Prevalence of Lyme Disease in *Ixodes scapularis* Ticks in Southeastern Minnesota and West Central Wisconsin

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Abstract

Lyme Disease is a common, widespread disease in the U.S. that is transmitted to humans through the bite of an infected *Ixodes* tick. The majority of Lyme Disease cases are found in Minnesota and Wisconsin, as well as along the East Coast in states ranging from Maine to Virginia. It was hypothesized that the Mississippi River acts as a barrier against the spread of Lyme Disease between Wisconsin and Minnesota. *Ixodes* ticks were collected from both sides of the river during legal white-tailed deer hunts from 2005-2012. A PCR was developed that amplified *Borrelia burgdorferi* (Lyme Disease) in ticks. In order to determine if the DNA was intact and viable, a multiplex PCR was developed to amplify both *Ixodes* and *Borrelia* DNA simultaneously. The use of 0.5uL of Scap *I. scapularis* specific primer and 1uL of JS *B. burgdorferi* specific primer resulted in successful visualization of simultaneous bands (**Figure 5**). In further research, multiple samples of female *Ixodes* ticks will be tested using this combination of primers to determine if the ticks are positive or negative for *Borrelia* DNA.

Introduction

Lyme Disease is a common, widespread disease that is transmitted to humans through the bite of an infected *Ixodes* tick. Signs and symptoms of this disease is a red ringed rash around the bite site, fatigue, stiffness, facial paralysis, and fever. At the early stages of Lyme Disease it can be easily treated with antibiotics and recovery can occur fast and completely. Each year, there are about 30,000 cases reported to the CDC. With recent estimates, through data surveillance, it suggest that 300,000 people may get Lyme Disease each year in the U.S. Most cases of this disease are found in Minnesota and Wisconsin, as well as the East Coast in states ranging from Maine to Virginia.

The research at hand aims to determine if the Mississippi river acts as a type of barrier against the transfer of Lyme Disease from the state of Wisconsin to Minnesota. The subject of interest is DNA from female *Ixodes scapularis* ticks that are found in Minnesota and Wisconsin. This is due to the larger blood meal female nymphs (immature nymphs) take from mice and deer, thus higher possibility of *Borrelia burgdorferi* infection in comparison to male ticks. Between the years 2012-2017 there has been a significant increase in reported cases of Lyme Disease in the states of Minnesota and Wisconsin. In 2012, Minnesota had 911 cases, while in 2017 there were 1,408 cases, making a 54% increase in reported cases of Lyme Disease. In 2012, Wisconsin had 1,368 cases, while in 2017 there were 1,794 cases, making a 31% increase in reported cases of the disease (Centers for Disease Control and Prevention). Although it has been noted that there are higher cases of Lyme Disease on the Wisconsin side of the Mississippi River, there is no definitive proof that the Mississippi River acts as a barrier preventing the spread of this disease.

Materials and Methods

Ixodes scapularis ticks were collected from two locations during legal white-tailed deer hunting seasons 2005-2012. Minnesota ticks were collected from deer being processed at the Ledebuhr meat processing plant (Goodview, MN). Wisconsin ticks were collected at a DNR check station located at a Kwik Trip gas station in Buffalo county, where a maximum of 5 ticks per deer were collected. These ticks were identified as male or female, sorted by location, and numbered. DNA extraction was performed using the Ammonium Hydroxide extraction method from 2005-2010 (Rymaszewska et. al, 2003), and then by Chelex Extraction method from 2010-2012 (personal communication). The bacteria positive control, *Borrelia burgdorferi*, was donated by a microbiology research lab at Gundersen Lutheran Hospital. DNA was quantified using a Nanodrop Spectrophotometer.

Polymerase chain reaction (PCR), was used to amplify certain gene sequences located on *I. scapularis* and *B. burgdorferi* DNA depending on primers used. It was important that when primer sets were chosen for *I. scapularis* and *B. burgdorferi*, that their segments were not too close in size, otherwise they would be unable to differentiate during gel electrophoresis. Types of *I. scapularis* and *B. burgdorferi* primers can be observed in **Table 1**. Many different master mixes were tested to determine the best concentration of reagents. Master Mixes were prepared and followed a format closely related to that observed in **Table 2**. Positive controls contained DNA while negative controls were ran with water rather than DNA.

Amplification was performed on a BioRad thermocycler. Conditions of thermocycler can be observed in **Table 3**. Upon completion of PCR, the products were frozen or prepared for gel electrophoresis. Gel electrophoresis was used to visualized targeted gene sequences amplified by PCR on a 0.75% agarose gel in TAE or TBE buffer. A 100 bp ladder DNA marker (Biosciences) was used to help visualize the size of gene products. Visualization of the completed gel was done by the use of UV transilluminator (Unique Value Proposition).

Table 1. Primer Sequences Used to Amplify *Borrelia* and *Ixodes* DNA

Tick DNA Primers	Primer	Primer Sequence
	IxSeq-5.8SF (28S)	5' - TCG ATG AAG AAC GCA GCC AG - 3'
	28S R1/1	5' - TTC TAT GCT TAA ATT CAG GGG GTT GTC - 3'
	Scap_f2.2	5' - GCG TTA GAA ACG GAG ATT TGA - 3'
	Scap_r2.2	5' - CCA CGA GAT TTA CAT TTG CC - 3'
	16S F	5' - CTG CTC AAT GAT TTT TTA AAT TGC TGT - 3'
	16S R	5' - GTC TGA ACT CAG ATC AAG T - 3'
Borrelia DNA Primers	JS1 F	5' - AGA AGT GCT GGA GTC GA - 3'
	JS2 R	5' - TAG TGC TCT ACC TCT ATT AA - 3'
	5S F	5' - GAG TTC GCG GGA GAG TAG GTT ATT - 3'
	5S R	5' - TCA GGG TAC TTA GAT GGT TCA CTT - 3'

Table 2. Baseline Master Mix Ingredients for Amplifying *Borrelia* and *Ixodes* DNA

Component	Concentration	Volume Used (ul)	Manufacturer
Green DreamTaq	2x	12.5	Thermo Scientific
$MgCl_2$	25 mM	1	Fisher BioReagents
Borrelia F 1^{o}	0.6 umol	1.5	IDT
Borrelia R 1^{o}	0.6 umol	1.5	
Ixodes F 1^{o}	0.4 umol	1	IDT
Ixodes R 1^{o}	0.4 uml	1	
DNA	~	~	~
Sterile Water	-	**Fill to 25ul**	Thermo Scientific

Table 3. Thermocycler Conditions Used to Amplify *Borrelia* and *Ixodes* DNA

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	95	4 min	1
Denaturation	95	30 sec	35
Annealing	50	1 min	
Extension	72	1min	
Final Extension	72	5 min	1

Results

Various PCR products were created to observe the condition of both *B. burgdorferi* and *I. scapularis* DNA samples to determine if they were adequate enough for research use by creating positive controls for each DNA sample being tested. Two different *B. burgdorferi* positive controls were created using the same JS primer but different concentrated DNA samples. The same was done for *I. scapularis*, using the same 16S primer but different tick DNA samples. In addition, two different PCR solutions containing both bacteria and tick primers with combinations of different bacteria and tick DNA was also created to determine if master mix uniformly expresses both bands sufficiently. (**Figure 1**).

Due to inadequate results in **Figure 1**, a series of PCR products containing different *I. scapularis* DNA samples were each spiked with 1 ul of 1:10 diluted *B. burgdorferi* (#9, 8.4 ug/uL) DNA. PCR products contains both JS and 16S primers and results were expected to show two bands for each solution. A negative control was also made, using the same mater mix, but with sterile water in place of DNA (**Figure 2**). In **Figure 2**, expected bands were expressed in lanes 2, 3, 4, and 5. Two bands were expected for lane 6 but only a band for *I. scapularis* was present. PCR products expressed in **Figure 2** were repeated however, with only 0.5 uL of 16S in each solution. The addition of a positive control for I. scapularis with 16S primer and B. burgdorferi with JS primers where also included (**Figure 3**). In **Figure 3**, bands were expected in lanes 2, 3, 4, 5, 6, 7 and were not expected in lane 9. Lane 8 was expected to show a single band for *B. burgdorferi*, however no band was shown. A different approach was taken by using tick DNA supplied by another student in which infective status was already known. PCR products contained both 16S and JS primers with a series of different tick DNA samples. No sterile water was used. The addition of *B. burgdorferi* positive control with both 16S and JS primers and a negative control of the same master mix for all the solutions including 16S and JS primers and strictly water supplemented for DNA, was also performed (**Figure 4**). In **Figure 4**, Known positive ticks in lanes, 2, 3, 4, 5, 6, 8, and 9 should have two bands. Only lane 4 results in two bands. Known negative ticks in lanes, 10 and 11 resulted in single bands which was expected. A series of different combinations of primers specific for *I. scapularis* and *B. burgdorferi* were attempted to determine successful separation of bands using tick DNA that was unnaturally spiked with *B. burgdorferi* DNA. In **Figure 5**, lane 4 successfully showed two isolated bands simultaneously. The top band is referred to as the *B. burgdorferi* DNA fragment while the bottom band is referred to the *I. scapularis* DNA fragment.

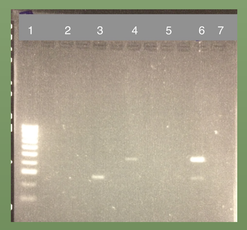

Figure 1. Contents of samples in each lane are comprised as follows, while JS primer refers to *B. burgdorferi* specific and 16S primers as *I. scapularis* specific, **Lane 1**: Ladder, **Lane 2**: *B. burgdorferi* DNA with JS primer positive control (#9, 8.4 ug/uL), **Lane 3**: *B. burgdorferi* DNA with JS primer (#9) and *I. scapularis* DNA with 16S primer (#F493, 179.7 ug/uL), **Lane 4**: *I. scapularis* DNA with 16S primer positive control (#F493), **Lane 5**: *B. burgdorferi* DNA with JS primer positive control (#1, 5.3 ug/uL), **Lane 6**: *B. burgdorferi* DNA with JS primer (#1) and *I. scapularis* DNA with 16S primer (#F494, 974 ug/uL), **Lane 7**: *I. scapularis* DNA with 16S primer positive control (#F494).

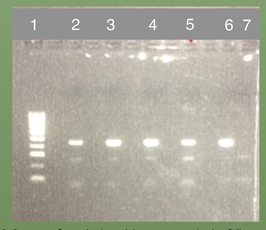

Figure 2. Contents of samples in each lane are comprised as follows, while JS primers refers to *B. burgdorferi* specific and 16S primers as *I. scapularis* specific. Spiked DNA refers to the addition of *B. burgdorferi* DNA in known concentration to each tick sample. **Lane 1**: Ladder, **Lane 2**: Spiked *I. scapularis* DNA with 16S and JS primer (#M211, 4.1 ug/uL), **Lane 3**: Spiked *I. scapularis* DNA with 16S and JS primer (#M199, 87.3 ug/uL), **Lane 4**: Spiked *I. scapularis* DNA with 16S and JS primer (#M192, 33.4 ug/uL), **Lane 5**: Spiked *I. scapularis* DNA with 16S and JS primer (#M203, 136.8 ug/uL), **Lane 6**: Spiked *I. scapularis* DNA with 16S and JS primer (#M195, 30.1 ug/uL), **Lane 7**: Negative control, sterile water with 16S and JS primer.

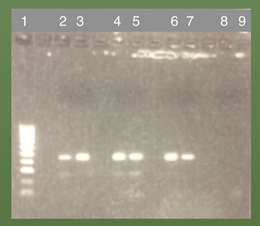

Figure 3. Contents of samples in each lane are comprised as follows, while JS primer refers to *B. burgdorferi* specific and 16S primers as *I. scapularis* specific. Spiked DNA refers to the addition of *B. burgdorferi* DNA in known concentration to each tick sample. **Lane 1**: ladder, **Lane 2**: Spiked *I. scapularis* DNA with 16S and JS primer (#M211, 4.1 ug/uL), **Lane 3**: Spiked *I. scapularis* DNA with 16S and JS primer (#M199, 87.3 ug/uL), **Lane 4**: Spiked *I. scapularis* DNA with 16S and JS primer (#M192, 33.4 ug/uL), **Lane 5**: Spiked *I. scapularis* DNA with 16S and JS primer (#M203, 136.8 ug/uL), **Lane 6**: Spiked *I. scapularis* DNA with 16S and JS primer (#M195, 30.1 ug/uL), **Lane 7**: *I. scapularis* DNA with 16S primer (#M211), **Lane 8**: *B. burgdorferi* DNA with JS primer (#9, 8.4 ug/uL), **Lane 9**: Negative control, sterile water with 16S and JS primer.

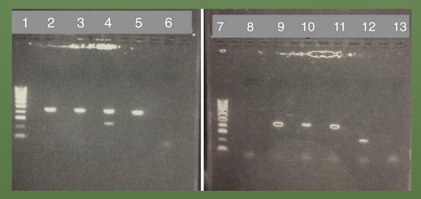

Figure 4. Contents of samples in each lane are comprised as follows, while JS primer refers to *B. burgdorferi* specific and 16S primers as *I. scapularis* specific, **Lane 1**: Ladder , **Lane 2**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#15, 10.4 ug/uL), **Lane 3**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#16, 44.5 ug/uL), **Lane 4**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#24, 16.7 ug/uL), **Lane 5**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#28, 14.7 ug/uL), **Lane 6**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#29, 12.6 ug/uL), **Lane 7**: Ladder, **Lane 8**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#40, 16.0 ug/uL), **Lane 9**: Known positive infected *I. scapularis* DNA with 16S and JS primer (#41, 16.8 ug/uL), **Lane 10**: Known negative infected *I. scapularis* DNA with 16S and JS primer (#45, 13.1 ug/uL), **Lane 11**: Known negative infected *I. scapularis* DNA with 16S and JS primer (#47, 15.8 ug/uL), **Lane 12**: Positive control, *B. burgdorferi* DNA with 16S and JS primer (#9, 8.4 ug/uL), **Lane 13**: Negative control, sterile water with 16S and JS primer.

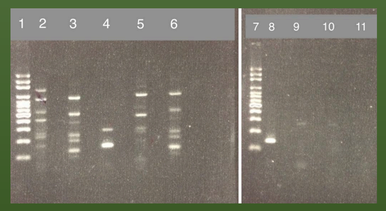

Figure 5. Contents of samples in each lane are comprised as follows, while JS and 5S primers refers to *B. burgdorferi* specific and 16S, Scap, and 28S primers as *I. scapularis* specific. Spiked DNA refers to the addition of *B. burgdorferi* DNA in known concentration to each tick sample. **Lane 1**: Ladder , **Lane 2**: Spiked *I. scapularis* DNA with 28S and JS primer, **Lane 3**: Spiked *I. scapularis* DNA with 28S and 5S primer, **Lane 4**: Spiked *I. scapularis* DNA with Scap and JS primer, **Lane 5**: Spiked *I. scapularis* DNA with 28S and JS primer. *I. scapularis* DNA in place of sterile water, **Lane 6**: Spiked *I. scapularis* DNA with 28S and 5S primer. *I. scapularis* DNA in place of sterile water, **Lane 7**: Ladder, **Lane 8**: Spiked I. scapularis DNA with Scap and JS primer. *I. scapularis* DNA in place of sterile water, **Lane 9**: Negative control, sterile water with 28S and JS primer, **Lane 10**: Negative control, sterile water with 28S and 5S primer, **Lane 11**: Negative control, sterile water with Scap and JS primer.

Discussion

The initial step taken place during this experiment was to determine if *I. scapularis* and *B. burgdorferi* DNA samples were viable to use to proceed with research. DNA samples were questioned due to freezing and thawing over many years of research which may alter the condition of the DNA giving tampered results. Figure 1 showed partially successful results. Single simultaneous bands were visualized for both *I. scapularis* (#F494) and *B. burgdorferi* (#1) in lane 6, concluding that DNA was viable (**Figure 1**). Two bands in lane 6 represented that with the use of 16S, *I. scapularis* specific primers, and JS, *B. burgdorferi* specific primers, allowed the visualization of DNA fragments to be shown simultaneously (**Figure 1)**. Neither *B. burgdorferi* positive controls appeared successful. This is a possible outcome due to experimental error, inadequate PCR conditions, or due to the use of 0.5 ul of JS primers (**Figure 1**).

PCR conditions similar to that of lane 6 in **Figure 1** helped to determine the adequate PCR conditions used to visualize bands simultaneously for *B. burgdorferi* and *I. scapularis*. Male tick DNA was spiked unnaturally with *B. burgdorferi* DNA. A 1:10 dilution of *B. burgdorferi* with water was used to simulate the amount of DNA found naturally within ticks. The amount of JS primers were increased to 1 ul due to the possibility that the concentration of *I. scapularis* DNA in PCR products was preventing *B. burgdorferi* primers from finding their target. The visualization of simultaneous bands were partially successful (**Figure 2**). Lane 6 was unsuccessful in the visualization of two bands. Only the *I. scapularis* band was present indicating that *B. burgdorferi* was unable to bind to the JS primers due to a large presence of tick DNA in the sample (**Figure 2**).

To increase the chances of *B. burgdorferi* binding to its primers, the experiment was revised using only 0.5ul of 16S *I. scapularis* specific primers instead of a full microliter. Partially successful results were observed in **Figure 3**. Lanes 2-5 showed expected results of simultaneous bands for both *I. scapularis* and *B. burgdorferi*. Lane 6, as well as the positive control in lane 8, was unsuccessful in the visualization of a *B. burgdorferi* band most commonly due to experimental error, or bands were too light to be observed (**Figure 3**).

DNA supplied by another student in which infective status was already known was used to determine if the protocol from **Figure 3** would be adequate in showing simultaneous bands. Using 1.5uL of *B. burgdorferi* specific JS primer, and 0.5uL of *I. scapularis* specific 16S primer, we expected all *I. scapularis* and *B. burgdorferi* DNA bands to appear in lanes 2-6, 8, and 9 (**Figure 4**). *B. burgdorferi* was successfully visualized in lane 4, while all other known positive samples were unsuccessful (**Figure 4**). It was unknown as to why the visualization of B. burgdorferi in these samples was absent. A different approach using new primers for both *I. scapularis* and *B. burgdorferi* were thought to help better present simultaneous bands.

Due to a multiplicity of unsuccessful trials, new *I. scapularis* and *B. burgdorferi* primers were ordered and tested in different combinations. The protocol used for **Figure 4** was altered to the following conditions, 0.5ul of *I. scapularis* and 1ul of *B. burgdorferi* primers. The most successful primer combination was visualized in lane 4 using JS *B. burgdorferi* specific primers and Scap *I. scapularis* specific primers (**Figure 5**). This combination will be used in future research to test different amounts of tick DNA along with different amounts of JS and Scap primers in a PCR solution, until *I. scapularis* and *B. burgdorferi* bands appear simultaneously on a gel consistently. It should be noted that other primer combinations were inadequate for proceeding in research due to improper binding and multiple visualized bands (**Figure 5**).

Future Research

To continue these studies, the protocol from **Figure 5**, lane 4 would make for the cleanest results in terms of lowest background "noise" (exactly two bands, one from *Borrelia*, one from *Ixodes*) when it comes to selecting for the two DNA types-- The Scap primer would work for *Ixodes* and produces the smaller base pair band, the JS primer selected the *Borrelia* DNA and created the larger (slower traveling) band. Furthering research upon this model would allow for future experiments involving the incorporation of these DNA samples in conjunction with other DNA or being able to test ticks directly for *Borrelia* infections for diagnostic purposes in medical laboratories.

Literature Cited

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