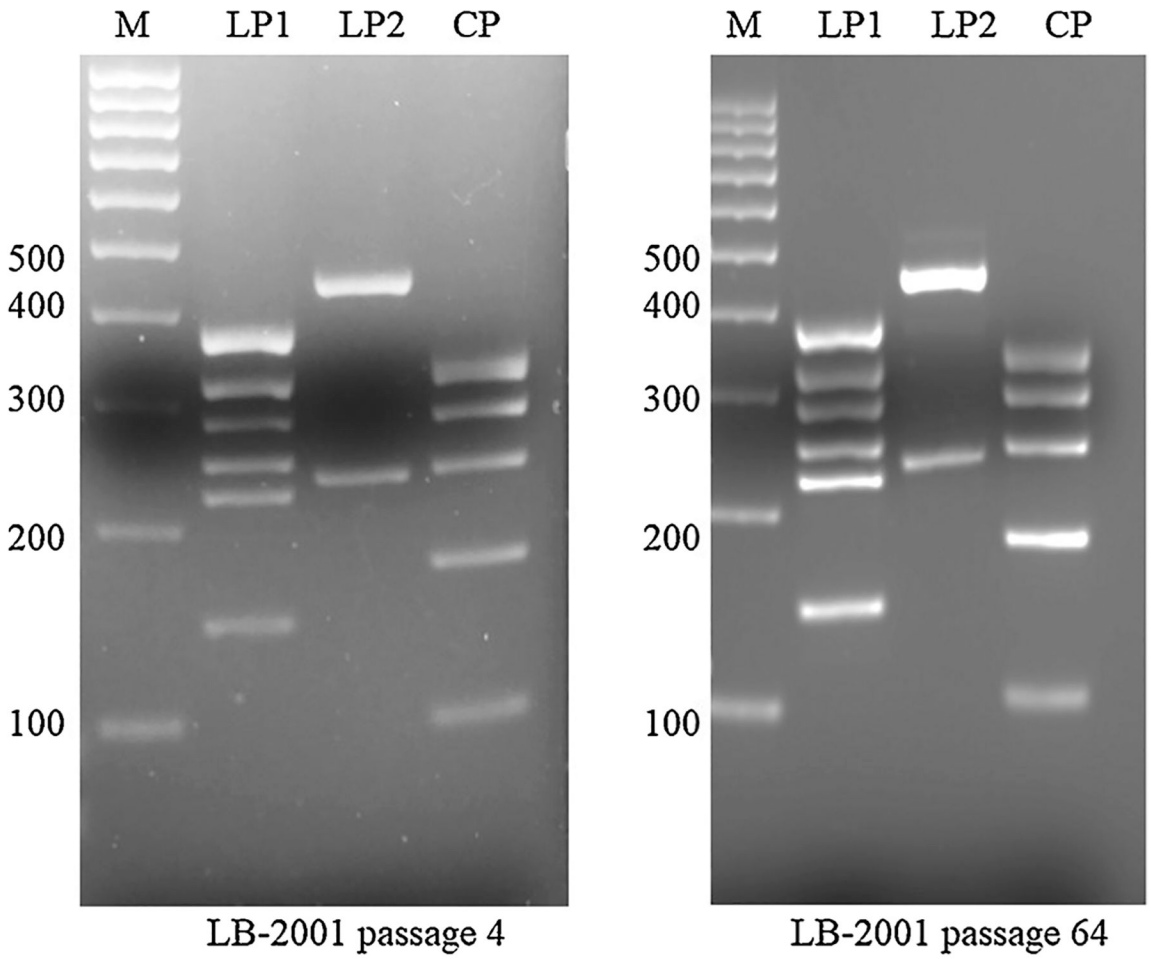

Fig. 5.

Multiplex PCR performed on *B. miyamotoi* strain LB-2001 passages 4 and 64. Amplicons electrophoresed on 4% TAE agarose gel. Multiplex reactions are designated by lanes. M, molecular weight markers; base pairs designations are left of panels.

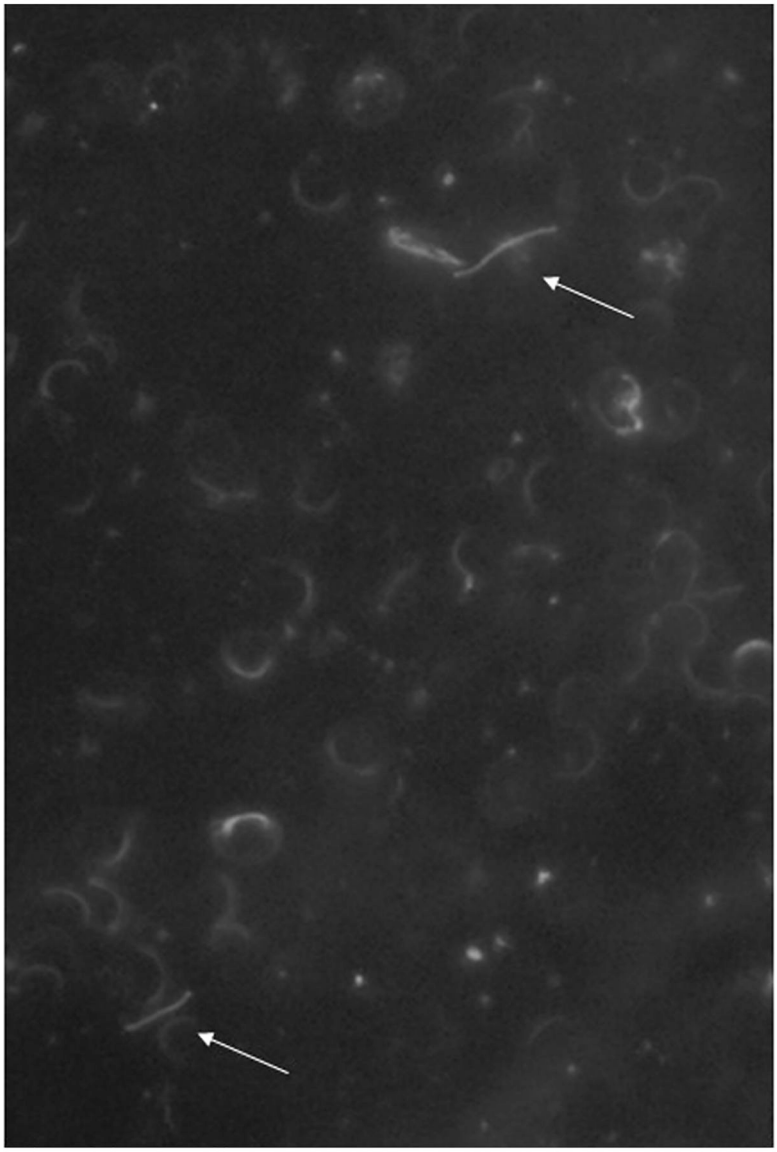

Fig. 6.

Representative dark field microscopy image of *B. miyamotoi* strain LB-2001 from mouse blood. Arrows point to spirochetes among mouse erythrocytes.

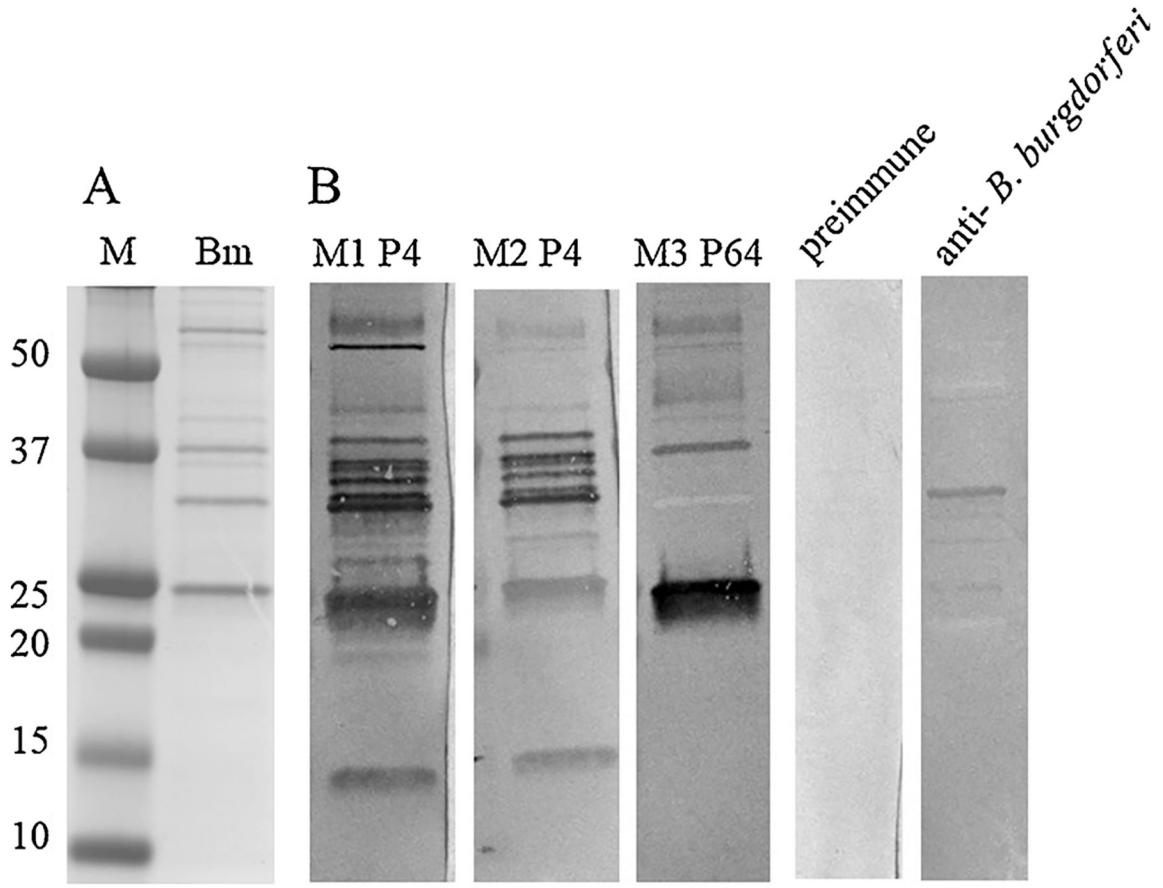

Fig. 7.

Western blots demonstrating seroconversion of mice inoculated with *B. miyamotoi* strain LB-2001 high and low passage. A) GelCode Blue staining SDS-PAGE. Lanes: M, molecular weight markers; Bm, *B. miyamotoi* whole cell lysate. Molecular weight in kilodaltons designated on left of panel. B) Western blots against *B. miyamotoi* strain LB-2001 whole cell lysate probed with mouse serum collected at day 42 post-inoculation of *B. miyamotoi* passage 4 mouse 1 and 2 (M1-P4, M2-P4) and passage 64 (M3-P64), preimmune mouse serum, and mouse anti- *B. burgdorferi.*

Table 1

Primers used in multiplex PCR.

primer-plasmid target	sequence	coordinates in CT13–2396	amplicon size (bp)
Group LP1			
lp23_7707_F	GCAGTTGCTCCAGCTAAA	7707–7724	146
lp23_7853_R	GTCGTTACAGCCTCATCTTC	7853–7833	
lp6_5150_F	CCTCAGGAGAGTGAGAGATATT	5150–5172	225
lp6_5375_R	CTCAACTTTGCCCTTTCTTTG	5375–5354	
lp41_3812_F	GGCAGAAAGAGAACCCATAC	3812–3832	252
lp41_4064_R	GTCGCAGGAGTTGGAATAC	4064–4045	
lp19_6359_F	TCTGTCCCTTTCGCTTATTATT	6359–6381	289
lp19_6648_R	GCGGAGATTCCAGTGATTT	6648–6629	
lp30_24962_F	GCAGATGGCACTAAGGATAAA	24962–24983	323
lp30_25285_R	TGCCTCCTTCTCATCCTATAA	25285–25264	
lp72_28752_F	AACACTACGGCCACAATAAG	28752–28772	370
lp72_29122_R	CCAAGGGTAATGGGTACATAAA	29122–29100	
Group LP2			
lp20–1_11269_F	CTGCGAAGAATGCCGATATAA	11269–11289	488
lp20–1_11756_R	CTAGCAGTAGTTGCACCAATAG	11756–11735	
lp26_957_F	GCAGTGTATCATTAGCCATAGA	957–979	435
lp26_1392_R	AGAAGTGGCAGCAGTAAAG	1392–1373	
lp20–2_16488_F	TGAACTTGTCCCTAATGAACC	16488–16509	236
lp20–2_16724_R	GAATCACCGCAATTTACTTCAG	16724–16702	
Group CP			
cp1_1938-F	GGTATCTCTATTGGCATAATAACAG	1938–1962	292
cp1_2229_R	GGGAGTGTTAAAGAAGCAGTA	2229–2209	
cp2_20293_F	GCTTCAATGGATGATACTGAGA	20293–20314	325
cp2_20617_R	TAACTGCTCGTTTATACGGAAA	20617–20596	
cp3_13269_F	CCAATACTTCACTATATTCGGCAA	13269–13292	244
cp3_13512_R	TGGTCTGTAGAAGCATTTGAAG	13512–13491	
cp4_8348_F	GGGTAGGTTTAGGTGATAATAAGG	8348–8371	178

primer-plasmid target	sequence	coordinates in CT13–2396	amplicon size (bp)
cp4_8525_R	CTACTAGCTATACCCGTTGAGA	8525–8504	
cp5_18025_F	TAAAGACATCTCTGTTGAACCATC	18025–18048	100
cp5_18124_R	TAGTAATAATATCGAAACCTCCTAAGGG	18124–18097	
additional lp20–1 primers			
lp20–1 F1	GGTTCTAGTGGTGTTGGTAGAATTAG	19,916	260
lp20–1 R1	CTCTACTTCTTTCTTTGCAGCTAAAGT	20,176	
lp20–1 F2	TCCCTAGCTCGGTATCTTAAA	6901	310
lp20–1 R2	GAAGATTCAGGGACTTGTACTC	7211	
lp20–1 F3	CTGATGGCTTAGGTTGATTCT	11,440	296
lp20–1 R3	GCTACTGTTCCTGCTTCATTA	11,736	
lp20–1 F4	TCTTTCTCTGTCAAATAGTCGCCTG	15,663	310
lp20–1 R4	GCAAATTTGATGATGTTCCTAATAAAGG	15,973	

Table 2

Mouse infectivity results from inoculation with high and low passage *B. miyamotoi*.

		Direct observation from blood	Blood culture microscopy	PCR from blood culture	PCR from tissues	Serology
Mouse (passage inoculated)						
1 (passage 4)	day 3	neg	neg	neg	ND	ND
	day 7	**pos**	neg	**pos**	ND	ND
	day 14	neg	neg	**pos**	ND	ND
	day 21	neg	neg	**pos**	ND	ND
	day 30	**pos**	neg	ND	ND	**pos**
	day 42	neg	neg	ND	**pos**	**pos**
2 (passage 4)	day 3	**pos**	neg	**pos**	ND	ND
	day 7	**pos**	neg	**pos**	ND	ND
	day 14	neg	neg	**pos**	ND	ND
	day 21	**pos**	neg	**pos**	ND	ND
	day 30	neg	neg	ND	ND	**pos**
	day 42	neg	neg	ND	**pos**	**pos**
3 (passage 64)	day 3	**pos**	neg	**pos**	ND	ND
	day 7	**pos**	neg	**pos**	ND	ND
	day 14	neg	neg	**pos**	ND	ND
	day 21	neg	neg	**pos**	ND	ND
	day 30	neg	neg	ND	ND	**pos**
	day 42	neg	neg	ND	**pos**	**pos**

pos = positive.

neg = negative.

ND = not done.