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Detection of *Borrelia americana* in the Avian Coastal Tick, *Ixodes auritulus* (Acari: Ixodidae), Collected from a Bird Captured in Canada

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Abstract

We document the first record of *Borrelia americana* in Canada. This *Borrelia* was detected in an avian coast tick, *Ixodes auritulus* (Acari: Ixodidae), collected from a Varied Thrush, *Ixoreus naevius*, along coastal British Columbia. Using real-time PCR and DNA sequencing of the *flagellin* gene, we determined that the borrelial amplicon from the *I. auritulus* nymph was 99% homologous with *B. americana* type strain SCW-41. Because patients infected with *B. americana* can be seronegative for Lyme disease, medical professionals should be willing to pursue molecular analyses and consider treatment for patients with Lyme disease-like symptoms.

Keywords

***Borrelia americana*, Lyme Disease, Avian Coastal Tick, *Ixodes auritulus*, Varied Thrush, Songbird, Bird Parasitism, Canada**

1. Introduction

Ticks are blood-sucking ectoparasites that transmit a diverse array of protozoan, viral, bacterial, and fungal pathogens [1]. These tick-borne microorganisms cause pernicious diseases in animals, including humans. The Lyme disease spirochete, *Borrelia burgdorferi* sensu lato (s.l.) Johnson, Schmid, Hyde, Steigerwalt & Brenner is typically carried and transmitted by several hard-bodied ticks (Acari: Ixodidae) [2]. At least 23 genospecies and

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genomospecies of the *B. burgdorferi* s.l. complex are recognized globally. In North America, at least 9 *B. burgdorferi* s.l. genospecies are characterized, namely *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* sensu stricto (s.s.), *B. californiensis*, *B. carolinensis*, *B. garinii*, *B. kurtenbachii*, and *B. mayonii* [3]-[10]. Of these genospecies, *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* s.s., *B. garinii*, *B. kurtenbachii*, and *B. mayonii* are pathogenic to humans [10]-[14]. Worldwide, Lyme disease has been diagnosed in over 80 countries [15].

The avian coastal tick, *Ixodes auritulus* Neumann, is indigenous along many seacoasts, including the Western Hemisphere, Australia, New Zealand and the islands south of Africa [16]-[18]. Ecologically, *I. auritulus* is found exclusively on birds [19], and parasitizes members of at least 8 bird orders [18], including Falconiformes, Galliformes, and Passeriformes in Canada [20]-[22]. Scott *et al*. [21] documented that 31% of *I. auritulus* (larvae, nymphs, adults) in far-western Canada are infected with *B. burgdorferi* s.l. This tick species greatly helps to perpetuate the enzootic transmission cycle of Lyme disease spirochetes along coastal British Columbia (B.C.). Any *I. auritulus* (larva, nymph, or female), which is infected with *B. burgdorferi* s.l., can transmit Lyme disease spirochetes to avian hosts.

Banerjee *et al*. [23] discovered *B. burgdorferi* s.l. in the western blacklegged tick, *Ixodes pacificus* Cooley & Kohls and *Ixodes angustus* Neumann collected from North American deer mice, *Peromyscus maniculatus* Wagner in southwestern B.C., including the Metchosin area. Of note, Lyme disease spirochetes were isolated from *I. angustus* larvae, and later delineated as *B. burgdorferi* s.s. and *B. bissettii*. In addition, Scott *et al*. [24] reported four different *B. burgdorferi* s.l. genospecies/genotypes in B.C. Epidemiologically, any Lyme disease vector ticks (i.e., *I. pacificus*, *Ixodes spinipalpis* Hadwen & Nuttall), which fed on spirochetemic birds, can acquire *B. burgdorferi* s.l. infection and, subsequently, transmit diverse spirochetes to other birds and mammalian hosts, including humans.

The Varied Thrush, *Ixoreus naevius* Gmelin (Turdidae), has a home range in far-western North America from southern California to north-central Alaska, northern Yukon, and northwest region of the Northwest Territories. Biogeographically, this thrush has a year-round range in the Pacific Northwest, including Vancouver Island. This ground-frequenting thrush breeds in dense, humid coniferous and mixed forests along the Pacific Coast.

Previously, the *B. americana* type strain, SCW-41^{T}, was isolated from a nymphal *Ixodes minor* Neumann collected from a Carolina Wren, *Thryothorus ludovicianus* (Latham), in South Carolina [7]. As well, California strains (CA-8B-89, CA-29-91), which were isolated from *I. pacificus* adults, have also been characterized as *B. americana* [7] [25].

Here we provide the first description of *B. americana* in Canada, and its presence in an *I. auritulus* nymph parasitizing a Varied Thrush. This novel bird-tick-pathogen discovery helps to demonstrate the wide distribution of *B. americana* in the Western Hemisphere, and adds to the known genetic diversity *of B. burgdorferi* s.l. in Canada.

2. Materials and Methods

2.1. Tick Collection

A Varied Thrush struck a window at Saanich, Vancouver Island, British Columbia, and was brought to BC SPCA Wild ARC, an animal rehabilitation centre for wildlife, located near Metchosin, B.C. Upon presentation, the thrush was thoroughly examined, and placed in a screened, outdoor enclosure which provided natural enrichment. After 18 d, the thrush was euthanized due to a fractured jaw and, at this time, an attached tick was found under the beak. Using super-fine, stainless steel forceps, wildlife rehabilitators removed the tick. The engorged tick was then put in a round-bottom, 8.5 mL polypropylene tube (15.7 mm × 75 mm) (Sarstedt, Montreal, Quebec) with a label specifying background information (host, geographic location, date collected, collector's name). A 7-mm hole in the polyethylene push cap (15.7 mm) provided ventilation for the tick and, to prevent the tick from escaping, fine tulle netting was stretched over the mouth of the vial before the push cap was inserted. The vial, which contained the tick, was placed in a self-sealing, double-zipper, plastic bag with a slightly moistened paper towel. The live tick was sent directly to the laboratory (JDS), and identified using a taxonomic key [19]. Since the partially engorged tick had only been attached to the bird for 3 days, the tick was acquired onsite.

2.2. Spirochete Detection

After identification, the tick was put in a 2 mL micro tube (Sarstedt, Montreal, Quebec) containing 94% ethyl alcohol, and sent by courier to the PCR amplification laboratory (JEF). DNA was extracted from the tick using an ammonium hydroxide technique as described previously [26]. Screening of the *I. auritulus* nymph for *Borre-*

lia species was performed using real-time PCR (TaqMan) as described previously [27]. We modified the protocol to use only the forward and reverse primers and the probe identified in that paper specific for *B. burgdorfer*i s.s. (*in silico* analysis and/or unpublished data indicate that both primers are generic for borreliae; the probe has 100% homology to almost all *B. burgdorferi* s.l. genospecies). The reaction was run in a combined thermocycler/fluorometer (ABI Prism 7700, Applied Biosystems, Foster City, California). A water negative control was included in each run and, likewise, DNA from cultured strains of *B. burgdorferi* s.s. and *B. bissettii* were employed as positive controls. The sample was considered positive if it had a cycle threshold (CT) <40, and a characteristic amplification curve. A strongly PCR-positive sample with a CT <35 was evaluated using conventional PCR for the *flagellin* gene as described [28], with modification to use GoTaq® Green Master Mix (Promega, Madison, Wisconsin). Following PCR and agarose gel electrophoresis, DNA was purified from gels using a kit (QiaQuick, Qiagen, Valencia, California), and submitted for sequencing on a ABI 3730 sequencer (UC Davis Sequencing, Davis, California) using the forward PCR primer; DNA from this tick (15-5A94) underwent bidirectional sequencing (forward and reverse). Electropherograms were corrected by visual examination and end-read errors trimmed. The sequenced amplicon was evaluated by BLAST search of GenBank (NCBI; http://blast.ncbi.nlm.nih.govBlast.cgi). Alignment of various sequences from the database with the *I. auritulus*-derived sequence, and construction of a maximum likelihood phylogenetic tree, were completed using the program CLC Workbench (Aahus, Denmark).

2.3. Nucleotide Sequences

In order to confirm the identity of the *B. burgdorferi* s.l. from Vancouver Island, we obtained and sequenced borrelial amplicons from the *I. auritulus* nymph (15-5A94). DNA sequences for *B. americana* Can94 were deposited in GenBank database, and the respective accession number is KX022979.

3. Results

A live, partially engorged *I. auritulus* nymph was collected from a male Varied Thrush at BC SPCA Wild ARC, Metchosin, Vancouver Island, British Columbia on 31 October 2015 (**Figure 1**). This songbird had struck a window at Saanich, B.C., and had a fractured jaw. It was examined carefully at presentation, and no ticks were found. This bird could fly well. After the Varied Thrush had been in rehabilitation for 18 days, a tick was found, and collected. Based on engorgement, the partially engorged nymph had only been feeding for 3 d. Since ticks have unlimited access to the enrichment enclosure, the *I. auritulus* nymph was acquired onsite at the rehabilitation centre.

Using real-time PCR, the nymph tested positive for *B. burgdorferi* s.l. with a CT of 29.05, which indicates a strongly positive sample. DNA sequencing of the *flagellin* gene yielded the expected 325-bp fragment characteristic of *B. americana*. After end-trimming, the amplicon from the *I. auritulus* nymph (15-5A94) collected fromthe Varied Thrush was 99% homologous with *B. americana* type strain SCW-41 located in the GenBank data

Figure 1. Varied Thrush, male, parasitized by a partially engorged *I. auritulus* nymph. This nymph was infected with *B. americana*, strain Can94. Photo credit: Vanessa Williams.

base (**Figure 2**). Based on phylogenetic analysis, the amplicon only had 97% homology with *B. burgdorferi* s.s. strain B31. This is the first record of *B. americana* in Canada and, likewise, the first account of *B. americana* in *I. auritulus*. Additionally, this is the first record of *B. americana*-infected tick parasitizing a bird in Canada. Moreover, this account provides the northernmost record of *B. americana* in North America, and constitutes a new distribution record for this borrelial genospecies.

In addition, 4 of 5 *I. auritulus* nymphs collected from a Spotted Towhee, *Piplio maculatus*, on 24 February 2016 at Sooke, B.C., tested positive for *B. burgdorferi* s.l.; DNA sequencing and a BLAST search characterized them as *B. americana*.

4. Discussion

We now provide the first account of *B. americana* in Canada. Our discovery follows earlier reports of this *B. burgdorferi* s.l. genospecies in California and the southeastern United States. Notably, *B. americana* has been detected in songbird-transported ticks, which indicates this *Borrelia* could well be widely dispersed across North America. However, in the present study, travel was not a factor; the bird parasitism occurred directly at a rehabilitation centre. Since *B. americana* is pathogenic to humans, and patients can test seronegative using the standard 2-tiered Lyme disease testing [12] [29]-[32], medical professionals must be steadfast in implementing clinical diagnoses. Health-care providers must be aware that *B. americana* can be widely dispersed in North America by migratory songbirds, and can exhibit many of the clinical manifestations associated with Lyme disease.

4.1. Geographic Distribution of *B. americana* by Bird-Feeding Ticks

Migratory songbirds play a significant role in the wide dispersal of bird-feeding ticks in North America. Currently, we know that *B. americana* is present in southeastern United States and California, and now British

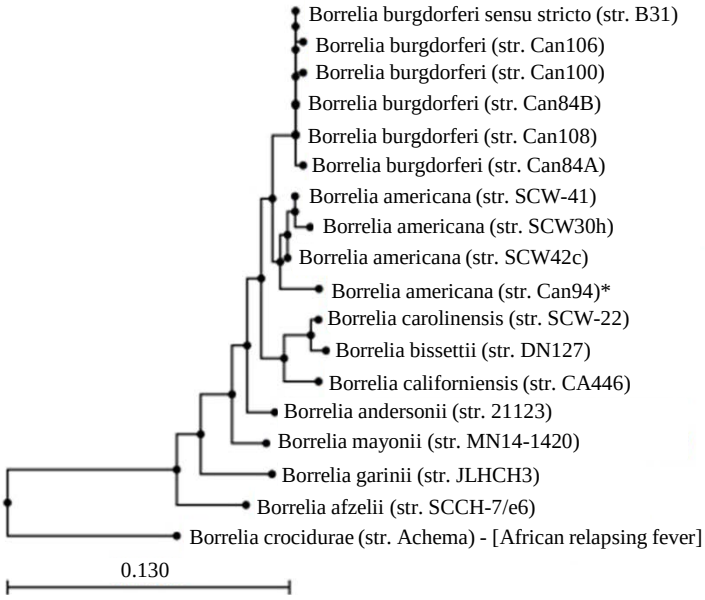

Figure 2. Phylogenetic tree of *Borrelia* spp. based on a portion of the *flagellin* gene obtained from *Ixodes* ticks in Canada and related tick species from GenBank. The tree was constructed using the maximum likelihood method (CLC Workbench). The scale bar represents the number of nucleotide substitutions. *Borrelia americana* strain Can94, with GenBank accession number KX022979, is indicated with an asterisk (*). The GenBank accession numbers for the non-Canadian reference strains are: *B. burgdorferi* s.l., B31: NC_001318.1; *B. americana* SCW-41: EU081292; *B. americana* SCW30h: HM802232; *B. americana* SCW42c: EU081295; *B. carolinensis* SCW22: KF422810; *B. bissettii* DN127: NC_015921.1; *B. californiensis* CA446: KF422809; *B. andersonii* 21123: D82764; *B. mayonii* MN14-1420: KR154295; *B. garinii* JLHCH3: DQ188915; *B. afzelii* Scch-7/e6: EU220781.1; *B. crocidurae* Achema: NC_017808.1.

Columbia. Our study reveals that *B. americana* is now connected with *I. auritulus*, a tick species that is exclusively on birds. Since *B. americana* is associated with birds, it is likely that it has extensive distribution in North America and, to our knowledge, has not been detected by standard borrelial screening. When birds migrate, they have great capacity to fly hundreds of kilometres, and widely disperse bird-feeding ticks, including *I. auritulus*. Because the Pacific flyway and the Atlantic flyway converge in Central and South America, there is ample opportunity for a replete tick to drop off a passerine migrant at an overwintering tropical stopover, and moult to the next life stage, and then parasitize another migratory songbird that is connected with another flyway. In essence, *B. americana*-infected ticks, which are linked with one flyway, can switch to migratory songbirds associated with another flyway. Such transfers provide a way for ticks from southbound fall migrants from the East Coast to switch to northbound spring migrants destine for the West Coast, and *vice versa*.

On the other hand, domestic travel could have been the mode of transport of *B. americana*-infected ticks from coast to coast. For example, a dog, which was infested with *B. americana*-infected *I. pacificus* ticks, could have transported this borrelial genospecies from California to the East Coast during long-distance, vehicular or airplane travel. Upon arrival, these replete ticks dropped off the dog in a tick-conducive habitat, and moulted to the next life stage, and subsequently bite a suitable host, including humans. On the contrary, a dog infested with *B. americana*-infected *I. scapularis* could have transported engorged ticks from the southeastern United States to California via travel by automobile or airplane. Such epidemiological events provide alternate mechanisms for the transcontinental dispersal of *B. americana*.

4.2. Epidemiology of *B. americana* in Canada

We provide the northernmost record of *B. americana* in North America. Prior to our study, the closest known location for *B. americana* was California. The *B. americana* strain, Can94, is 99% homologous to *B. americana* type strain SCW-41, and is well within the genetic proximity of this genospecies. In addition, Can94, only has 97% homology with *B. burgdorferi* s.s. strain B31 and, therefore, does not belong to the commonly occurring *B. burgdorferi* s.s. genospecies. Epidemiologically, the uncovering of the *Borrelia* variant, Can94, in an *I. auritulus* tick on Vancouver Island, B.C.'s a novel occurrence of *B. americana* in Canada.

In the present study, travel does not have any direct relationship with the *B. americana*-infected *I. auritulus* nymph because the Varied Thrush had been in a rehabilitation enclosure for 18 d before the nymph was collected. Any birds, which were previously in the rehabilitation compound, could have dropped fully engorged ticks and, these replete ticks would moult and, later, parasitize subsequent avian hosts. In late October, the day length is too short and the degree-days are insufficient for ticks to moult. Since the Varied Thrush was parasitized in the enclosure, the questing *I. auritulus* nymph must have entered the screen wall from the surrounding environs. This unique situation suggests that *B. americana* is cycling enzootically in the vicinity.

Scott *et al*. [21] found that 31% of the *I. auritulus* (larvae, nymphs, adults) collected along Vancouver Island's South Coast were infected with *B. burgdorferi* s.l. Since borrelial genospecies characterization was not conducted in this particular study [21], we do not know if any of the *I. auritulus* ticks were infected with *B. americana*. With respect to the distribution of *I. auritulus* along B.C.'s coast, tick researchers reported *I. auritulus* locally on the southern fringe of Vancouver Island and, also, unveiled the presence of this tick species at more northerly locations *(i.e.*, Haida Gwaii) [21] [22] [24] [33]. Ecologically, whenever birds are heavily infested with ticks, they can start new Lyme disease foci [34] [35]. Based on earlier tick studies, established populations of *I. auritulus* are certainly present along southern Vancouver Island. In our particular case, knowing the travel history of the Varied Thrush, and whether it was a resident or a migrant, is not helpful in determining the source of *B. americana*. Perhaps, a bird, which was previously in rehabilitation, could have dropped a replete *I. auritulus* larva, and this tick crawled outside the enclosure to the surrounding vegetation, and moulted, and re-entered the enclosure as an unfed nymph. Since the *B. americana*-infected *I. auritulus* was acquired at the rehabilitation centre, there is substantial evidence to suggest that *B. americana* is established locally in this bioregion.

4.3. Dispersal of *B. americana*-Infected Ticks in North America

The presence of *B. americana* on both sides of North America is intriguing. In southeastern United States, *B. americana* has been isolated from *I. minor* immatures collected from ground-frequenting songbirds [7] and, in California, from *I. pacificus* adults [25]. Since *Ixodes affinis* Neumann, *I. minor*, and *I. scapularis* infest rodents and birds in southeastern United States, there is every likelihood that north-bound passerine migrants are trans-

porting *B. americana*-infected ticks to central and eastern Canada. Similarly, *I. auritulus*, *I. pacificus*, and *I. spinipalpis* feed on birds in far-western North America, and widely disperse these *Ixodes* species. Because *I. pacificus* and *I. spinipalpis* feed both on birds and rodents, there is ample opportunity for *B. americana* to be maintained in Lyme disease foci in the Pacific Northwest by rodents and, ultimately, be dispersed by resident and long-distance avifauna.

In far-western North America, *I. spinipalpis* serves as a maintenance vector of *B. burgdorferi* s.l., and *I. pacificus* acts as a bridge vector to humans [7]. Likewise, in southeastern U.S.A., *I. affinis* and *I. minor* act as maintenance vectors in the enzootic cycle of *B. burgdorferi* s.l, and *I. scapularis* serves as a bridge vector to humans [36]. It is noteworthy that *B. americana* has been obtained from *Ixodes* ticks that have birds as hosts, and many of these avian hosts have long-range dispersal capabilities. Based on current epidemiological information, people residing on both the West Coast and East Coast can be exposed to *B. americana*.

During spring migration, several *Ixodes* ticks that originated from southern latitudes have been reported in Canada: an *I. minor* larva was collected from a Common Yellowthroat, *Geothlypis trichas* (Linnaeus) [37], *I. affinis* immatures have be collected from both Common Yellowthroat and Swainson's Thrushes, *Catharus ustulatus* (Nuttall) [22] [38], and many *I. scapularis* immatures have been collected from migratory songbirds during spring migration [22] [24] [33] [39]-[41]. Since all of these tick species are songbird-transported ticks, they can be imported into Canada annually during northward spring migration. Since *B. americana* is associated with each of these bird-feeding ticks, we anticipate that this borrelial genospecies has wider distribution in Western Hemisphere than previously thought.

4.4. Songbirds Act as Reservoirs of *B. burgdorferi* s.l.

Songbirds are known to harbour *B. burgdorferi* s.l. in their bodies and, subsequently, transmit Lyme disease spirochetes to feeding ticks [42]. Based on a host competency study involving spirochete-free *I. scapularis* larvae, Richter *et al*. [43] revealed that the American Robin, *Turdus migratorius* Linnaeus, is a competent reservoir of *B. burgdorferi* s.l. As well, Hamer *et al*. [44], found that the Swainson's Thrush, which is another member of the thrush family, is a reservoir-competent host. With respect to the *I. scapularis*, there is no transovarial transmission of *B. burgdorferi* s.l. from eggs to larvae, and any fed larvae that are infected with *B. burgdorferi* s.l. after a blood meal must have acquired spirochetes from the host [45]. Analogous to the present study, *B. americana* spirochetes could either have come directly from the host bird (Varied Thrush), or indirectly from the *I. auritulus* larva that had fed on a *B. americana*-infected bird. Previous studies with *I. auritulus* reveal that there is transstadial transmission of spirochetes from larvae to nymphs and, similarly, from nymphs to adults [21] [22]; however, there is no evidence to suggest transovarial transmission of *B. burgdorferi* s.l. from eggs to larvae.

Anderson *et al*. [42] cultured *B. burgdorferi* s.l. from the liver of a Veery, *Catharus fuscescens* (Stephens), and, likewise, isolated Lyme disease spirochetes from the blood of the American Robin and Common Yellowthroat and, additionally, from attached larvae [34]. As well, Durden *et al*. [46] detected *B. burgdorferi* s.l. in skin biopsies from songbirds which suggests that certain birds are competent reservoir hosts. Further studies along Canada's West Coast have reported *B. burgdorferi* s.l. infected *I. auritulus* larvae from birds (Passeriformes, Accipitriformes) which suggests that several species of avifauna can be spirochetemic, and act as competent reservoirs [20] [24] [37]. In far-western Canada, there is substantive evidence that birds are the spirochetal reservoir of *B. americana*.

4.5. Transmission of *B. americana* to Humans

When a *B. americana*-infected *I. auritulus* tick parasitizes a bird, it can start a chain of events that leads to human infection. The spirochetemic bird now becomes a reservoir of *B. americana* for other bird-feeding ticks. If an *I. pacificus* larva bites this infected bird, it can acquire *B. americana*, and moult to a nymph in 5 - 8 weeks. Then the *B. americana*-infected *I. pacificus* nymph can transmit spirochetal infection to dogs, cats, and humans. When people become infected, they will normally exhibit symptoms associated with Lyme disease. Because these patients are infected with *B. americana*, they will likely be seronegative, and may not be able to obtain therapeutic treatment.

4.6. Medical Significance of *B. americana*

The presence of *B. americana* in Lyme disease vector ticks has important medical significance. Using PCR am-

plification, Clark *et al*. [12] detected *B. americana* in the blood and skin tissue of patients residing in Florida. Although these patients were seronegative and culture-negative (BSK II culture medium), the PCR results were positive for *B. americana* using the *flagellin* gene. The patients displayed a wide array of clinical symptoms, including fatigue, headache, vertigo, chills, and rashes (single and multiple). Interestingly, these symptomatic patients were treated with standard courses of antibiotics, and symptoms improved for a while, but then returned after treatment was halted. These febrile, clinical manifestations indicate that *B. americana* can evade the immune system, and sequester in deep-seated tissue, and develop into persistent Lyme disease. Even though patients are infected with *B. americana*, they can be seronegative and, regrettably, may be misdiagnosed, and not be able to get proper treatment. Many Lyme disease infections are undiagnosed and neglected, particularly if they do not have the characteristic skin rash or do not remember a tick bite. Furthermore, inconsistent or negative serological results might be connected to the use of *B. burgdorferi* s.s. as the antigen during serological testing. Moreover, a co-infection of *B. burgdorferi* s.l. strains or associated tick-borne pathogens will normally induce a diverse spectrum of clinical manifestations [31] [47]. Currently, patients will have to obtain PCR testing and DNA sequencing to obtain definitive proof of *B. americana* infection. Because *B. americana* is pathogenic to humans, medical practitioners must be aware that patients can have Lyme disease without having a typical rash, or exhibiting positive serology, or traveling to an endemic area. Our findings suggest that *B. americana* in ticks in British Columbia is one of the reasons why some patients are seronegative using standard 2-tiered Lyme disease serology.

In conclusion, we demonstrate the presence of *B. americana* in *I. auritulus* in Canada for the first time. This discovery highlights the northernmost documentation of *B. americana* in North America, and the first record of this *B. burgdorferi* s.l. genospecies in Canada. The *flagellin* amplicon in the present study is genetically consistent with other *B. americana* isolates collected previously in California and southeastern United States. The presence of *B. americana* along coastal B.C. provides further evidence that there is considerable genetic heterogeneity among *B. burgdorferi* s.l. in far-western Canada. Because migratory songbirds widely disperse Lyme disease vector ticks, dogs, cats, and people can be bitten by *B. americana*-infected ticks throughout much of the Western Hemisphere. Since *B. americana* is pathogenic to humans, health-care providers should be aware that *B. americana* infection will cause Lyme disease in patients.

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