Development of a PCR Panel for Tickborne Pathogens

Veterinary Research Scholars Program
University of Missouri

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1. What primers should we use?

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

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

Target Pathogen	Primer			Target Amplicon				Positive Control				Reference
	Target Gene	Forward Sequence	Reverse Sequence	Size	T_m (°C)			Size	T_m (°C)			
					Basic	Salt Adjusted	Nearest Neighbor		Basic	Salt Adjusted	Nearest Neighbor	
Anaplasmataceae	16s rDNA	AACACATGCAAGTCGAACGA	CCCCCGCAGGGATTATACR	145 (*A. marginale*)	80.3	93.3	81.44	640	84.2	98.1	85.88	Yoo-Eam et al.
				153 (*E. ewingii*)	77.4	90.4	79.19					
Borrelia burgdorferi	OspA	TGAAGGCGTAAAAGCTGACAAAA	TTCTGTTGATGACTTGTCTTTGGAAG	142	73.7	86.7	76.7	568	84.4	98.4	86.12	Hodzic et al.
Borrelia spp.	FlaB	CTAATGTTGCAAATCT	GTTGTAACATTAATTGG	145	79.8	92.9	82.48	601	84	97.6	85.8	This Study
Rickettsia spp.	OmpA	ATGAGATAACGGCTGCAGG	CAGCATTCGCTCCCCCTAAA	345	80.2	93.7	82.96	679	84.3	98.2	86.09	
	OmpA	GCTTTATTCACCACCTCAAC	TAATCACCACCGTAAGTAAAT	212	79.7	93.1	82.22	429	85.7	99.5	86.74	Kidd et al.

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.

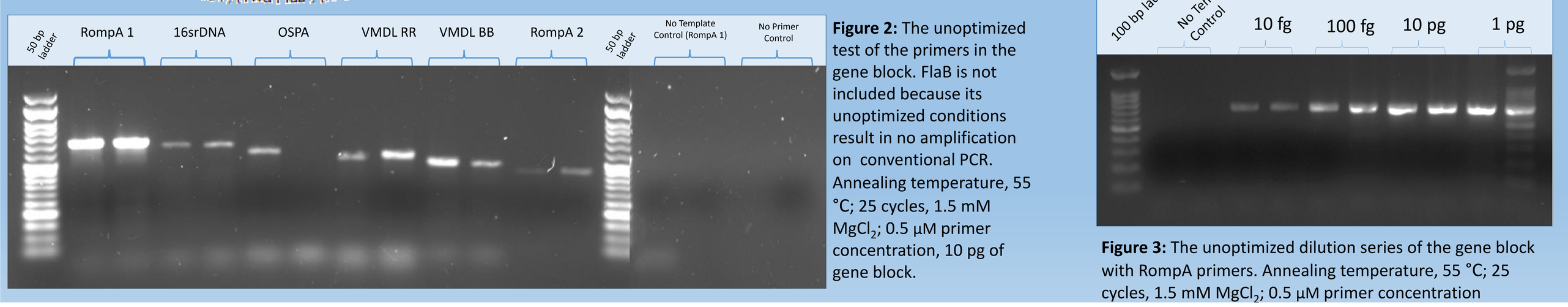


Figure 1: A representation of the sequences and their locations in the 5' to 3' strand of the gene block.

The MU Veterinary Medical Diagnostic Laboratory (VMDL) requested that we compare our primers to the primers currently used in their diagnostic testing.
This experiment will take place in the near future.

Figure 2: The unoptimized test of the primers in the gene block. FlaB is not included because its unoptimized conditions result in no amplification on conventional PCR. Annealing temperature, 55 °C; 25 cycles, 1.5 mM $MgCl_2$; 0.5 µM primer concentration, 10 pg of gene block.

Figure 3: The unoptimized dilution series of the gene block with RompA primers. Annealing temperature, 55 °C; 25 cycles, 1.5 mM $MgCl_2$; 0.5 µM primer concentration

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

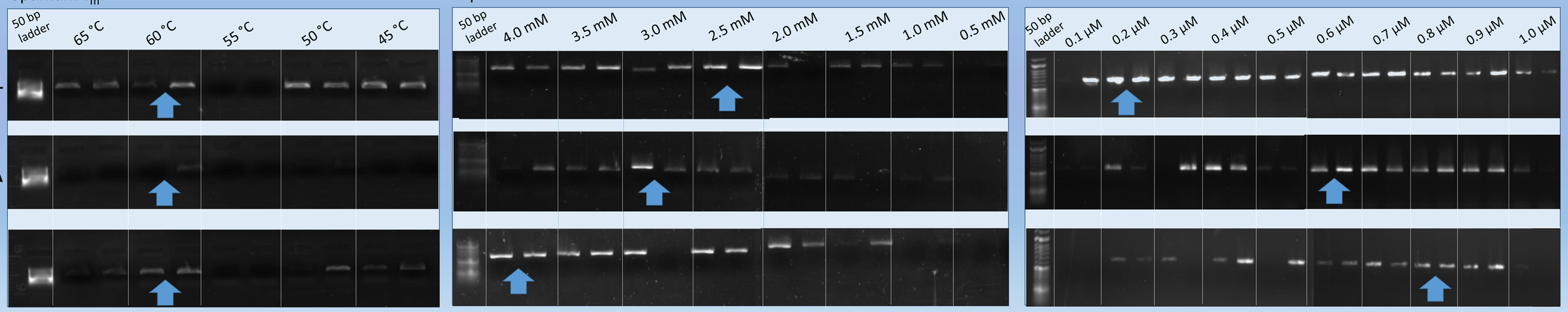
Amblyomma americanum, otherwise known as the Lone Star Tick, is the most common tick species found in Missouri. It is a known vector of *Ehrlichia chaffeensis* and *Ehrlichia ewingii*. [5]

Figure 4: Initial optimization of our top priority primers using conventional PCR

Figure 4A: Optimization of annealing temperature (T_m). Because the ultimate goal of this project is to develop a diagnostic test, we chose to determine a single annealing temperature for all primers in order to screen for multiple pathogens simultaneously. Arrows designate the chosen optimum T_m.

Figure 4B: Optimization of $MgCl_2$ with optimized T_m. 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 $MgCl_2$ already added, so ideally the optimized $MgCl_2$ concentrations would be at or above this value. Arrows designate the chosen optimum concentration.

Figure 4C: Optimization of primer concentration with optimized T_m and $MgCl_2$. Arrows designate the chosen optimum concentration.



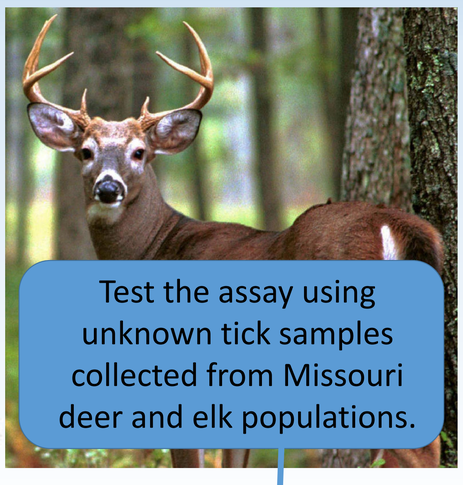
4. What are our next steps?

Repeat above optimization steps to establish confidence in final parameters.

Compare sample types and preparation methods in order to increase DNA yield prior to PCR amplification.

Transfer method to real-time PCR and determine analytical sensitivity.

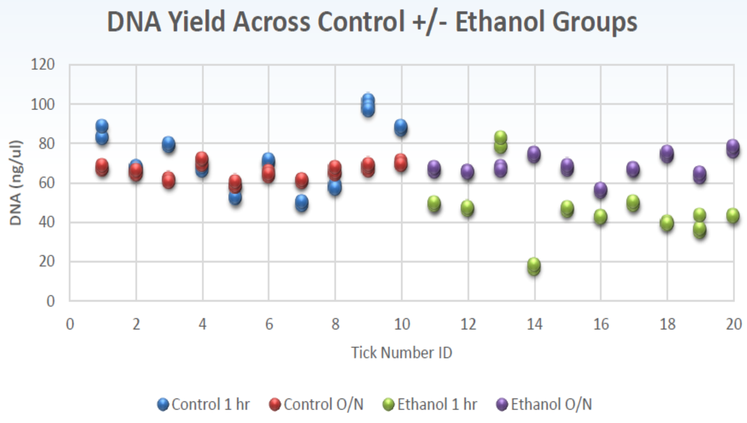
The current hurdle we must overcome to begin using real-time is combatting the contamination caused by aerosolizing of the gene block.



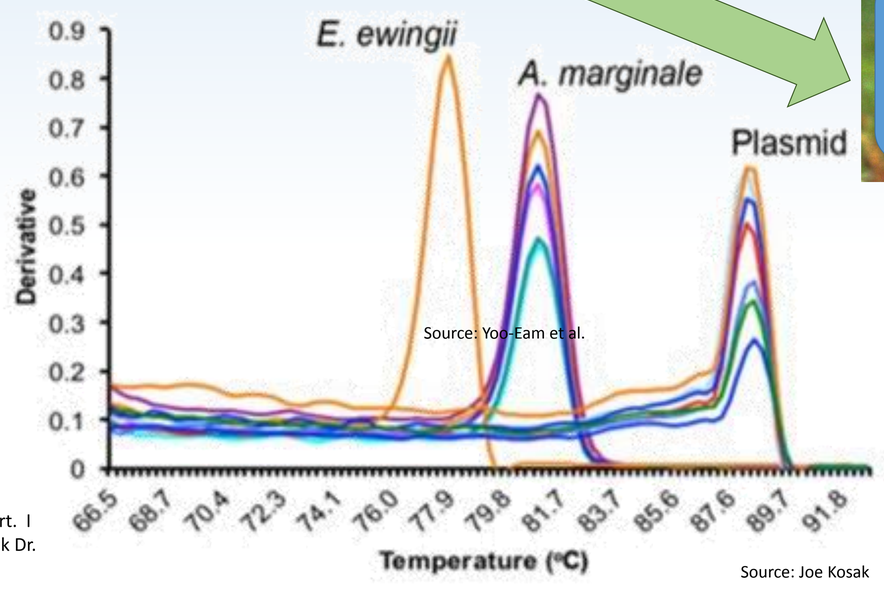
Test the assay using unknown tick samples collected from Missouri deer and elk populations.

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

Inconsistencies among the repeated reaction tubes in Figure 4 suggest that pipetting error could lead us to false conclusions.



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



Source: Joe Kosak

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