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Detection of *Borrelia* spp., *Ehrlichia canis*, *Anaplasma phagocytophilum*, and *Dirofilaria immitis* in Eastern Coyotes (*Canis latrans*) in Nova Scotia, Canada

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ABSTRACT: *Borrelia burgdorferi* and *Borrelia miyamotoi* are tickborne zoonotic pathogens in Canada. Both bacteria are vectored by ticks, *Ixodes scapularis* in Atlantic Canada, but require wildlife reservoir species to maintain the bacteria for retransmission to future generations of ticks. Coyotes (*Canis latrans*) are opportunistic feeders, resulting in frequent contact with other animals and with ticks. Because coyotes are closely related to domestic dogs (*Canis lupus familiaris*), it is probable that coyote susceptibility to *Borrelia* infection is similar to that of dogs. We collected livers and kidneys of eastern coyotes from licensed harvesters in Nova Scotia, Canada, and tested them using nested PCR for the presence of *B. burgdorferi*, *B. miyamotoi*, and *Dirofilaria immitis*. Blood obtained from coyote livers was also tested serologically for antibodies to *B. burgdorferi*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, and *D. immitis*. *Borrelia burgdorferi* and *D. immitis* were detected by both nested PCR and serology tests. Seroreactivity to *A. phagocytophilum* was also found. *Borrelia miyamotoi* and *E. canis* were not detected. Our results show that coyotes in Nova Scotia have been exposed to a number of vectorborne pathogens.

Key words: *Anaplasma*, *Borrelia*, Canada, coyote, *Dirofilaria immitis*, *Ehrlichia*, ticks.

Eastern coyotes (*Canis latrans*) are one of the dominant predators in Nova Scotia, Canada, since extirpation of breeding populations of wolves (*Canis lupus*). The closest evolutionary relatives to coyotes within the province are domestic dogs (*Canis lupus familiaris*; Gompper 2002). As with dogs, coyotes are at risk for tickborne infection and heartworm (Kazmierczak and Burgess 1989; Sacks et al. 2002). Populations of black-legged ticks, *Ixodes scapularis*, a vector for *Borrelia burgdorferi*, *Borrelia miyamotoi*, *Ehrlichia* spp., and *Anaplasma* spp., are

increasing in Nova Scotia (Gasmi et al. 2017). *Dirofilaria immitis* is a canine heartworm that requires a mammalian host and is transmitted by mosquitos (Lee et al. 2010). A serosurvey and direct testing of coyotes using a nested PCR allowed for a province-wide analysis of the prevalence and distribution of infection or exposure to these pathogens in wild coyotes in Nova Scotia.

Lyme borreliosis and other tickborne illnesses have been increasing in humans and companion animals throughout Canada, because increasing temperatures cause habitat to become more suitable for vectors (Lieske and Lloyd 2018). Nova Scotia is the Canadian province with the highest infections of *B. burgdorferi* per capita in humans and dogs (Gasmi et al. 2017; Littman et al. 2018). Propagation of *Borrelia* between generations of ticks involves a mammalian host (Barbour 2017). Our study used molecular and antibody detection of tickborne pathogens, and molecular and antigen detection of the mosquito-borne *D. immitis* pathogen to determine the regions in which coyotes are exposed to these infections in Nova Scotia, Canada. Coyotes are a suitable study species because coyote and tick habitats overlap, increasing the potential risk of coyotes contracting tickborne illnesses (Patterson and Messier 2001). Coyotes are closely related to dogs, meaning they also mount a robust immune response to *B. burgdorferi*, *Ehrlichia* spp., and *Anaplasma* spp. that can be detected using tests developed for dogs (IDEXX Snap 4Dx, IDEXX Laboratories, Westbrook, Maine, USA; Kazmierczak and Burgess 1989; Sacks et al. 2002).

TABLE 1. Primer sequences used for PCR detection of *Borrelia burgdorferi* and *Dirofilaria immitis*. For the nPCR *Borrelia* reactions, Rrs and Rrl served as outer primers for both *B. burgdorferi* and *B. miyamotoi*. Inner primers were Burg23s for *B. burgdorferi* and Miya23s for *B. miyamotoi*. For *D. immitis*, the D. imm28F and D. imm28SR primers were used in repeated “self-nested” reactions.

Primer name	5'-3' sequence	Annealing temperature (C)	Amplicon (base pairs)	Source
Rrs	GTATGTTTACTGAGGGGGGTG	50	588–1,029	Dibernardo et al. (2014)
Rrl	GGATCATAGCTCAGGTGGTTAG			
Burg23s_In_F	ATGTATTCCATTGTTTAAATTACG	51	340	This work
Burg23s_In_R	GACAAGTATTGTAGCGAGC			
Miya23s_In_F	ATAAACCTGAGGTCGGAGG	60	447	This work
Miya23s_In_R	AAAGTGTGGCTGGATCACC			
D.imm28F	AGTGCGAATTGCAGACGCATTGAG	64	545	Rishniw at al. (2006)
D.imm28SR	AGCGGGTAATCACGACTGAGTTGA			

Coyote carcasses ($n=173$) were submitted to the Nova Scotia Department of Lands and Forestry through the coyote management program, which used voluntary collection of carcasses from January 2017 to January 2018. Approval for the use of necropsy tissues was given by the Animal Care Coordinator of Mount Allison University (protocol no. NEC 2016-01). Liver and kidney samples were collected from coyote carcasses upon routine dissection. Liver and kidney DNA samples were extracted using the AquaGenomic (MultiTarget Pharmaceuticals, Colorado Springs, Colorado, USA) tissue protocol. We used PCR for direct molecular detection of *B. burgdorferi*, *B. miyamotoi*, and *D. immitis*. For *Borrelia*, outer primers designed by Dibernardo et al. (2014) were used with species-specific inner primers (Table 1). Round one amplification was run as described by Dibernardo et al. (2014), and both round two amplifications followed the same program of 5 min at 95 C, 40 cycles of 30 s at 95 C, annealing temperature (Table 1) for 30 s, and 72 C for 30 s followed by 72 C for 10 min. *Dirofilaria immitis* PCR was performed using primers for the 5.8S-ITS2-28S region reported by (Rishniw at al. 2006) and the same thermocycler program as mentioned earlier, except that the annealing time was 45 s, amplification time was 1 min, and annealing temperature was 64 C (Table 1). Double amplification was performed (with the same primers) to produce clear amplicons. All

reactions were run with 12.5 μ L of GoTaq-Green (Promega, Madison, Wisconsin, USA), 8.5 μ L of nuclease-free water, 1 μ L of the forward and reverse primers (Sigma, St. Louis, Missouri, USA), and 1 μ L of either input DNA for round one or 1 μ L of round one PCR product for round two, for a total of 25 μ L. The product was resolved on a 1.2% agarose gel and Sanger sequenced (McGill University, Montreal, Quebec, Canada) for confirmation. An enzyme-linked immunosorbent assay (ELISA; SNAP 4Dx, IDEXX Laboratories, Westbrook, Maine, USA) was run on blood samples to test for *B. burgdorferi*, *A. phagocytophilum*, *E. canis*, and *D. immitis*. Blood was recovered by centrifuging liver samples at $1,000 \times G$ for 1 min. The SNAP tests were used following the manufacturer’s protocol. Of the 173 coyote samples, 134 had enough blood recovered to test by this method.

Of 173 coyotes tested by nested PCR, one kidney sample was sequence-confirmed positive for *B. burgdorferi*, and two kidney samples produced amplicons of a size consistent with the presence of *D. immitis* DNA (presumably from microfilaria trapped in kidney vasculature), but these results must be interpreted with caution because nonspecific amplification of the abundant host DNA prevented sequence confirmation. We did not detect *B. miyamotoi*. Testing of blood samples by ELISA yielded the following positive samples: eight for *B. burgdorferi*, seven for

TABLE 2. Enzyme-linked immunosorbent assay (ELISA) and PCR results for coyote infection prevalence. The pathogens being tested for, the method, and the numbers found positive are shown. The percent positives for PCR were calculated using the total number of coyotes in this study ($n=173$), and ELISA percentages were calculated using the 134 coyotes from which blood could be recovered.

Pathogens	Method	Count (percentage \pm 95% confidence interval)
<i>Borrelia miyamotoi</i>	nPCR	0 (0.0 \pm 0.0)
<i>Borrelia burgdorferi</i>	nPCR	1 (0.6 \pm 0.6)
<i>Borrelia burgdorferi</i>	ELISA	8 (6.0 \pm 2.0)
<i>Anaplasma phagocytophilum</i>	ELISA	7 (5.2 \pm 1.9)
<i>Ehrlichia canis</i>	ELISA	0 (0.0 \pm 0.0)
<i>Dirofilaria immitis</i>	PCR	2 (1.2 \pm 0.8)
<i>Dirofilaria immitis</i>	ELISA	8 (6.0 \pm 2.0)
<i>Borrelia burgdorferi</i> + <i>Anaplasma phagocytophilum</i>	ELISA	3 (2.2 \pm 1.3)
<i>Borrelia burgdorferi</i> + <i>Dirofilaria immitis</i>	ELISA	1 (0.7 \pm 0.7)

A. phagocytophilum, and eight for *D. immitis*, including coinfections (Table 2). Coinfections of *B. burgdorferi* and *A. phagocytophilum* were more frequent than expected by chance (Fisher exact test, $P=0.003$), whereas there was no evidence of either synergies or antagonisms for *B. burgdorferi* and *D. immitis* ($P=0.300$) or for *D. immitis* and *A. phagocy-*

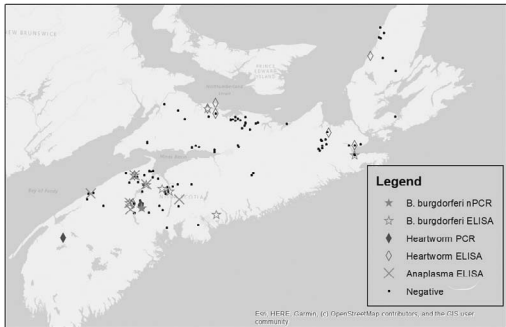


FIGURE 1. Location and test status of coyotes ($n=173$) in Nova Scotia, Canada. The test result of each coyote is superimposed on a map of Nova Scotia. Coyotes negative for all infections are shown as small squares. Coyotes positive for *Borrelia burgdorferi* by PCR or enzyme-linked immunosorbent assay (ELISA) are shown as stars (filled or unfilled, respectively). Coyotes positive for *Dirofilaria immitis* by PCR or ELISA are shown as diamonds (filled or unfilled, respectively). Coyotes positive for *Anaplasma phagocytophilum* by ELISA are shown as crosses. Coinfections are indicated by two symbols directly overlapping. Where multiple coyotes were collected from the same location, the points can represent multiple individuals.

tophilum ($P=0.709$). The coyote found positive for *B. burgdorferi* by nested PCR was ELISA-negative, giving a total of nine *B. burgdorferi*-positive coyotes, and the two *D. immitis* PCR-positive coyotes were antigen-negative, giving a possible total of 10 *D. immitis*-positive coyotes.

Our results showed that coyotes in Nova Scotia have been exposed to a number of vectorborne pathogens throughout the province. *Borrelia burgdorferi* and *D. immitis* responses were found across the province (Fig. 1), whereas *A. phagocytophilum* exposure results were clustered in the Annapolis Valley region of Kings, Lunenburg, and Annapolis counties, although this is also the region where most coyotes were collected.

Under the influence of climate change and other factors, ticks and tickborne pathogens are becoming increasingly common in Nova Scotia, as they are throughout Canada (Gasmi et al. 2017). *Borrelia burgdorferi* is the most common tickborne pathogen, and our findings suggest it is widely distributed in coyotes. The physical size and territorial range of coyotes make them ideal hosts to feed and disperse adult ticks, though their potential as reservoirs for *B. burgdorferi* appears to be unstudied (Mather et al. 1994; Patterson and Messier 2001; Levi et al. 2016).

Coinfections with *B. burgdorferi* and *A. phagocytophilum* were not unexpected because they share the same vector and reservoir

species. Finding a significant occurrence of coinfections in coyotes reinforces the risk of coinfecting ticks in Nova Scotia. While it is possible that two separate infected ticks caused exposures at different times, the two bacteria are present in the same geographic regions. *Ehrlichia canis* is most commonly found in domestic dogs. There is experimental evidence for American dog tick or wood tick (*Dermacentor variabilis*), also common in Nova Scotia (Johnson et al. 1998), to act as a vector (Ferrolho et al. 2016). We also recovered abundant, if temporary and localized, brown dog tick (*Rhipicephalus sanguineus sensu lato*). This species is the primary vector (Ferrolho et al. 2016), and abundant ticks have been obtained from both dwellings and outdoor areas, presumably due to importation of the ticks by domestic dogs exposed to endemic regions. With increased coyote presence in peri-urban and rural areas, contact between these imported ticks and coyotes is possible but less likely than exposure to *I. scapularis* ticks; in conjunction with the low occurrence of *E. canis* in domestic dogs across Canada (Villeneuve et al. 2011), it is unsurprising that coyotes were not exposed. Unlike the other pathogens, *B. miyamotoi* could only be assessed by nested PCR, and positives were not found. The PCR is generally less sensitive than serological detection, and *B. miyamotoi* is also less common than *B. burgdorferi* and has a lower prevalence within reservoir hosts (Barbour et al. 2009) and in ticks in Canada (Dibernardo et al. 2014).

An important caveat with our results is that, of necessity, blood was collected by (gentle) centrifugation of previously frozen tissues. This is not the optimal process for collection of sera. Hemolysis and the contents of other lysed cells might have affected the detection of antibodies or antigens, and hence, the specificity of the serological tests. The PCR test should not be affected by the use of previously frozen tissue, but its sensitivity is compromised by the vast excess of host DNA, so false negatives are possible, and this may well be represented by the animals with amplicons of appropriate size for both *B.*

burgdorferi and *D. immitis* that were not able to be confirmed by sequencing. While the negative results from the negative (no DNA) controls and most animals, as well as repeatable amplification from positive animals, suggested that false positives were rare, false positives from laboratory contamination cannot be excluded. Antibody-positive results indicated that coyotes were exposed to the pathogen, but not necessarily actively infected. The discrepancy between direct and antibody detection of *B. burgdorferi* and *D. immitis* is not unexpected. The presence of the bacteria does not mean there will be a detectable antibody or antigen level, as was seen in the PCR-positive coyotes. Conversely, antibodies can persist after an infection, and the limitation in the sensitivity of PCR tests in samples with an excess of host DNA and the possibility that the bacterial infection load is higher in one organ than another can also lead to a failure to detect infections (Bowman et al. 2009; Stone and Brissette 2017).

Exposure to *D. immitis* is uncommon in companion animals in Nova Scotia (Villeneuve et al. 2011). The presence of *D. immitis* antigens and the possibility of *D. immitis* DNA in coyotes distributed across Nova Scotia suggest that there is heartworm in the province, possibly due to introduction by infected domestic dogs. Priest et al. (2018) reported French heartworm (*Angiostrongylus vasorum*) in coyotes from Nova Scotia, but flushing of over 250 samples of coyote hearts and lungs from 2016 to 2019 did not recover any adult *D. immitis* specimens. Morphological detection of adult heartworms would be required to unambiguously document the presence of *D. immitis* in coyotes in Nova Scotia; however, if confirmed, our report would constitute the northernmost record for *D. immitis* from coyotes. Canids provide a reservoir for *D. immitis*, from which it can then infect local mosquito populations (Brown et al. 2012). As the climate change continues to favor increased mosquito population growth and expansion of their northern range, the risk for *D. immitis* to spread through and between wild and domestic canids will likely increase.

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