DESIGNING AND EXPRESSING A RECOMBINANT TICK SALIVA CHIMERA

An Undergraduate Research Scholars Thesis

by

TAYLOR DROST

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Albert Mulenga

May 2013

Major: Biomedical Sciences
Entomology

TABLE OF CONTENTS

Page

TABLE OF CONTENTS……………………………………………………………………….1

ABSTRACT………………………………………………………………………………….2

ACKNOWLEDGEMENTS…………………………………………………………………….3

CHAPTER

I INTRODUCTION……………………………………………………………..……..4

Ticks and tick borne diseases.………………….………………………………..4
Control of ticks and tick borne diseases..………………………………………...5
Hypothesis and objectives…………………………………………………………7

II METHODS…………………………………………………………….…………………8

Ticks and tick cDNA templates…………………………………………………...8
Primer design, PCR optimization and transcription analysis……………………...8
Gene cloning and sequencing…………………………………………….........9
Protein chimera design………………………………………………………...11

III RESULTS…………………………………………..…………………………….12

PCR Optimization………….………………………………………………12
Transcription analysis……………………………………………………...….14
Analysis of Sequences and Chimera Design……………………………………15
Restrictions……………………………………………………………………...17

IV CONCLUSION……………………………………….………...………………….…...18

REFERENCES…………………………………………………………………….…20

ABSTRACT

Designing and Expressing a Recombinant Tick Saliva Chimera (May 2013)

Taylor Drost
Department of
Entomology
Texas A&M University

Research Advisor: Dr. Albert Mulenga
Department of
Entomology

When ticks feed, they inject numerous proteins into the host that modulate the host's defense mechanism to tick feeding activity. When animals are repeatedly infested with ticks, they develop an immune response to tick saliva proteins. On these animals ticks have difficulties to complete feeding, acquire, and transmit disease agents, and reproduce. Thus immunization of animals with recombinant tick saliva proteins has been proposed. The strategy of this research is to deliver chimeric tick saliva protein antigens. Three cross-tick species conserved genes that encode for Lone Star tick, *Amblyomma americanum* tick saliva proteins were identified. cDNA regions that code for putative immunogenic regions were spliced together to make a chimeric tick saliva protein. The recombinant tick saliva protein chimera will be expressed and used to immunize rabbits against tick feeding. .

ACKNOWLEDGEMENTS

I would like to thank Dr. Albert Mulenga above all for helping me design and work through this project, as well as teaching me all he has for the years I've been working in his lab. I would never have gotten involved in research at all if it weren't for him.

I would also like to thank the graduate and undergraduate students working in Dr. Mulenga's lab for showing me the ropes and helping me with this project, each step of the way.

A great deal of thanks goes out to the Honors and Undergraduate Research office, as well as the coordinators of the Undergraduate Research Scholars program, for helping me get involved in honors and research and to get as much as I possibly can from my undergraduate experience. The paper would literally not be possible without you.

And of course, I would like to thanks my parents, who encouraged me to pursue a quality education and made it possible for me to accomplish so much. Without them, I would never have developed the drive to better myself not only by attending college, but also by taking advantage of everything once I arrived.

Thank you all.

CHAPTER I

INTRODUCTION

Ticks and tick borne diseases

Ticks are among the most successful blood feeding arthropods that transmit agents of human and animal diseases. In terms of disease impact ticks are second to mosquitoes, they surpass any other known blood feeding arthropod in terms of numbers and diversity of human and animal disease agents they transmit (Jongejan et al., 2004; Sonenshine, 1993). There are more than 850 species of ticks that are currently known (Sonenshine 1993). Ticks transmit various animal and human disease agents, including protozoa, bacteria, spirochaetes and viruses (Jongejan et al., 1994). Some tick species also inject toxins into animals and humans that cause paralysis (Kaire, 1966; Espinoza-Gomez et al., 2011). The cost of tick control, treatment and management of tick borne diseases was estimated to cost the livestock industry billions of dollars annually (Jongejan et al., 2004; Sonenshine, 1993). Since the 1980s when *Borrelia burgdoferri* was described as the causative agent for Lyme disease, the impact of ticks and tick borne diseases in public health has been expanding (Childs and Paddock, 2003). In the United States the majority of reportable human arthropod borne diseases are tick borne (Childs and Paddock, 2003).

The Lone Star tick, *Amblyommma americanum*, the focus of my undergraduate research project, is among principle ticks that are distributed in the United States. *Amblyomma americanum,* historically widely distributed in the Southeastern United States (Mixson et. al., 2006) was also found in the Northeastern region as far as Maine (Keirans et al., 1998). This tick is an aggressive feeder that bites a wide range of animals, from birds to humans (Childs and Paddock, 2003).

Heavy infestations of this tick caused mortality in white tailed fawns (Yabsley et al., 2005) and low productivity in cattle (Pegram and Chizyuka, 1990). In Southern states of the US, *A. americanum* ticks were found attached on humans in 83% of reported cases (Felz et al., 1996). *A. americanum* is known to transmit numerous animal and human disease agents. *A. americanum* is the main vector tick for *Ehrlichia chaffensis*, the causative agent of human monocytic ehrlichiosis, *E. ewingii,* which causes human granulocytic ehrlichiosis, *Francisella tularensis,* the causative agent of tularemia, and an unknown causative agent that creates a rash similar to that of Lyme disease called southern tick associated rash illness (STARI) (Anderson et al., 1991, Anderson et al., 1993, Murphy et al., 1998; Buller et al., 1999; Wolf et al., 2000, Hopla et al., 1953; Taylor et al., 1991, Armstrong et al., 2001; James et al., 2001). There is also evidence that *A. americanum* transmits *E. ruminantium*-like organism referred to as the Panola Mountain *Ehrlichia* (PME) (Reeves et al., 2008; Yabsley et al., 2008) and *Rickettsia amblyommii* to humans (Apperson et al., 2008). In animals, *A. americanum* transmits *Theileria cervi* to deer (Laird et al., 1988), and *E. ewingii* in dogs (Little et al., 2010).

Control of ticks and tick borne diseases

There are no vaccines against most tick borne diseases, and thus prevention of both animal and human tick borne diseases is heavily dependent on tick control. At present, tick control programs depend on the use of chemical acaricides. Acaricides are effective in the short term, but they do not offer a long-term solution because of serious limitations, such as ticks developing resistance and acaricide contamination of the environment and the food chain (Sonenshine, 1993). Several alternative tick control methods have been attempted (Sonenshine 1993). Immunization of animals against tick feeding using sub-unit recombinant tick antigens has been validated a

sustainable and environmental friendly alternative tick control method (de la Fuente et al., 2007, Willadsen 2001, 2004 2006a, 2006b). The idea of immunizing animals against tick feeding grew out of observations that repeated tick infestations of animals provoked protective anti-tick immunity (Miller et al., 2011, Brown 1977, Heller-Haupt et al., 1981). Subsequently numerous studies utilizing single tick saliva protein anti-tick vaccine design strategies achieved protection levels that were significant academically, but not good enough practically at field level. With a goal to improve on the efficacy tick saliva protein based vaccines, the goal of the research is to investigate design and delivery of chimeric tick saliva proteins as anti-tick vaccine antigens.

The current bottleneck towards design of tick saliva protein chimeras is discovery of immunogenic tick saliva proteins. Recently the Mulenga lab (Radulovic et al., *In preparation*) conducted a biopanning experiment to screen a phage display cDNA expression library using antibodies to 24h tick saliva proteins. In this experiment, the first step was to generate antibodies to 24h tick saliva proteins. This was accomplished by repeatedly infesting rabbits for 24h until an antibody response was detected. Routinely, rabbits were infested with 20 unfed female *A. americanum* ticks every 24h. Every 24h ticks were manually detached and then 20 unfed fed ticks put on the animal. The idea was to generate antibodies specifically to tick saliva proteins that are injected into the animal during the first day of tick feeding. Once, antibodies to 24h tick saliva proteins were validated, they were used to screen 24h and 48h phage display cDNA expression libraries. This biopanning experiment allowed for cloning of the immunogenic phage display cDNA library. This library was amplified by PCR, purified and submitted for next generation sequencing. Subsequently, sequence reads were assembled using the CLC software. The objective of this research project was to design, synthesize and express a recombinant tick

saliva protein chimera of three selected tick saliva proteins. These proteins were selected on the basis of being conserved in multiple tick species.

Hypothesis and objectives

This research tested the hypothesis that the selected three genes were associated with tick feeding and that immunogenic epitopes from multiple proteins will be preserved in the chimeric tick saliva protein. The overall goal of this project was to design and express a recombinant multi-antibody epitope tick saliva protein chimera. If successful, the tick saliva protein chimera developed in this project will in the future be utilized to protect animals from tick infestation and tick borne diseases. The research had two objectives.

Objective 1

Transcription analysis, cloning and sequencing of cDNA encoding putative tick saliva proteins, BP6C8, BP6C30 and BP6C78. The rationale is that the selected sequences were assembled from short sequence reads. There was a chance they were assembled in error. Thus it important to verify that they were expressed during tick feeding and that the nucleotide sequence was correct.

Objective 2

To assemble, synthesize and express recombinant chimeric tick saliva protein. The rationale is to fuse together immunogenic epitopes identified from each of the candidate tick saliva proteins.

CHAPTER II

METHODS

Ticks and tick cDNA templates

Ticks used in this research were fed on New Zealand White rabbits by the Mulenga lab. Ticks were purchased from the tick labs at Texas A & M University and/or Oklahoma State University. Total RNA from unfed, and ticks that were fed for 24, 48, 72 96 and 120h was extracted using the Trizol reagent as previously described (Mulenga et al., 2007). OligodT primed cDNA was synthesized from ~5µg of total RNA using the qScript cDNA supermix according to instructions by the manufacturer (Quanta Bioscience, Gaithersburg, MD). I would like to note here that, graduate students in the laboratory did tick feeding, dissections, total RNA extractions, and cDNA synthesis.

Primer design, PCR optimization and transcription analysis

Open reading frame PCR primers for BP6C genes (Figure 1) were designed manually. Forward primers were designed starting with the start codon, while reverse primers were designed starting from stop codon. The forward primer was synthesized in the sense orientation, while the reverse primer was synthesized in the anti-sense orientation. Primers were reconstituted to 100µM stock solution. The stock primer solution was diluted 10 fold to make 10µM working solution. If needed, gradient PCR was done to determine the optimum primer annealing temperature range. Routinely, a 10µL PCR reaction volume containing PCR primers up to 1µM concentration, ~50-100ng unfed tick, or 24h fed tick cDNA template added to 5µL PCR MyTaq (BioLine, Taunton, MA) or GoTaq (BioLine, Taunton, MA) PCR master mix was done. Once the annealing

temperature was optimized, candidate genes were amplified in 20µL reactions containing unfed tick, and 24, 48, 72, 96 and 120hr partially fed tick cDNA. PCR products were electrophoresed on 2% agarose gels containing 1µg/mL ethidium bromide. Amplification of target cDNA bands was visualized using the UV source and documented using the image analyzer.

ORF Primers
BP6C8
F: 5'- ATG GTG GCT TTC AAG GCG GCC CTC CTC -3'
Tm = 70.2
R: 5' - GGT AGT CCA TGC AGG CCT GGT ACA GC - 3'
Tm = 69.5
BP6C30
F: 5'- ATG CTG ACA TTG CGT CAG ATT GTG TTC - 3'
Tm = 59.8
R: 5' - TAA AGC AAG AAG CCA ATA TAG CAG TC - 3'
Tm = 57.1
BP6C78
F: 5' - ATG CAG CAG TAC TGC CTC ATC GCC CTT - 3'
Tm = 65.8
R: 5' - TCA GGC GCG TGG GAA TGC TGG CG - 3'
Tm = 68.7

Figure 1. Primers designed to amplify the open reading frame of the desired genes. Tm is the melting temperature of the primers, or the estimated temperature at which the double stranded DNA primer will lose stability and dissociate into single strands of DNA.

Gene cloning and sequencing

Purified PCR products were routinely cloned into the pGEMT (Promega, Madison, WI) vector using routine TA cloning method. To purify amplified cDNA bands, PCR products were electrophosed onto agarose gels containing 1µL/mL ethidium bromide. A thin gel slice around the PCR band was cut using a sterile razor blade. To recover amplified DNA, the gel slice was processed using a gel extraction kit (Agilent Techonologies, Englewood, CO). Purification of the amplified DNA was verified by agarose electrophoresis as described above. The purified DNA

was cloned into the pGEMT vector (Promega, Madison, WI). The ligation mixture contained the pGEMT vector, purified DNA, and DNA ligase. The recombinant pGEMT-DNA plasmid was used to transform DH5α *E. coli* bacteria using routine heat shock method. Transformed bacteria were plated on Luria agar (RPI) plate containing 75μg/mL ampicillin for selection. Plates were incubated overnight (ON) at 37°C.

Following ON incubation, transformation was confirmed by insert check PCR. Essentially, PCR conditions used to amplify the candidate DNA insert were employed. The reaction mixtures contained the same basic components, except the DNA in this case came from inoculating the reaction mixture with the bacteria from a specific colony. The PCR product was run on an agarose gel (same as before) and visualized under UV light to show which colonies had the insert.

For sequencing, selected insert positive bacteria colonies were grown in SOB ON at 37°C. Subsequently recombinant plasmids were purified using the Qiagen Miniprep protocol (Qiagen, Germantown, MD). Purified plasmids were quantified using the U640 spectrometer (Beckman, Brea CA). Approximately 500-600ng of plasmid DNA was used in the sequencing reaction, which contained the BigDye reaction mixture (Applied Biosystems, Grand Island, NY) and the one promoter primer, SP6 or T7. The BigDye PCR reaction product was prepared for sequencing by running the reaction through BigDye purification columns obtained from the TAMU GTL. The final product was dried in a SpeedVac and sent to the Borlaug Institute for sequencing. DNA sequence analysis was done using MacVector software.

Protein chimera design

The protein chimera is designed using B-lymphocyte immunoglobulin epitope predicting algorithms online (http://ailab.cs.iastate.edu/bcpreds/predict.html) that identify the regions of the query protein sequence that can bind to an antibody and thus evoke an immune response. The found epitope regions are then spliced together from numerous proteins that are expressed at similar times in the host, and sent out to GenScript that will send back the synthesized chimera gene.

CHAPTER III

RESULTS

PCR Optimization

The initial annealing temperature for each reaction was chosen to be about 5°C lower than the average Tm (melting temperature) of the two primers, and was chosen as such to allow the primers to bind without too much specificity to the cDNA, but to not bind too broadly as to amplify unwanted sequences. After the reactions were run on a 2% gel, BP6C30 was found to have no bands, while BP6C78 had bands of the expected size (670bp) for each time except for 120hr (shown in Figure 2).The optimal time of expression for BP6C78 was 48 hours of feeding, as it had the strongest band, meaning that the most template DNA with the desired gene was initially present. This early expression of the protein is a desirable result, as proteins expressed early in the ticks feeding cycle would be more useful in stimulating an immune response against the tick than would proteins expressed later in the feeding cycle, as the host would benefit more from an early immune response to the tick than a later response. The bands from this gel for BP6C78 were used for gel extraction.

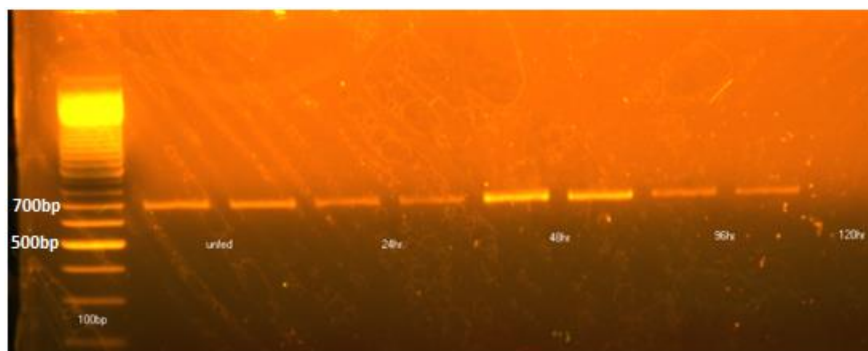


Figure 2. 2% electrophoresis gel run with BP6C78 PCR. The band, about 670bp in size, is the desired size for this primer pair. The different times of cDNA refer to the length of tick feeding on the host, in 24 hour increments beginning with unfed ticks.

Because BP6C30 did not have a band, the annealing temperature was changed from a single temperature to a gradient from 45-60°C in order to determine the optimal temperature for the primers. Unfed and 24 hours whole tick cDNA was used for this reaction, but only 24 hour cDNA produced any bands (shown in Figure 3). This shows that this protein is only expressed once the tick begins to feed, and is not constitutive like BP6C78. This makes the protein a more ideal candidate for useful immune response because a protein expressed only during feeding is more likely to have a necessary effect for the tick to feed successfully on the host. The optimal temperature was determined to be around 58°C, so this temperature was used for any subsequent PCR. The entire band for the 24hr gradient was cut out and used for gel extraction.

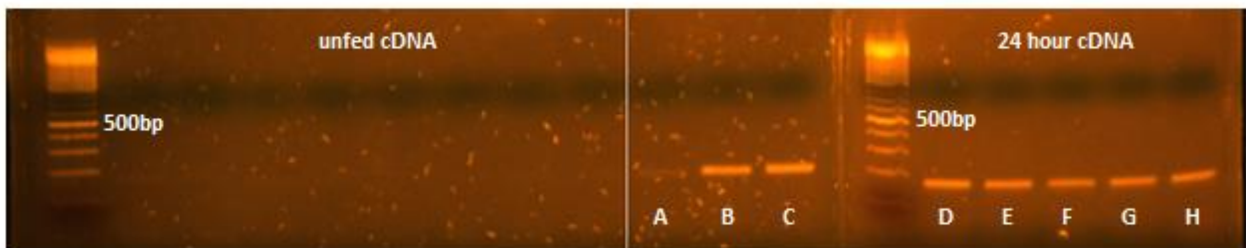


Figure 3. BP6C30 PCR gradient, from 45-60°C. Temperatures for each lane are as follows; A:45°C, B:46°C, C:47.8°C, D:50.7°C, E:54.2°C, F:56.9°C, G:58.8°C, H:60°C.

The PCR procedures were the same for BP6C8, but the annealing temperature was changed to 58°C. Upon running the reactions on a 2% electrophoresis gel, the desired band, at around 420bp (see Figure 4), was visualized and cut out for gel extraction. BP6C8 expression was strongest at 72hr, as this time point produced the strongest band.

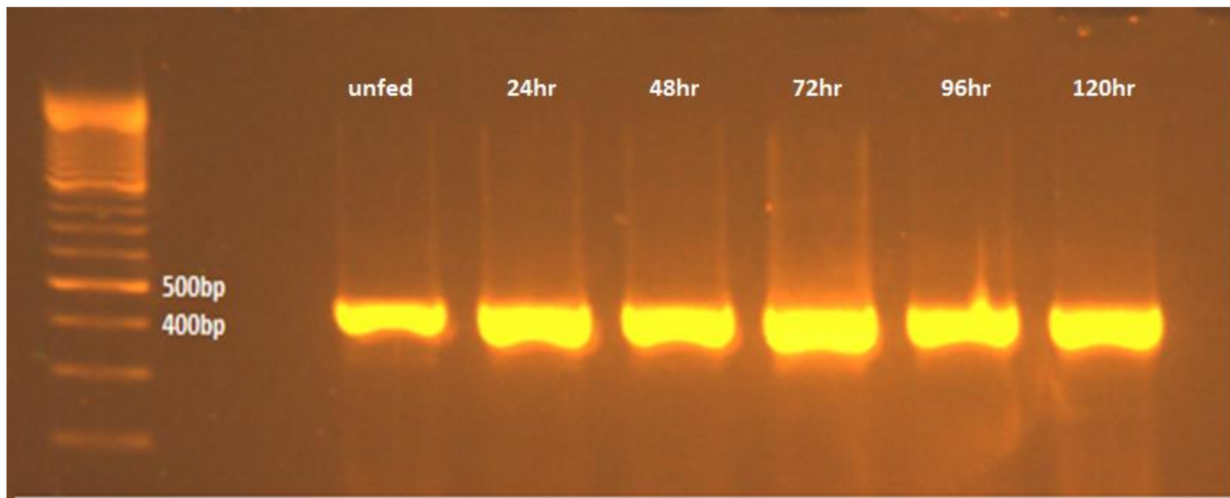


Figure 4. 2% electrophoresis gel for BP6C8. The band displayed, about 420bp in size, is of the desired size.

Transcription analysis: BP6C8 and BP6C78 mRNA are constitutively expressed, while BP6C30 mRNA is induced in response to feeding from the 24h feeding time point

It is known that there is error when contigs or sequences are assembled from short sequence reads. Thus, an important first step when working with such sequences is to verify transcription. Thus, it was significant for this project to verify expression as summarized in figures 2-4. Results summarized in figures 2 and 4 suggests that BP6C78 and BP6C8 mRNA were constitutively expressed in that mRNA was detected in unfed ticks, and those that were partially fed for 24 through 120h. In contrast, BP6C30 mRNA was apparently induced in response to feeding in that it was not expressed in unfed ticks (figure 3). It is important to note here, that BP6C30 transcription analysis was not done in 48-120h samples, because of time constraints. Additionally, the experiment in figure 4 (BP6C8) was by Ms. Taelor Pastine, undergraduate researcher in the Mulenga lab.

An important for objective 1 was in part to confirm that sequences that were assembled from short sequence reads were correct. Pairwise alignment analysis revealed that assembled sequences and those generated through sequencing of the cloned DNA were exact matches (results not shown). This finding was significant in that it allowed for the design of the tick saliva chimera to proceed.

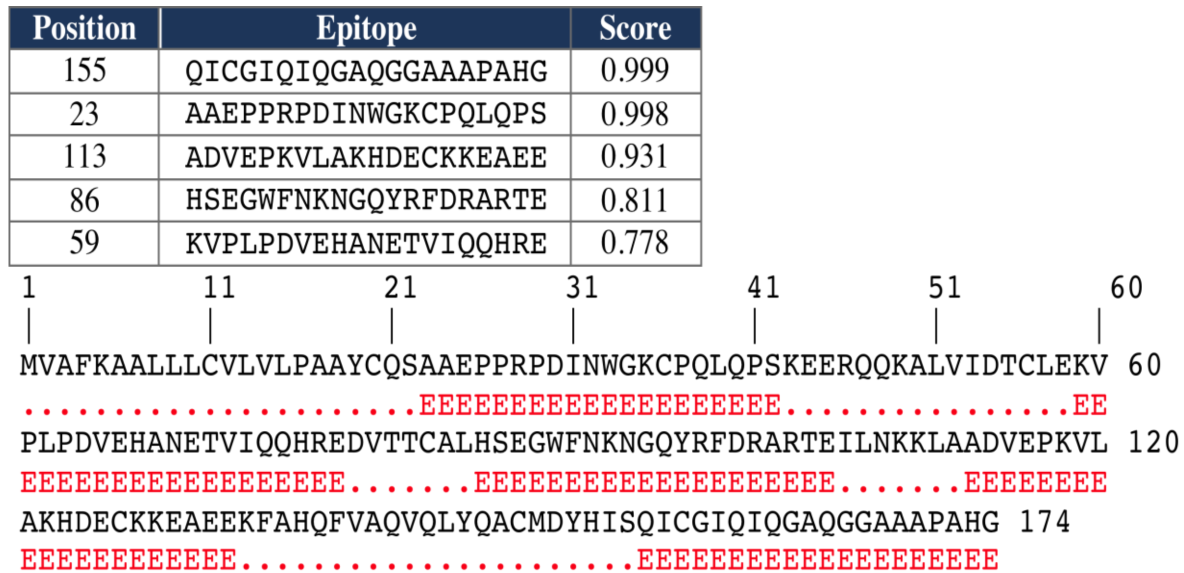
Analysis of Sequences and Chimera Design

The chimera will be synthesized from the regions of the three genes that are predicted to produce antibody response. Therefore, the sequences were run through an online B-cell epitope prediction algorithm (AI Lab, Iowa State Univeristy), and the predicted binding regions will all be combined into one protein. Because this chimera protein will contain numerous B-cell antibody binding regions, it will be expected to illicit a larger, more comprehensive immune response than would the individual proteins illicit on their own.

BP6C30 was a short sequence, and as such, it only had one punitive immunogenic region (Figure 5). BP6C8 had five punitive immunogenic regions predicted in its sequence (Figure 6) and BP6C78 had four predicted punitive immunogenic regions (Figure 7). The predicted epitopes were then combined, with some amino acids between them, into a protein chimera sequence (Figure 8). This is the sequence that will be sent to GenScript for synthesis.

Position	Epitope	Score
21	AMAAPAKKCPSFCKKECLNP	0.987

Figure 5. The predicted B-cell epitope for BP6C30, denoted by the red E sequence directly below.



Position	Epitope	Score
155	QICGIQIQGAQGGAAAPAHG	0.999
23	AAEPPRPDINWGKCPQLQPS	0.998
113	ADVEPKVLAKHDECKKEAEE	0.931
86	HSEGWFNKNGQYRFDRARTE	0.811
59	KVPLPDVEHANETVIQQHRE	0.778

Figure 6.. The predicted B-cell epitopes for BP6C8, denoted by the red E sequence directly below.

Position	Epitope	Score
179	DDREEQFEENIERRSGTSSS	0.997
86	RLGNCDPPMLIDGNTTVNCV	0.943
33	QHLPPLVRGSHALYPGARIP	0.91
114	TLTATTKGDSLVGTIKTIWV	0.907

Figure 7. The predicted B-cell epitopes for BP6C78, denoted by the red E sequence directly below it.

Putative immunogenic tick saliva protein chimera
AMAAPAKKCPSFCKKECLNPAAEPPRPDINWGKCPQLQPSKVPLPDVEHANET VIQQHREADVEPKVLAKHDECKKEAEEQICGIQIQGAQGGAAAPAHGQHLPPLV RGSHALYPGARIPRLGNCDPPMLIDGNTTVNCVTLTATTKGDSLVGTIKTIWVD DREEQFEENIERRSGTSSS

Figure 8. The putative immunogenic tick saliva protein chimera, designed from the ten B-cell epitopes.

Restrictions

Different genes were initially chosen for the project: Ama-190 and Ama-804. These genes were chosen in the same manner as the BP6C genes, but they were primarily based off of genes found in *Amblyomma maculatum*, and the primers chosen were designed for internal binding sites in the gene, not the ORF. The same amplification and cloning procedures were applied to these genes, but the primers were not adequately amplifying the DNA. At first, many tests were run to determine the optimal annealing temperature of the primers, and time point in tick feeding and tissue of expression for these genes, concentrating primarily on the midgut and salivary glands of the tick. When the primers were used with the optimal settings determined in lab, the primers still did not amplify the DNA well enough for sequencing. After these primers continued to fail with subsequent attempts, ORF primers were ordered, but the same obstacles were encountered. Next, RACE (Rapid Amplification of cDNA Ends) cDNA was tried, which is designed to increase the amplification success, as well as allowing capture of cDNA sequences past the end of the gene, as it amplifies to a certain region designed into the RACE cDNA instead of to the point of the paired primer. When all of these attempts to amplify the 190 and 804 genes failed, the BP6C genes were chosen for candidates in the chimera instead.

CHAPTER IV

CONCLUSION

A. americanum ticks are long-term blood feeders that remain attached onto the animal for up to 14 days in the case of adult ticks, and 4-7 days for immature stages, larva and nymphs (Sonenshine, 1993). Male ticks feed minimally, while females taken host blood up to ~300X their initial weight (Sonenshine). The female adult tick feeding process has been divided into three phases, preparatory, slow and rapid feeding. During the preparatory feeding phase, which lasts for ~24-36h, the ticks inserts its hyposthome (mouthpart) into the animal skin, secretes an adhesive substance (cement) to secure itself onto animal skin, and creates the feeding lesion by lacerating host tissue and blood vessels (Sonenshine, 1993). During the slow feeding phase, which may last up to 7 days, ticks grow new tissue to prepare for the massive amounts of blood that the female tick takes in during the rapid feeding phase. Results presented here show that BP6C8 and BP6C78 mRNA are constitutively expressed, while BP6C30 mRNA is induced in response to feeding from the 24h feeding time point. It is important to note here that the analysis done in this study was qualitative and not quantitative. Thus, whether or not the three genes are up regulated or down regulated in response to feeding cannot remain unknown. A quantitative analysis with appropriate internal controls is required to gain insight on the relationship of mRNA expression to tick feeding. Candidate cDNAs, BP6C8, BP6C30 and BP6C78 were assembled from short sequence reads. In these approaches there is always a possibility that there was an error in the assembly. Thus, the finding that sequencing results in this project were matched 100% with assembled sequences was significant.

It is apparent that anti-tick immunity conferred by repeated tick infestation of animals provoked by many proteins. When subjected to western blotting analysis using antibodies provoked by repeated tick infestations, multiple bands were detected on tick salivary gland blots (Mulenga, *unpublished*). This observation suggested that anti-tick resistance was conferred by multiple tick saliva proteins. In an attempt to mimic anti-tick immunity conferred by multiple tick saliva proteins this project designed a chimeric tick saliva protein. The synthesis and expressing the recombinant chimera is actively in progress.

In conclusion, this research exposed me to how different technologies can be integrated to solve a problem in life science. This project involved the use of knowledge that when ticks feed, animals can develop antibodies to proteins that facilitate feeding. So in order to identify these proteins, antibodies induced by repeated tick feeding were used to screen cDNA expression libraries to fish cDNAs. From these cDNAs, I learnt the identity of three tick saliva proteins. In the next technique, computer applications life science research was used to identify putative immunogenic epitopes. These epitopes were used to construct the chimeric tick saliva protein. In the final step, recombinant protein production technology will be used. I also learned that scientists cannot commit to one idea, and must adapt to the situation, because not everything will go according to plan. My initial road-blocks taught me to adapt and keep moving, not being discouraged by repeated failure.

REFERENCES

Anderson, B. E., Sims, K. G., Olson, J. G., Childs, J. E., Piesman, J. F., Happ, C. M., Maupin, G. O. and Johnson, B. J. (1993). Amblyomma americanum: a potential vector of human ehrlichiosis. *Am. J. Trop. Med. Hyg.* **49,** 239-244.

Apperson, C. S., Engber, B., Nicholson, W. L., Mead, D. G., Engel, J., Yabsley, M. J., Dail, K., Johnson, J. and Watson, D. W. (2008). Tick-borne diseases in North Carolina: is "Rickettsia amblyommii" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? *Vector Borne Zoonotic Dis.* **8,** 597-606.

Apperson, C. S., Engber, B., Nicholson, W. L., Mead, D. G., Engel, J., Yabsley, M. J., Dail, K., Johnson, J. and Watson, D. W. (2008). Tick-borne diseases in North Carolina: is "Rickettsia amblyommii" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? *Vector Borne Zoonotic Dis.* **8,** 597-606.

Brown SJ. (1977). Feeding performance, survival and appearance of Dermacentor variabilis nymphs (Acari: Ixodidae), as influenced by host resistance. *J Med Entomol.* 14, 497-498.

Buller, R. S., Arens, M., Hmiel, S. P., Paddock, C. D., Sumner, J. W., Rikhisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F. A., Liddell, A. M. et al. (1999). Ehrlichia ewingii, a newly recognized agent of human ehrlichiosis. *N. Engl. J. Med.* **341,** 148-155.

Childs, J.E. and Paddock, C.D. (2003). The ascendancy of Amblyomma americanum as a vector of pathogens affecting humans in the United States. *Annu. Rev. Entomol.* **48**, 307-337

de la Fuente, J., Almazán, C., Canales, M., Pérez, de la Lastra JM, Kocan KM, Willadsen P. (2007). A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Anim Health Res Rev*. **8**, 23-8.

Espinoza-Gomez, F., Newton-Sanchez, O., Flores-Cazares, G., De la Cruz-Ruiz, M., Melnikov, V., Austria-Tejeda, J. and Rojas-Larios, F. (2011). Tick paralysis caused by Amblyomma maculatum on the Mexican Pacific Coast. *Vector Borne Zoonotic Dis.* **11,** 945-946.

Felz, M. W., L. A. Durden, and J. H. Oliver, Jr. 1996. Ticks parasitizing humans in Georgia and South Carolina. *J. Parasitol*. 82, 505-508.

Heller-Haupt A, Varma MR, Langi AO. (1981). Acquired resistance to ixodid ticks in laboratory animals. *Trans R Soc Trop Med Hyg*. **75**, 147-148.

Hopla CE, D. C. (1953). The isolation of *Bacterium tularense* from the tick *Amblyomma americanum.. journal of kansas entomological society* **26,** 71-72.

James, A. M., Liveris, D., Wormser, G. P., Schwartz, I., Montecalvo, M. A. and Johnson, B. J. (2001). Borrelia lonestari infection after a bite by an Amblyomma americanum tick. *J. Infect. Dis.* **183,** 1810-1814.

Jongejan, F. and Uilenberg, G. (2004). The global importance of ticks. *Parasitology* **129**, S1–S12

Jongejan, F. and Uilenberg, G. (2004). The global importance of ticks. *Parasitology* **129 Suppl,** S3-14.

Kaire, G. (1966). Isolation of tick paralysis toxin from Ixodes holocyclus. *Toxicon* **4,** 91-97.

Keirans, J. E. and Lacombe, E. H. (1998). First records of Amblyomma americanum, Ixodes (Ixodes) dentatus, and Ixodes (Ceratixodes) uriae (Acari: Ixodidae) from Maine. *J. Parasitol.* **84,** 629-631.

Laird, J. S., Kocan, A. A., Kocan, K. M., Presley, S. M. and Hair, J. A. (1988). Susceptibility of Amblyomma americanum to natural and experimental infections with Theileria cervi. *J. Wildl. Dis.* **24,** 679-683.

Little, S. E., O'Connor, T. P., Hempstead, J., Saucier, J., Reichard, M. V., Meinkoth, K., Meinkoth, J. H., Andrews, B., Ullom, S., Ewing, S. A. et al. (2010). Ehrlichia ewingii infection and exposure rates in dogs from the southcentral United States. *Vet. Parasitol.* **172,** 355-360.

Miller RJ, White WH, Davey RB, George JE, Perez de Leon A. (2011). Efficacy of spinosad against acaricide-resistant and -susceptible *Rhipicephalus (Boophilus) microplus* and acaricide-susceptible *Amblyomma americanum and Dermacentor variabilis*. J Med Entomol. 48, 358-365.

Mixson, T. R., Campbell, S. R., Gill, J. S., Ginsberg, H. S., Reichard, M. V., Schulze, T. L. and Dasch, G. A. (2006). Prevalence of Ehrlichia, Borrelia, and Rickettsial agents in Amblyomma americanum (Acari: Ixodidae) collected from nine states. *J. Med. Entomol.* **43,** 1261-1268.

Murphy, G. L., Ewing, S. A., Whitworth, L. C., Fox, J. C. and Kocan, A. A. (1998). A molecular and serologic survey of Ehrlichia canis, E. chaffeensis, and E. ewingii in dogs and ticks from Oklahoma. *Vet. Parasitol.* **79,** 325-339.

Reeves, W. K., Loftis, A. D., Nicholson, W. L. and Czarkowski, A. G. (2008). The first report of human illness associated with the Panola Mountain Ehrlichia species: a case report. *J. Med. Case Rep.* **2,** 139-1947-2-139.

Sonenshine, D.E. (1993). *Biology of ticks*, Oxford, Oxford University Press.

Taylor, J. P., Istre, G. R., McChesney, T. C., Satalowich, F. T., Parker, R. L. and McFarland, L. M. (1991). Epidemiologic characteristics of human tularemia in the southwest-central states, 1981-1987. *Am. J. Epidemiol.* **133,** 1032-1038.

Willadsen P. (2001). The molecular revolution in the development of vaccines against ectoparasites. *Vet Parasitol.* **101,** 353-368.

Willadsen P. (2006a) Vaccination against ectoparasites. *Parasitology.* **133,** Suppl:S9-S25.

Willadsen P. (2006b) Tick control: thoughts on a research agenda. *Vet Parasitol.* **138,** 161-168.

Willadsen, P. (2004). Anti-tick vaccines. *Parasitology*. **129,** S367-87. Review.

Wolf, L., McPherson, T., Harrison, B., Engber, B., Anderson, A. and Whitt, P. (2000). Prevalence of Ehrlichia ewingii in Amblyomma americanum in North Carolina. *J. Clin. Microbiol.* **38,** 2795.

Yabsley, M. J., Loftis, A. D. and Little, S. E. (2008). Natural and experimental infection of white-tailed deer (Odocoileus virginianus) from the United States with an Ehrlichia sp. closely related to Ehrlichia ruminantium. *J. Wildl. Dis.* **44,** 381-387.

Yabsley, M. J., Quick, T. C. and Little, S. E. (2005). Theileriosis in a white-tailed deer (Odocoileus virginianus) fawn. *J. Wildl. Dis.* **41,** 806-809.