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14. ABSTRACT
Mosquito-borne alphaviruses such as Venezuelan equine encephalitis virus (VEEV) are of great concern due to their potential to cause severe acute human disease and the absence of effective treatment options. Cationic antimicrobial peptides (CAMPs) of innate immunity are a promising resource for the development of new antiviral therapeutics. We aim to develop a bioprospecting-inspired process for the discovery of antiviral CAMPs, using VEEV as a model arbovirus. Our process combines novel antiviral peptide harvesting microparticles, advanced mass spectrometry, and data analysis tools to identify potential antiviral peptides. Our studies focus on CAMPs from alligators and snakes, which have been suggested as potential reservoir species. We have produced particles for the identification of peptides that target VEEV-infected leukocytes. Development of particles to identify peptides that target VEEV is nearly complete. Development of conditions for stimulating reptile blood are nearly complete and development of our statistical analysis approach has progressed. In the coming quarter, antiviral CAMP discovery efforts will increase.

15. SUBJECT TERMS
Reptile, Venezuelan equine encephalitis virus, innate immunity, cytokine, chemokines, host defense peptides, bioprospecting, antiviral, and antiviral peptide discovery.

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TABLE OF CONTENTS

1. INTRODUCTION:

The rapid, worldwide spread of arthropod-borne viruses (arboviruses) that were previously limited to remote tropical and sub-tropical regions, including mosquito-borne alphaviruses (e.g. chikungunya virus and Venezuelan equine encephalitis virus-VEEV), is of great concern due to their potential to cause severe acute human disease. The absence of FDA approved vaccines or therapeutic agents for almost all arboviruses makes development of effective treatment options for this class of pathogens a significant national security and public health issue. Here we focus on the study of VEEV as a model arbovirus. Cationic antimicrobial peptides (CAMPs) of innate immunity represent a promising resource for the development of new antiviral therapeutics. There is a need to develop a system for the specific purpose of discovering peptides with antiviral activity. We aim to develop a bioprospecting-inspired process for the identification of antiviral peptides against VEEV, using novel antiviral peptide harvesting microparticles. These particles will incorporate elements from healthy host cells, infected host cells and virions in order to enable the identification of peptides that preferentially target infected cells and virus over healthy host cells. We will focus on alligators and snakes CAMPs, since these species have been suggested as potential arbovirus reservoir species. Our process employs an integrated workflow that begins with the preferential enrichment of CAMPs that target viral elements from reptile serum or plasma, followed by advanced mass spectrometry and data analysis in order to establish their sequences and identify those peptides with potential antiviral properties.

2. KEYWORDS:

Reptile, Venezuelan equine encephalitis virus, innate immunity, cytokine, chemokines, host defense peptides, bioprospecting, antiviral, and antiviral peptide discovery.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Develop microparticles for capturing antiviral peptides.

Major Task 1: Develop VLP-based capture particles (12 months: 09/30/18-09/29/19)

Milestones:

1.) Reproducible protocol for synthesizing and purifying PP2MA or similar click-chemistry-compatible monomer. (delivery date: 03/29/19 – completed)

2.) Preparations of VEEV virus like particles. (delivery date: 03/29/19 – completed)

3.) Protocol for preparing VLP-modified hydrogel particles using click chemistry and preliminary harvesting data for the particles. (delivery date: 09/29/19 – 80% complete)

4.) GMU IACUC Approval (delivery date: 03/29/19 – completed 11/20/18)

5.) ACURO Approval (delivery date: 05/29/19 – completed 03/20/19)

Major Task 2: Develop cell-membrane based capture particles (9 months: 09/30/18-06/29/19)
Milestones:

1.) Protocols for preparing and assessing hydrogel particles encapsulated in membranes from healthy host cells, with preliminary harvesting data. (delivery date: 06/29/19 – completed)

2.) Protocols for preparing and assessing hydrogel particles encapsulated in membranes from infected host cells, with preliminary harvest data. (delivery date: 06/29/19 – completed)

Specific Aim 2: Capture, analysis and identification of peptides from reptile plasma.

Major Task 3: Perform plasma harvests (12 months: 03/30/19-03/29/20)
Milestones:

1.) VEEV challenge conditions for stimulating release/production of peptides by reptile blood cells. (delivery date: 09/29/19 – 80% complete)

2.) Harvest protocol and conditions that afford efficient capture and identification of known model antiviral CAMPs from plasma. (delivery date: 09/29/19 – completed)

3.) Harvests performed from stimulated and unstimulated plasma using panel of particles developed in Aim 1 for the purpose of identifying antiviral peptides. (delivery date: 03/29/20 – 15% complete)

Major Task 4: Analyze harvested peptides (12 months: 03/30/19-03/29/20)
Milestones:

1.) Workflow for efficiently analyzing harvest samples via tandem mass spectrometry and determination of the peptide sequences. (delivery date: 09/29/19 – completed)

2.) Mass spectrometry data and sequences for peptides harvested for the purpose of identifying novel antiviral peptides. (delivery date: 03/29/20 – 30% complete)

3.) Statistical methods for analyzing harvested peptide sequences to afford statistical significance values of potential antiviral peptides. (delivery date: 03/29/20 – 50% complete)

4.) Predictive model(s) based on machine learning for analyzing harvested peptides to aid in identification of peptides likely to have antiviral properties. (delivery date: 03/29/20 – not started)

Major Task 5: Assess performance of likely antiviral peptides (6 months: 09/30/19-03/29/20)
Milestones:

1.) One or more novel alligator or snake peptides that exhibit antiviral properties against VEEV. (delivery date: 03/29/20 – 10%)

What was accomplished under these goals?

Specific Aim 1: Develop microparticles for capturing antiviral peptides.
Major Task 1: Develop VLP-based capture particles: (12 months: 09/30/18-09/29/19)

Technical challenges associated with quantifying propargyl monomer incorporation in particles and in preparing suitable amounts of VLP for incorporation in virus modified-particles have delayed development of virus-particle coupling conditions and achieving Major Task 1 deliverables by 09/29/2019 as originally projected in the SOW. Both of these problems have been overcome, and we are presently assessing conditions for efficiently and consistently coupling virus to the hydrogel microparticles via click chemistry. As can been seeing below, our initial studies have offered promising results, and we are presently optimizing conditions. Based on these results, we anticipate completing Major Task 1 objectives and initiating harvests studies with virus-modified particles by mid-November, 2019. We do not anticipate this delay that will impact our ability to achieve the final goals of the project as projected in the SOW.

Preparation of propargyl-PEG monomer

Incorporation of propargyl-PEG monomers in particle formulations allows chemoselective attachment of VEEV or VEEV VLP's to the particles under mild conditions via Click chemistry. Towards this end, we successfully synthesized and purified propargyl-PEG-methacrylamide and propargyl-PEG-methacrylate derivatives. We decided to focus our efforts on the methacrylate derivative propargyl-polyethylene-glycol-(4)-methacrylate (PP4ME), because the PEG-4 linker places the propargyl group further from the polymer backbone than would shorter linkers. Initially we explored the use of flash chromatography to purify the completed monomer, but we found that using Dowex anion-exchange resin provided a more efficient and scalable purification strategy. As seen in the NMR spectrum below, this approach affords very pure PP4ME (Figure 1). Accordingly, we developed a robust process for the synthesis and purification of PP4ME in amounts suitable to support the preparation of particle incorporating the monomer. We have used this process to prepare multiple batches of the PP4ME monomer.

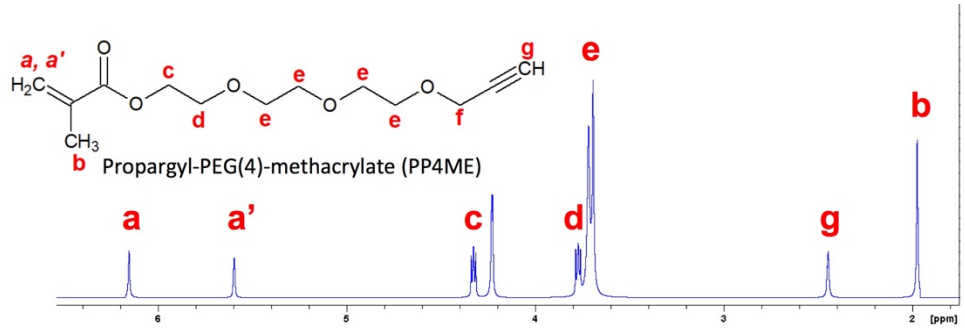

Figure 1. Assigned NMR spectra for PP4ME monomer establishing that the reaction was successful and purification scheme yielded very pure PP4ME.

Preparation of particles containing propargyl-PEG monomer (PP4ME)

We have synthesized multiple batches of PP4ME monomer for coupling to VEEV or VEEV-VLP's via click chemistry. Core-shell microparticles incorporating PP4ME were generated using RAFT polymerization, which consisted of an inert cross-linked tert-butylmethacrylamide core and shells based on cross-linked copolymers of *N*-methacryloyl-6-aminohexanoic acid (MA6AHA) and PP4ME. We generated a series of particles that incorporated 10% PP4ME and 20, 10, 5 and 0%

cross-linking with MA6AHA providing being the main component in their shells (N4.3, 4.4, 4.5, and 4.6 respectively) in order to identify shell architectures/formulations that are suitable for modification with virus and VLP's. Analysis of the particles via NMR showed incorporation of the monomers with levels of PP4ME in the particles relative to MA6AHA being somewhat lower than they were in the feed stock (See Table 1). The sizes of the N4.3 (20% cross-linking : 10% PP4ME) and N4.4 (10% cross-linking : 10% PP4ME) particles, determined via dynamic light scattering (DLS) were consistent with the sizes we have seen with other particles with MA6AHA-based shells (Table 2). These particles are currently being evaluated for their ability to bind virus and the viral loads that can be achieved.

		Particle Preparations (Mol %)			
	Monomer	**N4.3**	**N4.4**	**N4.5**	**N4.6**
Theoretical Formulation	PP4ME	10.0%	9.0%	10.0%	10.0%
	MA6AHA	70.0%	81.0%	85.0%	90.0%
	BIS	20.0%	10.0%	5.0%	0.0%
	PP4ME/MA6AHA Ratio	0.143	0.111	0.118	0.111
NMR Analyses of MA6AHA and PP4ME Content	MA6AHA (2.311 ppm, 2H)*	2.00	2.00	2.00	2.00
	PP4ME (3.679 ppm, 10H)	0.732	0.663	0.884	0.343
	PP4ME/MA6AHA Ratio	0.0732	0.0663	0.0884	0.0343

Table 1. Comparison of theoretical PP4ME content, in terms of mole fraction of PP4ME vs MA6AHA, based on reaction feed stock formulation and that determined by NMR. The NMR data suggests that the mole% PP4ME in the particles is higher in all formulations than that predicted based on feed stock stoichiometry.
* MA6AHA integral set to 2.00.

	Short Name		**N4.3**	**N4.4**	**N4.5**	**N4.6**	**N4 (Core)**
DLS	Z-Average	(d.nm)	294.3	302.2	TBD	TBD	215
	PdI		0.052	0.129	TBD	TBD	0.044
	Peak 1	(d.nm)	310.5	319.9	TBD	TBD	227.7

Table 2. Summary of DLS information indicating approximate size of particles. The 5% and 0% BIS crosslinking have difficulty being sized via DLS and are to be determined (TBD).

Preparations of VEEV virus like particles

We have developed semi-quantitative western blot methodology to quantitate our VLP preparations. For this we serially diluted VEEV TC83 (pfu/ml) and performed western blot analysis followed by densitometry analysis (Figure 2). Our standard curve gave us an R^2 value of 0.9852. This method will allow us to compare the amount of glycoprotein (GP) present in our VLPs to that found in our viral stocks; thereby giving us a semi-quantitative determination of the amount of particles present in our VLP preparations.

We have successfully produced VEEV VLPs by transfecting 293T cells with the VEEV structural polyprotein plasmid (Figure 3). Viral E2 and E1 proteins (the major constituent of the VLPs) are detected in cellular lysates as well as in supernatants, indicating that VLPs are being released from these cells. Experiments determined that production of VLPs in the presence of fetal bovine serum (FBS) interfered with detection of our VLPs. VLPs were found to enter cells as determined through fluorescent microscopy (data not shown). We attempted to quantitate our VLPs through flow cytometry, but this approach was not successful likely due to the non-specific binding of antibodies to the negative control nanoparticles. As an alternative, VLPs will be quantitated using semi-quantitative western blot analysis via densitometry measurements as described above.

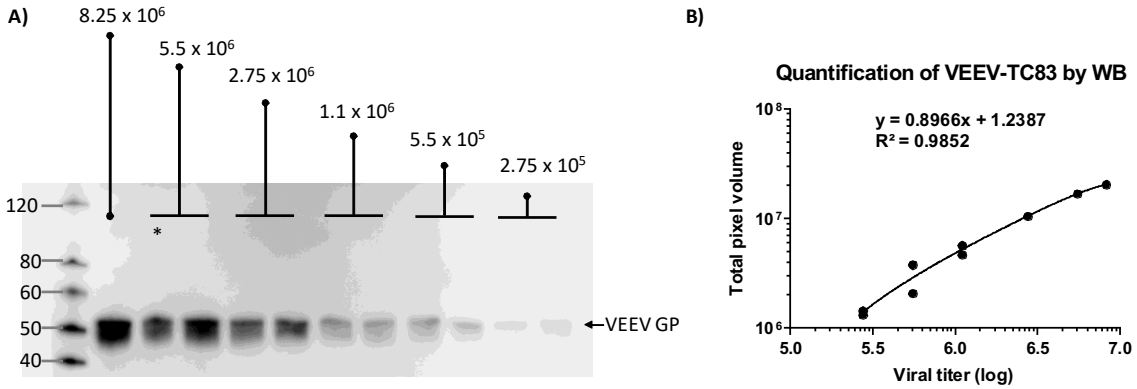

Figure 2: Semi-quantitative western blot analysis of GP concentrations. A) Western blot (WB) of serially diluted VEEV TC83 (pfu/ml). WB was performed with anti-VEEV glycoprotein antibody. B) Densitometry analysis was performed of the WB image and total pixel volume plotted vs. the viral titer (log pfu/ml).

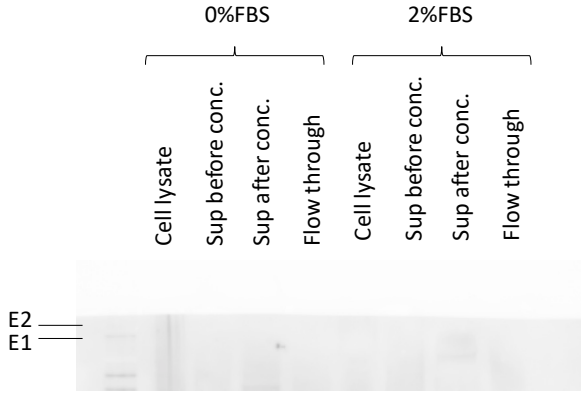

Figure 3: 293T cells were transfected with VEEV structural polyprotein in the presence of 0% or 2% FBS. Cell lysates and cell free supernatants (containing VLPs) were harvested at 48 hours post-transfection. Supernatants were concentrated using an Ambicon concentrator. Samples were separated via SDS-PAGE followed by western blot analysis using antibodies specific for VEEV E1 and E2 glycoproteins.

<u>Protocol for preparing VLP-modified hydrogel particles using click chemistry and preliminary harvesting data for the particles.</u>

There are some limitations of producing VLPs through transfection including the large expense of the transfection reagents and the difficulty in scaling-up production to enable large scale coupling of VLPs to the microparticles. To address these issues, we are working on large-scale production of VLPs through use of a pseudoinfectious virus (PIV) which has a mutation in the capsid gene that significantly impacts the ability of capsid to bind to viral RNA, resulting in the release of VLPs (PMID: 23221545). We have successfully cloned VEEV PIV and are in the process of producing infectious PIV. While we are awaiting the production of the PIV, we are using VEEV TC-83 as a model to work on coupling VLP-based capture particles.

As a first step, we have optimized a sucrose-cushion based purification method for virus purification. The method uses 20-50% sucrose-cushion where following ultracentrifugation, VEEV TC-83 can be found at the interface between these sucrose layers. Analysis of fractions from the sucrose cushion indicated that VEEV did accumulate in the lower fractions, F11 and F12 as expected (Figure 4). This is indicated by VEEV glycoprotein detection (panel A) and infectious virus detected by plaque assays (panel B).

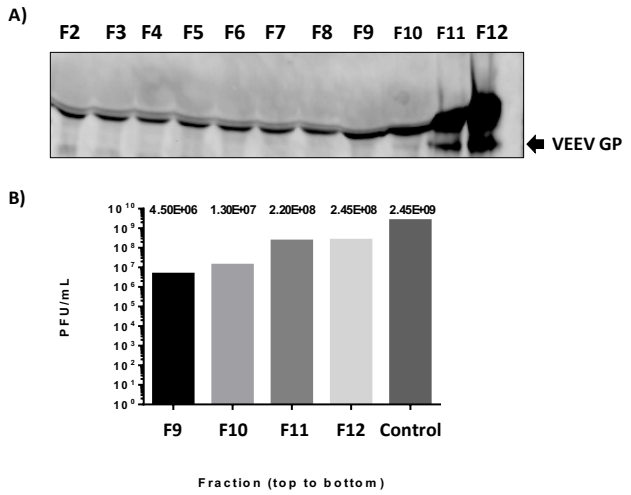

Figure 4: Sucrose-cushion purification of VEEV. 293T cells were infected with VEEV and supernatants collected at 48 hours post-infection. Supernatants were layered on top of a 20-50% sucrose cushion and centrifuged at 36,000 RPM for 1.5 hours at 4^0C. One mL fractions were collected from the top and an aliquot separated via SDS-PAGE followed by western blot analysis using antibodies specific for VEEV glycoproteins (panel A). Upper non-specific band is FBS. Fractions F9-F12 were also subjected to plaque assays to confirm that the lower fractions had infectious virus and not just glycoproteins (panel B).

An initial pilot experiment was performed to determine our ability to produce VLP-particles using sucrose-cushion purified VEEV TC-83. Following the click-chemistry reaction, samples were analyzed by RT-qPCR and western blot analysis. Initial PEG modified VEEV (VLP-PEG Azide) had high levels of both viral RNA and VEEV GP present (Figure 5). However, little viral RNA and no VEEV GP was detected in the VLP-particles (ssCTA-1 and N4.3-2) and any of the particle washes, indicating that the click-chemistry reaction was not optimal.

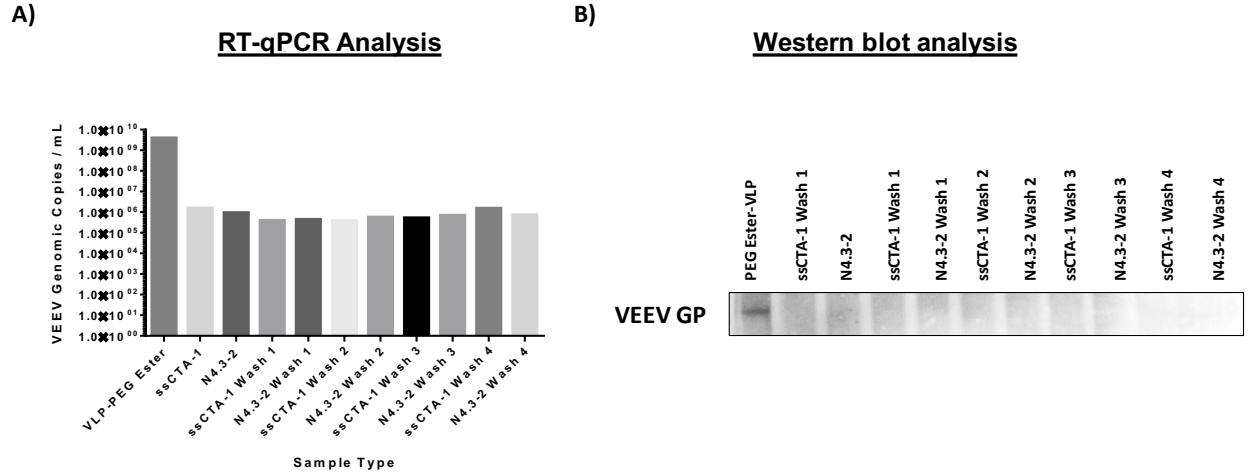

Figure 5: Pilot Study #1 on VLP-particle production. Sucrose-cushion purified VEEV TC-83 had PEG added and click-chemistry performed to link the PEG Azide modified virus to the particles. An aliquot of particles and washes were analyzed by RT-qPCR (panel A). An aliquot of particles and washes were also separated via SDS-PAGE followed by western blot analysis using antibodies specific for VEEV glycoproteins (panel B). In figure labels – "VLP-PEG Ester" and "PEG-Ester-VLP" refer to PEG Azide modified VEEV

A second pilot experiment was performed where a number of conditions were altered to help increase the coupling efficiency. All reactions were performed at room temperature (as opposed to 4^0C in the original experiment), the amount of PEG Azide used in modifying the virus was increase by 10-fold, and the particle concentration used in the subsequent click coupling reaction was increased from 1 mg of particles per reaction volume to 5 mg of particles per reaction volume. In addition, the sucrose was removed by dialysis in PBS prior to the addition of PEG Azide to the virus. There was detectable viral RNA and VEEV GP in the PEG-modified VEEV (Figure 6). Two different particles were used in this study (N4.4-3 and N4.3) which contain different amounts of cross-linking. Particle N4.4-3 had the greatest amount of viral RNA present with some VEEV GP detectable (albeit more of a smear on the gel). The results are encouraging as they suggest that some virus is coupled to the particles; however additional optimizations are required.

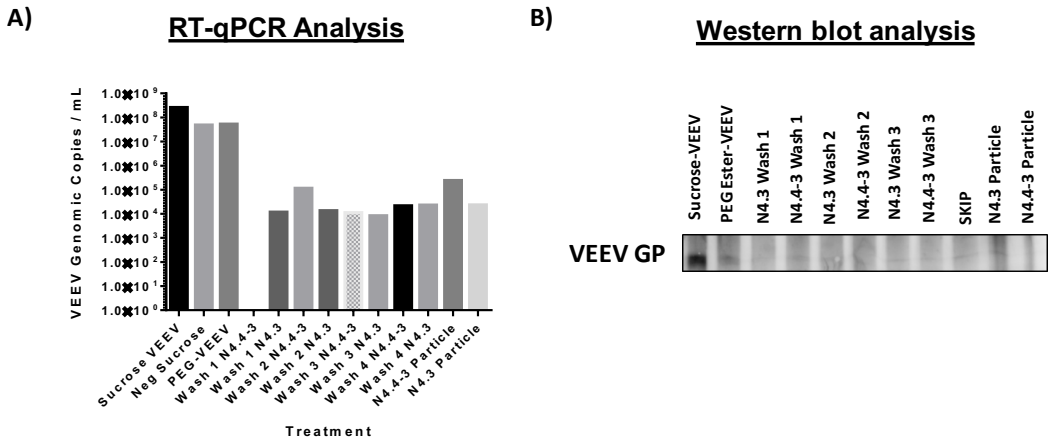

Figure 6: Pilot Study #2 on VLP-particle production. Sucrose-cushion purified VEEV TC-83 had PEG added and click-chemistry performed to link the PEG Azide modified virus to the particles. An aliquot of particles and washes were analyzed by RT-qPCR (panel A). Neg sucrose = sample after dialysis to remove the sucrose. An aliquot of particles and washes were also separated via SDS-PAGE followed by western blot analysis using antibodies specific for VEEV glycoproteins (panel B). In figure labels – "PEG-VEEV" and "PEG Ester-VEEV" both refer to PEG Azide modified VEEV

Major Task 2: Develop cell-membrane based capture particles: (9 months: 09/30/18-06/29/19)
1.) Protocols for preparing and assessing hydrogel particles encapsulated in membranes from healthy host cells, with preliminary harvesting data.

2.) Protocols for preparing and assessing hydrogel particles encapsulated in membranes from infected host cells, with preliminary harvesting data.

Particles encapsulated in healthy and infected cell membranes were prepared and assessed simultaneously.

A.) In the first quarter, we synthesized two series of core-shell hydrogel particles with functionalized cross-linked polymer shells. These particles explored reaction conditions used to generate the particles (heating via oil bath and hot plate vs water and a circulating immersion heater) as well as shell formulation (MA6AHA vs. N-isopropylmethacrylamide (NIPMAM) and methacrylic acid (MAAc) copolymers). Replicate batches of particles were produced in order to establish reproducibility of particle syntheses and resulting particle physical properties. Based on these studies, it was determined that particles with harvesting outer shells based on cross-linked poly-MA6AHA afforded the most consistent performance. Therefore, we elected to focus on these particles for use in first round general harvests.

B.) We tested 4 different plasma membrane preparation protocols to find the one that was optimal for plasma membrane isolation from U937 cells. CD45 was used as a marker of successful plasma membrane isolation (data not shown). Based on CD45 expression in our preparations, we selected a method whereby U937 cells are incubated with a hypotonic buffer (50mM Tris-HCl, 5mM EDTA, 100mM NaCl, 2mM MgCl, 3mM CaCl2, 5mM KCl, and protease inhibitor cocktail) for 10 minutes on ice. Cells are then disrupted with a dounce glass homogenizer and centrifuged at 4ºC at 400g for 10 minutes. The pellet is discarded and supernatant containing plasma membranes is saved for analysis.

To determine the purity of our preparations we performed western blot analysis with other cellular organelle markers (Figure 7A). Cadherin and CD45 (both plasma membrane markers) were found in our preparations, with detection enhanced by not subjecting the reduced protein to boiling (lanes 1-4). Boiling can result in protein aggregation and sticking to tubes, thus resulting in loss of protein. KPNB1 (nuclear protein) and Calnexin (ER protein) were not detected in our plasma membrane preparations (lanes 4-8). However, we were able to detect Calnexin and KPNB1 in U937 whole cell extracts via western blot (Figure 7B). These results demonstrate our ability to isolate plasma membranes from U937 cells.

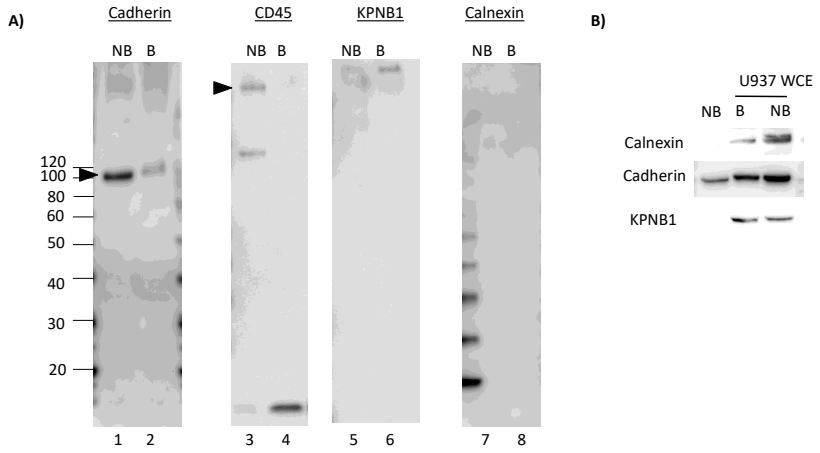

Figure 7: Plasma membrane preparation purity analysis. A) Plasma membranes were isolated from U937 cells and purity assessed by western blot analysis using anti-Cadherin, anti-CD45, anti-KPNB1 and anti-Calnexin antibodies. NB: no boiling; B: boiling at 95ºC B) Western blotting was performed with plasma membrane preps (lane 1) and U937 whole cell extracts (WCE). NB: no boiling; B: boiling at 95ºC

C.) Hydrophobically modified NIPMAm/MAAc particles (particles with cross-linked NIPMAm/MAAc copolymer outer shells) were prepared and encapsulated within leukocyte membranes (lipobeads). In generating these lipobeads, leukocyte cytoplasmic membranes were passed through an extruder to generate liposomes 100 nm in diameter. The liposomes were then incubated overnight with the hydrophobically modified particles at 4°C. The lipobeads that were formed were then pelleted by centrifugation and the supernatant collected in order to remove any free liposomes remaining in solution. The particles were suspended in buffer and the process repeated so as to remove any remaining unbound liposomes. This process was used to generate lipobeads encapsulated in membranes from healthy leukocytes (Lipobead-Healthy Leuk.), healthy cells that had been treated with trypsin (Lipobead-Tryp-Healthy Leuk.), VEEV-infected leukocytes (Lipobead-Infected Leuk.) and infected cells that had been treated with trypsin (Lipobead-Tryp-Infected Leuk.). Light scattering data (sizes) for particles are provided below in Table 3. Results from initial harvests from commercial Alligator plasma are provided below in Aim 2 data below.

	Core	Core-Shell (20% NIPMAm/MAAc)	Lipobead - Healthy Leuk.	Lipobead - Tryp - Healthy Leuk.	Lipobead - Infected Leuk.	Lipobead - Tryp - Infected Leuk.
Avg. Diameter (nm)	228.3	352.2	411.3	422	509.1	440
PDI	0.081	0.05	0.19	0.203	0.26	0.299

Table 3. Comparison of Particle Sizes: The diameters (nm) of the core particles, core-shell particles and varied lipobeads as determined through light scattering are provided.

Specific Aim 2: Capture, analysis and identification of peptides from reptile plasma.

Major Task 3: Perform plasma harvests

VEEV challenge conditions for stimulating release/production of peptides by reptile blood cells.

Efforts to optimize conditions for stimulating reptile blood cells with VEEV have taken longer than originally projected due to complications in scheduling collection of blood samples due to Hurricane Dorian, which had been projected in different forecasts to hit Florida where our collaborators at the St. Augustine Alligator Farm Zoological Park are located. Their need to prepare for the potential storm to protect the animals disrupted our schedule for getting blood in September, which in turn delayed our efforts to optimize stimulation conditions beyond our original projected 09/29/12019 deliver date. That being said, we received a fresh shipment of alligator blood on 10/23/2019, PBMCs have been collected and stimulation studies are underway. We anticipate establishing conditions and a protocol for stimulating reptile blood with VEEV by the end of October, completing this Task 3 deliverable.

Pilot experiments were performed to determine the optimal conditions for stimulating antiviral peptides in reptile blood. First, primers were designed to enable RT-qPCR analysis of innate immune response genes of the American Alligator. OASL, CXCL10, Mx1, and ISG20 were selected based on a published study indicating these genes were altered in Crocodile cells in culture following poly I:C stimulation (PMID: 29213275). American Alligator blood was incubated without or with VEEV TC83 at 1:10, 1:100, 1:1000 dilutions. Samples were collected at 2, 8, and 24 hours post stimulation. RNA was extracted and RT-qPCR analysis performed. Issues with RNA sample quantity were encountered and therefore data regarding the induction of gene expression was inconclusive. However, our data did indicate that our primers were able to successfully detect OASL, ISG20, and Mx1 gene expression with no primer dimers detected (data now shown). Repeated RT-qPCR analysis showed that these genes were not being stimulated by VEEV when exposed in this manner.

Stimulation experiments were repeated, however this time we opted to stimulate peripheral blood mononuclear cells (PBMCs) isolated from American Alligator blood. PBMCs were isolate using a Ficoll gradient and cells stimulated with VEEV TC83 (MOI ~100). Cells were collected at 2, 8 and 24 hours post-VEEV addition and RNA isolated. Mock-infected cells were collected in parallel. Interestingly, we observe a strong induction of Mx1 and OASL at 8 and 24 hours post-VEEV exposure (Figure 8B and C) and CXCL10 only at 24 hours (Figure 8D). No change in ISG20 expression was observed (Figure 8A). These data indicate that innate immune response genes are stimulated in PBMCs exposed to VEEV. For harvesting experiments, we are opting to utilize the 2- and 8-hour samples. The 2-hour samples may contain a population of antiviral peptides that are stimulated independent of transcriptional regulation, while the 8-hour samples should include peptides stimulated through transcriptional changes as the innate immune response genes we examined. We believe the 24-hour samples are too late in the time course, with cell death likely being induced which would confound our analysis.

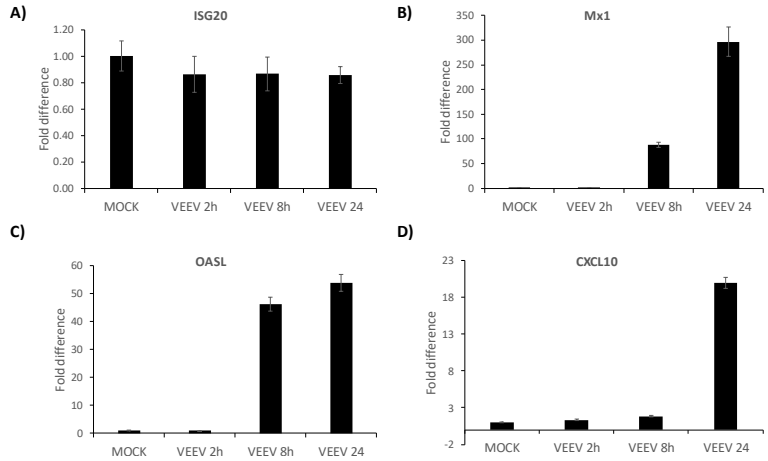

Figure 8: Innate immune response genes are stimulated in American Alligator PBMCs exposed to VEEV. RNA was extracted from PBMCs exposed to VEEV TC-83 (MOI ~100) and RT-qPCR analysis performed using a one-step SYBR Green kit including primers for ISG20, Mx1, OASL, CXCL10 and GAPDH (endogenous control). Gene expression changes were calculated using the ΔΔCt method, with Mock infected cells set to 1.

We also determined if VEEV RNA was present in the PBMCs, which would be indicative of a successful infection. RT-qPCR for viral RNA indicated that VEEV RNA levels increased over time; however, viral RNA levels were quite high at the start of the infection (7 logs). Therefore, a repeat experiment with lower amounts of VEEV would be required to determine if viral replication is occurring. At this point, we can conclude that VEEV viral RNA persists in American Alligator PBMCs.

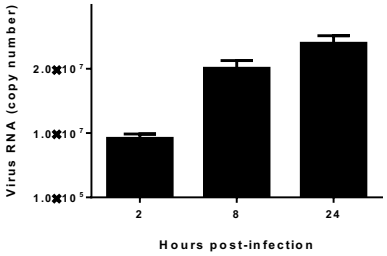

Figure 9: VEEV persists in American Alligator PBMCs. RNA was extracted from PBMCs exposed to VEEV TC-83 (MOI ~100) and RT-qPCR analysis performed using a one-step kit including primers and a probe for VEEV RNA. Virus RNA copy number was determined using absolute quantitation with known amounts of viral RNA used for the standard curve.

<u>Harvest protocol and conditions that afford efficient capture and identification of known model antiviral CAMPs from plasma. (delivery date: 09/29/19 – complete)</u>

In the third quarter, peptides were harvested from alligator plasma using the two-stage harvesting process to assess their harvesting properties and develop harvesting protocols. The first round of harvesting was performed using core-shell particles with cross-linked MA6AHA shell, and captured peptides were eluted using 1:1:0.1 TFE/H_2O/TFA. The resulting peptide eluents were aliquoted, and solvent then removed using a speed-vac. A second round of harvesting was then performed from these aliquots, using the four different membrane encapsulated particles, by dissolving the dried aliquots in buffered aqueous particle suspension (10 mM Tris, pH 7.4). The harvested peptides were then analyzed by tandem mass spectrometry (LC-MS/MS with ETD and HCD). Harvests were performed in triplicate and duplicate injections of captured peptides were performed on the mass spectrometer. The results of these harvests are presented in Table 4 and Figure 10. These initial harvests yielded promising results.

	General Harvest (20% MA6AHA)	Lipobead - Healthy Leuk.	Lipobead - Tryp - Healthy Leuk.	Lipobead - Infected Leuk.	Lipobead - Tryp - Infected Leuk.
No. Unique Database Search Peptide Spectrum Match Sequences	138	19	66	13	26
No. Unique De Novo Only Sequences	569	161	393	66	103
No. Database Peptides Unique to Particle	121	8	53	5	12
No. De Novo Peptides Unique to Particle	566	152	387	61	98

Table 4. Results from initial round of harvests using new particles encapsulated within cell membranes. The initial general harvest using MA6AHA-based particles yielded the greatest number of identified peptide sequences, both those with matches in the reference proteomic database as well as *de novo* sequences. Harvests using membranes from healthy and infected leukocytes yielded the fewest peptide identifications, with harvests using membranes from cells treated with trypsin yielded an intermediate number of peptide identifications. In all cases, the number of de novo only peptides greatly outnumbered those with database matches.

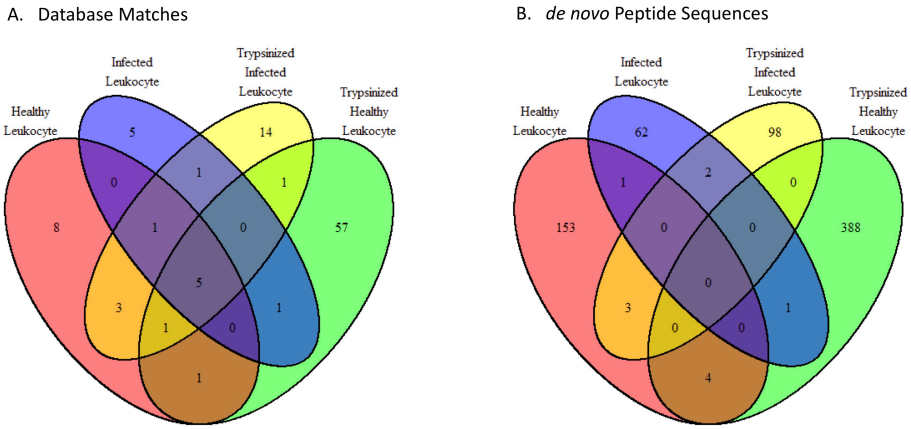

Figure 10. Venn diagrams illustrating distribution of peptides based on the four leukocyte membrane-encapsulated particle types. Diagram A.) peptide sequences with matches in the reference sequence database, and B.) *de novo* sequences with no identified database matches.

We detected reference AMPs in multiple harvests. While the reference peptides are not always detected in direct analyses of the general harvest MA6AHA-based particles, they are detected in subsequent harvests using the membrane-encapsulated particles, which verifies that the reference AMPs are indeed captured and enriched in the first-round harvests. The fact that these peptides are not detected in all of the harvests performed using membrane-encapsulated particles is not surprising as these peptides are anticipated to have a low propensity to bind to host cells.

<u>Harvests performed from stimulated and unstimulated plasma using panel of particles developed in Aim 1 for the purpose of identifying antiviral peptides.</u> (delivery date: 03/29/20 – 20%)

In the last quarter, we performed harvests from plasma derived from both healthy blood and blood that had been inoculated with VEEV and the analyses of the results from these harvests are presently underway. Additionally, we began performing harvests form media recovered from alligator PBMCs, both healthy cells and those that had been infected with VEEV (see description of infection studies above). The harvests from these samples are in the queue for analysis on the Fusion Orbitrap mass spectrometer.

Major Task 4: Analyze harvested peptides (12 months: 03/30/19-03/29/20)

Milestones:

1.) Workflow for efficiently analyzing harvest samples via tandem mass spectrometry and determination of the peptide sequences. We have refined the sample processing workflow to optimize it for analysis via tandem mass spectrometry with ETD and HCD. The initial harvests begun in Major Task 3 provided an opportunity for refining the sample processing workflow. At this time, we have a working framework for performing harvests from plasma or culture media using the first-round general harvest core-shell particles as well as membrane-encapsulated hydrogel particles.

2.) Mass spectrometry data and sequences for peptides harvested for the purpose of identifying novel antiviral peptides. In the third quarter, we analyzed peptides harvested from commercial plasma. Initial LC-MS/MS data yielded several peptide sequences of varied lengths. We observed a substantially greater number of de novo peptide sequences than sequences with matches in the reference sequence database. We have used this harvest and mass spectrometry data to refine mass spectrometry, chromatography, and MS data analysis parameters.

3.) Statistical methods for analyzing harvested peptide sequences to afford statistical significance values of potential antiviral peptides.

Preliminary analyses: Peptide sequence data from initial harvests and replicate injections were sent to collaborators at PNNL and they used this data to begin framing their analyses. The team at PNNL analyzed data for these harvested peptides in the 3rd quarter evaluating run-to-run (five replicate runs on the mass spectrometer of the same sample) variability. Initial analyses indicate relatively high percentage of common peptides based on the Jaccard Index (Figure 11) and percentage of identical peptides. In respect to proteome coverage, two replicates will identify approximately ~73% of all of the peptides identified from the combined replicate injections. This increases dramatically to ~93% for 4 replicates.

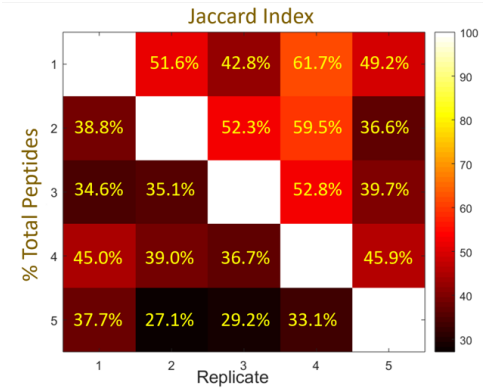

Figure 11: Image of two related similarity metrics (1) % total peptides and (2) Jaccard Index.

Healthy Alligator Plasma Initial Analysis: The team at PNNL analyzed peptide sequences identified from harvests performed on healthy alligator plasma beginning with a general using MA6AHA particles followed by harvests using the panel of membrane-encapsulated particles (described above under Major Task 3). Initially, the similarity and differences between harvests based solely on the observed peptides was evaluated. Again, the Jaccard index was used to evaluate similarity between samples, which evaluates the size of the intersection of observed peptides relative to the total peptides observed (Figure 12B). The data quality and similarity were excellent as seen in Figure 12C based on a standard hierarchical clustering based on the Jaccard similarity of the data. From this analysis it was observed that the healthy and infected leukocyte membranes were the most similar while the general harvest was the most distinct. Furthermore, we evaluated if there were differences in the total number of captured peptides for each of the harvests. As seen in

Figure 13, the healthy trypsinized leukocyte membranes have the highest counts of observed peptides, but is only statistically larger than the infected leukocyte membranes based on a Friedman test ($\chi 2$ statistic; p ~0.0013)

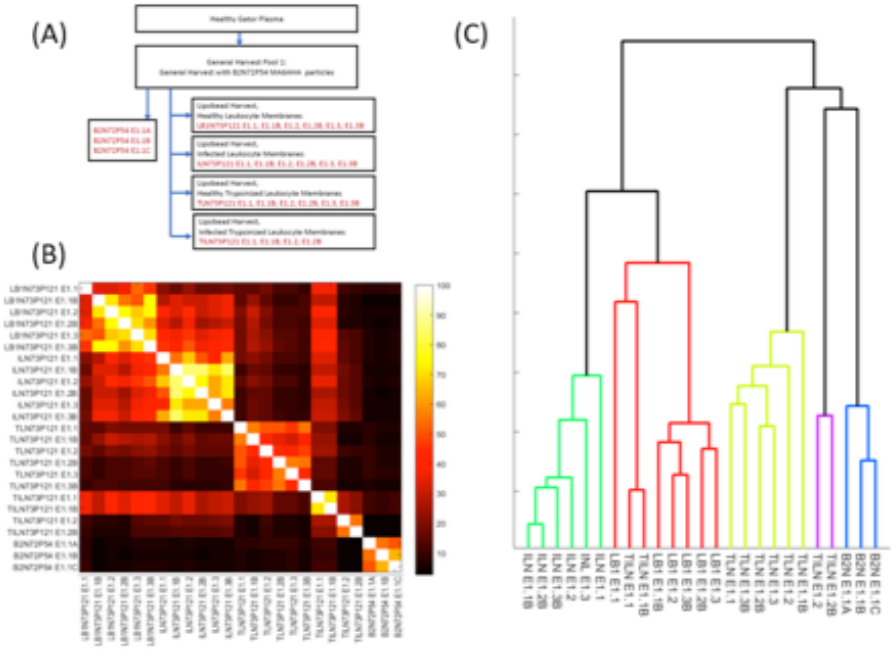

Figure 12: The (A) overall design showing relationship of the general harvest with B2N72P54 MA6AHA particles with the associated (B) JA measurements from the measured samples and (C) hierarchical clustering of samples into the expected groupings.

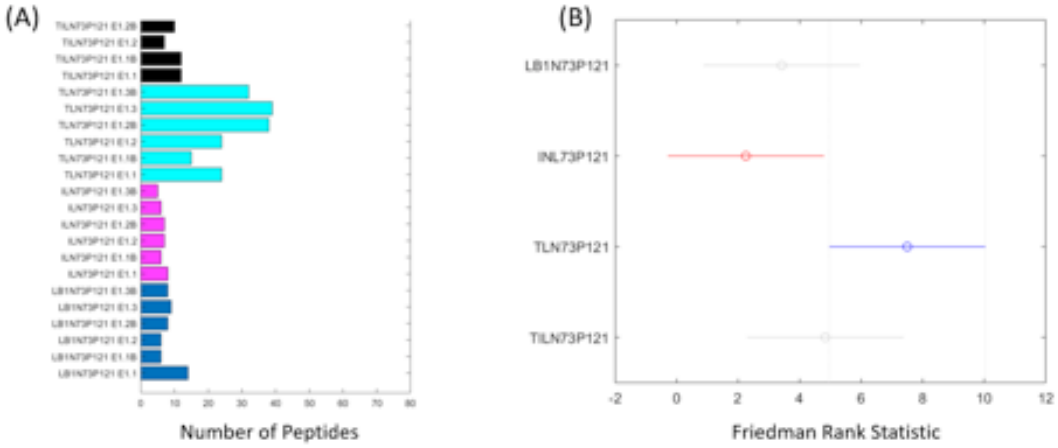

Figure 13: The (A) number of peptides per sample and (B) the post-hoc test for the statistical comparison of the number of peptides by harvest type.

A statistical analysis was performed that focused on the longest peptide variants (LPVs) in each harvest using a G-test (1) to evaluate if a specific peptide sequence in observed in one or more of the harvests than expected by random chance. This analysis identified several peptides of interest as unique to specific harvests. PEPAKSAPAPKKGSKKAVTK" and "GTGASGSFKLNKK" were both unique to infected leukocyte membranes with a p-values of approximately 0.002 and 0.007, respectively. Specifically, in this case PEPAKSAPAPKKGSKKAVTK was observed in 83.3% of the infected leukocyte membranes samples and none of the other samples. GTGASGSFKLNKK was observed in 66.7% of the infected leukocyte membranes samples and none of the other samples. The peptide NKRSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKY was unique to infected trypsinized leukocyte membranes samples (p-value approximately 0.007), observed 100% of the time in these samples and in none of the other groups. Lastly, ATPVKIRIENSNAFLSR was significantly higher in to infected cells (p-value approximately 0.032). It was observed in 100% of the infected samples, but only 62.5% of the samples. Thus, it was seen more frequently in the infected samples than expected by chance, but it was not unique. Expanded statistical analysis of of the peptide sequences (not limited to LPVs) from each harvest revealed two additional peptide sequences (RPPGFTPFRS and KPMKDSTVLPHF) that were associated with harvests performed using membranes from infected cells (p-values of 0.0065 and 0.018, respectively).

References:

Webb-Robertson et al., (2010) Combined statistical analyses of peptide intensities and peptide occurrences improves identification of significant peptides from MS-based proteomics data. J Proteome Res, 9(11):5748-56.

4.) Predictive model(s) based on machine learning for analyzing harvested peptides to aid in identification of peptides likely to have antiviral properties. (delivery date: 03/29/20 – not started)

Major Task 5: Assess performance of likely antiviral peptides (6 months: 09/30/19-03/29/20)
In our initial analyses described above in Major Task 4, we identified 6 novel peptides that were determined to be statistically unique to harvests performed using particles encapsulated in membranes from infected leukocytes, which had not been treated with trypsin. The discovery of these peptides is very encouraging and occurs ahead of the projected schedule. We have ordered five of these peptides to be chemically synthesized so that we may evaluate whether they have antiviral properties against VEEV directly or against VEEV infected leukocytes.

What opportunities for training and professional development has the project provided?

One student (Victoria Callahan) and a research associate (Shih-Chao Lin) have been involved in this project from the Kehn-Hall Lab. Two graduate students (Amy Carfagno and Samuel Garvey), a research assistant (Sabrina Lamont), and a research associate (Liana Soares) have been involved on this project from the Bishop Lab. Dr. Bishop, Dr. Kehn-Hall and researchers from both labs meet frequently to plan and coordinate their efforts on the project.

This project provides a unique training environment because it is a multidisciplinary project, which allows researchers from the biology/virology side to be exposed to the application of chemistry to address a biological question. They are also getting experience stimulating reptile blood cells with virus, which is outside the scope of the typically human focused research in the Kehn-Hall Lab. Similarly, the project provides researchers with backgrounds in organic chemistry, materials science and proteomic mass spectrometry the opportunity to work with virologists and biologists to gain experience and understanding of the biology that is at the heart of the project.

Researchers on the project contribute in the preparation of the reports and will also be included in the preparation of manuscripts produced by the project. We also anticipate they will present their data future conferences.

How were the results disseminated to communities of interest?

We presented a poster at the MHSRS conference in Orlando in August, 2019. The poster presented the overall goals and rationale for the project, as well as results from our efforts developing hydrogel particles that incorporated membranes from healthy and VEEV-infected leukocytes as baits for the differential capture of peptides in order to identify those, which were unique to harvests using membranes from VEEV-infected leukocytes. The title of the poster was "Bioprospecting Host Defense for New Antiviral Agents", and it received a lot of interest and favorable comments from attendees.

What do you plan to do during the next reporting period to accomplish the goals?

Major Task 1: Develop VLP-based capture particles.
We will produce large quantities of VLP using VEEV-PIV. VEEV PIV will be purified and quantitated using our establish protocols, followed by coupling to our particles. We anticipate completing Major Task 1 objectives and initiating harvests studies with virus-modified particles by mid-November, 2019.

Major Task 3: Perform plasma harvests.

1. We will confirm our VEEV harvest stimulation conditions with a second batch of blood, verifying stimulation by RT-qPCR detection of innate immune response genes.
2. We will harvest and analyze peptides generated from VEEV-stimulated American alligator PBMCs using panel of membrane encapsulated particles and VEEV-modified particles.
3. We will perform harvests from plasma from VEEV-stimulated snake blood or cell culture media from VEEV stimulated snake PBMCs. We anticipate that these harvests will result in the identification of new potential antiviral peptides, which will be chemical synthesized and evaluated.

Major Task 4: Analyze harvested peptides.

1. We will analyze the alligator and snake peptides and proteins using tandem mass spectrometry and tools that we have developed in order to prepare and sort data for statistical analyses at PNNL.
2. We will share data generated from additional harvests from both American alligator and snake plasma or leukocyte secretions with team at PNNL for their statistical analyses to identify likely potential antiviral peptides that target VEEV.

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3. This data will also be used to begin refining their statistical methods and developing predictive models for identifying likely antiviral peptides against VEEV.

Major Task 5: Assess performance of likely antiviral peptides.
We will test lead peptides for antiviral activity using cell-based assays.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The ultimate goal of this project is to identify novel peptides that have antiviral activity against Venezuelan equine encephalitis virus (VEEV). VEEV is an important human and veterinary pathogen with no current treatment options. Therefore, discovery of antivirals is an urgent unmet need. Due to the nature of antiviral peptides, the discovered peptides will likely have broad-spectrum activity, being able to inhibit multiple viruses within the alphavirus family that cause significant disease, such as chikungunya virus (CHIKV) and eastern equine encephalitis virus (EEEV). The peptide discovery process and resources developed here can in the future be adapted to target other viruses or classes of virus. Moreover, this work will provide basic immunological information about the peptide response to VEEV infection in reptile species.

What was the impact on other disciplines?

We are developing novel particles that can be used to isolate peptides associate with the innate immune response. These particles can be used as discovery tools for multiple other disciplines including cancer biology, neurobiology and immunology. There are also implications for using these particles as sensors.

What was the impact on technology transfer?

Nothing to Report; technology is yet to be transferred.

What was the impact on society beyond science and technology?

In humans, Venezuelan equine encephalitis virus (VEEV) infection causes a febrile illness typified by fever, malaise, and vomiting. In some cases, infection progresses to the central nervous system (CNS) with severe cases resulting in death. Neurological cases have a mortality rate as high as 35% in children and 10% in adults, with long-term neurological deficits observed in 4-14% of VEEV infection survivors. Neurological sequelae include convulsions, somnolence, confusion, photophobia, coma, intellectual disability, and emotional instability/behavioral changes. There are currently no specific antiviral therapeutics or FDA-approved vaccines for human use for the treatment or prevention of VEEV infection. Thus, it has been recognized that there is a clear need to develop therapeutic strategies for VEEV. Our current work addresses this problem by developing and utilizing novel antiviral-peptide harvesting microparticles to identify antiviral peptides from biofluids. In published work we have discovered novel CAMPs from both American alligator and Komodo dragon plasma and demonstrated that these peptides have antimicrobial activity against multiple bacterial species. We hypothesize that large-scale analysis of the innate host-defense peptide response to arbovirus exposure in reptiles will reveal valuable insights into their ability to resist or tolerate important human viral pathogens and result in the identification of novel therapeutics to protect the warfighter from these pathogens. To our knowledge there is currently no comparable platform that can differentially select for peptides with antiviral activity.

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We are developing a new class of hydrogel particles for the purpose of differentially enriching host-defense peptides that target VEEV and distinguishing between those peptides that are specific for VEEV and those that may impact host cells. While the immediate beneficiaries of this research would be members of the military who currently work with or may come into contact with VEEV, ultimately it would be the entire human population who would benefit from the fruits of the proposed research. Furthermore, our long-term goal is to extend this approach to other arboviruses of significance including Zika virus, chikungunya virus, and West Nile virus.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

1. Technical challenges associated with quantifying propargyl monomer incorporation in particles and in preparing suitable amounts of VLP for incorporation in virus modified-particles have delayed development of virus-particle coupling conditions and achieving Major Task 1 deliverables by 09/29/2019 as originally projected in the SOW. Both of these problems have been overcome, and we are presently assessing conditions for efficiently and consistently coupling virus to the hydrogel microparticles via click chemistry. Our initial studies have offered promising results, and we are presently optimizing conditions. Based on these results, we anticipate completing Major Task 1 objectives and initiating harvests studies with virus-modified particles by mid-November, 2019. We do not anticipate that this delay will impact our ability to achieve the final goals of the project as projected in the SOW.
2. Efforts to optimize conditions for stimulating reptile blood cells with VEEV have taken longer than originally projected due to complications in scheduling collection of blood samples due to Hurricane Dorian, which had been projected in different forecasts to hit Florida where our collaborators at the St. Augustine Alligator Farm Zoological Park are located. Their need to prepare for the potential storm to protect the animals disrupted our schedule for getting blood in September, which in turn delayed our efforts to optimize stimulation conditions beyond our original projected 09/29/12019 deliver date. That being said, we received a fresh shipment of alligator blood on 10/23/2019, PBMCs have been collected and stimulation studies are underway. We anticipate establishing conditions and a protocol for stimulating reptile blood with VEEV by the end of October, completing this Task 3 deliverable.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Protocol [ACURO Assigned Number]: ACURO Log Number = DM171301
Title: Translational Peptide Research for Personnel Protection
Target required for statistical significance: Proposed 20 crocodilians and 15 snakes
Target approved for statistical significance: Approved 03/20/2019

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

A poster was presented at MHSRS in August 2019.
Bishop, B. and Kehn-Hall, K. "Bioprospecting Host Defense for New Antiviral Agents" MHSRS, Orlando, FL, August, 2019

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Technologies associate with this project are currently still in development stages.

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Barney Bishop*
Project Role: *Principle Investigator*
Researcher Identifier (e.g. ORCID ID): *0000-0002-6626-9251*
Nearest person month worked: *5*
Contribution to Project: *As principle investigator, Dr. Bishop manages the project and coordinates research efforts between the GMU teams and the team at PNNL. He is also directly involved with particle and peptide harvest development as well as analysis of harvested peptides via mass spectrometry.*

Name: *Kylene Kehn-Hall*
Project Role: *co-Principle Investigator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *As co-principle investigator, Dr. Kehn-Hall has worked closely with Dr Bishop in coordinating research efforts. She is directly involved with microbiology/virology aspects of the project, including cell culture, preparation of virus like particles, blood stimulation and infection studies.*

Name: *Bobbie-Jo Webb-Robertson*
Project Role: *PNNL co-Principle Investigator*
Researcher Identifier (e.g. ORCID ID): *0000-0002-4744-2397*
Nearest person month worked: *1*
Contribution to Project: *Dr. Webb-Robertson is responsible for performing the statistical aspects of the projects and leads the PNNL efforts. She has been analyzing the technical replicate data.*

Name: *Paul Russo*
Project Role: *Affiliated Faculty*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Dr. Russo's area of expertise and contribution is in protein/peptide mass spectrometry. He is responsible for analyzing the harvested peptides via LC-MS/MS and developing methods/protocols for that purpose. He is also working with students in Dr. Bishop's lab in analyzing the mass spectrometry data for the harvested peptides.*

Name: *Sabrina Lamont*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*
Contribution to Project: *Ms. Lamont supported research efforts by Dr. Bishop and his graduate students in the area of particle development and evaluation. She helped with coordinating research efforts in the lab.*

Name: *Liana Soares*
Project Role: *Research Associate*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *5*
Contribution to Project: *Dr Soares is an experienced polymer chemist with experience in materials science and nanotechnology. Dr. Soares has been working with Dr. Bishop and his graduate students to improve particle production and developing new particle technologies for the targeted capture of peptides and proteins of interest from plasma. She has also contributed in performing harvests from Alligator plasma using the hydrogel particles and processing samples for mass spectrometry analysis. She has helped to provide much needed bandwidth in this area. Simultaneously, she is introducing multiple advancements in to the particle harvest technology, enhancing their performance and versatility.*

Name: *Shih-Chao Lin*
Project Role: *Research Associate*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*
Contribution to Project: *Dr. Lin is responsible for performing the virological aspects of the projects. He has been optimizing the VLP production, plasma membrane isolations and blood stimulation protocols.*

Name: *Victoria Callahan*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Victoria has been assisting with VLP-particle coupling conditions and optimization of VLP production.*
Funding Support: *Volunteer position*

Name: *Amy Carfagno*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Amy has been assisting with development of the membrane-encapsulated particles, harvests, and mass spectrometry analyses of the harvested peptides.*
Funding Support: *GMU Presidential Graduate Fellowship (over the summer received some wages support from the grant for efforts on the project.)*

Name: *Samuel Garvey*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Sam has been assisting with synthesis of the PP4ME monomer and particles containing the monomer, as well contributing in development of the virus-modified particles.*
Funding Support: *GMU Graduate Teaching Assistantship*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Kylene Kehn-Hall has received the following active support since the time of the last reporting period:

GMU Grant # 204570
Award # HDTRA11810040
Role: Co-Investigator
Principal Investigator: Narayanan, Aarthi
Project Title: Genome wide mapping of host proteome: pan alphavirus proteome interactions for discovery of broad spectrum countermeasures
Sponsor: US Department of Defense
Total Award Amount: $500,000 **Total Award Period:** 8/1/2018 – 10/31/2019
Commitment in Person-Months per Year: 0.20 Summer; 0.2 Calendar Months

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GMU Grant # 204578
Award # HDTRA1-18-1-0045
Role: Principal Investigator
Project Title: EGR1 regulation of neuronal survival and inflammation following VEEV infection
Sponsor: US Department of Defense
Total Award Amount: $499,995 **Total Award Period:** 8/1/2018 - 10/31/2019
Commitment in Person-Months per Year: 0.20 Summer; 0.10 Calendar Months

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Dr. Kylene Kehn-Hall is no longer supported by the following funding:

Project Title: Blood Brain Barrier Advanced Fundamental Research
Role: Co-Principal Investigator
Principal Investigator: Monique Van Hoek
Sponsor: US Department of the Navy
Total Award Amount: $175,000 **Total Award Period**: 02/28/2017 - 02/07/2019
Commitment in Person-Months per Year: 0.05 Summer, 0.05 Calendar Months

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What other organizations were involved as partners?

Organization Name: Pacific Northwest National Laboratory (PNNL)

Location of Organization: 902 Battelle Blvd, Richland, WA 99354

Partner's Contribution: Dr. Bobbie-Jo Webb-Robertson, of the Biological Sciences Division at PNNL, is collaborating with us on the project via a subcontract. She is primarily contributing in Major Task 4 efforts and her focus is on statistical analyses of the peptides identified from the harvests in Major Task 3 in order to identify those peptides uniquely associated with harvests performed using virion-modified particles as well as particles encapsulated in membranes from VEEV-infected Leukocytes. These peptides are expected to be potential antiviral and will by synthesized and evaluated.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.