AWARD NUMBER: W81XWH-19-1-0137

TITLE: Development of Recombinant Viral Vaccines for HFRS-Causing Hantaviruses

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REPORT DATE: August 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2021	Final	01May2019-30Apr2021

4. TITLE AND SUBTITLE

Development of Recombinant Viral Vaccines for HFRS-Causing Hantaviruses

5a. CONTRACT NUMBER
W81XWH-19-1-0137

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Hemorrhagic fever with renal syndrome (HFRS) is a deadly disease caused by a several species of widely distributed (throughout Eurasia) Old World hantaviruses. Most prominent are Hantaan (HTNV) and Seoul (SEOV) viruses in Asia and Puumala (PUUV) virus in Europe. Worldwide it is estimated that between 60,000-100,000 cases occur annually with a small percentage of these cases resulting in death. As a starting point for an effective vaccine against these HFRS-causing viruses, we will use a different, nonpathogenic virus, vesicular stomatitis virus (VSV), to express a protein from HTNV or PUUV by producing a recombinant VSV (rVSV). Although the VSV virus does normally cause disease in humans and the genetically altered vaccine rVSVs tested thus far also appear safe in normal animals, when VSV itself or some rVSVs enter the central nervous system or are used to infect animals that have immune deficiencies, severe disease or death can result. Due to the concerns raised by these findings we are also engineering rVSVs that are designed to be safer and less likely to cause disease even if they enter the CNS or infect a severely immunocompromised individual.

15. SUBJECT TERMS

Hantavirus, Hantaan virus (HTNV), Puumala virus (PUUV), recombinant vesicular stomatitis virus (rVSV), vaccine

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION**: Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by "Old World" viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "New World" viruses of the Americas (Sin Nombre and Andes viruses). Although the research under this funding was interrupted by the COVD pandemic, we made significant accomplishments. Novel vesicular stomatitis virus (VSV) vectors with altered transcription patterns and incorporating ribosome skipping sites were generated. It is anticipated that these vectors will have diminished potential pathogenicity as a vaccine platform compared to parental VSV vectors. Additionally, a panel of VSV pseudotyped viruses bearing eight different bunyavirual glycoproteins was produced for analysis of cross neutralization upon vaccination with a single hantavirus glycoprotein. Finally, a rapid, efficient, and accurate pseudotype neutralization assay was developed. When COVID shutdowns occurred, we pivoted and utilized this assay to analyze serum samples from vaccinated animals and people and various patient cohorts resulting in 14 high profile publications that employ the assay developed under this award. We summarize below the accomplishments on this project.
2. **KEYWORDS**: Hantavirus, Hantaan virus (HTNV), Puumala virus (PUUV), vesicular stomatitis virus (VSV).

3. ACCOMPLISHMENTS:

- What were the major goals of the project?
- The major goals of this one-year Discovery project are as follows:
- 1) Provide proof-of-concept for generation of neutralizing responses by the rVSV-PUUV and rVSV-HTNV as vaccine vectors
- 2) Determine the cross reactivity of the antibodies generated in mice immunized with rVSVs for different hantaviral strains using VSV pseudotypes bearing glycoproteins from a panel of hantaviruses.
- 3) Develop potentially safer 2nd and 3rd generation rVSV expressing PUUV or HTNV glycoprotein immunogens.

In completion of part of Major Aim 1, we amplified, concentrated and characterized the 1st generation rVSV-PUUV and rVSV-HTNV virus stocks. Also as part of major task 1, we wrote the animal protocols and received UPenn IACUC approval for this work. However, in large part due to disruptions caused by the COVID pandemic, we did not complete the immunizations with these viruses.

As part of Major Task 2 we constructed and characterized expression vectors for numerous different viral glycoproteins for use in analyzing cross neutralization in immunized mice. These include the Old World hantaviruses Puumala, Haantan, Seoul, Dobrava and the new world hantaviruses Andes and Sin Nombre as well as distantly related bunyaviruses SFTSV, Heartland, and LaCrosse. We established conditions for generation of VSV pseudotypes carrying these glycoproteins for use in neutralization assays. We validated the neutralization assays using sera from mice immunized against SFTSV where we observed sera concentration dependent neutralization curves. Based upon these accomplishments, we were poised to rapidly complete major goals #1 and 2 when COVID disruptions occurred.

Under Major Task 2 of this proposal, we developed a rapid and highly quantitative VSV pseudotype antibody neutralization assay. This rapid and highly quantitative assay was being developed for the hantaviruses when SARS-CoV-2 hit. The assay employs a replication-defective VSV reporter virus encoding mCherry which lacks a glycoprotein

gene. Introduction of this vector into cells expressing a foreign viral glycoprotein results in infectious virions carrying the foreign glycoprotein. At the beginning of the pandemic. due to the pressing need for thus type of assay for COVID patients and to analyze vaccine responses, we modified it for use with SARS-CoV-2 pseudotypes. This focus reduction neutralization titer (FRNT) assay has been extensively validated and employed to look at sera from SARS-CoV-2 vaccinated animals and patients resulting in numerous high impact publications[1-14]. This rapid assay is ready for use in evaluating hantaviral immune responses. An example of the results from this rapid neutralization assay are shown in Figure 1. Here the assay employed VSV pseudotyped with SARS-CoV-2 (the D614G alpha variant or the Omicron variant) and measured the antibody response in subjects receiving a COVID mRNA (Pfizer) booster vaccination. In this analysis COVID-naïve (blue) and COVID-experienced (orange) subjects were compared. Overall, we have used this assay to analyze >5000 samples from a variety of COVID projects, including analyzing responses to primary and secondary vaccination in vaccinated cohorts, and describing responses in patients with various immune deficiencies. To date, the results from this rapid assay have contributed to 14 high-profile publications in journals ranging from Cell to Nature Immunology [1-14].

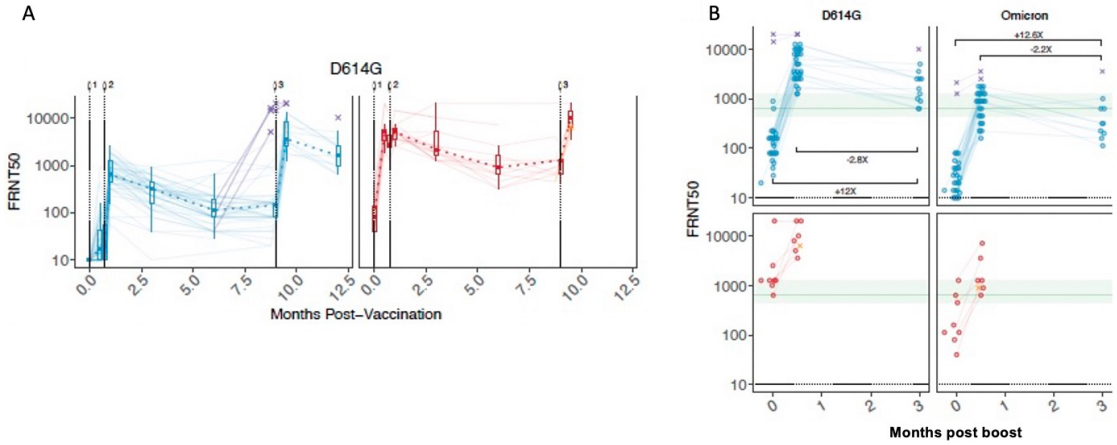

Figure 1. Antibody Responses in subject receiving Pfizer mRNA COVID Booster vaccine. *A. Pseudovirus neutralization titers against SARS-CoV-2 D614G Spike protein over time in plasma samples from vaccinated individuals. Data are represented as focus reduction neutralization titer 50% (FRNT50) values. B. Plasma neutralizing activity against D614G and Omicron before and after booster vaccination. Dotted lines indicate limit of detection for the assay. Green boxes and lines indicate interquartile range (IQR) and median of D614G neutralizing titers ~1 week following the second vaccine dose in SARS-CoV-2 naïve subjects. (from Cell. 2022 Apr 8:S0092-8674(22)00456-1.)*

For Major Task #3 (Specific aim 2 in the proposal) we have genetically engineered plasmids that encode the 2nd and 3rd generation rVSV-PUUV and HTNV that include ribosome skipping motifs (RSM) to alter the rVSV genome and ameliorate potential vaccine pathogenesis. Figure 2 depicts the (A) RSM protein junctions and (B) plasmid map for the rVSV vector that includes a T2A RSM between the P and M proteins and encodes the PUUV glycoproteins (VSV P-T2A-M PUUV mCherry). Unlike wt VSV where each gene has its own transcription start site, this vector we have generated expresses the P and M proteins from one transcript. The use of the T2A RSM produces a VSV P protein with a C-terminal 20 residue extension (Fig 2A) and the addition of a proline residue to the

amino terminus of the VSV M protein. Similar constructs that encode a related RSM (P2A) in place of the transcription start site between the N and P genes has also been constructed (not shown). A third type of construct that includes both the P2A and T2A sequences between N and M and M and P respectively has also been constructed. For this double RSM rVSV vector three genes are fused and expressed from one transcript that initiates upstream of the N gene. rVSV carrying RSM sequences and encoding PUUV and HTNV have been generated and are awaiting launch.

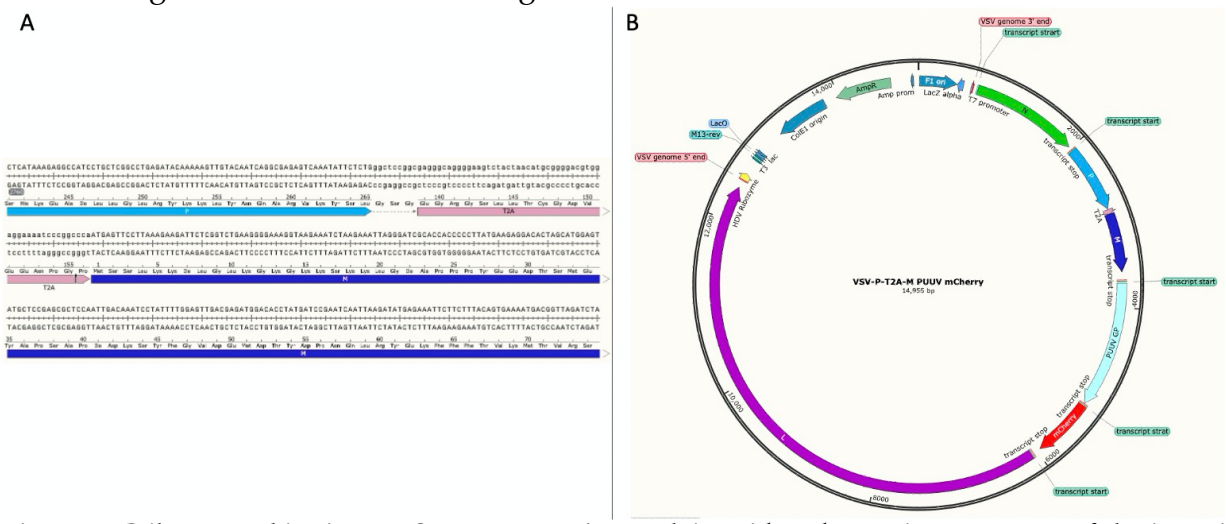

Figure 2. Ribosome skipping rVSV vectors. A. Nucleic acid and protein sequences of the junction between the VSV P and M genes where the transcription stop and start site in wt VSV have been replaced by a T2A ribosome skipping motif. Region in pink denotes the T2A protein sequence with the arrow indicating the site where the ribosome "skips and terminates the upstream protein (P, teal) and initiating the downstream one (M, purple). B. Map of the complete plasmid carrying the P-T2A-M fusion gene.

We struggled to get these rVSV to launch despite employing various plasmid ratios and transfection conditions. We Illumina sequenced the entire 17kb plasmids for all of these vectors to confirm that the sequences are correct, however this has not revealed the source of the launch difficulty. We changed the helper plasmids encoding VSV-N, VSV-P, VSV-L VSV-M and VSV-G and also Illumina sequenced these in their entirety – again this did not reveal the source of the problem. To overcome this problem, we will re-build the vector backbone using a rVSV that we have previously launched to determine if this will remedy the problem. Also, rVSV carrying foreign glycoproteins are not as readily launched as are rVSV encoding the cognate VSV-G. Therefore we will replace the PUUV gene with VSV-G. If we are still unable to launch these vectors, it will imply that VSV does not tolerate the RSM sites. Finally, because we have not yet launched the 2nd and 3rd generation vaccine vectors, the immunizations and analysis described within Major task #4 were not accomplished.

- What opportunities for training and professional development has the project provided?
- "Nothing to Report."
- How were the results disseminated to communities of interest?
- "Nothing to Report."
- What do you plan to do during the next reporting period to accomplish the goals?
- Once animal use is authorized, we will immunize mice with the in-hand rVSV-PUUV

and HTNV. For antibody response analysis blood will be collected 28 days post immunization or boost.

- We will also continue to attempt launching the 2nd and 3rd generation rVSV-PUUV and rVSV-HTNV.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- What was the impact on the development of the principal discipline(s) of the project?
- The described Puumala and Hantaan recombinant VSV's developed in this project are to our knowledge the only recombinant viruses currently being tested as vaccines for these deadly infections.
- What was the impact on other disciplines?
- "Nothing to Report."
- What was the impact on technology transfer?
- "Nothing to Report."
- What was the impact on society beyond science and technology?
- The neutralization assay that was initially being developed for this project, was modified to use in analyzing COVID vaccine and patient responses. Our work characterizing responses in immunized individuals has been communicated to the public and has helped to confirm the efficacy of mRNA vaccination technology to the general public.

5. **CHANGES/PROBLEMS**: The SARS-CoV-2 pandemic has significantly, negatively affected this research program. As was the case for most university and government labs, for several months UPenn prohibited all "non-essential" researchers from working on campus. All animal work was prohibited unless directly related to SARS-CoV-2. Also, because of our expertise in producing VSV pseudotyped viruses (developed in part for this proposal), we were tasked with developing a robust neutralization assay to study immune responses to SARS-CoV-2. We did so and have this assay in hand and ready to look at hantaviral neutralizing responses. For several months after the campus re-opened, the school of medicine required reduced laboratory density and social distancing within the labs. This has made the 2nd and 3rd generation rVSV launch and trouble-shooting this complex process extremely difficult because the PI or grad student with expertise and the technician working on this project were often not in the lab together.

- Changes in approach and reasons for change
  A significant change is that we have developed a much faster and high throughput neutralization assay the utilizes VSV pseudotyed viruses. This new FRNT assay will be used in lieu of the antibody neutralization assay described in the proposal. While this assay was developed for analysis of SARS-CoV-2 neutralization during the pandemic research interruption, we have adapted it for use in analyzing the antibody responses induced by the rVSV-PUUV and rVSV-HNTV vectors. Overall, this overnight assay, that is readily conducted in 96 well plates, will accelerate our analysis of the humeral responses in vaccinated mice. The technician who worked on this project has become proficient in this new neutralization assay.
- Actual or anticipated problems or delays and actions or plans to resolve them.
  Launching 2nd & 3rd generation vaccine vectors as described above
- Changes that had a significant impact on expenditures.
-
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
- Animals were not used as was originally planned due to COVID research

interruption and problems associated with production of the 2nd and 3rd generation rVSV vaccines.

- Significant changes in use or care of human subjects
- Nothing to Report
- Significant changes in use or care of vertebrate animals.
- Nothing to Report
- Significant changes in use of biohazards and/or select agents
- Nothing to Report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- Publications, conference papers, and presentations
- Journal publications. NA
- Books or other non-periodical, one-time publications. NA
- Other publications, conference papers, and presentations. NA
- Website(s) or other Internet site(s) NA
- Technologies or techniques We are generating novel rVSV with the potential to alter the pathogenic side effects of the vaccine vector. This technology will be shared freely with the scientific community.
- Inventions, patent applications, and/or licenses NA
- Other Products We have generated recombinant VSV viruses that carry various hantaviral glycoproteins. These reagents will be shared freely with the scientific community

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- What individuals have worked on the project?

▪ Name:	Paul Bates
▪ Project Role:	PI
▪ Researcher Identifier (e.g. ORCID ID):	PFBATES (eRA Commons ID)
▪ Nearest person month worked:	12
▪ Contribution to Project:	Directed Project, performed experiments, analyzed data
Funding Support:	NIH 1R21 AI 129531, this award
▪ Name:	Kendall Lundgreen
▪ Project Role:	Technician
▪ Researcher Identifier:	NA
▪ Nearest person month worked:	12
▪ Contribution to Project:	Performed experiments, analyzed data
Funding Support:	this award,
Name:	Robert (Teddy) Steinbock
Project Role:	Rotation graduate student
Researcher Identifier:	

Nearest person month worked:	4
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	UPenn university funding from BGS
Name:	Phil Hicks
Project Role:	Graduate Student
Researcher Identifier:	NA
Nearest person month worked:	8
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	NIH T32 training grant, this award

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- Yes, awarded NIH R01 to study mRNA and rVSV vaccination for SFTSV.
- What other organizations were involved as partners?
- Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- COLLABORATIVE AWARDS:
- Nothing to Report.

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