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Identification of Carriers of Babesia microti From Local Populations of Borrelia-infested Ticks

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Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
TIdentification of carriers of Babesia microti from local populations of Borrelia-infested ticks

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Abstract
Babesia microti is the causative agent for babesiosis, a blood-based parasitic disease that causes malaria-like symptoms in humans. It is passed through Ixodes scapularis (deer ticks) and is rising in incidence in the upper Midwest. Ticks that carry Borrelia, the causative agent for Lyme disease, may also carry Babesia microti. There are two major goals in this project: determine the prevalence of Babesia microti in ticks in the Winona area and determine whether those ticks that carry Borrelia are more likely to carry Babesia microti. The first part of the project requires development of a PCR assay that works well in detecting B. microti DNA. The second part involves development of a quantitative PCR (QPCR) assay that is more sensitive than regular PCR in order to detect B. microti DNA. DNA isolated from ticks by Dr. Kim Bates and others in her laboratory will be tested for the presence of B. microti. An update will be presented on progress in the project.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature _____ Date _____

Faculty Sponsor Signature _____ Date _____

PCR Analysis of Ixodes *scapularis* DNA Isolates for the Presence of *Babesia* Microti

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BIOL 427

Dr. Weber

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Introduction

Babesia microti is the causative agent for Babesiosis, a blood-based parasitic disease that causes malaria-like symptoms in humans. It is passed through *Ixodes scapularis* (deer ticks) and is rising in incidence in the northeast and upper Midwest. Ticks that carry *Borrelia*, the causative agent for Lyme disease, may also carry *Babesia microti*. There are two major goals in this project: determine the prevalence of *Babesia microti* in ticks in the Winona area and determine whether those ticks that carry *Borrelia* are more likely to carry *Babesia microti*. The presence of *Babesia microti* was identified using PCR from tick DNA isolates obtained from Dr. Kim Bates and other sources.

Research Methodology

The research goal for this semester was to develop a PCR reaction that works well for *Babesia microti* identification. We learned and improved the process of making PCR solutions, running the PCR reaction, preparing agarose gels, running gel electrophoresis, and using the UV Transilluminator for gel imaging. The main focus of each reaction was to determine the concentrations of the varieties of components of the PCR reaction that would allow for identification of conditions that produced maximum sensitivity to *Babesia microti*.

Solutions were made for the PCR reaction. The following solutions were made: 100mM Tris-HCl and 500mM KCl buffer at a pH of 8.3; 100mM Tris-HCl, 500mM KCl buffer at a pH of 8.3 with the addition of 0.01% gelatin; 100mM $MgCl_2$ stock solution; 1M Betaine stock solution; and a 10X, 2mM dNTP stock mixture. (Garcia, 2006) (Henegariu, 1997) (Musso, 2006) (Calculations can be seen in Attachment 1). PIRO A and PIRO B are primers that amplify the 435bp fragment of *Babesia microti* (Duh et. al., 2001). *Babesia* DNA was provided from their previous experimental use. This sample was not clearly labeled, so the concentration is unknown.

The master mix for the creation of 1 25 µL PCR tube was developed. This mixture can be seen below. The individual amounts in this mixture are multiplied by the number of tubes (adding 1 extra tube for every 8 tubes) in order to make the master mix. Therefore, for 22 tubes, (22+3) x 2.5 µL of 10XBuffer would go into the master mix. The water amount varies in order to reach final volume of 25µL depending on if any of the concentrations are altered for individual solution amounts.

2.50µL Tris-HCl 100mM pH 8.3/ 500mM KCl Buffer (without gelatin)
0.40µL DNA Taq Polymerase – 2 Units
0.75µL 50mM $MgCl_2$ solution
6.25µL 1M Betaine solution
2.50µL 10x Stock of 2mM dNTP Solution
1.00µL 0.4µM PIRO A forward primer
1.00µL 0.4µM PIRO B reverse primer
2.00µL DNA

8.60µL stddH_2O

The following PCR protocol was used: 3 minutes at 94°C; 1 minutes at 94°C, 40 seconds at 55°C, 30 seconds at 72°C for 30 rounds; and 2 minutes at 72°C. Five successful PCR reactions were run this semester. Varying dilutions of *Babesia microti* DNA were used. The purpose of this was to determine the maximum amount that the DNA can be diluted and still be clearly detectable by PCR. The dilution factors that were used were 1:10, 1:20, 1:30, 1:40, 1:50 and 1:100. The concentration of $MgCl_2$ was also varied throughout the reactions and for different dilution factors. $MgCl_2$ significantly increases the specificity, intensity, and robustness of the reaction, but it is necessary for $MgCl_2$ to be at the optimal concentration for this to occur (Henegariu, 1997). The concentrations ranged from 1.25-1.88mM (0.60-0.90µL). PCR reactions were run with and without the presence of betaine in order to determine if it was necessary to this specific reaction. Betaine has been proven to improve the amplification of G-C rich regions (Musso et. al., 2006).

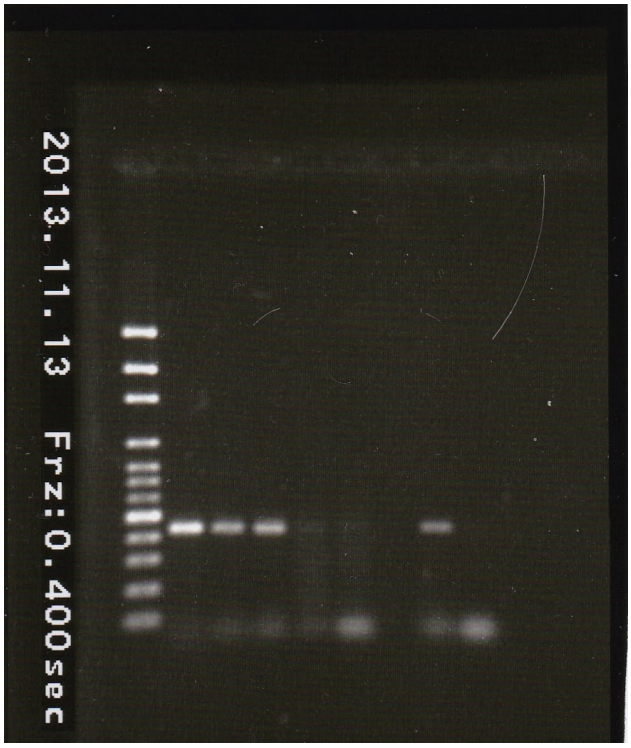
Once the PCR reaction was complete, the samples were placed in the freezer overnight. The following day, the samples were analyzed by doing gel electrophoresis. A 1.2% gel was made using 0.30g of agarose and 25mL of 1xTAE running buffer containing Tris base, acetic acid, and EDTA. A 12-pronged sample comb was placed into the gel, and the gel was placed in the cold room to harden. The combs were removed, and TAE running buffer was added to the MINICELL EC370M electrophoresis chamber, manufactured by E-C Apparatus Corporation, until it covered the top of the gel completely. Then, 4.0µL of a Axygen Biosciences 100bp ladder DNA marker containing dye was pipetted into the first lane. 10µL of each PCR sample was mixed with 2.0µL of a 10x orange DNA loading dye in separate PCR tubes. 5.0µL of each PCR sample solution was pipetted into the remaining lanes on the agarose gel. The gel was run at approximately 55 milliamps (100 volts) for 55-60 minutes. After electrophoresis was complete, the gel was stained with ethidium bromide (1.59mM), while rocking on an orbital shaker for 15 minutes. Then, the gel was de-stained for 5 minutes with distilled water while rocking. The gel was imaged using a UV transilluminator.

Results

11/13/13

Six reactions were run with a 1:10 DNA dilution and varying concentrations of $MgCl_2$ ranging from 1.4-1.6 mM (0.70µL to 0.80µL in 0.5µL increments). Each concentration of $MgCl_2$ was run with and without betaine. Two reactions with 1:20 DNA dilutions and 0.75µL $MgCl_2$ were ran with and without betaine (Figure 1). This gel showed that the concentration of $MgCl_2$ that provided the clearest results was 0.70µL(Figure 1: Lane 2 & 5). Furthermore, Betaine is required for clearly detectable results (Figure 1: Lane 2-4, 8).

Figure 1. 11/13/13 PCR Reaction Gel Image

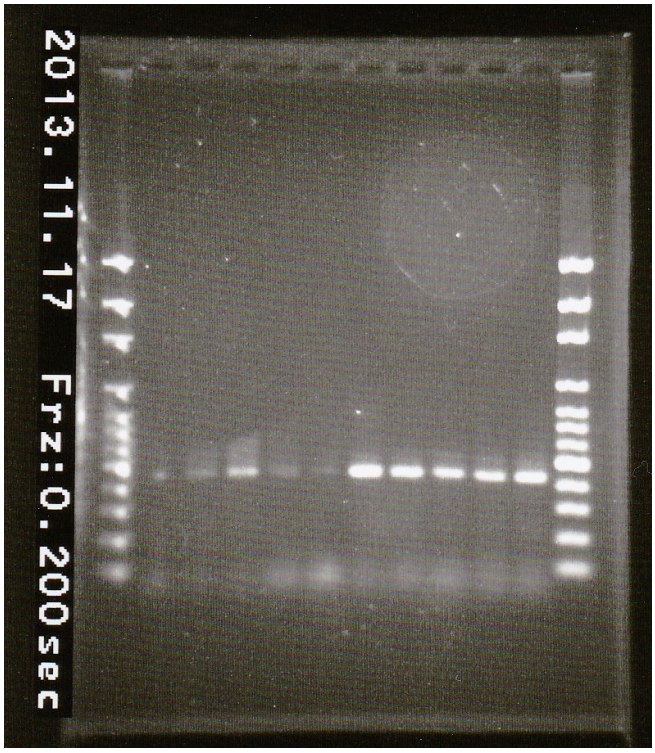


(Lanes listed left to right). ***Lane 1***: 100bp DNA ladder. ***Lane 2***: 0.70µL $MgCl_2$ with Betaine 1:10 DNA dilution. ***Lane 3***: 0.75µL $MgCl_2$ with Betaine 1:10 DNA dilution. ***Lane 4***: 0.80µL $MgCl_2$ with Betaine 1:10 DNA dilution. ***Lane 5:*** 0.70µL $MgCl_2$ without Betaine 1:10 DNA dilution. ***Lane 6***: 0.75µL $MgCl_2$ without Betaine 1:10 DNA dilution. ***Lane 7***: 0.80µL $MgCl_2$ without Betaine 1:10 DNA dilution. ***Lane 8***: 0.75µL $MgCl_2$ with Betaine 1:20 DNA dilution. ***Lane 9***: 0.75µL $MgCl_2$ without Betaine 1:20 DNA dilution.

11/17/13

This PCR reaction used a 1:20 DNA dilution and had concentrations of $MgCl_2$ ranging from 1.25 to 1.88mM (0.60µL to 0.90µL). DNA dilution factors of 1:30, 1:40 and 1:50 were also tested using 0.70µL of $MgCl_2$. All the reactions contained betaine (Figure 2). The 1:30, 1:40 and 1:50 dilutions were the most detectable (Figure 2: Lane 9-11). 0.70µL $MgCl_2$ still proved to be the ideal concentration because of the strong band in Lanes 4 and 9. Concentrations below 1.6mM (above 0.70µL) also were clearly detectable.

Figure 2. 11/17/13 PCR Reaction Gel Image

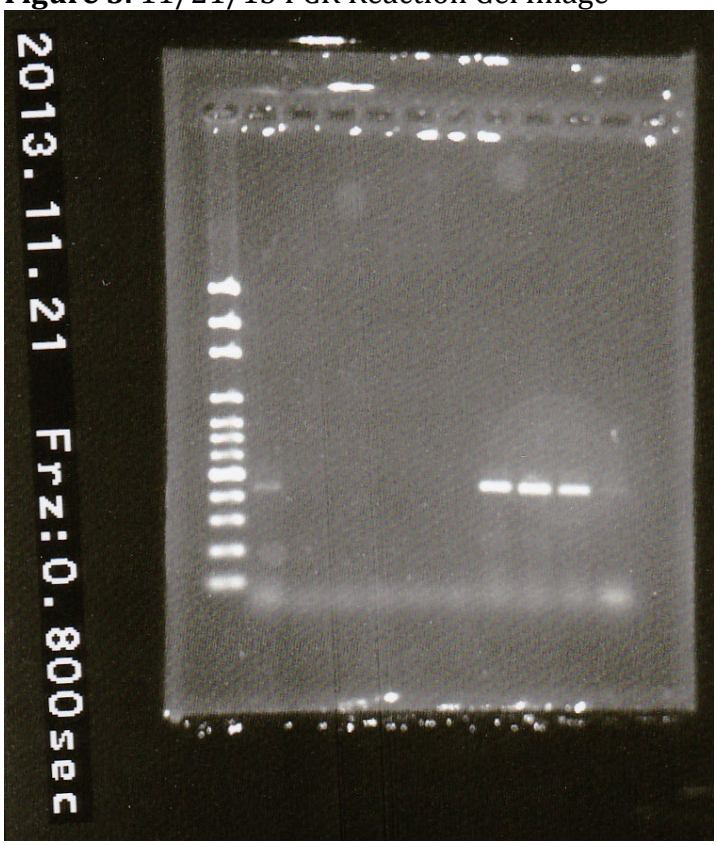


(Lanes listed left to right). ***Lane 1 and 12***: 100bp DNA ladder. ***Lane 2***: 0.60µL $MgCl_2$ ***Lane 3:*** 0.65µL $MgCl_2$. ***Lane 4***: 0.70µL $MgCl_2$. ***Lane 5***: 0.75µL $MgCl_2$. ***Lane 6***: 0.80µL $MgCl_2$. ***Lane 7***: 0.85µL $MgCl_2$. ***Lane 8***: 0.90µL $MgCl_2$ (Lanes 2-8 each had 1:20 DNA dilution factors). ***Lane 9***: 0.70µL $MgCl_2$ 1:30 DNA dilution. ***Lane 10***: 0.70µL $MgCl_2$ 1:40 DNA dilution. ***Lane 11***: 0.70µL $MgCl_2$ 1:50 DNA dilution.

11/21/13

1:40 and 1:50 DNA dilutions were run in this PCR reaction in order to validate the results of the previous dates reaction. The $MgCl_2$ concentrations ranged from 1.4-1.6 mM(0.70µL to 0.90µL) for each dilution (Figure 3). The 1:50 DNA dilution was clearly visualized on this gel, providing that this dilution factor is still detectable by PCR amplification (Lane 7-11).

Figure 3. 11/21/13 PCR Reaction Gel Image

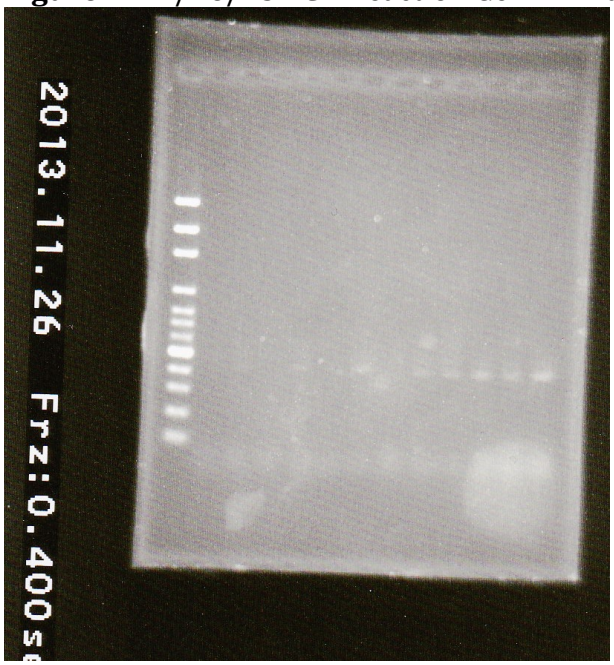


(Lanes listed left to right). ***Lane 1***: 100bp DNA Ladder. ***Lane 2***: 0.70µL $MgCl_2$. ***Lane 3***: 0.75µL $MgCl_2$. ***Lane 4***: 0.80µL $MgCl_2$. ***Lane 5***: 0.85µL $MgCl_2$. ***Lane 6:*** 0.90µL $MgCl_2$. (Lanes 2-6 had DNA dilution factors of 1:40). ***Lane 7***: 0.70µL $MgCl_2$. ***Lane 8***: 0.75µL $MgCl_2$. ***Lane 9***: 0.80µL $MgCl_2$. ***Lane 10***: 0.85µL $MgCl_2$. ***Lane 11:*** 0.90µL $MgCl_2$ (Lanes 7-11 had DNA dilution factors of 1:50).

11/26/13

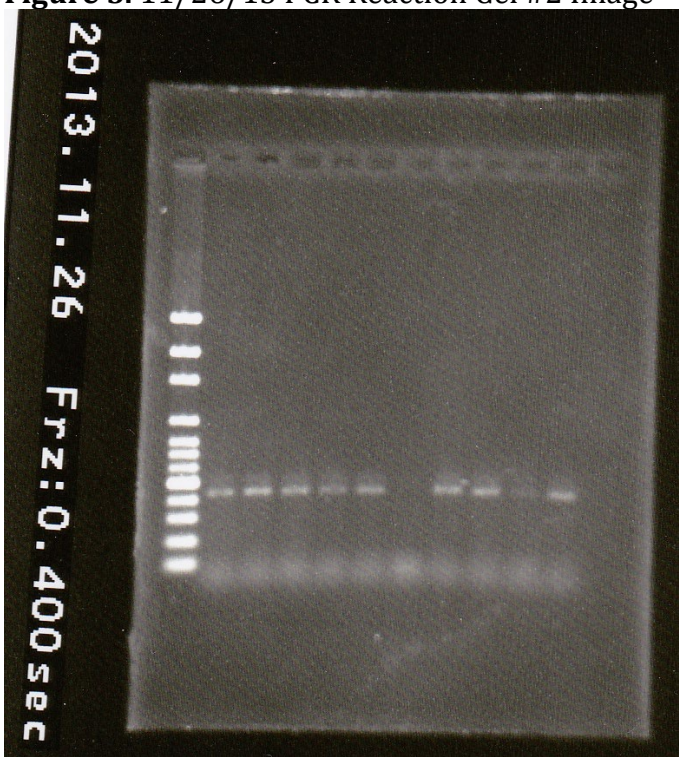
Twenty samples of isolated tick DNA collected in 2006 were used in this PCR reaction. This was done in order to test for the presence of *Babesia microti*. These samples were previously found to be negative for *Babesia microti*. 1.5 mM (0.75µL) of $MgCl_2$ was used for each of these samples. A positive control with a 1:50 dilution of *Babesia* DNA and 0.75µL $MgCl_2$ was also run along with a 1:100 dilution of *Babesia* DNA (Figures 4 and 5). The 1:50 dilution positive control showed a very faint band (Figure 4: Lane 2). Gel #1 showed faint to no bands (Figure 1). Nearly all the samples on gel #2 produced bands indicating that they were positive for *Babesia microti*, meaning that there may be some contamination present (Figure 2).

Figure 4. 11/26/13 PCR Reaction Gel #1 Image



(Lanes listed from left to right) ***Lane 1:*** 100bp DNA ladder. ***Lane 2*****:** positive control. ***Lane 3:*** 1:100 DNA dilution. ***Lanes 4-8:*** DNA samples M65-M71 respectively. ***Lanes 9-12***: DNA samples M73-M76 respectively. ***Lane 13***: DNA sample M79.

Figure 5. 11/26/13 PCR Reaction Gel #2 Image

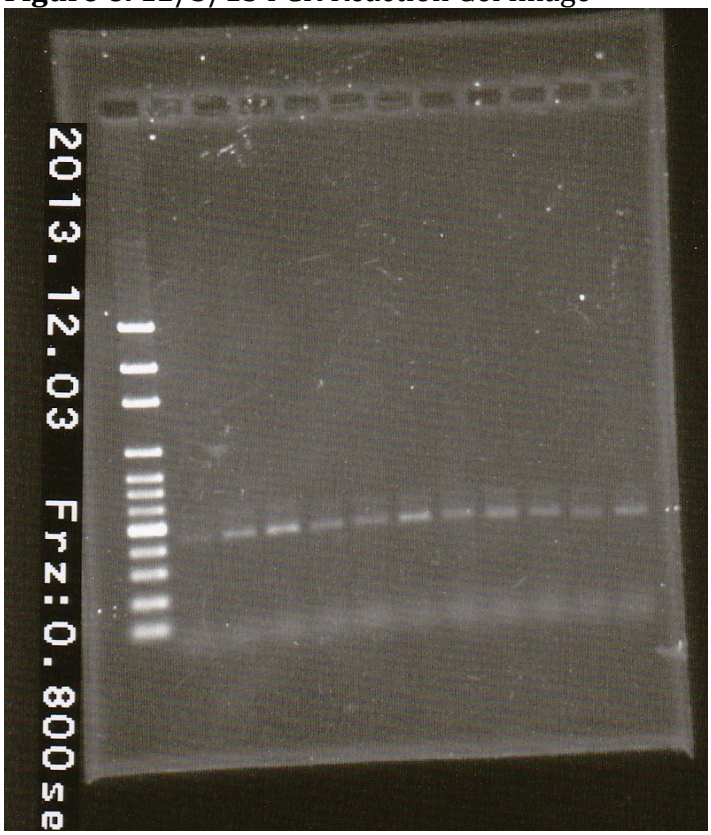


(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder. ***Lane 2:*** DNA sample M80. ***Lane 3***: DNA sample M81. ***Lanes 4-11***: DNA samples M83-M90 respectively.

12/3/13

The main purpose of this reaction was to test for contamination since it was suspected from the positive results above. A negative control without any DNA was used to test for contamination. The following DNA dilution factors were re-ran 1:20, 1:30, 1:40, 1:50 and 1:100 with 1.5mM and 1.6mM concentrations of $MgCl_2$ (Figure 6). The faint band in Lane 2 proved that contamination was present.

Figure 6. 12/3/13 PCR Reaction Gel Image

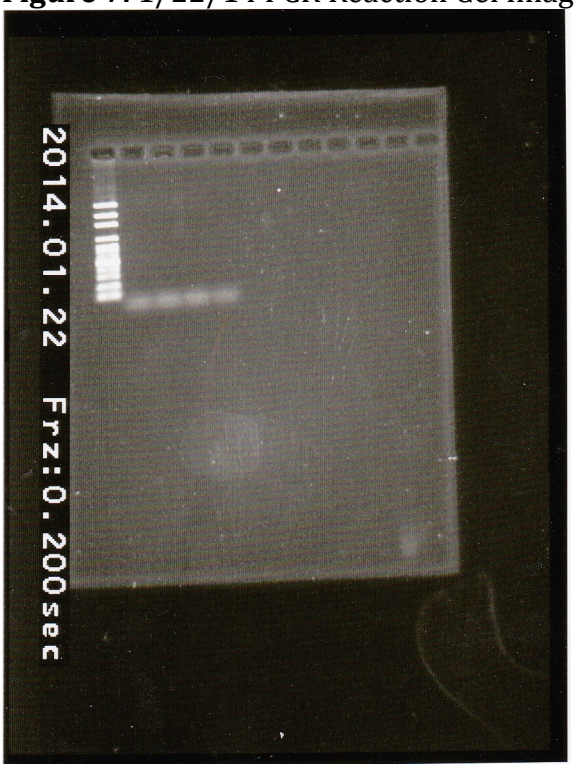


(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder ***Lane 2:*** Negative control ***Lane 3:*** 0.70µL $MgCl_2$ 1:20df ***Lane 4:*** 0.75µL $MgCl_2$ 1:20df. ***Lane 5***: 0.70µL $MgCl_2$ 1:30df. ***Lane 6***: 0.75µL $MgCl_2$ 1:30df. ***Lane 7:*** 0.70µL $MgCl_2$ 1:40df. ***Lane 8***: 0.75µL $MgCl_2$ 1:40df. ***Lane 9***: 0.70µL $MgCl_2$ 1:50df. ***Lane 10:*** 0.75µL $MgCl_2$ 1:50df. ***Lane 11:*** 0.70µL $MgCl_2$ 1:100df. ***Lane 12***: 0.75µL $MgCl_2$ 1:100df

1/22/14

This PCR reaction had a DNA dilution factor of 1:20 and 0.75µL $MgCl_2$. This reaction had two positive controls and two negative controls in order to test for the elimination of contamination. No bands were visualized on the gel, meaning that contamination was eliminated (Lane 2-4).

Figure 7. 1/22/14 PCR Reaction Gel Image

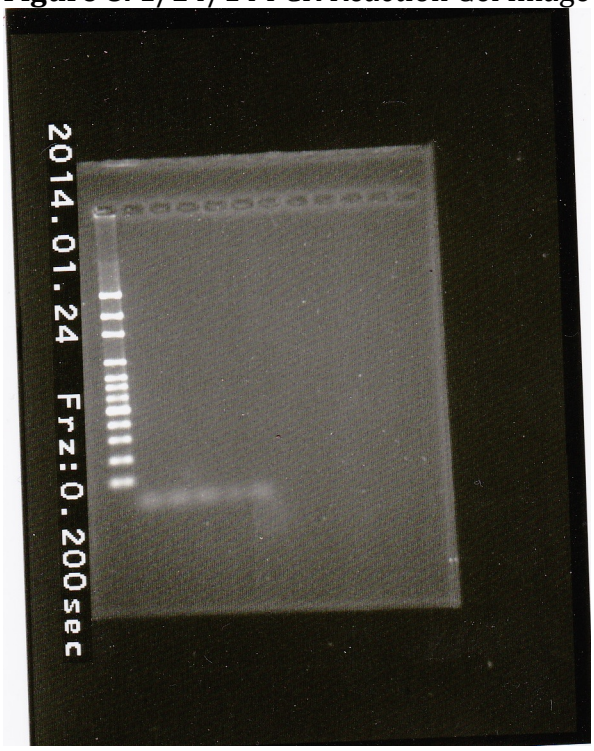


(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder ***Lane 2:*** 0.75µL $MgCl_2$ 1:20df ***Lane 3:*** 0.75µL $MgCl_2$ 1:20df ***Lane 4:*** Negative control ***Lane 5:*** Negative Control.

1/24/14

A 1:5 DNA dilution was used in this reaction at varying concentrations (2.5µL-10µL). No bands were visualized on the gel after the PCR reaction (Lane 2-5).

Figure 8. 1/24/14 PCR Reaction Gel Image

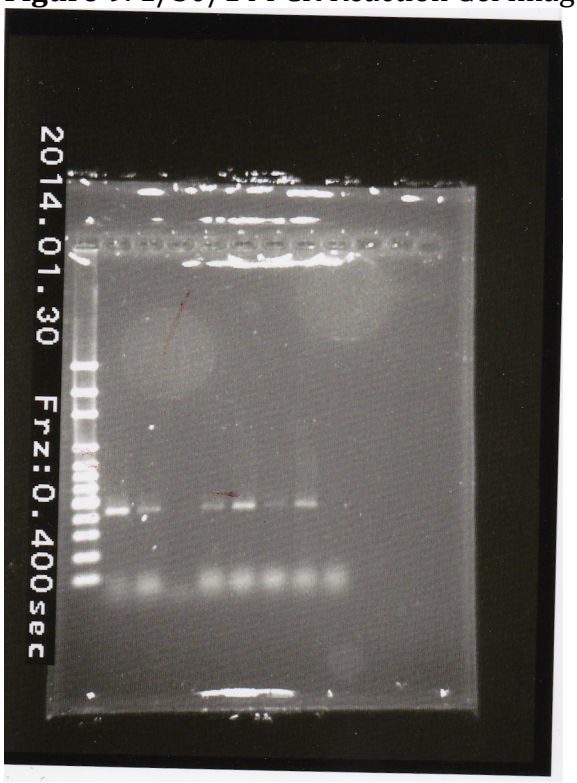


(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder ***Lane 2:*** 0.75µL $MgCl_2$ 10uL DNA ***Lane 3:*** 0.75µL $MgCl_2$ 5uL DNA ***Lane 4:*** 0.75µL $MgCl_2$ 2.5uL DNA. ***Lane 5***: 0.75µL $MgCl_2$ 1:5df. ***Lane 6***: Negative Control.

1/30/14

This reaction was run with a 1:5 DNA dilution factor with 5µL of DNA per PCR reaction tube. $MgCl_2$ concentrations ranged from 1.25-1.88mM (0.60-0.90µL). The extension and annealing time were changed in order to see if that would help to amplify the PCR results. The annealing time was changed to 1 minute at 52°C and the extension time was changed to 1 minute at 72°C. The 0.6µL $MgCl_2$ band was the clearest visualized band (Lane 2).

Figure 9. 1/30/14 PCR Reaction Gel Image

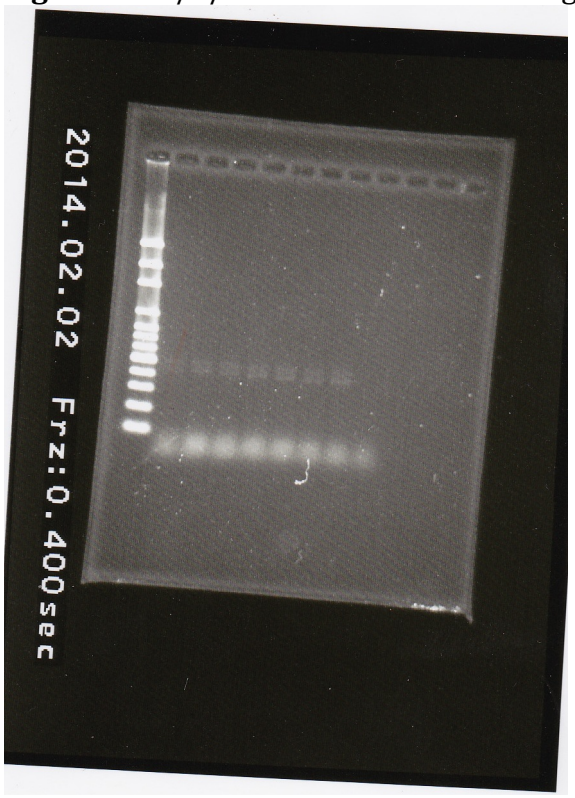


(Lanes listed left to right). ***Lane 1***: 100bp DNA Ladder. ***Lane 2***: 0.60µL $MgCl_2$. ***Lane 3***: 0.65µL $MgCl_2$. ***Lane 4***: 0.70µL $MgCl_2$. ***Lane 5***: 0.75µL $MgCl_2$. ***Lane 6:*** 0.80µL $MgCl_2$. ***Lane 7***: 0.85µL $MgCl_2$. ***Lane 8***: 0.9µL $MgCl_2$. (Lanes 2-8 had DNA dilution factors of 1:5 with 5µL of DNA per reaction tube). ***Lane 9***: Negative Control.

2/2/14

The previous reaction (Figure 9) was run again in order to confirm the results. All the bands were visualized the same, providing differing results that were suspected to be from using different PCR machines (Lanes 2-8).

Figure 10. 2/2/14 PCR Reaction Gel Image

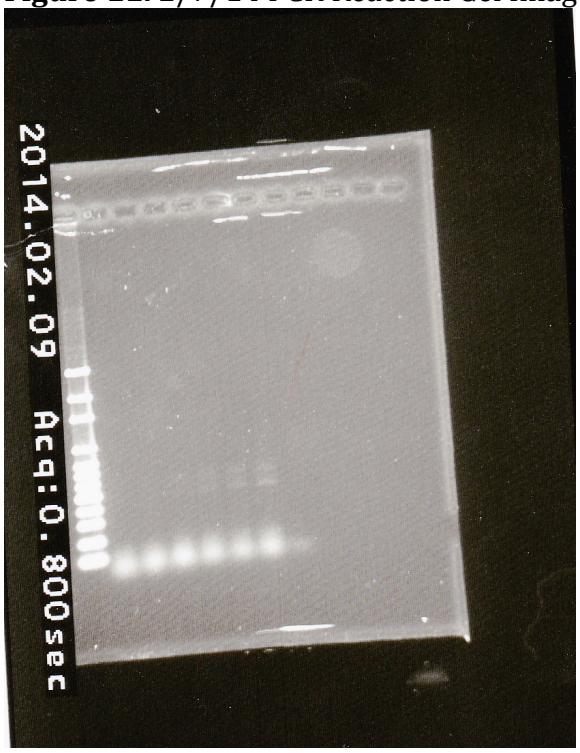


(Lanes listed left to right). ***Lane 1***: 100bp DNA Ladder. ***Lane 2***: 0.60µL $MgCl_2$. ***Lane 3***: 0.65µL $MgCl_2$. ***Lane 4***: 0.70µL $MgCl_2$. ***Lane 5***: 0.75µL $MgCl_2$. ***Lane 6:*** 0.80µL $MgCl_2$. ***Lane 7***: 0.85µL $MgCl_2$. ***Lane 8***: 0.9µL $MgCl_2$. (Lanes 2-8 had DNA dilution factors of 1:5). ***Lane 9***: Negative Control.

2/7/14

A 1:10 DNA dilution factor was used in varying concentrations for this reaction (2.5µL-10.0µL). Undiluted DNA was also used (1.25µL and 1.5µL). The $MgCl_2$ concentration was 0.75µL. The clearest visualized band was the undiluted 1.5µL of DNA (Lane 7).

Figure 11. 2/7/14 PCR Reaction Gel Images

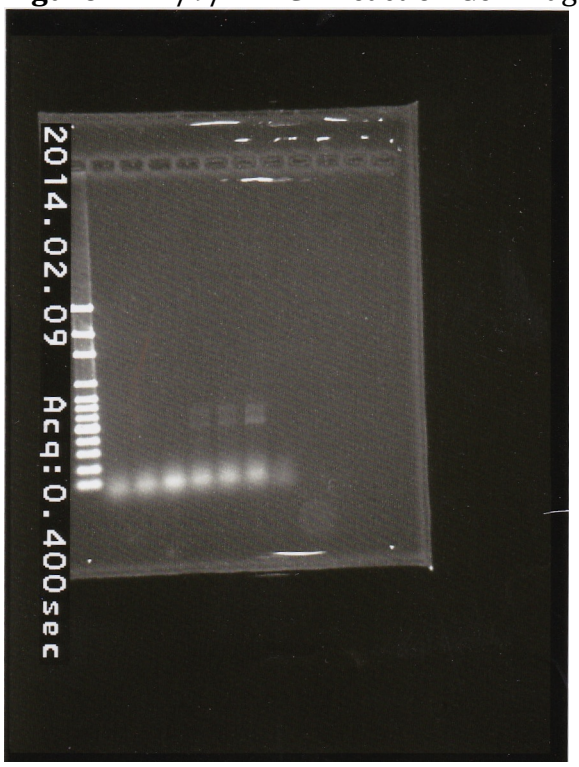


(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder ***Lane 2:*** 0.75µL $MgCl_2$ 1:10df. ***Lane 3:*** 0.75µL $MgCl_2$ 2.5ul DNA. ***Lane 4:*** 0.75µL $MgCl_2$ 5.0µL DNA. ***Lane 5***: 0.75µL $MgCl_2$ 10.0µL DNA. ***Lane 6***: 0.75µL $MgCl_2$ 1.25µL DNA. ***Lane 7:*** 0.75µL $MgCl_2$ 1.5µL DNA. ***Lane 8***: Negative Control.

2/9/14

The purpose of this reaction was to confirm the results of the previous reaction and see if they could be amplified even more. The previous reaction was run again (Figure 11). The annealing time was changed to 30 seconds for 35 rounds, and the extension time was changed to 30 seconds. The results of the previous reaction were confirmed since the 1.5µL of undiluted DNA still was visualized the clearest (Lane 7).

Figure 12. 2/9/14 PCR Reaction Gel Image



(Lanes listed from left to right) ***Lane 1***: 100bp DNA ladder ***Lane 2:*** 0.75µL $MgCl_2$ 1:10df. ***Lane 3:*** 0.75µL $MgCl_2$ 2.5ul DNA. ***Lane 4:*** 0.75µL $MgCl_2$ 5.0µL DNA. ***Lane 5***: 0.75µL $MgCl_2$ 10.0µL DNA. ***Lane 6***: 0.75µL $MgCl_2$ 1.25µL DNA. ***Lane 7:*** 0.75µL $MgCl_2$ 1.5µL DNA. ***Lane 8***: Negative Control.

Discussion

The results from the first reaction showed that the concentration of $MgCl_2$ that provided the clearest results was 1.6mM (0.70µL) for the 1:10 and 1:20 DNA dilution. Also, it was determined that Betaine is necessary for more sensitive detection of the *Babesia* DNA. Since the 1:20 DNA dilution bands were seen on the gel, we decided to run varying concentration of $MgCl_2$ for the next reaction, along with 1:30, 1:40, and 1:50 dilutions with 0.70µL of $MgCl_2$. This reaction produced some unexpected results. The 1:30, 1:40 and 1:50 dilution bands were clearer than the 1:20 dilution bands. It was determined from this gel that the concentration of $MgCl_2$ must be 0.70µL or higher in order to achieve clear bands. We decided to test 1:40 and 1:50 dilutions for our third PCR reaction at concentrations of $MgCl_2$ ranging from 0.70µL to 0.90µL. The 1:50 dilution produced the clearer results, which was also unexpected.

It was decided that the 1:50 DNA dilution with 0.75µL $MgCl_2$ would be used as a positive control for the fourth PCR reaction because it showed consistently clear bands in the previous two reactions. In order to determine if the DNA could be diluted even more, a 1:100 dilution with 0.75µL of $MgCl_2$ was also tested. 20 isolated tick DNA samples were ran in this reaction. These samples were previously shown to be negative for *Babesia microti*. These were tested as non-spiked to see if we achieved the same negative results. The 1:50 dilution positive control barely showed a band. Gel #1 showed very faint bands for the tested tick DNA samples, and nearly all the samples on gel #2 had bands indicating that they were positive for *Babesia microti*. These positive results indicated that something might be contaminated with *Babesia microti* DNA or with the amplified product.

We decided to re-test the DNA dilutions of 1:20, 1:30, 1:40, 1:50, and 1:100 in order to determine if the 1:50 dilution still works the best. Each of these dilutions was tested using 0.70 and 0.75µL of $MgCl_2$. A negative control was also run in order to determine if contamination was present. The 1:20 dilution with 0.75µL DNA appeared to be the clearest, indicating that this is the ideal dilution factor. A faint band was seen in lane two, which was the negative control, indicating that there was contamination present. It is difficult to determine where and when the contamination occurred because a negative control had not been used previously and there are many different solutions used in each PCR reaction that could be contaminated.

Since contamination was present we used a new dNTP solution, primers, and Taq solution to see if we could eliminate the contamination. The PCR reaction gel showed no bands, which indicated that the contamination was gone. Since no bands showed up, it was suspected that the *Babesia microti* DNA had a low concentration. The DNA was nanodropped and the DNA concentration was 1.06 ng/dL. For the next reaction, we decided to use a 1:5 dilution of DNA and use different concentrations of that DNA dilution to see what worked best. Still, no bands were visualized on the PCR reaction gel.

We decided to change the annealing and extension time for the next reaction. The annealing time was changed to 1 minute at 52°C and the extension time was changed to 1 minute at 72°C. Also, we tested $MgCl_2$ concentrations ranging from

1.25-1.88mM (0.60-0.90µL). The clearest results were 1.25mM (0.60µL). In order to confirm these results, the same PCR reaction was ran again. This time all bands were visualized the same. These differing results were suspected to be from using two different PCR machines.

The results from the eleventh reaction showed that the concentration of DNA that provided the clearest results was 1.5µL of DNA, which was undiluted. For the next reaction we decided to see if we could make the bands even clearer, so the same PCR reaction was run again on the same machine, but the extension and annealing times were changed again. The annealing time was changed to 30 seconds for 35 rounds, while the extension time was changed to 30 seconds. The results from this reaction confirmed that 0.75µL of $MgCl_2$ and 1.5µL of undiluted DNA were the optimal reaction conditions.

Future Experiments

Since the contamination was eliminated and the PCR reaction was mastered, the next step will be run the 20 previously tested DNA samples spiked and non-spiked. Also, we will add even more accuracy to the PCR reaction by making sure to record and note all concentrations. Then, we will learn to perform quantitative PCR (QPCR). After this technique is mastered, QPCR will be used to test hundreds of previously collected tick DNA samples for the presence of *Babesia microti*. This data will be used to identify the prevalence of *Babesia microti* in the Winona area using statistical analysis.

Attachment 1

100mM Tris-HCl pH 8.3, 500mM KCl

grams = (molarity of solution) x (molecular weight of chemical) x (mL of solution) ÷ 1000

Amount of Tris-HCl : (0.1M) x (157.6 g/mol) x (100mL) ÷ 1000 = 1.576 g

Amount of KCl: (0.5M) x (74.5g/mol) x (100mL) ÷ 1000 = 3.728 g

100mM Tris-HCl pH 8.3, 500mM KCl with 0.01% Gelatin

Amounts of Tris-HC and KCl used were the same as above (1.576 and 3.728 g respectively), with the addition of 0.1 g of gelatin.

50mM $MgCl_2$ Stock Solution

Amount of $MgCl_2$: (0.1M) x (20.3 g/L) x (50mL) ÷ 100 = 1.015 g $MgCl_2$
*added 100mL of ddH_20 for a final concentration of 100mM

1M Betaine Stock Solution

Amount of Betaine: (1M) x (117.1 g/mol) x 10mL ÷ 1000 = 1.171g of Betaine

10x Stock of 2mM dNTP Solution

10µL of each dNTP from 100mM stock of each dNTP + 460µL stddH$_2$0

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