

Prevalence of *Babesia* in the Maritime Canada

by

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Abstract

Babesia is a tick-vectored apicomplexan protozoan parasite that infects red blood cells of vertebrate hosts which can cause acute or relapsing chronic babesiosis. With the lack of surveillance efforts on the parasite within the Maritime provinces of Canada, our study aims to determine the prevalence of *Babesia odocoilei*, *Babesia duncani* and *Babesia microti* from locally acquired *Ixodes scapularis*, *Ixodes cookei*, *Dermacentor variabilis* and *Dermacentor albipictus* ticks. Our study also looked at *Babesia* in cow blood collected from Nova Scotia and New Brunswick dairy farms using Giemsa and Acridine Orange staining of blood smears. A direct detection of the pathogen using Polymerase Chain Reaction (PCR) test was done for both blood and tick samples. Frozen blood samples were of poor quality to detect *Babesia* using both methods. We found there is a higher prevalence of *B. odocoilei* among adult ticks in all tick species tested. *B. microti* was detected in 2 out of 339 ticks tested: 1 in adult *I. cookei* and 1 in adult *D. albipictus*. *B. duncani* was not detected. To assess how long *Babesia* has been established in the region, tick samples collected from two time periods (2013-2016 and 2017-2022) were compared. *B. odocoilei* was detected as early as 2013 and was consistently detected over the 9- year period. In the recent years, *B. odocoilei* also doubled in prevalence and had a wider geographic distribution, with the highest proportion detected in ticks from Prince Edward Island, followed by Nova Scotia, then New Brunswick suggesting that *B. odocoilei* is establishing and undergoing range expansion. *B. odocoilei* prevalence does not correlate with tick abundance and presence deer, an important host, as is the case with Prince Edward Island. Further surveillance of the pathogen in human, agricultural animal and wildlife is yet to be determined. The detection of *Babesia* pathogens is of medical and veterinary importance as it brings the possibility of acquiring babesiosis in the Maritimes.

Introduction

1.1 *Babesia* and Epidemiology

Babesiosis is a zoonotic disease caused by an apicomplexan protozoan parasite belonging to the genus *Babesia*. Since the discovery in the 19th century, many *Babesia* species have been identified. Currently *Babesia* species comprise of more than 100 pathogens that infect many vertebrate hosts, including domestic (cattle, sheep, cats, dogs, horses) wildlife animals (mouse, deer, moose, birds, elks) and humans (Kumar et al., 2021). *Babesia* protozoans are closely related to *Plasmodium*, the causative agent of malaria although unlike *Plasmodium*, *Babesia* is specifically adapted to use ticks as a vector. *Babesia* is transmitted between hosts through the bite of ticks.

A meta-analysis has surveyed different *Babesia* species in different ticks across the globe and most are well-observed in temperate regions (Onyiche et al., 2021). They are also present in tropical and subtropical regions although their prevalence is less known, possibly due to misdiagnosis as malaria (Zhou et al., 2013). In Europe, the predominant strain is *B. divergens*, known to be vectored by *Ixodes ricinus*. *B. microti* has been thought to be the most common causative agent of human babesiosis in North America. It is commonly transmitted through the bite of *Ixodes scapularis*, the deer or black-legged tick. *B. microti* is endemic to the northeastern and upper midwestern United States, with about 16,000 cases that have been reported during 2011-2019 (Swanson, 2023).

There is evidence that *Babesia* species is being progressively introduced in Canada with northward introduction of the tick vectors. Between 2009 and 2014, active and passive tick surveillance demonstrated the presence of *B. microti* in Quebec, New Brunswick, Ontario and Manitoba (O'Brien et al., 2016). More recently, one human babesiosis caused by *B. microti* has been reported from southwest Nova Scotia (Allehebi et al., 2022). *B. duncani*, formally called WA-1, was presumed to occur in western regions of United States, although the first locally acquired human babesiosis case in southern Ontario have been presented (Swei et al., 2019) (Scott, 2017). Nationwide surveillance between 2011 and 2017 from Canadian patients reported highest *B. duncani* occurrence in the Pacific coast region, but it was also detected in eastern Canada (Scott & Scott, 2018). Another medically important *Babesia* species is *B. odocoilei*, which is endemic in southeastern parts of United States and it has recently been identified as a causative agent for cervid babesiosis in Ontario and Quebec (Waldrup et al., 1990, Milnes et al., 2019, Crandall et al., 2022). Along with the recent studies of *B. odocoilei* among cervids in

Canada, it has also been reported that *B. odocoilei* can infect bovids such as the desert bighorn sheep in California and musk oxen in Minnesota suggesting that *Babesia* is not necessarily limited to one particular host (Schoelkopf et al., 2005).

The northward expansion of *Ixodidae* ticks is likely responsible for many of the emerging cases of Babesiosis in Canada. Climate change allows northern regions to be more suitable for ticks including *Ixodes scapularis*, *Dermacentor variabilis* and *Ixodes cookei* (Lieske & Lloyd, 2018, Minigan et al., 2018, Gasmi et al., 2018). Increased humidity and milder winter conditions have been determined to be a strong predictor of tick occurrence, as was reported in New Brunswick, a province that is undergoing a range expansion of *I. scapularis* (Lieske & Lloyd, 2018). As would be expected, pathogens that are vectored by *I. scapularis* also follow this range expansion. *Borrelia burgdorferi*, the most common causative agent for Lyme disease, have been detected in areas with high exposure to *I. scapularis*. These Lyme disease endemic areas are located in Manitoba, Quebec, Ontario, Nova Scotia and New Brunswick (Canada, 2022). Shared by the same vector, there is a potential for *Babesia* to undergo range expansion as well. More broadly, the risk of transmission of Babesiosis much like other tick-borne diseases (TBD) depends on socio economic factors as well such as human population growth, movement, preventative behavior measurements, knowledge and risk perception toward TBDs.

The family Ixodidae ticks, or hard ticks, (the genera include *Ixodes*, *Rhipicephalus* and *Dermacentor*) are hematophagous arthropods that rely on feeding blood from vertebrates after hatching from eggs in order to develop into three life stages: larva, nymph and adult. Ticks can be vectors for bacterial, viral and parasitological pathogens that can be transferred between hosts. The spread of pathogens generally depends on several factors including the abundance, resistance and the suitability of host and tick vectors. The feeding preference of a tick can determine the range of vertebrates *Babesia* hosts. For example, *I. scapularis* is a generalist tick that feeds on a variety of hosts, and may be more efficient in presenting *Babesia* species to new hosts (Merenstein et al., 2020). On the other hand, a specialist tick would restrict *Babesia* to a narrow range of host. Specific interactions between *Babesia*, and its host can allow adaptations that result in long-lasting persistence of the pathogen in the environment. It has been well documented that reservoir hosts are efficient at maintaining *Babesia* at low levels to prevent detrimental effects on host, however, this prolongs the infection and increase the potential for transmission to another tick. On the other hand, if the immune system of an unsuitable host is able to stop *Babesia* from

replicating, or if the infection results in host mortality, it can prevent maintaining *Babesia* within the population (Chauvin et al., 2009).

1.2 Morphology, Life Cycle of and Modes of transmission of *Babesia*

Babesia is a unicellular (ranging from 1.5 μm - 5 μm in diameter) eukaryote belonging to the parasitic phylum Apicomplexa, a group that share an apical complex structure, for which they are named. Located at the tapered end of a cell, the apical complex consists of secretory organelles that mediate host cell invasion. These organelles secrete enzymes and proteins that helps the parasite to recognize and adhere to host erythrocytes (Jalovecka et al., 2019). *Babesia* are pleomorphic intraerythrocytic parasites whose forms appear differently depending on life stage. A trophozoite, its actively feeding stage, is mononucleated ring structure. Paired pyriform (bi-nucleated) are paired-pear-shaped structures that result from a binary fission. Tetrad forms are tetraploid that result from a duplicate binary fission. The completion of binary fission results into a ring-form merozoites (Rossouw et al., 2015).

The *Babesia* life cycle includes asexual reproduction in the reservoir host and sexual reproduction in tick vector (Jalovecka et al., 2019) (Figure 1.1). The life cycle of *Babesia* begins with the injection of *Babesia* sporozoites by an infected tick. Sporozoites penetrates the erythrocytes (red blood cells). Inside the erythrocytes, the parasite differentiates into trophozoites, and divide asexually into two or four merozoites by binary fission. At the end of division, they mature into gamonts or pregametocytes. The development of merozoites into gametes and formation of spores takes place inside the tick. When gamonts are taken up by a tick from an infected host during a blood meal, gamonts differentiate into gametes inside the tick's gut where they can fuse to form a diploid zygote. Zygotes undergo meiosis and form motile haploid kinetes from which they are able to invade other organs of the tick. They continue to further develop into invasive sporozoites in the salivary gland and are readily transferrable during the next blood meal (Jalovecka et al., 2019) (Figure 1.1).

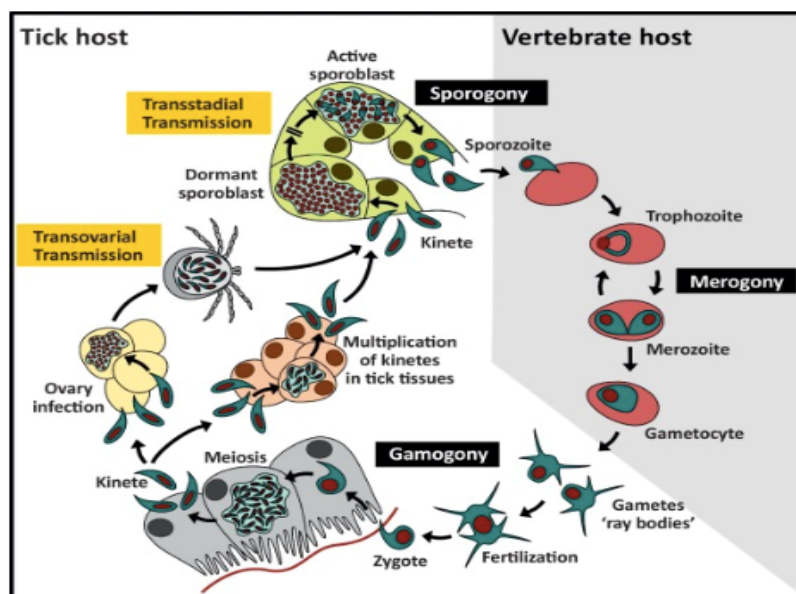


Figure 1.1 Life cycle of *Babesia* parasite, adapted from (Jalovecka et al., 2019)

The maintenance of *Babesia* relies on suitable vectors and hosts that facilitate different modes of transmission. Although most commonly transmitted through the bite of a tick between vertebrate hosts, *Babesia* can be transmitted through blood transfusion (Herwaldt et al., 2011) with an infected donor and through maternal transmission via the placenta (Joseph et al., 2012). *Babesia* parasites can persist between developmental stages of the same vector (transstadial passage) and can also infect developing eggs in female ticks which in turn can infect offspring (transovarial passage) (Jalovecka et al., 2019).

1.3 Signs of Babesiosis

Babesiosis in humans presents with symptoms that are similar to malaria. The incubation period is about 1 to 4 weeks after being exposed to the pathogen (Mareedu et al., 2017). Clinical symptoms start with a gradual onset of non-specific symptoms such as fever, fatigue, malaise, weakness, and muscle pain. Nausea, vomiting, diarrhea, high blood bilirubin level from erythrocytes breakdown are strong predictors of severe infection (Mareedu et al., 2017). *Babesia* hemolyzes and attacks additional red blood cells which further propagates the infection and can progress into hemolytic anemia, thrombocytopenia and jaundice. Some patients experience more severe complications such as liver, spleen and kidney failure or respiratory distress syndrome; all

of which may be fatal (Mareedu et al., 2017). Advanced age, asplenia, and autoimmune disorders are some risk factors for severe cases of babesiosis and require more hospitalization, and prolonged therapy. *Babesia* infection can cause acute or a persistent, relapsing chronic illness (Krause et al., 2008). Between 2010-2016, the overall health burden of babesiosis, a nationally notifiable disease in the United States, was assessed among hospitalized patients diagnosed with the illness (Bloch et al. 2022). The majority of these hospitalizations occurred in the Northeast and Midwest regions of the United States. It was found that over half of the patients experienced severe illness, while the mortality rate was about 2% (Bloch et al. 2022).

Bovine babesiosis, caused by *B. bovis* or *B. bigemina* in tropical and subtropical regions, poses a major threat to the cattle industry and can result in mortality (Springer et al., 2020). Infected animals suffer from reduced weight gain, meat and milk production resulting in substantial economic losses which can potentially reach \$1 billion annually in the U.S. (USDA, 2010). These economic losses are attributed to costs associated with control strategies, treatment and prevention (Esteve-Gasent et al., 2020). The reintroduction of tick vector *Rhipicephalus microplus* and the importation of live cattle from *Babesia* endemic regions of Mexico further adds to the continuous threat to the U.S. cattle industry (Giles et al., 2014). Bovine babesiosis present clinical symptoms that are similar to those in humans and wildlife babesiosis (Mathieu et al., 2018, Wagner et al., 1992, Milnes et al., 2019), but can also include increased heart rate, decreased appetite, decreased milk production, and accumulation of hemoglobin in the blood plasma and urine. Neurological symptoms can also present such as lack of coordination, teeth grinding and manic behavior. In bovine babesiosis endemic areas, calves are likely to be infected yet be asymptomatic and so develop immunity. Some species such as *B. bigemina* can maintain low levels of parasitemia in persistently infected cattle even after clinical recovery from babesiosis (Alvarez et al., 2019). Such established areas result in enzootic stability, whereby animals acquire strong immunity and resistance to the pathogen so the chances of observing clinical cases are low. Where this stability is disrupted due to changes in management strategies, climate and host introductions, an outbreak can occur and vaccination is recommended (Florin-Christensen et al., 2014).

1.4 Methods of *Babesia* detection

Diagnosis of babesiosis is made from clinical assessment with consideration of possible exposure to ticks, complemented by blood smear testing, molecular testing or antibody detection. Important considerations include the sensitivity, feasibility, cost and time required for testing.

The most common test for diagnosing *Babesia* infection is by a blood smear stained with Giemsa stain under light microscope or with acridine orange under fluorescent microscope. Blood smear technique is a common routine diagnostic laboratory procedure for detecting intracellular parasitic infections of blood cells. Giemsa stain is more widely used but staining with Acridine Orange can be a time saving technique (Gay et al., 1996). Thick blood smears allow for a larger sample of blood to be examined in the same area, whereas thin blood smears are used to better visualize parasite morphology and possibly allow the distinction between large *Babesia* (trophozoites >3µm) and small *Babesia* (trophozoites <3 µm) (Parija et al., 2015). While microscopy is low in sensitivity, it is helpful in detecting infected erythrocytes during the acute phase of the disease. However, if there are too few parasites circulating in the blood, detection becomes more difficult and can testing can return false negatives (Parija et al., 2015).

Blood smear examination and accurately identifying parasites require knowledge on the components of blood and parasite features. Blood comprises of three cell types: red blood cells, white blood cells and platelets. The cellular structure of RBC is a biconcave shape (about 5-6 µm for cattle and 6-8 µm for human) and as they mature, they lose their nucleus to maximize oxygen transport. Therefore, some immature red blood cells may contain nucleus for a short period of time (Leeds Histology Guide). White blood cells (WBCs) which are responsible for the immune response to infection consist of five types: granulocytes (neutrophils, eosinophils, basophils) and agranulocytes (monocytes and lymphocytes) (Leeds Histology Guide). The classification refers to the presence of granulocytes in the cytoplasm when stained. Using conventional staining methods such as Giemsa, different types of WBCs can be inferred by the shape of their nucleus and relative sizes. Neutrophils (12-14 µm) have a multilobed nucleus (between 2-5 lobes). Eosinophils (12- 17 µm) have nuclei that are bi-lobed. Their granules are acidophilic and thus, appear bright red or reddish purple. Basophils (14-16 µm) make up only 1% of white blood cells. Similar to eosinophils, their nucleus is bi-lobed, but in contrast, they consist of basic granules that stain deep blue. Lymphocytes (some small 6-9 µm, some larger 10-14 µm). They have a small spherical nucleus that takes up most of the space in the cell, and the cytoplasm is basic and thus appears blue. Monocytes (~20 µm) are the largest type of white blood cell and are the third

most common. They have a large bilobed, bean shaped nucleus and abundant cytoplasm with very faint pink granules. Finally, platelets (1.5-4.5 μm) are much smaller than red blood cells and do not have nucleus (Leeds Histology Guide). An important characteristic of *Babesia* is that they have ring structures/merozoites that are typically smaller than red blood cells. The parasite can also appear pear-shaped or tetrad structures also known as Maltese cross forms.

Microscopically, it is difficult to identify the *Babesia* pathogen to species, therefore DNA based molecular methods such as Polymerase Chain Reaction (PCR) can be used in detecting *Babesia* DNA. In one study, PCR detected parasitemia as low as 0.000001% and even in asymptomatic carriers (Alvarez et al., 2019). PCR is not without its limitations. While confirmation of the species is accomplished by sequencing the amplicons, the process of PCR involves primers targeted to the gene sequence in question, so unexpected non-target species may produce false negatives. To overcome this, nested PCR can be used, which involves two amplification reactions. The first reaction uses an outer pair of primers that anneal to sequences external to the sequence amplified by the second inner set of primers. The amplified product in the first round is used as a template for the second round to reduce non-specific binding of primers in DNA products as to increase sensitivity and specificity (Carr et al., 2010). On the other hand, over specificity can be a problem as it may yield negative results if the primers used in the reaction only amplify a single target therefore may fail to detect closely related pathogens and produce false negative which compromises disease diagnosis. In addition to the problem of over-specificity, cost for equipment and reagents can be an issue and the sensitivity of this approach is somewhat compromised by the risk for cross-contamination from additional sample handling (Alvarez et al., 2019).

Serological tests such as enzyme-linked immunosorbent assay is commonly used in screening blood donors in endemic areas and are used in seroprevalence studies. This is a technique that detects antibodies to *Babesia* antigens in serum, a measure of exposure to the pathogen. *Babesia* titers, the level of antibodies in the blood, increase within 2 to 4 weeks of illness and declines over 8-12 months (Parija et al., 2015). Therefore, the timing of the test is critical as levels of antibodies vary with timing of infection and if not interpreted carefully, it may not accurately reflect disease. The disadvantages of serological testing are that it only detects an exposure to a pathogen, it cannot distinguish between past and current infection, and that different *Babesia* antibodies may cross react and misidentify species (Parija et al., 2015).

1.5 Purpose of study

Within Canada, only a few nation-wide studies have been done to detect *Babesia* pathogens. Nationwide seroprevalence studies were conducted in 2013 and 2018, both of which screened for *B. microti* antibodies in Canadian blood donors and both suggested very low prevalence (O'Brien, 2016, Tonnetti et al., 2019). Seroprevalence studies involves serological testing which are associated with biological challenges mentioned previously. Thus, risk of disease may be erroneously misinterpreted and there is a need for a better testing that would accurately reflect disease prevalence. Furthermore, in provinces with established tick populations, such as New Brunswick and Nova Scotia, surveillance of the *Babesia* has not been done. Understanding that *Babesia* is not limited to a specific host led to the attempt to survey *Babesia* in cattle in this study. Cattles are economically important animals as they supply milk and beef. Currently in Canada, let alone in the Maritimes, there are no surveillance efforts on detecting *Babesia* from cattle, which might be problematic as cattle are exposed to ticks in Lyme disease endemic areas of Nova Scotia and New Brunswick (McGowan, 2019).

Recognizing that *Babesia* is a parasite of importance for human and veterinary medicine, surveillance of the pathogen is important and should not be limited to known endemic areas. This study aims to determine the prevalence of the *Babesia* species: *B. microti*, *B. duncani* and *B. odocoilei* in the Maritime Canada, particularly in cow blood samples collected from Nova Scotia and New Brunswick dairy farms, and in *Ixodes scapularis*, *Ixodes cookei*, *Dermacentor albipictus*, and *Dermacentor variabilis* ticks collected from New Brunswick, Nova Scotia and Prince Edward Island. Secondly, the study aims to assess how long these pathogens have been established in the region by assessing ticks collected during different time periods. Considering recent discovery of *B. odocoilei* and *B. duncani* in Canada, it is unknown if their emergence is recent or if they have been established but not discovered due to lack of surveillance. Finally, this study also looks at the presence of *Babesia* in different life stages of ticks: larvae, nymph, and adults to make inference on whether or not the pathogen can be transmitted between life stages. It is hypothesized that *Babesia* is present and will be more prevalent in southern New Brunswick and all mainland Nova Scotia as these are moderate to high risk areas for *I. scapularis* exposure and are in proximity to *Babesia*-endemic areas of the United States.

Methods

2.1 Cow and human blood collection

Blood samples from 220 outdoor-grazing dairy cows were previously collected in 2018 from farms located in northern New Brunswick (Restigouche, Gloucester and Northumberland Counties), southern New Brunswick (King's County) and northwestern Nova Scotia (King's, Hants, and Annapolis Counties) (Animal Care Protocol #102183). One sample was collected per cow. These samples were previously used for a *Borrelia* seroprevalence study, thus serum was removed from the blood collection tube with the residual cells stored in a -21 °C freezer (McGowan, 2019). One blood sample was obtained from a *Babesia* seropositive human (REB #2016-042). The sample of blood removed at autopsy was stored in a 4°C fridge (Research Ethics Board #2016-042). Control blood sample was collected from a finger prick from a healthy, non-symptomatic human.

2.2 Tick collection and sample selection

A total of 339 previously extracted tick DNA samples were selected from the Mount Allison Lloyd Lab and Geneticks tick bank. Ticks were submitted by the general public and each submission form contained collection city, and province to identify the areas from which ticks were recovered, as well as species name, life stage, sex, engorgement status and host of the tick. Submission that disclosed recent travel was excluded from sample selection. The first set of samples were collected from recent years (2017-2022), were primarily *Ixodes scapularis* adults and were geographically distributed throughout the provinces of New Brunswick, Nova Scotia and Prince Edward Island (Figure 2.1). The second set of samples that were collected from 2013-2016, included *Dermacentor variabilis*, *Ixodes cookei* and *Ixodes scapularis* at larvae, nymph and adult stages (Figure 2.2). Samples were chosen to be geographically dispersed as much as possible but also targeted areas from which *Babesia*-positive ticks were identified in the recent tick samples.



Figure 2.1 Location of tick samples collected from 2017-2022 selected from the Mount Allison Lloyd and Geneticks Tick Bank. N=175



Figure 2.2. Location of tick samples collected from 2013-2016 selected from the Mount Allison Lloyd Tick Bank. N=164

2.3 Blood smears preparation for Acridine Orange and Giemsa staining

Thick and thin blood smears were prepared on the same slide. A thick blood smear was prepared by placing a drop of blood at one end of the slide. A corner of another slide was used to spread the drop in a circular pattern until it was about 1.5 cm in diameter while applying a firm pressure to create small scratches for better adherence. To prepare a thin blood smear, a small drop of blood was placed at the other end of a slide. A second glass slide held at 30-45° angle above the drop was used to quickly spread the blood into a thin film across the slide. Smears were allowed to dry for 15-30 min. Smears were fixed by pipetting ~300µL methanol over the whole area of blood and were left to air dry at room temperature for ~10 min. Two sets of thick and thin blood smears were prepared, one for acridine orange staining and another for Giemsa staining.

Stains: Prior to staining, a phosphate buffer solution (PBS) was prepared containing 0.1 M of potassium dihydrogen phosphate (KH_2PO_4) (Sigma, #105K0138) and 0.1 M of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$) (ACP chemicals, #A1900) adjusted to pH 6. Basic acridine orange solution was prepared by mixing 0.1 g of acridine orange powder dye mixed with 10 mL dH_2O . To make a working acridine orange solution, basic acridine orange solution was dissolved in phosphate buffer solution in 1:10 ratio. Giemsa solution (Sigma, 48900-100ML-F Giemsa Stain, Modified Solution, #51811-82-6) was diluted with PBS in 1:20.

Staining: ~100 µL of acridine orange (Sigma Acridine Orange, C.I. 46005, Lot#69F3672) working solution was pipetted over the whole area of air-dried, fixed blood smears. The solution was allowed to sit for 2 min. The second set of slides were submerged in a Coplin jar full of Giemsa working solution for 45 min. All slides were rinsed with dH_2O until no residue stain was left and were air dried for 30 min or until dry.

2.4 Microscopic detection of *Babesia*

Microscopy (Leica) was performed to examine blood smears. Blood smears stained with Giemsa and acridine orange (as described above) was observed under brightfield and fluorescent microscopy respectively. To quantify *Babesia* parasites, the number of potentially parasitized red blood cells (RBC) was counted in 100 RBCs. These were characterized as ring-forms or pear-shaped merozoite forms inside red blood cells. These forms fluoresce green under acridine orange staining and stain dark purple under Giemsa staining.

2.5 DNA extraction of ticks and blood

Prior to performing DNA extraction, sterilization of equipment was applied by exposure to UV light for 15 min. Experiment was handled with appropriate personal protective equipment (PPE). Each tick specimen was cut in half; one half was used for DNA extraction and the other was stored in -20°C freezer. The half-tick was crushed and homogenized with a micropestle in a 1.5 mL microcentrifuge tubes containing 50 µL of AquaGenomic Solution (AGS) (Boca Scientific, #1011), followed by 45-min of immersion in a 60°C water bath (Fisher Scientific, #11-718). The tube with the homogenized tick was vortexed by repeatedly sliding the microcentrifuge tube against a rack held at an 45° angle for 10 seconds, then centrifuged (Spectrafuge 24D microcentrifuge, Labnet, #C2400) at 13,000 rpm for 4 min. The liquid supernatant was pipetted into 50µL of isopropanol in another sterile microcentrifuge tube, and the remaining pellet, which contained tick parts, were archived at -20°C. To precipitate DNA, the liquid supernatant and isopropanol solution was inverted 5 times then centrifuged at 13,000 rpm for 4 min. The supernatant was aspirated carefully to not disturb the pellet and was disposed into the liquid waste. Then, 50 µL of 70% ethanol was added to the tubes enough to submerge visible pellets to wash DNA, which was the pellet remaining the tube. Washing removes contaminating proteins and concentrate the DNA. Then, the tubes were air dried for 15 min before resuspending the DNA with 50 µL of 1mM TrisHCl and incubated in a 60°C water bath for one hour. This will solubilize DNA and protect from degradation. The DNA sample was then stored in -20°C freezer until use.

DNA extraction from blood followed the protocol from DNeasy Blood& Tissue Kit (50) (QIAGEN, #69504). Work surfaces and tools were sterilized and procedures were performed

with appropriate PPE as described above. For blood samples containing non-nucleated erythrocytes, as is the case for humans and cattle, 100 μL of blood was aliquoted. A 1.5 mL microcentrifuge tube containing 20 μL of 600 mAU/mL Proteinase K, 100 μL of cattle or human blood, 100 μL of Dulbecco's Phosphate Buffer Solution (50mM KH_2OPO_4 and 150 mM NaCl), and 200 μL of lysis Buffer AL was mixed by vortex and incubated at 56 $^\circ\text{C}$ for 10 min. This step was followed by the addition of 200 μL of 96% ethanol, and the tube was vortexed thoroughly (Scientific Industries, Vortex-Genie). This mixture was pipetted into a DNeasy Mini spin column imbedded in a 2 mL collection tube. The tubes were then centrifuged at 8,000 rpm for 1 min to permit binding of DNA to the DNeasy membrane of the spin column. The flow through and collection tube were discarded. Two washing steps were performed to remove remaining contaminants and enzyme inhibitors. The spin column was transferred to a new 2 mL collection tube and 500 μL of wash buffer AW1 was added. The column was centrifuged at 8,000 rpm for 1 min. The flow through and collection tube were discarded. The spin column was then transferred to a new 2 mL collection tube, and 500 μL of wash buffer AW2 was added, followed by centrifugation at 13,300 rpm for 3.5 min to allow DNeasy membrane to dry. Then, the spin column was placed to a clean 1.5 mL microcentrifuge tube and 200 μL of elution buffer AE was added, followed by incubation at room temperature for 1 min and centrifugation at 8,000 for 1 min. The eluate, containing the DNA, was then stored in -20°C freezer until use. Quality and concentration of DNA were measured with NanoDrop 1000 spectrophotometer (software version 3.8.1, Thermo Scientific, #C2400). The presence and integrity of DNA was tested using PCR amplification of the *cytochrome c oxidase subunit I (COI)* mitochondrial gene as described below (Table 2.1, Table 2.2).

2.6 Nested Polymerase Chain Reaction (nPCR)

Prior to performing nested polymerase chain reaction, all equipment and nuclease free water (nfH₂O) was sterilized by ultraviolet light. To prepare the master mix, the total amount of nfH₂O, GoTaq Green (GTG) (Promega, #M782B), and 10 μM forward and reverse primers was mixed in 1.2 mL microcentrifuge tube to make a master mix. The total amount of master mix necessary was calculated so that each sample, including negative control, contained 12.5 μL GTG, 9.5 μL nfH₂O, and 0.5 μL of each primer. There was no positive control available. A negative control was run at the same time as experimental amplifications to detect any contamination in the PCR hood or reagents. All 0.2 mL PCR tube (VWR, 20170-012) contained

23 μ L of master mix and 2 μ L of DNA sample. In the second-round amplification, an aliquot of 2 μ L of the first round PCR product was used as a template. All tubes were loaded into microcentrifuge to collect the mixture at the bottom of the tube before loading in to the thermocycler (Labnet, #TC9610). Stock and working solutions for all primers were prepared by S. Bishop and A. Berthold. The screening primers detected the 18s ribosomal RNA small subunit gene sequence of *B. microti*, *odocoilei* and *duncani* (Table 2.3). For the amplification of *B. odocoilei* and *B.duncani* the same outer primers and inner reverse primer were used (Table 2.3). The PCR thermocycler protocols are described below (Table 2.4). After the PCR program, samples were held at 4°C if being used immediately or stored at -20°C until use.

Table 2.1 PCR primers used for amplification of *cytochrome c oxidase subunit (COI)* mitochondrial gene in extracted DNA blood samples (Folmer et al., 1994, Zuccon et al., 2012).

Primer name	Amplified fragment	Amplicon size (bp)	Primer sequence (5' → 3')
FOLM F	<i>CoI</i>	710	GGTCAACAAATCATAAAGATATTGG
FOLM R			TAAACTTCAGGGTGACCAAAAAATCA

Table 2.2 Thermal cycler PCR program for *cytochrome c oxidase subunit (COI)* amplification (Folmer et al., 1994, Zuccon et al., 2012).

	5 cycles				35 cycles			
	Initial denaturation	Denaturation	Annealing	Elongation	Denaturation	Annealing	Elongation	Final Elongation
<i>COI</i>	94 °C for 5 min	94 °C for 30 sec	45°C for 30 sec	72°C for 1 min	94 °C for 30 sec	51 °C for 30 sec	72 °C for 1 min	72 °C for 10 min

Table 2.3. PCR primers used for amplification of *B. microti*, *B. odocoilei*, and *B. duncani* in tick DNA and blood DNA samples (Persing et al., 1992, Holman et al., 2003, Bloch et al., 2012).

Primer name	Primer Type	Amplified fragment	Amplicon size(bp)	Primer sequence (5' → 3')	Melting temperature °C
BabMicO utF	Outer		238	CTTAGTATAAGCTTTTATA CAGC	50.73
BabMicO utR	Outer		238	ATAGGTCAGAACTTGAAT GATACA	56.05
BabMicIn F	Inner		155	GTTATAGTTTATTTGATGTT C	45.96
BabMicIn R	Inner	<i>18s</i> rRNA	155	AAGCCATGCGATTCGCTAA T	58.4
BabGenF	Outer		488	GTCTTGTAATTGGAATGAT GG	52.57
BabGenR	Outer		488	TAGTTTATGGTTAGGACTA CG	51.88
odo53	Inner		311	CCGTATTTTGACTTTTGTCG ACTGT	60.28
dun689	Inner		280	GGTGGTTCTCCATTTGCCA G	59.11
BabGenIn R1	Inner		280 or 311	TCTGATCGTCTTCGATCCCC	58.68

Table 2.4. Thermal cycler PCR program *B. odocoilei*, *B. duncani* and *B. microti* amplification (Persing et al., 1992, Holman et al., 2003, Bloch et al., 2012).

	Initial denaturation	Denaturation	Annealing	Elongation	Cycles	Final Elongation
<i>1st round</i>	95°C for	95°C for	55°C for	72°C for	40	72°C for
<i>B. duncani</i>	5 min	30 sec	30 sec	45 sec		10 min
<i>B. odocoilei</i>						
<i>2nd round</i>	95°C for	95°C for	63°C for	72°C for	40	72°C for
<i>B. duncani</i>	5 min	30 sec	15 sec	20 sec		10 min
<i>B. odocoilei</i>						
<i>1st B. microti</i>	94°C for	94°C for	55°C for	72°C for	35	72°C for
	5 min	30 sec	30 sec	30 sec		5 min
<i>2nd B. microti</i>	94°C for	94°C for	55°C for	72°C for	30	72°C for
	5 min	30 sec	30 sec	30 sec		5 min

Preparation of putative positive samples for sequencing required nested PCR with a different set of primers (National Microbiology Laboratory Protocols) with the original DNA sample used as a template for the first round. The primers amplified the 18s ribosomal RNA small subunit gene sequence of *Babesia* species (Table 2.5). The PCR thermocycler protocols are described below (Table 2.6). PCR samples were electrophoresed on 1.2% agarose gels as described below. Separation of the amplicons produced multiple bands on gel, thus required gel purification to isolate the 343 bp amplicon using the EZ-10 Spin Column DNA Gel Extraction Kit (Biobasic, #BS354). Then, a final amplification of the purified DNA product was performed with the inner forward and reverse primers to concentrate the amplicon for sequencing. If the putative positive samples did not produce a 343 bp band, samples amplified with the screening primers were sent.

Table 2.5. PCR speciation primers used for amplification of *Babesia* species to prepare putative positive samples for sequencing (National Microbiology Laboratory Protocols).

Primer	Primer type	Amplified fragment	Amplicon size(bp)	Primer sequence (5' → 3')	Melting temperature °C
Bab1F	Outer		767	CCGTCGTAGTCCTAAC YATA AAC	58.41
Bab4R	Outer	18s		CCTTGTTACGACTTCTCCTTC C	58.65
Bab2F	Inner		343	TTCTTGATTCTYTGGGTRGT GG	57.93
Bab3R	Inner			CTAGGCATTCCCTCGTTCAWG AT	57.76

Table 2.6 Thermal cycler PCR program for *Babesia* species amplification for sequencing confirmation (National Microbiology Laboratory Protocols).

	Initial denaturation	Denaturation	Annealing	Elongation	Cycles	Final Elongation
1 st round <i>Babesia</i> species	95°C for 5 min	95°C for 30 sec	52°C for 1 min	72°C for 1 min	30	72°C for 10 min
2 nd round <i>Babesia</i> species	95°C for 5 min	95°C for 30 sec	55°C for 15 sec	72°C for 20 sec	30	72°C for 10 min

2.7 Gel Electrophoresis and Sequencing

An agarose gel (1.2 % g/mL) was prepared by mixing 1.2 g of powdered agarose (Froggarose LE,#A87-500G) and 100 mL of 0.5M 1X sodium borate (SB) buffer (10mM NaOH and 40mM H₃BO₃) to maintain environmental pH, and was heated in a microwave for 2 min, with intermittent stirring until all agarose particles had dissolved. When cooled enough to touch, 10 µL of DNA stain diluted in a 1:10000 ratio (Eco-Stain, Bio-Basic, #DT81413) was added. The mixture was poured into a mold, 20-well combs were inserted and allowed to solidify at room temperature for 15 min. Once solidified, the electrophoresis apparatus (Owl Separation Systems, Thermo Fisher Scientific, #116532) was prepared by submerging the gel in 1X SB Buffer. Once submerged, 5 µL of DNA ladder (100-1000bp DNA Ladder RTU, #DM001-R500 or 200-1500 bp (BioBasic #L3C00560K), 7 µL of negative control and 7 µL of round 2 PCR DNA products were loaded, separately, into the wells. The gel was electrophoresed at 250 V for 15 min. The gel was removed from the electrophoresis unit and viewed using a UV transillumination (Labnet DyNA Light Dual Intensity UV Transilluminator) and an image of a gel was taken for analysis. Each lane that corresponded to a sample was documented as either putatively positive or not by presence or absence of a DNA band of the predicted size. A potentially positive sample for any *Babesia* pathogen was identified by measuring the length of DNA bands with reference to the DNA ladder. Putative *B. odocoilei*, *B. duncani* and *B. microti* amplicons were indicated by the presence of a band at 311 bp, 280 bp, and 155 bp respectively. To confirm PCR results, putative positive samples were sent for Sanger or bidirectional sequencing at the Génome Quebec Innovation Center Mar. A total of 41 putative positive samples were sent. An additional 5 samples were sent for sequencing due to the presence of a non-specific band at 500 bp. The resulting forward and reverse sequence chromatograms were processed in FinchTV software and aligned with MEGA 11. To confirm species, a Nucleotide Basic Local Alignment Search Tool (BLAST) was performed using the National Center for Biotechnology Information (NCBI) Gen Bank.

Results

The goal of this study was to identify the presence of *Babesia* species namely *B. microti*, *B. duncani* and *B. odocoilei* in dairy cattle blood samples using microscopy and nested PCR. Molecular surveillance of all pathogens was also conducted in DNA samples of *I. scapularis*, *I. cookei*, *D. albipictus* and *D. variabilis* recovered from New Brunswick, Nova Scotia and Prince Edward Island in the years 2013-2016 and 2017-2022. Here I assess the prevalence, distribution and establishment of *Babesia* species in the Maritimes region and describe the presence of the pathogen in adult, nymph and larval ticks. It was hypothesized that *Babesia* is present and will be more prevalent in southern New Brunswick and all Nova Scotia. Finally, I report sequencing analysis of the PCR-confirmed positive samples.

3.1 *Babesia* identification in blood smear

A total of 57 stained cow blood smear samples were observed under the microscope to detect *Babesia* parasites. For blood smear examination and accurately identifying parasites, it is useful to have knowledge on features of blood components and *Babesia* which appear differently under different staining. In Giemsa-stained blood, red blood cells, which have biconcave shape, stain light pink and white blood cells, which have different nucleus structures, stain deep blue (Figure 3.1A). Neutrophils (multilobed nucleus) and lymphocytes (one spherical nucleus) and small platelets were recognized (Figure 3.1 A and B). In acridine orange staining, red blood cells have circular green autofluorescence and white blood cells have nucleus that fluoresce orange (Figure 3.1B). *Babesia* have ring structures/merozoites that are typically smaller than red blood cells. *Babesia* may also be in the form of pear-shaped structures and tetrad structures also known as Maltese cross forms. These forms fluoresce green under acridine orange staining and stain dark purple in Giemsa staining.

The resulting blood film quality of samples was poor despite attempts to reduce or prolong staining time, renewing acridine orange stock solution, and reducing the frequency of freeze-thaw cycles for the samples. Acridine orange-stained slides of cow blood samples revealed morphologically unidentifiable small fluorescent dots, and stain artifacts against a hazy green background (Figure 3.2 B). Giemsa-stained cow blood samples and the positive control revealed dark blue staining, presumably nuclei of white blood cells, but no staining of red blood

cells (Figure 3.2A and Figure 3.3A). Cow blood also lacked recognizable cellular components (Figure 3.2) unlike in the negative control human blood sample (Figure 3.1).

In acridine orange-stained positive control blood film, red blood cells appeared as ghostly green clumps in the background, with small fluorescent green dots formed singly or in clumps within or on top of red blood cells (Figure 3.3B). These may be a characterized feature of *Babesia* merozoites, but it would require molecular methods to confirm this. The Giemsa stained negative control showed no identifiable forms of parasites (Figure 3.1).

Cow blood samples were stored frozen prior to the study and it is possible that cells have been lysed due to the freeze-thaw process, and the small amount of water that may have remained during the staining process. As a result, examination of cow blood films was not pursued due to the poor quality of blood cells. Therefore, I moved on to trying DNA extraction and nested PCR for the detection of *Babesia spp.*

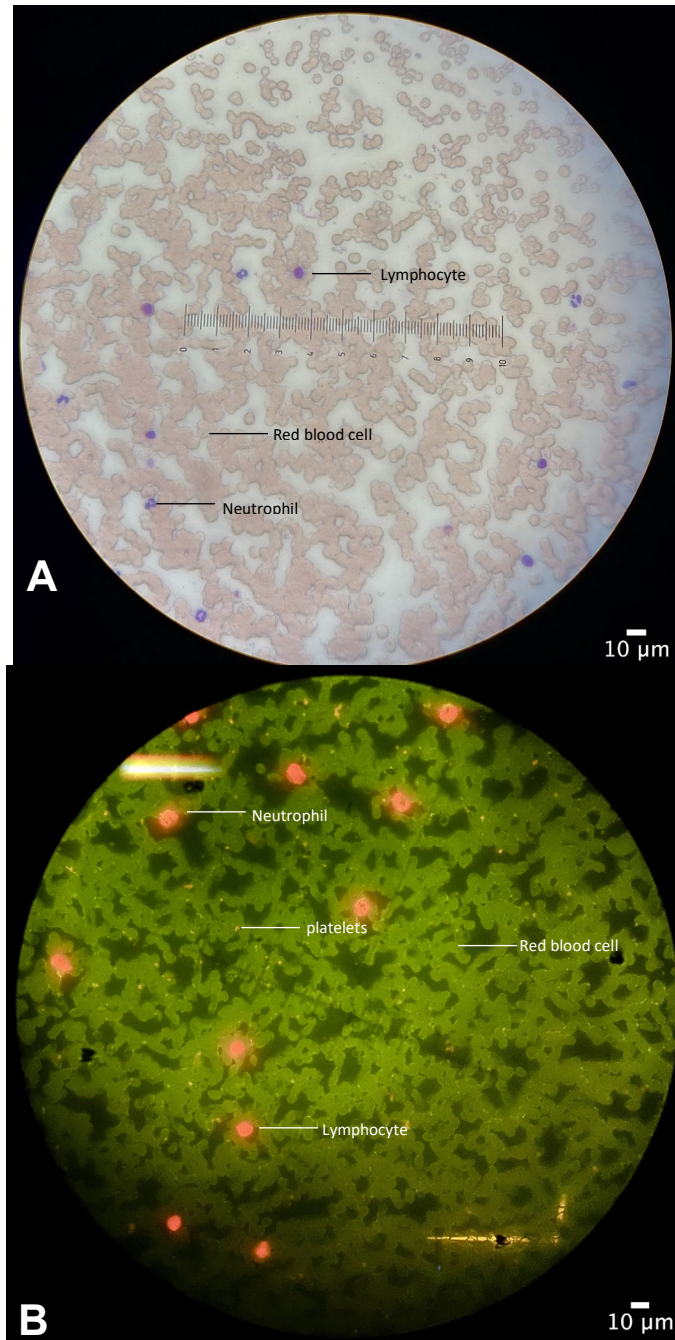


Figure 3.1. Control (presumed negative) blood sample from human stained with (A) Giemsa observed under a light microscope (400X magnification) (B) Acridine orange observed under a fluorescent microscope (400X magnification)

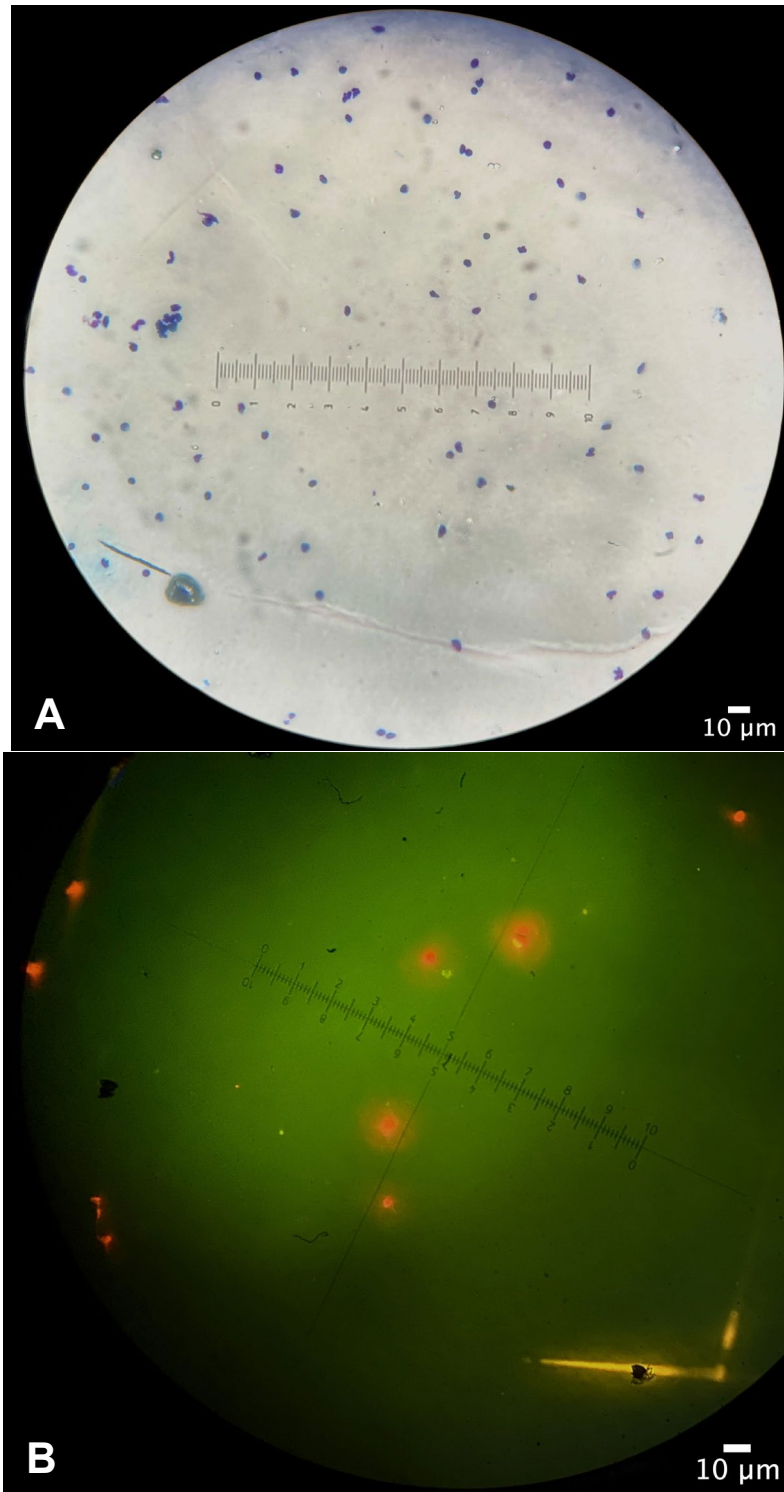


Figure 3.2. Cow blood smear sample from Farm D, stained with (A) Giemsa observed under a light microscope (400X magnification) (B) Acridine orange observed under a fluorescent microscope (400X magnification)

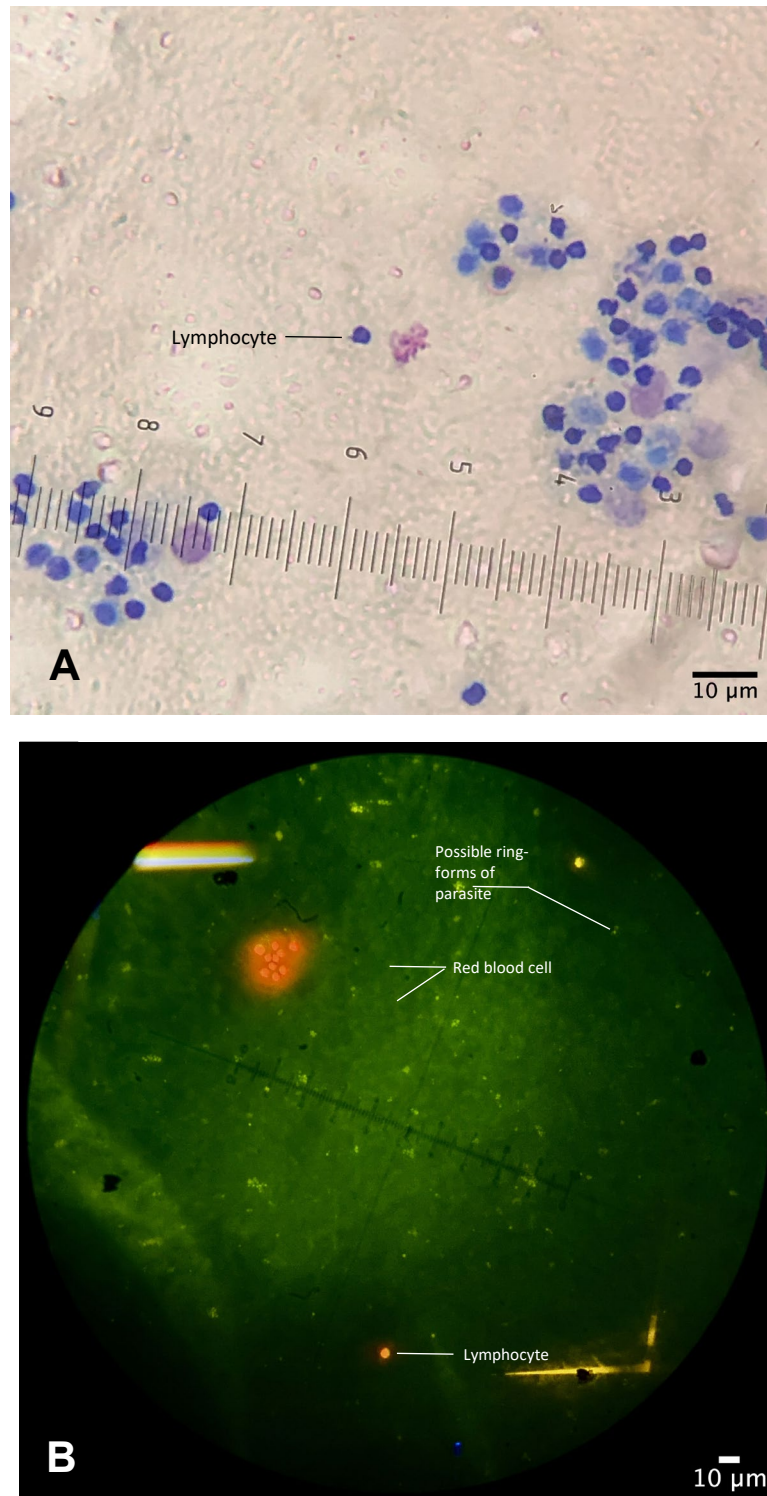


Figure 3.3. Human blood sample seropositive for *Babesia* stained with (A) Giemsa observed under a light microscope (400X magnification) (B) and acridine orange observed under a fluorescent microscope (400X magnification).

3.2 *Babesia* identification in blood samples using nested PCR

Fourteen blood samples were selected for DNA extraction, some of which were selected randomly but D420, D464, and D469 were particularly chosen as they revealed morphologically unidentifiable fluorescent dots in reference to Figure 3.3 B although these samples did not reveal identifiable red blood cells. Extracted DNA was tested for presence and integrity by attempting to amplify for the *cytochrome c oxidase subunit I (COI)* mitochondrial gene. No band was observed in any of the samples, even the positive control sample (Figure 4).

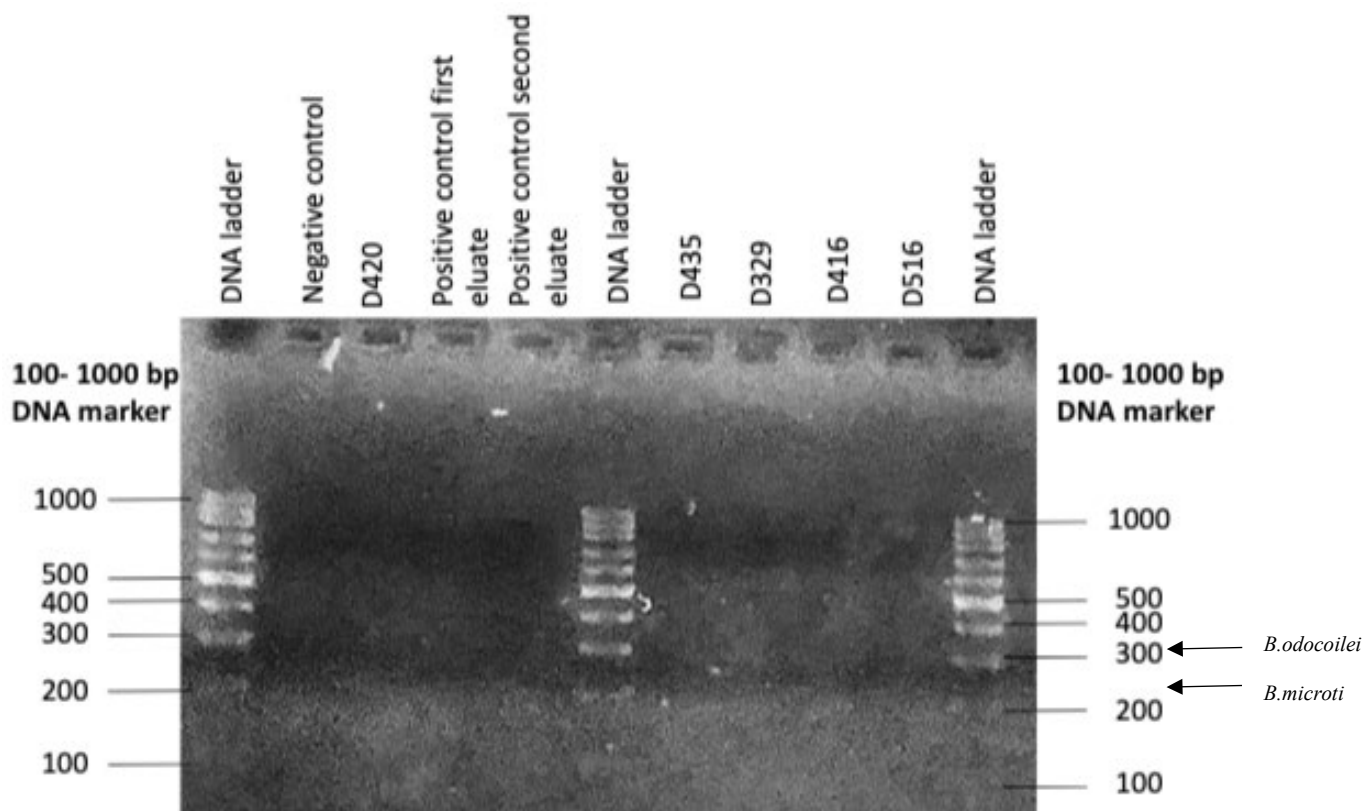


Figure 3.4. Image of a gel showing blood DNA samples amplified for *cytochrome c oxidase subunit I (COI)*.

Additionally, DNA concentration and purity of the blood samples was assessed. DNA concentration was low and quality was highly impure. There was a low ratio of 260/280 (optimal is 1.80) which indicates a higher amount of protein relative to nucleic acid. There was also a low ratio of 260/230 (optimal ratio is 2 or above) which indicates a higher amount salt relative to nucleic acid. Due to this high impurity, the rest of the samples were presumed to have low quality and were not extracted in this preliminary assessment.

Table 3.1. Purity of extracted DNA blood samples reflected in 260^a/280^b and 260/230^c ratios and DNA concentrations

Sample ID	260/280	260/230	DNA(ng/ μ L)
D516	1.78	0.11	2.5
D482	1.18	0.14	4.7
D494	0.87	0.14	4.1
D381	1.11	0.15	4.2
D416	0.64	0.07	1.5
D329	1.4	0.24	7.9
D417	1.13	0.2	8.8
D435	1.19	0.17	4.6
D384	1.08	0.15	6.9
D382	1.16	0.2	6.4
Positiveeluate1	0.99	0.13	5.0
Positiveeluate2	1.01	0.24	3.0
D464eluate1	1.4	0.24	12.2
D464eluate2	1.17	0.18	3.6
D420eluate1	1.61	0.58	17.1
D420eluate2	1.25	0.19	6.3
D469eluate1	1.45	0.28	6.0
D469eluate2	1.12	0.13	3.7

^aabsorbance of DNA at 260 nm wavelength

^babsorbance of protein at 280 nm wavelength

^cabsorbance of salt at 230 nm wavelength

Nested PCR amplification for *Babesia spp.* was nevertheless attempted for blood DNA samples. Putative *B. odocoilei*, *B. duncani* and *B. microti* amplicons would be indicated by bands at 311 bp, 280 bp, and 155 bp, respectively. The seropositive control blood DNA did not generate a positive PCR test result for any of the *Babesia* pathogens (Figure 3.5).

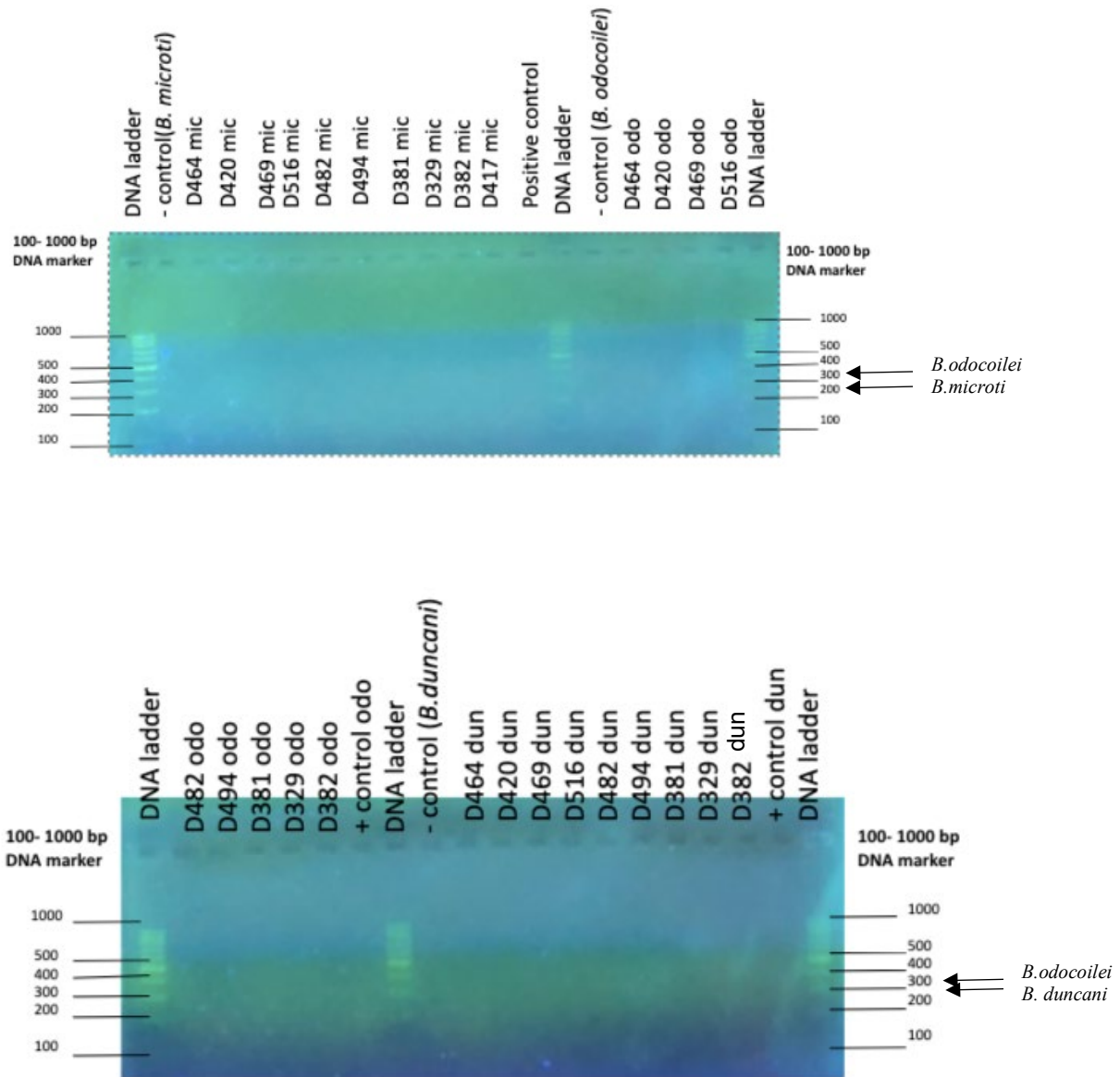


Figure 3.5. Image of a gel showing blood DNA samples amplified with primers for *B. microti*, *B. odocoilei* and *B. duncani*. Lanes correspond to blood sample number and primer it was tested for. “Odo” means the sample was tested for *B. odocoilei* and “dun” means that the sample was tested for *B. duncani* and “mic” means the sample was tested for *B. microti*.

3.3 *Babesia* identification in ticks using PCR

Ticks are known vectors of *Babesia* species, therefore in this study, tick DNA samples were tested with PCR for the presence of the parasite. *B. odocoilei* was detected in tick DNA samples evident by bands at approximately 300 bp (Figure 3.6B). Some samples such as 2021-0026 that were suspected positive for *B. odocoilei* also revealed band at approximately 500 bp when tested for *B. duncani* (Figure 3.6A). This sample was confirmed to contain *B. odocoilei* as revealed by genetic sequencing (Table 3.6). Four samples were also found to contain amplification of 500 bp DNA when amplified with the *B. odocoilei* primers (Figure 3.6C). This varied from the expected amplicon size (~300 bp) and therefore was also sent for sequencing. The presence of the expected amplicon visually assessed from gel was qualified for prevalence.

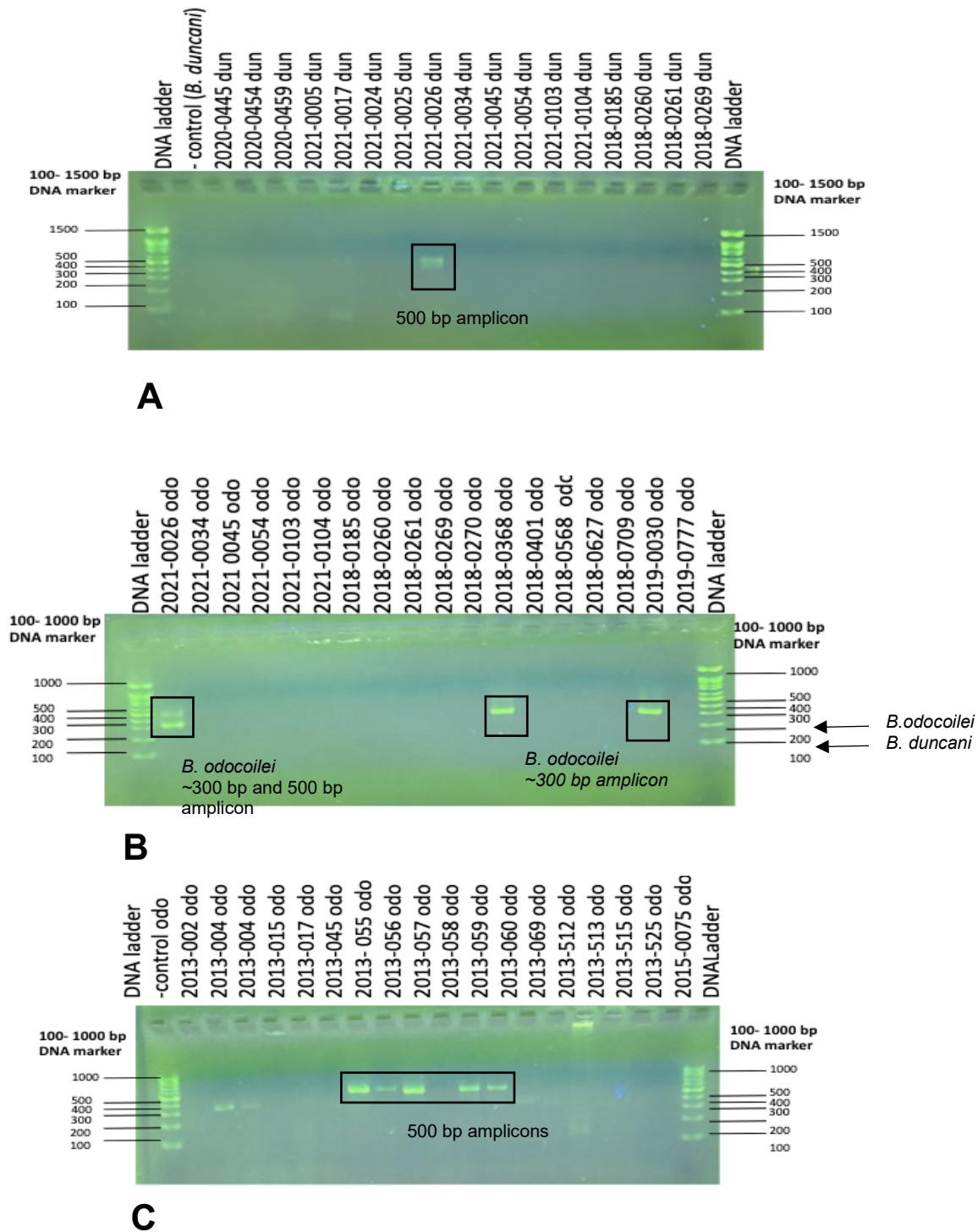


Figure 3.6. Image of a gel showing tick DNA samples amplified with primers for *B. microti*, *B. odocoilei* and *B. duncani*. Each lane corresponds to a tick sample number, and the primer it was tested for. “Odo” means the sample was tested for *B. odocoilei* and “dun” means that the sample was tested for *B. duncani*.

3.4 Prevalence, distribution and establishment of *Babesia species* in ticks in the Maritimes

Of the 175 tick DNA samples collected from 2017-2022, 25 produced amplicons consistent with the presence of *B. odocoilei*. *B. microti* and *B. duncani* were not detected. About 19% of the samples from Prince Edward Island, 16% from Nova Scotia and 10% from New Brunswick were putative positives for *B. odocoilei*, (Table 3.2).

To assess how long *Babesia* has been in the Maritimes, early ticks were tested. Of the 164 tick DNA samples collected from 2013-2016, 13 were considered PCR-confirmed for containing *B. odocoilei* amplicons and two were PCR-confirmed for containing *B. microti* . About 11% of the samples from PEI, 9% from Nova Scotia and 5% from New Brunswick were putative positives for *B. odocoilei* (Table 3.3). *B. odocoilei* was detected as early as 2013, and was consistently detected every year over the decade (Figure 3.7, Table 3.6). To sum, the prevalence of *B. odocoilei* doubled in ticks that were recovered in the recent years.

B. odocoilei was detected in ticks from all 3 provinces. *B. odocoilei* in recent ticks were more geographically dispersed stretching all the way across Nova Scotia and central and eastern Prince Edward Island and southern and northern New Brunswick (Figure 3.7). In addition to ensuring that early samples were geographically dispersed as much as possible, I considered testing early ticks near the areas from which *Babesia* positives were identified in the recent samples. Not all ticks from these areas had *B. odocoilei* in the early samples. *B. odocoilei* in ticks acquired from 2016-2022 were present in the southeastern New Brunswick close to the New Brunswick-Nova Scotia border, in central-eastern PEI and central Nova Scotia. *B. microti* was detected in central and eastern New Brunswick (Figure 3.7).

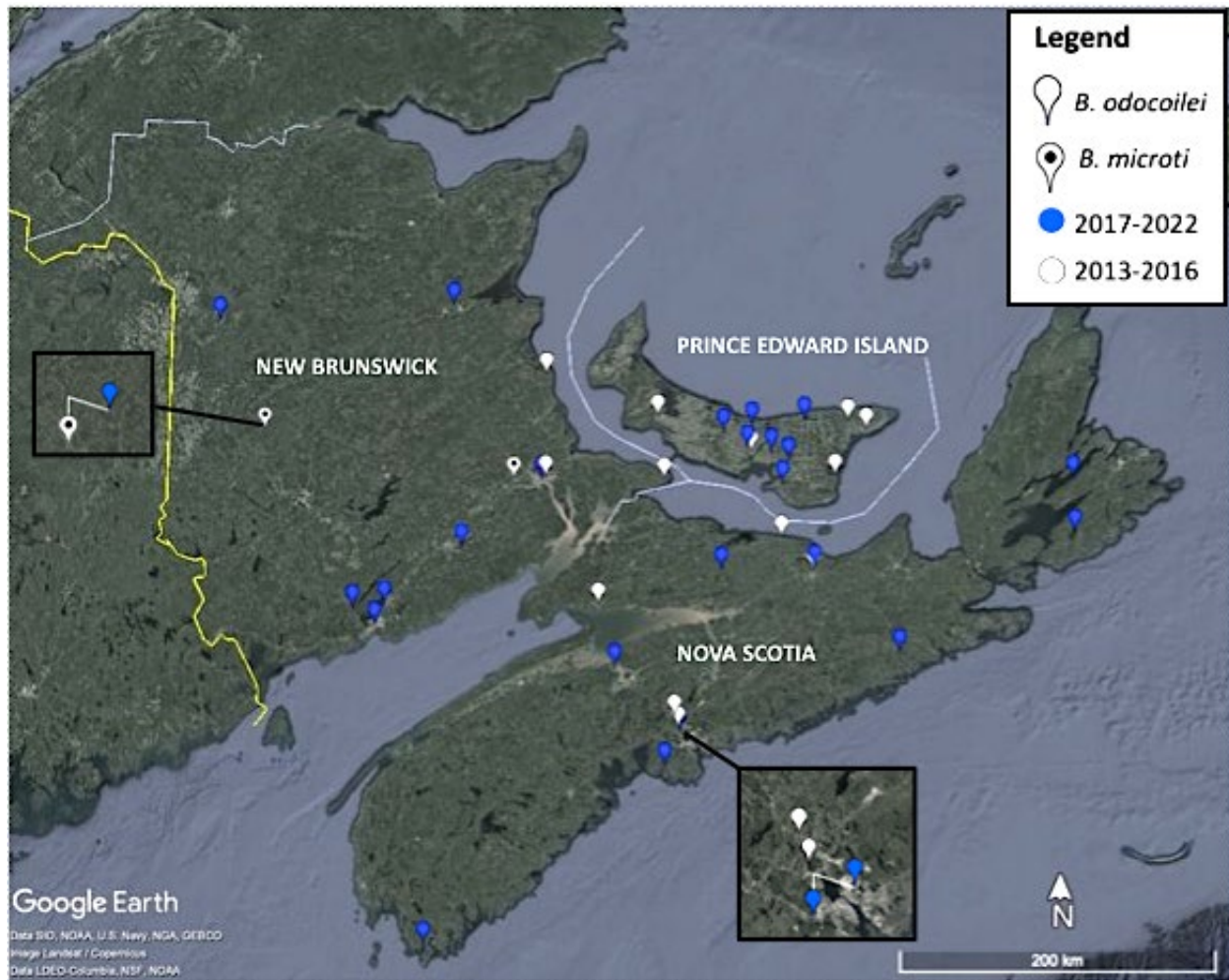


Figure 3.7. Location of collection of ticks PCR-confirmed positive for *B. odocoilei* and *B. microti*.

Table 3.2. Percentage of tick DNA samples (from 2017-2022) that are PCR confirmed positive for *Babesia* spp.

Province	<i>B. microti</i> % pos (95%CI)	<i>B. odocoilei</i> % pos(95%CI)	<i>B. duncani</i> % pos(95%CI)
New Brunswick (n=77)	0 (0-4.8)	10.39 (5.4-19.1)	0(0-4.8)
Nova Scotia (n=61)	0 (0-5.9)	16.4(9.2-27.6)	0 (0-5.9)
Prince Edward Island (n=37)	0(0-9.406)	18.92(9.48-34.2)	0(0-9.406)

Table 3.3. Percentage of tick DNA samples (from 2013-2016) that are PCR confirmed positive for *Babesia* spp.

Province	<i>B. microti</i> % pos(95%CI)	<i>B. odocoilei</i> % pos(95%CI)	<i>B. duncani</i> % pos(95%CI)
New Brunswick (n=65)	3.0(0.8-10.54)	4.6(1.6-12.7)	0(0-5.6)
Nova Scotia (n=53)	0(0-6.8)	9.4(4.1-20.3)	0(0-6.8)
Prince Edward Island (n=46)	0(0-7.7)	10.9(4.7-23.0)	0(0-7.7)

3.5 *Babesia* in different tick species and developmental stages

B. duncani was not detected in any of the ticks. *B. microti* was detected in *I. cookei* and *D. albipictus* while *B. odocoilei* was detected in all four species tested (Table 3.4). In general, *B. odocoilei* and *microti* were detected in adult and nymph ticks (Table 3.5). More specifically, *B. microti* was detected in an adult *D. albipictus* and *I. cookei* ticks. *B. odocoilei* was detected in nymph *I. scapularis* and adult of all tick species (Table 3.5).

Table 3.4. Number of ticks at different species that are PCR confirmed positive for *Babesia* spp.

Province	<i>B. microti</i>	<i>B. odocoilei</i>	<i>B. duncani</i>
<i>Ixodes scapularis</i> (n=277)	0	29	0
<i>Ixodes cookei</i> (n=56)	1	4	0
<i>Dermacentor variabilis</i> (n=27)	0	3	0
<i>Dermacentor albipictus</i> (n=26)	1	2	0

Table 3.5. Number of ticks at different developmental stages that are PCR confirmed positive for *Babesia* spp.

Province	<i>B. microti</i>	<i>B. odocoilei</i>	<i>B. duncani</i>
Adult (n=279)	2	33	0
Nymph (n=39)	0	4	0
Larvae (n=20)	0	0	0

3.6 *Babesia* sequence confirmation

Following sequencing and BLAST analysis of 41 PCR-confirmed *Babesia* positive samples, 36 samples showed strong sequence identity to *B. odocoilei* and two to *B. microti* (Table 3.6). Additional five samples that had amplicons measuring at 500 bp were also analyzed. One sample showed strong sequence identity to *B. microti*, three showed slightly lower percent identity to “*Babesia* sp. FXK-2016a clone T14” and one showed strong identity to *B. odocoilei* and Piroplasmida (Table 3.6). A few chromatogram sequencings revealed high amounts of ambiguous peaks which made it difficult to attain good sequencing result from BLAST. Three samples produced inadequate sequence match. Sample “2020-10039” had a failed forward sequence read, but the reverse sequence read showed strong identity to *B. odocoilei*. Seventeen samples were also likely contaminated by nearby wells.

Table 3.6. BLAST sequencing analysis of tick samples PCR confirmed positive for *B. odocoilei*, and *B. microti*^a and four samples. Forward and reverse sequences were compared. Samples with “FR” are forward and reverse sequences that were aligned, and assembled into one contiguous sequence to resolve any sequencing errors.

Sample	18s rRNA sequence result	% Query Cover	% Identification	Accession ID
2013_2-FR	<i>B. odocoilei</i>	100	100	<u>MK986474.1</u>
2013_4-FR	<i>B. odocoilei</i>	99	98.95	<u>MK986474.1</u>
*2013_55-FR ^a	<i>B. microti</i>	100	99.13	AY144698.1
*2013_56F-R ^b	<i>B. microti</i>	100	98.83	<u>AY144698.1</u>
*2013_57-FR ^b	<i>Babesia</i> sp. FXK-2016a clone T14	100	95.59	<u>KX168695.1</u>
*2013_59-FR ^b	<i>Babesia</i> sp. FXK-2016a clone T14	100	95.29	<u>KX168695.1</u>
*2013_60-FR ^b	<i>Babesia</i> sp. FXK-2016a clone T14	95.01	95.01	<u>KX168695.1</u>
*2013_69F	<i>B. odocoilei</i>	100	100	MT539381.1
*2013_69R	<i>B. odocoilei</i>	100	99.59	MK986473.1

*2013_194F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
*2013_194R	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
*2013_223F ^b	Piroplasmida sp	100	98.65	<u>EF057099.1</u>
*2013_223R ^b	<i>B.odocoilei</i>	100	99.58	<u>MH899097.1</u>
2013_406F	<i>B.odocoilei</i>	100	98.46	<u>MH899097.1</u>
2013_406R	<i>B.odocoilei</i>	99	99.57	<u>MH899097.1</u>
2014_285FR	<i>B. microti</i>	100	98.83	<u>AY144698.1</u>
2015_223FR	<i>Babesia</i> species	100	95.29	<u>AB566229.1</u>
2015_248F	<i>B.odocoilei</i>	99	99.64	<u>MH899097.1</u>
2015_248R	<i>B.odocoilei</i>	100	99.59	<u>MH899097.1</u>
2016_47F	<i>B.odocoilei</i>	100	100	<u>MT539381.1</u>
2016_47R	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
2016_53F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2016_53R	<i>B.odocoilei</i>	100	99.58	<u>MH899097.1</u>
2016_55F	<i>B.odocoilei</i>	100	99.64	<u>MH899097.1</u>
2016_55R	<i>B.odocoilei</i>	100	99.59	<u>MH899097.1</u>
*2016_67F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2016_67R	<i>B.odocoilei</i>	100	100	<u>MH899097.1</u>
2016_68F	<i>B.odocoilei</i>	100	99.64	<u>MH899097.1</u>
2016_68R	<i>B.odocoilei</i>	100	99.59	<u>MH899097.1</u>
2016_355FR	<i>B.odocoilei</i>	99	100	<u>MK986474.1</u>
*2017_8FR	<i>B.odocoilei</i>	100	100	<u>MT539381.1</u>
2018_368F	<i>B.odocoilei</i>	99	99.6	<u>MH899097.1</u>
2018_368R	<i>B.odocoilei</i>	100	99.18	<u>MH899097.1</u>
2018_638F	Sequencing failed	NA	NA	NA
2018_638R	Sequencing failed	NA	NA	NA
2018_859FR	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
2019_30F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2019_30R	<i>B.odocoilei</i>	100	99.17	<u>MH899097.1</u>
2019_59F	Sequencing failed	NA	NA	NA
2019_59R	Sequencing failed	NA	NA	NA
2019_45F	<i>B.odocoilei</i>	100	99.61	<u>MH899097.1</u>
2019_245R	<i>B.odocoilei</i>	100	99.59	<u>MH899097.1</u>

2019_426F	<i>B.odocoilei</i>	99	99.64	<u>MH899097.1</u>
2019_426R	<i>B.odocoilei</i>	100	99.57	<u>MH899097.1</u>
2019_648FR	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
*2019_789F	<i>B.odocoilei</i>	100	99.28	<u>MH899097.1</u>
*2019_789R	<i>B.odocoilei</i>	100	99.61	<u>MH899097.1</u>
2019_814F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2019_814R	<i>B.odocoilei</i>	100	98.77	<u>MH899097.1</u>
2019_907F	<i>B.odocoilei</i>	100	99.63	<u>MH899097.1</u>
2019_907R	<i>B.odocoilei</i>	100	100	<u>MH899097.1</u>
2020_273F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2020_273R	<i>B.odocoilei</i>	100	99.18	<u>MH899097.1</u>
*2020_459FR	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
*2020_10033FR	<i>B.odocoilei</i>	100	99.67	<u>MK986474.1</u>
*2020_10035FR	<i>B.odocoilei</i>	100	99.67	<u>MK986474.1</u>
*2020_10039F	Sequencing failed	NA	NA	NA
*2020_10039R	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
*2020_10082FR	<i>B.odocoilei</i>	100	100	<u>MK986474.1</u>
2021_17F	<i>B.odocoilei</i>	100	99.62	<u>MH899097.1</u>
2021_17R	<i>B.odocoilei</i>	100	99.58	<u>MH899097.1</u>
2021_26F	<i>B.odocoilei</i>	100	99.61	<u>MH899097.1</u>
2021_26R	<i>B.odocoilei</i>	100	100	<u>MH899097.1</u>
2021_10063F	Sequencing failed	NA	NA	NA
2021_10063R	Sequencing failed	NA	NA	NA
2021_10218F	<i>B.odocoilei</i>	100	99.57	<u>MH899097.1</u>
2021_10218R	<i>B.odocoilei</i>	100	99.56	<u>MH899097.1</u>
*2021_10277F	<i>B.odocoilei</i>	100	99.61	<u>MH899097.1</u>
*2021_10277R	<i>B.odocoilei</i>	100	100	<u>MH899097.1</u>
		100	99.64	<u>MH899097.1</u>
2021_10292F	<i>B.odocoilei</i>			
2021_10292R	<i>B.odocoilei</i>	100	99.59	<u>MH899097.1</u>
2021_10383F	<i>B.odocoilei</i>	99	99.62	<u>MH899097.1</u>
2021_10383R	<i>B.odocoilei</i>	100	99.57	<u>MH899097.1</u>
2022_10040F	<i>B.odocoilei</i>	100	99.23	<u>MH899097.1</u>
2022_10040R	<i>B.odocoilei</i>	100	99.57	<u>MH899097.1</u>

^a Samples that produced a band at ~200 bp consisted with *B.microti*

^b Samples that produced a band at 500 bp following PCR with the *B. odocoilei* screening primer

*Samples that were likely cross-contaminated in nearby wells as a result of unsealed cover

Discussion

The study aimed to identify *Babesia* parasites in cow blood and ticks using microscopy and nested PCR, respectively. Currently there is a lack of surveillance of the parasite within the Maritime provinces of Canada. In assessing the prevalence, distribution and establishment of *B. odocoilei*, *B. duncani* and *B. microti*, locally recovered tick samples from New Brunswick, Nova Scotia and Prince Edward Island were selected from two time periods: 2013-2016 and 2017-2022. PCR, gel electrophoresis and DNA sequencing were used to distinguish between *B. odocoilei*, *B. duncani* and *B. microti*. In the later tick samples, the prevalence of *B. odocoilei* had almost doubled and the geographic distribution of the species was wider. The pathogen was detected in all four tick species tested: *Ixodes scapularis*, *I. cookei*, *Dermacentor albipictus* and *D. variabilis*. *B. microti* was detected in *Ixodes cookei* and *Dermacentor albipictus* ticks. *B. duncani* was not detected.

4.1 *Babesia* detection by staining and microscopy

This study sought to identify *Babesia* in New Brunswick and Nova Scotia dairy cows. There has been no surveillance for this parasite, despite the cows' risk of tick exposure. Detection of *Babesia* was not possible in this study likely due to the poor quality of blood samples. Samples had been stored in -21 °C since collection in 2018 and the blood cells were lysed. Giemsa staining and Acridine orange staining was used to differentiate cellular structures but microscopic observations suggest that cells were hemolyzed (Lovelock, 1953). A human blood sample collected more recently and not frozen, thus produced higher quality blood film. Some features of *Babesia* may have been observed in acridine orange stained positive control blood film but PCR failed to confirm the presence of the pathogen.

Babesia species were also not detected in blood samples following DNA extraction and nested PCR. This too may have been due to degradation of DNA from repeated thawing in between the preparation of the same aliquots for staining and DNA extraction. This was indicated by spectrometry readings in the samples which demonstrated low DNA concentration. The effect of temperature and preservative after prolonged storage of blood samples on yield and quality of genomic DNA is important to consider. Ethylenediaminetetraacetic acid or EDTA blood stored long term at -20°C, with the addition of DNA preservative agent, can result in high DNA extraction yield (Bulla et al., 2016). Previous studies determined that collection and storage of blood samples may influence the sensitivity in detection of malarial parasites by PCR and that

freezing and thawing reduced the sensitivity (Färnert et al., 1999). Thus, my study reinforces the value of proper storage of blood which is crucial for isolating nucleic acids to ensure adequate performance of downstream applications such as PCR.

4.2 Prevalence of *B. odocoilei*, *B. microti* and *B. duncani* in the Maritimes

It was hypothesized that *Babesia* will be more prevalent in regions that are closer to northern *Babesia* endemic areas of the United States and in regions with well-established tick population: southern New Brunswick and all throughout Nova Scotia. This hypothesis was partially supported as *Babesia* is widespread across Nova Scotia. *Babesia* was also more prevalent in southern New Brunswick than the northern part of the province, however, it is important to consider sampling bias in favour of the southern region due to limited availability of ticks from northern region. Nevertheless, this can be biologically explained by the higher density of *Ixodes scapularis*, which vector *B. odocoilei*, in southern New Brunswick and all mainland Nova Scotia (Canada, 2022).

One of the goals of this study was to assess how long *Babesia* may have been established in the Maritimes. This study detected *B. odocoilei* in ticks collected as early as 2013, and the prevalence increased over the ensuing decade suggesting that *Babesia* is not a recently emerging pathogen and may be expanding in the region. The expansion and geographic distribution of tick populations may be one of the factors contributing to the range of *B. odocoilei* over the 9-year period surveillance in this study. The geographical location of *B. odocoilei* lie mostly within the Nova Scotia- New Brunswick border and the Halifax area in the early ticks. This timeframe coincides with another study from 2002-2013, which highlighted that the Halifax area had become a region at high risk for *I. scapularis* exposure and Lyme disease by 2010 (Hatchette et al., 2015). During 2012- 2020, New Brunswick also experienced an increasing tick abundance starting from the south east of the province and expanding westward and northwards the province by the end of the 9-year period (Lewis et al., 2021). Surveillance for *Babesia* in Prince Edward Island ticks is more limited in this study, with the earliest tick samples obtained only in 2016 as part of a passive surveillance study of ticks (Foley-Eby et al., 2020). However, there is evidence to suggest that *B. odocoilei* may be establishing in Prince Edward Island as it was present in the province as early as 2016, and has since shown a higher prevalence compared to other provinces.

Although other studies have demonstrated pathogen prevalence coincides with vector abundance (Walk et al., 2009), the range of suitable hosts may also play a role. Interestingly, Prince Edward Island had the highest prevalence of *B. odocoilei*. Despite the island having no deer populations, an important host for *I. scapularis* and reservoir host of *B. odocoilei* (Crandall et al., 2022), there is a possibility that wildlife and agricultural animals can support tick populations and *B. odocoilei*, and potentially other *Babesia* species. In addition, researchers have reported that migratory bird species such as Common Yellowthroat, and Veery can transport *B. odocoilei*-infected *I. scapularis* to eastern Canada (Scott et al., 2020). Therefore, it may be worth it to study ticks carried by migratory birds (State of Wildlife Report, 2007) that possibly facilitate the emergence of *B. odocoilei* in Prince Edward Island. Another explanation for the higher prevalence in Prince Edward Island may be attributable to the lower sample size. Nevertheless, *B. odocoilei* is present in the island. Another *Babesia* species, *B. vulpes*, is also present in the island, which was recently reported to cause canine babesiosis (Arsenault et al., 2022).

This study detected *B. microti* from New Brunswick in 2013, in the same year as the first known instance of tickborne *B. microti* infection in Canada, documented in Manitoba (Bullard et al., 2014). *B. microti* human babesiosis cases seems sparse, or at least less reported, with only one reported in the Maritime regions so far (Allehebi et al., 2022). The higher prevalence of *B. odocoilei* is noteworthy. With the potential for *Babesia* to be transmitted by blood transfusion, *B. microti* has been tested in Canadian blood donors (Tonnetti et al., 2019, O'Brien et al., 2016), but testing for the presence of other *Babesia* species is lacking. Indeed, it was only in 2021 that *B. odocoilei* was confirmed to cause human babesiosis (Scott et al., 2021), therefore surveillance of this pathogen in humans would be advisable given the data on its prevalence in the Maritime regions, presented here.

4.3 *Babesia* in different tick species

The study looked at *Babesia* in different tick species that are commonly found feeding from humans in the Maritimes to determine if they carry *Babesia*. In this study *B. odocoilei* was detected in multiple tick species: *I. scapularis*, *I. cookei*, *Dermacentor variabilis* and *D. albipictus*. The most commonly reported vector of *B. odocoilei* is *I. scapularis*, which was also found in this study (Milnes et al., 2019, Waldrup et al., 1990, Crandall et al., 2022). *B. odocoilei* has not yet been reported in the other tick species tested here, and vectoring by additional tick

species might extend the geographical risk of *B. odocoilei* infection as different species of ticks are abundant in different regions of the country. Certainly, this data warrants investigation of whether these tick species are vector-competent for *B. odocoilei*. Studies have demonstrated other *Babesia spp.* can be vectored by other *Dermacentor spp.*; such as *B. vulpes* and *B. canis* can be vectored by *D. reticulitis* (Mierzejewska et al., 2021) and *B. divergens*, closely related to *B. odocoilei*, is found in *D. marginatus* in Europe (Hilpertshauser et al., 2006). Although *B. duncani* was not detected in this study, *D. albipictus* has been documented to be a vector for *B. duncani*, which has mule deer as its reservoir in the western United States (Swei et al., 2019). This study appears to be the first to report *B. microti* in *D. albipictus*, however, other studies have reported *B. microti* in *D. reticulitis* (Dwuznik et al., 2019). This study also detected *B. microti* in *I. cookei*, which is supported by previous studies from Ontario (Scott et al., 2019).

4.4 Babesia in adult, nymph and larval ticks

Testing of ticks at adult, nymph and larval stages for *Babesia* was conducted in this study. *B. odocoilei* was detected in nymph and adult ticks and *B. microti* was detected in adult ticks. The dataset for the samples included the tick's life stage, engorgement status and host which can be useful to infer transstadial transmission of the *Babesia* species and the potential risk for transmitting *Babesia* to a host (appendix). Two ticks that were confirmed to contain *B. odocoilei* in this study were non-engorged, which is suggestive of transstadial transmission of the pathogen. There is evidence in the literature that *B. odocoilei* can be transstadially transmitted from larval-nymphal stages and nymphal to adult stages of *I. scapularis* (Waldrup et al., 1990, Scott et al., 2020). Though *B. odocoilei* was not detected in larvae in this study, *B. odocoilei* is also capable of vertical transmission, at a low incidence (Zembsch et al., 2021). The current study also detected *B. odocoilei* and *B. microti* in engorged ticks that had fed on humans or pets. This could indicate that *Babesia* was acquired after the blood meal of a host or that the tick had already acquired the pathogen from another host during a previous stage. The latter is more likely as humans and pets might be expected to be symptomatic and, ideally, treated if infected.

4.5 Limitations and Future Directions

As mentioned above, obtaining properly preserved blood samples may enhance the quality of DNA and blood smears which would aid in the detection of *Babesia*. Microscopic identification of the parasite also requires considerable technical expertise and having poor quality samples adds challenge in detecting the pathogen by microscopy. Resampling to obtain an even representation from ticks at each life stage and species would also enhance the research. Given the unbalanced sample size among the provinces, resampling may also provide a better estimate of the pathogen prevalence. Genetic sequencing allowed me to confirm that ticks that tested *Babesia* positive from PCR did contain the pathogens, although a few sequence reads were of too poor quality to generate adequate sequence match and technical sequence failure reduced the number of samples that could be sequence-confirmed. Sequencing analysis of the samples that showed strong identity to *Babesia spp.* suggests that there is not enough variability in the 500 bp amplicon to differentiate into *Babesia* species thus require resequencing with different set of primers. Given the time constraints of the study, resequencing of the samples was not performed.

While this study provides evidence that *B. odocoilei* is establishing in the Maritimes, ongoing surveillance for the pathogen in field-acquired ticks, wildlife, agricultural and domestic hosts would be important future studies to ascertain whether *Babesia* is enzootic in the region. *B. odocoilei* and *B. microti* were also detected in tick species at different life stages, some of which that have not been noted in the literature and thus less is known about their specific transmission dynamics. For example, experimental studies such as allowing *Babesia*-fed ticks to molt in the laboratory would be needed to ascertain transstadial transmission of the pathogen (Scott et al., 2020). Tick abundance and absence of previously noted reservoir hosts do not necessarily coincide with *B. odocoilei* prevalence as is the case with Prince Edward Island, thus other factors such as tick-infested migratory birds or other animals that might serve as reservoir hosts are worth exploring.

4.6 Conclusions

This is the first study to comprehensively survey *Babesia* pathogens across the Maritimes. It provides relevant information about the prevalence, establishment, distribution of *B. odocoilei*, *B. duncani* and *B. microti* as a reference for future studies. *B. odocoilei* is the more prevalent of these 3 *Babesia* pathogens and is likely established in parts of the Maritimes and expanding its distribution. Between the provinces, Prince Edward Island had the highest proportion of the pathogen, followed by Nova Scotia, then New Brunswick. *B. odocoilei* and *B. microti* were also detected in tick species at adult and nymph stages, finding of *B. odocoilei* in *D. variabilis* and *D. albipictus*, and *B. microti* in *D. albipictus* are the first such reports in the literature. Prevalence in cows and wildlife is yet to be determined. Though the detection of *Babesia* was not possible in the blood samples, Acridine Orange and Giemsa staining proved to be successful, at least in fresh or unfrozen samples providing valuable information on the feasibility of conventional microscopic detection of *Babesia*. Of great concern is that the ticks tested in this study were submitted by the public and obtained feeding on people or pets, indicating that there is already an exposure to the pathogen to humans and pets, further demonstrating the risk for babesiosis in the Maritimes.

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Appendix

File link containing tick and sequencing data

