1. What primers should we use?

We chose to focus on tickborne pathogens which were of epidemiologic importance to Missouri and the Midwest.

2. What will be our positive control?

Rather than create an individual positive control for each primer set, we chose to manufacture a synthetic gene block containing all of the primers in tandem. The distinctive large size of the resulting positive control amplicon will allow us to distinguish between the positive control and a positive sample.

3. How can we improve the specificity and sensitivity of this assay?

Figure 4A: Initial optimization of our top priority primers using conventional PCR. The results indicate that we may not need to test all of our primers on conventional PCR.

Figure 4B: Optimization of MgCl₂ with optimized Tₘ. While this is performed on conventional PCR, we expect to transition this assay to real-time PCR. The kit we will use for this (Qiagen Quantitect SYBR Green) supplies a master mix with 2.5 mM MgCl₂ already added, so ideally the optimized MgCl₂ concentrations would be at or above this value. Arrows designate the chosen optimum concentration.

Figure 4C: Optimization of primer concentration with optimized Tₘ and MgCl₂. Arrows designate the chosen optimum concentration.

4. What are our next steps?

Test the assay using unknown tick samples collected from Missouri deer and elk populations.

Here, we will compare our primers selected from the literature with the primers currently used by the VMDL.

The MDC has provided us with ticks stored in 20% ethanol. We plan to compare this DNA preservation method with traditional ethanol storage.

The current hurdle we must overcome to begin using real-time PCR is combating the contamination caused by aerosolizing of the gene block.

Here is a list of the tick species commonly found in Missouri and the Midwest:
- American Dog Tick (Dermacentor variabilis)
- Blacklegged Tick (Ixodes pacificus)
- Lone Star Tick (Amblyomma americanum, otherwise known as the Lone Star Tick, is the most common tick species found in Missouri. It is a known vector of Ehrlichia chaffeensis and Anaplasma phagocytophila)
- Deer Tick (Dermacentor andersoni)

Table 1: Primary primer sequences currently being tested for the assay.

<table>
<thead>
<tr>
<th>Target Pathogen</th>
<th>Target Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Size (bp)</th>
<th>Tₘ°C</th>
<th>Nearest Neighbor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaplasma spp.</td>
<td>16sDNA</td>
<td>YCTTTTGYGGGACTAG</td>
<td>AATAGGCTCTCTTTCTC</td>
<td>125</td>
<td>77.4</td>
<td>90.4</td>
<td>Zhang et al.</td>
</tr>
<tr>
<td>Borrelia burgdorferi</td>
<td>DotA</td>
<td>TCGGAGGCATCTGAGCAGG</td>
<td>CAGGTCACAGGTTTACAGQ</td>
<td>153 (A. marginale)</td>
<td>80.3</td>
<td>91.2</td>
<td>81.44</td>
</tr>
<tr>
<td>Borrelia spp.</td>
<td>FlaB</td>
<td>CAGGACATCTTTCTTCTCTC</td>
<td>TATATGCTATTATTATTTT</td>
<td>142</td>
<td>73.7</td>
<td>86.7</td>
<td>76.7</td>
</tr>
<tr>
<td>Rickettsia spp.</td>
<td>DotA</td>
<td>TCGGAGGCATCTGAGCAGG</td>
<td>CAGGTCACAGGTTTACAGQ</td>
<td>145</td>
<td>79.8</td>
<td>92.3</td>
<td>82.48</td>
</tr>
<tr>
<td></td>
<td>FlaB</td>
<td>CAGGACATCTTTCTTCTCTC</td>
<td>TATATGCTATTATTATTTT</td>
<td>345</td>
<td>80.2</td>
<td>93.7</td>
<td>82.96</td>
</tr>
</tbody>
</table>

Acknowledgments:
I would like to thank Dr. Zhang for her mentorship and support, Dr. Stich for his humor and easygoing attitude, and Dr. Straka for her infectious enthusiasm.

The VRSP provided us with this wonderful opportunity and the Department of Veterinary Pathobiology requested that we compare our primers to the primers currently used in their diagnostic testing.