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Expression of Glycine-Rich Proteins Found In Salivary Glands of the Lone Star Tick (*Amblyomma americanum*) Using a Mammalian Cell Line

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The University of Southern Mississippi

Expression of glycine-rich proteins found in salivary glands of the Lone Star Tick
(*Amblyomma americanum*) using a mammalian cell line

By

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Abstract

Ticks play an important ecological role as well as a growing role in human health and veterinary care. Ticks are hosts to a plethora of microbial pathogens that can be transferred during feeding to cause tick-borne diseases in humans and many animals. Ticks may in large part owe the success of the transfer of these pathogens between hosts to their complex saliva. The saliva secreted upon a tick's attachment to a host serves the following, among other, functions: anti-hemostasis of the blood pool, preventing an inflammatory response at the bite site, and serving as a natural anti-microbial substance. An important component of this functional saliva is multiple Glycine Rich Proteins (GRPs). We hypothesize that the GRPs found in the saliva produced by the Lone Star Tick, *Amblyomma americanum*, are responsible for these observed functions in the saliva; therefore, the aim of this research is to express and purify one particular protein, GRP 34358, to be used in further studies and assays to determine its role in anti host-response defenses. The DNA sequence corresponding to GRP 34358 was transformed into a plasmid containing a His-tag. The plasmid was then put through a restriction digestion to determine presence of the gene insert, and then sequenced to determine the orientation of the insert. The plasmid was transfected into a Vero cell line for protein expression. Expression and purification of this GRP would lead to valuable further studies of its function and structure—information that may lead to discoveries of multiple commercial uses such as a component of a biological adhesive or an anti-tick topical product.

Key terms: *Amblyomma americanum*, glycine-rich protein, protein expression, transfection, saliva, Lone Star Tick

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Chapter 1: Introduction

Ticks are hematophagous ectoparasites that can be found in almost any place on Earth. Ticks play an important ecological role as well as a growing role in human health and veterinary care. Ticks are hosts to a plethora of microbial pathogens that can be transferred during feeding to cause tick-borne diseases in humans and many animals. Some of the most prominent being *Lyme borreliosis*—agent of Lyme disease, species of *Rickettsia*—agents of Rocky Mountain Spotted Fever and its derivatives, and species of *Ehrlichia*—agents of *Ehrlichiosis* and Heartwater disease. One particular species of tick, *Amblyomma americanum*, also known by the common name Lone Star Tick, is common in southern Mississippi and the surrounding Gulf Coast region. This species are particularly common hosts for *Ehrlichia chaffeensis*, Heartland virus, Bourbon virus, and the newly described Tacaribe virus. This species is responsible for a large amount of tick-borne diseases in the southern Mississippi region.

Ticks may in large part owe the success of the transfer of these pathogens between hosts to their complex saliva. The saliva secreted upon a tick's attachment to a host serves several functions. The first being anti-hemostatic—some components in the tick's saliva prevent blood clotting in the host's tissue surrounding the bite site. If undisturbed by outside forces such as scratching or biting, one tick may be able to feed on the same host for over two weeks. For the entirety of this feeding period, blood must remain able to flow through the bite wound to be taken up by the tick. The second main function of tick saliva is that of preventing an inflammatory response in the host. In undisturbed feeding ticks, the site around the bite wound on the host remains uninflamed,

no redness is observed, and no itching sensation is present. Third, the saliva acts as a natural anti-microbial substance. Throughout the course of feeding the bite site does not become infected and no fungal growth is observed. This is particularly interesting considering the type of environmental conditions usually surrounding a normal host; a cow or dog may spend most of its time outside surrounded by an unmeasurable amount of microbial life.

An important component of this very functional saliva is multiple Glycine Rich Proteins (GRPs) (Karim and Ribeiro 2015; Bullard et al., 2016). Recent research has suggested that these molecules possess structural functions and may also serve as stress response molecules upon the tick's encounter of stressors such as unfavorable temperature or injury (Lewis, 2006; Francischetti et al., 2009; Bullard et al., unpublished). One would hypothesize that the GRPs found in the saliva produced by the Lone Star Tick have also evolved antimicrobial, anti-inflammatory, and anti-hemostatic functions in addition to their structural and stress response roles; therefore, the aim of this research is to express a particular GRP found in the saliva of the Lone Star Tick, GRP 34358, in a mammalian cell line for the purpose of the further study and determination of the function of this particular protein.

Chapter II: Literature Review

Ticks: An overview

Ticks are hematophagous ectoparasitic arthropods in the family *Parasitiformes*. Ticks are native to all fifty of the United States, and to any part of the world from the tropics to the subarctic regions [Anderson 2008]. They are common in woody areas or open, grassy fields, but can be found in nearly any type of environment and may feed on any type of vertebrate host. Depending on the species and its current life stage, a tick may feed on anything from a reptile or bird to a large mammal, including a human. The life cycle of a tick is made up of three life stages: larval, nymph, and adult. Larval ticks possess six legs, while nymph and adult ticks possess eight. Unfed adult ticks may range from 2–20 mm in length, and engorged adults may extend up to 30 mm in length and can weigh up to 100 times their original weight. A blood meal is required for the tick to develop into the next life stage, and for reproduction. Female ticks cannot oviposit until after a blood meal [Anderson 2008]. Ticks obtain nutrients solely by taking a blood meal from a host, and do not feed on plants or other organisms between meals.

Because of their parasitic nature and almost universal distribution, ticks pose as a major environmental and health concern. Ticks are vectors for a multitude of disease-causing agents which can affect both animals and humans. Tick-borne diseases are a major threat to cattle and livestock; millions of dollars are costed each year either as a result of financial losses due to death of livestock or as money spent on preventative measures and pesticides [Doube 1980]. Not only can tick-borne diseases directly affect

the health of the host, but they can lead to contamination of consumer products from large livestock such as dairy and meat.

In addition to the numerous tick-borne diseases that affect animal hosts, ticks are also vectors for a large number of biological agents that can infect humans. Common diseases in the U.S. include Anaplasmosis, Babesiosis, Ehrlichiosis, Lyme Disease, Rocky Mountain Spotted Fever, and Tularemia. Symptoms of these diseases range from a minor rash to meningitis, cardiac manifestations, and/or death in some cases [CDC]. Tick-borne diseases can have a large economic and environmental impact as well as result in a substantial infringement on public health both worldwide and in the U.S.

Anatomy of the tick, feeding mechanisms, and an introduction to saliva

Ticks feed by remaining attached to the exterior of its host for a variable amount of time and continuously taking up the host's blood until engorged. To accomplish this, several events must take place. First, the tick must attach to a host that is within its species-specific range. The mechanism of host specificity remains a topic of ongoing research, but many authors have suggested that the tick's specificity for a certain host or range of hosts is in large part correlated with evolutionary development of the tick species in accordance to the ecological characteristics of the hosts themselves. For example, host species that largely aggregate to breed or that live in tight communities tend to be host to tick species with an increased specificity to that animal as opposed to a roaming or non-social species. Host selection is also correlated with light and thermal cues, and is dependent on the fed-state of the tick as well as the tick's location and environment [McCoy 2015].

After engaging with a host, the tick searches for a place to physically attach itself. It does so by inserting its mouth parts into the skin of its host. The tick's entire body consists of two parts: the capitulum which contains the mouthparts, and the body to which the legs are attached. There is no distinct head. The mouthparts are a long, cylindrical apparatus that extends from the basis capituli and includes several structures: Two palps lay on the outer left and right of the entire structure. These palps are not inserted into the skin during feeding, but lay laterally against the skin. The chelicera (mandible) extends outward from the basis and contains two cylindrical shafts on the end of which reside two sharp digits that are used for tearing the skin of the host. A flexible hypostome also extends outward from the basis and is shielded dorsally by the chelicera. The hypostome acts as the canal through which the host's blood enters the mouth during feeding. Its ventral surface is covered in tiny, sharp teeth that act to anchor it into the skin of the host [Anderson 2008., Kemp 1982., Gregson 1960a].

When a tick bites a host, it first grips the host with its legs and leverages its body at an angle to the skin, then the insertion of the mouthparts into the skin occurs in four phases [Kemp 1982., Lees, 1948]. The first is a surface grazing step in which the sharp cheliceral digits come into initial contact with the skin and the chelicerae bend to part slightly. During the second phase, the cheliceral digits begin to twitch. The third phase involves the actual shearing of the skin by the digits; the chelicerae flex back and forth, first in an alternative then a synchronized movement causing the sharp digits on the end to slice through the skin. In the final stage, the hypostome is inserted into the skin as the chelicerae flex in an outward "V" fashion several times, pushing the hypostome further

into the skin with each movement. At the end of the fourth stage, the entire hypostome and chelicerae are inserted into the host [Richter 2013].

Once the mouthparts have been inserted, saliva is immediately secreted into the bite wound by the tick's salivary glands and surrounds the mouthparts [Kemp 1982, Gregson 1960]. The saliva secreted by ticks has many functions that act as defenses against the host's bodily reactions to the bite. The saliva also contains special proteins that harden into a strong cement cone that surrounds the mouthparts. This cement cone serves as a means of prolonged attachment, allowing a feeding tick to remain imbedded into its host for the duration of the feeding period despite events of movement, scratching, biting, etc. by the host, and to protect the hypostome from extensive damage [Kemp 1982]. While this particular function is paramount to the physical attachment of the tick to the host, it is the aforementioned defensive properties of the saliva that are the focus of this study.

The saliva observed and a look at Glycine-rich proteins

As a result of exposure to the tick's saliva, the vast majority of tick bite wounds do not exhibit redness, swelling, itching, pain, infection, or clotting [Francischetti 2009]. Multiple studies have been done to investigate which components of the saliva may contribute to its apparent ability to resist or cancel out any host responses that would otherwise lead to ejection of the tick from the surface of the host. It has been determined that within the sialotranscriptome (all salivary transcripts) of several related tick species, there exists an abundance of a family of proteins named Glycine-rich Proteins (GRPs)

[Karim and Ribeiro 2015]. GRPs are characterized by semi-repetitive glycine-rich motifs and are generally composed of at least 20% glycine.

GRPs have been identified and studied in several places in nature. GRPs were first studied in plants during the late 1990's and were found to serve as structural components of the cell wall and to be involved in stress response cascades [Mangeon 2010]. GRPs have since also been identified as a component of spider silk fibroin and of the protein matrix of the shell of certain pearl oysters [Ringli 2001, Yano 2006]. Aside from these discoveries, it has recently been hypothesized that GRPs may be able, at least in part, to perform many of the functions in a ticks' saliva that are essential for their successful feeding. A wide variety of molecules involved in host platelet aggregation and blood coagulation have been found to contain glycine-gated channels and glycine-rich peptides more commonly known as gloverins and attacins have been classified as anti-microbial peptides [Giambelluca 2007, Schemmer 2013, Yi 2014].

Glycine-rich proteins in depth

From the moment a tick bites, it is subject to certain responses from the host. The first of these is a hemostatic (blood clotting) response. Before the uptake of blood by the tick, blood pools under the skin and remains liquid from the time it is pooled until the time of mouthpart detachment which can be upwards of two weeks in some species [Kemp 1982]. During this time, the tick continuously secretes saliva, some components of which must inhibit the formation of platelet aggregation and blood coagulation, otherwise the tick would not be able to feed [Martiz-Olivier 2007]. Platelets can be activated by ADP and collagen [Francischetti 2009]. Once activated, platelets begin to

clump together and release signal molecules that recruit more platelets to the forming clump and other platelet-derived factors which contribute to a buildup of thrombin, which increase the rigidity of the platelet plug [Ribiero 1985]. Another component of blood clotting is the formation of a fibrinogen network—the structural scaffolding of the blood clot. Fibrinogen is a molecule present in the blood plasma that is activated by thrombin to contribute to platelet adhesion and aggregation and to the formation of the network [Perez 2014]. Certain platelet inhibitors, such as prostacyclins and disintegrins, that contain a glycine motif have been found to inhibit the formation of the fibrinogen network and the binding of fibrinogen to platelets [Francischetti 2009].

The second host response encountered by the tick upon attachment is an inflammatory response. During and after the majority of tick bites, the host does not experience pain, any itching sensation, or inflammation or redness at the bite site. Studies have shown that no innate inflammatory response is present unless the host has developed a humoral resistance to the tick—presumably specifically to the components of the saliva [Wikel 2013]. The anti-hemostatic property of the saliva may double as an anti-inflammatory mechanism. An example of this is the molecule apyrase. Apyrase acts to inhibit hemostasis by degrading ADP used to activate platelets and may also contribute to reduced inflammation. The reduction in the size of the platelet plug itself may also contribute to less inflammation [Ribiero 1985]. Inflammation is the result of the induction of epidermal keratinocytes. These special cells are activated by UV light or a contact allergen and illicit the inflammatory response by imposing pro-inflammatory adhesion molecules and cytokines [Barker 1991]. An assay can be used to test for some of these cytokines to determine the presence of an inflammatory response from a sample.

Using this method, a study determined that dietary glycine reduced inflammatory response and joint swelling in mice independent of the model used and that the magnitude of the effect was dependent upon the concentration of glycine ingested [Hartog 2007]. This finding strongly supports the hypothesis that GRPs may play a significant role in the anti-inflammatory properties of tick saliva.

Another obstacle encountered by the tick is keeping the bite wound uninfected and uninhabited by microbial life. The large majority of bites remain uninfected for the duration of time that the tick is physically attached to the host. Anti-microbial peptides have been identified in *Ixodes scapularis* and *Dermacentor variabilis* ticks. In *D. variabilis*, a strong anti-microbial response was observed when microbes were introduced to the tick's hemocoel which resulted in a near lack of microbial presence within one hour [Johns 2001, Pichu 2009]. In addition, gloverins and attacins are families of anti-microbial peptides that are glycine rich. Gloverins have only been identified in species of Lepidoptera, but attacins have been found in some tick species and may play a role in the anti-microbial functionality of tick saliva [Yi 2014]. The expression of these peptides is stimulated by the recognition of a pathogen by the receptors of some signaling pathways in the tick [Ezzati-Tabrizi 2013]. Due to the targeting mechanisms of these peptides, bacteria are not able to evolve quickly or efficiently enough to build any noticeable resistance, therefore making antimicrobial peptides an efficient means of preventing infection, and possibly a major future subject of clinical research [Illić 2013].

Species of interest: The Lone Star Tick, Amblyomma americanum

The majority of the tick-borne diseases that are of concern to the inhabitants and cattle-raisers of the south-central and southeastern regions of the U.S. are vectored by the Lone Star tick, *Amblyomma americanum*. The most common disease-causing agents vectored by this species include several strains of *Ehrlichia*—agents of human monocytotropic ehrlichiosis and human and canine granulocytic ehrlichiosis, *Francisella tularensis*—agent of tularemia, and *Borrelia lonestari*—the bacterium thought to be responsible for southern-tick associated rash illness [Masters 2008]. This species is of particular concern because of its rapidly growing range. Historically, the distribution of this species ranged from the Gulf coast to northern parts of Missouri and Kentucky, and from the Atlantic coast to central regions of Texas. Throughout the past several decades, however, its native distribution has extended upwards into northern parts of Maine and the New England region, and westward into western regions of Texas, Nebraska, and Iowa. A vast expansion of range has resulted in an increase in the risk of exposure to tick-borne diseases for citizens and livestock in the U.S. This tick now poses as even more of a threat to public health in the last decade and has become one of the most economically important tick species in the U.S., making it an ideal subject of investigation for this study [Goddard 2009].

The Lone Star Tick is a 3-host tick that, after reaching the adult life stage, may be capable of reproducing a new generation each year [Tick App]. This species is a generalist species at all three life stages. Larvae and nymphs typically feed on ground-frequenting birds and small mammals. Adults feed on white-tail deer, cattle, horses, sheep, and dogs. All three life stages will bite humans [Goddard 2009]. Temporal gene

expression in the salivary glands of this species were recently determined and recorded. According to results published by Bullard, et al., several glycine-rich proteins displayed increased expression during different time points of feeding, as compared to normal gene expression during periods of fasting. Included in this set of highly expressed proteins was GRP 34358. This particular protein was expressed at several of the feeding time points tested, as would be expected of a protein whose function is to carry out the previously discussed defenses against host responses upon the tick's attachment to the host. GRP 34358 was highly expressed at the following time points during feeding: 24 hours, 48 hours, and 96 hours. [Bullard 2016]. Based on these findings, GRP 34358 was the protein chosen for investigation and expression in this study.

Significance of this study

The goal of this research is to express *Amblyomma americanum* Glycine-rich protein 34358 in a mammalian cell line. The purpose of doing so is to have a purified sample of this protein with which to perform further assays and experiments that may provide further insight into the specific function of this particular protein. By performing research on this topic, and on this protein specifically, we will be able to gain insight and knowledge towards determining the precise function of the multiple Glycine-rich proteins that are a component of the saliva of several species of ticks. By gaining this knowledge, future researchers may have the opportunity to manipulate and utilize these findings for the purpose of creating new methods of disease and pest prevention. Ultimately, the research performed on salivary GRPs could lead to a commercial breakthrough in the fields of public health and commercial and environmental economics.

Chapter III: Methods

Tick acquisition

Adult male and female *Amblyomma americanum* were purchased from the tick facility at Oklahoma State University. All ticks were kept in a plastic container at room temperature (25° C) and 90% relative humidity with a photoperiod of 14 hrs light / 10 hrs dark. 20-25 adult female ticks were fed on sheep and removed at 24 hour intervals during feeding (24 hr, 48 hr, 96 hr, 120 hr, 144 hr, 168 hr, and 192 hr). All procedures were carried out using IACUC approved protocols from the University of Southern Mississippi and were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institute of Health.

Tick dissection and salivary gland collection

Partially fed ticks were removed from a sheep at 24 hour intervals. These ticks along with unfed ticks were dissected under a dissecting scope. From each tick, the salivary glands were removed and cleaned in cold M199 buffer. Salivary glands from the same feeding interval were pooled together and preserved in RNAlater solution. Each sample was stored at -80° C.

RNA isolation

Frozen salivary gland samples were thawed on ice. The RNAlater solution was removed from each sample by pipetting then each sample was tittered using a plastic pestle. RNA was isolated from the tissue samples using the Illustra RNAspin MiniKit

(GE Healthcare Lifesciences) and the included manufacturer's protocol. Once isolated, the concentration of RNA in each sample was recorded using a Nanodrop spectrophotometer. Each sample was stored at -80°C .

cDNA synthesis

2 μg of RNA from each sample was added to separate 20 μL reverse transcription reactions from the iScript cDNA Synthesis Kit (Bio-Rad) using the included manufacturer's protocol. Each reaction was heated in a Bio-Rad thermocycler under the following conditions: 25°C for 5 min, then 42°C for 30 min, then 85°C for 5 min, followed by a 4°C hold. The cDNA concentration of each sample was then measured using a Nanodrop spectrophotometer and each sample was diluted to a concentration of 25 $\text{ng}/\mu\text{L}$ using nuclease free water. The samples were stored at -20°C .

PCR of GRP 34358

3 μL of cDNA was added to 1 μL of gene specific forward primer, 1 μL of gene specific reverse primer, 7.5 μL H_2O , and 12.5 μL of One-Taq polymerase Master Mix. The reaction was heated in the Bio-Rad thermocycler under the following conditions: 95°C for 5 min; 95°C for 30 sec, 62°C for 30 sec, 72°C for 45 sec (x 35); 72°C for 7 min, followed by a 4°C hold. The presence of the PCR product was confirmed by electrophoresis using a 2% agarose gel. PCR product was isolated using the QIAquick PCR Purification Kit (Qiagen) and the enclosed manufacturer's protocol.

Transformation of GRP 34358 into pcDNA 3.1/V5-His Plasmid

A TOPO cloning reaction using competent *E. coli* cells was performed using the manufacturer's protocol included with the pcDNA 3.1/V5-His TOPO TA Expression Kit (Invitrogen). 200 μ L of transformation reaction was spread onto selective LB agar plates and incubated overnight at 37° C. Ten colonies from the plates were selected to be cultured in LB medium containing 50 μ g/mL ampicillin. Plasmid DNA was isolated from the successful cultures using the QIAprep Spin Miniprep Kit (Qiagen) and the included manufacturer's protocol. The concentration of each plasmid DNA sample was measured using the Nanodrop spectrophotometer.

Restriction digestion of pcDNA 3.1/V5-His plasmids

The presence of the GRP 34358 gene insert in the pcDNA plasmids was confirmed using a restriction digestion reaction on three samples of purified plasmid DNA. Restriction enzymes specific for genes neighboring the gene insert were used. For the first round of restriction digestion, 1000 ng of plasmid DNA was added to 2 μ L 20X BSA buffer, 4 μ L Fast Digest buffer, and 2 μ L Hind III restriction enzyme. The reaction was heated in a Bio-Rad thermocycler under the following conditions: 37° C for 10 min, then 80° C for 10 min. For the second round of restriction digestion, 4 μ L NE Buffer 2 and 2 μ L Xho I restriction enzyme was added to the completed first round reaction. The second reaction was heated in the thermocycler under the following conditions: 37° C for 45 min, then 65° C for 20 min. After both reactions were completed, each sample was run under electrophoresis on a 2% agarose gel to confirm the presence and size of the

restriction cuts. Samples displaying bands on the gel at the same size as the size of the gene of interest were sent for sequencing to confirm the orientation of the gene insert.

Vero cell culture

Vero cells were cultured in T-75 flasks in 5 mL DMEM media supplemented with 10% L-glutamine and 10% Fetal Bovine Serum. Culture splitting was accomplished using a trypsin digestion method.

Transfection of Vero cells with plasmid containing GRP 34358

Adherent Vero cell cultures were grown for one day in 1 mL of supplemented growth media in sixteen wells of a 24-well plate. After one day of incubation at 37° C, the cultures were transfected with plasmids containing GRP 34358 gene inserts using Jet PEI transfection reagent. The wells of the plate were prepared as follows: Row 1 contained cultures transfected with plasmids containing GRP 34358. Added into well 1 was 1 ng plasmid DNA and 1 µL Jet PEI reagent. Added into well 2 was 2 ng plasmid DNA and 1 µL Jet PEI reagent. Added into well 3 was 2 ng plasmid DNA and 2 µL Jet PEI reagent. Added into well 4 was 2 ng plasmid DNA and 4 µL Jet PEI reagent. Row 2 contained cultures transected with a control plasmid containing no gene inserts. Added into well 5 was 1 ng control plasmid DNA and 1 µL Jet PEI reagent. Added into well 6 was 2 ng control plasmid DNA and 1 µL Jet PEI reagent. Added into well 7 was 2 ng control plasmid DNA and 2 µL Jet PEI reagent. Added into well 8 was 2 ng control plasmid DNA and 4 µL Jet PEI reagent. Row 3 contained cultures only introduced to Jet PEI reagent and no plasmid DNA. Added into well 9 was 1 µL Jet PEI reagent. Added

into wells 10 and 11 was 2 μ L Jet PEI reagent. Added into well 12 was 4 μ L Jet PEI reagent. Row 4 contained negative control cultures. Each well was observed over a period of three days and media was collected from each well containing live cells at the end of the third day.

SDS-PAGE

The media from the transfected cell cultures, and the control cultures was collected. A protease inhibitor was added to each of the samples. The concentration of protein in each sample was quantified using a Bradford Assay. 3 μ g of protein from each sample was then pipetted into a separate well on a 4-20% SDS gel. Two gels were filled and run in the SDS-PAGE apparatus. After electrophoresis, one of the two gels was removed and stained with Gel Code Blue stain, then de-stained with distilled water. The gel was then imaged.

Western Blot

The second of the SDS-PAGE gels containing the separated protein samples was transferred to a nitrocellulose membrane and kept moist in PBS buffer. The membrane was covered with PBST buffer for 30 min, then rinsed twice with PBS for 5 min. The blot was then blocked with 10 mL TBST buffer and 20 mL BSA buffer and left on a shaker for 1 hour. Next, the blot was washed twice with TBST buffer for 5 min. The blot was then covered with 1 mL of His detector antibody, 2 mL BSA buffer, and 10 mL TBST buffer. The blot was then washed 4-5 times with Pico Substrate Signal solution and imaged.

Chapter IV: Data and Results

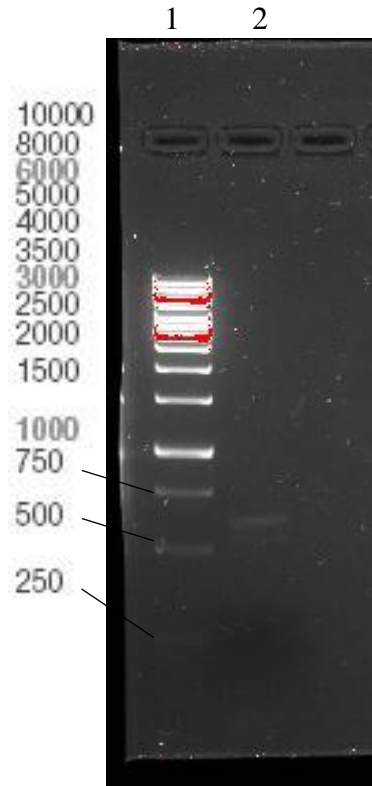


Figure 1 Imaged gel containing GRP 34358 gene PCR product replicated from 24 hr-fed *A. americanum* cDNA.

The approximate weight of the gene coding GRP 34358 is 600 bp. A solid band is visible on the gel pictured in lane 2 at an approximate size of 600 bp, as determined by comparison with the gene ladder in lane 1.

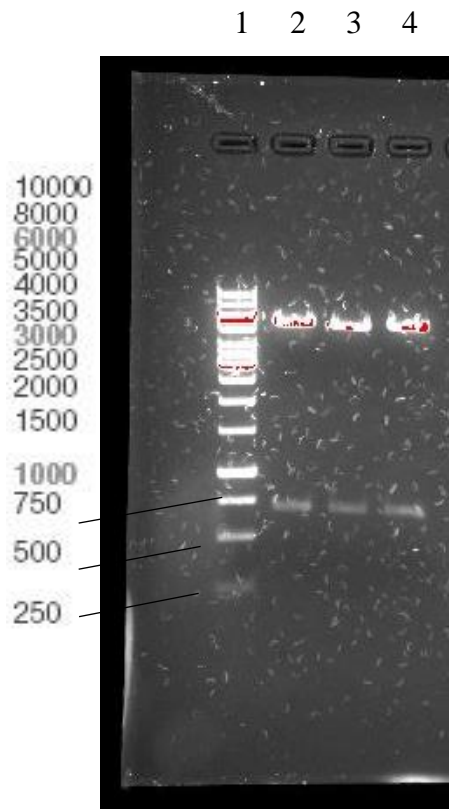


Figure 2 Image of the electrophoresis gel containing restriction digestion cuts of plasmids containing the gene inserts for GRP 34358.

Solid bands are visible in lanes 2, 3, and 4 at two different sizes—the smallest at approximately 600 bp in size and the larger at approximately 4500 bp. The smallest band represents the cut-out gene insert; the size of the cut gene insert on this gel corresponds with the weight of the gene of interest. The larger band represents the remaining piece of the pcDNA 3.1 plasmid after being cut by restriction enzymes Hind III and Xho I.

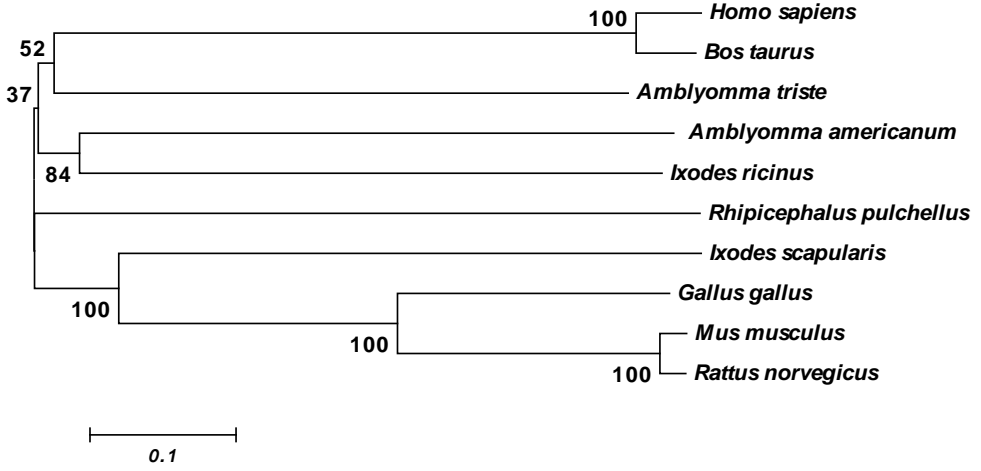
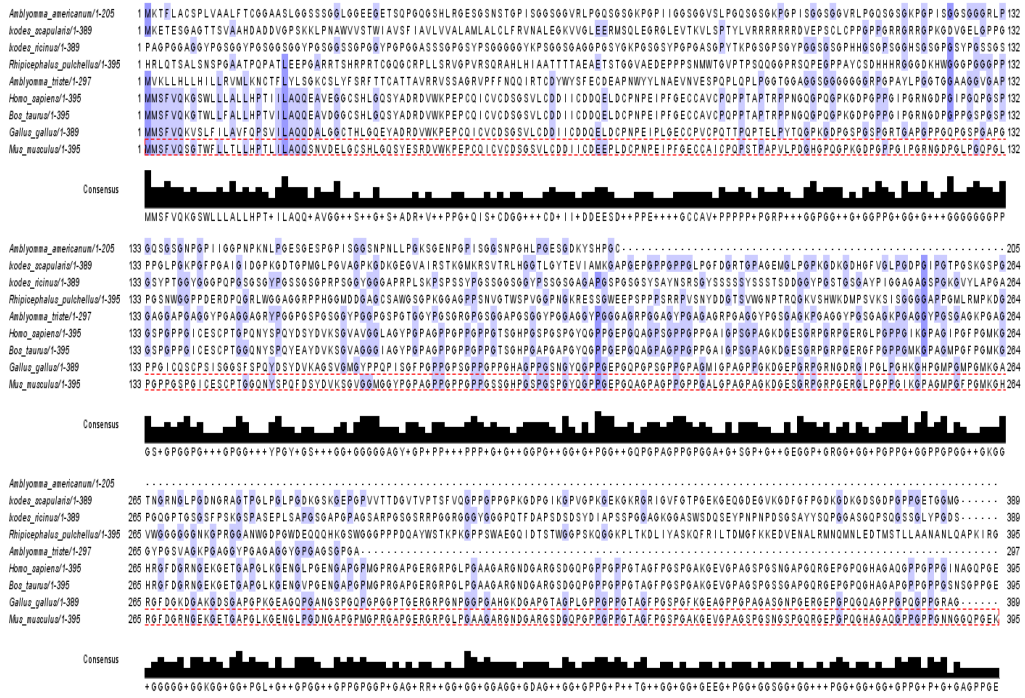


Figure 3 Multiple sequence alignment and phylogenetic tree comparing *A. americanum* 34358 with a similar protein found in several species of ticks and other insects.

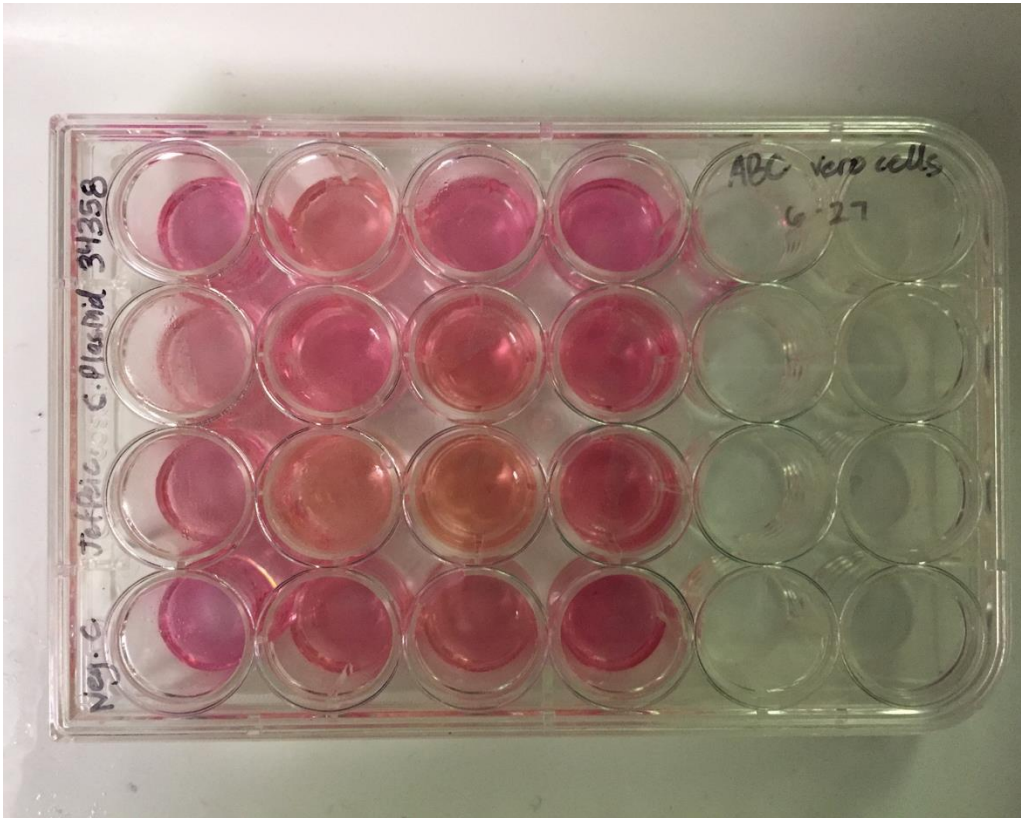


Figure 4 Transfection of pcDNA 3.1 plasmid containing gene insert for GRP 34358 into Vero cells using Jet PEI transfection reagent.

Row 1: Transfection with plasmid containing 34358. Left to right: 1 ng DNA to 1 uL Jet PEI, 1 ng DNA to 2 uL Jet PEI, 2 ng DNA to 2 uL Jet PEI, 2 ng DNA to 4 uL Jet PEI.

Row 2: Transfection with pcDNA 3.1 control plasmid. Ratios same as Row 1.

Row 3: Un-transfected Vero cells plus Jet PEI reagent. Left to right: 1 uL Jet PEI, 2 uL Jet PEI, 2 uL Jet PEI, 4 uL Jet PEI.

Row 4: Negative control Vero cells.

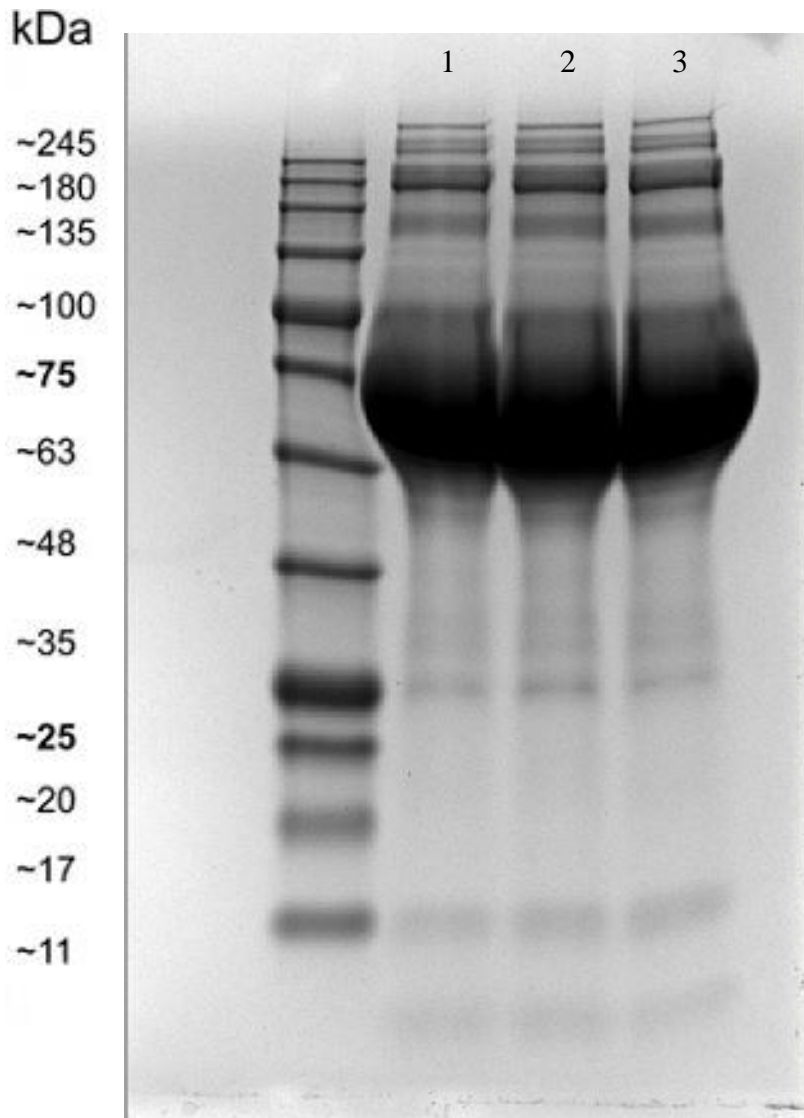


Figure 5 SDS-PAGE gel containing samples of transfected Vero cell culture media.

Lane 1 contains the protein ladder. Lane 2 contains media collected from vero cells transfected with the plasmid containing the gene insert for GRP 34358. Lane 3 contains media collected from Vero cells transfected with a control plasmid. Lane 4 contains media collected from negative control Vero cells. Banding patterns are identical in lanes 2, 3, and 4.

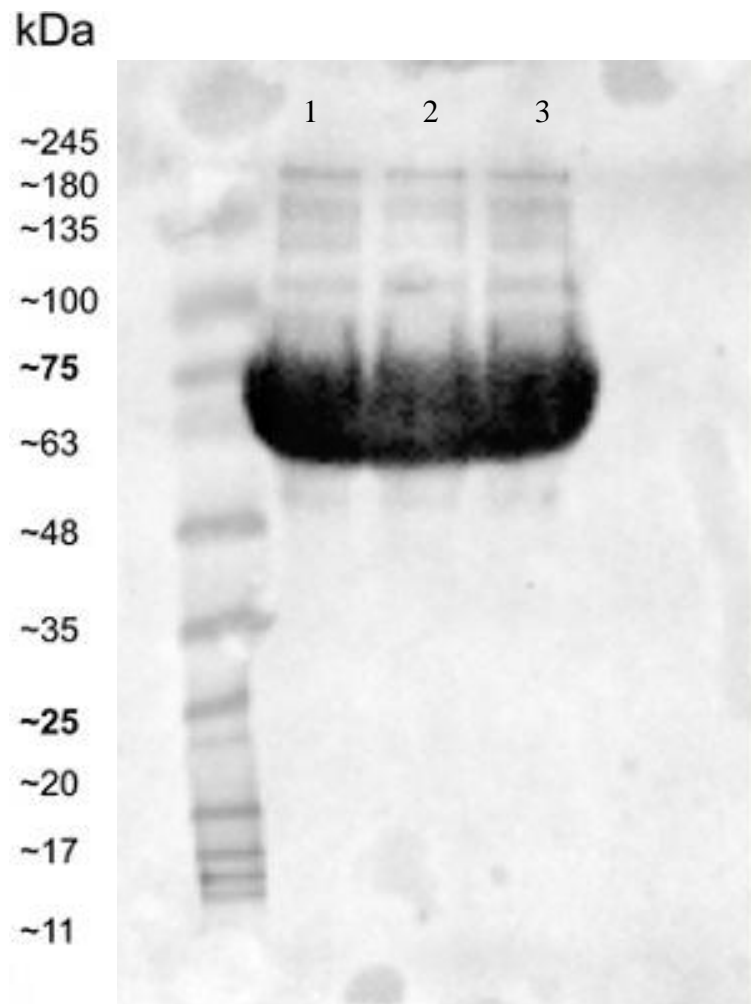


Figure 6 Western blot blocked with His-detector antibodies. Lane 1 contains the protein ladder. Lane 2 contains media collected from vero cells transfected with the plasmid containing the gene insert for GRP 34358. Lane 3 contains media collected from Vero cells transfected with a control plasmid. Lane 4 contains media collected from negative control Vero cells. Banding patterns are identical in lanes 2, 3, and 4.

Chapter V: Discussion

Tick samples being collected at various time points allowed for temporal gene expression in the salivary glands to be determined. In theory, a protein whose primary function is to defend against the innate defensive responses of the host at the bite wound, such as preventing blood clotting, inflammation, and infection, would be expressed in all or nearly all of the time points during the feeding period. Since the host's defenses are a continuous obstacle throughout the course of feeding, the tick's defense against them—assumed to be the glycine-rich proteins within the saliva—would also need to be expressed throughout the entire time frame. Expression of the GRP that was chosen for investigation, GRP 34358, fell in line with this assumption. It was highly expressed during six of the seven feeding time points.

Amplification of GRP 34358 by PCR allowed for a larger working sample and for purification of the gene of interest from the sample of salivary gland cDNA. Purifying the gene of interest from the other DNA allowed for increased confidence that the specific gene of interest would be transformed into the pcDNA plasmid, rather than any other genes within the sialotranscriptome. Once purified from the remaining DNA, the sample containing the PCR product was loaded into a gel and run through electrophoresis to determine its size. As shown in Figure 1, the PCR product migrated to a position on the gel which indicated its size to be approximately 600 bp. This is the size that was expected, as it is also the size of the gene for GRP 34358, which had been determined by outside studies.

The restriction digestion of the pcDNA plasmid containing the GRP 34358 gene insert utilized restriction enzymes for two neighboring genes within the plasmid, Hind III and Xho I. The purpose of this method was to make restriction cuts as close as possible to each end of the gene insert. Once digested, the samples containing the restriction cuts were run through electrophoresis to be separated by size. As shown in Figure 2, two bands are visible in each of the sample lanes. The heavier (top) band contains the larger portion of the plasmid that was cut—this is the portion that does not contain the gene insert. The smaller (bottom) band contains the gene insert. This band is located on the gel at a slightly larger size than the original PCR product. This is due to the fact that the neighboring genes that were cut by the restriction enzymes were not immediately to the left or to the right of the gene insert. The entire smaller band includes the previously inserted PCR product plus several additional genes that were located close enough to the insert to be included in the final restriction cut. Nonetheless, the size of the smaller band was similar to the previously determined size of the gene of interest to provide enough reason to assume that the correct gene had been inserted into the pcDNA plasmid.

Once the results of the restriction digestion were analyzed, the plasmid containing the ~600 bp gene insert was sent for sequencing, and this sequence was compared to that of a similar protein found in several other species of ticks and other insect. The results of this sequencing are shown in Figure 3. These results confirmed that the gene inserted into the pcDNA plasmid was in fact the gene coding for GRP 34358. The sequencing results also confirmed that the gene of interest had been inserted into the plasmid in the correct orientation.

Once the sequence and orientation of the gene insert were confirmed, several Vero cultures were conditioned for transfection. As shown in Figure 4, the plasmid was transfected into the cells in various quantities and in the presence of various quantities of transfection reagent. The purpose of this method was to confirm the ideal ratio of DNA to transfection reagent so that a successful transfection may be performed in future experiments. The ideal ratio in this experiment was determined to be 1 ng plasmid DNA to 2 μ L Jet PEI reagent. The other ratios used resulted in cell death which was determined by the absence of any color change in the culture media from pink to orange.

The purpose of the transfection into a mammalian cell line was to introduce the gene of interest into a line of cells that possessed the cellular machinery to first transcribe the gene of interest into mRNA which could then be translated into a functional protein. Once expressed by the mammalian cells, the protein was expected to be secreted into the growth media.

After transfection, the culture media from each well was collected and a small volume of each sample was used in a Bradford assay to determine the presence and concentration of protein in each sample. The samples (one each from Vero cells transfected with the plasmid containing the gene of interest, Vero cells transfected with a control plasmid, and a negative control) were the run through SDS-PAGE. If the transfection were successful, an additional protein, in this case the secreted GRP 34358, would be present in the sample of growth media collected from the Vero cells transfected with the plasmid containing the gene of interest, resulting in an extra band in the lane containing that sample (lane 1). As shown in Figure 5, the banding pattern in all three

lanes was identical. These results indicate that the transfection was unsuccessful and that GRP 34358 was never expressed and/or secreted by the Vero cells.

The results of the Western blot, shown in Figure 6, confirm these findings. The plasmid into which the gene of interest was inserted contained a sequence coding for a polyhistidine tag. Therefore, the His-detector antibody used in the Western blot would have bound to any proteins expressed by the mammalian cells whose genes were contained within the plasmid, again leaving an extra band within the lane on the gel containing the sample of media collected from the Vero cells that were transfected with the plasmid containing the gene insert. As shown in the figure, the banding pattern for all three lanes containing media samples is identical. The gel does display large portions to which the protein dye bound—this is assumed to be due to the albumin within the Fetal Bovine Serum that was added to the growth media. Were this experiment to be repeated, the cell cultures should be grown in a serum free media before the growth media is collected and analyzed for protein.

The results of this study may be used in the future as a reference for the transfection of GRP 34358 into a mammalian cell line. Although the transfection in this study was unsuccessful, the procedures used could be helpful in determining parameters for a successful transfection. Were GRP 34358 to be successfully expressed in a mammalian cell line, the protein could be purified using a nickel affinity column, due to its attached His-tag. The protein could then be used in several biological assays to determine its effects, if any, on mammalian hemostasis, cellular inflammation, and microbial growth. The results of those assays could lead to a better understanding of the proteins that make up the saliva of ticks, and if GRPs in general contribute to any of the

saliva's properties that aid the tick in defending against its host's defensive responses at the bite site.

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