

Prevalence of the Zoonotic Diseases

Leptospirosis and Borreliosis in the Maritimes of Canada

By

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To everyone who encouraged me along the way.

Abstract

Leptospirosis and Lyme borreliosis are two globally important infectious diseases caused by the transmission of either the *Leptospira* spirochaete or the *Borrelia* spirochaete, respectively. Although the spirochaetes have similarities in their shape, size, and movement, their transmission to animals is quite different. Infection of pathogenic leptospires requires environmental exposure to infected water or soil. The main route of exposure is through mucous membranes, such as drinking from infected water sources. Once infected, some of the leptospires remain in the host's body and replicate, whereas others pass through the renal tubules to be shed back into the environment through the individual's urine, continuing the infectious cycle. *Borrelia* infection, on the other hand, is a tick-borne disease in which an individual becomes infected through the bite of an infected tick.

The risk of contracting leptospirosis in Canada has been increasing over the last 30 years, with New Brunswick reporting an increase in infected dogs, an important sentinel species for the infection, with fatal outcomes. To determine the prevalence of *Leptospira* spp. in the province, multiple methodologies and areas of interest were examined. Three methodologies (centrifugation, filtration, and growth in media) were attempted to detect leptospires in stagnant water sources around areas of recent flooding events. Unfortunately, all three methods returned negative results, although it is unclear if they were unsuccessful in finding the leptospires or if the leptospires were not present in these samples. Molecular testing of local wildlife necropsies, including suspected maintenance hosts as well as accidental hosts, and ticks was carried out. Testing small mammals showed 3.2% specimens were positive for *Leptospira borgpetersenii*. Testing large mammals showed 1.8% positive for *L. borgpetersenii*. Infected animals included moose (*Alces alces*), snowshoe hare (*Lepus americanus*), meadow vole (*Microtus pennsylvanicus*), jumping mouse (*Napaeozapus insignis*), deer mouse (*Peromyscus maniculatus*), maritime shrew (*Sorex maritimensis*), and black bear (*Ursus americanus*). Testing of ticks (*Dermacentor variabilis*, *Ixodes cookei*, and *Ixodes scapularis*) showed 3.6% were positive

for *L. borgpetersenii*. Finally, dog serum was collected from 14 veterinary hospitals across the province with 19.3% testing positive for the presence of antibodies against *Leptospira* spp. Together, these results elucidate a widespread rate of infection in the wildlife species, sentinel species, and tick species of New Brunswick.

The risk of contracting Lyme borreliosis in New Brunswick has also been increasing over the last few years due to expanding tick populations. The risk of transmission of *Borrelia* species, such as *B. bissettiae*, through the bite of an infected tick within New Brunswick began with the identification of the bacteria in local tick species, yet transmission of *B. bissettiae* to local wildlife species was still unknown. Of the small wildlife species tested in this study, one meadow vole (*M. pennsylvanicus*) and one deer mouse (*P. maniculatus*) were sequence positive for *B. bissettiae*. Of the large mammals tested, none were positive for the presence of *B. bissettiae*. This evidence suggests that the bacteria has established maintenance hosts, but evidence for the ability to infect accidental hosts has not yet been found in New Brunswick.

Understanding the risk of infection by *Borrelia* spp. of our local wildlife species becomes directly relevant to human health, as it can cause Lyme disease in humans. In its disseminated form, it can affect organs such as the heart, causing what is known as Lyme carditis, a rare but potentially fatal outcome when diagnoses are missed. This thesis presents a case of fatal severe heart disease following suspected untreated Lyme carditis of a 17-year-old female from Nova Scotia, Canada. Formalin fixed paraffin-embedded sections of the heart, lung, kidney, adrenals, and pancreas showed florescent structures through immunohistochemistry staining in similar size and shape of *Borrelia* bacteria. Due to the nature of these samples, however, these findings could not be confirmed through polymerase chain reaction followed by Sanger sequencing and therefore cannot be confirmed as *Borrelia*.

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Chapter 1

Zoonotic Diseases

Zoonotic Diseases

Of all emerging infectious diseases, approximately 70 % of them originate from wildlife, feral, captive, or farmed animals (Haider *et al*, 2020). These globally emerging infectious diseases are caused by the transmission of a pathogen from an animal to human; known as a zoonotic disease (Jones *et al*, 2008; Rahman *et al*, 2020). Vector-borne and zoonotic diseases have caused immense harm to human health throughout history as they invade novel regions and continue to re-emerge globally. Of all known infectious diseases, approximately 17 % are vector-borne, meaning pathogens, whether parasites, bacteria, or viruses, are transmitted to humans by arthropods, such as mosquitoes, flies, or ticks. Such diseases, like malaria, dengue, and tick-borne encephalitis, cause over 700,000 deaths annually (WHO, 2020 a).

Infectious diseases can spread rapidly around the globe with little regard to geographic barriers. This is primarily due to the fact that these diseases, when new to humans, have recently evolved or have moved into novel areas, resulting in delayed diagnoses, and delayed treatment, allowing the disease to spread rapidly (Rahman *et al*, 2020; WHO, 2020 b). There are many specific factors that increase the spread of these infectious diseases, but all these factors are united by the concept of change. Change in human demographics such as increased density of human populations, change in human behaviour such as suburban living, change in location such as through increases in international travel and the movement of goods, changes in global technology and increased industry needs, microbial evolution and adaptation, ecological changes due to human interference in agriculture, water ecosystems, deforestation, and the reduction of public health interactions, among other factors, can all increase risk of emerging diseases (Institute of Medicine, 1992; Morse, 1995; Morse and Schluederberg, 1990). Climate change, in particular, has allowed the migration of pathogen vectors and their hosts into new

areas, resulting in the geographic expansion of zoonotic diseases. As climate changes, temperatures are prolonged, heavy rainfall and flooding events increase in frequency, among other factors (Vincent *et al*, 2020). As these changes occur, many animals and accompanied parasites and arthropods, as well as many vectors including ticks and mosquitoes, are forced into new habitats as they migrate to escape environments that no longer accommodate them (Rushing *et al*, 2020; Platts *et al*, 2019; Alto and Juliano, 2001; Lieske and Lloyd, 2018). This process introduces parasites, bacteria, and viruses into novel areas, pushing many zoonotic diseases into new areas (Alto and Juliano, 2001). In addition, range expansion is accompanied by an increase in human interaction with animals (Jones *et al*, 2008). Understanding how these changes will affect future movement of humans and wildlife, along with increased interactions between host and pathogen, are important considerations to determine the potential threat of future outbreaks of severe diseases, such as Borreliosis and Leptospirosis (Jones *et al*, 2008; Bartlow *et al*, 2019).

Spirocheates

The spirochaetales is a diverse group of spiral-shaped pathogenic bacteria that includes species that are free-living and those that are obligate parasites, and all variations between. The order is split into the *Spirochaeta*, *Cristispira*, *Treponema*, *Borrelia* and *Leptospira* genera based on morphology and physiology (Haake, 2009; Holt, 1978). Pathogenic spirochaetes are flagellated bacteria that can easily move through the many barriers' of the mammalian or arthropod body, such as skin, blood-brain barrier, gut epithelia, and circulatory epithelia (Wolgemuth, 2015; Radolf *et al*, 2012). The spirochaete rotates its flagella, causing a series of ripples and allows the body to undulate, which propels the bacteria forward, or backwards, depending on the direction of flagellar rotation (Wolgemuth, 2015; Chang *et al*, 2021). The causative agents of the globally important zoonotic diseases, Borreliosis and Leptospirosis, are the *Borrelia burgdorferi sensu lato* and *Leptospiraceae* spirochaetes; both lead the

spirocheatales in causing human disease (Wolgemuth, 2015; Haake, 2009; Gil *et al*, 2005; Thibeaux *et al*, 2018).

Leptospirosis

Causative agent

The *Leptospira* genus comprises 64 species, including pathogenic, saprophytic, and intermediate categories. Each complex and diverse species have been further sub-divided based on the individuals' arrangement of its major agglutinogens and placed into serovars groups, in which there have been over 200 pathogenic serovars recorded to date (Reagen & Skyes, 2019; Wynwood *et al*, 2014; Costa *et al*, 2015). The cells of the *Leptospira* species, called leptospire, are typically 6-20 μm long and 0.1 μm in diameter, usually with one or both ends hooked. The morphology of leptospire differs from other spirochaetes; their ends switch between a hook-shaped end and a spiral-shaped end (Wolgemuth, 2015).

Transmission & Symptoms

Infection in mammals requires environmental exposure to the pathogenic leptospire, commonly encountered in infected water or soil and introduced through small cuts, abrasions, or through mucous membranes (Wynwood *et al*, 2014). The bacteria invade the vascular system of the host and eventually encounter the kidneys. Some leptospire pass through the renal tubules and are shed back into the environment through the urine of the infected individual (Temeiam *et al*, 2020; Costa *et al*, 2015), while others persist in the body and replicate there.

Symptoms of Leptospirosis were first described by Landouzy (1883), who determined the disease common among sewage workers in 1883 (Landouzy, 1883 as reviewed in Tarantola & Goarant, 2018). The disease was later named Weil's syndrome, after Adolph Weil in 1886, who likened the symptoms to an "abortive form of typhus" (Weil, 1886 as reviewed in Tarantola & Goarant, 2018). Many names for what we now recognize as leptospirosis were used in reports from Europe and the United States; each

was believed to represent a different disease, complicating discovery of the etiological agent (Edwards & Domm, 1960). By 1915, the causative agent of the disease was discovered by Inada, Ido, Kaneko, Hoki, and Ito (1916), which led to the discovery of the source of infection and its pathology (Inada *et al*, 1916 as reviewed in Kobayashi, 2001). The first recorded case of Leptospirosis in humans in the United States was in 1922 but awareness of the disease took time, and it wasn't until 1941 when an extensive review on the disease was published and recognition of the signs and symptoms increased (Edwards & Domm, 1960). Eventually, the correlation between the prevalence of infection among labourers and those who engaged in recreational outdoor swimming with their exposure to contaminated water by the urine of an infected host or carrier, was made (Edwards & Domm, 1960; Buchanan, 1927; Alston, 1935). With this understanding, human leptospirosis cases worldwide are estimated to be 1.03 million annually (Costa *et al*, 2015).

Leptospirosis is biphasic in humans. The time between exposure and presentation of symptoms ranges from 3 days to 4 weeks (Morgan *et al*, 2002). Beginning abruptly, the first phase of the disease, referred to as the acute or septicaemic phase, generally presents with fever, chills, headache, muscle aches, vomiting and/or diarrhea, although symptoms vary between individuals (Picardeau, 2013; Haake & Levett, 2015). The second phase, referred to as the immune phase, begins roughly during the second week post infection, during which the host's humoral immune system develops detectable antibodies targeting the lipopolysaccharide of the spirochaete while the bacterial load within the bloodstream diminishes (Alia *et al*, 2019; Silveira *et al*, 2017; Guerriero *et al*, 2001; Picardeau, 2013; Levett, 2001). This phase is considered more severe, as the infected individual can suffer from kidney or liver damage or failure, meningitis, and respiratory distress (Plank & Dean, 2000).

In animals, clinical signs of leptospirosis may not always be apparent and similarly to humans, symptomatic cases can range from mild to severe (Bunnell *et al*, 2000). Generally, male dogs between four and ten years old are more commonly affected, and those which herd and hunt are at a higher risk

of infection due to increased contact with water infected by wildlife (van de Maele *et al*, 2008; Stokes & Forrester, 2004; Ward *et al*, 2002). In dogs, the clinical signs of leptospirosis vary and are nonspecific. The first stage, known as the invasive or peracute stage, happens shortly after infection, usually 5 to 15 days later, and can either be accompanied by lethargy and anorexia or result in sudden death with no noticeable symptoms (Byrne, 1955; Langston & Heuter, 2003). The second stage, known as the primary renal or subacute stage, follows from one to three weeks post infection. This is generally when illness is clinically detected, as symptoms become more noticeable, including fever, vomiting, abdominal pain, diarrhea, refusal to eat, severe weakness and depression, stiffness, severe muscle pain, weight loss, fluid build-up in the abdomen, jaundice, a decline in brain cell function due to severe liver disease, labored breathing, cough, acute respiratory distress, hemorrhage, congestion, edema, neutrophilic and lymphocytic infiltration, bronchopneumonia, and others (Byrne, 1955; van de Maele *et al*, 2008; Langston & Heuter, 2003). These problems arise as the bacteria enter and impair the kidney tubules. The third stage, known as the secondary renal stage, is characterized by chronic interstitial nephritis (Byrne, 1955). The main fatal outcomes for dogs with leptospirosis stem from acute hepatic failure or severe renal disease (White *et al*, 2017). Cats can also become infected by the bacteria yet appear less susceptible and their immune response is able to rapidly clear the infection in most cases, with outdoor cats at the highest risk of infection due to their predatory nature and hunting rodents (van de Maele *et al*, 2008; Watson, 1994; Hartmann *et al*, 2013). The majority of feline cases present with renal and hepatic inflammation, although there are few reported cases of cats presenting with symptoms similar to the canine subacute phase (Beaudu-Lange & Lange, 2014; Hartmann *et al*, 2013). Importantly, with or without symptoms, an infected animal is still able to transmit the bacteria, infecting other animals as well as owners and veterinary professionals (van de Maele *et al*, 2008; Hartmann *et al*, 2013; Watson, 1994).

Animal Reservoirs

An animal can only become a reservoir for *Leptospira* if the kidney supports colonization of the bacteria, otherwise, the animal clears the bacteria without shedding leptospire into the environment, ending the bacterial infection and clonal lifecycle. The main wildlife carriers of the pathogenic leptospire are rodents, marsupials, and chiropterans, with mice being the main asymptomatic carriers (Bunnell *et al*, 2000; Boey *et al*, 2019). Testing the renal carriage of 739 rats found an infection prevalence of 52.9% in *Rattus norvegicus*, although only 4.4% in *Rattus rattus* (Costa *et al*, 2015; Biscornet *et al*, 2017).

Numerous host species ranging across several orders demonstrates the global trend of mass species infection. A meta-analysis of 300 publications documented that *Leptospira* was common in “all eight orders and 21 families of the class Mammalia” (Andersen-Ranberg *et al*, 2016). Additional reservoirs are still being discovered; another recent meta-analysis of 34 publications documented isolation of *Leptospira* from species not previously recognized as reservoirs (Cilia *et al*, 2021). These species included carnivora and rodentia but also Cetacea, including a southern right whale and a common bottlenose dolphin, as well as Reptilia, including a Prado’s lancehead snake and a hognose snake, and Amphibia, including marine toads and whistling frogs (Cilia *et al*, 2021). In Canada, Shearer *et al* (2014) sampled 460 kidneys from various wildlife species, including beavers, coyotes, deer, foxes, opossums, otters, racoons, and skunks. No deer, otter, or coyote were found infected; however, infection was identified in skunks, racoons, beavers, foxes, and opossums with the highest prevalence of infection in skunks and racoons, at 42% and 33% infection prevalence, respectively. Of note, many infected species were those which are tolerant of human populations and reside in or close to areas with high human populations (Shearer *et al*, 2014).

This proximity of infected wildlife and human populations raises the risk of transmission to both companion animals and to humans and in many cases, leptospire can be transmitted to humans by

pets. Transmission can occur by contact, for example through licking or bed sharing. With 14-62% of humans reportedly sharing a bed with their pets, the potential for transmission from pet to human is high (Chomel & Sun, 2011; Rahman *et al*, 2020). Less intimate contact can also allow infection. A study on infection prevalence of dogs and their handlers in Malaysia revealed 26.3% of dogs and 34.5% of dog handlers sampled from working dog organizations and dog shelters were seropositive for *Leptospira*. While seropositivity in the dogs might be confounded by vaccination, the human cases represent exposure or active infection as vaccination of humans is rare (Goh *et al*, 2020). The risk of pet to human transmission is not restricted to dogs; cats can be infected and could also transmit infection to owners. Recently, 10% of the feral cat populations in Prince Edward Island, Canada tested positive for antibodies against at least one type of *Leptospira* serovar, suggesting previous or current infection (Bourassi *et al*, 2021). Our increasing understanding of *Leptospira* host species shows that many animals, wild or domestic, can potentially transmit infection to humans.

Water

The ability of leptospires to survive months in fresh water sources and weeks in stagnant sources, such as puddles and wet soil, increases its ability to infect animals and so increases the risk posed to animal and human health (Wynwood *et al*, 2014). Survival of leptospires depends on the viscosity and salt concentrations of the environment; *Leptospira* survived in distilled water for 110 days, while more viscous fresh water increased the survival to 347 days (Trueba *et al*, 2004). Further, pathogenic strains have been isolated from large bodies of water, such as rivers and lakes (Wynwood *et al*, 2014).

The majority of both urban and rural communities in North America collect drinking water from rivers, streams, or underground aquifers, which is then stored in reservoirs (Environmental Protection Agency, 2008; Federal-Provincial-Territorial Committee on Drinking Water, 2002; Kot *et al*, 2011). Hospital data from the United States has shown that over 70% of patients admitted for leptospirosis

infections were infected by contact with contaminated water sources (Wynwood *et al*, 2014). As there are no current validated protocols for testing leptospirosis in water samples, contamination in drinking water has not been documented in Canada (Wynwood *et al*, 2014). However, outbreaks of infectious diseases such as *Giardia*, *Campylobacter*, *Cryptosporidium*, Norwalk-like viruses, *Salmonella*, and hepatitis A virus in Canada have been documented (Schuster *et al*, 2005; Wilson *et al*, 2009). Severe weather, proximity to wildlife, malfunction or poor maintenance of treatment systems were associated with the outbreaks (Schuster *et al*, 2005; Wilson *et al*, 2009). While water treatments, such as chlorine treatment, reduces the probability of leptospirosis infection, these systems need to be constantly monitored and water distribution networks well maintained. Smaller systems (serving up to 5000 people) that service 80% of communities in Canada are at a higher risk of chemical- or bacterial-based water advisories due to aging infrastructure and limited budgets, particularly in small Indigenous communities where extended drinking water advisories can be in place for months to years (Health Canada, 2013; Smith *et al*, 2006; Galway, 2016; McLeod *et al*, 2020). In addition to drinking water contamination, direct connections have been seen between leptospirosis outbreaks and water exposure in environmental disasters such as flooding. As well, recreational use of water and occupational exposure to bodies of water can increase risk (Morgan *et al*, 2002; Haake & Levett, 2015; CDC, 1997; Brown & Murray, 2013).

Diagnostics

There are several laboratory tests that can either directly or indirectly detect leptospires in a sample. Direct detection is of leptospires themselves, indirect involves detecting the prior presence of leptospires via host antibodies (Temeiam *et al*, 2020). Each approach has its own advantages and limitations. Direct diagnostics approaches include culturing and PCR-based detection. Indirect or serological tests include microagglutination test (MAT) and ELISA based tests (Alia *et al*, 2019; Temeiam *et al*, 2020; Prescott, 2008).

Indirect Detection

The limitations of direct diagnostic techniques mean that confirmation of clinical diagnosis generally relies on serology; testing sera for antibodies. This approach is rapid and relatively inexpensive, and most healthcare systems are well provisioned to support this methodology. However, serological tests rely on host-produced antibodies that take at least a week to develop; the tests are not sensitive or specific when performed in the early stage of infection, with under 50% of cases being correctly diagnosed (Limmathurotsakul *et al*, 2012; Smythe *et al*, 2009).

Serological testing includes microscopic agglutination test (MAT), in which multiple dilutions of serum from a suspected infected individual is incubated with a suspension of locally relevant serovars of live antigens. Antibody titre, which determines the presence and level of antibodies within the blood, is then calculated under a dark-field microscope through the confirmation of serovar-specific agglutination (Coico *et al*, 2005; Musso & Scola, 2013; Chirathaworn *et al*, 2014). As the degree of agglutination is determined by eye, there is variability not only from person-to-person, but between laboratories as well. Although research is being conducted on machine learning models to automatize the MAT procedure in confirming positive or negative results of MAT images (Temeiam *et al*, 2020; Alia *et al*, 2019; Oyamada *et al*, 2021). Other difficulties associated with the MAT include the availability, preparation, and maintenance of a complete panel of live leptospire; an incomplete panel can result in false negative results. The timing of when testing is performed, cross-reactivity with multiple serovars, and the inability to distinguish between an active and past infection are also significant challenges with this approach (Coico *et al*, 2005; Musso & Scola, 2013).

Serological analysis by enzyme-linked immunosorbent assay (ELISA) tests were developed to either replace or aid the laborious and time-consuming MAT, ideally while improving the sensitivity and specificity of these diagnostic tests. ELISA testing uses specific antigens, to which antibodies in the infected serum bind, followed by visualization through colour change or fluorescence, which occurs

once a labelled and specific antibody is added to the sample; the colour or fluorescence is detected by instrumentation rather than an individual which reduces variability and supports higher throughput testing (Fenner *et al*, 1987). Specificity and sensitivity of ELISA tests in comparison to MAT, report between 64 to 100% specificity and 90 to 100% sensitivity (Hartleben *et al*, 2013; de Souza *et al*, 2014; Bomfim *et al*, 2005; Bourhy *et al*, 2013; Penna *et al*, 2017; Rosa *et al*, 2017; Niloofa *et al*, 2015). The main disadvantage of ELISAs is the need for costly reagents and a higher possibility of false positives (Sakamoto *et al*, 2018).

Due to the overlapping limitations of each of these tests, it is not uncommon for multiple tests of the same or different kind performed to decrease the risk of false positive or negative results. However, poorer countries do not always have access to the materials or equipment needed to conduct said experiments (Temeiam *et al*, 2020).

Direct Detection

Culturing is not typically used in a clinical setting due to the slow growth of the leptospirae; as with most spirochaetes, it can take weeks to months for the bacteria to multiply enough for detection (Alia *et al*, 2019). In one study where patient blood was tested for leptospirae through culturing, the longest duration for a single sample to show presence of leptospirae was 109 days (Chideroli *et al*, 2017). This approach also entails biosafety concerns for researchers, although the risk is low if mitigated using a class II biosafety cabinet (BSC) to provide protection from potential aerosols, personal protective equipment, and disinfecting work areas with 70% ethanol (Arduino *et al*, 2020, Burnett *et al*, 2009).

PCR-based detection also directly detects the bacteria; it is typically able to provide rapid confirmation of infection but can be limited by the need for somewhat concentrated target samples and the need for specialized molecular equipment and expertise. Further, if the serovar being tested is novel, new protocols with newly designed and validated primers may need to be developed to assess sensitivity and specificity. For example, two highly sensitive primers were developed for the conserved

16SrRNA and LipL32 gene. However, Merien *et al* (1992) found that although 16SrRNA gene target allowed sensitive detection of leptospires, the protocol did not differentiate between pathogenic and non-pathogenic. In contrast, the LipL32 is a major lipoprotein found on the outer membrane protein of only the pathogenic species and expressed during mammalian infection, but not found on the non-pathogenic species, making this gene a more specific target for detecting pathogenic species (Haake *et al*, 2000; Guerreiro *et al*, 2001). These technical complexities, as well as the cost and safety of both direct detection methods, are obstacles to the clinical use, however, both approaches are invaluable in research (Alia *et al*, 2019; Temeiam *et al*, 2020).

Environmental Detection

As difficult as it is to detect leptospires in a host, finding the bacteria in the environment is even more challenging. Leptospirosis is usually transmitted through water; however, finding leptospires in water is extremely difficult due to their low concentration in water. There is also the problem of contamination of water and culture samples from other bacteria that are normally present in the water sources (Wynwood *et al*, 2014). There are a few approaches used to isolate leptospires from water: isolation from unprocessed water (eDNA approaches), enrichment by physical processes, and enrichment by culture. Optimized DNA extraction is a method that quantifies the leptospires in environmental waters through a specific process that allows for concentration of the samples to increase the amount of potential DNA to then be extracted. This method is suitable for the detection of leptospires in the environment, but there is no way in differentiating viable from non-viable cells (Riediger *et al*, 2016; Muñoz-Zanzi *et al*, 2014). One approach, prior to DNA extraction to reduce contamination by other bacteria naturally occurring in the water body is differential filtration. Using centrifugation or filtration to isolate leptospires requires a large amount of water to ensure that enough bacterial cells are isolated for successful DNA extraction and analysis (Wynwood *et al*, 2014). This can create further problems if trying to concentrate the samples through filtration, especially when samples

are collected from highly sedimented sources. When attempting to culture leptospires, it is important to remove as many possible contaminants by filtration, as many other bacteria can out-grow the slow-growing leptospires (Goarant *et al*, 2020).

Prevention and Treatment

The best treatment approach is preventative. Vaccines have been approved for dogs and agricultural animals such as cattle, swine, and sheep (Waldner *et al*, 2019; Wilson-Welder *et al*, 2020; Hodges *et al*, 1985). Vaccines are sero-specific, meaning there are multiple vaccines required for full protection against leptospirosis, as a single dose may only protect against one to four serotypes (Ward, 2002). As a result, vaccine for the specific serovars considered the main threat for that species is used. For example, North American cattle are the maintenance host for the hardjo serovar of *Leptospira borgpetersenii*, so the bovine vaccine uses inactivated whole-cell vaccine containing hardjo, canicola, Pomona, and icterohaemorrhagiae serovars (Naiman *et al*, 2001; Giles *et al*, 1983; Hanson *et al*, 1972; Faine *et al*, 1999). Similarly, a '4-way' vaccine has been offered to dogs in Canada since 2001 that covers the *Leptospira interrogans* serovars canicola, icterohaemorrhagiae, and pomona, and *Leptospira kirschneri* serovar grippotyphosa (Prescott, 2008; LaFleur *et al*, 2016). Due to these vaccines, the serovar canicola has largely been eradicated from dogs in North America (Prescott, 2008).

If infection does occur, humans, companion animals, or agricultural animals may receive treatment with antibiotics, which is recommended to start as quickly as possible after diagnosis (Haake & Levett, 2015). Prophylactic use for travellers at risk or those with occupational risk also occurs (Sejvar *et al* 2003; Takafuji *et al* 1984; Pappas & Cascio 2006). In some cases, people can appear to recover from the infection, but if treatment is inadequate, relapse can occur. Evidence of relapse post-treatment in dogs, however, is lacking (Spichler *et al*, 2011; Goris *et al*, 2013; Nicolescu & Andreescu, 1984; Sykes *et al*, 2011).

Risk in New Brunswick

The risk of contracting leptospirosis in Canada has been increasing over the last 30 years. Prescott (2008) reported positive canine tests rose ten-fold and 30-fold in Quebec and Ontario, respectively, between 1998 and 2006. In the Maritimes, there has also been an increase in reports of infected dogs, an important sentinel species for the infection. Sadly, these cases have usually resulted in fatality. These cases may correlate with record high monthly rainfall events, events that have been increasingly affecting the Maritimes; the two historic flooding events occurred in subsequent years of the Saint John River (Mallet *et al*, 2018; Rokaya *et al*, 2018). Another risk factor arises from much of the province being densely forested or covered in grasslands. Based on Statistics Canada data, of the total 7.1 million hectares of land that make up the province, approximately 6.1 million hectares is currently treed. Three hundred and thirty-eight thousand hectares are reserved as parks and protected areas, and another 338 thousand hectares of land is dedicated to farming. These areas are natural landscapes that are rodent friendly. Collectively, this allows direct and environmental interactions between humans and wildlife or agricultural animals. Although this is concerning for transmission of leptospires, they are not the only spirochaete we need to monitor in the province.

Borreliosis

Causative agent

Borreliosis is a zoonotic tick-borne disease caused by spirochaetes of the *Borrelia* genus that has been recorded worldwide (Southern & Sanford, 1969). These unique spirochetes are large (12-22 µm long, 0.2-0.6 µm thick), loosely coiled in shape, and have a distinctive way of moving. Individual cells have been observed alternating between translocating, wriggling, and lunging while occupying the dermis of a mouse (Cutler *et al*, 1997; Paster, 2001; Harman *et al*, 2012; Nakamura, 2020). These movements, specifically the wriggling and lunging, are thought to aid the bacteria in evading the host immune system (Harman *et al*, 2012). The main difference between the movements of the *Borrelia*

spirochaete to the *Leptospira* spirochaete is that rotation of the periplasmic flagella provides a thrust during swimming in *Borrelia*, whereas in *Leptospira*, there are three rotating parts that need to communicate in order to swim (Harman *et al*, 2012; Nakamura, 2020).

The bacteria is named after the researcher, Wilhelm Burgdorfer, who isolated *Borrelia burgdorferi* sensu stricto (s.s.) from the midgut of the *Ixodes dammini* tick, a junior synonym for the *Ixodes scapularis* tick. *B. burgdorferi* was the first spirochaete in the United States connected to the novel disease affecting children in Lyme, Connecticut, USA (Burgdorfer *et al*, 1982). *I. dammini* were suspected to transmit the disease in the Northeastern and Midwestern States of America, whereas the *I. pacificus* was deemed responsible in the western states (Wallis *et al* 1978; Steere *et al* 1978; Burgdorfer *et al* 1982). For some time after its original discovery, the spirochaete *B. burgdorferi* was divided into two categories: *B. burgdorferi* s.s. and *B. burgdorferi* sensu lato (s.l.). Now, the *Borrelia* genus is split into three groups: the lesser known “reptile group” (REP) or Echidna-Reptile group (REPG), relapsing fever (RF) group, and Lyme borreliosis (LB) group, also known as *B. burgdorferi* s.l. (Binetruy *et al*, 2020). To date, it is believed that the reptile group do not cause infection in humans and has only been reported to infect amphibians and reptiles (Trevisan *et al*, 2021; Colunga-Salas *et al*, 2020; Kurtenbach *et al*, 2006). The RF group, split into the tick-borne relapsing fever and the louse-borne relapsing fever groups, based on transmission, is caused by a *Borrelia* spirochaete that causes fever and disease relapse in those affected (Barbour, 2005; Southern & Sanford, 1969). The tick-borne group is mainly vectored by soft-bodied ticks, like the *Ornithodoros* ticks, although some genera of hard ticks, such as *Ixodes*, *Amblyomma*, and *Rhipicephalus*, also vector some of these RF *Borrelia* species (Nunes *et al*, 2016; Talagrand-Reboul *et al*, 2018; Colunga-Salas *et al*, 2020). LB, also known as Lyme disease (LD), is caused by a cluster of related spirochaete species of the *Borrelia* genus and are split into three genomic groups: *B. burgdorferi* s.s., *Borrelia afzelii*, and *Borrelia garinii* (Dressler *et al*, 1994). The term *B. burgdorferi* s.s. defines a single species, *B. burgdorferi*, that can cause LB, whereas *B. burgdorferi* s.l. comprises a

complex group that contains at least 20 genospecies globally, with ten of those present in North America: *B. burgdorferi* s.s., *B. americana*, *B. andersoni*, *B. bissettae*, *B. californiensis*, *B. carolinensis*, *B. garinii*, *B. kurtenbachii*, *B. laneii*, and *B. mayonii* (Scott *et al*, 2016; Golovchenko *et al*, 2016; Rose *et al*, 2019). New species and variants were continuously recognized with seven genospecies being classified as infectious to humans (Rudenko *et al*, 2011). For the purpose of the research conducted, the remainder of the thesis will focus on the LB group.

Transmission and Hosts

The impact of ticks as vectors is second only to mosquitoes. This is due to their global presence and the diversity of pathogens they transmit (Dennis & Piesman, 2005; Minigan, 2018; Wallis *et al*, 1978; Steere *et al*, 1979). When a tick feeds on an infected host, the *Borrelia* spirochaetes respond to chemoattractants in the tick saliva and migrate through the host to the site of the feeding tick, allowing the tick to ingest the bacteria while consuming its blood meal. The spirochaetes are then deposited in the gut of the tick and tether themselves to gut wall proteins, avoiding digestion and exposure to the tick hemolymph, which is the primary tissue of the tick immune system (Kurokawa *et al*, 2020; Wolgemuth, 2015). The midgut, along with the bacteria, are retained through molting, thus the bacteria are transmitted through moults. When the tick starts feeding, the change in temperature and gut pH triggers detachment of the spirochaetes from the midgut, they migrate into the haemolymph and translocate into the salivary glands. The bacteria are then introduced into a new host along with tick saliva and its complex cocktail of anticoagulants and immune suppressants, that provides the bacteria with a good environment for the establishment of infection (Kurokawa *et al*, 2020). The horizontal transmission cycle, host to tick and tick to host, as well as the transstadial transmission cycle, maintaining the pathogen between tick life stages, are essential for the survival and continuation of *Borrelia*. Evidence suggests that vertical or transovarial transmission, the transmission of pathogens from parent to offspring, does not occur for *B. burgdorferi* (although it can for other *Borrelia* species)

meaning that recently hatched larvae are uninfected and need an infected host as a blood meal to become infected (Han *et al*, 2019; Rollend *et al*, 2013).

While ticks are wingless vectors and do not move much independently during their lives, they can be moved both short and long distances by mammals and birds (Margos *et al* 2010; Scott *et al* 2010; Morshed *et al*, 2005). The role of white-tailed deer (*Odocoileus virginianus*) is complex. Deer are considered as “dilution hosts” for *B. burgdorferi* as they do not contribute to the maintenance of the bacteria in the natural environment (Huang *et al*, 2019). The blood of certain ungulates, such as white-tailed deer in North America, and roe deer (*Capreolus capreolus*) and moose (*Alces alces*) in Norway, is borreliacidal, and the animals can clear *Borrelia* infections from themselves and even from infected *I. scapularis* ticks (Kjelland *et al*, 2011; Roome *et al*, 2016). However, deer are a good source of blood meals for ticks so they promote robust tick populations. Tick abundance has been positively correlated to the density of deer in an area (Wilson *et al*, 1990; Medlock *et al*, 2008). Additionally, deer are important for the dispersal of ticks. Birds are considered to be co-vectors for LB spirochaetes as they both transport infected ticks and some species are also important as bacterial reservoirs. *B. burgdorferi* has been found in 71 species of North American birds, and *B. garinii* and *B. valaisiana* have also been reported in several bird species (Brinkerhoff *et al*, 2009; Franke *et al*, 2010; Morshed *et al*, 2005). Birds can migrate long distances, from hundreds to thousands of kilometers, allowing the dispersal of attached ticks along migration routes. For example, the Blackpoll Warbler travels 2270-2770 km non-stop over three days, allowing the relocation of ticks along the migratory route (Reed *et al*, 2003; DeLuca *et al*, 2015). The migratory path of many songbirds overlaps with Lyme endemic areas in the United States, so ticks and their pathogens can be picked up and transported to Canada (Scott *et al*, 2018). The common yellowthroat, Golden-crowned sparrow, song sparrow, and Swainson’s thrush were all found importing infected tick species across Canada during their yearly migration (Scott *et al*, 2010). Not only *Ixodes scapularis* is transported by birds; Morshed *et al* (2005) documented *Ixodes denatus* in

Manitoba and Ontario, *Ixodes muris* in British Columbia, *Ixodes pacificus* from passerine species in Alberta and British Columbia. Further, the ticks themselves may be carrying multiple species of *Borrelia*. Collectively, migratory animals and multi-pathogen bearing ticks allow a wide range of pathogens to move between cities or countries (Hersh *et al*, 2014).

Symptoms

Lyme disease was first recognized as a cluster of unusual, primarily childhood cases and brought to the attention of researchers by a group of concerned mothers. The illness was described as patients experiencing rheumatoid arthritis with 25% reporting a red, circular lesion. Those affected were clustered within three communities in Lyme, Connecticut, with a total of 39 children and 12 adults noticing similar symptoms (Steere *et al*, 1977). In the four decades since its original discovery, the list of symptoms associated with LB has expanded enormously, splitting the disease into three stages: early localized, early disseminated, and late disseminated. One of the first signs within the early localized stage is the emergence of an erythema migrans (EM) rash, although it is important to note that the EM rash is highly variable the rash documented in 18-90% of patients, depending on the study (Sperling & Sperling, 2009; Tibbles & Edlow, 2007; Nigrovic *et al*, 2008; Agüero-Rosenfeld *et al*, 2005; Glaude *et al*, 2015). For many, serological tests are not offered to patients unless they present with an EM rash, yet these skin lesions are often misdiagnosed as eczema, roundworm, or bites from insects such as mosquitoes or spiders (Shapiro, 2014). Further, EM rashes may appear in different forms and patients are often dismissed or misdiagnosed when they present with other cutaneous manifestations (Cameron, 2007). Additional early signs and symptoms can be conjunctivitis, an acute onset of a variety of cardiac symptoms (disturbances in rhythm, myocarditis, or atrioventricular conduction disturbances), arthritis presenting as joint swelling in a few large joints, Borrelial lymphocytoma (painless deep purple nodules), or meningitis and facial palsy, specifically in children (Borchers *et al*, 2015). With or without EM, early stage infections may go unrecognized as many infected are otherwise asymptomatic, although non-

specific symptoms such as fever, malaise, headache, stiff neck, myalgia, or arthralgia are reported (Shapiro, 2014). The early disseminated stage follows within days to weeks of infection, typically affecting the nervous system, heart, or joints (Steere, 2001). Symptoms that follow are similar to those in the early localized stage, with some cases reporting cranial nerve palsies, meningitis, carditis, and complete heart block (Shapiro, 2014; Steere, 2001). The late disseminated stage occurs weeks to months after infection, with arthritis as the main symptom and a notably high count of leukocytes in the synovial fluid (Steere, 2001; Shapiro, 2014).

Diagnostics and Treatment

The diagnostic challenges of Lyme disease remain a serious problem. Rapid diagnostics can result in earlier treatment, reducing bacterial dissemination and damage. When treatment was delayed, treatment failure was twice as likely as for early treatment (Cameron, 2007). There are many options when it comes to laboratory-based diagnostic tools that could be used to assist a healthcare provider in their clinical diagnosis. All techniques differ in specificity, sensitivity, cost, and availability with only specific serological tests being accepted within most healthcare settings.

For Lyme disease, a two-tiered diagnostic system is used to increase specificity, arguably at the expense of sensitivity. The first tier uses an ELISA, and only a positive result is followed up by the second tier, an immunoblot (Western Blot), which is considered both more sensitive and specific than the ELISA (Sperling and Sperling, 2009; Steere *et al*, 2008). The disadvantage of this methodology is that false positives are a possibility for patients who have been infected but treated in previous years, as antibodies persist even after a clinical cure (Shapiro *et al*, 2014). More importantly for the patient, false negatives are a possibility. For patients in the earliest stages of infection, antibodies have not yet formed. Further, some genetic, pharmacological, or environmental conditions can result in antibody levels below the limit for detection. Antibody titres also decline over time, limiting the use of this

diagnostic for long-standing infections (Sperling and Sperling, 2009). False negatives result in either delayed treatment, absence of treatment, or misdiagnosis (Shapiro *et al*, 2014).

The PCR method is viewed as a rapid and specific method of detection (Schwaigera *et al*, 2001). PCR can not only detect infection in the early stages of illness, albeit with limited sensitivity, but it also allows identification of the bacterial to the species level, including those that are difficult to culture (Santino *et al*, 2008; Schwaigera *et al*, 2001; Cerar *et al*, 2008). As with all techniques, these are disadvantages. PCR reactions are sufficiently sensitive that contamination in the lab environment can be an issue and obtaining tissue samples can be invasive (Venczel *et al*, 2016; Shapiro *et al*, 2014). Less invasive biological samples, such as blood or spinal fluid, will often produce false negative results, as the bacterial counts in these fluids are low (Shapiro *et al*, 2014). Lastly, as PCR can detect DNA from dead bacteria, some have suggested that this could lead to false positives, if the bacteria was in the patient's system, but killed by antibiotics (Aberer *et al*, 2007).

Unlike other diseases caused by bacterial infections, culture is not often used as a diagnostic tool because the growth of *Borrelia* is so slow. For example, growth of the bacteria from a biopsied skin sample can take an average of three weeks, which delays treatment leading to further dissemination in the body and more serious disease (Coulter *et al*, 2005; Cameron, 2007). The sensitivity of this method in the early stages of disease is also low. Only 33% of patients in the acute phase who also presented with an EM rash had a positive culture result, although, this number jumped to 66% when retested three to four weeks later, but this is still too low for satisfactory diagnostic power (Steere *et al*, 2008). Culture from chronic disease patients is much more successful (Middelveen *et al*, 2018). The type of sample used for culture is also an important factor. Culturing skin biopsies of an EM were only 51% sensitive, plasma samples were only 45% sensitive, and cerebrospinal fluid and synovial fluid varied between 10 to 50% sensitive depending on timing of sample collection and duration of symptoms (Aguero-Rosenfeld *et al*, 2020; Wilske, 2005). Despite the time taken for this procedure and its difficulty, culture offers the

best confirmation of an active infection and is invaluable in the research context (Aguero-Rosenfeld *et al*, 2020).

Detection of *Borrelia* spirochaetes through staining and light microscopy, with or without associated molecular techniques, is another possible method for spirochaete identification. Results vary, which impairs utility in clinical settings. There are multiple stains and methods available, each with their own advantages and disadvantages. Silver staining may detect spirochaetes; however, it is not specific enough to be considered as a primary diagnostic tool (Schwan *et al*, 1991). Immunohistochemistry has been reported as low sensitivity compared to real-time PCR assays when used as a diagnostic tool, whereas FISH (fluorescent in situ hybridization) staining is considered to be highly sensitive in comparison to other staining techniques (Briciu *et al*, 2016; Shah *et al*, 2020). Depending on the stain used and the protocols followed, some FISH diagnostic tools can provide a result in 2 hours and is species-specific (Shah *et al*, 2020). For both molecular techniques, sensitivity and specificity vary depending on whether they are being used on cultured bacteria or bacteria in the context of tissues. When using staining as a diagnostic tool, it is also important to consider the type of tissue that is being used; for example, blood smears are easy to perform bedside, however, they are not considered as sensitive when compared to the buffy coat, anticoagulated blood sample containing white blood cells and platelets (Telford *et al*, 2019; Eldin *et al*, 2019). Further, bacteremia is short lived, only occurring at the beginning of dissemination and sporadically during further waves of dissemination in untreated infections, so looking for bacteria in the blood is likely to return a negative result (Eldin *et al*, 2019). Overall, staining *Borrelia* in human tissues is made difficult by the bacteria being scarce leading to both false negatives and false positives (Raffetin *et al*, 2020; Aguero-Rosenfeld *et al*, 2020).

Risk in New Brunswick

The risk of contracting Lyme disease increases with expanding tick populations throughout New Brunswick as climate change develops suitable environments through milder winter conditions and

higher levels of precipitation. Based on these changes, Lieske and Lloyd (2018) predict by the year 2080 one third of the province will become a highly suitable environment for *Ixodes scapularis*. Although the three main tick species collected from dog, cat, and human hosts in NB are the *I. scapularis*, *Ixodes cookei* and *Dermacentor variabilis*, *I. scapularis* is the most likely to carry *B. burgdorferi* (Lewis *et al*, 2021). *Ixodes scapularis* has also been documented to carry *B. bissettiae* (Lewis & Lloyd, 2018) and *B. miyamotoi* (Dibernardo *et al*, 2014) in New Brunswick. *Borrelia bissettiase* and *B. miyamotoi* are of important note to human health as they have been documented in patients with Lyme disease in Europe (Rudenko *et al*, 2009) and North America (Krause *et al*, 2013). To determine risk to animal and human health in the Canadian Maritimes, transmission of the bacteria to wildlife needs to be documented. Transmission of *B. burgdorferi* to animals has been well documented over time (Lane *et al*, 1991; Crippa *et al*, 2002; Piesman *et al*, 1987). In New Brunswick, *B. burgdorferi* and *B. miyamotoi* transmission to wildlife in 2016 and 2017 was documented by Zinck and Lloyd (2022). It was originally believed that *I. scapularis* ticks were unable to transmit *B. bissetti* to animals (Leydet 2014), however, multiple studies have shown a multitude of animals, including humans, have been affected by the bacteria (Oliver Jr., Golovchenko and Rudenko, unpublished observations as reported by Golovchenko *et al*, 2016; Postic *et al*, 1998; Rudenko *et al*, 2015). Collectively, the risk of *Borrelia* infection is increasing over time as infected migrating ticks introduce these bacteria into novel areas of the province.

Conclusion

The level of endemicity of an area can be predicted based on local indicators. As the range of animals and ticks shifts due to climate change, and the ingression of humans, companion and agricultural animals into former wildlife habitats increases, the spread of infection is expected to follow. Determining the prevalence of leptospirosis and borreliosis in the province of New Brunswick is essential for predicting and controlling future outbreaks. By testing water, canine sentinals, small and large mammals, and ticks as biosentinals, this will allow us to determine the best approach for

surveillance. These findings lead to conclusions about the best methodological approaches for surveillance that are needed to reduce health impacts on humans, companion animals, and agricultural animals and protect our local communities.

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Chapter 2 – Statement of Authorship and contributions to the work

This study took advantage of the small wildlife biobank, the tick biobank, and a collection of large mammal specimens. The initial collection of many of the small wildlife samples in the wildlife biobank were conducted by Chris Zinck in 2016 and 2017. DNA extraction was also performed by Chris Zinck. All molecular testing using the 16SrRNA primer set to detect leptospires DNA and leptospira analysis was completed by Samantha Bishop. Collection of the large mammal samples was done by government scientists at Natural Resources and Energy Development and provided to this study by Dr. Brian Hayden. Subsampling of these samples were done with the help of Micaïla Abboud and Patrick O'Reilly of the University of New Brunswick, Fredericton. Initial collection and DNA extraction of all tick samples were conducted by Andrea Kirby from 2016 to 2019, Alexandra Foley-Eby in 2019, and Julie Lewis in 2020. Molecular testing for leptospirosis was done by Mackenzie Scott for her independent study in 2021 winter term. Collection of dog samples was completed by Natalie Bjurman and Dr. Vett Lloyd in 2013 and 2014 and sample preparation and mailing was done by Chris Zinck. The remainder of the work completed for this chapter (data analyses, creation of figures and tables) was completed by Samantha Bishop, with editing from Dr. V. Lloyd. Publication plans include two papers, one regarding *Leptospira* surveillance in wildlife and one regarding *Leptospira* prevalence in tick species.

Chapter 2

Leptospira in water sources, wildlife, ticks, and dogs from the Canadian Maritimes

Abstract

Leptospirosis is a disease recognized globally and infectious to a wide range of animal species. In recent years, outbreaks of leptospirosis have been reported in New Brunswick with fatal outcomes in local dogs. The objective of this study is to determine the prevalence of *Leptospira* spp. in the province through molecular testing of stagnant water sources around areas of recent flooding events, local wildlife necropsies, such as suspected maintenance hosts and accidental hosts, sentinel species such as dogs, and parasitic arachnids, ticks. Three methodologies were used to test water samples, and all three (centrifugation, filtration, and growth in media) returned negative results. Testing of small mammals (n=264) showed 3.2% of specimens tested positive for *Leptospira borgpetersenii*, with meadow vole (*Microtus pennsylvanicus*) showing the highest prevalence found. Of the 283 large mammals tested, 1.8% tested positive for the presence of *L. borgpetersenii*. Infected animals include moose (*Alces alces*), snowshoe hare (*Lepus americanus*), meadow vole (*M. pennsylvanicus*), jumping mouse (*Napaeozapus insignis*), deer mouse (*Peromyscus maniculatus*), maritime shrew (*Sorex maritimensis*), and black bear (*Ursus americanus*). Of the 99 white-tailed deer (*Odocoileus virginianus*) tested, none were positive for the bacteria, suggesting that they may be able to clear the infection, as they do for *Borrelia*, another infectious spirochaete. Dog serum samples (n=647) were collected from 14 veterinary hospitals spread across the province with 19.3% (n=130) testing positive for the presence of antibodies against *Leptospira* spp. Due to the timing in which these samples were collected, it is believed that these positive cases are related to current or past infections, rather than vaccination, as it was uncommon during this time. Of the 110 ticks tested (*Dermacentor variabilis* n=22; *Ixodes cookei* n=36; *Ixodes scapularis* n=52) 3.6% were positive for *L. borgpetersenii* spread between 4 of the 23 wildlife

management zones in which they were collected. Together, these results elucidate a widespread rate of infection in the wildlife species, sentinel species, and tick species of New Brunswick.

Introduction

Leptospirosis is a globally present zoonotic disease caused by the pathogenic spirochaete in the *Leptospira* genus. Eight pathogenic *Leptospira* species are the main agents of leptospirosis in mammals and humans; *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri*, *L. alexanderi*, and *L. mayottensis* (Evangelista & Coburn, 2010; Bierque *et al*, 2020). These species are further classified by their surface lipopolysaccharide and grouped into over 200 serovars and 24 serogroups. Although serovars have no taxonomic standing, they are still important for certain diagnostic tools, such as microscopic agglutination tests (MAT), and proper classification when paired with a species (Levett, 2001; Evangelista & Coburn, 2010; Levett & Galloway, 2019; Cerqueira & Picardeau, 2009).

The *Leptospira* infectious cycle is maintained by the endemic infection of the renal tubules of maintenance hosts that shed leptospires into the environment through urine. These environmental leptospires can infect new maintenance hosts or cause infection in incidental hosts through contact with infected water or soil. The bacteria enter the host through small cuts, abrasions, or mucous membranes (typically through drinking infected water sources), migrating through the bloodstream to colonize target organs, such as the liver, lungs, heart, and kidneys (Wynwood *et al*, 2014; Levett, 2001; Temeiam *et al*, 2020; Costa *et al*, 2015; Plank & Dean, 2000). This can lead to infection by consumption of contaminated drinking waters or exposure through environmental disasters, such as flooding and heavy rainfall events (Morgan *et al*, 2002; Haake & Levett, 2015). Leptospires can survive months in fresh water sources and weeks in stagnant source, with isolation of pathogenic strains from large bodies of waters, such as rivers and lakes (Wynwood *et al*, 2014).

The significant global impact of leptospirosis is due to the diversity of animal species susceptible to the bacteria. A review of 34 papers regarding the incidental hosts of *Leptospira* by Cilia *et al* (2021) reported isolation from “Carnivora, Didelphimorphia, Rodentia, Cetacea, Cingulata, Afrosoricida, Chiroptera, and Primate orders, as well as in Reptilia and Amphibia classes”. In Canada, it has been detected in beavers, coyotes, deer, foxes, opossums, otters, raccoons, and skunks (Shearer *et al*, 2014; Kassim *et al*, 2018) and human exposures have been reported as far north as Nunavik, Quebec (Messier *et al* 2012). With the diverse and abundant maintenance and incidental hosts, it is no surprise that domestic animals, in particular dogs, are seeing increased infection rates as well. Between 1998 and 2005, Prescott (2008) reported an increase in canine leptospirosis cases in Canada. Consistent with this report, in the last five years (2017 through 2021), the Maritime provinces (New Brunswick, Nova Scotia, and Prince Edward Island) have reported leptospirosis outbreaks leading to death in local dogs (CTV Atlantic, 2017; Bosse, 2019; Bourassi *et al*, 2021).

There are a multitude of tests available for canine leptospirosis, including polymerase chain reaction (PCR), microscopic agglutination test (MAT), and culture-based tests. However, it can still be difficult to diagnose the infection in animals and some serovars may be missed; Canada typically only tests for serogroups autumnalis, bratislava, canicola, icterohemorrhagiae, grippityphosa and pomona (Reagan & Skyes, 2019; Prescott, 2008). Further problems are posed by the nature of clinical testing. Confirmation of infection typically relies on antibody testing, yet there are many limitations of this type of analysis (Chirathaworn *et al*, 2014). Antibodies can be detectable from either present and/or past infection, early antibiotic treatment can suppress development of antibodies, and tests need to be performed at least 5 to 10 days post infection otherwise the antibody titres will be too low for detection (Coico *et al* 2005; Chirathaworn *et al*, 2014; Lizer *et al*, 2018). Samples from wildlife may offer the advantage of PCR testing of necropsied organs, such as liver and kidney. The kidney contains higher concentrations of the bacteria (36.6 bacteria/ μ L) than the liver (4.3 bacteria/ μ L), as reported by

Fornazari *et al* (2012). PCR targeting the 16SrRNA gene allows for efficient, sensitive results as it allows detection of many different species and serovars of the *Leptospira* spp. from pathogenic and saprophytic clades (Perez *et al*, 2020; Fouts *et al*, 2016; Backstedt *et al*, 2015). Testing water sources for leptospires is an obvious approach but one that can be challenging. Methods used to optimize DNA extraction, such as filtration or centrifugation, may still leave leptospires undetectable due to the low concentration of bacteria (Wynwood *et al*, 2014; Bierque *et al*, 2020; Tansuphasiri *et al*, 2006). Further, naturally occurring bacteria or other inhibitors (such as heavy metals) can easily contaminate cultures from water and interfere with PCR detection.

A previously unexplored option for detection is to use environmental indicators. Insects and other arthropods have been used as indicator species for environmental changes in water, soil, and air quality (Parikh *et al*, 2021). In the case of transmission of *Borrelia*, another species of spirochaete, an arthropod vector is needed for transmission so is an obvious target for surveillance. Although there is limited research on the transmission of leptospires from arthropods, there is documentation of the presence of *Leptospira* spp. in *Ixodes ricinus*, *Ornithodoros turicata*, *Amblyomma maculatum*, *Dermacentor andersoni*, *Dermacentor marginatus*, and *Rhipicephalus sanguineus* tick species (Wójcik-Fatla *et al*, 2012; Burgdorfer, 1956; Burgdorfer, 1959; Krepkogorskaia, 1957; Van Der Hoeden, 1958). Further, Burgdorfer reported leptospires persisted in *O. turicata* ticks for more than 518 days, but they only persisted in the *D. andersoni* and *A. maculatum* ticks for 10 days post-bloodmeal from an infected hamster (Burgdorfer, 1959). This information provides a potentially powerful new approach for environmental detection and surveillance of leptospirosis. Tick species such as *Ixodes scapularis* and *Dermacentor variabilis* have already dispersed across Canada and are predicted to continue to expand throughout North America (Minigan *et al*, 2018). The typical host species for ticks in Canada are similar to those for *Leptospira* ranging from small to large mammals (Bishopp & Trembley, 1945). Therefore,

ticks found feeding from wild and companion animals should be investigated to determine their potential for surveillance as well as potential transmission risk.

This increase in knowledge of *Leptospira* host species has suggested that all animals, wild or domestic, can become a transmission source, increasing the risk to public health. Using a combination of diagnostic tools and types of samples, from wildlife to domesticated animal, water sources to ticks, we can determine the rate of infection and environmental risks within the province. This could lead to the implementation of control measures.

Materials & Methods

Leptospira in water

Sample collection & DNA optimization

Method One – Citizen Science Water Collection

Water samples were collected in 50 mL sterile containers from various locations across the province by multiple undergraduate students that were taking the course “Applied Genetics”, transported within a week of collection, and stored at -20 °C until processed. Samples were centrifuged in the Eppendorf 5430 Centrifuge (ACME Eppendorf) at 3000 rcf for 20 minutes at 20 °C to create a concentrated pellet of bacteria and debris.

Method Two - Filtration

Water samples were collected in duplicate in 50 mL sterile containers from stagnant sources, such as puddles, resulting from the receding spring freshet flood waters along the Saint John River (Appendix 1). Samples were transported on ice and stored at -20 °C. To optimize DNA extraction, thawed samples were filtered under sterile conditions in a biosafety cabinet first through 0.45 µm polyether sulfone membrane filters (VWR International, CAT# 28145-505) and again through 0.22 µm low protein binding durapore (PVDF) membrane filters (Millipore, Cork, Ireland, CAT# SLGVM33RS). The

filters were cut and removed from their plastic encasings using sterilized wire cutters and placed in individual 1.5mL centrifuge tubes before DNA extraction.

Method Three - Culture

Sites from the spring freshet flood collection (visited for method two above) were revisited in the fall of 2020 to recollect 50 mL water samples, as described above, although, many sites were not able to provide a similar stagnant source due to the lack of recent flooding events. The samples collected were transported on ice and stored at -20 °C as described for method two. To optimize DNA extraction, filtration was performed as mentioned above, followed by bacterial culture. The filters were cut and removed from their plastic encasings using sterilized wire cutters and placed in individual 25 mL glass tubes. Barbour-Stoener-Kelly (BSK-H) rich bacterial medium (Darlynn Biologicals) was prepared with antibiotics Rifampicin (50 µg/ml in dimethyl-sulfoxide (DMSO)) (Sigma, CAT# R3501-IG), phosphomycin (20 µg/ml in DMSO) (Sigma, CAT# P5396-1G), and amphotericin B (2.5 µg/ml in DMSO) (Sigma, CAT# A9528-100MG). Twenty-two mL of BSK-H growth solution was inoculated with the filter disks and placed at 34°C (VWR international, model 1545) without agitation. Samples were monitored every other day for 12 days to monitor bacterial growth using the Bright-Line hemacytometer (Reichert-Jung) and Leitz Wetzlar condenser microscope before DNA extraction. All handling was done in a Biosafety Cabinet (BSC) in a Biosafety level 2 (BSL2) lab.

DNA extraction

DNA extraction was performed in a fume hood using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's recommendation for gram-negative bacteria. Samples were vortexed before a 1 mL aliquot was removed to a 1.5 mL Eppendorf tube. The samples in Eppendorf tubes were then centrifuged at 15,000 x g for 20 minutes to harvest cells and the supernatant discarded into a biohazard waste jar. The pellet was resuspended in tissue lysis buffer with 20 µL of proteinase K and incubated at 56 °C for 20 minutes, with periodic vortexing. After 20 minutes and a final vortex, 200 µL of

a lysis buffer and 200 µL of 96% ethanol was added to the sample, then immediately and thoroughly vortexed. The mixture was added to the DNeasy Mini Spin Column with a collection tube (Qiagen, #1113885) and centrifuged at 6,000 x g for 1 minute in a microcentrifuge. The collection tube containing the flow-through was discarded into the suitable biohazard waste container (the collection tube was placed in a biohazard materials waste bag and the flow-through was added to a biohazard liquid waste container) and the spin column was transferred to a new collection tube, where 500 µL of the first wash buffer was added, followed by centrifugation at 6,000 x g for 1 minute. Again, the collection tube containing the flow-through was discarded and the spin column transferred with 500 µL of the second wash buffer added. The sample was centrifuged at 16,300 x g for 3 minutes to dry the membrane, with an additional centrifuge for 1 minute after the flow-through was discarded, if needed. The spin column was transferred for a final time to a clean 1.5 µL microcentrifuge tube with an additional 100 µL of elution buffer and allowed to incubate at room temperature for 1 minute. After a final centrifugation at 6,000 x g for 1 minute, the spin column was discarded and the eluate was stored at -20 °C until PCR analysis could be performed. All biohazard material, solid and liquid, were autoclaved before disposal.

Leptospira in Wildlife

Sample collection

Small Mammals & Birds

Members of the public donate small rodents and various bird species to the University. These specimens were the result of a lethal attack by pets, trapping (i.e. mouse traps), window collisions, car strikes and related misfortunes (Animal Care Committee approval by Mount Allison University, protocol number NEC 2016-01; specimen collection approval from the government of New Brunswick, Fish and Wildlife branch, permit number SP16-006; migratory bird specimen collection approval from the Canadian Wildlife Service, permit number SS 2025). Samples used in this study were donated from 2016

through 2020. Species identification was attempted for all samples, however for damaged specimens (i.e. specimens with missing body parts), specimens were labelled as “unknown”. Samples donated between 2016 and 2017 were previously catalogued, dissected and the DNA extracted from the liver, kidney, bladder, muscle, and skin by Chris Zinck as part of his M.Sc. research work testing for the presence of *Borrelia burgdorferi* and *Borrelia miyamotoi*.

Large Mammals

Researchers at the New Brunswick Natural Resources and Energy Development government offices collected tongues from black bear, white-tailed deer, and moose carcasses, when kills were registered by hunters. Organ availability was limited as tongues were the easiest and fastest organs to collect when processing animals harvested by hunters. Subsamples of this material was generously provided to Dr. Brian Hayden, University of New Brunswick (Fredericton). Samples were stored at -20°C until they were ready for dissection or subsampling. Dissections were performed in a biosafety cabinet, and a ~3 mm subsection of the tissue removed for DNA extraction. The remaining sample was stored at -20°C for future use.

DNA extraction

DNA extraction was performed using the AquaGenomic™ tissue protocol (Multi target Pharmaceuticals). Under sterile conditions in a biosafety cabinet, each subsection was added to AquaGenomic solution with an additional 10 µl of 2.0 µg/µl of proteinase K. The samples were incubated at 65 °C for 90 minutes followed by 95 °C for 10 minutes. Debris was pelleted by centrifugation at 16,300 x g in a tabletop microcentrifuge (Spectrafuge 24D). The supernatant was then transferred to a new tube with 100% isopropanol and DNA was pelleted by centrifugation at 16,300 x g for 5 minutes. The supernatant was discarded, and the pellet rinsed with 70% ethanol before resuspension using 50 µl of tris(hydroxymethyl) aminomethane (TRIS buffer). All DNA samples were then stored at -20 °C until use.

Leptospira in ticks

Sample collection

All tick DNA samples were taken from the Mount Allison Tick Bank, an archive of ticks and their extracted DNA from 2012 to present day (Lewis *et al*, 2021). These samples were collected through passive surveillance, primarily from the Canadian Maritime provinces. Upon arrival, ticks were assigned an identification number based on their order of arrival within each calendar year and photographed using a Leica EZ4D dissecting microscope, with magnification ranging between 12.5X and 35X, depending on the size of the tick. These photographs as well as records of the date and location of collection, host species, recent travel history, tick species identification, life stage, sex, and state of engorgement were documented.

This metadata was used to select tick DNA samples based on their geographical origin, species, and host. To ensure a uniform coverage of NB, we aimed to get nine samples (an *Ixodes scapularis*, *Ixodes cookei*, and *Dermacentor variabilis* from a dog, cat, and human host) from each of New Brunswick's 27 wildlife management zones. Unfortunately, we could not meet these requirements for each WMZ as samples meeting these conditions were not always available (Appendix 2). Ticks from years 2016-2020 were used. One tick from each of the three most common species of tick recovered from humans or pets in New Brunswick, *Dermacentor sp.*, *Ixodes scapularis*, and *Ixodes cookei*, were selected, one from each of humans, cats, and dogs were used. All DNA extraction steps were carried out by previous students and lab technicians as the ticks were submitted as described by protocol described by Wills *et al.* (2018).

Positive Control

As a positive control, two plasmids containing the partial (549bp) 16S ribosomal RNA gene sequence from *Leptospira borgpetersenii* serovar Ballum strain CRAN13 (Accession: HM776722.1) and the Lipoprotein L32 (LipL32) gene sequence from *Leptospira interrogans* serovar lai strain 56601

(Accession: AE010300) (Appendix 3) were found using NCBI genebank (NCBI Resource Coordinators, 2016) and synthesized in vector pUC57 (2710bp) (BioBasic). The lyophilized plasmids were rehydrated and transformed into *E.coli* strain DH5alpha (DNA Invitrogen, strain DH5alpha) by heat shock. The cells were resuspended in 50mM CaCl₂ pH 6.1 and 2 µL of the plasmid. The samples were incubated on ice for 15 minutes, followed by heat shock in a hot water bath at 45 °C for 50 seconds, then recovery in an ice bath for 2 minutes. The samples received 250 µL of LB broth (Invitrogen, CAT# 12780-052) and were incubated at 35 °C for 2 hours. The solution was then transferred to sterile agar plates made with 6.4 g LB agar powder (Invitrogen, CAT# J12-67) to 200 mL distilled water and 20 µl of 100 µg/µl ampicillin (Sigma, CAT# 085K0484) and incubated overnight. A single colony was selected and grown in liquid LB broth with 100 µg/µl ampicillin overnight followed by DNA extraction. DNA extraction was performed in a fume hood using the DNeasy Blood and Tissue kit (Qiagen) following manufacturer's recommendation for gram-negative bacteria, as outlined above. Colonies were prepared for long term storage by briefly centrifuging for 1 minute to pellet bacteria, discarding supernatant, and added to 20% glycerol in a 1:1 ratio, then frozen at -80 °C. All bacterial samples were prepared for disposal in the fume hood by adding a 10% bleach solution for 30 minutes before they were autoclaved, whereas the agar plates were autoclaved before disposal.

PCR

The two primers chosen for this study were selected from the multi-locus sequencing typing (MLST) from Oxford University primer list (<http://pubmlst.org>) to amplify a portion of the 16SrRNA gene repeat as confirmation of *Leptospira* spp. The multi-locus sequencing typing (MLST) database (Oxford University <http://pubmlst.org>), provides primers for species-specific housekeeping genes, including seven different genes of the *Leptospira* genus. The 16SrRNA and Lipoprotein L32 (LipL32) genes were chosen as targets as they have been reported as being useful for determining all the *Leptospira* species

(Fouts et al, 2016). The 16S primer set was used in this study as the LipL32 primers did not amplify the positive control plasmid.

Samples were prepared for the thermal cycler (BioRad, MyCycler; Eppendorf, Mastercycler) in 0.5 mL tubes with 2 μ L of the extracted DNA and 23 μ L of master mix; containing 8.5 μ L of nuclease-free water (Promega), 2.5 μ L of GoTaqGreen polymerase (Promega), 1 μ L of the 16SrRNA forward primer (forward: 5' – CATGCAAGTCAAGCGGAGTA, melting temperature 64 °C, annealing temperature 58 °C) (Sigma) and 1 μ L of the 16SrRNA reverse primer (reverse: 5' – AGTTTGAGCCCGCAGTTTTC, melting temperature 64.3 °C, annealing temperature 58 °C) (Sigma) per sample. Negative controls to detect DNA contamination in the reagents or due to aerosolized amplicons were performed by replacing input DNA with 2 μ L of nuclease-free water. A positive control was performed using 2 μ L of DNA extracted from the synthesized plasmid mixed with 23 μ L of master mix. The tubes were then added to the thermal cycler and the amplification process was carried out as follows: 5 minutes at 95 °C, 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds, with a final elongation period of 72 °C for 7 minutes (<http://pubmlst.org>). To increase amplicon amount, a second round of PCR was completed as above but with 2 μ L of the first-round product used as input DNA.

Gel electrophoresis

A 1.2% (w/v) agarose gel was prepared using 100 mL of 0.05M sodium borate (SB) buffer (boric acid, sodium hydroxide) and 5 μ L of “ECO-STAIN” (BioBasic, CAT# DT81413), and 7 μ L of each PCR product was loaded into the wells. A 200bp DNA ladder (BioBasic, cat # GM401) was added to the first and last well of each row. Gels were electrophoresed at 107V for approximately 50 minutes and imaged using a UV transilluminator (Labnet DNA Light) in a dark room and photographed using a cell phone. The brightness and contrast levels were altered for optimal visualization of bands. Bands at 541 bp amplicon length were identified as positive amplicons.

Sequencing

Amplicons of the appropriate size by agarose gel electrophoresis were prepared for sequencing. The second round of PCR was repeated with volumes doubled and sent to Génome Québec Innovation Centre at McGill University (Montréal, QC) for confirmation by Sanger sequencing. Results were viewed using FinchTV chromatogram viewer (Geospiza Inc.) and each sample sequence was compared to the sequence database using the nucleotide Basic Local Alignment Search Tool (nBLAST) provided by the National Center for Biotechnology Information's (NCBI Resource Coordinators, 2016) GenBank. Any sequences that returned a sequence comparison to anything other than *Leptospira* spp. was considered a false positive.

Leptospirosis in dogs

Canine sera samples were collected from 21 New Brunswick veterinary clinics in the fall of 2013 and the spring of 2014 as part of a study conducted by Natalie Bjurman to determine antibodies against *Borrelia burgdorferi* in dogs in New Brunswick (Bjurman *et al*, 2016). Residual sera was sent to IDEXX (IDEXX Laboratories, Markham, Ontario) for testing using their rapid ELISA test, referred to as a SNAP® test, to detect antibodies against *Leptospira* in canine serum samples (Curtis *et al*, 2015).

Results and Discussion

This study investigated the prevalence of *Leptospira* spp. in New Brunswick using four approaches: testing water from stagnant sources along the Saint John River, molecular testing of local wildlife necropsies and parasitic arachnids, ticks, and determining the presence of antibodies against *Leptospira* in dog sera.

Methodology

To determine the lowest concentration of DNA that our PCR method was able to detect, both in pure water and in the presence of competing DNA, the DNA concentration for the 16S rRNA plasmid

was measured using a NanoDrop 1000 UV-Vis Spectrophotometer (ThermoFisher). A six-step ten-fold serial dilution was performed, and samples were amplified by PCR and visualized as described in the materials and methods section. The visual detection limit was calculated to be 4.8×10^5 copies of target DNA in both 1 μ L of extracted DNA and 1 μ L of water (Fig 1). 16S primers worked and allowed detection of target DNA in water and competing DNA.

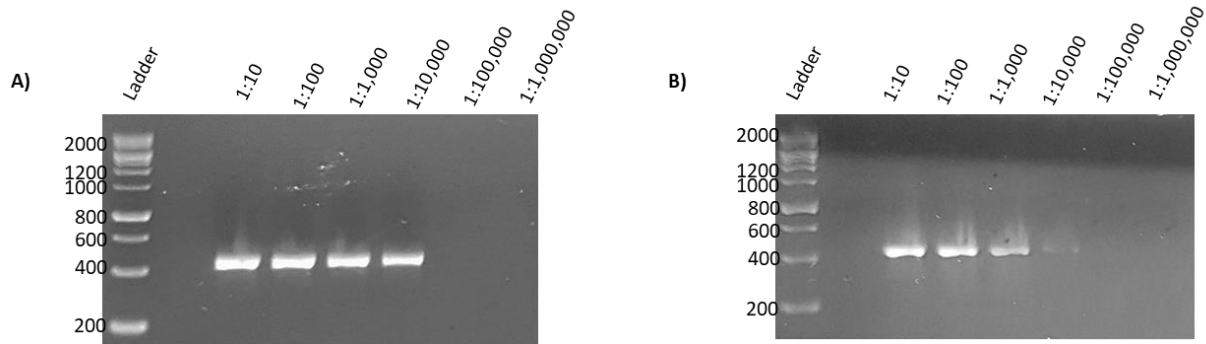


Figure 1: PCR amplification of serial dilutions of the 16S rRNA gene plasmid to determine the detection limit of *Leptospira* DNA in A) extracted mammalian DNA and B) water. A 200-2,000bp ladder was used for band size reference.

Detection of Leptospires in water

As leptospirosis outbreaks initiate through exposure to leptospire-contaminated water, testing water samples was an obvious approach to determine the location of potential sources of infection. To sample as many areas throughout the province as possible and assess different detection methodologies, two different methods of sample collection and three methods of DNA detection were attempted.

Method One - Citizen Science Water Collection

The first method relied on a citizen science approach. Undergraduate students in the “Applied Genetics” course of the fall 2019 semester were asked to collect 50mL of water from stagnant sources during their travel over reading week, or from the Sackville area if they were not traveling. This type of

sampling method has the advantage that multiple samples can be collected at the same time throughout the province with limited logistical burden. As with all citizen science initiatives, there was variability in sample collection and storage. This could have resulted in contamination or degradation of bacterial DNA.

Centrifugation was used to concentrate bacteria and so optimize DNA recovery. However, this method only produced a visible pellet in samples with a large amount of sediment. DNA extraction was attempted on each sample, with or without a visible pellet, followed by PCR and gel electrophoresis. Bands only appeared for the positive controls (Fig. 2).

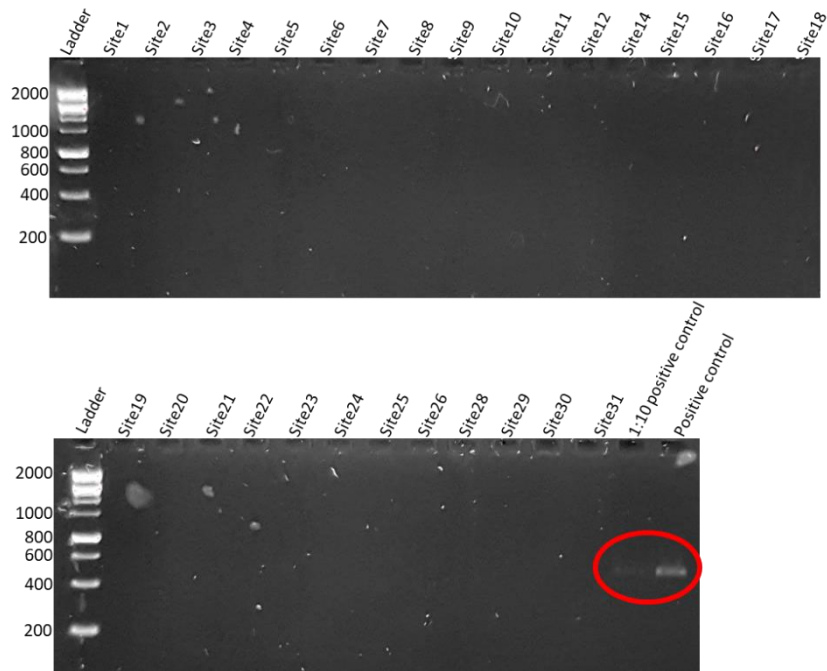


Figure 2: PCR amplification of samples collected by citizen scientists during the fall of 2019 with amplicons produced from the two positive controls containing *Leptospira* plasmid DNA, highlighted by the red circle. A 200-2,000bp ladder was used for band size reference.

Method Two - Filtration

The second method involved the collection of samples by one individual, myself, in a single day along a targeted region of the Saint John River (SJR). This region was chosen due to its history of flooding

events and reports of dogs in the area becoming affected with leptospirosis (Wright, 2018; Bosse, 2019). Samples were collected during the spring freshet in May 2020 as the floodwaters receded and were kept on ice until storage at -20°C. Filtration was subsequently used to collect cells in order to optimize recovery of DNA. Again, none of the samples, apart from the positive control, returned a positive band on the agar gel (Fig. 3).

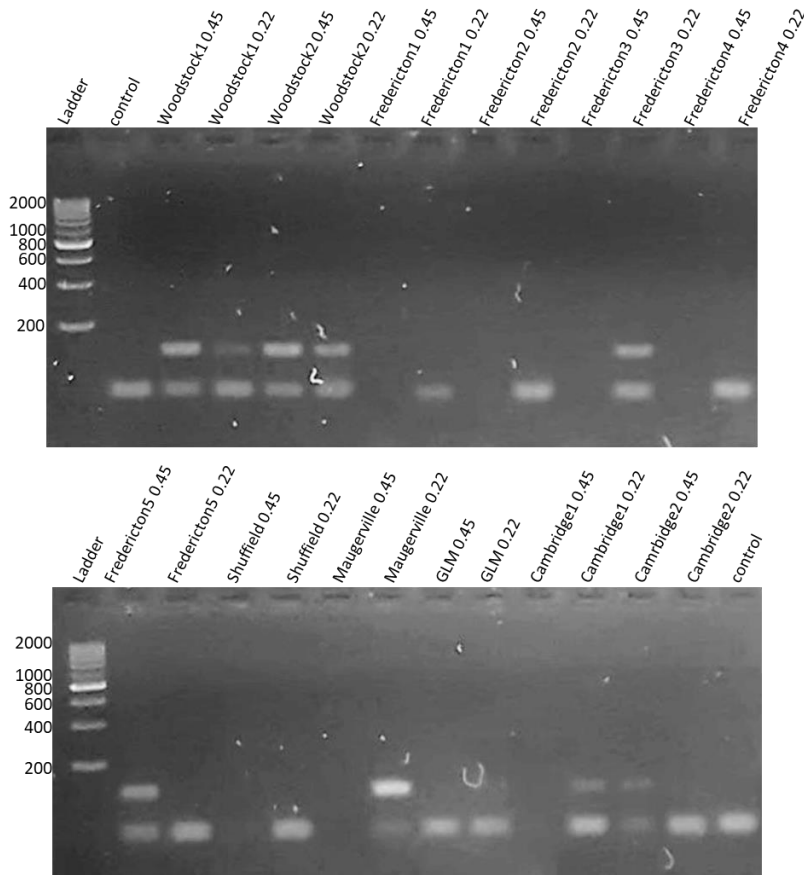


Figure 3: PCR amplification of samples collected along the SJR in the spring of 2020 and passed through either a 0.45 µm or 0.22 µm filter.

Method Three – Culture

Collection of samples was nearly identical to that used in the second method, however, as this collection was performed in the fall of 2020 and not after a flooding event, some collection sites were

not included as they no longer offered sources of stagnant water. Collection was repeated during the fall because there is literature that suggests the seasonal higher rainfall and cooler temperatures lead to a higher incidence of leptospirosis outbreaks (Haake & Levett, 2015). Sample filtration followed by culture in BSK-H media growth did produce amplicons from several samples, but these amplicons were present as multiple amplicons, none at the correct size (Fig 4). Sanger sequencing of two amplicons returned sequences indicative of *Alcaligene faecalis*, bacteria commonly found in soil and water (Appendix 4), but not *Leptospira* spp. The quality of the other two samples, “Woodstock2 0.45” and “Fredericton4 0.45”, were deemed poor and sequences were not provided.

In summary, while direct testing of water for leptospires is an obvious and direct approach, more work would be needed to optimize the collection and testing of water sources. As leptospires are mainly transferred through contaminated water sources (Wynwood *et al*, 2014), this should be a research priority for those active in protecting public and wildlife health.

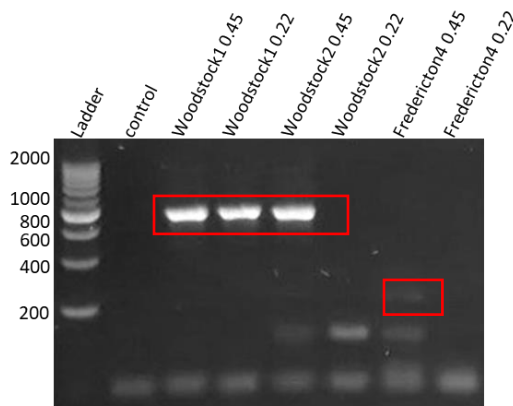


Figure 4: Agarose gel of PCR positive samples using the 16SrRNA gene primers with bands of varying lengths from filtration and culture of water samples; bands highlighted by a red box are not the correct length, however, these amplicons were sequenced. A 200-2,000bp ladder was used for band size reference.

Wildlife surveillance

A wide range of species from various sources were used to maximize diversity and sample size. As the collection method relied on the donations of accidentally killed wildlife from the public, in particular for small specimens, the geographic distribution of samples was non-random and biased towards the region of the University. On the other hand, this citizen science collection method made lethal collection of wildlife, an activity inconsistent with wildlife preservation concerns, unnecessary. In contrast, the large mammal samples were more systematically collected and so were more evenly spread across the province. These were categorized by species and the wildlife management zone (WMZ) in which each sample was collected.

Of the 263 small wildlife samples previously collected, the majority were in WMZ 25, the most commonly donated animal was the meadow vole, which made up 52.5% of the collection. Other frequent submissions were deer mice (11%), maritime shrew (9.9%), jumping mice (5.3%), unidentifiable species (7.2%), mostly various bird species, and finally species for which there were fewer than 10 animals per species collected (14%). Where possible, at least two organs were tested from each animal; the organs collected were the liver (n=233), kidney (n=237), brain (n=37), muscle (n=21), and skin (n=15) (Table 1). For the large mammals, 283 tongue samples were collected from 99 white-tailed deer, 99 moose, and 85 black bears spread across 22 wildlife management zones. Samples were not available from zones 3, 4, 5, 9 and 27.

A total of 76 *leptospira* PCR-positive wildlife samples (including small and large wildlife species) were found by PCR using the 16S rRNA gene; only 30% of these amplicons were sequence confirmed (Appendix 5). All samples were found to have *L. borgpetersenii*, with the exception of one meadow vole which returned a sequence for *Leptospira interrogans* for the forward PCR reaction but *Leptospira kirschneri* for the reverse reaction, declining by a total of 69.7%. Using only the sequence confirmed

samples, 2.7% of NB wildlife specimens were infected with *L. borgpetersenii* at the time of their death/collection (Table 1, Fig 5). While the unequal species and geographic distribution of the small mammals affects the overall predictive power of this data, the large mammals were collected from a wider geographic area. There appears to be very little overlap in high Leptospirosis infection prevalence and flood hazard areas or protected watersheds of New Brunswick (Fig 5B), although, these areas only indicate the 30 protected surface watersheds which supply drinking water to 21 communities (Government of New Brunswick, 2022) and do not show the entire watershed areas. As watersheds include three zones surrounding the protected area, the watersheds of New Brunswick are much larger than pictured on this map, covering 78% of the province (Government of New Brunswick, Canadian Rivers Institute, 2017). When looking at the overlap between positive cases and the larger watershed areas, there does appear to be a connection between WMZ 12, with the highest positive cases in large mammals, and the headwaters of the Miramichi watershed.

The numbers reported in this section are a conservative estimation due to the restricted geographic spread of the small wildlife samples and the limited organ availability from the large mammals. To better understand the true spread of *Leptospira* spp. in the province, specifically in the small wildlife, samples would need to be provided from multiple wildlife management zones. In addition, an equal number of samples from varying species would also be beneficial to creating a more accurate estimation of *Leptospira* spread. In the large mammals, samples would have ideally been collected from the kidneys, as bacterial colonization occurs in the kidneys and indicates that the animal is a reservoir for the bacteria. It is possible that the positive tongue samples do not indicate an active infection, but rather could indicate environmental exposure, as bacteria can be present due to drinking from an infected source. This is not expected, however, as PCR detection is not likely to pick up enough DNA from an exposure to result in a positive test result.

Table 1: Number, species, and infected organ of animals with sequence-confirmed *L. borgpetersenii*.

Species	Common Name	Number of samples tested	Sequencing Positives	Kidney		Liver		Brain		Muscle		Skin		Tongue	
				Tested	Sequence Positive	Tested	Sequence Positive	Tested	Sequence Positive	Tested	Sequence Positive	Tested	Sequence Positive	Tested	Sequence Positive
<i>Acanthis flammea</i>	Common redpoll	6	0 (0%)	0	-	3	0 (0%)	3	0 (0%)	0	-	0	-	0	-
<i>Alces alces</i>	Moose	99	3 (3%)	0	-	0	-	0	-	0	-	0	-	99	3 (3%)
<i>Archilochus colubris</i>	Ruby-throated hummingbird	2	0 (0%)	0	-	0	-	1	0 (0%)	1	0 (0%)	0	-	0	-
<i>Haemorhous mexicanus</i>	House finch	2	0 (0%)	0	-	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-
<i>Lepus americanus</i>	Snowshoe hare	4	1 (25%)	2	0 (0%)	2	1 (50%)	0	-	0	-	0	-	0	-
<i>Marmota monax</i>	Groundhog	2	0 (0%)	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-	0	-
<i>Megaceryle alcyon</i>	Belted kingfisher	2	0 (0%)	0	-	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-
<i>Microtus pennsylvanicus</i>	Meadow vole	285	9 (3.1%)	128	3 (2.3%)	123	4 (3.3%)	18	1 (5.6%)	10	0 (0%)	6	1 (16.7%)	0	-
<i>Mustela erminea</i>	Short-tailed weasel	2	0 (0%)	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-	0	-
<i>Napaeozapus insignis</i>	Jumping mouse	26	3 (11.5%)	14	3 (21.4%)	12	0 (0%)	0	-	0	-	0	-	0	-
<i>Odocoileus virginianus</i>	White-tailed deer	99	0	0	-	0	-	0	-	0	-	0	-	99	0 (0%)
<i>Peromyscus maniculatus</i>	Deer mouse	62	1 (1.5%)	29	1 (3.4%)	27	0 (0%)	2	0 (0%)	2	0 (0%)	2	0 (0%)	0	-
<i>Picoides pubescens</i>	Dawny woodpecker	2	0 (0%)	0	-	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-
<i>Procyon lotor</i>	Raccoon	4	0 (0%)	2	0 (0%)	1	0 (0%)	0	-	1	0 (0%)	0	-	0	-
<i>Rattus norvegicus</i>	Brown rat	33	0 (0%)	9	0 (0%)	9	0 (0%)	5	0 (0%)	5	0 (0%)	5	0 (0%)	0	-
<i>Sciurus carolinensis</i>	Eastern grey squirrel	6	0 (0%)	3	0 (0%)	3	0 (0%)	0	-	0	-	0	-	0	-
<i>Sciurus vulgaris</i>	Red squirrel	7	0 (0%)	2	0 (0%)	2	0 (0%)	1	0 (0%)	1	0 (0%)	1	0 (0%)	0	-
<i>Sorex maritimensis</i>	Maritime shrew	51	2 (3.8%)	25	0 (0%)	24	2 (8.3%)	0	-	1	0 (0%)	1	0 (0%)	0	-
<i>Spinus pinus</i>	Pine siskin	4	0 (0%)	0	-	2	0 (0%)	2	0 (0%)	0	-	0	-	0	-
<i>Spinus tristis</i>	American goldfinch	2	0 (0%)	0	-	1	0 (0%)	1	0 (0%)	0	-	0	-	0	-
<i>Tamias striatus</i>	Chipmunk	4	0 (0%)	2	0 (0%)	2	0 (0%)	0	-	0	-	0	-	0	-
<i>Ursus americanus</i>	Black bear	85	2 (2.4%)	0	-	0	-	0	-	0	-	0	-	85	2 (2.4%)
Unknown	Unknown	47	2 (5.4%)	19	2 (10.5%)	17	0 (0%)	1	0 (0%)	0	-	0	-	0	-
TOTAL		836	23 (2.7%)	237	9 (3.8%)	233	7 (3%)	37	1 (2.7%)	21	0 (0%)	15	1 (6.7%)	283	5 (1.8%)

Canine seroprevalence surveillance

Due to their closeness to humans, dogs act as sentinel species for many parasites, becoming a tool to detect the risk of disease to humans. Positive cases were seen from 13 of the total 14 participating veterinary clinics; Moncton was the only location with no positive tests. Out of the 647 serum samples tested province-wide, 19.3% showed the presence of antibodies against *Leptospira* spp. However, this test does not distinguish between serovars and it picks up both pathogenic and saprophytic leptospires. Further, it cannot determine if there is an active infection or antibodies from either a previous infection or vaccination. Although there is no obvious correlation with areas reporting high chances of flooding, there is higher sero-reactivity in dogs in the rural parts of the province (Fig 6A). Based on the data provided by Statistics Canada from the 2016 census, the beta regression does indicate a correlation between areas with lower population densities per kilometer² and the number of dogs exposed to *Leptospira* spp. (z-statistic P-value = 0.0325) (Fig 6B). In the counties containing lower population densities, it is likely that the dogs tested in this study have higher chances of overlapping areas with wildlife, therefore increasing their chances of infection (White *et al*, 2017; Ward *et al*, 2004).

Ticks

As ticks are external parasites, feeding on the blood of animals, they become a useful tool for monitoring blood-borne pathogens. The previously extracted DNA from 110 ticks were selected from the Mount Allison tick bank to be tested for *Leptospires*. Selection was based on the WMZ in which the ticks were believed to be encountered, the host in which they were found on (cat, dog or human), and the tick species (*Ixodes scapularis*, *Ixodes cookei* or *Dermacentor variabilis*). No other factors were considered when picking samples, such as level of engorgement, approximate feeding time, or sex. In total, 86 females, 9 males, and 15 with sex labelled as unknown were chosen; of these, 9 were non-engorged, 46 were engorged, and 55 were highly engorged. Of the 110 ticks tested, 15 tick samples produced amplicons consistent with *Leptospira*; 4 of these were confirmed as *Leptospira borgpetersenii*

(Fig 7). These included an engorged adult female *I. scapularis* collected from a cat in WMZ 18, a highly engorged adult female *I. cookei* from a dog host in WMZ 17, an engorged adult female *I. cookei* from a human host in WMZ 24, and a non-engorged adult male *D. variabilis* from a dog host in WMZ 9. Of the remaining 11 PCR confirmed samples that were sent away for sequencing, six were poor quality, one found no significant similarity, two were identified as *Mus musculus*, one was identified as different *Bacillus* strains, and one was identified as *Borrelia burgdorferi*.

This project was a small pilot project to determine if *Leptospira* could be detected in ticks; clearly it can be. This raises the question to whether ticks can carry the bacteria because they have recently sampled the blood on an infected animal or are able to maintain the bacteria through moults after having fed from an infected animal in an earlier life stage. As it seems likely that ticks can retain leptospires through moults, this raises the question of whether ticks could transmit leptospires to naïve animals during feeding. Burgdorferi (1956) determined transmission of *L. pomona* from an infected animal to an argasid tick, a soft bodied tick, *Ornithodoros turicata*, where the leptospires retained in the ticks for 232 to 518 days. Furthermore, he reported that leptospires could penetrate the gut wall and were discovered in the salivary glands, among other organs.

At a minimum, these results mean that ticks could be used as a convenient means of surveying wildlife species. These results also make urgent investigation of whether ticks can vector leptospires, which would make leptospirosis another tick-vectored disease.

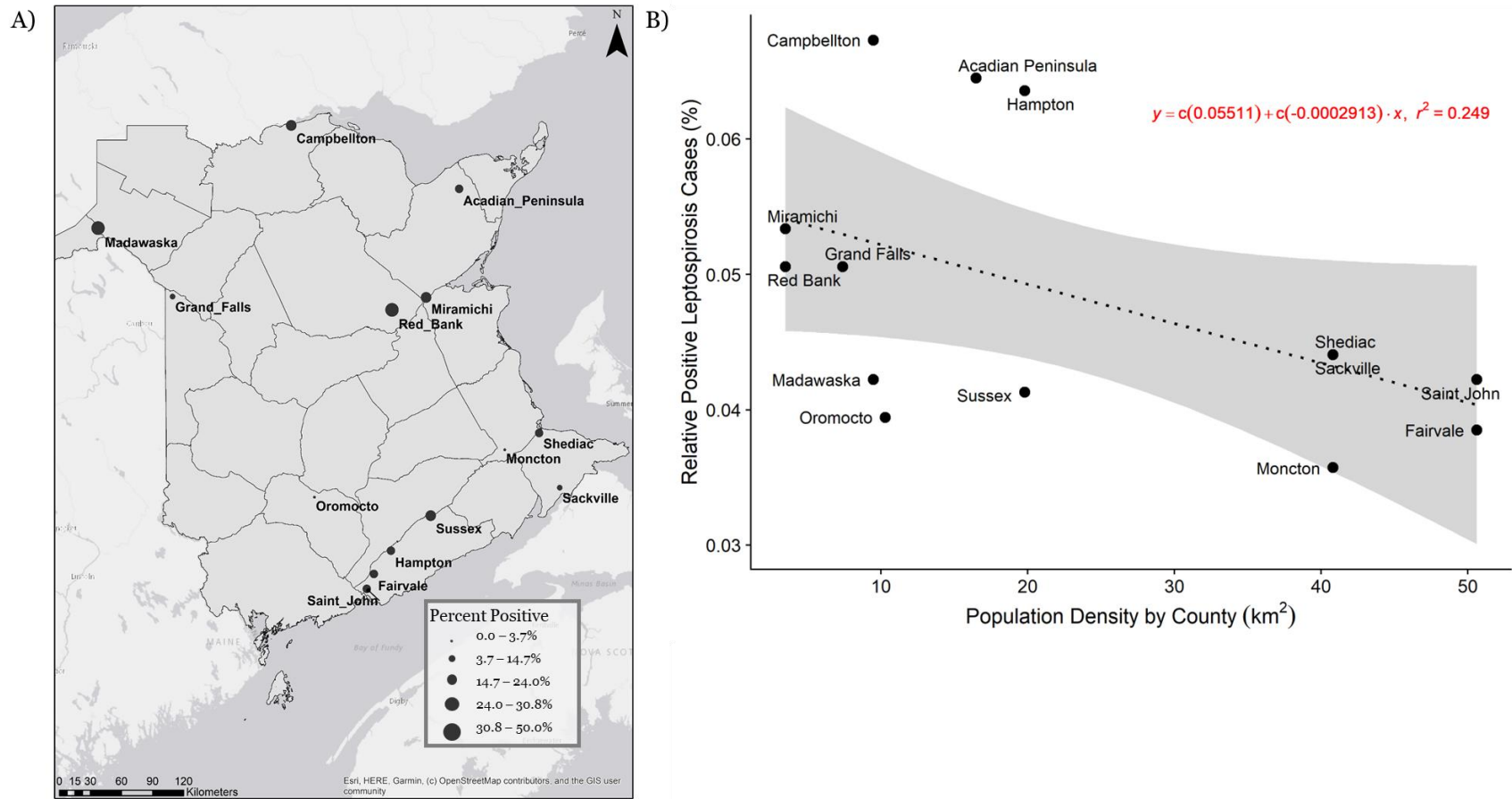


Figure 6: A) Location of Leptospira seropositive dogs based on total dogs tested for that location. **B)** Beta regression model showing relationship between population density per kilometer-squared of county (numbers taken from Statistics Canada 2016 Census) and percentage of positive Leptospira results. P-value=0.0325, std error=0.0016. Model visualization was produced using GGPlot2 package on R-studio software.

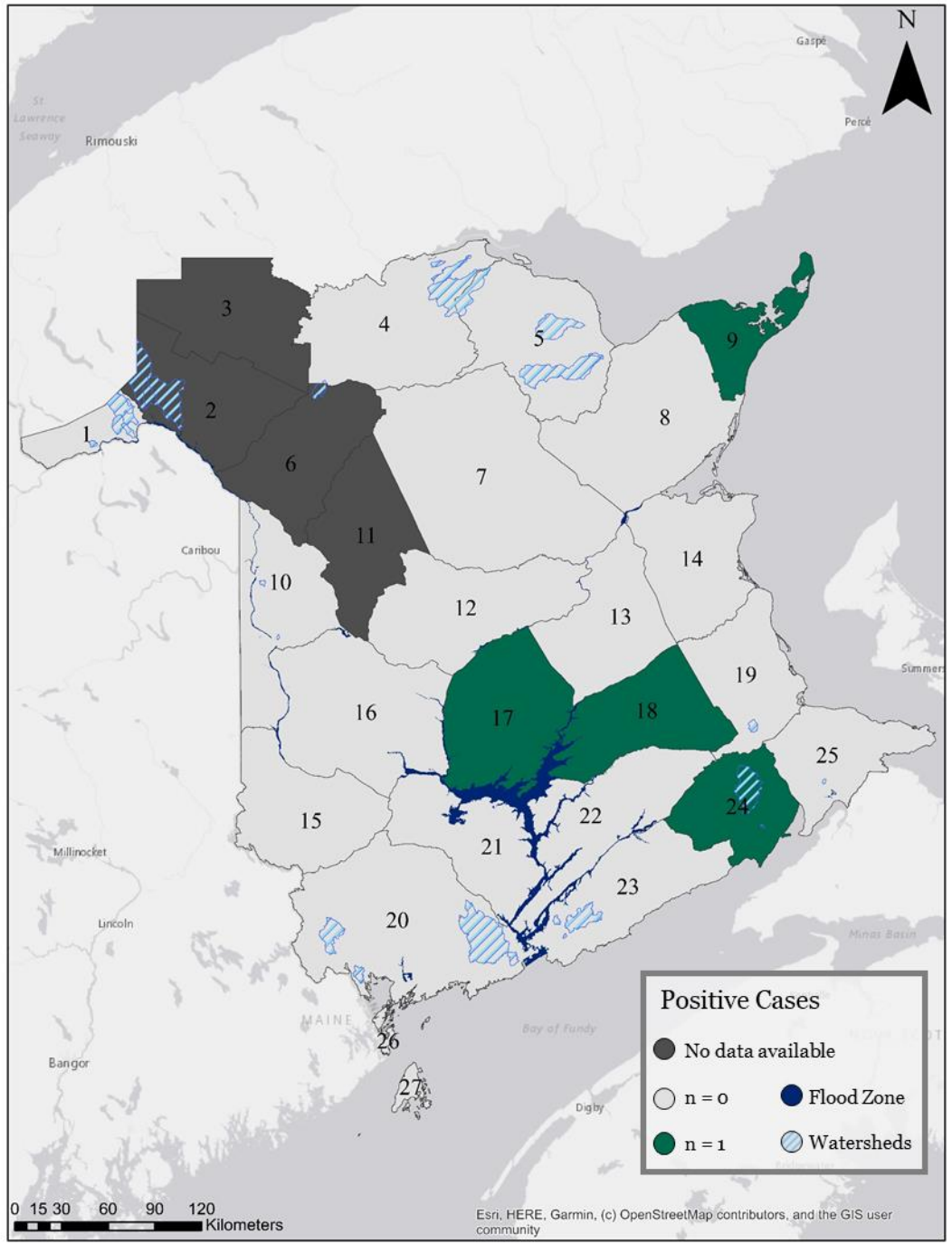


Figure 7: Distribution of sequence-confirmed positive cases of *L. borgpetersenii* from tick species *Ixodes scapularis*, *Ixodes cookei*, and *Dermacentor variabilis*. The flood hazard areas are shown in dark blue along the Saint John River, and protected watersheds are shown by blue stripes.

Conclusion

This study used three approaches to assess the incidence of *Leptospira* in New Brunswick. DNA sequencing confirmed the presence of *L. borgpetersenii* cases in 3.2% of small wildlife tested and 1.8% of large wildlife tested, or 2.7% of all wildlife tested; three moose, one snowshoe hare, nine meadow voles, three jumping mice, one deer mouse, two maritime shrews, two black bears, and two animals of unknown species. Similarly, 3.6% of the 110 ticks were sequence confirmed to be carrying *L. borgpetersenii*. Consistent with these results, 19.3% of dogs tested from 13 of the 14 participating veterinary clinic locations across the province had antibodies against *Leptospira* spp. Although we could not confirm the presence of *Leptospira* in water, outbreaks with canine fatalities indicates that it is present in the environment. There does appear to be correlation between the wildlife management zone with the highest positive large mammal cases and the Miramichi headwaters as well as between canine exposures to the bacteria and counties containing lower population densities per kilometer-squared. To solidify these correlations, further investigation with larger sample sizes would be desirable.

Moving forward, surveillance should be continued to determine if *Leptospira* species increase in incidence or change in location over time as the climate warms and flooding and rainfall events increase. Community donated samples are a powerful way of monitoring wildlife, and a citizen science approach could be extended to collect small mammals from larger parts of the province. Methods for direct sampling of water require optimization. Use of environmental DNA (eDNA) sequencing might provide increased sensitivity, although cost limits the use of this methodology for routine surveillance. Obtaining a better understanding of the *Leptospira* genus and how it enters the environment and hosts will help manage this zoonotic disease.

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Chapter 3 – Statement of Authorship

The initial collection and DNA extraction of the small wildlife samples were conducted by Chris Zinck in 2016 and 2017. All molecular testing using the *Borrelia bissettiae* nested primer set was completed by Samantha Bishop. Collection of the large mammal samples was done by government scientists at Natural Resources and Energy Development and provided to this study by Dr. Brian Hayden.

Subsampling of these samples were done with the help of Micaïla Abboud and Patrick O'Reilly of the University of New Brunswick, Fredericton. The remainder of the work completed for this chapter (data analyses, creation of figures and tables) was completed by Samantha Bishop, with editing from Dr. V. Lloyd.

Chapter 3

Borrelia bissettiae in wildlife from the Canadian Maritimes

Acknowledgements

We would like to thank N. Rudenko for the suggestion of this study.

Abstract

Borrelia bissettii is part of the *Borrelia burgdorferi* sensu lato complex, the causative agent of Lyme borreliosis (LB), also known as Lyme disease. *B. bissettiae* has been isolated from patients in Europe with symptoms of LB and has therefore been considered as one of the species to cause LB in humans. In order to determine the risk to public and veterinary health, the prevalence within New Brunswick needs to be identified. Previous studies have found the presence of the spirochaete in the local tick species, *Ixodes scapularis*. This study looks at the presence of *B. bissettiae* in the local wildlife, from small rodents and migratory birds to large mammals. Of the 264 small wildlife species tested, two tested positive for *B. bissettiae*: the meadow vole (*Microtus pennsylvanicus*) and the deer mouse (*Peromyscus maniculatus*). None of the 283 large mammals tested positive for the presence of *B. bissettiae*. This evidence suggests that the bacteria has established maintenance hosts, but the ability to infect accidental hosts has not been found in New Brunswick yet.

Introduction

Lyme borreliosis, also known as Lyme disease, is an infection caused by the pathogenic members of the Lyme Borreliosis group of *Borrelia* spirochaetes, also known as the *Borrelia burgdorferi* sensu lato (sl) complex. The disease, and its tick vectors are expanding throughout the northern hemisphere (Stanek *et al*, 2012; Steere *et al*, 2016). *Borrelia bissettiae* (formerly *B. bissettii*), one of the 23 genospecies within the *B. burgdorferi* sl complex, was first documented by Bissett and Hill in 1987 in Northern California, US after they found an isolate of *Borrelia* similar to those found in Europe; the

spirochaete did not react with the monoclonal antibodies to the common surface proteins, OspA or OspB, something all other isolates from the United States had been found to do until that time (Bissett & Hill, 1987). *Borrelia bissettiae* shares many similarities to *B. burgdorferi*, including host species, such as the eastern wood rat (*Neotoma floridana*), downy woodpecker (*Picoides pubescens*), Carolina wren (*Thryothorus ludovicianus*), and northern waterthrush (*Parkesia noveboracensis*) in North America (Golovchenko *et al*, 2016; Richter *et al*, 2004), and geographic range; it is one of the globally dispersed *Borrelia* being found through North America and Europe (Golovchenko *et al*, 2016). Its pathogenicity was highlighted when presence of *B. bissettiae* was found in the cardiac valve tissue from a patient with endocarditis and in patients experiencing symptoms of Lyme disease in Europe (Rudenko *et al*, 2008; Rudenko *et al*, 2009).

In eastern Canada, the primary vector for *Borrelia* bacteria that parasitizes humans and companion animals is the *Ixodes scapularis* or black-legged tick. Other *Ixodes* ticks can also vector *Borrelia* but do so less effectively or feed from humans less frequently (Lindquist *et al*, 2016). *Dermacentor variabilis* also parasitizes humans and companion animals in eastern Canada, however it is not believed to be a competent host for *Borrelia* spirochaetes, rather these ticks can harbour and transmit bacteria that cause relapsing or spotted fevers and tularemia (Minigan *et al*, 2018; Lewis *et al*, 2021; Fritzen *et al*, 2011). *B. bissettiae* has been primarily reported in the south-eastern and western United States (Margos *et al*, 2010) in *I. pacificus*, *I. spinipalpis*, or *I. affinis* (Lin *et al*, 2001; Lin *et al*, 2003), although it has been found in *I. scapularis* as well (Lin *et al*, 2003; Leydet, 2014; Lewis & Lloyd, 2019). Due to climate change, milder winter conditions in New Brunswick have resulted in the increased survival of *I. scapularis*, and their pathogens (Lieske & Lloyd, 2018). Recently, Lewis and Lloyd (2019) reported *B. bissettiae* infection in *I. scapularis* ticks in southern New Brunswick, confirming the presence of this bacteria in the province. Nevertheless, Leydet (2014) showed that while *I. scapularis* is able to

acquire *B. bissettiae* from infected murine hosts, the bacterial load is smaller than for *B. burgdorferi* and transmission was not detected from this tick species to their murine model animals.

In order to see if *B. bissettiae* was present in New Brunswick wildlife, which would raise the possibility of transmission risk, for wildlife, human, and companion and agricultural animals, this study examined small and large mammals, as well as birds, for current *B. bissettiae* infections using direct detection by nested PCR. By determining the presence of this spirochaete in New Brunswick, we will be able to assess the risk to both public and veterinary health.

Materials & Methods

See chapter 2, materials & methods section, for information on sample collection and DNA extraction.

PCR

Extracted DNA was prepared for nested PCR using a set of primers as outlined by Lewis and Lloyd (2018) to amplify a portion of the *OspA* gene of *Borrelia bissettii*. Samples were prepared for the thermal cycler (BioRad, MyCycler; Eppendorf, Mastercycler) in 0.5 mL tubes with 2 μ L of the extracted DNA and 23 μ L of master mix; containing 8.5 μ L of nuclease-free water (Promega), 2.5 μ L of GoTaqGreen polymerase (Promega), 1 μ L of the forward primer (outer: 5'-GCAAAACATTAGTGTCAAAAAAG, annealing temperature 60 °C; inner: 5'-CAATGAAAAAGGCGAATTAGTTGA, annealing temperature 59 °C) and 1 μ L of the reverse primer (outer: 5'-CTTCCAAGTTGGTTCCTGC, annealing temperature 60 °C; inner: 5'-TATTTTTGTGATGTAATTGTGTCTTG, annealing temperature 59 °C) (Sigma) per sample. Negative controls were created using 2 μ L of nuclease-free water in place of extracted DNA to 23 μ L of master mix to detect any DNA contamination in the reagents or presence of aerosolized DNA. Lastly, a positive control was created using 2 μ L of DNA extracted from an *I. scapularis* tick that was previously sequence confirmed for carrying the spirochaete (provided by J Lewis from the Lloyd lab) mixed with 23 μ L of master mix to confirm the solutions were

viable and machinery did not malfunction. Samples were placed in a thermocycler with an initial denaturation period of 95 °C for 5 minutes, followed by 40 cycles consisting of a 15 s denaturation step at 95 °C, a 30 s annealing step at 60 °C for the outer round or 59 °C for the inner round, and a 45 s elongation step at 72 °C, followed by a single cycle for a final elongation of 5 min at 72 °C.

DNA extracted from the large mammals were also tested for *Borrelia burgdorferi* using nested primers targeting the 23s gene, as these samples are newly added to our biobank and not previously tested as the small wildlife samples were. The outer primer set was designed by DiBernardo *et al* (2014) to detect multiple *Borrelia* species and the inner primer set was designed by Zinck and Lloyd (2022) targeting the 16-23s intergenic spacer, a conserved region of the *Borrelia* genus that can detect Lyme- and relapsing fever-causing species. Samples were prepared for the thermal cycler in 0.5mL tubes for the first round of PCR containing 12.5 µL of GoTaqGreen polymerase (Promega), 8.5 µL of nuclease-free water, 1 µL of the forward primer (outer: 5'- GTATGTTTAGTGAGGGGGGTG, annealing temperature 50 °C) and 1 µL of the reverse primer (outer: 5'- GGATCATAGCTCAGGTGGTTAG, annealing temperature 50 °C) (Sigma), and 2 µL of sample DNA to target the 588-1029bp amplicon. The program followed one round at 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 50°C for 1 min, and 72 °C for 30 sec, followed by a final elongation cycle of 72 °C for 10 min (DiBernardo *et al*, 2014). The second round of amplification used 1 µL of the forward primer (inner: 5'- ATGTATTCCATTGTTTTAATTACG, annealing temperature 51 °C) and the 1 µL of the reverse primer (inner: 5'- GACAAGTATTGTAGCGAGC, annealing temperature 51 °C) (Sigma) and 2 µL of the PCR product from round one. The program followed one round at 95 °C for 5 min, 40 cycles of 95 °C for 30 sec, 50°C for 1 min, and 72 °C for 1 min, followed by a final elongation cycle of 72 °C for 10 min (Zinck & Lloyd, 2022).

Gel electrophoresis

A 1.2% (w/v) agarose gel was prepared using 100 mL of 0.05M sodium borate (SB) buffer, stained with 5 µL of ECO-STAIN (Biotech, CAS# 163795-75-3), and 7 µL of each PCR product was loaded

into the wells. A 100bp DNA ladder (FroggaBio, cat # DM003-R500) was added to the first and last well of each row. Gels were electrophoresed at 107V for approximately 50 minutes and imaged using a UV transilluminator (Labnet DNA Light) in a dark room and photographed using a cell phone. The brightness and contrast levels were altered for optimal visualization of bands. Fluorescent bands in the samples tested for *B. bissettiae* at 366 bp amplicon length were identified as a positive result (Lewis & Lloyd, 2019), and the samples tested for *B. burgdorferi* at 340 bp amplicon length were identified as a positive result (Zinck & Lloyd, 2022).

Sequencing

Samples considered positive on the agarose gel were then prepared for sequencing, repeating the inner round of PCR with volumes doubled, and sent to Génome Québec Innovation Centre at McGill University (Montréal, QC) for confirmation by Sanger sequencing. Results were viewed using FinchTV chromatogram viewer (Geospiza Inc.) and each sample sequence was compared to the sequence database using the nucleotide Basic Local Alignment Search Tool (nBLAST) provided by the National Center for Biotechnology Information's (NCBI) GenBank. Any sequences that returned a sequence comparison to anything other than *Leptospira* was considered a false positive.

Results and Discussion

Of the 264 small wildlife samples tested, 20 produced amplicons of the appropriate size (366 bp) to potentially identify *B. bissettiae*. A total of 138 meadow voles, 30 deer mice, 26 maritime shrew, 14 jumping mice, nine brown rats, three eastern grey squirrels, two chipmunks, one groundhog, two pine siskins, two raccoons, two red squirrels, one short-tailed weasel, two snowshoe hares, 13 bird species (one American goldfinch, one belled kingfisher, three common redpolls, one downy woodpecker, one house finch, one ruby-throated hummingbird, and five unknowns), and 14 unidentifiable non-bird species from which 239 liver samples, 243 kidney samples, 37 bladder samples, 21 muscle samples, and

15 skin samples were tested. Of these, 12 positive amplicons came from the liver, seven from the kidney, and one from the bladder; muscle and skin samples did not show a positive amplicon from PCR although only 36 such samples were tested. Of these 20 putative positives, only three were able to be reamplified well enough to produce sufficient DNA for sequence confirmation; of these three, two returned a positive *B. bissettiae* result. Of the 283 large mammals, white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), and black bears (*Ursus americanus*), that were tested, none of the samples were PCR positive for *B. bissettiae* (or *B. burgdorferi*). These results are consistent with other published results showing that animals such as white-tailed deer are able to clear the infection, as their blood is borreliacidal (Kjelland *et al*, 2011; Roome *et al*, 2016). Kjelland *et al* (2011) reported that this may apply to moose studied in southern Norway, as feeding nymphs collected from the moose were not infected with *B. burgdorferi*, however, moose in Minnesota were sero-prevalent for *B. burgdorferi* (Durrani & Goyal, 2011) and moose in North Dakota had antibodies to *B. burgdorferi* as well (Bahnsen *et al*, 2021). Bears have not been well studied as reservoir species for Borrelia, although Zolnik *et al* (2015) reported that they were not able to detect *B. burgdorferi* in tick-infested black bears. More research on the reservoir potential of moose and bear are needed, however, this preliminary data suggests that in New Brunswick these animals, as well as deer may not be reservoirs for either *B. burgdorferi* or *B. bissettiae*.

One of the positive samples was from the bladder of a meadow vole (*Microtus pennsylvanicus*) collected from Sackville, NB, and the other from the kidney of a deer mouse (*Peromyscus maniculatus*) collected from Jolicure, NB (Appendix 6; Table 1). As *Leptospira* spp. and *B. burgdorferi* sl have similar host species, it is not uncommon to find co-infections occurring in wildlife (Rodríguez-Rojas *et al*, 2020). It is possible that these two spirochetes can coinfect a host but may out compete each other for survival in an individual organ. The effects this may have on a host are unclear, however, coinfections can affect the disease outcome and transmissibility (Hoarau *et al*, 2020). When the *B. bissettiae* results were

compared to the *Leptospira* results, no sequence confirmed co-infection appeared. When comparing PCR positives, there were three animals that showed possible co-infection. *Leptospira borgpetersenii* was sequence confirmed in the kidney of a jumping mouse but the liver was PCR negative for *Leptospira* spp. yet PCR positive for *B. bissettiae*. *Leptospira* spp was PCR confirmed in the liver of a brown rat and the kidney was PCR positive for *B. bissettiae*. *L. borgpetersenii* was sequence confirmed in the liver of a meadow vole as well as PCR confirmed for *B. bissettiae*. As these samples were previously collected and tested for the presence of *B. miyamotoi* and *B. burgdorferi* (Zinck & Lloyd, 2022), PCR positives and sequence confirmed cases were compared in individual samples for the presence of coinfection. Four *B. bissettiae* PCR positive deer mice were also PCR positive for *B. miyamotoi* and one bird (species unknown) was PCR positive for *B. bissettiae* and *B. burgdorferi*, although none were sequence confirmed for any of the *Borrelia* species mentioned. The two *B. bissettiae* sequence confirmed samples did not return a sequence positive or PCR positive result for *B. miyamotoi* or *B. burgdorferi*. Data on which organs were infected with *B. miyamotoi* or *B. burgdorferi* was not available, therefore, coinfection comparisons within an individual was not possible.

Lewis and Lloyd (2019) reported of the 189 *I. scapularis*, eight *Dermacentor variabilis*, and three *I. cookei* tested for *B. bissettiae*, seven were infected with the bacteria. This might suggest that the bacteria would have been identified in more wildlife samples. Some possible reasons for this discrepancy may be differences in the collection locations and times of the tick and small wildlife samples. In Lewis and Lloyd (2019) the tick samples analysed were collected over three years from 2014 to 2016 and whereas this study looked at wildlife collected over two years from a much more restricted range. As their study covered the Saint John, greater Moncton/Dieppe area, Port Elgin, and Bathurst, ours mainly covered the greater Sackville area, extending the zone of small mammal collection would provide a better understanding of the infection incidence for *B. bissettiae*, and other zoonotic pathogens. Additionally, detecting *Borrelia* in a tick might be easier than a mammal due to differences in

bacterial load in the organism as well as competing host DNA. Nevertheless, this study was able to identify two wildlife species with an active infection of *B. bissettiae*. This means that the pathogen has been introduced to local wildlife species; neither the meadow vole nor deer mouse were likely to be long distance migrants or intentionally imported. Although *B. bissettiae* may continue to be maintained only in enzootic cycles, these results still show that *B. bissettiae* may be a threat to both human and animal health.

Conventional testing for *B. burgdorferi* uses a two-tiered serological approach; generally, a positive ELISA followed by an immunoblot (western blot) (Lloyd & Hawkins, 2018). These tests are optimized for highly specific, if not necessarily sensitive, detection of one strain of *B. burgdorferi* (Lloyd & Hawkins, 2018); their ability to detect *B. burgdorferi* strains infecting people in Canada varies by region (Ogden *et al*, 2015) and their sensitive towards other *Borrelia* species, such as *B. bissettiae* would be expected to be lower. Additionally, Tjernberg *et al* (2009) confirmed that ELISAs were unreliable when it came to the confirmation of multiple species of *Borrelia* in Swedish patients.

Conclusions

This study looked at the presence of *Borrelia bissettii* in the local wildlife, small and large, mammals and birds, in New Brunswick and documents the first instances of *B. bissettiae* in Canadian wildlife. Consistent with studies in the United States and Europe, rodents appear to be the primary reservoirs for this pathogen. With the northward expansion of ticks populations in the province, and throughout Canada, and the presence of the bacteria in the local *Ixodes scapularis* ticks, this finding is not unexpected. It is, however, of concern for the health of humans and companion and agricultural animals. *B. bissettiae* has been shown to be pathogenic in humans and current Lyme disease testing system does not address a wide range of *Borrelia* strains and species, increasing the likelihood of these infections being missed. The significance for veterinary medicine needs to be explored. With *B.*

bissettiae found in wildlife in an Eastern Canadian province, wider scale surveillance in Canada is required. Further, the evolution of our diagnostic systems is required to ensure that any human and veterinary infections are detected.

Table 1: Total number of samples tested from each organ compared to the number of PCR positive results and sequence confirmed results for *Borrelia bissettiae* in wildlife.

Species	Kidney			Liver			Bladder			Muscle			Skin			Tongue		
	Total Tested	PCR positive	Sequence Positive	Total Tested	PCR positive	Sequence Positive	Total Tested	PCR positive	Sequence Positive	Total Tested	PCR positive	Sequence Positive	Total Tested	PCR positive	Sequence Positive	Total Tested	PCR positive	Sequence Positive
<i>Acanthis flammea</i>	0	-	-	3	0	-	3	0	-	0	-	-	0	-	-	0	-	-
<i>Alces alces</i>	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	99	2	0
<i>Archilochus colubris</i>	0	-	-	0	-	-	1	0	-	1	0	-	0	-	-	0	-	-
<i>Haemorrhous mexicanus</i>	0	-	-	1	1	0	1	0	-	0	-	-	0	-	-	0	-	-
<i>Lepus americanus</i>	2	0	-	2	0	-	0	-	-	0	-	-	0	-	-	0	-	-
<i>Marmota monax</i>	1	0	-	1	0	-	0	-	-	0	-	-	0	-	-	0	-	-
<i>Megascyle alcyon</i>	0	-	-	1	0	-	1	0	-	0	-	-	0	-	-	0	-	-
<i>Microtus pennsylvanicus</i>	128	2	0	123	2	0	18	1	1	10	0	-	6	0	-	0	-	-
<i>Mustela erminea</i>	1	0	-	1	0	-	0	-	-	0	-	-	0	-	-	0	-	-
<i>Napaeozapus insignis</i>	14	1	0	12	1	0	0	-	-	0	-	-	0	-	-	0	-	-
<i>Odocoileus virginianus</i>	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	99	0	-
<i>Peromyscus maniculatus</i>	29	3	1	27	8	0	2	0	-	2	0	-	2	0	-	0	-	-
<i>Picoides pubescens</i>	0	-	-	1	0	-	1	0	-	0	-	-	0	-	-	0	-	-
<i>Procyon lotor</i>	2	0	-	1	0	-	0	-	-	1	0	-	0	-	-	0	-	-
<i>Rattus norvegicus</i>	9	1	0	9	0	-	5	0	-	5	0	-	5	0	-	0	-	-
<i>Sciurus carolinensis</i>	3	0	-	3	0	-	0	-	-	0	-	-	0	-	-	0	-	-
<i>Sciurus vulgaris</i>	2	0	-	2	0	-	1	0	-	1	0	-	1	0	-	0	-	-
<i>Sorex maritimensis</i>	25	0	-	24	0	-	0	-	-	1	0	-	1	0	-	0	-	-
<i>Spinus pinus</i>	0	-	-	2	0	-	2	0	-	0	-	-	0	-	-	0	-	-
<i>Spinus tristis</i>	0	-	-	1	0	-	1	0	-	0	-	-	0	-	-	0	-	-
<i>Tamias striatus</i>	2	0	-	2	0	-	0	-	-	0	-	-	0	-	-	0	-	-
<i>Ursus americanus</i>	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	85	1	0
Unknown species	19	0	-	17	0	-	1	0	-	0	0	-	0	0	-	0	-	-
Total	237	7 (2.9%)	1 (0.4%)	233	12 (5.1%)	0	37	1 (2.7%)	1 (2.7%)	21	0	0	15	0	0	283	3 (1.1%)	0

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Chapter 4 – Statement of Authorship

The formalin-fixed paraffin embedded blocks and the autopsy report was provided to our lab by the department of pathology and laboratory medicine of the QE2 in Halifax, NS, Canada. The remainder of the work completed for this chapter (slide preparation, staining, imaging, creation of figures and tables) was completed by Samantha Bishop, with editing and supervision by Dr. V. Lloyd.

Chapter 4

A case study of Lyme Carditis

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Abstract

Lyme disease, caused by the *Borrelia burgdorferi* sensu lato complex, that affects the heart in its disseminated form is known as Lyme carditis. This outcome is rare, with approximately 1-2% of all Lyme disease cases being diagnosed as Lyme carditis. Missed diagnoses in these cases can become fatal. This study presents a case of fatal severe heart disease following suspected untreated Lyme carditis of a 17-year-old female from Nova Scotia, Canada. Immunohistology of sections of the heart, lung, kidney, adrenals, and pancreas showed fluorescent structures in similar size and shape to *Borrelia* bacteria. Results from in situ hybridization using DNA probes were unclear. As the samples provided were formalin-fixed paraffin embedded tissues, confirmatory testing through polymerase chain reaction followed by Sanger sequencing was not an available option. Therefore, these structures cannot be confirmed as *Borrelia*.

Introduction

Lyme borreliosis, also known as Lyme disease (LD), results from infection with the *Borrelia burgdorferi sensu lato* (s.l.) (Bbsl), or Lyme borreliosis, cluster of spirochaete bacteria, with the majority of human cases in North America caused by *B. burgdorferi sensu stricto* (s.s.) (Bbss) (Marques, 2010; Sperling & Sperling, 2009). Lyme disease progression is divided into three stages; early localized, early disseminated, and late disseminated (Depietropaolo *et al*, 2005). When the initial signs, such as an erythema migrans (EM) or skin rash, are missed or do not appear on the patient, the dissemination progresses to its disseminated form, where the heart, nervous system and joints are affected (CPHLN,

2007). When the heart is involved, this manifestation is termed Lyme carditis (LC) (Johnson *et al*, 2018). In addition to direct pathology arising from cellular damage from the bacteria, immune response targeting infected cells can target host cells, tissues, and organs (Lochhead *et al*, 2021). LC is considered a rare outcome with only 1-2% of LD cases diagnosed as LC (Cardenas-de la Garza *et al*, 2019). Similarly, based on a 2018 study looking at the clinical manifestations of self- and clinician-reported LD cases in Ontario from 2005 to 2014, these included 75% of clinical manifestations being flu-like and only 6% were cardiac symptoms (Johnson *et al*, 2018). Although the atrioventricular block, with which LC is generally identified, is considered a sign of early disseminated LD, in cases where LD is not identified and/or treated, ongoing cardiac compromise can be expected (Robinson *et al*, 2015; Yeung & Baranchuk, 2019).

The current recommended diagnostic in Canada, and other countries, in support of a disseminated LD diagnosis involves a two-tiered serology test; the first-tier includes an enzyme immunoassay (EIA) with a positive result required before moving to the second-tier western/immuno blot as confirmation (CPHLN, 2007). There are many limitations to relying on this type of testing, it is an indirect test, relying on both the production of host antibodies and the sensitive and specific detection of those antibodies. Host production of antibodies is not useful as a diagnostic tool very early in infection, and attenuates with ongoing infection (Shapiro, 2014). Host genetics, physical conditioning, use of pharmaceuticals, and many other factors may compromise the ability of the host to produce the required level of antibody response for a positive serological result (Shapiro, 2014). Not only are children likely to have lower antibody titres than adults, but antibody titres also decline over time, limiting the use of this diagnostic for long-standing infections (Singh & Girschick, 2004; Sperling & Sperling, 2009). A further problem is posed by diversity of the pathogen. The *B. burgdorferi* strain B31 was used to design the commercial tests that underlie the two-tiered testing system. This strain of Bbss has been shown to cause arthritic symptoms in a higher proportion of patients than other Bbsl species,

leaving other forms of the infection potentially undetectable (Sperling & Sperling, 2009). This is highlighted by regional reactivity with this testing protocol shown by Public Health Agency of Canada (PHAC) researchers (Ogden *et al*, 2017). All these limitations can lead to false negatives, resulting in either delayed treatment, absence of treatment, or misdiagnosis (Shapiro, 2014).

The case study presented here focuses on the immunohistochemical and DNA in situ direct detection of *Borrelia* in autopsied tissues of a 17-year-old female who died of severe heart disease following suspected untreated Lyme disease.

Short History

In September of 2008, the subject, 8 years old at the time, presented with flu-like symptoms at the local outpatients and was sent home after being seen but without medical intervention. The individual experienced severe headaches, vomiting and nausea, fever, rash covering the neck, chest, and back, and dyspnea before returning to outpatients. Initial diagnosis was either a hole in the heart or viral infection; the patient was then taken to the IWK Health centre in Halifax, NS for further testing. The patient was clinically diagnosed with congestive heart failure presumably due to complications resulting from viral myocarditis and was placed on intravenous immune globulin (IVIG) and diuresis. Once the patient's health improved, she was discharged from the hospital and an unknown heart medication was prescribed.

Over the next ten years symptoms worsened and others, including dyslexia (not clinically diagnosed), patella dislocation and hyper mobile knees, heart palpitations, skin rashes, insomnia, anxiety, formication, leg cramps, light, noise, smell and taste sensitivities, derealization, aches and pains throughout the body, chronic heartburn, increase in food, dust and seasonal allergies, cardiomyopathy, dizziness, nightmares, brain fog, overactive bladder, and mumbled speech developed. In addition to the lengthening list of symptoms, her left ventricular function did not recover (persistent dilatation, with the

left ventricular ejection fraction, LVEF, in the 40-55% range) and she remained in mild congestive heart failure.

Although a tick bite was not recalled, the patient did live in an endemic area for LD. A blood test using EIA for Lyme was done at the Victoria General Hospital in Halifax in August of 2017 with a negative result. As the first tier of the two-tiered testing was negative, the second-tier western blot was not performed. The patient was then referred to the company DNA Connexions in the United States by a Naturopath and a urine test identified the DNA of *Borrelia burgdorferi* Osp-C surface protein in the urine. By July of 2018, the patient followed up with another blood test using an EIA at the Victoria General Hospital, but it again returned a negative result. Antibiotics were not prescribed.

Over the years, multiple tests were completed at the hospital, including an exercise stress tests, MRI, CT scans of head and sinuses, and X-rays of head, knees, chest, spine, neck, and abdomen. Visits to their family doctor, pediatricians, cardiologists, internal medicine specialists, physiotherapists, psychologists, naturopath, osteopath, massage therapists, and emergency room doctors occurred frequently. The individual, with full parental support, regularly visited their cardiologist and was compliant in taking heart medications and supplements, in addition to a restricted diet (removal of all grains, sugars, and dairy) and daily exercise to reduce symptoms and stay healthy. The patient took multiple natural supplements to manage symptoms; these supplements included magnesium, vitamin c, vitamin d, fish oil, coq10, 5HTP, tincture of knotweed, cat's claw, stevia, chase tree, dong quai, wild yam, in addition to regular massages to manage the body aches.

In July of 2018 the patient returned to the emergency department at the Valley Regional Hospital after 4 days of vomiting, chest heaviness, and shortness of breath. She was diagnosed with acute decompensated congestive heart failure and transferred to the Pediatric Intensive Care Unit at the IWK. The following day she was transferred to the Cardiac Care Unit of the QE2 Health Sciences Center where troponin levels were reported as 'high'. Implantation of bi-ventricular assist devices,

tracheostomy, and bilateral chest tube insertion was required, but was complicated by a liver laceration, requiring laparotomy and washout. During this time, she developed ventilator-associated pneumonia, with *Staphylococcus aureus* and *Enterobacter cloacae*. Progress was steady with physical rehabilitation, and she was weaned from the ventilator with the tracheostomy decannulated after 21 days. Nearly 4 months later, the patient developed sudden respiratory distress, alleviated by suctioning of bronchial blood clots through the tracheostomy site. As oxygenation worsened, oro-tracheal intubation was required and the right ventricular assist device filled with air, ultimately failing. The patient passed away the following morning with the official cause of death reported as severe biventricular failure due to the consequence of myocarditis, significant respiratory distress, pulmonary and cardiovascular collapse.

Autopsy confirmed dilated cardiomyopathy leading to severe pulmonary vasculopathy, causing fatal respiratory distress due to acute pulmonary hemorrhage. The etiology of her dilated cardiomyopathy was still unclear after autopsy; however, viral myocarditis or genetic cause was suggested. Neuropathologic examination revealed evidence of a peripheral neuropathy, with loss of ascending fibers in the spinal cord, which could share a possible etiological link with dilated cardiomyopathy. The parents, with support of the IWK, requested molecular testing for *Borrelia* in formalin fixed paraffin-embedded organ samples.

Methods

Tissue donor, informed consent, and sample tissues

This study was initiated at the request of the tissue donor's family. Signed informed consent for use of the tissues for LD research was obtained from the donor's family. All results reported here are from testing performed in the laboratory of Dr. Vett Lloyd at Mount Allison University, Sackville, New Brunswick and approved by the Mount Allison Research Ethics Board (2016-042/ 101796). The laboratory is an academic laboratory specializing in molecular genetics. The laboratory is not a clinical laboratory. The consent for this donation specifies that this testing was performed as part of a larger

research project and individual results, while returned to the donor's family cannot be used to direct clinical treatment or for legal purposes. Health history and redacted versions of patient copies of some medical documents were provided by the donor's family; no medical documentation from the NS health authority was obtained with the exception of the autopsy final report, which was provided by the department of pathology and laboratory medicine of the QE2 in Halifax, NS, Canada. The autopsied tissues arrived on March 2nd, 2020 as formalin-fixed paraffin embedded (FFPE) blocks and were derived from the heart, larynx, tracheostomy site, lungs, liver, kidneys, adrenals, ovaries, spleen, thyroid, pancreas, brain, and spinal cord.

Positive and Negative Control Slides

B. burgdorferi strain B31 (ATCC, 35210) were used to infect mammalian HeLa cell lines (ATCC, CCL-2) to create a positive control for immunohistology staining. *Borrelia* cells were grown in BSK-H medium with 6% rabbit serum (Darlynn Biologicals, lot# BB83-08251C) in PYREX™ Screw Cap Culture tubes (25ml, CAT 14-933C, Fisher Scientific) at 34 °C. Cell growth was monitored by cell counting with a Leitz phase contrast microscope. HeLa cells were grown in a T75 flask (Celltreat, CAT 229341) in DMEM media (Lonza, CAT 12-733F) supplemented with 10% v/v heat-inactivated FBS (Rockland, CAT FBS.02-0500), each 1% v/v L-glutamine (Lonza, CAT 17-605E), sodium pyruvate (Lonza, CAT 13-115E), and penicillin/streptomycin (Sigma-Aldrich, CAT P4333). HeLa cells were thawed and cultured according to standard cell culture proceedings under sterile conditions following the protocol outline by American Type Culture Collection (ATCC, 2022), and incubated at 37°C, 5% CO₂ and humidified air (Nuair, Model NU-5500). Cell growth was monitored daily with an inverted cell culture microscope (Motic, AE31) with camera set up and media was replaced of medium about every three days until cell density reached 8×10^5 cells/cm². At this point cells were washed with phosphate-buffered saline (DPBS) (Thermofisher, CAT 14190144), were detached from the cell surface with trypsin (Lonza, CAT CC5012) and seeded for experiments. In each well (growth area of 9.6 cm²) poly-L-lysine coated coverslips (Mandel Scientific,

CAT NEU-H-20-PLL) were placed and cells in a density of 4000 cells/cm² were added. Cells were incubated at 37 °C, 5% CO₂ and humidified air (Nuair, Model NU-5500) until 48 hours post seeding, when cells were infected with *Borrelia*. The number of *Borrelia* cells and HeLa cells were determined with the aid of a hemocytometer (Hausser Scientific, CAT 1492).

For the positive control, HeLa cell monolayers on coverslips were prepared for infection by removal of the medium and rinsed three times with PBS. The *Borrelia* were pelleted at 4,500 X g for five minutes to remove bacteria media and resuspended in antibiotic-free DMEM before being added to the HeLa cells. A multiplicity of infection (MOI) of 100 was used. For the negative control, the medium was replaced with fresh DMEM. Both infected and uninfected cells on coverslips were incubated for 72 hours at 37 °C.

Negative and positive control cells for immunohistology staining, as described above, were prepared by removing media and fixating the cells using ice-cold 100% methanol (Fisher Scientific, CAT A412-1) for five minutes at room temperature, followed by three rinses with ice-cold Phosphate buffered saline (PBS) (2.7 mM potassium chloride, 137 mM sodium chloride and 10 mM Phosphate Buffer) (Sigma, CAT PD0435). PBS was removed and coverslips with cells were incubated with 10% goat serum (Gibco by life technologies, CAT 16210-064) in PBS with 1% bovine serum albumin (BSA) (Sigma, CAT A7906). Following the removal of the goat serum, coverslips with cells were incubated with the primary antibody. The primary antibody which was a rabbit polyclonal antibody to *Borrelia burgdorferi* (abcam, ab20950) was used in 1:100 dilution in PBS with 1% BSA. Incubation took place in a humidifying chamber for 24 hours at 4 °C. The solution was decanted, and coverslips with cells were washed in PBS three times for five minutes. The following steps were carried out in a dark room to prevent photobleaching. Coverslips with cells were incubated with the secondary antibody. The secondary antibody was goat anti-rabbit IgG H&L (FITC) (abcam, ab6717) and diluted 1:100 in PBS with 1% BSA. Incubation took place in a humidifying chamber for one hour room temperature. The solution was

decanted, and the coverslips with cells were washed in PBS three times for five minutes. Coverslips were mounted (cells facing down) using 30% glycerol and sealed with nail polish before imaging with the ImageXpress® Pico imaging system (Molecular Devices).

Tissue Preparation

Tissues were prepared for staining by creating 5-7 μm sliced sections using a Leica RM 2135 BioCut Rotary Microtome. The cut tissue was floated in a Fisher Scientific Paraffine Bath (Model 168) at 5-7 °C to remove wrinkles and distortion in the tissues before adhered to electrostatically treated microscope slides (Fisherbrand™ Superfrost™ Plus, CAT# 22-037-246). To prepare the slides for staining, the paraffin sections were dewaxed and rehydrated following the IHC deparaffinization protocol outlined by abcam (Abcam, IHC deparaffinization protocol) with a few alterations. To begin the dewaxing, slides were baked at 60 °C for 20 minutes, followed by three 100% xylene baths to completely dewax the samples. Then, three serial ethanol baths were used at varying concentrations (95%, 70%, and 50% ethanol) to rehydrate the samples. The incubation time for each bath was three minutes. Samples were then placed in the final bath of RO water for three minutes, or until they were ready to be stained. From this step onward, slides were not allowed to dry to prevent non-specific antibody binding which can lead to high background staining.

Borrelia Immunohistology

Borrelia immunohistology was performed as described by abcam's IHC staining protocol (Abcam, IHC staining protocol; paraffin, frozen and free-floating sections). The tissue sections were washed twice for five minutes using PBS plus 0.025% of Triton X-100 with gentle agitation followed by blocking with 10% normal goat serum (Life technologies) in 1X PBS with 1% bovine serum albumin (BSA; Sigma-Aldrich) in a humidifying chamber for two hours at room temperature. The slides were then washed three times using 1X PBS with 1% BSA and incubated at 4 °C overnight in a humidifying chamber with 1:100 dilution (in 1X PBS with 1% BSA) of the primary antibody; rabbit polyclonal antibody to

Borrelia burgdorferi (abcam, ab20950). The technical negative control slides used nuclease-free water in place of the primary antibody in 1X PBS with 1% BSA. All following steps remain the same for negative control slides. After overnight incubation, the slides were washed twice using 1X PBS with 0.025% Triton X-100 with gentle agitation. The following steps were performed in a dark room to prevent photobleaching; the secondary antibody, goat anti-rabbit IgG H&L conjugated with Alexa Fluor® 488 (abcam, ab150077), was diluted 1:100 in 1X PBS with 1% BSA for 1 hour at room temperature in a humidifying chamber, then rinsed three times for 5 minutes using 1X PBS with gentle agitation, and drained to remove excess solution. To reduce background fluorescence, 1X TrueBlack Lipofuscin Autofluorescence Quencher (Biotium, 23007) diluted in 70% ethanol was added to each sample for 30 seconds before rinsing three times with 1X PBS. Thirty % glycerol was added, and the section covered by a coverslip, sealed with quick dry nail polish. Stained sections were examined using the ImageXpress® Pico imaging system (Molecular Devices).

Tissue in situ DNA detection using Molecular Beacon and FISH probes

Two molecular beacons probes were used to target, respectively, the *B. burgdorferi* *OspA* gene with the single labelled (5' – [6FAM]ATATATTC AAGGAATTCGATGACATC-3') probe and the *B. burgdorferi* *FlaB* gene targeted by the 23-base-long double-labelled hairpin molecular beacon probe (5'- [6FAM]CGCGATCTGGGAGTTTCTGGTAAGATTAATGATCGCG-(BHQ®-1) - 3') (Sigma). The staining protocol as described by Middelveen *et al* (2014), was used with minor alterations. A working solution containing 108µl of nuclease-free water, 12µl of stock beacon solution (*OspA* or *FlaB* beacon probe diluted 1:10 in nuclease-free water), and 12µl of 10X Promega Buffer H was added to each slide, followed by 10 µl of 100% dimethylformamide (DMF). A working solution containing 120µl of nuclease-free water and 12µl of 10X Promega Buffer H was added to each negative control slide to assess any autofluorescence. A section of plastic cut from a Ziploc® bag was used to cover the section and prevent the area from drying while it was heated to 90 °C for 10 minutes to denature DNA, followed by 72 °C for 30 minutes before

gradually cooling the slides to room temperature overnight. The next day, samples were rinsed with 1X PBS three times and 30% glycerol was added before applying a coverslip. Stained sections were examined using the Zeiss Axioskope 2 plus epi-fluorescence microscope using the filter set 09, with 450-490 nm BP excitation filter, and images were taken using the AxioCam HR camera with AxioVision version 4.9.1.0 software.

Results

Positive and Negative HeLa Control Cells

Immunohistology of infected compared to noninfected HeLa cells showed the spirochetal and round body forms of *Borrelia* in and around the HeLa cells (Fig. 1). It is possible that the spirochaetes were beginning to form into a biofilm-like colony, as seen in figure 1C, shown by the red circle. These figures aided in visual identification of *Borrelia* in the autopsied samples as other forms of confirmation, such as PCR, were not an available option.

Borrelia Immunohistology

Immunohistology showed fluorescent structures in similar size and shape to *Borrelia* bacteria in many areas of the heart (left anterior descending artery, right coronary artery, left ventricle lateral and inferior, right ventricle anterolateral apex and mid, right ventricle inferior and right ventricular outflow tract) (Fig. 2, Fig. 3). Additionally, the right middle lobe of the lung, the kidney, adrenals, and pancreas (Fig. 4) showed immunofluorescent structures. Autofluorescence appearing in the technical negative controls, as seen in Figure 3, E through H, is typical of lipids, red blood cells that have pooled in section vessels, and protein aggregates, with differentiation made based on shape and size of fluorescent structures. The tissues that did not show any fluorescent structures, or only autofluorescence, were the ovaries, spleen, thyroid, and tracheostomy site (Fig. 6). Due to time constraints, only the right superior and middle temporal gyri, left dentate nucleus and cerebellum, and the cervical/thoracic and lumbosacral regions of the spinal cord were stained with no resulting immunofluorescent structures.

In situ hybridization using DNA probes

Due to time constraints and technical issues with imaging, only eight samples were examined using this method; four from the heart (left circumflex artery, left ventricle anterior mid, left ventricle inferior mid, and right ventricle anterolateral mid), two from the lung (right upper lobe and right middle lobe), and two from the spinal cord (cervical/thoracic and lumbosacral). As there were signs of autofluorescence and/or non-specific binding, it is unclear whether any fluorescence seen in Fig. 5 A-D can be confidently reported as *Borrelia*.

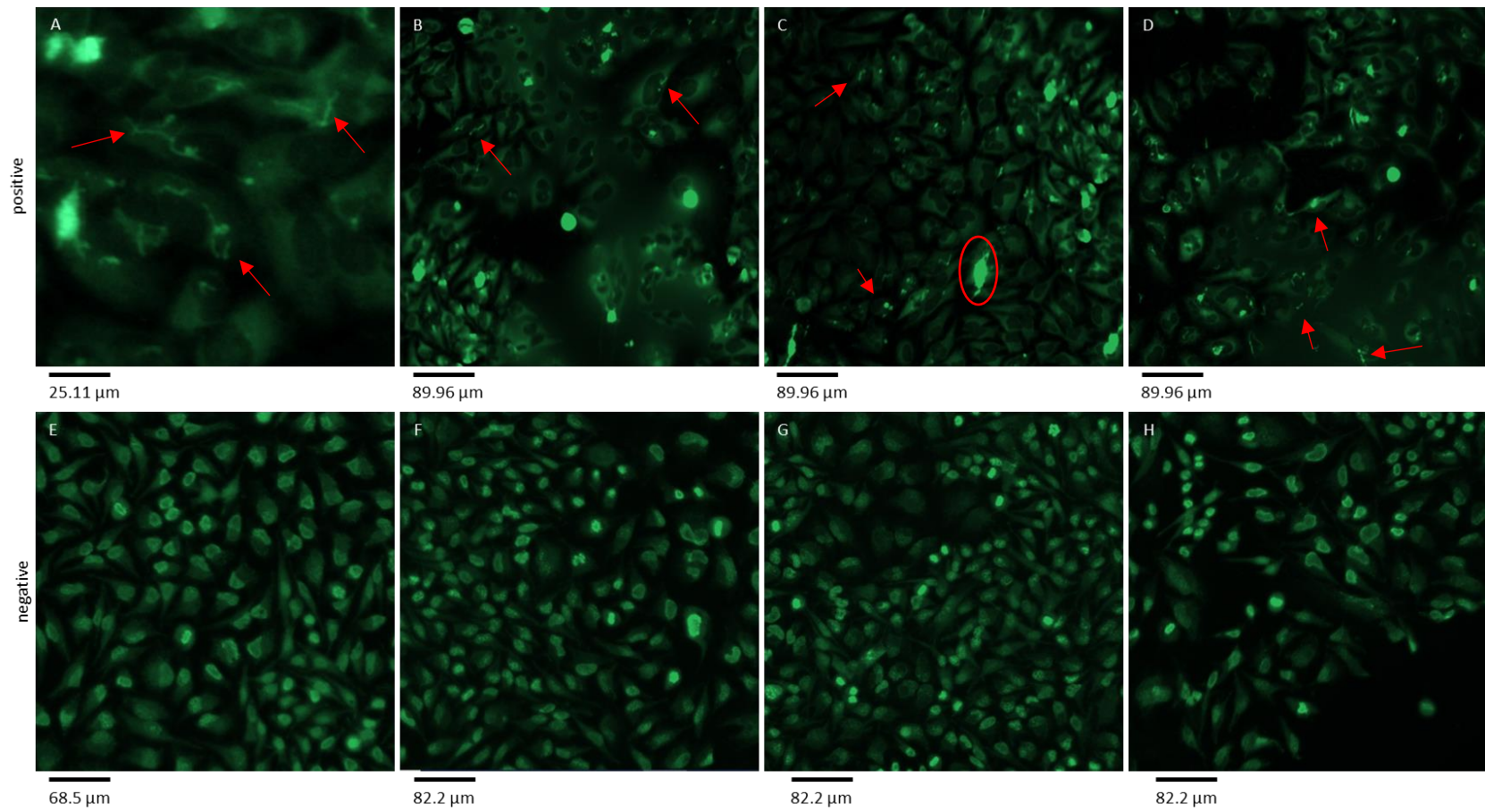


Figure 1: HeLa cells infected with *B. burgdorferi* strain B31 (A, B, C, D) and uninfected HeLa cells (E, F, G, H), 200X magnification with digital zoom. Arrows indicate spirochaete-like structures whereas the circle indicates a biofilm-like colony.

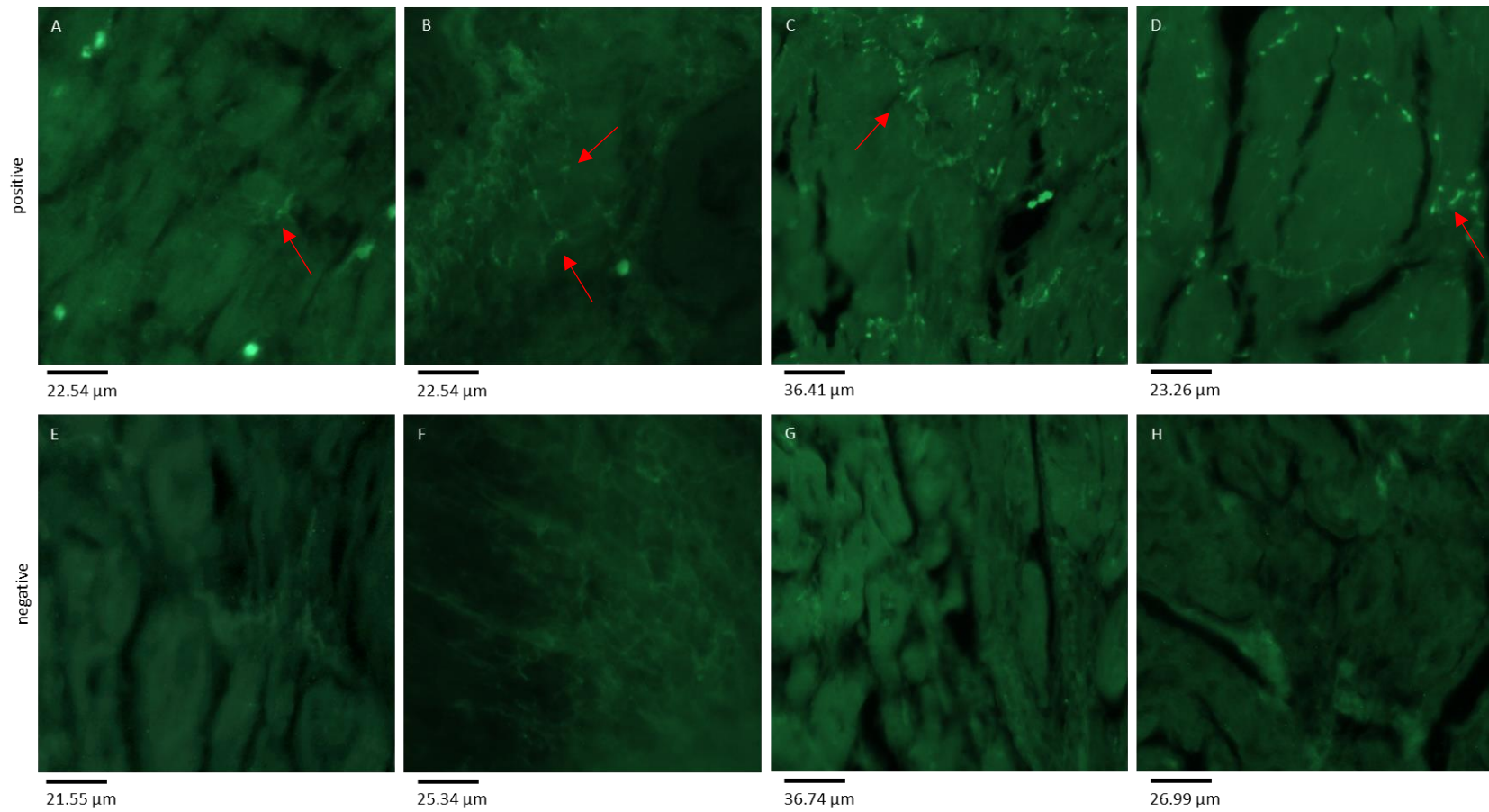


Figure 2: *B. burgdorferi* in spirochetal form within the heart, left ventricle (LV) inferior, mid (**A, B, C, D**) and the corresponding negative controls directly below (**E, F, G, H**) at 200X and imaged using digital zoom. Arrows indicate spirochaete-like structures.

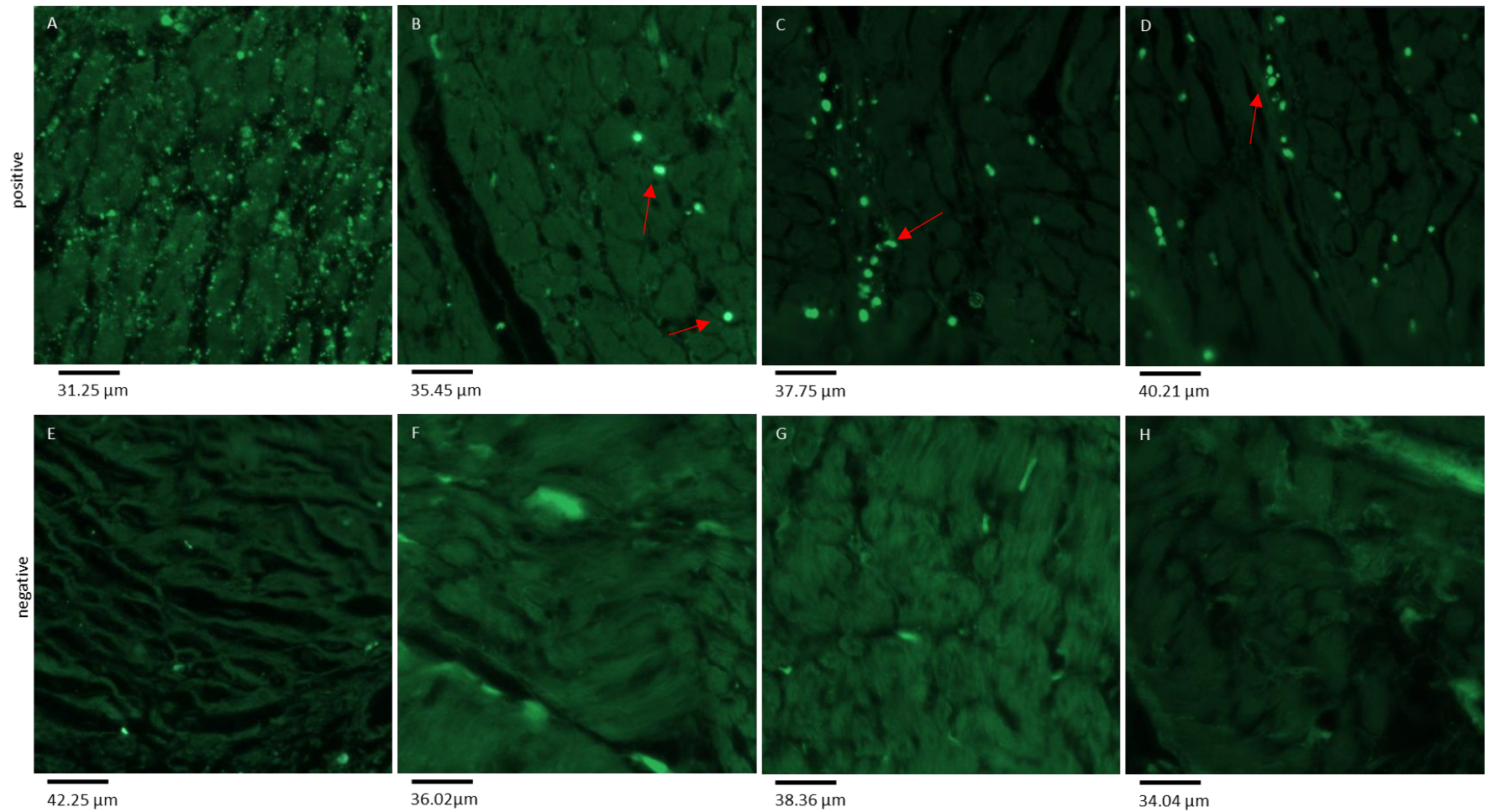


Figure 3: *B. burgdorferi* round bodies in the heart, right ventricle (RV) anterior mid (**A, B, C, D**) and the corresponding negative controls directly below (**E, F, G, H**) at 200X and imaged using digital zoom. Arrows indicate round body-like structures. Autofluorescence seen in the technical controls (**E, F, G, H**) are likely lipids, pooled red blood cells within sectioned vessels, and protein aggregates.

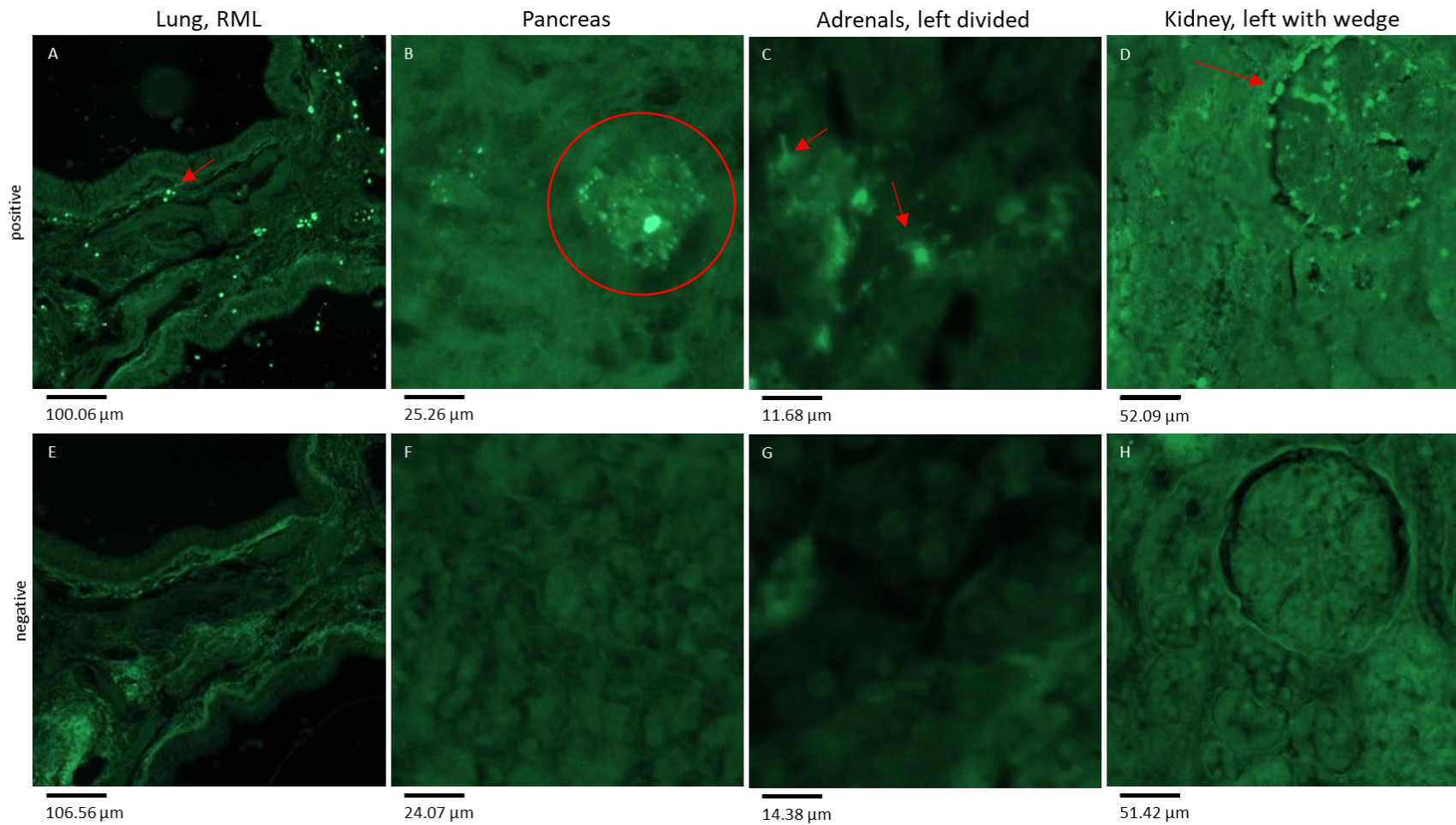


Figure 4: *B. burgdorferi* in non-cardiac organs at 200X and imaged using digital zoom; round bodies in the right middle lobe of the lung (A), biofilm-like structure in the pancreas (B), spirochaetes in the left adrenals (C), round bodies in the left kidney (D), and their technical negative controls directly below (E, F, G, H).

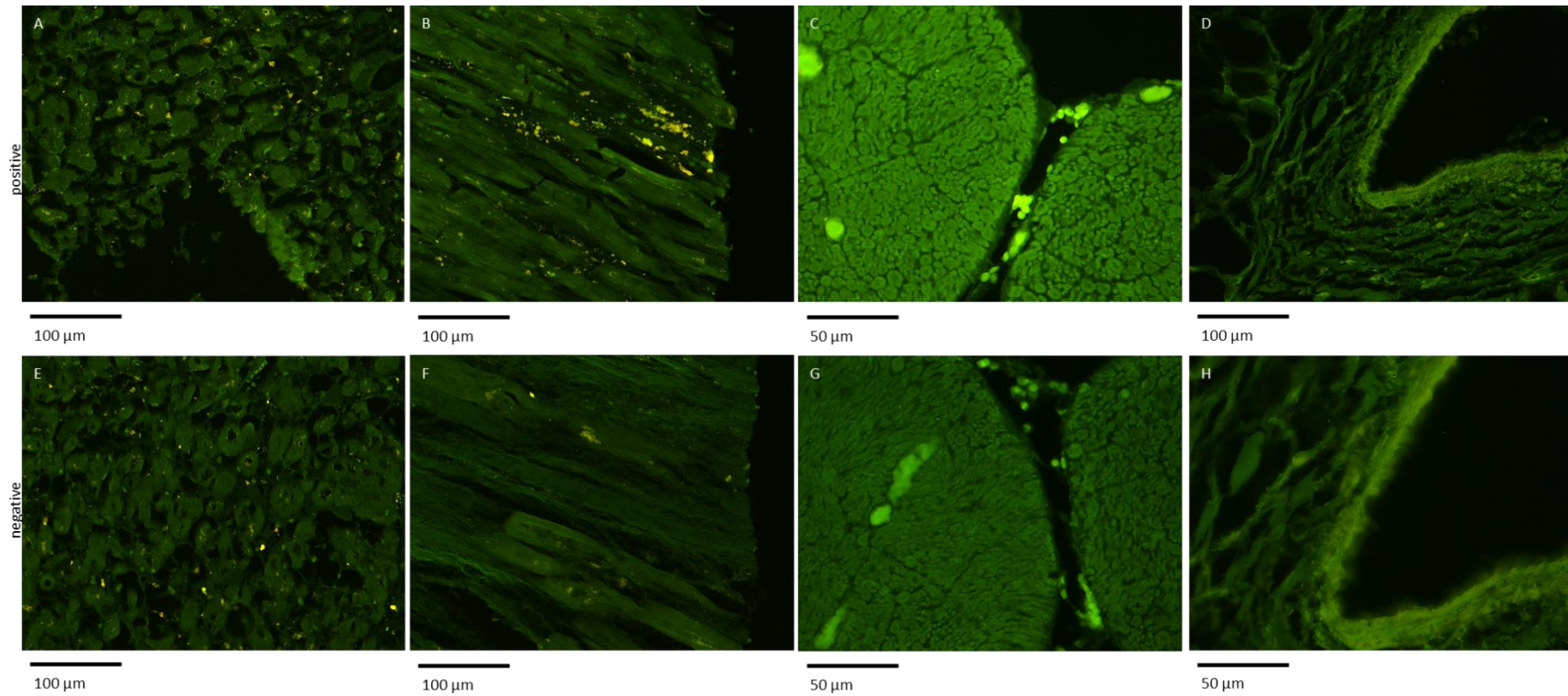


Figure 5: In situ hybridization using the *ospA* probe on the left ventricle of the heart (**A, B**) with the corresponding technical negative controls (**E, F**) directly below at 200X magnification. In situ hybridization using the *FlaB* probe on the cervical/thoracic portion of the spinal cord (**C**) and the corresponding negative control below (**G**) at 400X magnification and on the right ventricle of the heart at 200X magnification (**D**) and the corresponding negative control below at 400X magnification (**H**).

Discussion

Reported cases of LD in Canada have increased 20-fold within the last 13 years; a total of 14,616 human cases reported to the Public Health Agency of Canada, with the majority of these cases occurring in Eastern Canada (Government of Canada, 2022). These numbers are thought to be a serious underestimate as LD cases in Canada are likely about 10-fold under-detected, as they are in the USA and other countries (Lloyd & Hawkins, 2018). In Nova Scotia, the first endemic region was recorded in 2003 after *B. burgdorferi*-infected *Ixodes scapularis* tick populations were recorded and currently the entire province of Nova Scotia is designated as a high-risk area for contracting LD (Government of Canada, 2022; Hatchette *et al*, 2015). Between 2002 and 2013, 329 cases of LD were reported in Nova Scotia, with 19.4% of cases occurring in individuals under 20 years of age, and 1.2% of all cases presenting with symptoms targeting the cardiovascular system (Hatchette *et al*, 2015). Although thought to be rare, LD can manifest into LC once *Borrelia* enters the heart, affecting atrioventricular conduction and rhythm disturbances, with potential myocarditis or pericarditis occurring (Stanek *et al*, 2012). LC can easily be misdiagnosed as symptoms tend to vary with individuals presenting with nonspecific flu-like symptoms from 4 days to 7 months post tick-bite (Costello *et al*, 2009; Fish *et al*, 2008).

The two-tiered testing system Canada uses to detect *Borrelia* infection in individuals using an EIA followed by an IgG/IgM immunoblot was developed to optimize detection of *B. burgdorferi* strain B31, a species and strain of *Borrelia* best known for inducing arthritic symptoms in patients with LD (Sperling & Sperling, 2009). As this system still had room for improvement, a modified two-tiered testing system with improved sensitivity was recently developed for laboratory diagnosis of LD in Canada. According to the study published by Hatchette and Lindsay (2020), both the standard two-tiered testing system and the modified two-tiered testing system lack the sensitivity needed to identify infection in LC cases, with sensitivity ranging from 48-60.2% to 61-66.8%, respectively.

Borrelia burgdorferi is the most common *Borrelia* species in ticks in Canada, although *Ixodes scapularis* ticks in Nova Scotia have been infrequently found carrying Human Granulocytic Anaplasmosis (HGA), *Borrelia miyamotoi*, Babesia, and Powassan virus (Government of Canada; Government of Nova Scotia). In this case study, infection of *B. burgdorferi* was assumed based on the area in which the patient resided and urine test results, although these were not supported by serological testing. The patient resided in an endemic region of Nova Scotia in which *B. burgdorferi*-infected *Ixodes scapularis* ticks have been documented by public health researchers (Government of Nova Scotia; Hatchette *et al*, 2015; Bouchard *et al*, 2015). Fluorescent structures produced after immunohistochemistry staining structures the expected size and shape for *B. burgdorferi* were observed. The patient had evidence of spirochetal (10-20 μm), round body (1.3-2.8 μm), and biofilm forms (colony of >10 spirochaetes/round bodies) (Meriläinen *et al*, 2015) of *B. burgdorferi* infection in autopsied sections in multiple organs, in addition to the heart. Like many other bacterial species *in vitro*, *B. burgdorferi* is pleomorphic, changing its morphology in response to its environment and to evade the immune system and antibiotics, transforming into spherical shapes (commonly referred to as round-bodies) and biofilm-like colonies in addition to the typical spirochetal form (Berndtson, 2013; Karvonen *et al*, 2021). When *B. burgdorferi* was grown in BSK-II medium, to simulate conditions within a human host, or mammalian cell culture medium RPMI, the number of spirochetal forms decreased to 10-24% after a 4-day incubation period, with the cells mainly morphing into round bodies (Meriläinen *et al*, 2015). This change in morphology is crucial to the survival of the bacteria in harsh environments and can allow persistent infection as the “persisters” typically form into antibiotic-tolerant-biofilms (Rudenko *et al*, 2019).

We cannot confirm the structures seen here are indeed *Borrelia*, as the typical confirmatory test, polymerase chain reaction (PCR) followed by Sanger sequencing, was not available due to the samples being formalin-fixed. FFPE tissues, while stable at room temperature for storage, does not preserve the DNA integrity needed for robust DNA amplification or sequencing technologies (Lou *et al*,

2014). Similarly, culture of these samples is not possible as the cells in the samples are dead. The need for analysis of forensic and historical specimens is pushing the development of methodologies to confirm the presence or absence of spirochaetes in autopsy and biopsy specimens and methodologies for the use of damaged DNA are being developed. A need for caution regarding the interpretations of these findings is due to the incompletely validated sensitivity and specificity of the DNA and antibody probes used. The DNA probes, while reportedly specific to *B. burgdorferi* (Middelveen *et al*, 2014), have not been well validated. The primary antibody used in this study does detect *B. burgdorferi*, however, it also cross-reacts with other spirochaetes, such as *Treponema pallidum*, *B. hermsii*, *B. parkerii*, and *M. tuberculosis* (Abcam). Thus, these results cannot unambiguously differentiate between the presence of *B. burgdorferi*, other *Borrelia* species, or other spirochaetes. Further staining of these sections using and monoclonal antibodies, which can provide increased specificity, homogeneity, consistency (Xi *et al*, 2003; Lipman *et al*, 2005), would be beneficial in resolving this question. Most importantly, a better understanding of the connection between *Borrelia* in a tissue and disease is needed. In the context of autopsy specimens or conventional histological stains in combination with *Borrelia* immunohistology would allow examination of any correlations between the presence and location of *Borrelia* and histopathological changes in the tissues. Additionally, staining with antibodies against disease-associated proteins, and DNA probes against other pathogens, RNA probes against transcripts, chemical probes for other cellular processes (i.e., DNA damage, apoptosis, mitochondrial malfunction), would allow a more sophisticated understanding of the correlation between *Borrelia* presence and cellular, tissue, and organ function or malfunction. Ultimately, to decipher a casual relationship, examination of *in vitro* models or animal models, in which controlled experiments can be performed, would be needed.

Although mortality is rare, there are case reports of fatal LC (Yoon *et al*, 2015; Semproni *et al*, 2020; CDC, 2013). The prognoses for LC, if treated early, is favourable with most patients and children recovering following antibiotic treatment (Yeung & Baranchuk, 2018; Costello *et al*, 2008). A case study

from 2019 analyzed immunohistology and FISH stained biopsied heart sections obtained due to an aortic valve replacement from a patient previously diagnosed and aggressively treated with antibiotics for LD (Haque, 2019). Similar to our case study, the patient did not recall a tick bite and had a negative Canadian serology test but positive US-based testing, in that case, a western/immune-blot. Based on exposure history and clinical presentation, the patient was treated with multiple antibiotics and natural supplements were prescribed until their health was restored. The only tissues that showed immune-positive structures indicating presence of *Borrelia burgdorferi* were in the connective tissue associated with the left internal thoracic artery, hypothesized to be due to residual dead *Borrelia* cells. In contrast, the individual in our study had evidence of immune-positive structures throughout multiple sections of the heart, as well as other organs. Although these reflect only two case studies, they highlight different health outcomes associated with antibiotic treatment in the early stages of Lyme disease.

Conclusion

This case study reports the presence of immunopositive structures in similar size and shape to *Borrelia* bacteria in the heart, lung, kidney, adrenals, and pancreas of an individual who died due to cardiac failure, suspected to be a case of LC. These results highlight the need for additional methodologies suitable for analysis of FFPE specimens and more fundamental work on the relationship between *Borrelia* presence and cellular, tissue, and organ damage. Ultimately, these findings, in comparison with outcomes in cases where treatment did occur, demonstrate the critical need for improved diagnostics and treatment for borreliosis.

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Conclusion to the Thesis

The research described in this thesis took a One Health approach, which integrates environmental, animal, and human health. In this thesis, I focused on the environment, animal health, and human health as it relates to Leptospirosis and Borreliosis. In chapter two, I tested water as a source of *Leptospira* infection within wildlife populations and companion animals. An increase in rainfall and flooding events is likely to increase the frequency of leptospirosis outbreaks in communities. Climate change has also been shown to be affecting the natural environment. Climate change has produced milder temperatures throughout the years that have allowed the northward expansion of tick populations, and the pathogens they transmit, as well as wildlife species that act as pathogen reservoirs. Tick species establishing in New Brunswick includes *Ixodes scapularis*, which is responsible for the transmission of many diseases including Borreliosis (Lyme disease). The risk posed by this tick vector of zoonotic disease was highlighted in Chapter Two for Leptospirosis and Chapter Three for *Borrelia bissettii*. Wildlife featured prominently in this work, on both Leptospirosis and Borreliosis, in both Chapters Two and Three. Chapter Four emphasized the human health impact of zoonotic diseases through a case study of *Borrelia* and Lyme carditis.

The *Leptospira* and *Borrelia* spirochaetes pose a health risk to both humans and their animal companions. As the climate changes within New Brunswick, as is true throughout Canada and the rest of the world, the risk of infection from these zoonotic diseases rises. Understanding not only the geographic range of these diseases, but also knowing which species of wildlife animals are infected allows us to create a better understanding of the risk. This study shows the importance of a One Health approach to integrate and understand the connectedness of human health, animal health, and environmental health. If we do not maintain the health of our environment and our wildlife, humans become more vulnerable to diseases and the attendant suffering.

Appendices



Appendix 1 Sites along the Saint John River that provided a water sample for the spring 2020 session (as shown by the circles and triangles) as well as the sites that provided a sample for the fall 2020 session (as shown by the triangles only). Flood risk areas are highlighted in light blue.

New Brunswick				
WMZ 1	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human				2019
WMZ 2	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human				
WMZ 3	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human				
WMZ 4	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2020	2018	2020	
Cat		2019	2020	
Human				
WMZ 5	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2018	2020	2020	
Cat		2019	2019	
Human			2020	
WMZ 6	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human				
WMZ 7	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human			2018	
WMZ 8	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				2018
Cat				2017
Human				
WMZ 9	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2016	2018	2019	
Cat			2017	
Human		2017	2020	
WMZ 10	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog		2019	2020	
Cat		2018	2020	
Human			2019	
WMZ 11	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog				
Cat				
Human				
WMZ 12	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog			2018	
Cat			2019	
Human				
WMZ 13	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2017			
Cat				
Human				
WMZ 14	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog		2018	2018	
Cat			2019	
Human	2017	2019		
WMZ 15	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog		2020	2020	
Cat		2019	2019	
Human	2019		2017	
WMZ 16	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2020	2020	2020	
Cat		2020	2020	
Human	2020		2018	
WMZ 17	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog		2016?	2019	
Cat			2020	
Human				
WMZ 18	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog			2019	
Cat		2020	2017	
Human	2018		2020	
WMZ 19	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2019	2020	2020	
Cat		2020	2020	
Human	2018		2020	
WMZ 20	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2020	2020	2020	
Cat		2019	2020	
Human	2020	2019	2020	
WMZ 21	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2020	2019	2020	
Cat			2019	
Human	2020		2019	
WMZ 22	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2019	2020	2020	
Cat		2019	2019	
Human		2018	2020	
WMZ 23	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2019	2020	2020	
Cat		2019	2020	
Human	2018		2020	
WMZ 24	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2018	2019	2020	
Cat		2018	2020	
Human	2018	2018	2020	
WMZ 25	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog	2020	2020	2020	
Cat	2017	2020	2020	
Human	2020	2020	2020	
WMZ 26	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog			2017	
Cat				
Human				
WMZ 27	<i>Demacentor variabilis</i>	<i>Ixodes cookei</i>	<i>Ixodes scapularis</i>	
Dog			2019	
Cat			2017	
Human			2019	

Appendix 2 Tick samples selected divided into Wildlife Management Zones (WMZ) and chosen based on host and tick species including the year they were donated.

Appendix 3 Gene sequences from NCBI genebank of the partial (549bp) 16S ribosomal RNA gene sequence from *Leptospira borgpetersenii* serovar Ballum strain CRAN13 (Accession: HM776722.1) and the Lipoprotein L32 (LipL32) gene sequence from *Leptospira interrogans* serovar lai strain 56601 (Accession: AE010300) sent to BioBasic to synthesize into vector pUC57. Forward and reverse primer sequences obtained from the multi-locus sequencing typing (MLST) database (Oxford University) highlighted in yellow.

>HM776722.1 *Leptospira borgpetersenii* serovar Ballum strain CRAN13 **16S ribosomal RNA (rrs2)** gene, partial sequence

AAACATGCAAGTCAAGCGGAGTAGCAATACTCAGCGCGAACGGGTGAGTAACACGTGGGTAATCTTCCTCTGAG
TCTGGGATAACTTTCCGAAAGGGGAGCTAATACTGGATAGTCCCGAGAGGTCATAGGATTTTTCGGGTAAAGATT
TATTGCTCGGAGATGAGCCCGCGCCGATTAGCTAGTTGGTGAGGTAATGGCTACCAAGGCGACGATCGGTAGC
CGGCCTGAGAGGGTGTTCCGCCACAATGGAAGTACGACACGGTCCATACTCCTACGGGAGGCAGCAGTTAAGAA
TCTTGCTCAATGGGGGGGAACCCTGAAGCAGCGACGCCGCGTGAACGATGAAGGTCTTCGGATTGTAAAGTTCAA
TAAGCAGGGAAAAATAAGCAGCGATGTGATGATGGTACCTGCCTAAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTATGGTGCAAGCGTTGTTCCGGAATCATTGGGCGTAAAGGGTGCCTAGGCGGACATGTAAGTCA
GGTGTGAAAAGTGCAGGGCTCAACTCGC

>NC_004342.2 *Leptospira interrogans* serovar Lai str. 56601 chromosome I, complete sequence

Lipoprotein L32 (LipL32)

GCTATCTCCGTTGCACTCTTGC AAGCATTACCGCTTGTGGTGCTTTCCGGTGGTCTGCCAAGCCTAAAAAGCTCTTT
TGTTCTGAGCGAGGACACAATCCAGGGACAAACGAAACCGTAAAAACGTTACTTCCCTACGGATCTGTGATCAA
CTATTACGGATACGTAAAGCCAGGACAAGCGCCGACGGTTTAGTCGATGGAAACAAAAAGCATACTATCTCTA
TGTTTGATTCTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCAACAGGCGAAATCGGTGAGCCAGG
CGACGGAGACTTAGTAAGCGACGCTTTCAAAGCGGCTACCCAGAAAGAAAAATCAATGCCACATTGGTTTGATAC
TTGGATCCGTGTAGAAAGAATGTCGGCGATTATGCCTGACCAAATCGCCAAAGCTGCGAAAGCAAAACCGTTCA
AAAATTGGACGATGATGATGGTGAC

Appendix 4 Top five nucleotide-BLAST results from the forward and reverse PCR reaction examples for two of the sequenced samples, as the quality of the other two samples were deemed “poor quality” and did not return a sequence.

Sample	Description	Scientific Name	Total Query		E value	Per. Ident	Acc. Len	Accession
			Score	Cover				
SJB_WOOD1_0.45_F	Alcaligenes faecalis strain AN70 chromosome, complete genome	Alcaligenes faecalis	883	79%	0	98.43	3922717	CP036294.1
	Alcaligenes aquatilis strain BU33N genome	Alcaligenes aquatilis	570	79%	1.00E-157	84.84	3838399	CP022390.1
	Alcaligenes aquatilis strain QD168 chromosome, complete genome	Alcaligenes aquatilis	561	79%	5.00E-155	84.45	4323879	CP032153.1
	Alcaligenes faecalis strain P156, complete genome	Alcaligenes faecalis	554	79%	2.00E-153	84.22	4047508	CP021079.1
	Alcaligenes faecalis strain J481 chromosome, complete genome	Alcaligenes faecalis	552	78%	3.00E-152	84.29	3866803	CP032521.1
SJB_WOOD1_0.45_R	Alcaligenes faecalis strain AN70 chromosome, complete genome	Alcaligenes faecalis	801	72%	0	98.08	3922717	CP036294.1
	Alcaligenes aquatilis strain BU33N genome	Alcaligenes aquatilis	514	72%	7.00E-141	84.43	3838399	CP022390.1
	Alcaligenes faecalis strain P156, complete genome	Alcaligenes faecalis	512	73%	2.00E-140	84.14	4047508	CP021079.1
	Alcaligenes faecalis strain J481 chromosome, complete genome	Alcaligenes faecalis	509	72%	8.00E-140	84.22	3866803	CP032521.1
	Alcaligenes aquatilis strain QD168 chromosome, complete genome	Alcaligenes aquatilis	505	72%	4.00E-138	84.01	4323879	CP032153.1
SJB_WOOD1_0.22_F	Alcaligenes faecalis strain AN70 chromosome, complete genome	Alcaligenes faecalis	881	79%	0	98.43	3922717	CP036294.1
	Alcaligenes aquatilis strain BU33N genome	Alcaligenes aquatilis	570	79%	1.00E-157	84.84	3838399	CP022390.1
	Alcaligenes aquatilis strain QD168 chromosome, complete genome	Alcaligenes aquatilis	561	79%	5.00E-155	84.45	4323879	CP032153.1
	Alcaligenes faecalis strain P156, complete genome	Alcaligenes faecalis	554	79%	2.00E-153	84.22	4047508	CP021079.1
	Alcaligenes faecalis strain J481 chromosome, complete genome	Alcaligenes faecalis	552	79%	3.00E-152	84.29	3866803	CP032521.1
SJB_WOOD1_0.22_R	Alcaligenes faecalis strain AN70 chromosome, complete genome	Alcaligenes faecalis	802	72%	0	98.08	3922717	CP036294.1
	Alcaligenes aquatilis strain BU33N genome	Alcaligenes aquatilis	516	72%	2.00E-141	84.47	3838399	CP022390.1
	Alcaligenes faecalis strain P156, complete genome	Alcaligenes faecalis	514	73%	7.00E-141	84.18	4047508	CP021079.1
	Alcaligenes faecalis strain J481 chromosome, complete genome	Alcaligenes faecalis	511	72%	2.00E-140	84.26	3866803	CP032521.1
	Alcaligenes aquatilis strain QD168 chromosome, complete genome	Alcaligenes aquatilis	507	72%	1.00E-138	84.04	4323879	CP032153.1

Appendix 5 Top five nucleotide-BLAST result for sequenced wildlife samples for the forward and reverse PCR reactions.

Sample	Sample Type	Description	Total Score	Query Cover	E Value	Per Ident	Accession
L_T203M_IF	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	791	99%	0	99.77	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	784	99%	0	99.54	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	784	99%	0	99.54	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	784	99%	0	99.54	CP047372.1
L_T203M_IR	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	793	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	780	99%	0	99.53	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	780	99%	0	99.53	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	780	99%	0	99.53	CP047372.1
L_T171M_IF	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	857	99%	0	98.35	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	845	99%	0	97.93	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	845	99%	0	97.93	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	845	99%	0	97.93	CP047372.1
L_T171M_IR	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	793	99%	0	94.83	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	787	99%	0	94.63	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	787	99%	0	94.63	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	787	99%	0	94.63	CP047372.1
L_T219M_IF	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	891	99%	0	99.39	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	878	99%	0	98.98	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	878	99%	0	98.98	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	878	99%	0	98.98	CP047372.1
L_T219M_IR	<i>Alces alces</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	900	99%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	887	99%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	887	99%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	887	99%	0	99.39	CP047372.1

L_C067L17_IF	<i>Lepus americanus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	815	99%	0	99.78	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	809	99%	0	99.55	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	809	99%	0	99.55	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	809	99%	0	99.55	CP047372.1
L_C067L17_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	902	99%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	891	99%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	891	99%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	891	99%	0	99.39	CP047372.1
L_C040L_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	521	54%	1.00E-143	98.97	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	516	54%	6.00E-142	98.63	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	516	54%	6.00E-142	98.63	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	516	54%	6.00E-142	98.63	CP047372.1
L_C040L_IR		No significant similarity found.					
L_C053L_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C053L_IR		No significant similarity found.					
L_C056L_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	904	97%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	893	97%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	893	97%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	893	97%	0	99.39	CP047372.1
L_C056L_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	898	98%	0	99.2	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	885	98%	0	98.8	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	885	98%	0	98.8	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	885	98%	0	98.8	CP047372.1
L_C060L_IF	<i>Microtus pennsylvanicus</i>	Poor quality					
L_C060L_IR		Poor quality					
L_C061L_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C061L_IR		No significant similarity found.					

L_C081L_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	902	98%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	891	98%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	891	98%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	891	98%	0	99.39	CP047372.1
L_C081L_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	850	99%	0	97.24	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	837	99%	0	96.85	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	837	99%	0	96.85	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	837	99%	0	96.85	CP047372.1
L_C094K_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii strain R6L chromosome 1	364	99%	8.00E-97	99.5	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	364	99%	8.00E-97	99.5	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	364	99%	8.00E-97	99.5	CP047372.1
		Leptospira borgpetersenii strain Mo4 chromosome 1	364	99%	8.00E-97	99.5	CP047334.1
L_C094K_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	510	99%	1.00E-140	98.94	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	505	99%	7.00E-139	98.59	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	505	99%	7.00E-139	98.59	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	505	99%	7.00E-139	98.59	CP047372.1
L_C061L17_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C061L17_IR		No significant similarity found.					
L_C010L17_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C010L17_IR		No significant similarity found.					
L_C027K_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C027K_IR		No significant similarity found.					
L_C145K16_IF	<i>Microtus pennsylvanicus</i>	Leptospira interrogans serovar Copenhageni strain SK1 chromosome I, complete sequence	1658	99%	0	98.52	CP048830.1
		Leptospira interrogans strain R11 chromosome 1	832	99%	0	98.52	CP047510.1
		Uncultured Leptospira sp. clone Lap1 16S ribosomal RNA gene, partial sequence	832	99%	0	98.52	MN545905.1
		Leptospira interrogans serovar Hardjo strain L53 chromosome 1, complete sequence	1653	99%	0	98.52	CP043041.1
L_C145K16_IR		Leptospira kirschneri strain CES 16S ribosomal RNA gene, partial sequence	854	99%	0	97.6	MK726123.1

		Leptospira kirschneri serovar Mozdok strain 3759 16S ribosomal RNA gene, partial sequence	854	99%	0	97.6	KP125531.1
		Leptospira kirschneri serovar Mozdok strain 61H 16S ribosomal RNA gene, partial sequence	854	99%	0	97.6	KP114456.1
		Leptospira kirschneri strain Grippotyphosa_DB35 16S ribosomal RNA gene, partial sequence	854	99%	0	97.6	JQ988856.1
L_C156K16_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	861	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	854	99%	0	99.79	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	854	99%	0	99.79	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	854	99%	0	99.79	CP047372.1
L_C156K16_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	907	99%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	896	99%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	896	99%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	896	99%	0	99.39	CP047372.1
L_C160K16_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C160K16_IR		No significant similarity found.					
L_C103L_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C103L_IR		No significant similarity found.					
L_C109K_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C109K_IR		No significant similarity found.					
L_C117B_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	797	99%	0	98.67	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	791	99%	0	98.44	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	791	99%	0	98.44	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	791	99%	0	98.44	CP047372.1
L_C117B_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	863	99%	0	98	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	852	99%	0	97.6	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	852	99%	0	97.6	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	852	99%	0	97.6	CP047372.1
L_C120K_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C120K_IR		No significant similarity found.					

L_C127L_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C127L_IR		No significant similarity found.					
L_C180L_IF	<i>Microtus pennsylvanicus</i>	No significant similarity found.					
L_C180L_IR		No significant similarity found.					
L_C191L_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	795	99%	0	95.98	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	784	96%	0	96.28	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	784	96%	0	96.28	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	784	96%	0	96.28	CP047372.1
L_C191L_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	802	99%	0	97.84	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	789	99%	0	97.4	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	789	99%	0	97.4	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	789	99%	0	97.4	CP047372.1
L_C003S16_IF	<i>Microtus pennsylvanicus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	883	99%	0	99.19	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	878	99%	0	98.98	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	878	99%	0	98.98	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	878	99%	0	98.98	CP047372.1
L_C003S16_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	902	99%	0	99.2	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	891	99%	0	98.8	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	891	99%	0	98.8	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	891	99%	0	98.8	CP047372.1
L_C064K17_IF	<i>Napaeozapus insignis</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	894	99%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	883	99%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	883	99%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	883	99%	0	99.39	CP047372.1
L_C064K17_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	900	99%	0	99.6	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	887	99%	0	99.19	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	887	99%	0	99.19	CP047504.1

		Leptospira borgpetersenii strain R6 chromosome 1	887	99%	0	99.19	CP047372.1
L_C013K17_IF	<i>Napaeozapus insignis</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	228	49%	3.00E-55	79.75	HM776722.1
		Leptospira interrogans serovar Canicola strain 611 chromosome 1, complete sequence	439	49%	1.00E-53	79.43	CP044513.1
		Leptospira interrogans serovar Sejroe strain 3705 16S ribosomal RNA gene, partial sequence	224	49%	1.00E-53	79.43	MK756318.1
		Leptospira interrogans serovar Bataviae 16S ribosomal RNA gene, partial sequence	224	49%	1.00E-53	79.43	KY075912.1
L_C013K17_IR		No significant similarity found.					
L_C015L17_IF	<i>Napaeozapus insignis</i>	No significant similarity found.					
L_C015L17_IR		No significant similarity found.					
L_C118K_IF	<i>Napaeozapus insignis</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	809	96%	0	99.11	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	802	96%	0	98.89	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	802	96%	0	98.89	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	802	96%	0	98.89	CP047372.1
L_C118K_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	883	99%	0	99.19	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	872	99%	0	98.78	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	872	99%	0	98.78	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	872	99%	0	98.78	CP047372.1
L_C062K17_IF	<i>Peromyscus maniculatus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	902	99%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	891	99%	0	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	891	99%	0	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	891	99%	0	99.39	CP047372.1
L_C062K17_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	832	99%	0	99.78	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	821	99%	0	99.34	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	821	99%	0	99.34	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	821	99%	0	99.34	CP047372.1
L_C012L17_IF	<i>Rattus norvegicus</i>	No significant similarity found.					
L_C012L17_IR		No significant similarity found.					

L_C001B16_IF	<i>Sciurus vulgaris</i>	Sciurus carolinensis genome assembly, chromosome: 15	548	97%	4.00E-72	84.74%	LR738605.1
L_C001B16_IR		Sciurus carolinensis genome assembly, chromosome: 15	248	88%	1.00E-61	82.99%	LR738605.1
L_C001K16_IF	<i>Sciurus vulgaris</i>	Sciurus carolinensis genome assembly, chromosome: 15	543	95%	5.00E-71	84.47%	LR738605.1
L_C001K16_IR		Sciurus carolinensis genome assembly, chromosome: 15	1133	92%	3.00E-84	83.67%	LR738605.1
L_C001M16_IF	<i>Sciurus vulgaris</i>	Sciurus carolinensis genome assembly, chromosome: 15	556	96%	2.00E-74	84.89	LR738605.1
L_C001M16_IR		Sciurus carolinensis genome assembly, chromosome: 15	248	88%	1.00E-61	82.99	LR738605.1
L_C048L_IF	<i>Sorex maritimensis</i>	No significant similarity found.					
L_C048L_IR		No significant similarity found.					
L_C059L_IF	<i>Sorex maritimensis</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	898	98%	0	99.59	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	885	98%	0	99.19	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	885	98%	0	99.19	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	885	98%	0	99.19	CP047372.1
L_C059L_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	915	98%	0	99.8	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	904	98%	0	99.4	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	904	98%	0	99.4	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	904	98%	0	99.4	CP047372.1
L_C065L17_IF	<i>Sorex maritimensis</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	706	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	699	99%	0	99.74	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	699	99%	0	99.74	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	699	99%	0	99.74	CP047372.1
L_C065L17_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	702	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	689	99%	0	99.47	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	689	99%	0	99.47	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	689	99%	0	99.47	CP047372.1
L_T198BM_IF	<i>Ursus americanus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	610	65%	3.00E-170	99.7	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	604	65%	1.00E-168	99.4	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	604	65%	1.00E-168	99.4	CP047504.1

L_T198BM_IR		Leptospira borgpetersenii strain R6 chromosome 1	604	65%	1.00E-168	99.4	CP047372.1
		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	608	64%	9.00E-170	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	595	64%	7.00E-166	99.39	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	595	64%	7.00E-166	99.39	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	595	64%	7.00E-166	99.39	CP047372.1
L_T168B_IF	<i>Ursus americanus</i>	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	592	67%	9.00E-165	98.79	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	588	67%	1.00E-163	98.49	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	588	67%	1.00E-163	98.49	CP047504.1
L_T168B_IR		Leptospira borgpetersenii strain R6 chromosome 1	588	67%	1.00E-163	98.49	CP047372.1
		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	573	67%	3.00E-159	97.58	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	562	67%	7.00E-156	96.97	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	562	67%	7.00E-156	96.97	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	562	67%	7.00E-156	96.97	CP047372.1
L_C088K_IF	Unknown	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	704	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	697	99%	0	99.74	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	697	99%	0	99.74	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	697	99%	0	99.74	CP047372.1
L_C088K_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	804	99%	0	97.27	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	791	99%	0	96.86	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	791	99%	0	96.86	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	791	99%	0	96.86	CP047372.1
L_C090K_IF	Unknown	Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	878	99%	0	99.38	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	867	99%	0	98.97	CP047520.1
		Leptospira borgpetersenii strain R14 chromosome 1	867	99%	0	98.97	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	867	99%	0	98.97	CP047372.1
L_C090K_IR		Leptospira borgpetersenii serovar Ballum strain CRAN13 16S ribosomal RNA (rrs2) gene, partial sequence	894	99%	0	100	HM776722.1
		Leptospira borgpetersenii strain R6L chromosome 1	881	99%	0	99.59	CP047520.1

		Leptospira borgpetersenii strain R14 chromosome 1	881	99%	0	99.59	CP047504.1
		Leptospira borgpetersenii strain R6 chromosome 1	881	99%	0	99.59	CP047372.1
L_C135K16_IF	Unknown	No significant similarity found.					
L_C135K16_IR		No significant similarity found.					

Appendix 6 Results from the NCBI Nucleotide Blast for the two samples that returned a positive sequence result for *Borrelia bissetii*.

Sample ID	Sample sequence & the corresponding Nucleotide Blast GeneBank Matches																																																																																																														
bis_C028K_IF	<p>TCTTTGTCTCATAAGTGATCAGCGAGGGACCCCGTCAATGCTGGGTGGAACCTCAAGGGTATGTTCTCAGCGGA ACTCTTACTGCTGAACAAGCAACCTTGGTGGTTAAGAAGGAACTGTTACTTTAAGTACGCACATTTCAAATCT GGAGAAGTAACAGCTGAACTTAATGACACTGACAGTACTCAAGCTACTAAAAAACTGGGAAATGGGATGCAGG CACTTCAACTTTAACAATTACTGTAAACAACAAAAAACTAATGCCCTTGATTTTACAAAAACAAGACACAATTAC ATCACAAAAATA</p> <table border="1"> <thead> <tr> <th data-bbox="422 505 464 579"></th> <th data-bbox="464 505 1234 579">Description</th> <th data-bbox="1234 505 1528 579">Scientific Name</th> <th data-bbox="1528 505 1591 579">Max Score</th> <th data-bbox="1591 505 1654 579">Total Score</th> <th data-bbox="1654 505 1717 579">Query Cover</th> <th data-bbox="1717 505 1780 579">E value</th> <th data-bbox="1780 505 1843 579">Per. Ident</th> <th data-bbox="1843 505 1906 579">Acc. Len</th> <th data-bbox="1906 505 2055 579">Accession</th> </tr> </thead> <tbody> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia sp. W97F51 outer surface protein A (ospA) gene, partial cds</td> <td>Borrelia sp. W97F51</td> <td>411</td> <td>411</td> <td>81%</td> <td>4e-110</td> <td>96.43%</td> <td>712</td> <td>AY884357.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetii outer surface protein A (ospA) gene, complete cds</td> <td>Borrelia bissetiae</td> <td>411</td> <td>411</td> <td>81%</td> <td>4e-110</td> <td>96.43%</td> <td>822</td> <td>AF230516.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA) gene, complete cds</td> <td>Borrelia burgdorferi</td> <td>402</td> <td>402</td> <td>81%</td> <td>2e-107</td> <td>95.63%</td> <td>822</td> <td>U20357.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetii DN127 plasmid lp54, complete sequence</td> <td>Borrelia bissetii DN127</td> <td>351</td> <td>351</td> <td>76%</td> <td>4e-92</td> <td>92.47%</td> <td>55219</td> <td>CP002761.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetii strain CA389 ospAB operon, partial sequence</td> <td>Borrelia bissetiae</td> <td>351</td> <td>351</td> <td>76%</td> <td>4e-92</td> <td>92.47%</td> <td>1619</td> <td>AF186845.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia sp. DN127 ospA gene, partial</td> <td>Borrelia bissetii DN127</td> <td>351</td> <td>351</td> <td>76%</td> <td>4e-92</td> <td>92.47%</td> <td>822</td> <td>Y10897.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetii strain CA128 ospAB operon, partial sequence</td> <td>Borrelia bissetiae</td> <td>346</td> <td>346</td> <td>76%</td> <td>2e-90</td> <td>92.05%</td> <td>1619</td> <td>AF186846.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetiae isolate Tick542-2015 outer surface protein A (ospA) gene, partial cds</td> <td>Borrelia bissetiae</td> <td>331</td> <td>331</td> <td>65%</td> <td>3e-86</td> <td>96.10%</td> <td>252</td> <td>MK033850.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetiae isolate Tick389-2016 outer surface protein A (ospA) gene, partial cds</td> <td>Borrelia bissetiae</td> <td>325</td> <td>325</td> <td>64%</td> <td>1e-84</td> <td>96.04%</td> <td>258</td> <td>MK033852.1</td> </tr> <tr> <td><input checked="" type="checkbox"/></td> <td>Borrelia bissetiae isolate Tick655-2015 outer surface protein A (ospA) gene, partial cds</td> <td>Borrelia bissetiae</td> <td>324</td> <td>324</td> <td>64%</td> <td>5e-84</td> <td>96.02%</td> <td>257</td> <td>MK033851.1</td> </tr> </tbody> </table>		Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession	<input checked="" type="checkbox"/>	Borrelia sp. W97F51 outer surface protein A (ospA) gene, partial cds	Borrelia sp. W97F51	411	411	81%	4e-110	96.43%	712	AY884357.1	<input checked="" type="checkbox"/>	Borrelia bissetii outer surface protein A (ospA) gene, complete cds	Borrelia bissetiae	411	411	81%	4e-110	96.43%	822	AF230516.1	<input checked="" type="checkbox"/>	Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA) gene, complete cds	Borrelia burgdorferi	402	402	81%	2e-107	95.63%	822	U20357.1	<input checked="" type="checkbox"/>	Borrelia bissetii DN127 plasmid lp54, complete sequence	Borrelia bissetii DN127	351	351	76%	4e-92	92.47%	55219	CP002761.1	<input checked="" type="checkbox"/>	Borrelia bissetii strain CA389 ospAB operon, partial sequence	Borrelia bissetiae	351	351	76%	4e-92	92.47%	1619	AF186845.1	<input checked="" type="checkbox"/>	Borrelia sp. DN127 ospA gene, partial	Borrelia bissetii DN127	351	351	76%	4e-92	92.47%	822	Y10897.1	<input checked="" type="checkbox"/>	Borrelia bissetii strain CA128 ospAB operon, partial sequence	Borrelia bissetiae	346	346	76%	2e-90	92.05%	1619	AF186846.1	<input checked="" type="checkbox"/>	Borrelia bissetiae isolate Tick542-2015 outer surface protein A (ospA) gene, partial cds	Borrelia bissetiae	331	331	65%	3e-86	96.10%	252	MK033850.1	<input checked="" type="checkbox"/>	Borrelia bissetiae isolate Tick389-2016 outer surface protein A (ospA) gene, partial cds	Borrelia bissetiae	325	325	64%	1e-84	96.04%	258	MK033852.1	<input checked="" type="checkbox"/>	Borrelia bissetiae isolate Tick655-2015 outer surface protein A (ospA) gene, partial cds	Borrelia bissetiae	324	324	64%	5e-84	96.02%	257	MK033851.1
	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession																																																																																																						
<input checked="" type="checkbox"/>	Borrelia sp. W97F51 outer surface protein A (ospA) gene, partial cds	Borrelia sp. W97F51	411	411	81%	4e-110	96.43%	712	AY884357.1																																																																																																						
<input checked="" type="checkbox"/>	Borrelia bissetii outer surface protein A (ospA) gene, complete cds	Borrelia bissetiae	411	411	81%	4e-110	96.43%	822	AF230516.1																																																																																																						
<input checked="" type="checkbox"/>	Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA) gene, complete cds	Borrelia burgdorferi	402	402	81%	2e-107	95.63%	822	U20357.1																																																																																																						
<input checked="" type="checkbox"/>	Borrelia bissetii DN127 plasmid lp54, complete sequence	Borrelia bissetii DN127	351	351	76%	4e-92	92.47%	55219	CP002761.1																																																																																																						
<input checked="" type="checkbox"/>	Borrelia bissetii strain CA389 ospAB operon, partial sequence	Borrelia bissetiae	351	351	76%	4e-92	92.47%	1619	AF186845.1																																																																																																						
<input checked="" type="checkbox"/>	Borrelia sp. DN127 ospA gene, partial	Borrelia bissetii DN127	351	351	76%	4e-92	92.47%	822	Y10897.1																																																																																																						
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<input checked="" type="checkbox"/>	Borrelia bissetiae isolate Tick655-2015 outer surface protein A (ospA) gene, partial cds	Borrelia bissetiae	324	324	64%	5e-84	96.02%	257	MK033851.1																																																																																																						
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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/> Borrelia sp. W97F51 outer surface protein A (ospA).gene._partial cds	Borrelia sp. W97F51	432	432	100%	1e-116	93.73%	712	AY884357.1
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bis_C185B_IF

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/> Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA).gene._complete cds	Borrelia burgdorferi	525	525	93%	1e-144	99.31%	822	U20357.1
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bis_C185B_IR	AAATTGTTAAAGTtGAGATGCCTGCATCCCATTtCCCAGTTTTTGTtTAGTAGCTTCAGTACTGTTAGTGTCATTAA GTTTCAGCTGTTACTTCTCCAGATTTTTGAAATGTGCTTACTTAAAGTAACAGNTTCCTTCTTTAACCACCAATGTTGC TTTTTCAGCAGTTAGAGTTCTTCAAGAACATATTCTTTTAAGGTTTCTTTAGCTTTTCCGGATCCATCGCTTTTAA TTTCTGTGATTCAAGTATGGTTCCGTTTGCTCTTGCCATTATTTTTTCAACTAATTCGCCTTTTTTCATT																																																																																																														
	<table border="1"> <thead> <tr> <th data-bbox="422 412 464 488"></th> <th data-bbox="464 412 1257 488">Description</th> <th data-bbox="1257 412 1530 488">Scientific Name</th> <th data-bbox="1530 412 1591 488">Max Score</th> <th data-bbox="1591 412 1652 488">Total Score</th> <th data-bbox="1652 412 1713 488">Query Cover</th> <th data-bbox="1713 412 1791 488">E value</th> <th data-bbox="1791 412 1869 488">Per. Ident</th> <th data-bbox="1869 412 1929 488">Acc. Len</th> <th data-bbox="1929 412 2045 488">Accession</th> </tr> </thead> <tbody> <tr> <td data-bbox="422 488 464 526">✓</td> <td data-bbox="464 488 1257 526">Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA) gene, complete cds</td> <td data-bbox="1257 488 1530 526">Borrelia burgdorferi</td> <td data-bbox="1530 488 1591 526">512</td> <td data-bbox="1591 488 1652 526">512</td> <td data-bbox="1652 488 1713 526">99%</td> <td data-bbox="1713 488 1791 526">8e-141</td> <td data-bbox="1791 488 1869 526">97.67%</td> <td data-bbox="1869 488 1929 526">822</td> <td data-bbox="1929 488 2045 526">U20357.1</td> </tr> <tr> <td data-bbox="422 526 464 563">✓</td> <td data-bbox="464 526 1257 563">Borrelia sp. W97F51 outer surface protein A (ospA) gene, partial cds</td> <td data-bbox="1257 526 1530 563">Borrelia sp. 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DN127 ospA gene, partial</td> <td data-bbox="1257 712 1530 750">Borrelia bissetii DN127</td> <td data-bbox="1530 712 1591 750">424</td> <td data-bbox="1591 712 1652 750">424</td> <td data-bbox="1652 712 1713 750">99%</td> <td data-bbox="1713 712 1791 750">4e-114</td> <td data-bbox="1791 712 1869 750">92.33%</td> <td data-bbox="1869 712 1929 750">822</td> <td data-bbox="1929 712 2045 750">Y10897.1</td> </tr> <tr> <td data-bbox="422 750 464 787">✓</td> <td data-bbox="464 750 1257 787">Borrelia bissetii isolate Tick368-2014 outer surface protein A (ospA) gene, partial cds</td> <td data-bbox="1257 750 1530 787">Borrelia bissetii</td> <td data-bbox="1530 750 1591 787">418</td> <td data-bbox="1591 750 1652 787">418</td> <td data-bbox="1652 750 1713 787">83%</td> <td data-bbox="1713 750 1791 787">2e-112</td> <td data-bbox="1791 750 1869 787">96.83%</td> <td data-bbox="1869 750 1929 787">254</td> <td data-bbox="1929 750 2045 787">MK033847.1</td> </tr> <tr> <td data-bbox="422 787 464 824">✓</td> <td data-bbox="464 787 1257 824">Borrelia bissetii strain M7p outer surface protein A (ospA) gene, partial cds</td> <td data-bbox="1257 787 1530 824">Borrelia bissetii</td> <td data-bbox="1530 787 1591 824">407</td> <td data-bbox="1591 787 1652 824">407</td> <td data-bbox="1652 787 1713 824">99%</td> <td data-bbox="1713 787 1791 824">4e-109</td> <td data-bbox="1791 787 1869 824">91.33%</td> <td data-bbox="1869 787 1929 824">504</td> <td data-bbox="1929 787 2045 824">KM269421.1</td> </tr> <tr> <td data-bbox="422 824 464 862">✓</td> <td data-bbox="464 824 1257 862">Borrelia carolinensis isolate SCW-21 plasmid lp54 lipoprotein 1 (ospA) gene, partial cds</td> <td data-bbox="1257 824 1530 862">Borrelia carolinensis</td> <td data-bbox="1530 824 1591 862">407</td> <td data-bbox="1591 824 1652 862">407</td> <td data-bbox="1652 824 1713 862">99%</td> <td data-bbox="1713 824 1791 862">4e-109</td> <td data-bbox="1791 824 1869 862">91.33%</td> <td data-bbox="1869 824 1929 862">560</td> <td data-bbox="1929 824 2045 862">EU085397.1</td> </tr> </tbody> </table>		Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession	✓	Borrelia burgdorferi C-1-11 outer surface protein A precursor (ospA) gene, complete cds	Borrelia burgdorferi	512	512	99%	8e-141	97.67%	822	U20357.1	✓	Borrelia sp. W97F51 outer surface protein A (ospA) gene, partial cds	Borrelia sp. W97F51	507	507	99%	4e-139	97.33%	712	AY884357.1	✓	Borrelia bissetii outer surface protein A (ospA) gene, complete cds	Borrelia bissetii	501	501	99%	2e-137	97.00%	822	AF230516.1	✓	Borrelia bissetii strain CA389 ospAB operon, partial sequence	Borrelia bissetii	429	429	99%	9e-116	92.67%	1619	AF186845.1	✓	Borrelia bissetii DN127 plasmid lp54, complete sequence	Borrelia bissetii DN127	424	424	99%	4e-114	92.33%	55219	CP002761.1	✓	Borrelia bissetii strain CA128 ospAB operon, partial sequence	Borrelia bissetii	424	424	99%	4e-114	92.33%	1619	AF186846.1	✓	Borrelia sp. DN127 ospA gene, partial	Borrelia bissetii DN127	424	424	99%	4e-114	92.33%	822	Y10897.1	✓	Borrelia bissetii isolate Tick368-2014 outer surface protein A (ospA) gene, partial cds	Borrelia bissetii	418	418	83%	2e-112	96.83%	254	MK033847.1	✓	Borrelia bissetii strain M7p outer surface protein A (ospA) gene, partial cds	Borrelia bissetii	407	407	99%	4e-109	91.33%	504	KM269421.1	✓	Borrelia carolinensis isolate SCW-21 plasmid lp54 lipoprotein 1 (ospA) gene, partial cds	Borrelia carolinensis	407	407	99%	4e-109	91.33%	560	EU085397.1
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