Evaluation of Biological and cDNA Clone-Derived Stocks of Venezuelan Equine Encephalitis Virus TC-83 Vaccine Strain

by

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Introduction:

Members of the *Togaviridae* family, alphaviruses are enveloped, single-stranded RNA viruses. Alphaviruses are globally distributed and most commonly transmitted by mosquito vectors. There have been frequent reported outbreaks of human and livestock infection, creating a public health concern. Venezuelan equine encephalitis virus (VEEV) is one of the most extensively studied alphaviruses due to its historical production as a biological warfare agent by both the United States and former Soviet Union during the Cold War. In humans, VEEV infection causes a debilitating acute febrile illness which may lead to encephalitis. A live attenuated TC-83 vaccine strain of VEEV was derived by serial passage of the equine-virulent, Trinidad donkey (TRD) strain in fetal guinea pig heart cells. However, the human vaccine has side effects and does not produce sterilizing antibodies (Burke et al., 1977). Despite decades of research, there is currently no FDA-approved vaccine or therapeutic for protection of humans against VEEV.

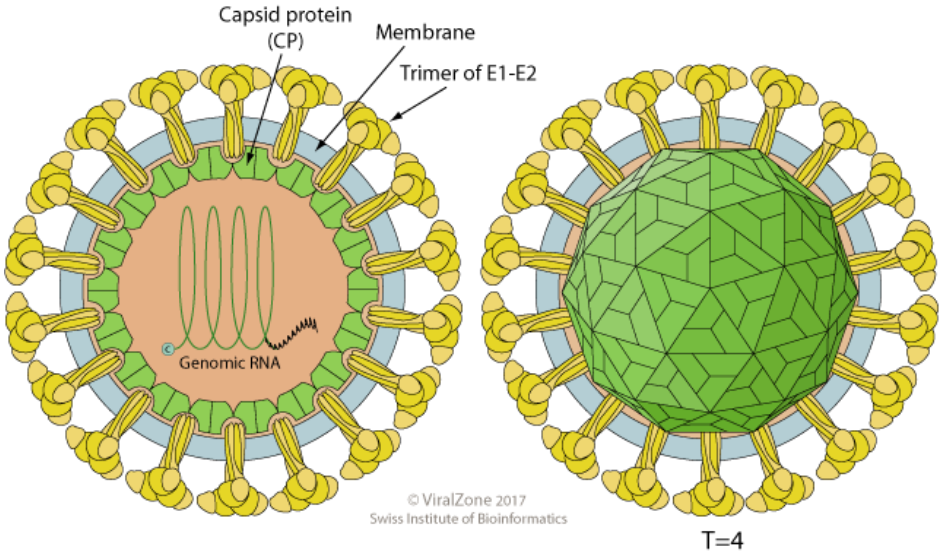

Figure 1. *Togaviridae* virion structure. This virus contains a single-stranded RNA genome enclosed in an enveloped protein capsid shell decorated with glycoprotein projections. Taken from SIB, Swiss Institute of Bioinformatics (2011).

The RNA genome of VEEV is surrounded by a protein capsid shell within a host-derived lipid envelope which contains virus encoded glycoprotein projections that facilitate virus binding to cells (Figure 1). The viral genome has two open reading frames (ORFs) (Figure 2). The first ORF is translated directly upon cellular entry and encodes the nonstructural proteins required for RNA synthesis. The second ORF encodes structural proteins that play a role in the assembly of new virus particles as well as attachment and entry into new host cells (Strauss et al., 1984). Alphavirus replication requires the function of all four nonstructural proteins (nsP1-4) both as individual components and as a polyprotein. Upon cellular entry, the alphaviral genomic RNA is translated to yield a nonstructural polyprotein, nsP123. However, suppression of termination at the stop codon located at the junction of nsP3 and nsP4 must occur for the production of the nsP1234 polyprotein. This nsP1234 polyprotein is incapable of initiating RNA synthesis until nsP2 proteolytically cleaves the polyprotein into nsP123 and nsP4. The nsP123 polyprotein and nsP4, as a complex, provide RNA-dependent RNA polymerase activity resulting in the production of minus-strand RNA. This full-length minus strand serves as a template not only for the production of additional genomic RNA but also for transcription of the subgenomic RNA. Further proteolytic processing of the nsP123 polyprotein into nsP1 and nsP23 results in the shift between the syntheses of minus-strand RNA to positive-strand RNA, resulting in the production of genomic and subgenomic RNAs (Lemm et al., 1994). The structural domain is translated as a polyprotein from subgenomic mRNA. The structural polyprotein is processed to produce the capsid protein, two small polypeptides E3 and 6K, and two glycoproteins E1 and E2. The transmembrane

glycoproteins of enveloped viruses carry out recognition of, and binding to, specific receptors on host cells, as well as initiation of entry into the host cell.

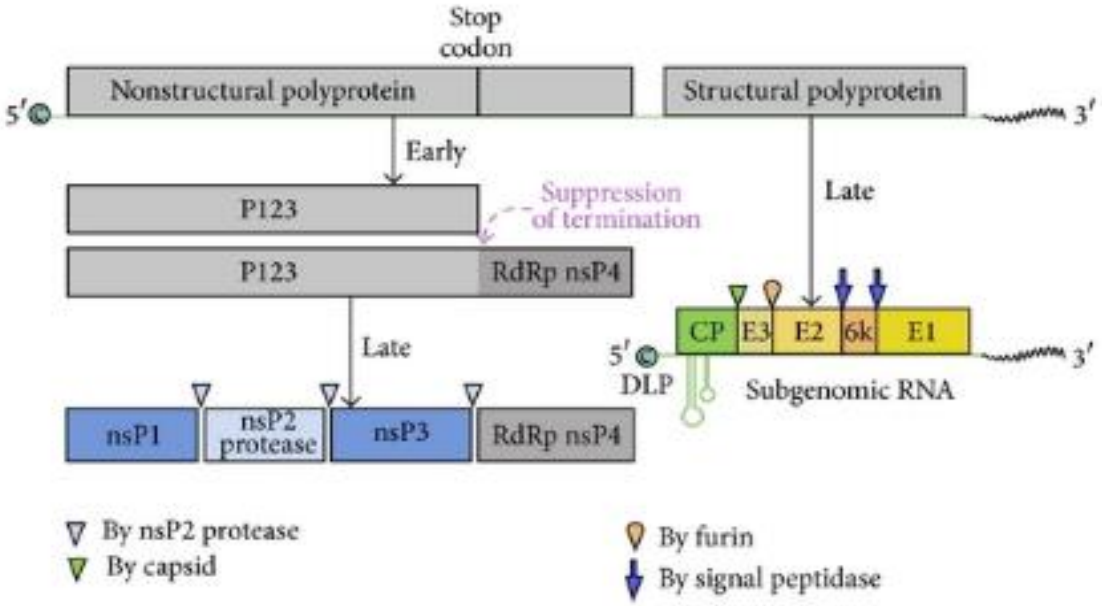

Figure 2. Organization of the alphaviral genome. Suppressing termination of the first ORF results in nsP1234 polyprotein. Translation of the second ORF results in the structural polyprotein which includes: envelope proteins, capsid proteins as well as glycoproteins E1 and E2. Taken from SIB, Swiss Institute of Bioinformatics (2011).

Structural proteins are the target of neutralizing antibodies that provide protection against VEEV infection. Numerous studies have demonstrated that administration of neutralizing antibodies are a prophylactic and therapeutic defense against VEEV in mice (Hu et al., 2010; Goodchild et al., 2012; O'Brien et al., 2012). The neutralization site on the E2 surface glycoprotein of the TC-83 vaccine strain of VEEV has been characterized using monoclonal antibodies. All epitopes contained within this neutralization site produced monoclonal antibodies that could protect mice from peripheral virus challenge. Two particular antibodies identified, 1A3B-7 and 1A4A-1 (Roehrig et al., 1985) have been extensively studied for their protection of mice from both

peripheral or aerosol exposure with VEEV. The humanized versions of the 1A3B-7 and 1A4A-1 monoclonal antibodies (mAbs) were recently used in a therapeutic efficacy study in non-human primates (NHPs) (Burke CW, unpublished data). In one study, NHPs were administered the neutralizing antibody 1A3B-7 or PBS 24 hours after exposure to aerosolized VEEV TRD strain. Administration of antibody 1A3B-7 reduced viremia in all animals (Figure 3). However, one NHP had a recurrence of viremia on day 4 post-exposure, despite treatment with the neutralizing antibody.

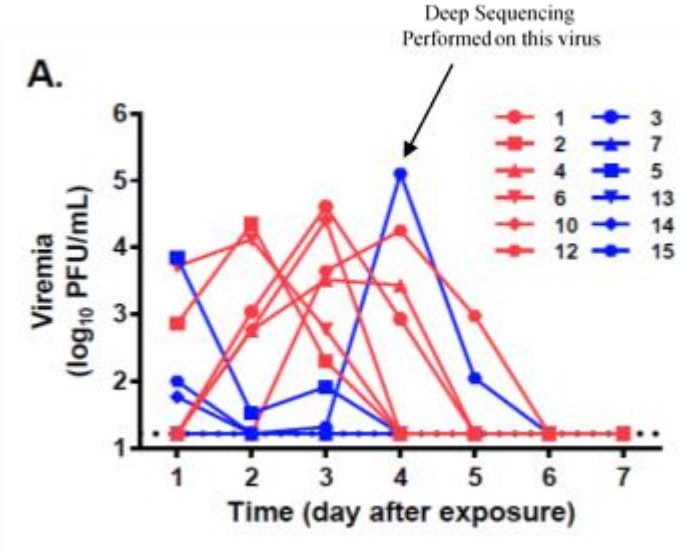

Figure 3. Detection of viremia. Viremia was evaluated in NHP following administration of mAb 1A3B-7 (blue) or PBS (red) Twenty-four hours after exposure to aerosolized VEEV. Each line represents an individual animal. Deep sequencing of virus was performed on serum collected from the NHP exhibiting high titer viral titer 4 days post-virus exposure.

One possible explanation for this recurrence of viremia is that the virus generated a mutation that allowed it to escape neutralization by the antibody. If this occurred, a virus particle with an altered neutralization site on the E2 glycoprotein would be produced thus leading to a lack

of reactivity to the neutralizing antibody 1A3B-7. Deep sequencing of the virus present in the blood revealed two nucleotide changes in the E2 glycoprotein, at nucleotide 9177 (cytosine to adenine) and 9189 (adenine to guanine). These nucleotide changes resulted in amino acid changes at positions 205 (lysine to threonine) and 209 (glycine to glutamine). These mutations may cause significant changes in the efficiency of antibody 1A3B-7 binding to the E2 glycoprotein, thereby causing the mutant virus to be undetected by this antibody. Infectious complementary DNA (cDNA) clones for alphaviruses were generated previously (Davis et al., 1989; Davis et al., 1991; Liljestrom et al., 1991; Davis et al., 1994). These cDNA clones were generated by taking the viral RNA sequence, which is positive-sense like mRNAs, and converting it into DNA via reverse transcription and then amplifying the cDNA sequence through polymerase chain reaction (PCR). The cDNA copy was inserted into a plasmid to provide the necessary components for plasmid DNA amplification and purification from E. coli and also encode necessary components, a RNA promoter sequence and linearization site, to allow for production of RNA transcripts. The RNA transcripts produced from the plasmid DNA serve as mRNAs when electroporated into mammalian cells and results in the production of virus particles. Alphavirus cDNA clones are used routinely for the production of virus to evaluate the effects of specific mutations on viral replication and pathogenesis (Davis et al., 1991; Liljestrom et al., 1991; Sjoberg and Garoff, 2003).

Research Objective:

The objective of this research study was to evaluate the potential antibody escape mutations identified. The specific aims of this study were as follows: Aim 1: Generate mutant virus stocks containing the identified mutations, singly or in combination; Aim 2: Characterize the mutant

viruses, along with the biological or clone-derived wild-type viruses, for fitness and ability to bind antibody 1A3B-7.

For Aim 1, a reverse genetics approach was utilized to introduce the identified mutations, singly and in combination, into the infectious cDNA clone of VEEV TC-83 vaccine strain. Primers were designed and purchased for use in a PCR mutagenesis reaction to introduce the mutations singly into the cDNA clone. Candidate cDNA clones containing the individual mutations were generated and prepared for sequencing to identify clones containing the expected mutations prior to preparing virus stocks. Sequencing confirmation is in progress.

For Aim 2, growth kinetics analysis of the mutants and wild-type, biological and clone-derived, viruses were to be compared. However, due to the delays encountered with mutant cDNA clone and virus stock productions, studies to compare the biological and clone-derived VEEV TC-83 viruses proceeded for future comparison with the mutant viruses. Comparing the clone-derived and biological TC-83 virus will serve as a 'control' to future studies evaluating clone-derived mutant viruses. Without results showing that both the biological and clone-derived VEEV TC-83 virus behave the same with regards to replication, antibody neutralization, and virulence, research evaluating clone-derived escape mutants could not be completed.

Evaluation of the biological and clone-derived wild-type VEEV TC-83 viruses followed the same experimental procedures as planned for the evaluation of the escape mutants. Replication kinetics were compared by plaque assay and growth kinetics analysis. Antibody neutralization was compared between the biological and clone-derived wild-type VEEV TC-83 virus by PRNT. The original plan was to characterize the mutants using two monoclonal antibodies, 1A3B-7 and 1A4A-1; however, only the 1A3B-7 antibody was used to evaluate the biological and clone-derived virus since this was the antibody administered in the study in which the mutations were

identified. Lastly, virulence of the biological and clone-derived wild-type VEEV TC-83 virus was compared in mice by observing lethality, weight change, and clinical signs of illness.

The studies completed were fundamental to ensure that both the biological and clone-derived wild-type VEEV TC-83 viruses behave similarly in regards to replication, antibody neutralization, and virulence. Furthermore, the results of this study will aid to the progression of evaluating potential escape mutants and the development of therapeutic strategies to combat VEEV. Prior studies demonstrate that therapeutic antibodies may offer post-exposure protection against an alphavirus infection; however, there is the need for further monoclonal antibody therapeutic development against this family of viruses. The results of the future studies characterizing escape mutants may suggest the most effective way to combat against alphaviruses is through the administration of a broadly neutralizing antibody (bNAb) or a cocktail of mAbs recognizing different epitopes on the surface of the virus. A bNAb is a type of antibody that can recognize and block many mutant or variant strains of VEEV from entering healthy cells. Researchers are investigating whether bNAbs could be used to develop a therapeutic HIV vaccine (Burton & Hangartner, 2016). Alphaviruses have an inherently high mutation frequency and will likely require the targeting of several epitopes on the surface of the virus to reduce the potential for escape from neutralization. A cocktail containing two or three mAbs would require mutations within each epitope sequence of the genome to escape virus neutralization. Cocktails of mAbs were effective against Ebola virus infection when administered to mice and NHPs (Olinger et al., 2012; Pettitt et al., 2013; Qiu et al., 2014; Wong et al., 2014).

Material and Methods:

Generation of Mutant Infectious cDNA Clone:

As part of Aim 1 to generate virus stocks containing potential escape mutations, the mutant cDNA clones had to be generated. To generate infectious cDNA clones with the incorporated changes, the mutations had to first be introduced via site-directed mutagenesis using PCR. The QuikChange Lightning Site-Directed Mutagenesis protocol developed by Agilent Technologies was followed (Figure 4). This procedure utilizes a dsDNA vector with an insert of interest and two synthetic primers both of which contain the desired mutation. This protocol allows the rapid introduction of point mutations into sequences of interest using a pair of complementary mutagenesis primers to amplify the entire plasmid in a single PCR. The dsDNA vector used in this procedure was the live attenuated VEEV TC-83 vaccine strain cDNA clone placed within the pUC18 plasmid to create a final construct, pVE/IC-92 (Kinney et al., 1993) (Figure 5). The two nucleotide changes introduced into pVE/IC-92 were at nucleotide 9177 (cytosine to adenine) and

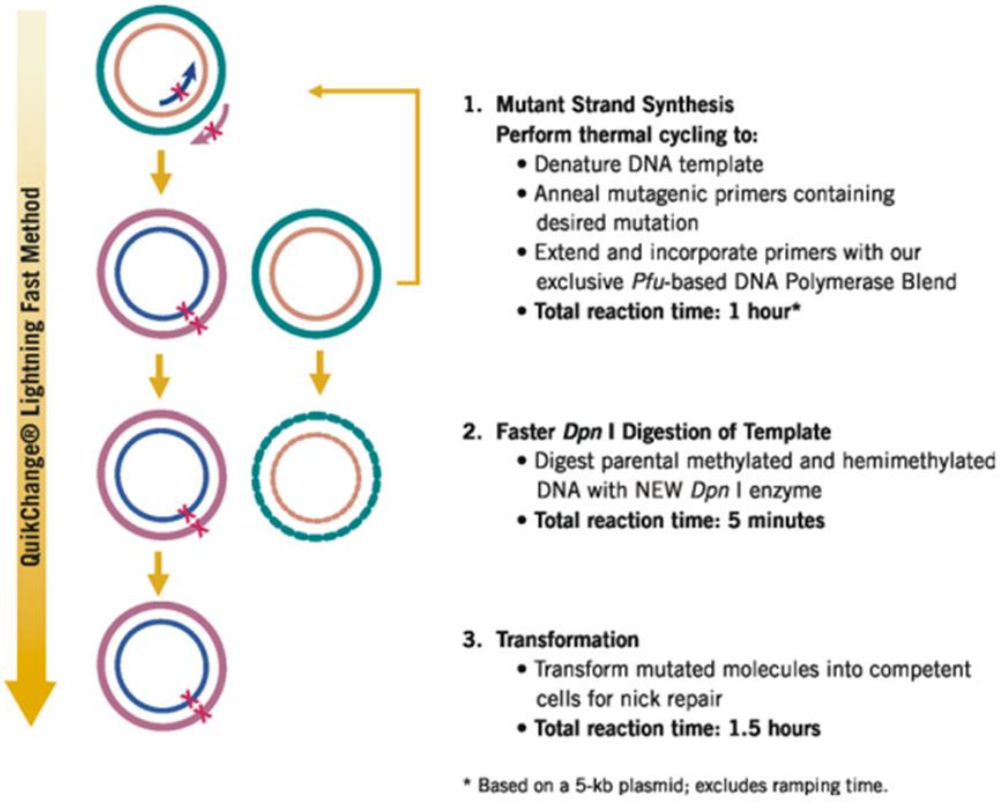

Figure 4. QuikChange Lightning site-directed mutagenesis method (Agilent Technologies, 2018). This figure provides an overview of the method utilized for introduction of the sequence changes into the cDNA clone.

nucleotide 9189 (adenine to guanine). The primers used in this procedure are shown in Figure 6; each primer was complementary to the opposite strands of the vector. Primers were redesigned following four unsuccessful attempts to amplify the mutant DNA to a concentration high enough to proceed to transformation. The redesigned primers had approximately ten additional

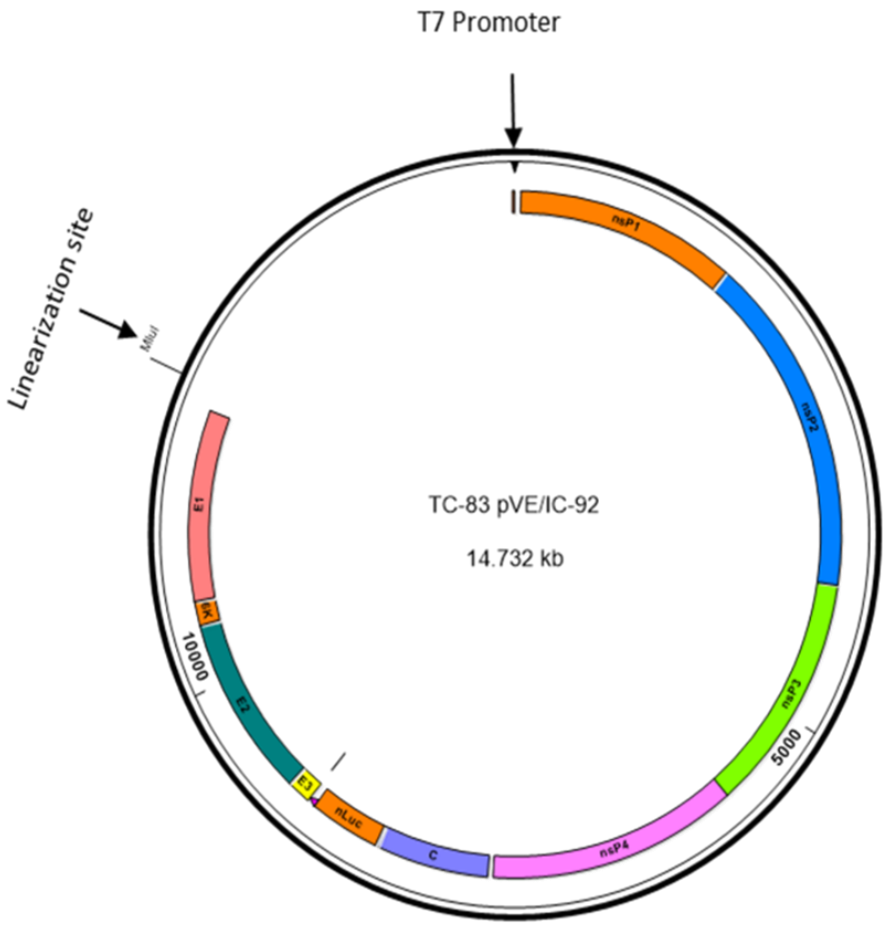

Figure 5. Infectious cDNA clone plasmid map. pVE/IC-92 plasmid with full length TC-83 virus genome encoded. The T7 promoter is the initiation site of transcription once the plasmid has been linearized via restriction digestion. Mutations were introduced within the E2 glycoprotein sequence. Diagram was generated in DNASTAR from sequence provided by Dr. Radoshitzky.

Original Primers:

TC83 9177 C to A-S	5’ GTGGCGGCA**A**AAAGATCTCCG 3’
TC83 9177 C to A-AS	5’ CGGAGATCTTT**T**TGCCGCCAC 3’
TC83 9189 A to G-S	5’ GATCTCCG**G**GACCATCAAC 3’
TC83 9189 A to G-AS	5’ GTTGATGGTC**C**CGGAGATC 3’

Redesigned Primers:

TC83 9177 C to A-S	5’ GCGAGTGTGGCGGCA**A**AAAGATCTCCGAGAC 3’
TC83 9177 C to A-AS	5’ GTCTCGGAGATCTTT**T**TGCCGCCACACTCGC 3’
TC83 9189 A to G-S	5’ CGGCACAAAGATCTCCG**G**GACCATCAACAAGACAA 3’
TC83 9189 A to G-AS	5’ TTGTCTTGTTGATGGTC**C**CGGAGATCTTTGTGCCG 3’

Figure 6. Primers for site-directed mutagenesis. Original and redesigned mutagenesis primers containing the two nucleotide changes to be incorporated into the VEEV E2 glycoprotein sequence.

nucleotides, five added to each side of the original primer, in order to increase the efficiency of complementary binding to the vector. The oligonucleotide primers were extended during thermocycling via a proprietary *Pfu*-based DNA polymerase exclusive to the QuikChange Lightning Site-Directed Mutagenesis kit. It is reported that the *PfuUltra* HF DNA polymerase has an 18-fold higher fidelity in DNA synthesis than Taq DNA polymerase (Agilent Technologies, 2015). Extension of the primers generated a mutated plasmid containing staggered nicks within the sequence. The mutated plasmid was then treated with the *Dpn* I endonuclease in order to digest the parental DNA template and to select for the mutation-containing amplified DNA.

The amplified mutant cDNA was transformed directly into β-mercaptoethanol treated XL10-Gold ultra-competent cells to allow for the production of more mutant cDNA for downstream steps. Transformation of the synthesized DNA was conducted according to the QuikChange Lightning site-directed mutagenesis protocol. Transformation of the DNA into

competent cells was achieved by a 45 second heat shock at 42°C followed by a two minute incubation on ice. One deviation to the QuikChange transformation protocol was the use of SOC media to the cells as opposed to NZY broth. These media are often used interchangeably and would not affect transformation efficiency. Cells were incubated for 60 minutes in a 37°C with shaking. Following incubation, the cells were plated on LB plates supplemented with 2X ampicillin, if making a new cDNA construct. To prepare DNA for the TC-83 wild-type cDNA clone, an aliquot from a glycerol stock of transformed cells was added to 25-30 mL of LB broth containing ampicillin (amp). The presence of the amp gene is commonly used as an indicator for the identification of bacterial clones that contain the transformed cDNA clone. The amp gene, amp^r, encodes for the enzyme β-lactamase which provides ampicillin resistance. This enzyme catalyzes the breakdown of ampicillin thereby conferring resistance to the antibiotic. By inoculating the transformed cells on an agar plate supplemented with ampicillin it is possible to identify whether the transformation was successful or not. If successful, the transformed cells, containing the plasmid, will grow on the agar plate regardless of the presence of ampicillin due to the resistance conferred by β-lactamase, if not successful no growth will occur due to inhibition of untransformed cells by ampicillin.

The mutant DNA clones were purified using the Promega Pureyield Miniprep protocol and the DNA concentration was determined. The bacterial cells harboring the plasmid DNA were lysed under alkaline conditions via cell lysis buffer. Following lysis, the bacteria chromosomal DNA and proteins were denatured while the plasmid DNA remained as a covalently-closed circle. The addition of Neutralization Solution caused the chromosomal DNA and protein to precipitate and the plasmid DNA to remain in solution thus, following centrifugation, plasmid DNA was present within the supernatant. The supernatant containing plasmid DNA was then transferred into a Pure

Yield Minicolumn. This column preferentially binds to DNA via hydrogen bonding to hydroxyl groups within the column membrane. The Minicolumn was then loaded with Endotoxin Removal Wash and Column Wash Solution to ensure that any intracellular components that did not pellet in the previous centrifugation would not be retained within the column. A final rinsing of the column with nuclease-free water allowed the purified plasmid DNA to be eluted through the column. The concentration of plasmid DNA, containing the VEEV TC-83 sequences, was then calculated using a Nanodrop 2000. Once DNA was isolated, it was sent for sequencing analysis to ensure the clone contained the desired mutation.

Generation of Clone-Derived Virus Stocks:

Clone-derived virus stocks were generated after the cDNA was linearized by restriction enzyme digest and RNA was transcribed in order to produce infectious viral genomes. A single restriction digestion was performed on the plasmid DNA in order to linearize the DNA. Restriction digestion is an enzymatic process that cleaves DNA at a specific sequence called a restriction site, each plasmid that contains the desired sequence has the sequence located at exactly the same position within the plasmid. In this procedure the plasmid was linearized at the 3' end of the VEE virus-specific cDNA insert by digestion with the MluI restriction enzyme. Figure 7 presents the restriction site sequence for MluI. Linearizing the DNA via restriction digestion is essential to the transcription of the plasmid DNA to increase accessibility of the transcriptional machinery and produce transcripts of only the desired insert. The linearized DNA was then cleaned and isolated using The Wizard SV Gel and PCR Clean-Up System. DNA was loaded into a minicolumn and treated with membrane wash solution with ethanol added. Ethanol was added to remove any

Figure 7. Digestion site for MluI restriction enzyme. Arrows designates site at which the MluI enzyme cuts the DNA.

excess water from the column; thereby only allowing DNA to bind to the column matrix. This PCR Clean-Up System is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. Following an initial centrifugation, the flow through was discarded and the minicolumn was treated with nuclease-free water. Treatment with water facilitated the release of DNA from the membrane as DNA preferentially will hydrogen bond with water and thereby exit the column as flow through. Up to 90% of the DNA is recovered using this method based on the 11,447-bp size of the cDNA clone of VEE TC-83 virus. Infectious, positive-sense, full-length viral genomic mRNA was then transcribed using a commercial *in vitro* transcription (IVT) kit (Promega). *In vitro* transcription requires a purified linear DNA template containing a promoter, an appropriate phage RNA polymerase, ribonucleoside triphosphates, and a buffer system that includes dithiothreitol (DTT) and magnesium ions used to stabilize the polymerase. Transcriptions were performed in a reaction containing a linearized cDNA clones, bacteriophage T7 RNA polymerase, and a mixture of ribonucleoside triphosphates (ATP, CTP, GTP, and UTP). Following the production of the RNA transcripts, these infectious, positive-sense RNA were transfected into eukaryotic cells. RNA was transfected into baby hamster kidney (BHK) cells through electroporation. Electroporation uses pulses of electricity to briefly increase the permeability of cellular membranes to allow for the entry of exogenous DNA or RNA into host cells. Exposing the cellular membranes to a high-voltage electric field results in their temporary breakdown and the formation of pores that are large enough to allow macromolecules (such as

nucleic acids) to enter or leave the cell. BHK cells were chosen as they are highly permissive to alphaviral infection and will produce the highest viral titer following transfection. The efficiency of electroporation is extremely important and a transfection efficiency of 95 to 100% must be achieved to ensure only a single round of viral replication for the production of virus stock. The transfection efficiency indicates what percent of BHK cells successfully took up the RNA transcripts. Multiple rounds of replication following low efficiency transfection would increase the potential for introduction of unwanted mutations by the viral-encoded RNA-dependent RNA polymerase. BHK cells were harvested and suspended in Opti-MEM for electroporation. The purified RNA transcripts (20 µg) were added to the cells for electroporation with the BioRad Gene Pulser with the capacitance extender. The cell + RNA mixture was shocked using two pulses of 220V and 1000 µF. The electroporated BHK cells were then incubated at 37°C in a mixture of minimal essential medium (MEM) and containing 10% fetal bovine serum (FBS) until cytopathic effects were clearly present in the cells.

Virus particles were harvested from the supernatant of the electroporated BHK cells after 18 to 24 hours of incubation. The harvested supernatant was centrifuged at 3500 rpm in order to separate the cellular debris and virus progeny; the cellular debris pelleted at the bottom of the centrifuge tube while the virus remained within the supernatant. This clarified supernatant was aliquoted in 250 µL aliquots. The electroporation supernatant is the p0 master stock of a clone-derived virus. The virus titer, a numerical expression of the quantity of virus in a given volume often expressed as plaque forming units per milliliter (PFU/mL), was determined by plaque assay on Vero76 cells (kidney epithelial cells originally from an African green monkey). Ten-fold dilutions of the virus stock were prepared, and 0.1 ml aliquots were inoculated onto Vero76 cells. After a 1 hour incubation period, the cells were covered with a nutrient rich agar. The purpose of

the nutrient-rich agar was to reduce virus spread to only cells in close contact to the infected cells. Upon incubation of cells overlayed with agar, the infected cells release viral progeny. The spread of the new viruses was restricted to neighboring cells by the solidified agar. Consequently, each infectious particle produced a clear circular zone of infected cells called a plaque. The titer of a virus stock was calculated by counting the number of plaques and taking into account the dilution factor at which the plaques were counted along with the volume used during the infection. This calculation determines the titer in units of PFU/mL. Using this calculated viral titer from the plaque assay, the volume of the p0 virus stock required for production of working virus stocks was calculated. To reduce multiple rounds of replication in preparation of working stocks and the potential for the introduction of unwanted mutations, cells were infected at a multiplicity of infection (MOI) of 1. The MOI is the average number of virus particles infecting each cell and determined by the following equation: [(the number of cells to be infected) x (MOI PFU/cell)] x [1/ virus titer (PFU/mL)]. Thus, for an MOI of 1, if there are one million Vero cells, one million TC-83 virions must be added and the volume that must be added can be calculated using the virus titer. Once determined, the required volume of the p0 master stock was used to infect Vero cells at a MOI of 1. The cell supernatant was harvested, this time 24-48 hours post infection to ensure the virus was replicated and released and the Vero cells were killed via viral induction of an apoptotic pathway. The cell debris was centrifuged and the supernatant containing the virus was aliquoted and stored at -80 ºC, this stock was designated the p1 working stock. The same protocol was followed for the preparation of wild-type clone derived VEEV TC-83.

Generation of Biological Virus:

Biological viruses was previously prepared in the lab. The TC83 vaccine lot 9 run 3 was passaged twice on Vero76 cells to make a master stock. Diluted master stock aliquots were created

to minimize the need for multiple freeze thaws of the master stock aliquots and for in vitro assays that did not require large amounts of virus.

Evaluation of Biological and Clone-derived Viruses.

Plaque reduction neutralization test (PRNT):

In vitro neutralization was measured using the plaque reduction neutralization test (PRNT). The design of the PRNT allows for virus-antibody interaction to occur in a microtiter plate. Antibody effects on viral infectivity are then measured by plating the virus-antibody mixture on virus-susceptible cells. The cells are overlaid with a semi-solid medium that restricts spread of progeny virus. Each virus that initiates a productive infection produces a localized area of infection (a plaque), that can be detected using stains or vital dyes. Plaques are counted and compared with the starting concentration of virus to determine the percentage reduction in total virus infectivity. In the PRNT, the serum or antibody-containing specimen being tested is subjected to serial dilutions prior to mixing with a standardized amount of virus. The concentration of virus is held constant such that, when added to susceptible cells and overlaid with semi-solid medium, individual plaques can be discerned and counted. In this way, PRNT endpoint titers can be calculated for each serum specimen at any selected percentage reduction of virus activity (Roehrig et al., 2008).

Antibody 1A3B-7 samples of known protein concentration were diluted in MEM and then serially diluted 1:2. Virus stocks were diluted to a concentration of 2.0×10^3 PFU/mL and added 1:1 to the serially diluted samples or control wells containing media alone for the virus only control. All samples were incubated overnight at 2-8ºC. Vero 76 cells seeded in 6-well plates were grown to ~90-100% confluence. Cells were infected with 0.1 mL per well of each serial dilution

in duplicate. Plates were incubated at 37ºC for about 1 hour with gentle rocking every 15 minutes. After 1 hour, cells were overlaid with nutrient-rich agar and incubated for 24 hours at 37ºC. A second overlay of nutrient-rich agar and 5% of total volume neutral red vital stain was added to wells and further incubated 18-24 hours for visualization of plaques. Plaques were counted following an 18 hour incubation with stain overlay. The total PFU input into the assay was determined by counting plaques in the virus only control wells. This value was used to determine the values at which 50% of the virus was neutralized (PRNT50) or 80% of the virus was neutralized (PRNT80). Plaques were counted in all wells for the samples containing antibody until the antibody concentration resulted in less than 50% neutralization of the virus. The PRNT80 and PRNT50 were expressed as the last dilution that resulted in either 80% or 50% percent neutralization compared to virus only control.

***In Vivo* Characterization of Viruses:**

Adult, female C3H/HeN mice (6 to 8 weeks old) were used for *in vivo* characterization of the biological and clone-derived TC-83 viruses. C3H/HeN mice are used in a wide range of studies including neuro-sensorial biology which would be appropriate for characterizing an encephalitic virus (Janvier Labs, 2017). This mouse model was described previously for evaluation of potential therapeutics against VEEV infection (Julander et al., 2008a; Julander et al., 2008b). Mice were infected via the intranasal route with either the biological or clone-derived strain of the VEEV TC-83 virus. Mice were observed twice daily for weight change as well as clinical signs of illness (ruffled fur, depression, anorexia, and/or paralysis) and death. Clinical scoring observed the outward appearance of the infected subject as the infection progresses, scoring includes: healthy, minor alteration in normal fur or soiled, ruffled fur, an outward curvature of the spine at the back causing hunching, lethargy and decreased activity levels, neurological signs such as hindlimb paralysis or unresponsive, even when stimulated, and death.

Results:

Generation of Mutant Infectious cDNA Clone:

The site-directed mutagenesis PCR followed digestion with DpnI and transformation was unsuccessful using the first set of primers in the reaction. Once the primers were redesigned, transformation of competent E. coli cells with the DpnI digested product resulted in the growth of clones on LB amp plates. Several clones were isolated and grown in LB broth. Plasmid DNA was purified from several clones for sequence analysis. Sequence analysis of the cDNA plasmid preparations is in progress. Because these clones could not be completed in time to prepare virus, studies to evaluate the biological and cloned-derived wild-type TC-83 viruses were completed and are presented in the following sections below.

Replication Kinetics of Biological versus Clone-Derived Wild-type VEEV TC-83 Viruses:

The number of virus particles generated by the biological or clone-derived VEEV TC-83 viruses were similar (Figure 8). Virus titer was calculated for the biological strain as well as both the p0 master stock and the p1 working stock. The results showed that at a multiplicity of infection 1.0 (1:1 ratio of virus particles and Vero cells) and 0.1 (1:10 ratio of virus particles and Vero cells) both the biological and clone-derived TC-83 virus produced similar viral titers. At both an MOI of 1.0 and 0.1, the biological, p0 master, and p1 working virus stocks of VEEV TC-83 all produced titers of approximately 9.2×10^9 PFU/mL. Furthermore, the kinetics of viral growth were identical for the biological and clone-derived VEEV TC-83 at the 6 hour and 24 hour time points post-infection (Figure 9).

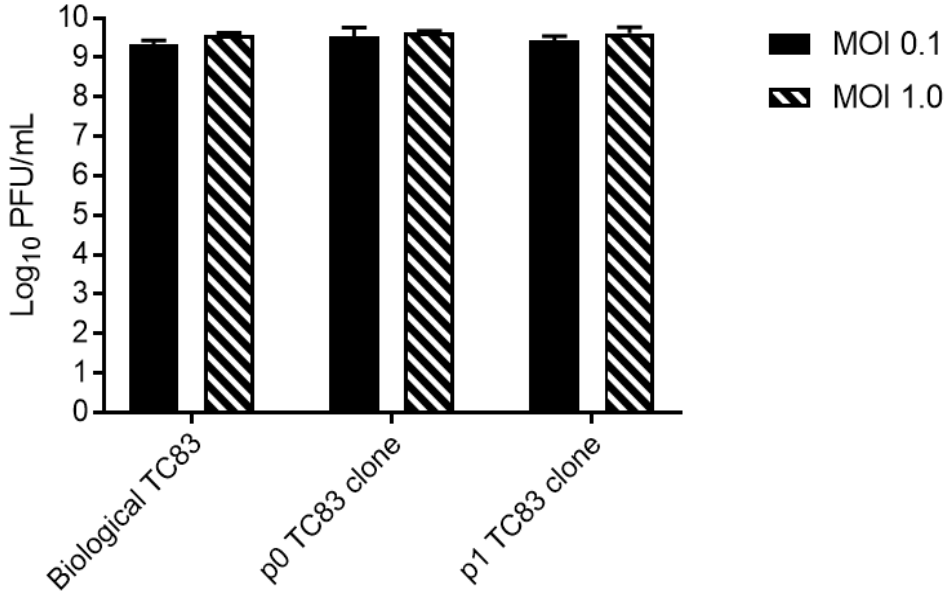

Figure 8. Virus yield following infection of Vero cells different MOIs. Vero cells were infected at a MOI of 0.1 or 1.0. Supernatants were harvested at 18 hours post-infection and virus yield (PFU/mL) was determined by plaque assay. Regardless of MOI, the biological and clone-derived VEEV TC-83 virus yielded equivalent amounts of virus.

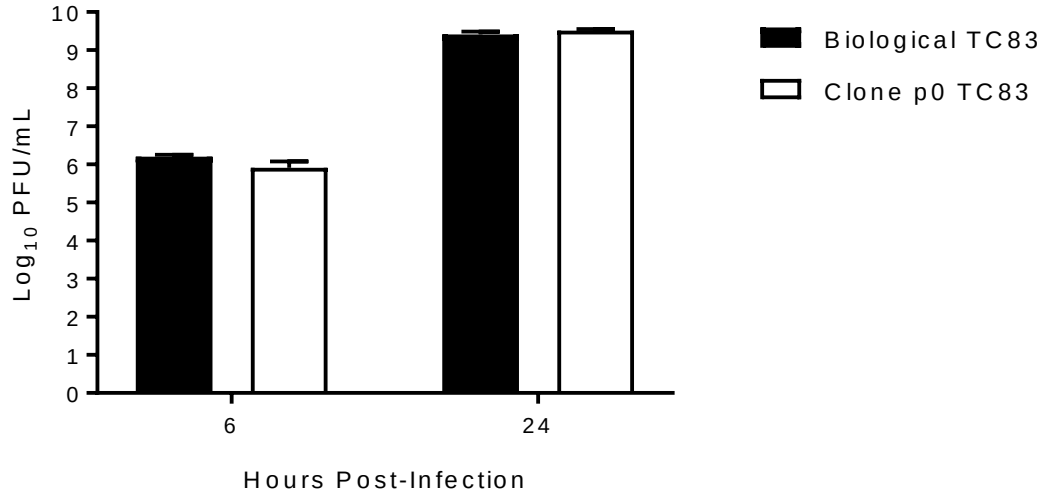

Figure 9. Kinetics of virus growth in Vero cells. Vero cells were infected at an MOI of 0.1. Supernatants (or an aliquot) were collected at 6 or 24 hours post-infection for virus yield evaluation. Virus yields at both times collected were identical for the biological and clone-derived VEEV TC-83 viruses.

Antibody Binding and Neutralization of Biological and Clone-Derived VEEV TC-83:

Results of the PRNT are presented in Figure 10. At concentrations above 0.5 µg/mL, the antibody 1A3B-7 was consistent in neutralizing both the biological and clone-derived VEEV TC-83 virus with similar percent neutralization values. At 50 µg/mL the antibody was effective at neutralizing nearly 100% of the biological or clone-derived VEEV TC-83. The PRNT80 for 1A3B-7 for both the biological TC83 and the p1 TC83 clone was 6.25ug/mL. The PRNT50 for 1A3B-7 for the biological TC83 was 0.78ug/mL and for the p1 TC83 clone was 0.39ug/mL. The difference in the PRNT50 between the two viruses is a 2 fold difference which is within the variability of the assay.

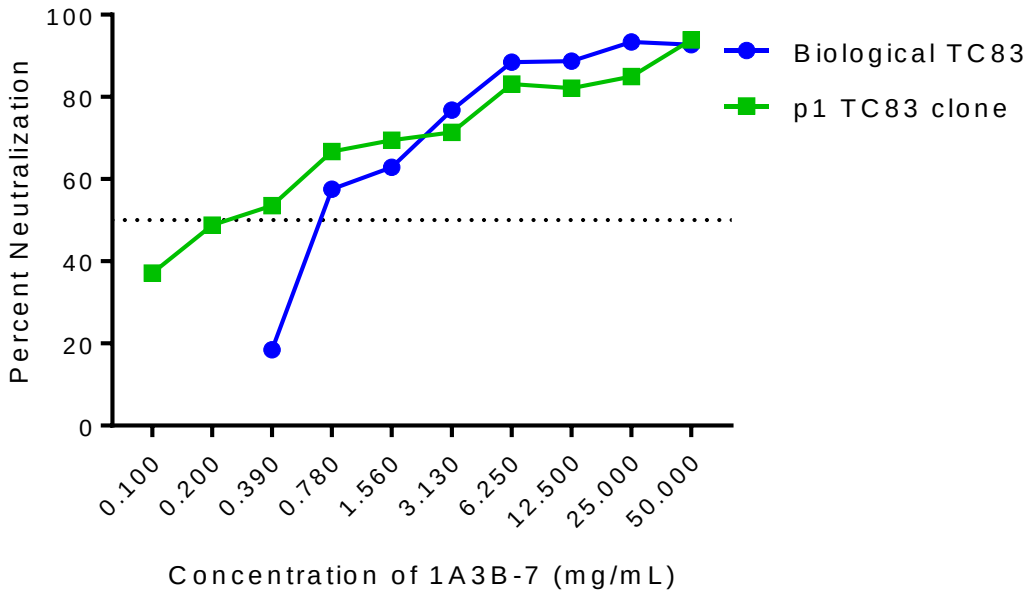

Figure 10. Neutralization of virus with antibody 1A3B-7. The ability of antibody 1A3B-7 to neutralize biological and clone-derived virus was evaluated in a PRNT.

***In-Vivo* Screening of Biological Versus Clone-Derived VEEV TC-83 viruses:**

In-vivo screening of the biological and clone-derived VEEV TC-83 viruses was accomplished through intranasal infection into C3H/HeN mice. The virulence of the biological and clone-derived VEEV TC-83 viruses was compared by evaluating weight loss, clinical signs of illness scoring, and lethality.

Weight loss, which is an observable measurement of virulence, was compared for the biological and clone-derived VEEV TC-83. Weight was measured in infected C3H/HeN mice up to 21 days post-infection. Weight loss was a much more consistent measurement when comparing the biological and clone-derived VEEV TC-83 virus (Figure 11-12). For both the biological and clone-derived VEEV TC-83 viruses, the greatest weight loss occurred between 9 and 10 days post-infection, after which the animals gained weight (Figures 11-12).

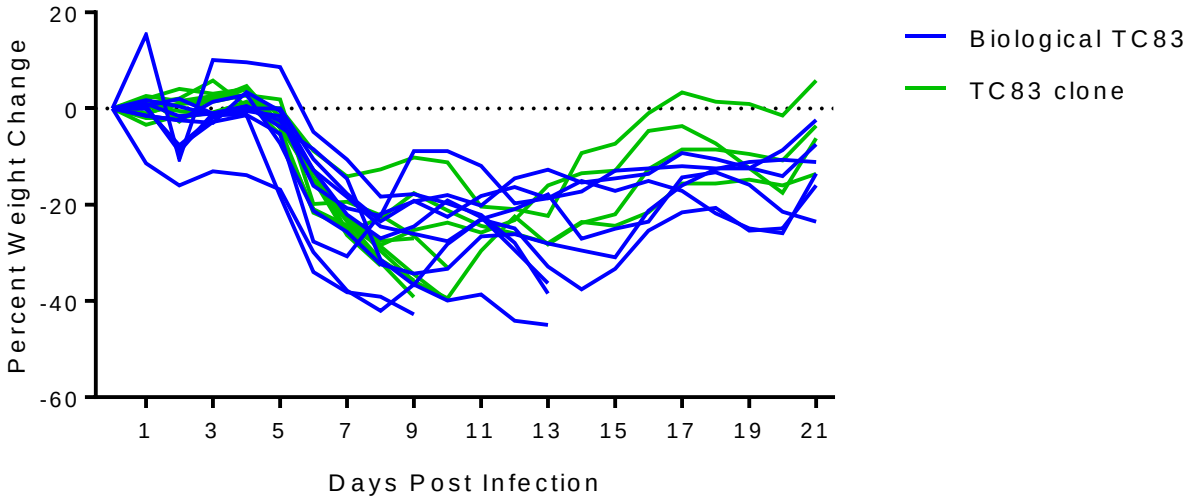

Figure 12. Change in body weight in individual mice. The percent weight change of C3H/HeN mice challenged with intranasal infection with either the biological or clone-derived VEEV TC-83 was similar regardless of virus received with the greatest weight change occurring between 9 and 10 days post-infection.

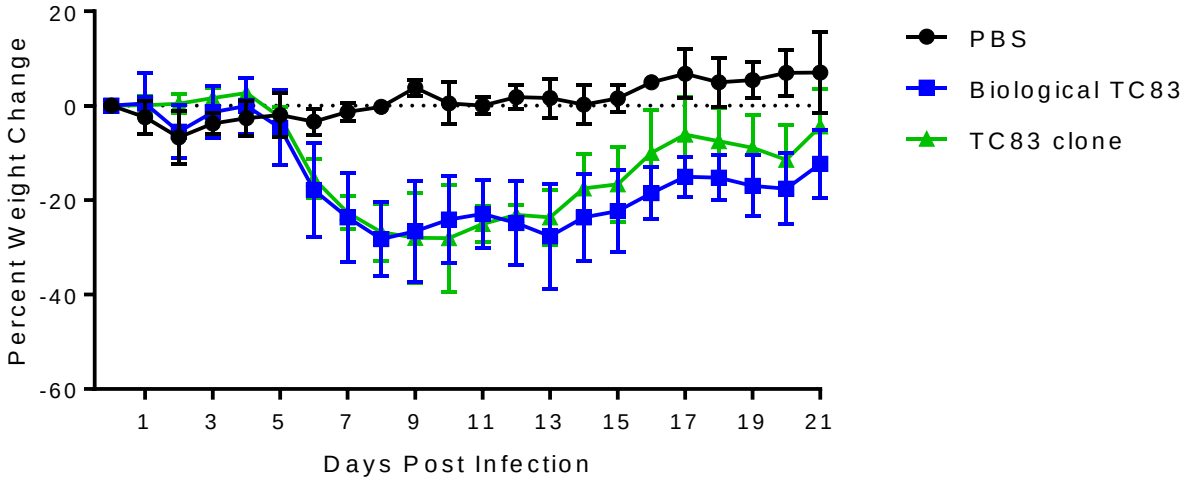

Figure 11. Average body weight following infection in mice. Average percent change in weight was similar following intranasal infection of C3H/HeN mice with the biological or clone-derived VEEV TC-83 virus. A mock-infected control group was administered PBS instead of virus.

Another facet used to compare virulence between the biological and clone-derived VEEV TC-83 viruses was clinical signs of illness observed in mice. The scoring key used to assess clinical illness is presented in the legends of Figures 13 and 14. All mice remained healthy up to 4 days post-infection. All infected mice were hunched and less active by day 4 or 5 post-infection regardless of whether infected with the biological or clone-derived VEEV TC-83 virus. Between days 7 and 10, all mice developed anorexia and lethargy, while some mice progressed demonstrating neurological signs or unresponsiveness and meeting the euthanasia criteria. Analysis of the average clinical score for groups infected with the biological or cDNA clone-derived virus revealed the score and duration of illness were virtually identical for these viruses in this mouse model (Figure 15).

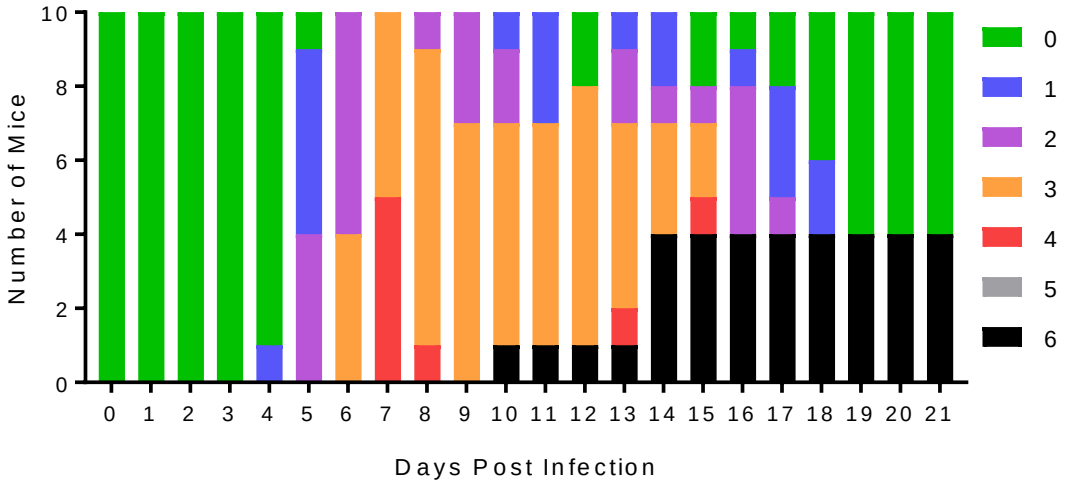

Figure 13. Clinical scoring of C3H/HeN mice infected with the biological VEEV TC-83 virus. Mice were observed twice daily for clinical signs of disease. Scoring was as follows: 0=Healthy, 1=Decreased grooming-minor alteration in normal fur or soiled, 2=Ruffled fur-severe alteration in normal fur; raised fur with ruffled appearance, 3=Hunched posture-outward curvature of the spine at the back causing hunching, 4=Lethargic-decreased activity, animal not moving as much as normal, 5=Neurological signs (circling/hind limb paralysis) or unresponsive, even when stimulated, 6=Dead. Bars indicate the number of mice that received a specific score for each day.

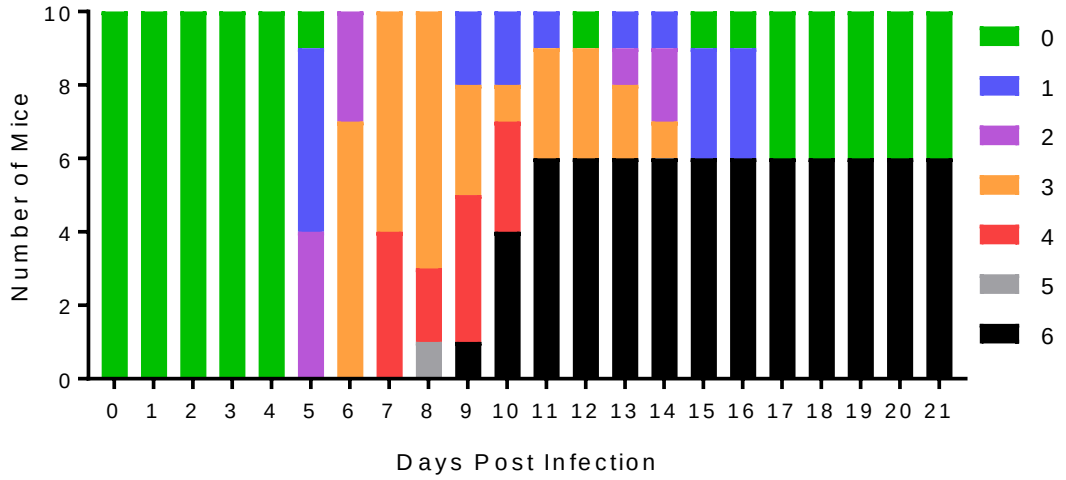

Figure 14. Clinical scoring of C3H/HeN mice infected with the clone-derived VEEV TC-83 virus. Mice were observed twice daily for clinical signs of disease. Scoring was as follows: 0=Healthy, 1=Decreased grooming-minor alteration in normal fur or soiled, 2=Ruffled fur-severe alteration in normal fur; raised fur with ruffled appearance, 3=Hunched posture-outward curvature of the spine at the back causing hunching, 4=Lethargic-decreased activity, animal not moving as much as normal, 5=Neurological signs (circling/hindlimb paralysis) or unresponsive, even when stimulated, 6=Dead. Bars indicate the number of mice that received a specific score for each day at the AM observation, since not all groups received a PM observation every day.

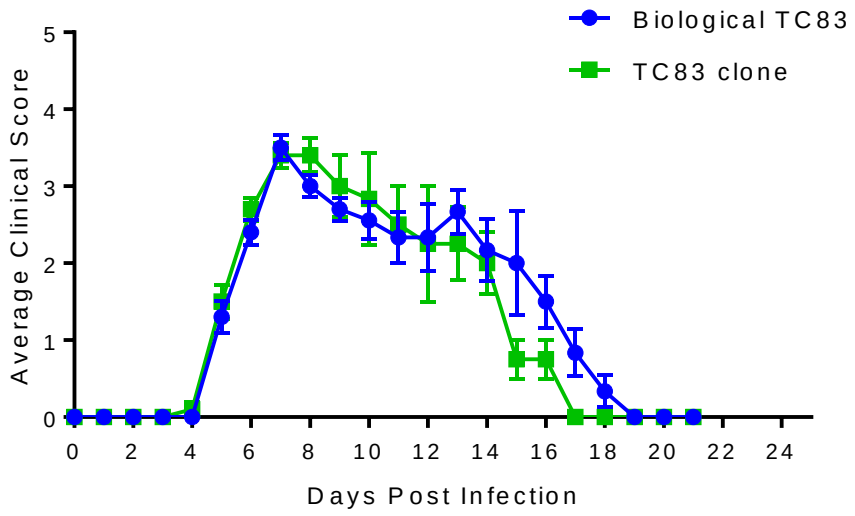

Figure 15. Average clinical scores of C3H/HeN mice infected with the biological or cDNA-clone derived virus. The average clinical score was determined for each group using the AM observation score, since not all groups had a PM observation every day. Each point is the average clinical score each day, error bars represent the standard error.

The final facet used to compare virulence was lethality associated with the biological versus the clone-derived VEEV TC-83 viruses (Figure 16). Infection with either biological or clone-derived VEEV TC-83 virus was only partially lethal resulting in 40% or 60% lethality, respectively. This difference was not statistically significant by Chi-square test or Mantel Cox log rank test. It was evident that the clone-derived virus had a slightly faster time to death, with the first fatality occurring eight days post-infection as opposed to 10 days for the biological VEEV TC-83 virus. This difference in average survival time was statistically different (Student's t-test, $p=0.007$).

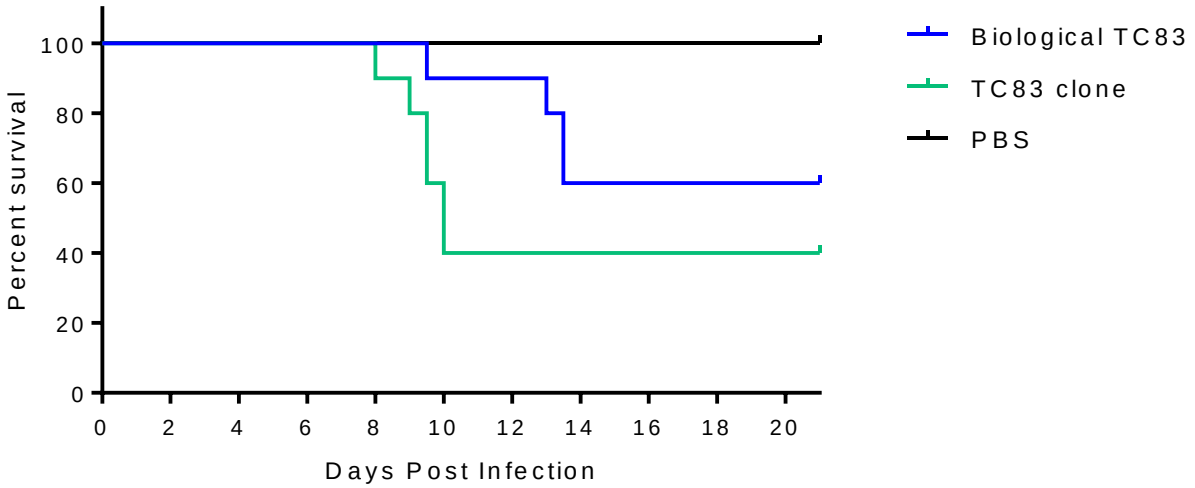

Figure 16. Survival in mice following infection with biological or clone-derived VEEV TC-83 virus. Lethality was greater as well as quicker in C3H/HeN mice following intranasal infection of the clone-derived strain of VEEV TC-83 virus as compared to the biological VEEV TC-83 virus.

Discussion:

The objective of this study was to evaluate variations in replication kinetics, antibody neutralization, and virulence in biological and cloned-derived viruses. While the full study intended to generate and characterize mutant VEEV TC-83 viruses and compare these to wild-type VEEV TC-83 viruses, the challenges that were faced during the construction of the mutant viruses delayed the ability to compare the mutant viruses. The cDNA clones containing the individual mutations were prepared and sequencing to confirm the introduction of the intended mutations is in progress. In spite of these delays, the comparison studies continued focusing on the evaluation of wild-type and clone-derived TC-83 viruses. The experimental design was exactly the same for the necessary evaluation of these 'controls' and provided the foundation required to move forward with the analysis of the potential antibody escape mutant viruses. It was expected that the

biological and clone-derived wild-type evaluation would be conducted concurrently with the mutant viruses as these samples provide critical information required to ensure that both the biological and clone-derived wild-type VEEV TC-83 viruses behave similarly with regard to replication, the ability of particular antibodies to neutralize the virus, and virulence within an infected host. Replication kinetics of the biological and clone-derived wild-type VEEV TC-83 viruses evaluated virus production over the life cycle of virus production. Neutralizing antibody concentrations were determined for each virus by PRNT. Lastly, virulence within an infected host was compared using lethality, weight loss, and clinical signs of illness scoring to ensure that both the biological and clone-derived wild-type VEEV TC-83 viruses elicited the same symptoms over a similar time frame.

The results of the virus yield and growth kinetic analysis revealed that both the biological and clone-derived VEEV TC-83 viruses replicated to a similar titers over the course of infection. Variations in MOI of 1.0 and 0.1 for both viruses did not result in differences in virus yield. 6wild-typeBased on these results, no difference was detected in virus replication between the biological and clone-derived wild-type VEEV TC-83 viruses.

Neutralization of the biological and clone-derived wild-type VEEV TC-83 viruses was evaluated using the PRNT. Percent neutralization of the biological and clone-derived wild-type VEEV TC-83 viruses were quite consistent with each other until the concentration of the 1A3B-7 antibody dropped below 0.5 µg/mL. At concentrations above 0.5 µg/mL of antibody, there was minimal variation in percent neutralization between the two viruses. Nearly complete neutralization was achieved with an antibody concentration starting at 50 µg/mL. The PRNT50 for both viruses were identical. While the PRNT80 values were not identical for the two viruses; they were within a dilution of each other which is within the variability of the assay. Therefore,

these results demonstrate that both the biological and clone-derived VEEV TC-83 viruses were neutralized equally by antibody 1A3B-7.

Virulence in a mouse model of VEEV infection was assessed by weight change, clinical signs of illness scoring, and lethality. Both viruses resulted in significant, yet similar weight loss in mice. Additionally, infection with these virues resulted in similar clinical signs of illness with respect to score and duration of illness. There was a significant difference in the average survival time; however, the difference in percent survival between the infected groups of mice was not statistically different. .

Taken together, the results of these studies demonstrated that the biological and clone-derived VEEV TC-83 viruses behaved similarly with regards to replication in cell culture and antibody neutralization. Though there appeared to be some differences in virulence in the C3H/HeN mouse model, the difference in survival was not statistically different. The results of these studies were important to provide the necessary foundation for characterization of the potential antibody escape mutations as the mutants viruses must be clone-derived from cDNA. Without ensuring that cDNA clone-derived virus behaved comparable to the biological virus there would be no control for future research that requires the use of clone-derived VEEV. Future research will observe the two escape mutant viruses with nucleotide changes in the E2 glycoprotein at nucleotide 9177 and 9189. Characterization of these mutants will follow the same methods presented in this work, including evaluation of replication in cell culture, antibody binding and neutralization, and virulence in a mouse model of VEEV infection. The study will also observe any changes in efficacy within infected hosts administered 1A3B-7 antibody treatment. Evaluation of the escape mutants may suggest the most effective way to develop a therapeutic against VEEV

would be through the administration of a broadly neutralizing antibody or a cocktail of monoclonal antibodies which recognize multiple epitopes on the surface of the virus particle.

Acknowledgements:

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

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