Lyme Disease Spirochete, *Borrelia burgdorferi*, Endemic in Epicenter at Turkey Point, Ontario

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ABSTRACT The Lyme disease spirochete, *Borrelia burgdorferi* Johnson, Schmidt, Hyde, Steigerwalt, and Brenner, was discovered in blacklegged ticks, *Ixodes scapularis* Say at Turkey Point, Ontario, Canada. We report the first isolation of *B. burgdorferi* from a vertebrate animal collected on mainland Ontario. During this 2-yr study, spirochetes were isolated from the white-footed mouse, *Peromyscus leucopus* Rafinesque, and attached *I. scapularis* larvae. Similarly, isolates of *B. burgdorferi* were cultured from blacklegged tick adults, and confirmed positive with polymerase chain reaction by targeting *OspA* and *rrf* (5S)-*rrl* (23S) genes. Moreover, all isolates of *B. burgdorferi* from this area had complementary genetic structure, and the second primer set amplicons confirmed the first primer set amplification products. These findings show an epicenter endemic for *B. burgdorferi* within an established population of *I. scapularis* at Turkey Point.

KEY WORDS Lyme disease, *Borrelia burgdorferi*, *Ixodes scapularis*, Turkey Point, Ontario

LYME DISEASE (LYME BORRELIOSIS) is a bacterial zoonosis caused by *Borrelia burgdorferi* Johnson, Schmid, Hyde, Steigerwalt, and Brenner that is transmitted to humans by certain ixodid ticks (Burgdorfer et al. 1982). In eastern and central Canada, the blacklegged tick, *Ixodes scapularis* Say (northern populations previously treated as *I. dammini* [deer tick]) (Oliver et al. 1993, Keirans et al. 1996), is a competent vector of the Lyme disease spirochete (Burgdorfer and Gage 1986, Piesman and Sinsky 1988, Sanders and Oliver 1995). The blacklegged tick also acts as a vector for tick-borne microorganisms that cause human granulocytic ehrlichiosis (Pancholi et al. 1995, des Vignes and Fish 1997), human babesiosis (Piesman et al. 1986, Mather et al. 1990), and deer tick virus (Telford et al. 1997, Ebel et al. 1999), which is a variant of Powassan virus (Kuno et al. 2001). Recently, *Bartonella henselae*, the causal organism of cat scratch disease, has also been detected in *I. scapularis* (Eskow et al. 2001).

Previously, three established populations of *I. scapularis* were reported on the north shore of Lake Erie. Watson and Anderson (1976) collected all three motile stages (larva, nymph, adult) of *I. scapularis* on white-tailed deer, *Odocoileus virginianus* Zimmerman on the Long Point sand spit. Later, Barker et al. (1988) first found *B. burgdorferi* in *I. scapularis* (reported as *I. dammini*) at Long Point, an endemic area for Lyme disease. Further west, along the north shore of Lake Erie, Morshed et al. (2003) discovered an epicenter endemic for Lyme disease at Rondeau Provincial Park, Ontario. As well, an established population of *I. scapularis* is present at Point Pelee National Park (Banerjee et al. 2000). Elsewhere, across Ontario, *B. burgdorferi*-positive blacklegged ticks were collected from domestic animals, which had no out-of-province travel, and were serologically positive for Lyme disease (Banerjee et al. 1995, 1996, 2000).

Turkey Point is located on the north shore of Lake Erie in the same vicinity as Long Point, but these two areas are parted by Inner Bay, and considered distinctly separate. The blacklegged tick coexists in the Turkey Point area with the American dog tick, *Dermacentor variabilis* Say, which is not a competent vector of Lyme disease spirochetes (Piesman and Sinsky 1988, Sanders and Oliver 1995, Johns et al. 2001). Normally, blacklegged ticks are reported by the public in the spring and fall, whereas American dog ticks are noted during late spring and summer months.

At Turkey Point, white-footed mice, *Peromyscus leucopus* Rafinesque, which are abundant in the area, act as competent reservoirs for *B. burgdorferi* (Bosler et al. 1983, Anderson et al. 1985, Donahue et al. 1987). White-tailed deer are common throughout the area, and play a role in the local distribution of *I. scapularis*. Both of these mammals act as hosts of *I. scapularis*, and they overlap in search of nuts produced by several species of oak and walnut trees in this area.

In this 2-yr study, we examined ticks and vertebrate hosts to determine the presence of *B. burgdorferi*, its

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endemicity, and whether this spirochete is cycling enzootically in the Turkey Point area.

Materials and Methods

Study Area. Sampling occurred in three general areas at Turkey Point (42° 40′ N, 80° 21′ W), which is located in the County of Norfolk, situated on the north shore of Lake Erie. The first study area is on the southwest side of the village, which is a sandy outwash lowland surrounded on two sides by marsh. It consists mainly of black ash, *Fraxinus nigra* Marshall, and a few black walnut, *Juglans nigra* L., chinquapin oak, *Querus muehlenbergii* Engelmann, swamp white oak, *Q. bicolor* Willdenow, and Scots pine, *Pinus sylvestris* L. The understory has pockets of scouring rush, *Equisetum hymale* L. The second study area is on the northwest side of the village on a well-drained upland bluff, part of the Norfolk Sand Plain, which comprises Turkey Point Provincial Park and St. Williams Crown Forest. This Carolinian hardwood/mixed forest is dominated by red oak, *Quercus rubra* L., white oak, *Q. alba* L., black oak, *Q. velutina* Lamarck, bur oak, *Q. macrocarpa* Michaux, shagbark hickory, *Carya ovata* (Mill.) K. Koch, sugar maple, *Acer saccharum* Marshall, and eastern white pine, *Pinus strobes* L. Third, minimal sampling was conducted in the village of Turkey Point.

Tick Collection. Adult blacklegged ticks were collected by flagging woodland understory using a 62 × 90-cm white, flannel-covered, waterproof crib sheet (Dundee Mills, New York, NY). These collections coincided with the blacklegged tick's bimodal activity at Turkey Point in the spring and fall. Live males and unfed females were stored in separate vials capped with tulle netting, and kept in ziplock bags with moist paper towel. These live ticks were sent promptly by courier for culturing to the British Columbia Centre for Disease Control (BCCDC). Adult *D. variabilis* were observed, but not collected. Some ixodid specimens were sent to Georgia Southern University for identification, and then forwarded to BCCDC.

Larval and nymphal ticks were collected from small mammals during early and late summer using 57–66 live traps (H. B. Sherman, Tallahassee, FL) per night. Fully engorged, immature ticks were kept alive for culturing, and handled in a similar way to adults; however, nonengorged or partially engorged ticks were put directly into microvials containing 70% isopropyl alcohol for polymerase chain reaction (PCR) testing. Ticks were tested in pools of up to five adults (normally three) or seven larvae.

Culturing of *B. burgdorferi* from Ticks. Live *I. scapularis* ticks were surface sterilized with 10% H_2O_2 for 10 min, followed by 70% isopropyl alcohol, and washed three times with sterile distilled water. The midgut was removed and placed in BSK II media, as described previously (Barbour 1984, Scott et al. 2001), incubated at 34°C, and checked weekly by dark-field microscopy for live spirochetes for up to 30 d.

Culture of *B. burgdorferi* from Organs. Small mammals were killed using CO_2, and directly soaked in chlorine bleach solution for 10 min, transferred to distilled water for 1 min, placed in 70% isopropyl alcohol for 5 min, dipped in distilled water for 1 min, and laid on cotton batting to absorb excess aqueous liquid. Organs and tissues were dissected from four mice from the lowland site, and placed promptly in BSK II media plus antibiotics (rifampicin, kanamycin), as described previously (Barbour 1984), and incubated at 34°C for 30 d. Vials were checked for live spirochetes weekly.

Polymerase Chain Reaction. DNA was extracted from pure or contaminated cultures using Qiagen tissue kits (Qiagen, Mississauga, ON, Canada). PCR was performed to amplify 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 *Osp*A gene (Persing et al. 1990).

The PCR mixture for the variable spacer region consisted of one commercial bead containing 1.5 U of *Taq* polymerase (Roche Diagnostics, Quebec, QC, Canada), 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP; Roche Diagnostics, Quebec, Canada), and stabilizers including bovine serum albumin (Amersham Pharmacia Biotech, Quebec, Canada), 1 μl (20 pmol) primer 1 (CTGCGAGTTCGCGGGAGA), and 1 μl (20 pmol) primer 2 (TCCTAGGCATTCACCATA), both from the same supplier (Sigma, Oakville, ON), and 10 μl extracted DNA in a total volume of 30 μl. Thermal cycling consisted of 5 min at 94°C, 50 cycles for 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, and a final 7-min extension at 72°C.

The PCR mixture for the *Osp*A gene consisted of one commercial bead containing 1.5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and stablizers, including bovine serum albumin, 1 μl (20 pmol) primer 3 (TTCTGACGATCTAGGTCAAA), and 1 μl (20 pmol) primer 4 (GCAGTTAAAGTTCCTTCAAG), and 10 μl extracted DNA in a total volume of 30 μl. Thermal cycling consisted of 5 min at 95°C, 50 cycles for 1.5 min at 95°C, 1 min at 55°C, and 1.83 min (110 s) at 72°C, and a final 7-min extension at 72°C.

Negative and positive controls were used in all PCRs. The negative control was sterile water, and the positive control used purified *B. burgdorferi* strain B31. Amplification products were analyzed by electrophoresis in 2.0% agarose gels, followed by staining with ethidium bromide and UV light illumination.

Results

Tick and Small Mammal Collections. During the 2-yr study (1 April 2001–30 November 2002), both host-seeking *I. scapularis* and *D. variabilis* ticks were collected. A total of 63 immature ticks (59 *I. scapularis*, 4 *D. variabilis*) were removed from 81 small mammals during three summer field trips, which consisted of 472 live-trap nights (Table 1). The majority of larval and nymphal ticks were collected from the lowland area. Immature *I. scapularis* (3 nymphs, 53 larvae) were collected primarily from

Table 1. Immature ticks collected from small mammal hosts at Turkey Point, Ontario, 2001–2002

	Hosts					Ticks				Overall prevalence of ticks on small mammals (%)
						I. scapularis		*D. variabilis*		
	P.l.	*B.b.*	*M.m*	*T.h.*	*T.s.*	Larvae	Nymphs	Larvae	Nymphs	
Early summer 23–27 June 2001	36	0	1	1	1	12	4	0	2	12/39 (31)
Late summer 27–30 Aug. 2001	26	1	0	0	1	4	0	0	0	2/28 (7)[a]
Late summer 6–7 Sept. 2002	14	0	0	0	0	39	0	1	1	13/14 (93)
Total	76	1	1	1	2	55	4	1	3	27/81 (33)

P.l., Peromyscus leucopus; B.b., Blarina brevicauda; M.m., Mus musculus; T.h., Tamiasciurus hudsonicus; T.s., Tamias striatus.
[a] Indicative of a very dry summer.

white-footed mice; however, one larva was removed from the single house mouse, *Mus musculus* L., and one nymph and one larva from the single red squirrel, *Tamiasciurus hudsonicus* Erxleben. No ticks were found on a single northern short-tailed shrew, *Blarina brevicauda* (Say), or on two eastern chipmunks, *Tamias striatus* (L.), that were caught. Overall, tick prevalence on small mammals was 33% (27 of 81). For *I. scapularis*, the prevalence on small mammals was 32% (26 of 81), with a mean intensity on infested hosts of 2.3 (range, 1–8). Specifically, the prevalence of *I. scapularis* on the most common host, *P. leucopus*, was 32% (24 of 76). Because the summer of 2001 was very dry, the frequency of immature ticks was minimal for the late summer collection.

A total of 254 adult blacklegged ticks (127 males, 127 females) were collected by flagging. An average of 5.7 adults were collected per hour during 44.5 h of flagging. Interestingly, one *I. scapularis* male was collected along the beach at the tree line; however, this dry microclimate is not conducive to tick establishment.

Spirochete Detection. At the epicenter located on the southwest side of Turkey Point, *B. burgdorferi* was cultured from the ear lobe and urinary bladder of one of four (25%) *P. leucopus* and from attached *I. scapularis* larvae. These isolates were compared using DNA sequencing of two *B. burgdorferi* primer sets (variable spacer region between 5S rRNA [*rrf*] and 23S rRNA [*rrl*]; *Osp*A gene), and were positive. As well, all isolates from *I. scapularis* adults were positive for *B. burgdorferi*. In each case, the second primer set amplicons confirmed the first primer set amplification products. In the fall of 2000, we obtained a *B. burgdorferi*-positive, engorged *I. scapularis* female that was removed from a dog from this locale, and it had no out-of-province travel, nor travel to Long Point or Rondeau Provincial Park. None of the immature *D. variabilis* were tested.

Based on all *I. scapularis* adults collected at the epicenter located on the southwest side of Turkey Point, 24 (45%) of 53 culture pools were PCR positive for *B. burgdorferi*. Two pools of *I. scapularis* adults collected from the upland bluff in St. Williams Crown Forest produced live cultures of *B. burgdorferi*. Notably, both immature and adult blacklegged ticks were collected at all three study areas, including Turkey Point Provincial Park.

Discussion

The isolation of *B. burgdorferi* from *I. scapularis* and *P. leucopus* collected at the lowland site confirms that this epicenter is endemic for Lyme disease. The *B. burgdorferi*-positive blacklegged tick adults collected from the upland bluff may have detached as nymphs from passerine birds or roving mammals, which had recently frequented the lowland epicenter. Because transovarial transmission of *B. burgdorferi* is rare or not apparent in *I. scapularis* (Patrican 1997), spirochetes are typically transmitted directly to attached *I. scapularis* larvae from *B. burgdorferi*-infected white-footed mice during engorgement.

At least three of the small mammal species that we caught are hosts for larval and nymphal *I. scapularis*, and are competent reservoirs of *B. burgdorferi:* white-footed mouse (Bosler et al. 1983, Anderson et al. 1985, Donahue et al. 1987), northern short-tailed shrew (Telford et al. 1990), and eastern chipmunk (McLean et al. 1993). In contrast, white-tailed deer are incompetent reservoirs of *B. burgdorferi* (Telford et al. 1988); however, they do act as amplifying hosts for all motile stages of *I. scapularis*, especially adults (Durden and Keirans 1996). Similarly, the raccoon, *Procyon lotor* (L.), common in the area, is an inefficient reservoir of the spirochete (Ouellette et al. 1993, Norris et al. 1996). Of direct significance, we report the first isolation of *B. burgdorferi* from a vertebrate animal in mainland Ontario.

Songbirds play a role in the dispersal of immature *I. scapularis* in Canada during northward spring migration (Morshed et al. 1999). Moreover, Scott et al. (2001) reported wide distribution of *I. scapularis* across Canada on passerine birds extending from northern Alberta to Nova Scotia, some of which are infected with *B. burgdorferi*. Notably, some species of birds act as reservoir-competent hosts of *B. burgdorferi*, and provide a means to introduce infected larvae and nymphs (Anderson et al. 1986, 1990; Weisbrod and Johnson 1989; Stafford et al. 1995; Richter et al. 2000).

Over the summer, these subadult ticks molt to the next developmental stage, either nymph or adult. Subsequently, they quest for domestic and feral hosts. Multiple *I. scapularis* ticks, detached in one location from a bird, could initiate a new population. Given its southward proximity across Inner Bay, Long Point could act as a source of *B. burgdorferi*-infected *I. scapularis* that parasitize avian hosts. Lindsay et al. (1991) reported a *B. burgdorferi* infection prevalence of 58% in *I. scapularis* adults (reported as *I. dammini*) at Long Point, an endemic area for Lyme disease. Because white-tailed deer are incompetent reservoirs for *B. burgdorferi* (Telford et al. 1988), ground-foraging passerine birds are more likely to be sources of infected ticks for Turkey Point, especially during northward spring migration.

Along the north shore of Lake Erie, daytime temperatures during winter sometimes climb above freezing and *I. scapularis* adults may be questing for a blood meal. These ticks have a marked increase in host-seeking activity at temperatures ≥4°C (Duffy and Campbell 1994); however, Carroll and Kramer (2003) collected adult *I. scapularis* in Maryland during the winter when the temperature was as low as −2°C. For most of the year, human residents and visitors should take tick-preventive measures during outdoor activities while frequenting local woodlands and unmowed vegetation.

In Canada, the endemic site at Long Point has been proclaimed as the only place in which a person can contract Lyme disease. Our new epidemiological findings are significant because Turkey Point and Long Point are parted by water and are physiographically different; these two sites are distinctly separate. Previously, the mainland area that encompasses Turkey Point was reported to be nonendemic for Lyme disease. However, our findings substantiate a local population of *I. scapularis* within which lies an epicenter for *B. burgdorferi*. This focal area not only presents a public health risk for local residents and domestic animals, but also for unsuspecting visitors involved in outdoor recreational activities. Although Lyme disease cases have been reported in Ontario, some physicians doubt the presence of *B. burgdorferi* in the province. In fact, some physicians are telling patients that there is no Lyme disease in North America. If physicians are not expecting Lyme disease, they are less likely to diagnose it, or to report actual and suspected cases. Moreover, Lyme disease cases become dispersed when travelers return home. Thus, potential pitfalls are exacerbated in regions in which the public and physicians have been told that Lyme disease does not exist. Physicians must be aware that their patients may encounter *B. burgdorferi*-infected *I. scapularis* in the Turkey Point area, and subsequently contract Lyme disease.

In conclusion, we provide direct evidence that the blacklegged tick is established in the Turkey Point vicinity with all three motile stages present. *B. burgdorferi* was isolated from both immature and adult *I. scapularis* collected at the focal area on the southwest side of Turkey Point. Furthermore, *B. burgdorferi*-positive *I. scapularis* attached to *B. burgdorferi*-infected *P. leucopus* were collected at this lowland epicenter. The Lyme disease spirochete is endemic at Turkey Point, as it is cycling enzootically between *I. scapularis* and *P. leucopus*.

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