

Coinfection of *Bartonella* spp. and *Borrelia burgdorferi* in *Ixodes Scapularis* Using PCR Assay, a Case Study in Nova Scotia

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Abstract

Coinfection of vector species can provide more insight into the complex relationship between zoonotic pathogens and its host. *Ixodes scapularis* (Say) or the deer-tick in particular is an important species in North America because of its exceptional ability as a vector that can transmit zoonotic diseases such as Lyme and Cat Scratch Disease (CSD). In recent years, many studies have suggested a possible link between the coinfection of *Borrelia burgdorferi*, the causative agent of Lyme, with other tick-borne bacteria such as *Bartonella* spp., the causative agent of CSD, as partly responsible for the symptoms associated with Chronic Lyme Disease or Post-Treatment Lyme Disease Syndrome. This study investigates the prevalence of *Bartonella* spp. and *Borrelia burgdorferi* in *Ixodes scapularis* using Polymerase Chain Reaction (PCR) assay to potentially find a link between the two of the most common tick-borne pathogens found in Nova Scotia. Standard PCR using primers targeted at the two bacterial species were conducted on 157 *I. scapularis* ticks collected in Nova Scotia. Overall, we found high prevalence for both bacteria at 75.16% for *Bartonella* spp. and 47.13% for *B. burgdorferi* with no significant differences between the sex of the ticks. Interestingly, all the ticks positive for *B. burgdorferi* were also positive for *Bartonella* spp. which implies that the coinfection rate between *B. burgdorferi* and *Bartonella* spp. is 47.13%. We report one of the highest coinfection rates for *B. burgdorferi* and *Bartonella* spp. in *I. scapularis*, consistent with the current trends of increasing tick presence in North America.

Keywords: *Bartonella* spp., *Borrelia burgdorferi*, coinfection, *Ixodes scapularis*, PCR, ticks

1. Introduction

1.1 Coinfection in Ticks

Coinfection in arthropod vectors is an ongoing topic of research. Studies range from investigating the effect of coinfection on transmission to and from the host (Levin and Fish, 2000; Steiner et al., 2014; Diuk-Wasser et al., 2016) to the clinical effects of coinfection (Eskow et al., 2001; Diuk-Wasser et al., 2016). One of the most well studied group of arthropod vectors with regards to coinfection is the ticks. Hard ticks (F. Ixodidae) in particular is of interest due to their clinical significance. In Eastern Canada, *Ixodes scapularis* (Say) is the species that is responsible for the majority of tick-borne diseases including Lyme disease (Curry et al., 2017; Lloyd and Hawkins, 2018; Carey et al., 2019; Foley-Eby et al., 2020).

1.2 *Ixodes Scapularis*

Ixodes scapularis, more commonly known as either the deer-tick or the black-legged tick, is found throughout eastern North America. They are common vectors for *Borrelia* spp., *Bartonella* spp. of bacteria, as well as an assortment of other infectious pathogens (Corona & Schwartz, 2015; Karasartova et al., 2018). One of the reasons that *I. scapularis* is able to accommodate a variety of pathogens is in part due to their life cycle and the unique requirements for each individual life stage. Once the egg hatches, each life stage (larva, nymph and adult) corresponds to distinct hosts (Edwards and Rawlings, 2012), including a variety of wild and domestic fauna. Larvae and nymphs tend to feed on birds or small rodents. Adults typically feed on larger fauna, for example, white-tailed deer, domestic dogs, and humans (Kim et al., 2016). By feeding on different hosts, the probability of the tick being infected by and/or transmitting pathogens increases (Parola and Raoult, 2001; Sonenshine, 1991). Among all the bacteria that can be transmitted, *Borrelia burgdorferi* sensu lato and *Bartonella* spp. are among the most documented bacteria species transmitted in Eastern Canada (Lloyd and Hawkins, 2018; Carey et al., 2019; Foley-Eby et al., 2020).

1.3 *Borrelia burgdorferi*

Borrelia burgdorferi is the causative agent for one of the most concerning tick-borne diseases, Lyme disease (Ginsberg et al., 2017). *B. burgdorferi* is a spirochete bacterium that enters the host through the blood meal of a tick. The bacterium can then infect several organs throughout the body through the bloodstream and cause Lyme borreliosis or Lyme disease (Ruef, 2011; Lloyd and Hawkins, 2018). Recent studies have suggested a link between symptoms of Lyme disease with coinfection of other bacteria that inhabit the tick vector including *Bartonella spp.* (Holden et al., 2006; Sytykiewicz et al., 2012).

1.4 *Bartonella Spp.*

Bartonella spp. are gram negative, aerobic, facultative intracellular bacteria (Müller et al., 2016). *Bartonella spp.* can cause various clinical symptoms within humans depending on the species or sub-species (Chang et al., 2001). While there is no evidence suggesting human transmission of *Bartonella spp.* from ticks there is the possibility of co-infection with *Borrelia burgdorferi* as previously found in mice (Hofmeister et al., 1998; Müller et al., 2016). Understanding the infection prevalence within ticks for these bacteria species is essential for better assessment of the exposure risk.

1.5 Aim of Study

This study aims to identify the coinfection rate of *Borrelia burgdorferi*, and *Bartonella spp.* in *I. scapularis* found in Nova Scotia, Canada. The number and variety of bacterial species found in each tick will be established via DNA isolation and PCR. This report will provide the foundation needed to investigate the relationships between bacterial co-infections and its host in an important local vector.

2. Methods

2.1 Sample Collection

Ixodes scapularis ticks were collected in the wild from various areas in Nova Scotia and tested for *Bartonella spp.* and *Borrelia burgdorferi* using Polymerase Chain Reaction (PCR) assay. The detailed protocols followed for sample collection, DNA extraction, PCR for *Bartonella spp.*, and gel electrophoresis can be found in Carey et al. (2019).

Table 1. A list of the specific primers used in this study to detect *Bartonella spp.*, and *Borrelia burgdorferi*

Bartonella spp.

Primer name	Gene	Sequence (5'-3')	Amplicon Size	Annealing Temperature	Source
P-24E	<i>16S-rRNA</i>	GGAATCCCTCCTTCAGTTAGGCTG G	279 bp	56 °C	Eskow et al. (2001);
P-12B	<i>16S-rRNA</i>	CGGGATCCCAGATGGCTTTTGGG GATTA			Carey et al. (2019)

Borrelia burgdorferi

Primer name	Gene	Sequence (5'-3')	Amplicon Size	Annealing Temperature	Source
OspA_Out_R1	<i>OspA</i>	GTTAGCAGCCTTGAC GAGA	272 bp	55 °C	Ogden et al. (2006);
OspA_Out_F1	<i>OspA</i>	GATACTAGTGTTTTGCCATC			
OspA_In_R1	<i>OspA</i>	GCGTTTCAGTAGATTTGCCTG	214 bp	58 °C	Patterson et al. (2017)
OspA_In_F1	<i>OspA</i>	TCAAGTGTGGTTTGACCTAG			
FlaB_Out_R1	<i>FlaB</i>	AATTGCATACTCAGTACTATCTTT ATAGAT	612 bp	55 °C	
FlaB_Out_F1	<i>FlaB</i>	AAGTAGAAAAAGTCT TAGTAAGAATGAAGGA			
FlaB_In_R1	<i>FlaB</i>	GAAGGTGCTGTAGCAGGTGCTGGC TGT	390 bp	58 °C	
FlaB_In_F1	<i>FlaB</i>	CACATATTCAGATGCA GACAGAGGTCTA			

2.2 Testing for *Bartonella spp.* Prevalence

Briefly, DNA from half ticks was extracted using AquaGenomic solution (Multitarget Pharmaceuticals, Colorado, USA) as per the developer's instructions. The extracted DNA samples were then stored in a -18 °C freezer until PCR (Carey et al., 2019). PCR was done using GoTaq Green (Promega, Maddison, USA) master mix. The primers P-24E and P-12B were used to amplify a region of the *16S rRNA* gene-specific to *Bartonella spp.* from each tick sample (Table 1). Agarose gel (2.0%) and SYBR Safe Green were used to visualize the amplicon.

2.3 Testing for *Borrelia burgdorferi* Prevalence

In addition to testing for *Bartonella spp.*, the same samples were tested for the prevalence of *Borrelia burgdorferi* using nested PCR procedure and primers outlined in Patterson et al. (2017) and later updated in Wills et al. (2018). Nested PCR is a modification of the standard PCR procedure to target smaller amplicons better using two rounds of PCR reactions, Primary and Nested (Massung et al., 1998). The Primary reaction will generate an amplicon from the DNA sample, while the Nested reaction will then generate a more specific amplicon using the Primary reaction's amplicon (Massung et al., 1998; Wills et al., 2018). All PCR amplifications were done on an Eppendorf Mastercycler ep Gradient S.

Two genes were used to assess the prevalence of *Borrelia burgdorferi*: *FlagellinB (FlaB)* and *Outer surface protein A (OspA)*, the former corresponding to the flagellum's primary filament protein while the latter is a lipoprotein that is involved in tick midgut colonization (Patterson et al., 2017; Wills et al., 2018). All the primers were obtained from Sigma-Aldrich and are outlined in Table 1.

After PCR, all amplicons were visualized on a 2.0% agarose gel (2 g agarose, 100 mL of 0.5x Tris-Borate-EDTA (TBE) buffer, and 5 μ L SYBR Safe Green). 5 μ L of samples and 5 μ L of 100 bp DNA Ladder (GeneDireX) were then loaded into the gel which ran for 1 hour at 100V. Each gel was then photographed for further analysis using a Canon EOS Rebel T5.

2.4 Assessing Co-infection and General Precaution

Co-infection was assessed based on the prevalence of both *Bartonella spp.* and *Borrelia burgdorferi* in a sample. Percent prevalence values were calculated for each as well as both bacteria (number of positive samples/number of tested samples x 100).

In the case of *B. burgdorferi*, if a positive amplicon for *OspA* is detected but not *FlaB*, we will consider the sample negative due to *OspA* being prone to false-positives; however if a positive amplicon for *FlaB* is detected but not *OspA*, we will consider the sample positive due to the chance that there may be related *Borrelia spp.* in the sample (more information in Wills et al., 2018).

A Chi-square goodness of fit test was done to determine a correlation between tick sex and the infection prevalence of both bacteria. To determine whether there is variance around the means for infection prevalence, a one-way ANOVA was done. In addition, to investigate the relationship between the two bacteria's infection prevalence, a linear regression analysis with confidence level of 95% was conducted. All statistical analyses were done in Microsoft Excel v2005 and R v.4.0.1.

All experimental reactions and solutions were prepared in a certified Biological Safety Cabinet. UV and 95% ethanol were used as sterilizing agents during sample preparation, DNA extraction, and PCR prep.

3. Result

3.1 Infection Prevalence and Sex of *Ixodes Scapularis*

A total of 157 *Ixodes scapularis* ticks (75 male and 82 female) were tested in this study. All 157 samples were tested using PCR for the presence of *Borrelia burgdorferi* and *Bartonella spp.*. Overall, *Bartonella spp.* has higher infection prevalence than *B. burgdorferi* in both sexes. Table 2 shows the results for the *B. burgdorferi* and *Bartonella spp.* PCR tests separated by sex.

Table 2. Table showing the number of *Ixodes scapularis* ticks tested using PCR of *OspA* and *FlaB* targeted primers for *Borrelia burgdorferi*, and *16S rRNA* targeted primers for *Bartonella spp.* separated by sexes

<i>Borrelia burgdorferi</i>			
	Positive	Negative	Total
Male	38	37	75
Female	36	46	82
Total	74	83	157
<i>Bartonella spp.</i>			
	Positive	Negative	Total
Male	55	20	75
Female	63	19	82
Total	118	39	157

Table 2 suggests that number of ticks that were positive with *Bartonella spp.* is around two thirds of the overall sample size. This pattern appears to be consistent regardless of the sex of the ticks. For *B. burgdorferi*, the positive samples were almost on par with the negative samples for the male *I. scapularis* samples but there is slightly less infection

prevalence in the female ticks. Looking at the total proportion of samples tested, there is almost triple the amount of positive *Bartonella spp.* samples compared to the negative samples, and there are slightly more negative samples for *Borrelia burgdorferi* compared to positive samples.

A visual representation of the ratio of the two bacteria infection (in percent) separated by sex is shown in Figure 1 below.

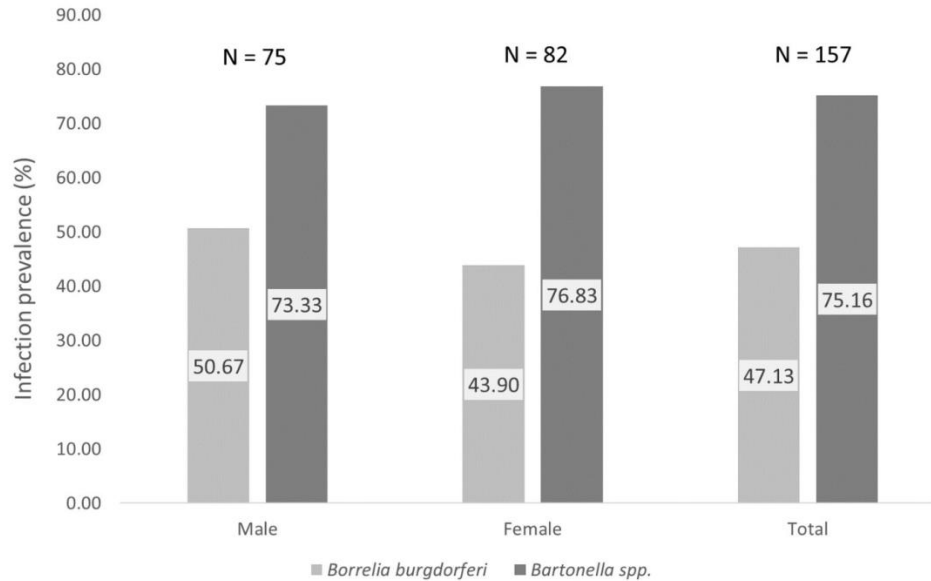


Figure 1. Bar graph showing the percent infection prevalence of *Borrelia burgdorferi* and *Bartonella spp.* in *Ixodes scapularis* samples separated into male (N = 75), female (N = 82), and total combined (N = 157). The light grey bars represent positive PCR products from the primers targeting *OspA* and *FlaB* genes for *B. burgdorferi*, while the darker bars indicate positive *Bartonella spp.* PCR results from primers targeting *16S rRNA* gene

Overall, the infection prevalence of *B. burgdorferi* is less than *Bartonella spp.*, which is consistent in both male and female ticks (Figure 1). A Chi-square goodness of fit test on the each of the bacteria data from Table 2 gave a P values of 0.719 for *Borrelia burgdorferi* and 0.068 for *Bartonella spp.* which suggests that the sex of the tick is independent to either bacteria’s infection prevalence.

3.2 Coinfection of *B. Burgdorferi* and *Bartonella Spp.*

Looking at the raw data alone, one interesting observation is that all the *Ixodes scapularis* ticks that were infected with *Borrelia burgdorferi* were also infected with *Bartonella spp.* (Data not shown). Figure 1 suggests a high coinfection rate of 47.13% (N = 157) between *Bartonella spp.* and *B. burgdorferi* in *Ixodes scapularis* ticks. Two statistical analyses were conducted to further explore the relationship between *B. burgdorferi* and *Bartonella spp.* infection in these tick samples (Table 3).

Table 3. (A.) A one-way ANOVA comparing the infection prevalence of *Borrelia burgdorferi* and *Bartonella spp.* in *Ixodes scapularis* ticks (N = 157). The P-value of 2.17E-07 suggest a significant difference between the two means. (B.) Part of the results for a linear regression analysis using *B. burgdorferi* as the dependent variable. The positive Coefficients value as well as a P value of 2.07E-13 suggests a statistically significant positive linear relationship between the two infection prevalence

A.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.16	1	6.16	28.1	2.17E-07	3.87
Within Groups	68.4	312	0.219			
Total	74.6	313				

B.

Linear Regression	Coefficients	Standard Error	t Stat	P-value
Intercept	1.66e-15	0.067	2.46	1
<i>B. burgdorferi</i> from <i>Bartonella spp.</i>	0.627	0.078	8.05	2.07E-13

A One-way ANOVA suggests a significant difference in infection prevalence between *Borrelia burgdorferi* and *Bartonella spp.* at a P value of 2.17e-7, and a linear regression analysis suggests a positive relationship for *B. burgdorferi* with *Bartonella spp.* (Table 3). Altogether, these statistical analyses suggest a potential correlation of coinfection between *B. burgdorferi* and *Bartonella spp.* and that the infection prevalence data between the two bacteria are statistically significant.

4. Discussion**4.1 General Findings and role of Sex in Prevalence**

Sex has been suggested as one of the variables that can influence the physiology of ticks (Meeüs et al., 2002; Wójcik-Fatla et al., 2006; El Nabbout et al., 2018; Batool et al., 2021). In this study, we tested 157 *Ixodes scapularis* ticks for *Borrelia burgdorferi* and *Bartonella spp.* using conventional PCR and gel electrophoresis techniques to determine coinfection between the two bacteria species and between the sexes of the ticks. When looking at *B. burgdorferi* infection between sexes, there were no significant differences between the male (50.67%) and female (43.9%) *I. scapularis* ticks (Chi-square goodness of fit test, P-value: 0.40). The same trend was observed for *Bartonella spp.* between male (73.33%) and female (76.83) *I. scapularis* ticks (Chi-square goodness of fit test, P-value 0.07). These results suggest that infection of these bacteria is consistent between sexes and is particularly important for the consideration of male ticks in further studies. Male ticks are often overlooked in studies due to their less prominent role as a vector of tick-borne diseases, but they can still act as reservoirs of bacteria and viruses (Sakamoto et al., 2016). A study of *I. scapularis* and *Ixodes affinis* microbiome by Van Treuren et al. (2015) in the East Coast and upper Midwest of United States found that male ticks had a wider variety of microbiome than females; similar results were observed in a study of *I. scapularis* in Texas by Thapa, Zhang, and Allen in 2019. In addition to being reservoir hosts, male ticks also play an important role in horizontal transmission of bacteria and viruses through processes such as co-feeding (Gonzalez et al., 1992; Labuda et al., 1993; Zemtsova et al., 2010; Belli et al., 2017). Here we show that male ticks carry just as much bacteria as female ticks and are therefore worthy of inclusion in future studies involving tick-borne diseases.

4.2 Infection Prevalence of *Bartonella spp.* and *B. Burgdorferi*

With regards to the two bacteria we tested, the data suggests a high prevalence of *Bartonella spp.* (75.16%, Figure 2) and *B. burgdorferi* (47.13%, Figure 2). The prevalence rate of *B. burgdorferi* will be discussed in the next section as it is synonymous with the coinfection rate of both bacteria. The results reported in this study is the highest reported prevalence rate for *Bartonella spp.* locally. A study by Carey et al. (2018) found 45.5% of *I. scapularis* ticks were infected with the bacteria compared to the 75.16% prevalence rate reported in this study. Another study by Curry et al. (2017) looked at female *I. scapularis* specifically and found 64% prevalence rate of *Bartonella spp.* compared to the 76.83% reported prevalence rate of female ticks reported in this study. In both cases, there appear to be an increase in prevalence rate over time although more analysis is needed to test this hypothesis. The caveat here is that we used genus-level primers for *Bartonella spp.*, which may factor into why we see a higher proportion of positive samples compared to the more specific *B. burgdorferi* primers. Further analysis using species-specific primers may provide a more accurate infection prevalence at the species level. However, for this study, we were interested in the concurrent infection of any form of *Bartonella* bacteria with *B. burgdorferi* and therefore the genus level primer was sufficient.

4.3 Coinfection of *Bartonella Spp.* and *B. Burgdorferi* in Ticks

Interestingly, all the ticks that had *B. burgdorferi* were also positive for *Bartonella spp.*, but not all ticks that had *Bartonella spp.* were infected with *B. burgdorferi*. The coinfection prevalence was the number of positive *B. burgdorferi* samples of 74 (Table 2) out of the 157 total samples tested or 47.13% (Figure 1). This may imply that the presence of *Bartonella spp.* is essential for the infection of *B. burgdorferi* in the host tick, however additional studies will be required to address this claim.

The coinfection of *Borrelia burgdorferi* with other bacterial species and viruses has been documented previously in not only tick hosts (Holden et al., 2006; Sytykiewicz et al., 2012; Moutailler et al., 2016; Cross et al., 2018), but also organisms that ticks feed on such as *Peromyscus leucopus* (Hofmeister et al., 1998) and humans (Eskow, Rao and Mordechai, 2001). Studies have found that *Borrelia burgdorferi* is often found in ticks that are also infected with other bacterial species (Holden et al., 2006; Sytykiewicz et al., 2012; Moutailler et al., 2016; Cross et al., 2018), and to our

knowledge, the 47.13% coinfection rate of *B. burgdorferi* and *Bartonella spp.* reported in this study is among the highest in the literature. The importance of the coinfection between these two bacteria extends beyond the host and even into a clinical setting in humans. A study by Eskow, Rao, and Mordechai in 2001 found that patients with neuroborreliosis conditions had DNA traces of *Bartonella henselae* and *Borrelia burgdorferi*, but no symptoms of cat-scratch disease.

4.4 Implications of Study

Surveillance of Lyme disease is becoming more prevalent but cat scratch disease is not being monitored consistently perhaps due to the asymptomatic nature in most infected individuals, something that is also a potential cause of under-diagnosed Lyme disease cases (Lloyd and Hawkins, 2018; Zangwill, 2021). Within Canada alone, cases of Lyme disease have increased from 144 cases in 2009 to 2025 cases in 2017 annually and is projected to continue increasing (Gasmi et al., 2017; Ogden et al., 2019). In contrast, cat scratch disease is not well documented in Canada whereas in the US, it is estimated to occur at a rate of 4-6 per 100,000 populations (Zangwill, 2021). Our high coinfection rates can be explained by the migration of ticks northward which has brought about various tick-borne diseases and increasing surveillance of these diseases and bacteria is warranted in order to better understand the spread and transmission of these diseases (Khatchikian et al., 2015; Sonenshine, 2018; Sagurova et al., 2019).

5. Conclusion

In this study, we determined the coinfection rate of *Borrelia burgdorferi* and *Bartonella spp.* that is consistent between sexes in *Ixodes scapularis* ticks. While both *B. burgdorferi* and *Bartonella spp.* are well studied individually, the relationship and interaction that these two bacteria have with each other and their host have not been thoroughly explored.

Here we outline several suggestions of research areas that may improve our understanding of the impact of coinfection in this system. The first is to look at the effects of bacterial load on the biology of the tick host. Focusing on the influence of the infection towards the metabolic pathways or behavior may contribute towards our understanding of parasite-host interaction and the mechanisms behind it. The findings that all the ticks that were positive for *B. burgdorferi* were also positive for *Bartonella spp.* but not the other way around is particularly fascinating. Further study on the molecular pathways of these bacteria inside the host and the role of coinfection in maintenance of *B. burgdorferi* should be considered. Finally, another aspect of this system that needs to be considered is the role that coinfection plays on the transmission of the bacteria. The high coinfection rate for both these bacteria may also imply a higher probability or bacterial load during transmission from the vector to a host and warrants further investigation. This is especially important from a clinical and public health point of view to manage the spread of Lyme disease and better understand the mechanism underlying the vector-host relationship.

To conclude, this study provides a foundation to further investigate the effect of coinfection in a highly successful vector system and raises the importance of considering other parasites when examining parasite-host interactions.

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