Development of a PCR for Simultaneous Amplification of *Borrelia burgdorferi* and *Ixodes* Tick DNA

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Abstract

The high prevalence of *Ixodes* ticks in Southeast Minnesota has serious implications as they are often vectors for infectious tick-borne agents, such as *Borrelia burgdorferi*, the causative agent of Lyme Disease (LD). Currently, our lab has developed a PCR that can amplify *Borrelia* DNA in infected ticks, however, when ticks fail to amplify it is unclear whether this is from lack of the bacterial agent or if the DNA is corrupted. A possible solution to this problem is to concurrently amplify a gene within the *Ixodes* tick in order to assess the quality of the DNA. The goal of this study was to efficiently develop a PCR that will simultaneously test for *Ixodes* and *Borrelia burgdorferi* DNA. This would allow our lab to test almost 3000 tick samples collected between 2005-2012 to be tested for *Borrelia* with the internal *Ixodes* control confirming the DNA was intact. Varying combinations of *Ixodes*/ *Borrelia burgdorferi* DNA, primers specific for 16S rRNA in *Ixodes* and 23S rDNA in *Borrelia burgdorferi*, and salts for efficient binding of DNA were prepared for PCR application and run on a 2% agarose gel. Currently, amplification of each of the genes has been successful when the organisms are amplified independently but co-amplification has resulted in only *Borrelia* being successfully amplified. Future research using these methods may include mapping of the prevalence of *Borrelia burgdorferi* infected *Ixodes* ticks in Southeast Minnesota and West Central Wisconsin.

Background

The hard-bodied *Ixodes* ticks have a widespread distribution across North America and Europe, with ranges that appear to be increasing (Wright et al., 2014: Nelson et al., 2015). These ticks inhabit a wide diversity of regions including mountains, forests, and plains, which includes much of the northern hemisphere. With the ability to inhabit such a wide range of habitats, *Ixodes* ticks are not only prevalent in the wilderness, but in urban habitats and outdoor recreational areas. Throughout all these habitats the *Ixodes* ticks infest not only wild, but domestic animals and humans too (Ebani et al., 2015). However, ticks of all species can be vectors to many tick-borne pathogens. One of the most important tick-borne bacterial infections around the world, is Lyme disease. LD is caused by the spirochete bacteria *Borrelia burgdorferi* (Figure 2), which is transmitted and spread primarily by ticks within the genus *Ixodes* (Chang et al., 2000)(Figure 1). What makes LD especially important is the wide range of hosts it can affect, from humans to wild and domestic animals.

Figure 1. *Ixodes* scapularis. Commonly referred to as Blacklegged tick or Deer tick. *Ixodes* is the vector for Borrelia burgdorferi. Photo provider TickEncounter Resource Center.

Figure 2. *Borrelia burgdorferi*, the causative spirochete bacteria of Lyme Disease.

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Objective

The goal of this study was to efficiently develop a PCR that will simultaneously test for *Ixodes* and *Borrelia burgdorferi* DNA. This would allow our lab to test almost 3000 tick samples collected between 2005-2012 to be tested for *Borrelia* with the internal *Ixodes* control confirming the DNA was intact.

Methods and Materials

An experimental method was developed to test multiple variables that contributed to the efficacy of the PCR being developed to simultaneously test for *Ixodes* and *Borrelia burgdorferi* DNA. This method explored the effects of spiked *Ixodes* DNA with a positive *Borrelia burgdorferi* DNA control, pure *Ixodes* DNA, and pure *Borrelia burgdorferi* DNA reacting with varying combinations of primers. Each experimental PCR trial had a final volume of 25µL. Each trial in both experiments contained 1X DreamTaq™ Hot Start PCR Master Mix, 0.4µM each of one or both sets of primers specific for 16S rRNA in *Ixodes* or 23S rDNA in *Borrelia burgdorferi*, 1-10ng/µL of one or both pure samples of *Ixodes* or *Borrelia burgdorferi* DNA (excluding experiment 1: trial 8 and experiment 2: trial 5 and 6 that contained no DNA), and 1mM $MgCl_2$ (exclusive to experiment 2 trials). Each PCR was amplified in a Bio Rad thermocycler using a hot start under the following conditions (Table 1).

Method:
- Trial 1: *Ixodes* DNA/ *Borrelia* primers
- Trial 2: *Ixodes* DNA/ *Ixodes* primers
- Trial 3: *Borrelia* DNA/ *Ixodes* primers
- Trial 4: *Borrelia* DNA/ *Borrelia* primers
- Trial 5: *Borrelia* and *Ixodes* DNA/ *Borrelia* primers
- Trial 6: *Borrelia* and *Ixodes* DNA/ *Ixodes* primers
- Trial 7: *Borrelia* and *Ixodes* DNA/ *Borrelia* and *Ixodes* primers
- Trial 8: ddH_2O (no DNA)/ *Borrelia* and *Ixodes* primers

Method 2: Experiment 2- with 1 mM $MgCl_2$
- Trial 1: *Borrelia* DNA- *Borrelia* Primer
- Trial 2: *Borrelia* DNA- *Ixodes* Primer
- Trial 3: *Ixodes* DNA- *Borrelia* Primer
- Trial 4: *Ixodes* DNA- *Ixodes* Primer
- Trial 5: ddH_2O- *Borrelia* Primer
- Trial 6: ddH_2O- *Ixodes* Primer

Step	Temperature, ^0C	Time	Number of Cycles
Initial denaturation	95	4 min	1
Denaturation	95	30 sec	30
Annealing	50	1 min	
Extension	72	1 min	
Final Extension	72	5 min	1
Hold	16	Up to 2 hours	1

Table 1: PCR Thermocycler Conditions for amplifying 16S rRNA gene segments in *Ixodes* ticks, and 23S rDNA gene segments in *Borrelia burgdorferi*.

Resources

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Chang, Y. F., Novosol, V., McDonough, S. P., Chang, C. F., Jacobson, R. H., Divers, T., ... & Lein, D. H. (2000). Experimental infection of ponies with *Borrelia burgdorferi* by exposure to Ixodid ticks. Veterinary pathology, 37(1), 68-76.

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Wright, C. L., Hynes, W. L., White, B. T., Marshall, M. N., Gaff, H. D., & Gauthier, D. T. (2014). Single-tube real-time PCR assay for differentiation of *Ixodes affinis* and *Ixodes scapularis*. Ticks and tick-borne diseases, 5(1), 48-52.

Discussion and Results

Amplified DNA has been successfully visualized on electrophoresis gels for both *Borrelia burgdorferi* DNA, at ~250bps and *Ixodes* DNA, at ~500bps, in trials where the DNA are separated. However, in the spiked *Ixodes* trials, though bands for both *Ixodes* and *Borrelia* DNA are present, the *Borrelia burgdorferi* DNA band at ~250bps is much more prominent. This indicates that in instances where *Borrelia* DNA is present along with the *Ixodes* DNA, the amplification in the PCR was more sensitive for detecting *Borrelia burgdorferi* DNA. This resulted in the skewed band distribution in the gel. Making changes in concentration of *Ixodes* DNA did not change results, as the *Borrelia burgdorferi* DNA bands still appeared more prominently in spiked trials across the gel. Additionally, the second method that included $MgCl_2$ to increase primer/DNA binding, resulted in bands for both *Borrelia burgdorferi* DNA and *Ixodes* DNA, in separate trials. However, this method, when combined in spiked trials, did not improve the results, and may have hindered the amplification. The next step will be to manipulate the concentration of each primer in the spiked trials. The concentration of both forward and reverse primers specific for 16S rRNA in *Ixodes* will be increased to 0.6µM from 0.4µM. While the concentration of both forward and reverse primers specific for 23S rDNA in *Borrelia burgdorferi* will be reduced to 0.2µM from 0.4µM. This will be done to discern if changes in primer volume will equalize the sensitivity for detecting both *Borrelia burgdorferi* DNA and *Ixodes* DNA in a single PCR trial.

Throughout the course of this experiment various errors needed to be remedied before constructive results were possible. There had been an early struggle in this research with getting the *Borrelia burgdorferi* DNA control to be successfully amplified in the PCR. Eventually new *Borrelia burgdorferi* DNA was extracted from intact dead bacteria and diluted to concentration between 1-10 ng/µL. Once extracted and diluted, the *Borrelia burgdorferi* DNA was able to be successfully amplified. Additional struggles were related to the efficacy of the initial set of *Ixodes* primers specific for 16S rRNA gene segments. The particular forward and reverse primers that were being used were two years old and seemed to have lost the ability to bind effectively and amplify the *Ixodes* DNA. Once these two significant problems were addressed, the research was able to continue with testing methods 1 and 2 of the PCR development.

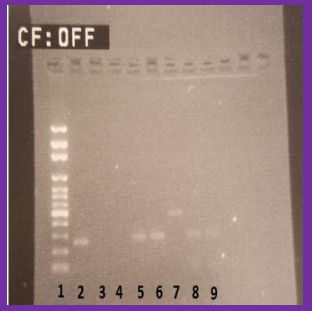

Figure 3. PCR method with equal 0.4µM concentrations of *Ixodes* and *Borrelia burgdorferi* primers. Lane 1: 100 bp ladder. Lane 2: *Ixodes* DNA/ *Borrelia* primers. Lane 3: *I/ I'*. Lane 4: *B/I'*. Lane 5: *B/B'*. Lane 6:*I B/B'*. Lane 7:*I B/I'*. Lane 8:*I B/I'B'*. Lane 9: ddH2O/*I'B'*. Gel was run with old *Ixodes* primers, and Lane 9 was accidently run with trial 7 spiked PCR Spiked trials show faint *Ixodes* band at ~500bps, and *Borrelia burgdorferi* band at ~250bps. The tick sample used is *Borrelia burgdorferi* positive.

Future Research

In the scope of this research almost 3000 tick samples have been collected from the Southeast Minnesota and West Central Wisconsin area, between the years 2005-2012. The PCR being developed will be used to test all the samples for the presence or absence of Lyme Disease. The 3000 tested tick samples may then be used for mapping the prevalence of *Borrelia burgdorferi* infected *Ixodes* ticks in Southeast Minnesota and West Central Wisconsin.