Epidemiological study of *Borrelia miyamotoi* disease in Japan

（我が国におけるボレリア・ミヤモトイ病に関する疫学研究）

２０１９

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Abbreviation list

BMD: *Borrelia miyamotoi* disease

CBB: Coomassie brilliant blue

GlpQ: glycerophosphodiester phosphodiesterase

HRP: horseradish peroxidase

LD: Lyme disease

NIID: National Institute of Infectious Diseases

PBS: phosphate-buffered saline

REP: Reptile-associated

RF: Relapsing fever

rGlpQ: recombinant GlpQ

SFTS: Severe fever with thrombocytopenia syndrome

Abstract

Borrelia miyamotoi, which is genetically classified to Relapsing fever (RF) borreliae, was identified as an etiological agent of emerging RF in Russia in 2011. Emerging RF, recently renamed "*B. miyamotoi* disease (BMD)", is a zoonosis transmitted by hard ticks from natural reservoir hosts. In this study, I discovered the first case of *B. miyamotoi* infection in Japan, and serological investigation further revealed that cases of BMD are distributed nationwide. Briefly, I demonstrated two cases of *B. miyamotoi* infection by retrospective surveillance of Lyme disease (LD) cases. In the investigation, I detected borrelial DNA from patient sera which was obtained in the acute phase, and sequence analysis revealed that the *B. miyamotoi* found was identical or closely related to that found from patients in Russia and *Ixodes* ticks in Hokkaido. This was the first report of BMD in Japan, and my data strongly suggested that BMD is widely distributed from Russia to Japan. In the next step, I conducted a retrospective cohort study using patient sera that were obtained from clinically suspected LD cases and a healthy cohort. In this study, I confirmed the presence of anti-RF borreliae-specific antibody in sera from clinically suspected LD cases, and the frequency was significantly higher than in the healthy cohort. Furthermore, I found that BMD cases were distributed nationwide. This finding suggested *B. miyamotoi* infection is a human health concern in Japan. These studies provide baseline information for public health regarding BMD in Japan.

Preface

Since 2013, three kinds of tick-borne infectious diseases (Severe fever with thrombocytopenia syndrome (SFTS), Anaplasmosis, and BMD) have emerged in Japan. The causative agents of these diseases are transmitted by hard ticks and thought to be zoonotic diseases. BMD is caused by *B. miyamotoi* infection. The genus *Borrelia* is comprised of three phylogenic groups: LD borreliae, which include the agents of LD, RF borreliae, and Reptile-associated (REP) borreliae (1) (Figure 1). LD and REP borreliae are transmitted by Ixodid (hard-bodied) ticks and most of the RF borreliae are transmitted by Argasid (soft-bodied) ticks, except for *Borrelia recurrentis*, which is transmitted by the human louse. Some RF borreliae such as *B. miyamotoi*, *Borrelia theileri* and *Borrelia lonestari*, however, are found in hard-bodied ticks: *Ixodes* spp., *Rhipicephalus* spp. and *Amblyomma* spp., respectively (2, 3). *B. miyamotoi*, one of the hard tick borne RF borreliae, has been found in *Ixodes scapularis* and *Ixodes pacificus* ticks in North America (4~6), *Ixodes ricinus* in Europe (7, 8), *Ixodes persulcatus* in Russia (9) and Asia (10, 11). In Japan, it has been found in *I. persulcatus*, *Ixodes pavlovskyi*, and *Ixodes ovatus*. (10, 11). Because these ticks are also known to transmit LD borreliae, concomitant infection with BMD has occurred in LD cases. Transmission cycle of *B. miyamotoi* is summarized in Fig. 2.

B. miyamotoi was originally isolated from field collected *I. persulcatus* ticks and the small Japanese field mouse *Apodemus argenteus* in Japan (12), but was considered a non-pathogenic *Borrelia* until 2011, when Platonov et al. reported the first evidence of human infections with acute febrile signs in Russia

(9). This was followed by reports from the United States of symptomatic cases showing influenza-like symptoms similar to the cases in Russia, as well as cases of meningoencephalitis (13~16). The most common clinical manifestations of BMD are fever, fatigue, headache, chills, myalgia, arthralgia, and nausea. Symptoms of BMD generally resolve within a week of initiating antibiotic therapy. Because clinical manifestations are non-specific, etiologic diagnosis requires confirmation by PCR, antibody assay, in vitro cultivation, and/or isolation by animal inoculation.

In this study, I conducted a retrospective investigation to identify potential cases of human infection with *B. miyamotoi* in Japan, based on detection of *B. miyamotoi* DNA from serum samples. I also performed a serological study to detect antibody to specific antigen of RF borreliae in patients clinically suspected of LD with comparison to a healthy cohort.

Figures

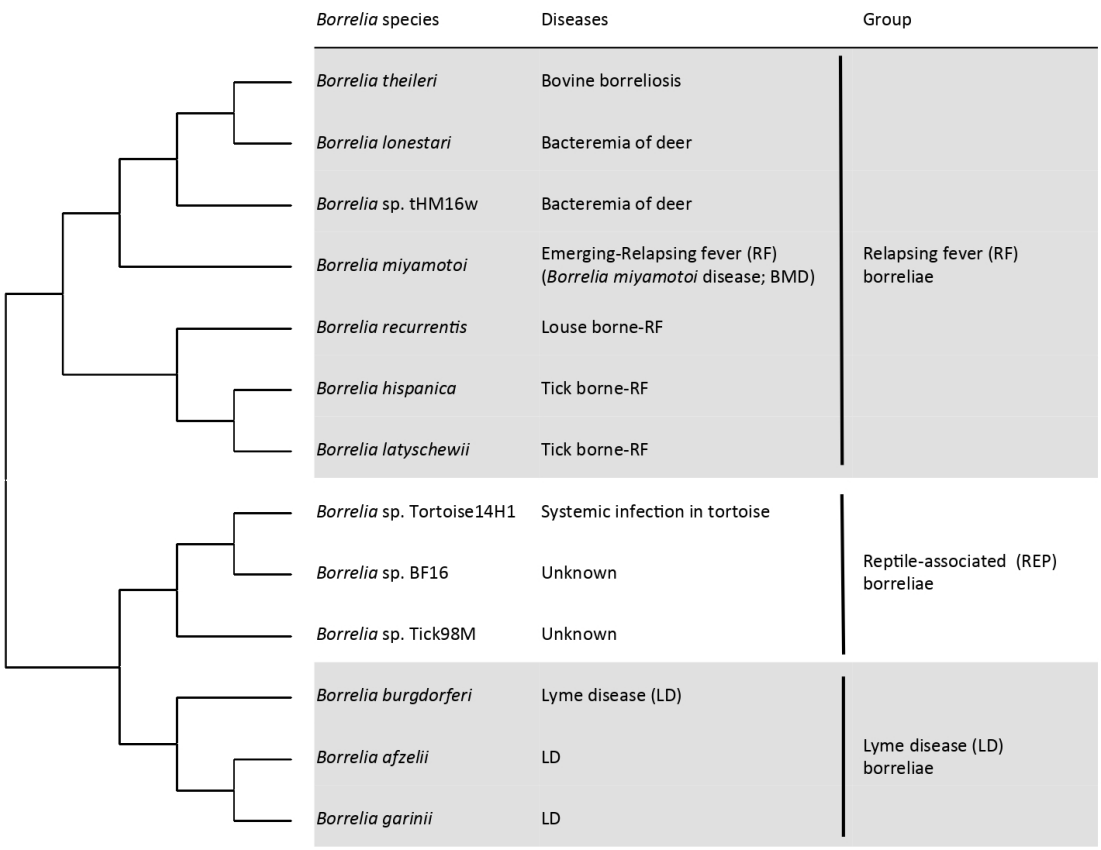


Figure 1. Phylogenetic relationship of *Borrelia* spp., disease association and classification.

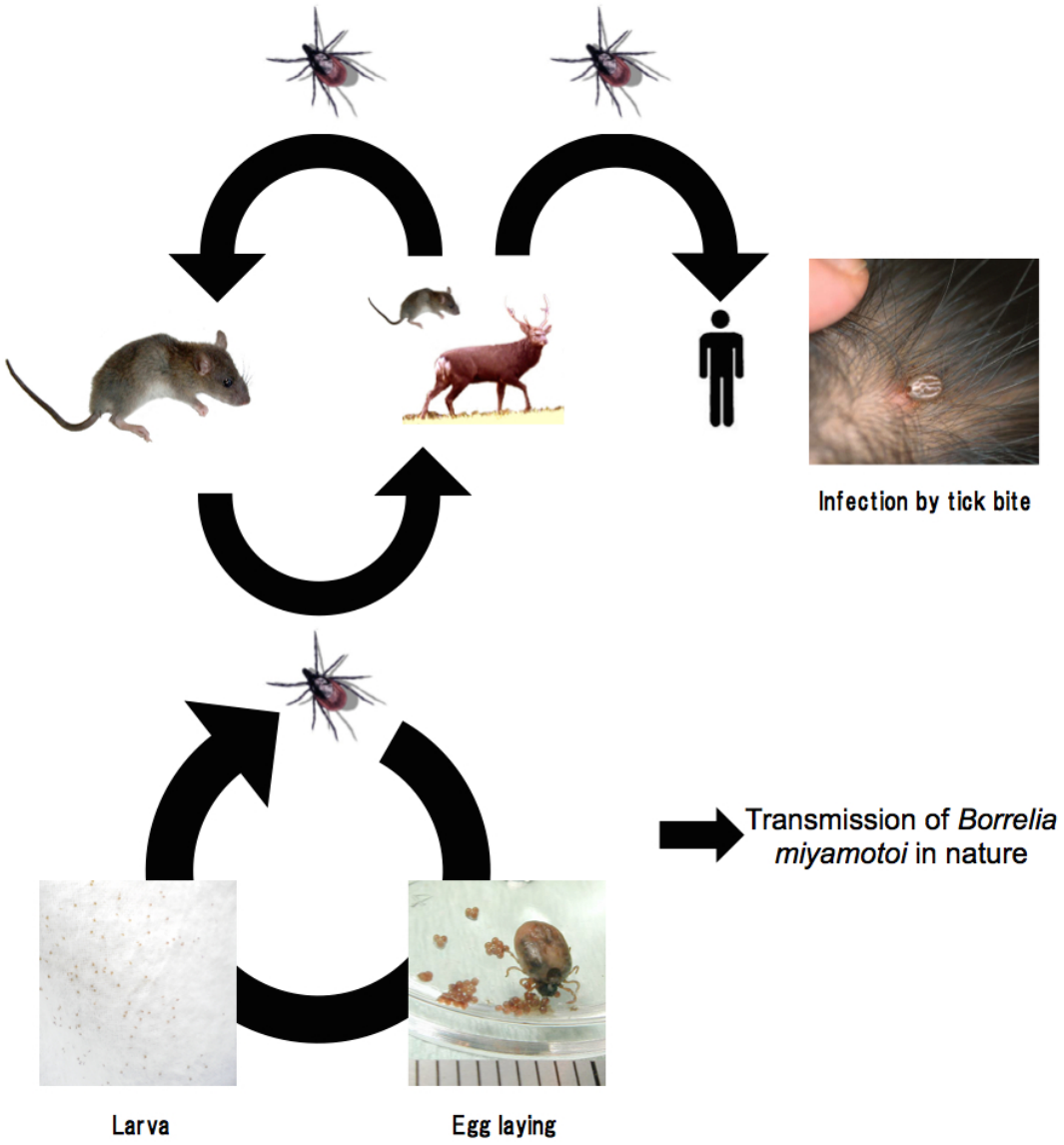


Fig. 2. Transmission cycle of *B. miyamotoi* in nature.

I. Discovery of BMD cases in Japan.

Summary

I confirmed infection of two patients with *B. miyamotoi* in Japan by retrospective surveillance of LD patients and detection of *B. miyamotoi* DNA in serum samples. One patient also showed seroconversion for antibody against recombinant glycerophosphodiester phosphodiesterase (rGlpQ) of *B. miyamotoi*. Indigenous BMD should be considered a health concern in Japan.

Introduction

B. miyamotoi, which is genetically classified to RF borreliae, was recently identified as a human pathogen in Russia (9), the United States (17), and Europe (15). Ticks of the *I. persulcatus* complex serve as transmission vectors. In Japan, this pathogenic borrelia was first discovered from *I. persulcatus* in 1995 (12). In a LD endemic area of Japan, wild rodents are infected with *B. miyamotoi* (18); however, no human infections have been confirmed. The Japanese isolates of *B. miyamotoi* are potential human pathogens because they formed a monophyletic lineage with Russian isolates from patients (10). In this study, a retrospective investigation was conducted to find potential cases of human *B. miyamotoi* infections in Japan.

Materials and methods

Patients sera

In total, 615 sera obtained from 408 individuals (Hokkaido-Tohoku 86 individuals; Kanto 134; Chubu 60; Kinki 52; Chugoku-Shikoku 34; Kyushu-Okinawa 44) in Japan including confirmed LD cases or unconfirmed, but clinically suspected LD, were used to detect *B. miyamotoi* DNA. The serum archive was established during 2008 to 2013 in the National Institute of Infectious Diseases (NIID), Japan.

Detection of borrelial DNA

All sera were centrifuged (15,000 x g, 10 min), and the sediments were used for DNA extraction. DNA extraction was performed by using DNeasy Blood&Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modification (The extraction column was incubated 10 minutes at 70°C prior to DNA collection). For detection of *B. miyamotoi* DNA, realtime PCR was performed using primers and probes previously described by Barbour et al. (19). The reaction was performed in 25 µl volume in single tubes at a final concentration of 1 µM for each primer and 0.25 µM for each probe. The PCR condition was set to 42 cycles of 95°C for 5 seconds and 60°C for 31 seconds on an Applied Biosystems 7000 Real-Time PCR apparatus. For confirmation of positive samples, flagellin gene (*flaB*)-nested PCR and sequencing of the amplicons were performed as reported previously (20).

The number of copies of DNA in a patient's serum was estimated by quantitative PCR (qPCR). Plasmid pBMrrs1 including a part of the 16S rRNA

gene for *B. miyamotoi* strain HT31 was prepared as previously described (20), and was used as a quantitative control.

Preparation of rGlpQ antigen

B. miyamotoi strain FR64b was cultivated using BSK-M medium at 30°C as previously described (10). Total DNA was purified using Wizard genomic DNA purification kit (Promega, WI, USA), and KOD FX DNA polymerase (TOYOBO Co., LTD., Osaka, Japan) was used for PCR amplification. PCR primers 1065 (5'-GACGACCGACAAGAAACAAGAAATGGGTTCAAA-3') and 1066 (5'-GAGGAGAAGCCCGGTTATTTTTTTATGAAGTTCA-3') were used for DNA amplification of the GlpQ gene (*glpQ*). The underlined portion of each DNA primer was a linker sequence for ligase-independent cloning to plasmid DNA. The amplified DNA fragment was purified using the High pure PCR product purification kit (Merck, Kenilworth, NJ, USA) and was used for in-fusion cloning with plasmid vector, pET46 Ek/Lic (Merck). *E.coli* strain Rosetta™(DE3) pLysS (Merck) was used for expression of the *N*-terminal 6 X Histidine(His) tagged rGlpQ antigen. The rGlpQ was assessed by SDS-PAGE and Coomassie brilliant blue (CBB) stain (21, 22), and reactivity to anti-His tag monoclonal antibody (Medical & Biological Laboratory Co. Ltd., Aichi, Japan). Quantification of rGlpQ was performed with the BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc., MA, USA). The *B. miyamotoi* strain FR64b was kindly provided by Minoru Nakao, Asahikawa Medical University.

Serodiagnosis of *B. miyamotoi*

Purified rGlpQ protein or whole lysate of *B. miyamotoi* strain MYK3 (0.5μg/well) was separated by 12.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and transferred to a Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (22, 23). Antigen blotted membrane was treated overnight at 4°C with blocking solution by the DIG wash and block buffer set (Merck). The blocked strips were then individually incubated with human serum at a 1:200 dilution at room temperature in blocking solution overnight. The strips were then washed 3 times and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-human IgM or IgG (Jackson Immuno Research Inc., West Grove, PA, USA) at a 1:10,000 or 1:20,000 dilution in blocking solution. After the strips were washed 3 times, bound antibodies were detected by chemiluminescence using the ECL Prime detection reagent (GE Healthcare UK Ltd Amersham Place, Little Chalfont, England).

Serodiagnosis of LD

Laboratory diagnosis for LD, immunoblotting was performed for these patients by using a commercial kit, recomLine borreliae IgM/IgG (Mikrogen GmbH, Neuried, Germany). Decisions on seropositivity were made based on the manufacturer's instructions.

Ethics statement

The use of the human samples was approved by The Ethical committee of the NIID for medical research using human subjects (Approval No. 360).

Results

From the retrospective investigation, the 2 cases (2.6%) out of 408 individuals were diagnosis with BMD by detection of *B.miyamotoi* DNA.

[Case 1]

Case 1 was a previously healthy, 72-year-old female resident of Hokkaido with no history of foreign travel. She developed myalgia and anorexia on July 13, 2011, and was subsequently admitted to a hospital on July 25, at which time she had a fever of 39°C. Physical examination revealed erythema migrans (EM), and the patient confirmed that she had been bitten by a tick 10 days prior. Laboratory tests on July 25 revealed elevated levels of C-reactive protein (44 mg/L), alanine aminotransferase (94 IU/mL), and aspartate aminotransferase (90 IU/ml). Procalcitonin was elevated to a positive level. Leucocytes were 3,900 cells/μl (87% neutrophils), and left shift was observed. Counts of red blood cells and platelets were within normal range. The patient was clinically diagnosed with acute LD because of typical EM with a history of tick bite. Antibiotic treatment with minocycline (100 mg/day) was continued for 5 days. The patient's symptoms improved rapidly, and she left the hospital on July 30. Borrelial DNA was detected from a serum sample collected on July 25 by both 16S rRNA gene based-real time qPCR and *flaB*-nested PCR. Sequencing of *flaB*-PCR amplicon DNA (294 bp) determined the infectious borrelia was *B. miyamotoi* (Accession Number AB921566), because the sequence was identical to *B. miyamotoi* HT31 (Acc. No. D43777). The number of copies of the borrelia genome in the serum was estimated to be 7.2 X 10^3 copies/ml by qPCR. Anti-GlpQ IgM antibody was elevated in her convalescent serum collected on

August 10 (Fig. 3A). In this case, I also found that antibodies were elevated in convalescent serum by immune blot analysis using a whole cell lysate of *B. miyamotoi* strain MYK3 (Fig. 3B).

[Case 2]

Case 2 was a previously healthy 37-year-old male, also a resident of Hokkaido, with no history of foreign travel. The patient was bitten by a tick on May 28, 2013, and was subsequently admitted to a hospital on June 11, at which time he had a fever of 39.8°C and EM at the location of the tick bite. The patient was clinically diagnosed with acute LD with EM. Antibiotic treatment with ceftriaxone (1 g/day) was continued for 7 days. Borrelial DNA was detected from a serum sample collected on June 11 by both qPCR and *flaB*-nested PCR. Sequencing of *flaB*-PCR amplicon DNA (294 bp) confirmed *B. miyamotoi* infection in the patient (Acc. No. AB921567) because the sequence was identical to *B. miyamotoi* HT31 (Acc. No. D43777). The number of copies of the borrelia genome in the serum was estimated to 2.8 X 10^4 copies/ml by qPCR. Anti-GlpQ antibody was not detected in his serum collected on June 11 (Fig. 3A).

IgM serology showed that serum from these patients reacted to several antigens for LD borreliae with a commercial kit (recomLine Borrelia IgG/IgM, Mikrogen) (Fig. 3C). Briefly, convalescent serum from Case 1 reacted to P100 originated to *Borrelia afzelii*, vmp-like sequence E (VlsE) originated to various *Borrelia* genomospecies, P41 originated to *Borrelia burgdorferi* sensu stricto, and Outer surface protein C (OspC) (*B. afzelii* and *Borrelia garinii*), and acute serum from Case 2 reacted to OspC (all of *Borrelia* spp. included in the kit).

Discussion

In this study, I confirmed infection of 2 patients with *B. miyamotoi* in Japan by retrospective surveillance of LD patients and detection of *B. miyamotoi* DNA in serum samples. However, the commercial serology test does not provide enough evidence to determine these patients were co-infected with LD borreliae, because the antigenic difference between *B. miyamotoi* and *B. burgdorferi* sensu lato has never been examined. Platonov and his colleagues reported that *I. persulcatus* is a transmission vector for *B. miyamotoi* and LD borreliae in Russia (9). This tick species is also ubiquitous in Hokkaido, and host-seeking behavior of the adult ticks is very active during the spring to late summer. Human tick bites in Hokkaido occur most often by *I. persulcatus*, and LD borreliae is transmitted to humans mainly through the bite of the adult tick (24). Although in the two cases herein described the causative tick species was not identified, circumstantial evidence suggests that *I. persulcatus* is a main transmission vector for *B. miyamotoi* in Hokkaido, as already shown in Russia (9).

BMD due to *B. miyamotoi* occurs in Russia, North America, and Europe, and *B. miyamotoi*-related meningoencephalitis has been reported in the United States, Holland and Germany (13~16). This study indicates that a human health threat from this BMD also exists in Japan. For risk analysis of this BMD, epidemiological surveys (e.g. determining the infection rates of host-seeking ticks belonging to the *I. persulcatus* complex in various localities in Japan) and improvement of serological diagnosis systems (especially early diagnosis) should be considered.

Figure

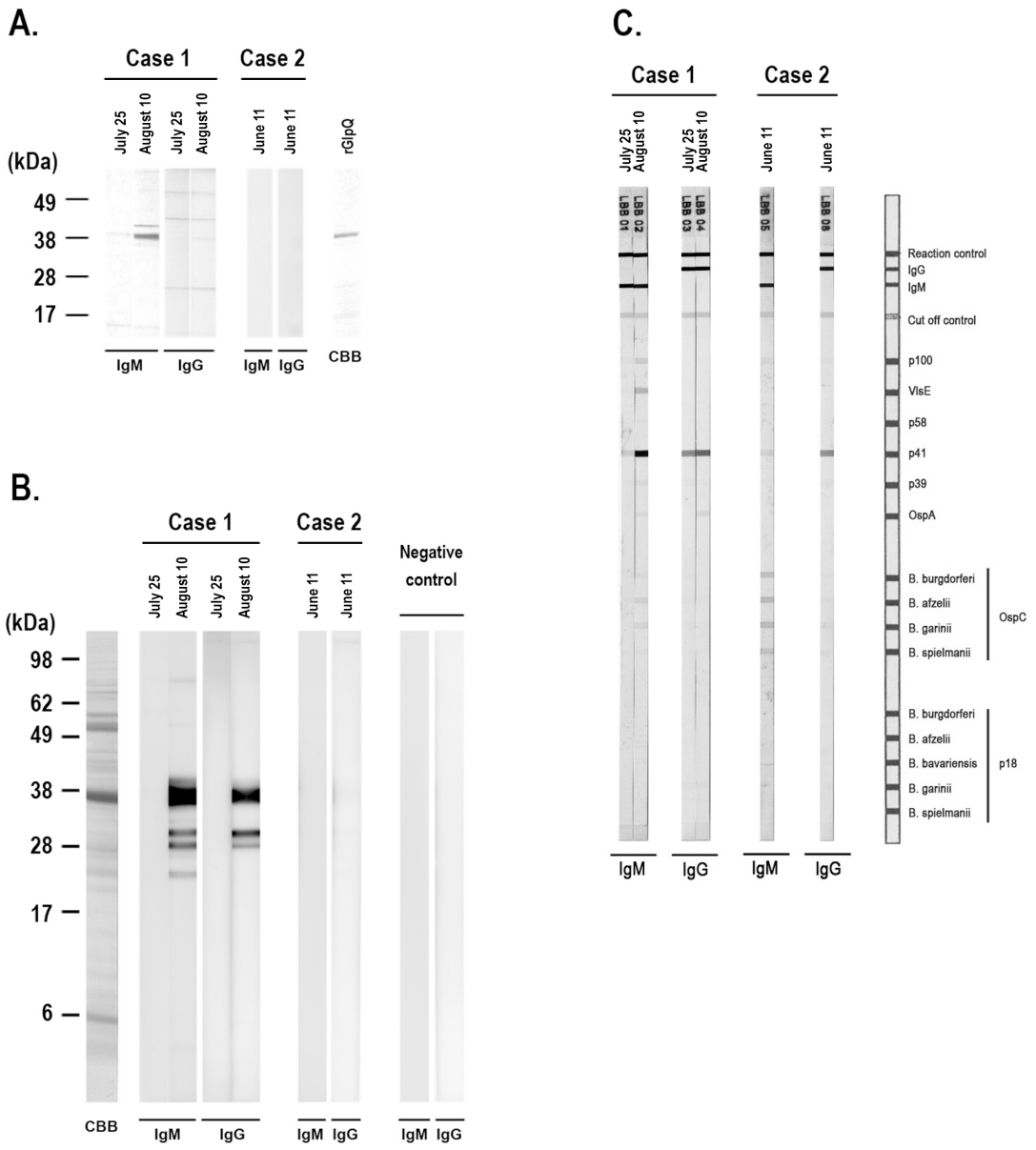


Fig. 3. Serum reactivity to antigens of *B. miyamotoi* and LD borreliae. Sera obtained from two patients were examined. In Case 1, acute serum (collected on July 25) and convalescent serum (collected on August 10) were used. In Case 2, acute serum (collected on June 11) was examined for the serological study. (A)

Reactivity to recombinant GlpQ antigen (rGlpQ). Crude rGlpQ were used for immunoblot analysis. Recombinant GlpQ was separated by 5-20% gradient Gel (Wako Pure Chemical Industries), and the antigen was strained by CBB (right). Molecular weight markers (49-17) are shown on the left in kilodaltons (kDa). (B) Reactivity of patient sera to whole cell lysate of *B. miyamotoi* antigens. Low passage strain of *B. miyamotoi* (strain MYK3) was used for the immunoblot analysis. The protein profile (CBB stain) is shown on the left. The negative control is from serum obtained from a healthy human (resident of a non-endemic area). (C) Serodiagnosis for LD with a commercial kit. A commercial kit (recomLine Borrelia IgG/IgM, Mikrogen) was used for this study. The test was performed according to the manufacture's instruction.

II. Case control study: Serological evidence that RF/BMD occurs nationwide in Japan

Summary

Since 2011, BMD has been reported in five countries in the northern hemisphere. The causative agent of BMD is transmitted by *Ixodes* ticks, which are also vectors of LD borreliae. In this study, I examined 459 cases of clinically suspected LD, and found twelve cases that were seropositive for the GlpQ antigen derived from *B. miyamotoi*. The retrospective surveillance revealed that the seroprevalence of anti-GlpQ in the clinically suspected LD cases was significantly higher than in a healthy cohort. Seropositive cases were observed from spring through autumn when ticks are active, and the cases were geographically widespread, being found in Hokkaido-Tohoku, Kanto, Chubu, Kinki, and Kyushu-Okinawa regions. Seropositive cases for GlpQ were most frequent in the Chubu region (6.3%) where *B. miyamotoi* has been found in *Ixodes* ticks. Out of the 12 seropositive cases that were found in the cohort clinically suspected of LD, three cases exhibited concomitant seropositivity to LD borreliae by western blot assay. This is the first report of serological surveillance for BMD in Japan, and I conclude that BMD occurs nationwide.

Introduction

RF is caused by infection with spirochetes of the genus *Borrelia*. The causative agents of RF are transmitted by the human louse or Argasid ticks, and RF is mainly reported in the Afrotropic and Palearctic ecozones (25). Louse-borne RF was endemic in Japan before World War II (26), however, no indigenous cases have been reported in recent decades (27). BMD, tentatively referred to as an emerging RF, is caused by infection with *B. miyamotoi*. Patients with BMD generally present with acute, nonspecific, flu-like symptoms, such as fever, headache, general malaise, myalgia, and arthralgia. However recurrent fever, a characteristic symptom of RF, is rare in BMD (9, 28, 29). Human infection was first reported in 2011 in Russia (9). Since then, patients have been reported in the United States, the Netherlands, Germany, Russia, and Japan (13-17, 29, 30). *B. miyamotoi* is transmitted by prostriate ticks of the genus *Ixodes* in Asia, Russia, Europe, and North America (31). The BMD agent, *B. miyamotoi*, has been detected in three species of *Ixodes* ticks in Japan (10, 11): *I. persulcatus*, the main vector of LD borreliae (*B. garinii, B. afzelii*, and *Borrelia bavariensis*) is found in Hokkaido and the mountainous regions of Kanto and Chubu regions (32, 33), *I. pavlovskyi*, a rare tick species in Japan , and *I. ovatus*, which is found throughout Japan and occasionally attaches to humans (34).

In the section I, I reported two cases of concomitant *B. miyamotoi* infection in patients with LD. Moreover, *B. miyamotoi* is known to share a transmission vector tick with LD borreliae (31). Therefore, while the clinical features of BMD are still unclear, I hypothesized that LD or suspected LD cases would be an appropriate cohort for the study of BMD. In this study,

epidemiological characteristics of human BMD were investigated and compared to a healthy cohort. I used a two-step *B*. *miyamotoi* GlpQ antigen-based antibody assay to test archived sera from confirmed and suspected LD patients nationwide.

Materials and Methods

Serum specimens used in this study

a) Suspected LD cases

Sera were obtained from patients who were clinically suspected of LD from 2005 through 2012. A total of 459 individuals were included in the group, and 221 (48.1%) of them were male. The mean age was 47 years (range, 1-84 years): ≦10 years 25 (5.4%); 11-20y 19 (4.1%); 21-30y 43 (9.4%); 31-40y 54 (11.8%); 41-50y 61 (13.3%); 51-60y 71 (15.5%); 61-70y 63 (13.7%); ≧71y 48 (10.4%); unknown 75 (16.3%). The distribution of patients used in this study is as follows: Hokkaido-Tohoku 86 individuals (18.7%); Kanto 166 (36.2%); Chubu 63 (13.7%); Kinki 62 (13.5%); Chugoku-Shikoku 30 (6.5%); Kyushu-Okinawa 52 (11.3%). Age group, sex, and residence of patients by region are listed in Table1 (Suspected LD cases is designated as “LD group” in this section). Sera from patients were stored at -20°C until use. Laboratory diagnosis for LD, immunoblotting was performed for these patients by using a commercial kit, recomBlot borreliae IgM/IgG or recomLine borreliae IgM/IgG (Mikrogen). Decisions on seropositivity were made based on the manufacturer’s instructions.

b) Healthy control

Sera from 542 healthy individuals were used for this cohort. These control sera were provided by the National Serum Reference Bank [NIID]. The ratio of control sera was adjusted for age group, sex, and region of patient residence to match the LD group (Table 1).

Preparation of rGlpQ antigen

The *N*-terminal His tagged rGlpQ antigen was prepared as previously described. Cobalt resin was used to purify the rGlpQ according to the manufacturer's instructions (Takara Bio, Shiga, Japan). The purity of rGlpQ was assessed by SDS-PAGE and CBB stain (21,22), and reactivity to anti-His tag monoclonal antibody (Medical & Biological Laboratory Co. Ltd., Aichi, Japan). Quantification of purified rGlpQ was performed with the BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc., MA, USA).

ELISA and western blot

a) In house rGlpQ-ELISA

A 96 well Maxisorp plate (Thermo Fisher Scientific Inc.) was used for rGlpQ-ELISA. We coated the ELISA plate with 0.5μg/well of purified rGlpQ in phosphate-buffered saline (PBS) and incubated the plate at 4°C overnight. After rGlpQ coating, the plate was treated with blocking buffer (PBS with 1% Skim milk) at room temperature for 2 hrs. The plate was then emptied, and human serum (1:100 dilution with blocking buffer) was incubated at 37°C for 1 hr. The plate was then washed 5 times with washing buffer (PBS with 0.05% Tween20). As the secondary antibodies, goat anti-human IgM HRP conjugate (40 ng/mL) (Jackson ImmunoResearch Inc.) or goat anti-human IgG HRP conjugate (20 ng/mL) (Jackson ImmunoResearch Inc.) were incubated at 37°C for 1 hr. To detect the HRP-labeled secondary antibodies, a 3,3',5,5'- tetramethylbenzidine solution (Nakarai tesque, Kyoto, Japan) was added and allowed to react for 15

min before absorbance was measured at 450/620 nm. The optimal IgM/IgG ELISA cut-off values were determined by comparing the mean ± 2 standard deviations of 91 negative sera. The negative control was determined by the following procedure. I randomly selected 92 samples from the LD group-sera and confirmed that there was no bias in the area where these patients resided. Then, western blotting using an rGlpQ antigen was performed for all sera. Of these 92 sera, 91 sera were judged to be negative for anti-GlpQ antibody and further used as the “Negative control” in this study. For each ELISA assay, sera that showed a titer around the cut-off value was used as a “cut off control” in every examination.

b) Western blot with rGlpQ

Purified rGlpQ protein (0.5μg/well) was separated by 12.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Wako Pure Chemical Industries) and transferred to a Sequi-Blot PVDF membrane (Bio-Rad Laboratories) as previously described (22). Antigen blotted membrane was treated overnight at 4°C with blocking solution by the DIG wash and block buffer set (Merck). The blocked strips were then individually incubated with human serum at a 1:200 dilution at room temperature in blocking solution overnight. The strips were then washed 3 times and incubated for 1 h with HRP-conjugated goat anti-human IgM or IgG (Jackson Immuno Research Inc.) at a 1:10,000 or 1:20,000 dilution in blocking solution. After the strips were washed 3 times, bound antibodies were detected by chemiluminescence using the ECL Prime detection reagent (GE Healthcare UK Ltd Amersham Place).

Statistic analysis

Prevalence of anti-GlpQ antibodies in each group from different categories were analyzed using Fisher's exact test. Comparison of age distribution between the BMD group and the LD group was analyzed using Student's *t*-test. A p-value less than 0.01 was considered to be statistically significant.

Ethics statement

Human serum specimens were all previously obtained as part of routine diagnostics, and the study using human specimens was approved by the Medical Ethics Committee of the NIID. (Approval Nos. 657 and 704).

Results

Calculation of cut off value of rGlpQ-ELISA

Ninety-one negative sera confirmed by rGlpQ-WB were used to determine a cut off value. These 91 sera showed IgM and IgG titers by the assay as follows: IgM titer median=0.193, SD=0.106, 2σ(upper)=0.427, IgG titer median=0.219, SD=0.066, 2σ(upper)= 0.356, respectively. From this data, I set cut off values for each ELISA at 0.427 for IgM and 0.356 for IgG, respectively.

Seroprevalence of rGlpQ antibody

Recombinant GlpQ was used for in-house rGlpQ-ELISA and rGlpQ-WB examination. Out of 459 cases from the LD group, 12 cases (2.6 %) were seropositive for BMD by the rGlpQ serological tests (Fig. 4). The mean age of rGlpQ-seropositive patients was 36.5 years. Of the 542 healthy individuals, one in their 30's (0.2%) was seropositive for rGlpQ (data not shown). Comparative analysis between the LD group and healthy cohort revealed that cases seropositive for rGlpQ were significantly higher in the LD group (p= 0.0009, Fisher Exact test, 2 tailed [Odds ratio=14.5, 95% CI: 1.9 to 112.1]). Out of 12 cases that were anti-GlpQ positive, three cases (25%) were also IgM positive by laboratory diagnosis for LD. For this reason, they were decided to be concomitant infections. Of these 12 cases, 11 cases were IgM positive by rGlpQ western blot and one case (case no. 12) was IgG positive by rGlpQ western blot (Table 2). Of the 11 cases, 6 had records for the number of days after onset of disease that serum was collected (Cases 1, 3, 8, 9, 10, and 11). Seropositivity (or seroconversion) was, on average, detected on day 22.7 (range 1-44 days)

after disease onset. In case no. 2, seroconversion was detected on day 29 after tick exposure in mountain in Kanto region.

Seasonal distribution

Seasonal distribution was analyzed based on submitted data of clinical specimens to NIID. Briefly, sera obtained from the LD group were sorted according to the submitted month. The number of anti-GlpQ antibody-positive patients and the number of LD antibody-positive patients were calculated for each month. Then, the number of anti-GlpQ antibody positive patients and the number of LD antibody positive patients were divided by the number of patients by month to calculate the monthly positive rate. These results are summarized in Fig.5. All of the anti-GlpQ positive patients in the LD group were observed from April to October, while cases of LD occurred in all months of the year. Of 459 cases in the LD group, 341 cases were submitted to NIID between April and October. Among these 341 cases, 12 cases were positive for anti-GlpQ antibody, and the frequency was 3.5% (range 1.5-6.3%). The number of LD and RF/BMD cases were highest in August and July, respectively.

Locality

The region of patient residence for each case that was seropositive to rGlpQ is listed in Fig. 6. Briefly, the antibody positive rate for each region was calculated by dividing the number of patients submitted from each region by the number of anti-GlpQ antibody positive patients. The seroprevalence in each region was: Hokkaido-Tohoku 1.2% (1 case / 86 individuals); Kanto 3.0%

(5/166); Chubu 6.3% (4/63); Kinki 1.6% (1/62); Chugoku-Shikoku 0% (0/30); Kyushu-Okinawa 1.9% (1/52). One case in the Kanto region was suspected to be due to exposure abroad during international travel. While seroprevalence of RF/BMD was high in the Chubu region, the difference was not statistically significant.

Discussion

In this study, retrospective surveillance revealed a high seroprevalence of anti-GlpQ in the LD group compared with the healthy cohort. The test procedures used in this study were based on classical methods used for GlpQ since *glpQ* is genetically conserved among RF borreliae, including *B. miyamotoi*, *B. theileri* and *B. lonestari*, but not in LD borreliae (35). Therefore the assay is specific for confirming infection with RF borreliae in humans. Previous investigations have uncovered several *Borrelia* spp. that are genetically classified as RF borreliae from environmental specimens in Japan. *B. miyamotoi* has been reported as a human pathogen, and it has been detected in *Ixodes* ticks and wild rodents (10, 11, 18). *Borrelia* sp. K64, which clusters with *Borrelia johnsonii* and *Borrelia turicatae* was detected from *Carios* ticks in colonies of streaked shearwater (*Calonectris leucomelas*) (36). *Borrelia* sp. tHM16w and its lineage has been found in *Haemaphysalis* ticks and wild deer (*Cervus nippon* and *C. nippon yesoensis*) (37~39). These *Borrelia* spp. have *glpQ* in their genome, and patient serum would react to GlpQ if infected with any of these RF *Borrelia* spp. However, ecological and epidemiological evidence suggests that sera reacting to *B. miyamotoi* GlpQ is most likely to have been exposed to *B. miyamotoi* than either *Borrelia* sp. K64 or *Borrelia* sp. tHM16w. *Borrelia* sp. K64 has only been found from *Carios* ticks, and the habitat of these ticks is limited to colonies of streaked shearwater on desert islands (e.g. Kutsujima island, Kyoto) (36). *Borrelia* sp. tHM16w, which is a member of the hard tick-borne RF borreliae, was recently identified in deer in Japan (37~39). The lineage of *Borrelia* sp. tHM16w is clustered with *B. theileri*. While *B. theileri* is a known pathogen of

cattle, human pathogenicity has not been reported. The tick vectors of *Borrelia* sp. tHM16w are also suggested to be a vector of SFTS virus. Epidemiological data indicate that human cases of SFTS are limited to the western part of Japan (40), which does not match the regional distribution of GlpQ-seropositive cases. The suspected tick vectors of BMD are *I. ovatus* and *I. persulcatus*, and regional distribution and seasonal activity of these ticks matches our serological assessment (33,34). From these findings, people seropositive to GlpQ are considered to have been exposed to *B. miyamotoi*.

Since 2013, serological investigations for BMD have been reported in the United States and the Netherlands. In the Northeast of the United States, it was reported that seroprevalence of BMD in suspected LD cases and healthy individuals were 3-9% and 1-4%, respectively (17, 41). In the Netherlands, the prevalence of anti-*B. miyamotoi* antibody among forestry workers was 10%, significantly higher than among blood donors (2%) (42). Although serological studies of human BMD infection are limited, these reports suggest unrecognized cases and/or asymptomatic cases may occur in habitat of the vector ticks. Compared with these reports from the United States and the Netherlands, seroprevalence in suspected LD cases and healthy individuals was slightly lower in Japan. The reason for this remains unknown; however, the prevalence of *B. miyamotoi* in ticks may be directly affecting the seroprevalence of BMD. Infection of *B. miyamotoi* in *I. ovatus,* a suspected vector, is 0.1-0.5% in Japan (10,11), which is lower than in *I. scapularis* in the Northeast of the United States (1.9-3.2%) (19, 43) or *I. ricinus* in the Netherlands (3.6-3.8%) (44, 45).

Before this study, it has reported that the prevalence of *B. miyamotoi* in *I. persulcatus* ticks, which is transmission vector of LD borreliae, is approximately 2%. In this study, I found that 3% of LD patients had co-infection with *B. miyamotoi*. This was considered to be well correlated between seroprevalence of BMD in LD cases and prevalence of co-infection in *I. persulcatus* ticks.

One patient in our study (Case 12 in Table 2), a female in her 70's and a resident of the Kanto region, exhibited IgG positivity to GlpQ. This patient was hospitalized for aseptic meningitis in October. Given that meningoencephalitis cases due to BMD have been reported in the United States (14) and Europe (15, 16), and that the patient was seropositive to GlpQ, the meningoencephalitis was attributed to BMD; however, it was not clear whether the patient had chronic BMD or a history of exposure to RF/BMD borreliae, because DNA detection was not attempted.

Between 2013 and 2017, 19 cases of indigenous BMD were reported in Japan. Out of the 19 cases, 17 cases (89%) were found in Hokkaido (46). Therefore, Japanese physicians thought BMD was a local disease in Hokkaido. Although the case number is limited, in this study I showed that human BMD is observed nationwide and infection occurs during the active season for ticks. *B miyamotoi* is increasingly recognized as the agent of a nonspecific febrile illness often misdiagnosed as acute LD without rash in the United States (31, 47). This report suggests that for patients who show febrile illness after tick exposure, physicians should consider BMD as a differential diagnosis, and laboratory testing for BMD should be performed.

Figures

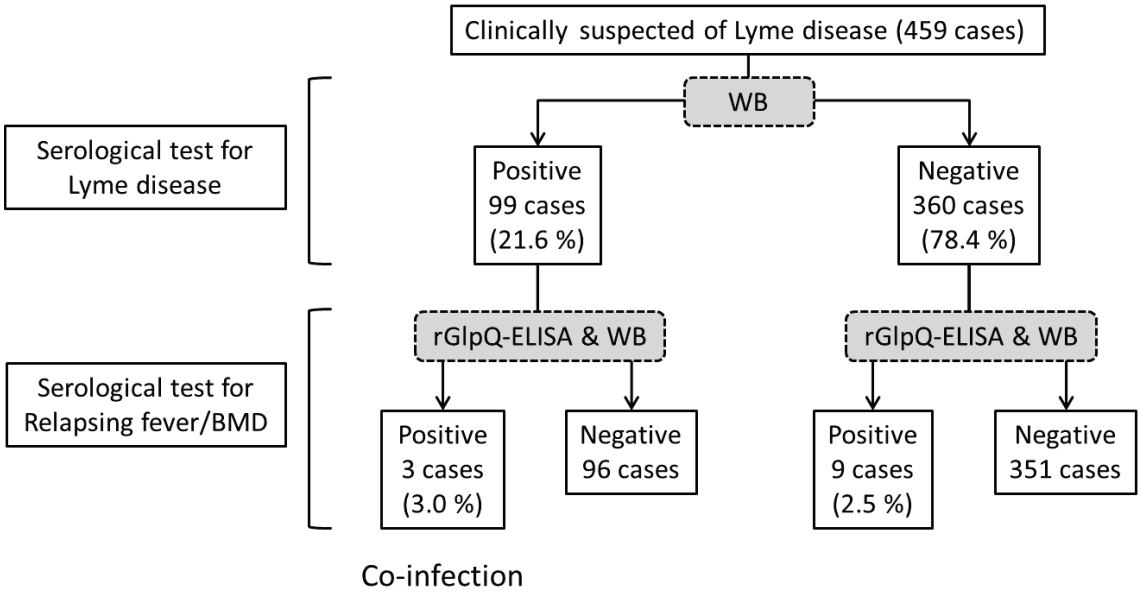
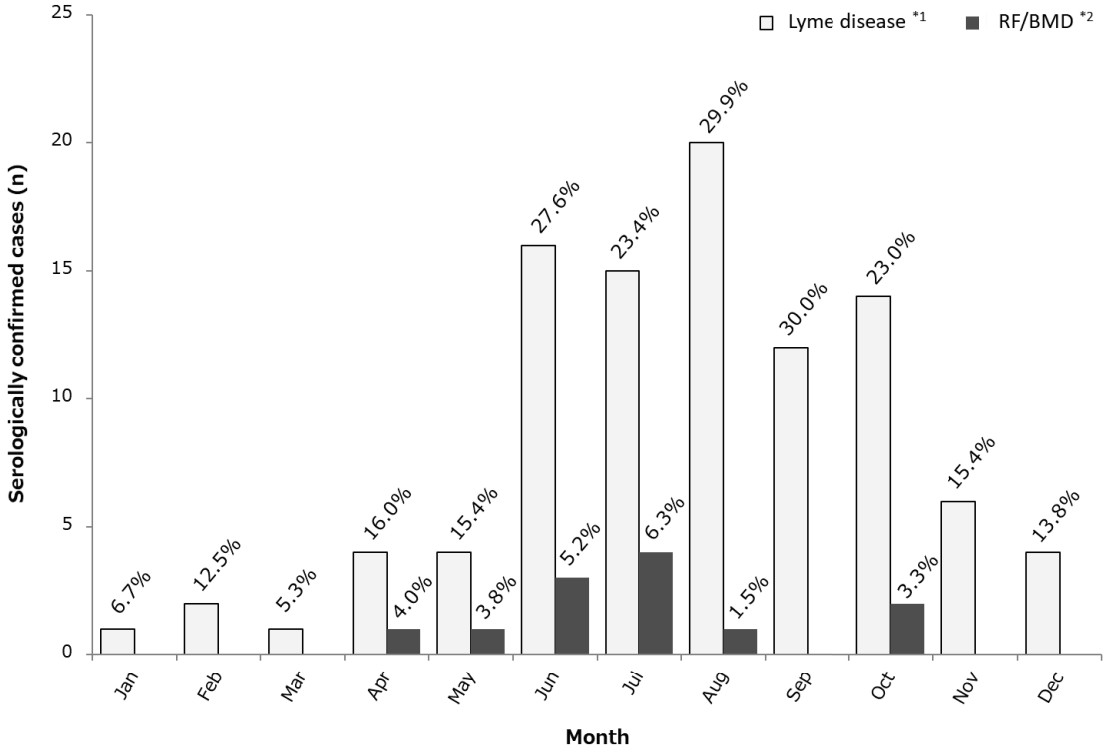


Fig. 4. Seroprevalence of LD and RF/BMD in suspected LD cases.



*1: Lyme disease (LD) was serologically confirmed by recomBlot/Line-western blot (WB)

*2: Relapsing fever (RF)/*B. miyamotoi* disease (BMD) was serologically confirmed by rGlpQ-WB.

Fig. 5. Seasonal distribution of cases of LD and RF/BMD in clinically suspected LD cases. Frequency (%) indicates positives in each month by recomBlot/recomLine-western blot (WB) or rGlpQ-WB, respectively.

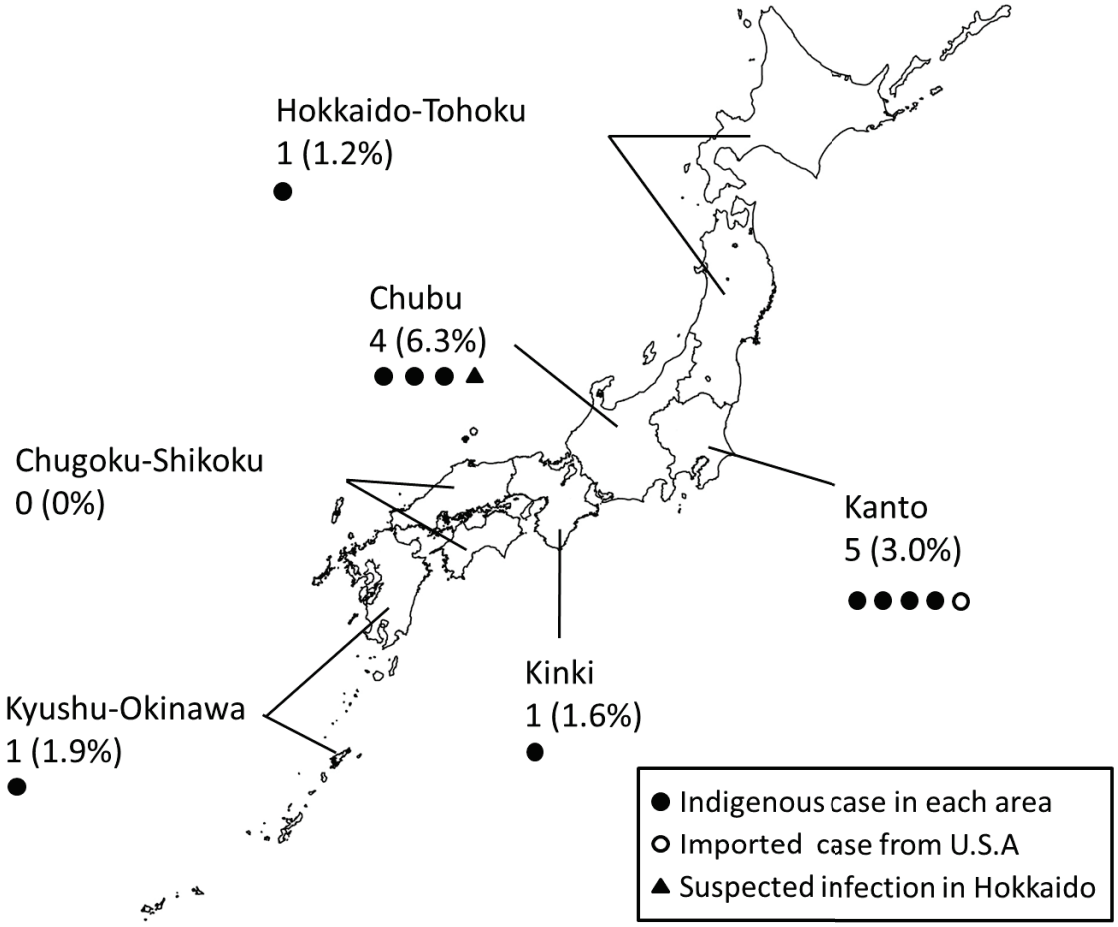


Fig. 6. Regional distribution of RF/BMD in clinically suspected LD cases. Case number and prevalence (%) in each area are shown. Black circles and whites circle indicate indigenous cases and imported cases in each area, respectively. The black triangle indicates a case with recent travel history to Hokkaido.

Tables

Table 1. Age, sex and locales of individuals used in this study.

Locales and Age of individuals	Lyme disease/suspected Lyme disease			Healthy control	
	Male	Female	Not reported	Male	Female
Hokkaido-Tohoku[-1]					
≦10	1	2	0	3	3
11-20	3	1	0	3	2
21-30	5	3	0	5	4
31-40	4	3	0	8	7
41-50	5	2	0	8	7
51-60	9	4	0	10	8
61-70	10	4	0	11	8
≧71	7	2	0	3	2
Not reported	1	0	20	0	0
Subtotal	45	21	20	51	41
Kanto[-2]					
≦10	7	6	0	7	5
11-20	2	2	0	6	5
21-30	13	6	0	10	8
31-40	8	17	0	17	13
41-50	11	15	0	17	13
51-60	11	10	0	20	15
61-70	11	7	0	22	17
≧71	4	6	0	14	11
Not reported	7	6	17	0	0
Subtotal	74	75	17	113	87
Chubu[-3]					
≦10	0	1	0	3	2
11-20	0	2	0	2	2
21-30	2	2	0	4	3
31-40	6	5	0	7	5
41-50	9	0	0	7	5
51-60	11	4	0	8	6
61-70	3	5	0	9	7
≧71	3	4	0	6	4
Not reported	2	2	2	0	0
Subtotal	36	25	2	46	34
Kinki[-4]					
≦10	2	5	0	2	2
11-20	1	2	0	2	2
21-30	5	3	0	4	2
31-40	1	1	0	6	4
41-50	2	5	1	6	4
51-60	5	4	0	7	5
61-70	2	0	0	7	6
≧71	5	6	0	4	3
Not reported	1	2	9	0	0
Subtotal	24	28	10	38	28

Chugoku-Shikoku[*5]					
≦10	0	1	0	2	1
11-20	2	1	0	1	1
21-30	1	2	0	2	1
31-40	2	1	0	4	3
41-50	3	0	0	4	3
51-60	0	1	0	5	3
61-70	5	3	0	5	4
≧71	4	0	0	3	2
Not reported	0	0	4	0	0
Subtotal	17	9	4	26	18
Kyushu-Okinawa[*6]					
≦10	0	0	0	2	2
11-20	2	1	0	2	1
21-30	1	0	0	3	2
31-40	5	0	1	5	4
41-50	4	4	0	5	4
51-60	5	7	0	6	5
61-70	6	7	0	7	5
≧71	2	4	1	4	3
Not reported	0	1	1	0	0
Subtotal	25	24	3	34	26
Total No.	221	182	56	308	234
		459		542	
Age, average (range)		47 (1-84)		46 (0-87)	
Sex, Male : Female		1.2 : 1		1.3 : 1	

*1: Hokkaido-Tohoku includes 7 prefectures: Akita, Aomori, Fukushima, Hokkaido, Iwate, Miyagi, and Yamagata.

*2: Kanto includes 7 prefectures: Chiba, Gunma, Ibaraki, Kanagawa, Saitama, Tochigi, and Tokyo.

*3: Chubu includes 9 prefectures: Aichi, Fukui, Gifu, Ishikawa, Nagano, Niigata, Shizuoka, Toyama, and Yamanashi.

*4: Kinki includes 7 prefectures: Hyogo, Kyoto, Mie, Nara, Osaka, Shiga, and Wakayama.

*5: Chugoku-Shikoku includes 9 prefectures: Ehime, Hiroshima, Kagawa, Kochi, Okayama, Shimane, Tokushima, Tottori, and Yamaguchi.

*6: Kyushu-Okinawa includes 8 prefectures: Fukuoka, Kagoshima, Kumamoto, Okinawa, Oita, Miyazaki, Nagasaki, and Saga.

Table 2. Seropositive cases for relapsing fever/*Borrelia miyamotoi* disease in clinically suspected Lyme disease cases.

Case No.	ID	Days after disease onset	rGlpQ-WB		Lyme disease	
			IgM	IgG	IgM	IgG
Co-infection (N=3)						
1	394	5	+	-	+	-
	403	NA*	-	-	+	+
2	880	13**	-	-	-	-
	881	29**	+	-	+	+
3	667	31	+	-	+	+/-
Relapsing fever/BMD (N=9)						
4	427	NA	+	-	-	-
5	482	NA	+	-	-	-
6	631	NA	+	-	-	-
7	876	NA	+	-	-	-
8	680	44	+	-	-	-
9	367	12	-	-	-	-
	368	27	+	-	-	-
	374	59	-	-	-	-
10	550	1	+	-	+/-	-
	551	15	+	-	-	-
11	844	8	-	-	-	+/-
	848	28	+	-	-	+/-
12	907	8	-	+	+/-	+/-
	911	18	-	+	+/-	+/-

*: Not available in record sheet from hospital or local health care center.

**: Days after tick exposure (days after disease onset was not reported in record sheet)

Conclusion

The purpose of this study was to clarify the current status of emerging RF/BMD in Japan, and provide information on this public health concern. From this study, I provided information regarding emerging RF/BMD in Japan as follows;

1: BMD cases were confirmed by retrospective study. *B. miyamotoi* detected from patient sera were identical or closely related to *B. miyamotoi* found in BMD patient and environmental specimens in Japan.

2: Anti-RF borrelia specific antibody was found in patients clinically suspected of LD. Seroprevalence is significantly higher among patients clinically suspected of LD than among the healthy cohort. It is likely that these patients were infected with *B. miyamotoi* rather than other RF borreliae. Because, if the infectious agent is *B. miyamotoi*, the distribution area of the tick vector and the resident area of the patient correlate well.

3: Suspected BMD cases were observed during the active season for ticks, and distribution is nationwide throughout Japan. This suggests that the tick vectors of *B. miyamotoi* are also widespread rather than limited to Hokkaido.

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References

1. Takano, A., Goka, K., Une, Y., Shimada, Y., Fujita, H., Shiino, T., Watanabe, H. and Kawabata, H. (2010). Isolation and characterization of a novel *Borrelia* group of tick-borne borreliae from imported reptiles and their associated ticks. Environ. Microbiol. 12, 134~146.
2. Yparraguirre, L.A., Machado-Ferreira, E., Ullmann, A.J., Piesman, J., Zeidner, N.S. and Soares, C.A. (2007). A hard tick relapsing fever group spirochete in a Brazilian *Rhipicephalus* (*Boophilus*) *microplus*. Vector Borne Zoonotic Dis. 7, 717~721.
3. Takano, A., Sugimori, C., Fujita, H., Kadosaka, T., Taylor, K.R., Tsubota, T., Konnai, S., Tajima, T., Sato, K., Watanabe, H., Ohnishi, M. and Kawabata, H. (2012). A novel relapsing fever *Borrelia* sp. infects the salivary glands of the molted hard tick, *Amblyomma geoemydae*. Ticks Tick Borne Dis. 3, 259~261.
4. Mun, J., Eisen, R.J., Eisen, L. and Lane, R.S. (2006). Detection of a *Borrelia miyamotoi* sensu lato relapsing-fever group spirochete from *Ixodes pacificus* in California. J. Med. Entomol. 43, 120~123.
5. Salkeld, D.J., Cinkovich, S. and Nieto, N.C. (2014). Tick-borne pathogens in Northwestern California, USA. Emerg. Infect. Dis. 20, 493~494.
6. Scoles, G.A., Papero, M., Beati, L. and Fish, D. (2001). A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic Dis. 1, 21~34.
7. Fraenkel, C.J., Garpmo, U. and Berglund, J. (2002). Determination of novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks. J. Clin. Microbiol. 40,

3308~3312.

8. Richter, D., Schlee, D.B. and Matuschka, F.R. (2003). Relapsing fever-like spirochetes infecting European vector tick of Lyme disease agent. Emerg. Infect. Dis. 9, 697~701.
9. Platonov, A.E., Karan, L.S., Kolyasnikova, N.M., Makhneva, N.A., Toporkova, M.G., Maleev, V.V., Fish, D. and Krause, P.J. (2011). Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg. Infect. Dis. 17, 1816~1823.
10. Takano, A., Toyomane, K., Konnai, S., Ohashi, K., Nakao, M., Ito, T., Andoh, M., Maeda, K., Watarai, M., Sato, K. and Kawabata, H. (2014). Tick surveillance for relapsing fever spirochete *Borrelia miyamotoi* in Hokkaido, Japan. PLoS One. 11, e104532.
11. Iwabu-Itoh, Y., Bazartseren, B., Naranbaatar, O., Yondonjamts, E., Furuno, K., Lee, K., Sato, K., Kawabata, H., Takada, N., Andoh, M., Kajita, H., Oikawa, Y., Nakao, M., Ohnishi, M., Watarai, M., Shimoda, H., Maeda, K. and Takano, A. (2017). Tick surveillance for *Borrelia miyamotoi* and phylogenetic analysis of isolates in Mongolia and Japan. Ticks Tick Borne Dis. 8, 850~857.
12. Fukunaga, M., Takahashi, Y., Tsuruta, Y., Matsushita, O., Ralph, D., McClelland, M. and Nakao, M. (1995). Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int. J. Syst. Bacteriol. 45, 804~810.
13. Chowdri, H.R., Gugliotta, J.L., Berardi, V.P., Goethert, H.K., Molloy, P.J., Sterling, S.L. and Telford, S.R. (2013). *Borrelia miyamotoi* infection presenting as human granulocytic anaplasmosis: a case report. Ann. Intern.

Med. 159, 21~27.

14. Gugliotta, J.L., Goethert, H.K., Berardi, V.P. and Telford, S.R. 3rd. (2013). Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. N. Engl. J. Med. 368, 240~245.
15. Hovius, J.W., de Wever, B., Sohne, M., Brouwer, M.C., Coumou, J., Wagemakers, A., Oei, A., Knol, H., Narasimhan, S., Hodiamont, C.J., Jahfari, S., Pals, S.T., Horlings, H.M., Fikrig, E., Sprong, H. and van Oers, M.H. (2013). A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. Lancet. 382, 658.
16. Boden, K., Lobenstein, S., Hermann, B., Margos, G. and Fingerle, V. (2016). *Borrelia miyamotoi*-Associated Neuroborreliosis in Immunocompromised Person. Emerg. Infect. Dis. 22. 1617~1620.
17. Krause, P.J., Narasimhan, S., Wormser, G.P., Rollend, L., Fikrig, E., Lepore, T., Barbour, A. and Fish, D. (2013). Human *Borrelia miyamotoi* infection in the United States. N. Engl. J. Med. 368, 291~293.
18. Taylor, K.R., Takano, A., Konnai, S., Shimozuru, M., Kawabata, H. and Tsubota, T. (2013). *Borrelia miyamotoi* infections among wild rodents show age and month independence and correlation with *Ixodes persulcatus* larval attachment in Hokkaido, Japan. Vector Borne Zoonotic Dis. 13, 92~97.
19. Barbour, A.G., Bunikis, J., Travinsky, B., Hoen, A.G., Diuk-Wasser, M.A., Fish, D. and Tsao, J.I. (2009). Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am. J. Trop. Med. Hyg. 81, 1120~1131.
20. Takano, A., Fujita, H., Kadosaka, T., Konnai, S., Tajima, T., Watanabe, H.,

Ohnishi, M. and Kawabata, H. (2011). Characterization of reptile-associated *Borrelia* sp. in the vector tick, *Amblyomma geoemydae*, and its association with Lyme disease and relapsing fever *Borrelia* spp. Environ. Microbiol. Rep. 3, 632~637.

21. Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 227, 680~685.
22. Masuzawa, T., Kawabata, H., Beppu, Y., Miyamoto, K., Nakao, M., Sato, N., Johnson, R.C. and Yanagihara, Y. (1994). Characterization of monoclonal antibodies for identification of *Borrelia japonica*, isolates from *Ixodes ovatus*. Microbiol. Immunol. 38, 393~398.
23. Saito, K., Ito, T., Asashima, N., Ohno, M., Nagai, R., Fujita, H., Koizumi, N., Takano, A., Watanabe, H. and Kawabata, H. (2007). Case report: *Borrelia valaisiana* infection in a Japanese man associated with traveling to foreign countries. Am. J. Trop. Med. Hyg. 77, 1124~1127.
24. Hashimoto, Y., Miyamoto, K. and Iizuka, H. (2003). Tick bites and Lyme disease in Hokkaido, Japan [in Japanese]. Hihubyo Shinryo. 25, 926~929.
25. Barbour, A.G. (2005). Relapsing fever. *In*: Goodman, J.L., Dennis, D.T. and Sonenshine, D.E., [eds] Tick-borne diseases of human, pp. 268~291. American Society for Microbiology, Washington, D.C.
26. Inoue, Z. (1907). Febris recurrens. *In*: Inoue, Z. [ed] Inoue Naika Shinsho, 4, pp. 287~295. Touhoudo Shoten, Tokyo.
27. Kutsuna, S., Kawabata, H., Kasahara, K., Takano, A. and Mikasa, K. (2013). The first case of imported relapsing fever in Japan. Am. J. Trop. Med. Hyg. 89, 460~461.

28. Molloy, P.J., Telford, S.R. 3rd, Chowdri, H.R., Lepore, T.J., Gugliotta, J.L., Weeks, K.E., Hewins, M.E., Goethert, H.K. and Berardi, V.P. (2015). *Borrelia miyamotoi* disease in the northeastern United States: a case series. Ann. Intern. Med. 163, 91~98.
29. Yamano, K., Ito, T., Kiyanagi, K, Yamazaki, H., Sugawara, M., Saito, T. and Kawabata, H. (2017). Case Report: Clinical Features of a Case of Suspected *Borrelia miyamotoi* Disease in Hokkaido, Japan. Am. J. Trop. Med. Hyg. 97, 84~87.
30. Sato, K., Takano, A., Konnai, S., Nakao, M., Ito, T., Koyama, K., Kaneko, M., Ohnishi, M. and Kawabata, H. (2014). Human infections with *Borrelia miyamotoi*, Japan. Emerg. Infect. Dis. 20. 1391-1393.
31. Krause, P.J., Fish, D., Narasimhan, S. and Barbour, A.G. (2015). *Borrelia miyamotoi* infection in nature and in humans. Clin. Microbiol. Infect. 21. 631-639.
32. Masuzawa, T. (2004). Terrestrial distribution of the Lyme borreliosis agent *Borrelia burgdorferi* sensu lato in East Asia. Jpn. J. Infect. Dis. 57, 229~235.
33. Okino, T., Ushirogawa, H., Matoba, K. and Hatsushika, R. (2009). Bibliographical Studies on Human Cases of Hard Tick (Acarina: Ixodidae) Bites in Japan. (3) Cases of *Ixodes persulcatus* infestation. Kawasaki Medical Journal. 35, 67-80. [in Japanese]
34. Hatsushika, R. (1998). Human cases of infestation with hard tick, *Ixodes ovatus* (*Acarina*: *Ixodidae*) in Japan: -A bibliographical review-. Med. Entomol. Zool. 49, 1~13. [In Japanese]
35. Schwan, T.G., Schrumpf, M.E., Hinnebusch, B.J., Anderson, Jr. D.E. and

Konkel, M.E. (1996). GlpQ: an antigen for serological discrimination between relapsing fever and Lyme borreliosis. J. Clin. Microbiol. 34, 2483~2492.

36. Takano, A., Muto, M., Sakata, A., Ogasawara, Y., Ando, S., Hanaoka, N., Tsurumi, M., Sato, F., Nakamura, N., Fujita, H., Watanabe, H. and Kawabata, H. (2009). Relapsing fever spirochete in seabird tick, Japan. Emerg. Infect. Dis. 15, 1528~1530.
37. Lee, K., Takano, A., Taylor, K.R., Sashika, M., Shimozuru, M., Konnai, S., Kawabata, H. and Tsubota, T. (2014). A relapsing fever group *Borrelia* sp. similar to *Borrelia lonestari* found among wild sika deer (*Cervus nippon yesoensis*) and *Haemaphysalis* spp. ticks in Hokkaido, Japan. Ticks Tick Borne Dis. 5, 841~847.
38. Furuno, K., Lee, K., Itoh, Y., Suzuki, K., Yonemitsu, K., Kuwata, R., Shimoda, H., Watarai, M., Maeda, K. and Takano, A. (2017). Epidemiological study of relapsing fever borreliae detected in *Haemaphysalis* ticks and wild animals in the western part of Japan. PLoS One. 12, e0174727.
39. Kumagai, Y., Sato, K., Taylor, K. R., Zamoto-Niikura, A., Imaoka, K., Morikawa, S., Ohnishi, M. and Kawabata, H. (2017). A relapsing fever group *Borrelia* sp. is widely distributed among wild deer in Japan. Ticks Tick Borne Dis. 9, 465~470.
40. Severe fever with thrombocytopenia syndrome (SFTS) in Japan, as of February 2016. (2016). Infectious Agents Surveillance Report. 37, 39-53.
41. Jobe, D.A., Lovrich, S.D., Oldenburg, D.G., Kowalski, T.J. and Callister, S.M. (2016). *Borrelia miyamotoi* Infection in Patients from Upper Midwestern United States, 2014-2015. Emerg. Infect. Dis. 22, 1471~1473.

42. Jahfari, S., Herremans, T., Platonov, A.E., Kuiper, H., Karan, L.S., Vasilieva, O., Koopmans, M.P., Hovius, J.W. and Sprong, H. (2014). High seroprevalence of *Borrelia miyamotoi* antibodies in forestry workers and individuals suspected of human granulocytic anaplasmosis in the Netherlands. New Microbes New Infect. 2, 144~149.
43. Crowder, C.D., Carolan, H.E., Rounds, M.A., Honig, V., Mothes, B., Haag, H., Nolte, O., Luft, B.J., Grubhoffer, L., Ecker, D.J., Schutzer, S.E. and Eshoo, M.W. (2014). Prevalence of *Borrelia miyamotoi* in *Ixodes* ticks in Europe and the United States. Emerg. Infect. Dis. 20, 1678~1682.
44. Fonville, M., Friesema, I.H., Hengeveld, P.D., Docters van Leeuwen, A., Jahfari, S., Harms, M.G., van Vliet, A.J., Hofhuis, A., van Pelt, W., Sprong, H. and van den Wijngaard, C.C. (2014). Human exposure to tickborne relapsing fever spirochete *Borrelia miyamotoi*, the Netherlands. Emerg. Infect. Dis. 20, 1244~1245.
45. Cochez, C., Heyman, P., Heylen, D., Fonville, M., Hengeveld, P., Takken, W., Simons, L. and Sprong, H. (2015). The Presence of *Borrelia miyamotoi*, A Relapsing Fever Spirochaete, in Questing *Ixodes ricinus* in Belgium and in The Netherlands. Zoonoses Public Health. 62, 331~333.
46. Infectious diseases weekly report (IDWR), National Institute of Infectious Diseases, Japan. https://www.niid.go.jp/niid/ja/idwr.html (In Japanese).
47. Telford, S.R. 3rd, Goethert, H.K., Molloy, P.J., Berardi, V.P., Chowdri, H.R., Gugliotta, J.L. and Lepore, T.J. (2015). *Borrelia miyamotoi* Disease: Neither Lyme Disease Nor Relapsing Fever. Clin. Lab. Med. 35, 867~882.