Rapid introduction of Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto, in *Ixodes scapularis* (Acari: Ixodidae) established at Turkey Point Provincial Park, Ontario, Canada

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ABSTRACT: *Borrelia burgdorferi* sensu stricto (s.s.) was isolated from questing adult *Ixodes scapularis* Say ticks collected from Turkey Point Provincial Park (TPPP), Ontario, Canada during 2005-2006. DNA from ten (67%) of 15 pools of ticks was confirmed positive for *B. burgdorferi* s.s. using polymerase chain reaction (PCR) by targeting the *rrf* (5S)-*rrl* (23S) intergenic spacer region and *OspA* genes. This significant infection rate indicates an accelerated development of *B. burgdorferi* s.s. in TPPP, because this pathogen was not detected five years previously during sampling of the three motile life stages of *I. scapularis*. Our study provides the initial report of the presence of *B. burgdorferi* s.s. in TPPP, which is now endemic for Lyme disease. Ultimately, people and domestic animals are at risk of contracting Lyme disease when they frequent this park. ***Journal of Vector Ecology* 33 (1): 64-69. 2008.**

Keyword Index: *Borrelia burgdorferi*, Lyme disease, ticks, *Ixodes scapularis*, Turkey Point Provincial Park, Ontario.

INTRODUCTION

Lyme disease is a bacterial tick-borne zoonosis caused by the spirochete *Borrelia burgdorferi* sensu lato (s.l.) typically transmitted by certain ixodid ticks (Burgdorfer et al. 1982, Johnson et al. 1984). *Borrelia burgdorferi* s.l. was initially identified as a single strain and currently consists of at least 12 recognized genospecies irregularly distributed in different parts of the world (Richter et al. 2006). Three genospecies (*B. afzelii*, *B. burgdorferi* sensu stricto [s.s.], and *B. garinii*) are pathogenic to humans, and four other genospecies (*B. bissettii*, *B. lusitaniae*, *B. spielmanii*, and *B. valaisiana*) have been isolated from and/or detected by polymerase chain reaction (PCR) in Lyme disease patients residing in Eurasia.

In central and eastern North America, the blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), is the primary vector of *B. burgdorferi* s.l.; at least 125 species of North American vertebrates act as hosts (Keirans et al. 1996). Similarly, the western blacklegged tick, *Ixodes pacificus* Cooley and Kohls, is a principal vector for *B. burgdorferi* s.l. in far-western North America (Burgdorfer et al. 1985, Lane and Lavoie 1988, Banerjee et al. 1994); this tick species has been found on 108 different vertebrates in California (Castro and Wright 2007). Across Canada, *B. burgdorferi* s.s., which is pathogenic to humans and domestic animals, is the dominant genospecies (Morshed et al. 2005, 2006). Recently, *B. garinii*, which is commonly pathogenic to people in Eurasia, has been reported in the seabird tick, *Ixodes uriae* White, collected from Atlantic Puffins in Newfoundland and Labrador (Smith et al. 2006). During a ten-year tick-host study conducted in Ontario, Morshed et al. (2006) found *I. scapularis* adults as far north as the 50th parallel on hosts that had no out-of-province travel, and these ticks had an infection prevalence of 12.9%. Correspondingly, Ogden et al. (2006) reported 12.5% infectivity for *B. burgdorferi* s.l. in *I. scapularis* retrieved from domestic and wildlife hosts from Manitoba to Newfoundland and Labrador. Collectively, *I. scapularis* has been reported on avian and mammalian vertebrates from northern Alberta to Newfoundland and Labrador (Scott et al. 2001, Ogden et al. 2006).

Several established populations of *I. scapularis* have been documented along the north shore of Lake Erie. Initially, *I. scapularis* was studied at Long Point (Watson and Anderson 1976) and, subsequently, *B. burgdorferi* s.l. was detected (Barker et al. 1988, Lindsay et al. 1991, Banerjee et al. 2000). Likewise, *B. burgdorferi* s.l. was isolated from *I. scapularis* at Rondeau Provincial Park (Morshed et al. 2003), and a colony of *I. scapularis* has been documented at Point Pelee National Park (Banerjee et al. 2000). Recently, two populations of *I. scapularis* were discovered in the Turkey Point locality: one in Turkey Point Provincial Park (TPPP) uplands, and one in the Turkey Point lowlands that is distant from TPPP (Scott et al. 2004). This two-year study revealed *B. burgdorferi* s.l. in *I. scapularis* in the lowland forest. However, Lyme disease spirochetes were not detected in larvae and nymphs collected from small- and medium-sized mammals or from adult ticks (males, females) obtained by flagging in any area of TPPP. The purpose of the current study was first, to determine if *B. burgdorferi* s.l. had become established in TPPP; second, to check the range expansion of Lyme disease spirochetes; and third, to discern whether people with work-related or recreational activities in this parkland are at risk of contracting Lyme disease.

MATERIALS AND METHODS

Tick collection

Questing adult *I. scapularis* were collected during October and November, 2005-2006, at Turkey Point Provincial Park uplands (42˚ 42' 19" N, 80˚ 19' 57" W) in Norfolk County, Ontario, situated on the north shore of Lake Erie. Unfed ticks were obtained along nature trails and from ecotone areas of the Carolinian deciduous and coniferous forest by flagging brush and low-level vegetation using a 62- by 90-cm white, flannel-covered crib sheet (Dundee Mills, New York, NY). Likewise, St. Williams Crown Forest, which is a neighboring forest habitat, was flagged for ticks. Live males and females were placed separately in ventilated polyethylene vials with tulle netting caps and put in a self-sealing plastic bag with a moist paper towel. These ticks were sent promptly via overnight courier to the British Columbia Centre for Disease Control (B.C.C.D.C.) for culturing and DNA testing.

Culturing *B. burgdorferi* s.l.

At B.C.C.D.C., live ticks were surface sterilized using 10% hydrogen peroxide followed by 70% isopropyl alcohol and then rinsed with sterile water. Midgut contents were cultured for mobile borreliae in Barbour-Stoenner-Kelly (BSK) II medium, as described previously (Barbour 1984). The cultures were incubated at 34° C and checked weekly by dark-field microscopy for 30 days. Spirochetes were subcultured when they were observed.

DNA extraction and PCR amplification

Total DNA was extracted from tick cultures using Qiagen tissue kits (QIAGEN, Mississauga, ON) according to manufacturer's instructions. PCR targeted amplicons of borrelial DNA and amplified a portion of the variable spacer region between two conserved structures, the 3' end of the 5S rRNA (*rrf*) and the 5' end of the 23S rRNA (*rrl*), as described previously (Postic et al., 1994) and, similarly, a portion of the *OspA* gene (Persing et al. 1990).

The PCR was performed using a HotStarTaq Master Mix kit (QIAGEN, Mississauga, ON). A total of 5 µl of extracted DNA was used for a 25 µl reaction. For the 5S-23S (*rrf-rrl*) reaction, primer 1 (CTGCGAGTTCGCGGGAGA) and primer 2 (TCCTAGGCATTCACCATA), both from the supplier (Sigma, Oakville, Ontario), were used. The conditions for thermal cycling were as follows: initial denaturation at 95˚C for 10 min, followed by 50 amplification cycles, which included a denaturing step at 95˚ C for 1 min and an annealing step at 52˚ C for 1 min, and extension at 72˚ C for 2 min, then ending with 72˚C for a 7-min extension. For the *OspA* reaction, primer 3 (TTCTGACGATCTAGGTCAAA) and primer 4 (GCAGTTAAAGTTCCTTCAAG) were used. The conditions for thermal cycling were as follows: initial denaturation at 95˚ C for 10 min, followed by 50 amplification cycles, which included a denaturation step at 95˚ C for 1 min and an annealing step at 55˚ C for 1 min, and extension at 72˚C for 2 min, then ending with 72˚ C for a 7-min extension.

Amplification was carried out using negative and positive controls for all PCR reactions. The negative control was sterile water and the positive control used purified *B. burgdorferi* s.s. strain B31. Amplification products were analyzed by electrophoresis in 2.0 % agarose gels followed by staining with ethidium bromide and ultraviolet light illumination.

Gene sequencing

The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Mississauga, ON). Nucleotide sequencing of all amplicons was determined by the dideoxy chain termination method using the same primer sets as described above with the BigDye Terminator Cycle Sequencing kit on GeneAmp PCR System 9700 (Applied Biosystems Co., Foster City, CA). The cycling reactions were cleaned by precipitation with a sodium acetate/ethanol mixture, formamide denatured, and then run on an ABI Prism 3100/3130 DNA Sequencer (Applied Biosystems Co., Foster City, CA). DNA sequence data were analyzed using SeqMan and MegAlign modules within the Lasergene Sequence Analysis software (DNASTAR Inc., Madison, WI) and compared with representative sequences of *Borrelia* species downloaded from GenBank (National Center for Biotechnology Information, Bethesda, MD).

RESULTS

Tick collection

During the 14-month study (16 October 2005 to 7 November 2006), 46 field-derived, questing *I. scapularis* adults (23 males, 23 females) were collected from the upland area of Turkey Point Provincial Park. For epidemiologic and ecologic comparison, an *I. scapularis* female was collected from the St. Williams Crown Forest, a neighboring parkland.

Spirochete detection

All *I. scapularis* adults were submitted live, and all live cultures had motile spirochetes. Spirochetes from ten (67%) of 15 pools of *I. scapularis* adults from TPPP were observed by dark-field microscopy, and stored at -80˚C for further DNA studies (Figure 1). Similarly, spirochetes were isolated from an *I. scapularis* female collected from St. Williams Crown Forest.

PCR amplification, sequencing, and genetic alignment analysis

Based on PCR amplification of the *rrf* (5S)-*rrl* (23S) intergenetic spacer and *OspA* genes, ten (67%) of 15 isolate pools were confirmed positive for *B. burgdorferi* s.l. Two of the ten *B. burgdorferi* s.l. isolates from the TPPP uplands were selected and genetically compared to isolates previously collected from a white-footed mouse, *Peromyscus leucopus* (Rafinesque), and an attached *I. scapularis* larva previously collected from the bottomland forest that is separate from TPPP, a distance of 5 km away (Scott et al. 2004). DNA sequencing of the 218-bp amplicons of the *rrf* (5S)-*rrl* (23S) intergenic spacer gene of TPPP upland isolates revealed a close similarity to bottomland isolates (AY363432 [*I. scapularis* larva] and AY363398 [*P. leucopus* ear lobe]) previously described (Morshed et al. 2006) and, likewise, to the *B. burgdorferi* s.s. reference strain, L30127 (B31). One isolate, ON06FTP933 (GenBank accession no. EU019128) from the TPPP uplands had a 2-bp difference with the *I. scapularis-P. leucopus* isolates, whereas another uplands isolate, ON06FTP934 (GenBank accession no. EU019129) was a sequence match (Figure 2). The B31 reference strain had a 1-bp difference compared to previously collected isolates from the endemic Turkey Point lowlands. The 218-bp amplicons had a sequence similarity, which ranged from 99.1 to 100%. Additionally, when we compared TPPP upland isolates with the B31 strain, we noted a sequence divergence of 1.0% for nucleotide substitution. All sequenced isolates from both TPPP uploads and St. Williams Crown Forest were confirmed positive positive for *B. burgdorferi* s.s. The DNA sequences of the two isolates (ON06FTP933, ON06FTP934) from the TPPP uplands were deposited in the GenBank database.

DISCUSSION

Using DNA analysis, we detected a 67% infection prevalence of *B. burgdorferi* s.s. in host-seeking *I. scapularis* adults in Turkey Point Provincial Park. Previously, Scott et al. (2004) observed a *B. burgdorferi* s.l. prevalence of 45% in *I. scapularis* adults at the Turkey Point lowlands; however, Lyme disease spirochetes were not detected in any of the post-embryonic stages of *I. scapularis* collected within the TPPP uplands. Since ecological changes have not been apparent in either of these focal meadow and forest habitats, our findings provide new evidence that *B. burgdorferi* s.s. has rapidly become established in TPPP uplands in the past five years.

Adult *I. scapularis* ticks were collected because they are the last development life stage, and climax the acquisition of *B. burgdorferi* s.l. during larval and nymphal blood meals. Canadian prognosticators have purported that *I. scapularis* nymphs are the main mode of transmission of *B. burgdorferi* s.l. to humans and domestic animals. In fact, recent Canadian studies reveal that *I. scapularis* females are the primary modality of *B. burgdorferi* s.l. transmission. In a ten-year tick-host study (Morshed et al. 2006), all *I. scapularis* collected from humans and domestic animals were adults. Similarly,

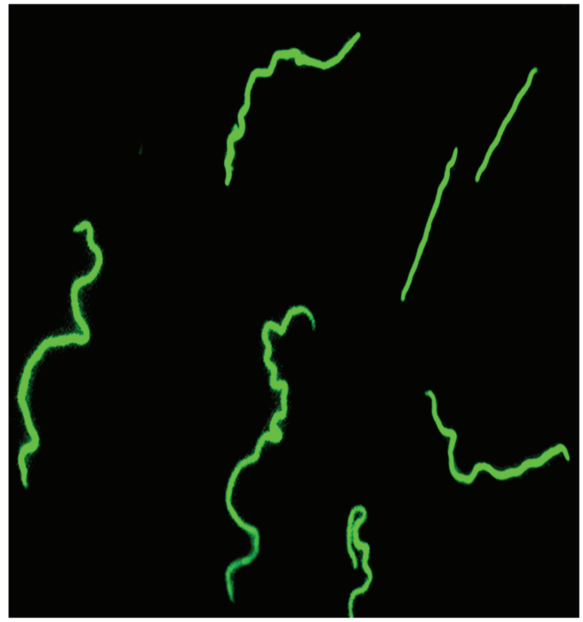

Figure 1. *Borrelia burgdorferi* sensu stricto spirochetes stained with anti-human IgG Fluorescein isothiocyanate (FITC) conjugate using IFA technique; original magnification, 500×. Typical spiral-shaped morphology is long (15 to 30 μm) and narrow (0.2 to 5 μm).

of the larval, nymphal, and adult blacklegged ticks collected from humans, domestic, and wildlife hosts from eastern and central Canada (Manitoba to Newfoundland and Labrador), only 1% were nymphs, and none of these nymphal ticks were infected with *B. burgdorferi* s.l. (Ogden et al. 2006). If, in fact, attached engorging nymphs play a role in *B. burgdorferi* s.l. transmission across Canada, it is not apparent.

Rather than test ticks individually, we pooled ticks to obtain the general level of endemicity for *B. burgdorferi* s.l. and also conserve laboratory resources. In Lyme disease endemic areas, infection levels have ever-changing enzootic dynamics. Objectively, pooling of ticks is a standard diagnostic method for assessing public health risk; individual testing does not provide any additional cautionary benefit.

Songbirds act as natural vehicles for the dispersal of *B. burgdorferi* s.l.-infected *I. scapularis* to new areas, including TPPP uplands. During northward avifaunal spring migration, passerine species, which overwinter in the southern United States, Central, and South America, make stopovers at Lyme disease endemic areas to rest and refuel (Weisbrod and Johnson 1989), coinciding with the time when *I. scapularis* larvae have elevated host-seeking activity and nymphs peak in their questing action (Scott et al. 2001, Morshed et al. 2005). Neotropical migrants release engorged ticks later across southern Canada in tick-friendly habitats that provide a supportive, moist microenvironment. Songbirds that are nesting and raising their brood are constantly circulating the local area in search of food. In particular, ground-foraging songbirds meander through low-lying vegetation and, in Lyme disease endemic areas, are in juxtaposition with covert, questing ticks. Some of these birds are attracted to the carcasses of dead *I. scapularis* females, which have completed

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                    10        20        30        40        50        60        70        80
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AY363432     GTAGGTTATTGCCAGGGTTTTTATTTTTATACTTTAAACTTTGATTTTATTTTTATGTTTTTTAAATATTGGTGTTTTTG
AY363398     GTAGGTTATTGCCAGGGTTTTTATTTTTATACTTTAAACTTTGATTTTATTTTTATGTTTTTTAAATATTGGTGTTTTTG
ON06FTP933   GTAGGTTATTGCCAGGGTTTTTATTTTTATACTTTAAACTTTGATTTTATTTTTATGTTTTTTAAATATTGGTGTTTTTG
ON06FTP934   GTAGGTTATTGCCAGGGTTTTTATTTTTATACTTTAAACTTTGATTTTATTTTTATGTTTTTTAAATATTGGTGTTTTTG
L30127(B31)  GTAGGTTATTGCCAGGGTTTTTATTTTTATACTTTAAACTTTGATTTTATTTTTATGTTTTTTAAATATTGGTGTTTTTG

                    90       100       110       120       130       140       150       160
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AY363432     AATGTGTTGTTTAAATAACATAAAAAATAAAATATATATTGACATGTATTAAACAAAGATATATATTATTTTTATGTTGTA
AY363398     AATGTGTTGTTTAAATAACATAAAAAATAAAATATATATTGACATGTATTAAACAAAGATATATATTATTTTTATGTTGTA
ON06FTP933   AATGGGTTGTTTAAATAACATAAAAAATAAAATATATATTGACATGCATTAAACAAAGATATATATTATTTTTATGTTGTA
ON06FTP934   AATGTGTTGTTTAAATAACATAAAAAATAAAATATATATTGACATGTATTAAACAAAGATATATATTATTTTTATGTTGTA
L30127(B31)  AATGTGTTGTTTAAATAACATAAAAAATAGAATATATATTGACATGTATTAAACAAAGATATATATTATTTTTATGTTGTA

                   170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|...
AY363432     TAAATAAATTGGCAAAATAGAGATGGAAGATAAAAATATGGTCAAAGTAATAAGAGTC
AY363398     TAAATAAATTGGCAAAATAGAGATGGAAGATAAAAATATGGTCAAAGTAATAAGAGTC
ON06FTP933   TAAATAAATTGGCAAAATAGAGATGGAAGATAAAAATATGGTCAAAGTAATAAGAGTC
ON06FTP934   TAAATAAATTGGCAAAATAGAGATGGAAGATAAAAATATGGTCAAAGTAATAAGAGTC
L30127(B31)  TAAATAAATTGGCAAAATAGAGATGGAAGATAAAAATATGGTCAAAGTAATAAGAGTC
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Figure 2. Comparison of 218-bp sequences of the *rrf* (5S)-*rrl* (23S) intergenic spacer gene collected from upland TPPP isolates (ON06FTP933, ON06FTP934) and bottomland isolates (AY363432, AY363398) and *B. burgdorferi* s.s. type strain L30127 (B31). Nucleotide substitutions are in boldface type.

oviposition (Milne 1950, J.D.S. unpublished data). Fat deposits in these tick morsels provide subcutaneous adipose energy reserves to sustain long-distance flight and ongoing metabolic activity. If a songbird traverses the spot where a gravid *I. scapularis* female laid eggs in the past year, very high larval tick parasitism can occur. Furthermore, if a songbird is infected with *B. burgdorferi* s.l., this avian reservoir host can infect a large number of larval ticks. If these spirochete-infected ticks drop off in a suitable habitat, there is likelihood that a new endemic area will be initiated. Moreover, because two preexistent Lyme disease endemic areas are situated in the Turkey Point and Long Point bioregions, local avian reservoir hosts are more apt to be harbingers of endogenous spirochetal infections. In earlier bird-tick studies in Connecticut, Anderson and Magnarelli (1984) removed 21 *I. scapularis* larvae (reported as *I. dammini*) from both a Gray Catbird and a Swamp Sparrow and, likewise, 19 *I. scapularis* nymphs from an American Robin at the time of capture. Although these high tick infestations are not unprecedented, they indicate the stark potential for a single songbird to act as a vanguard for *I. scapularis* and *B. burgdorferi* s.l. establishment.

A spirochetemic songbird can infect many larval and nymphal ticks and, logically, drop them near the nesting site to start a focal area for Lyme disease. In the TPPP bioregion, two Lyme disease endemic areas provide a viable source of *I. scapularis* during the nesting period. Depending on the passerine species, flight time from the bottomlands area would be approximately ten min. In our study, the newly-found presence of *B. burgdorferi* s.s.-infected *I. scapularis* in the TPPP uplands area suggests that songbirds are likely the transporting vertebrates and, therefore, the cause of the subtle increase of Lyme disease spirochetes in the enzootic cycle.

Alternatively, terrestrial mammals can act as transporting agents for vector ticks from the Turkey Point lowlands to the TPPP uplands. However, these two *I. scapularis* populations are 5 km apart and separated by several natural and man-made barriers, namely a main highway, the town of Turkey Point, a waterlogged swamp, a dense marsh, and a body of water. Because of these biogeographic obstacles and potential predator attack, transit across this vast forest floor and wetlands by a white-footed mouse during five days of tick engorgement is perilous. White-tailed deer, *Odocoileus virginianus* Zimmermann, which are common denizens in this Carolinian forest biome, act as amplifying hosts of all three motile stages of *I. scapularis*, especially adults (Durden and Keirans 1996). However, these cervid hosts only play a minimal role in non-endemic areas because they do not sustain viable *B. burgdorferi* s.l. in their bodies (Telford et al. 1988). Furthermore, because transovarial transmission of *B. burgdorferi* s.l. in *I. scapularis* is rare, or inapparent (Patrican 1997), gravid females would be unlikely purveyors of infection. The single collection of a *B. burgdorferi* s.s.-infected *I. scapularis* in St. Williams Crown Forest, which is contiguous to TPPP uplands, certainly appears to be an adventitious release of an immature *I. scapularis* tick from a roving small mammal in this parkland ecosystem.

Our genetic analysis reveals that upland *B. burgdorferi* s.s. isolates are tantamount to previously collected Turkey Point bottomland isolates. Based on DNA sequencing of the 218-bp amplicon of the *rrf* (5S)-*rrl* (23S) intergenic spacer gene, one of the TPPP upland isolates, ON06FTP934 (GenBank accession no. EU019129), matched a *B. burgdorferi* s.s. isolate (Cluster A group) cultured from a *P. leucopus* mouse and its attached, engorged *I. scapularis* larva collected from the bottomlands (Morshed et al. 2006). This close parity of isolates suggests that a reservoir-competent songbird (i.e., American Robin) provided transportation locally for *B. burgdorferi*-infected *I. scapularis* subadults to the TPPP uplands. Isolate ON06FTP933 (GenBank accession no. EU019128), which has a 2-bp difference, is genotypically similar to Cluster C group isolates from Rondeau Provincial Park and Long Point, Ontario; this genetic variance also suggests introduction by passerine hosts from further afield. Indeed, the marked increase of *B. burgdorferi* s.s. in the TPPP uplands over the previous five years clearly indicates that this focus is a fitting habitat for enzootic cycling.

To conclude, the results of our investigation show that the uplands of TPPP have rapidly developed into an endemic area for Lyme disease. The accelerated incorporation of *B. burgdorferi* s.s. in *I. scapularis* at TPPP uplands surged from an undetected level to 67% in five years. Given the genetic diversity of *B. burgdorferi* s.s. in this population of *I. scapularis*, songbirds are likely candidates for the introduction of spirochetal variants. Because of the clarion increase of *B. burgdorferi* s.s. infection in this established *I. scapularis* colony, park staff and visitors (i.e., hikers, campers, and mushroom collectors) should take precautionary measures when frequenting this woodland area. The medical profession and the general public must be cognizant that *B. burgdorferi* s.s.-infected *I. scapularis* are a public health risk in TPPP.

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