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Development of an Attenuated Zika Virus by Editing the 5' Untranslated Region

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DEVELOPMENT OF AN ATTENUATED ZIKA VIRUS BY EDITING THE 5' UNTRANSLATED REGION

by

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ABSTRACT

Zika Virus (ZIKV) is a mosquito-transmitted flavivirus that usually causes no symptoms to mild febrile in humans, and it has been regarded as an insignificant pathogen to public health. However, recent outbreaks of ZIKV infection have revealed that ZIKV can cause severe neurological effects in adults, such as Guillain-Barre Syndrome (GBS), and in infants whose mothers acquired the virus during pregnancy, causing Congenital Zika Syndrome (CZS). Currently, no approved vaccine is available and there is a critical need to develop an effective and safe vaccine. While most vaccine developmental strategies target the viral prM-E protein of ZIKV, we aimed to create an attenuated ZIKV by inserting several different nucleotide lengths into the 5' untranslated region (UTR) of the viral genome. We generated a viable mutated ZIKV virus (Z7) was after inserting a 50-nt sequence into the 5'UTR. We then characterized Z7 by comparing growth kinetics *in vivo* replication by using qPCR and immunostaining assays in cell cultures. We also measured the infectivity and immunogenicity of Z7 in *Ifnar1*$^{-/-}$ mice. We found that this mutated virus developed a lower viremia compared to WT ZIKV control but induced a similar level of antibody response. Importantly, one dose of Z7 inoculation can protect the mice from a secondary high dose of ZIKV infection in *Ifnar1*$^{-/-}$ mice. Together, these results suggest that we have successfully developed an attenuated ZIKV strain, which induces protective immune responses against ZIKV infection in mice.

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To determine the viral growth kinetics, $5x10^5$ Vero cells were plated in 6-well plates and were infected with 100 µL of virus-containing DMEM diluted with 900 µL of fresh DMEM and allowed the virus to attach for 1 hour at 37°C. Cells were collected on days 2, 3, 4, 5 and 6 post-infection. After collection and qPCR was performed to measure the expression levels of *ZIKVE*. The qPCR results showed that while Z1 peaked after longer incubation than Z7, Z1 replicated at much higher levels than Z7 consistently at all the tested time points (Figure 4.1A and B). To exclude the possibility of an effect of dose variation, $5x10^4$ cells were plated in 24-well plates and infected with MOI of 0.05 of viruses for one hour at 37°C. Cells were collected on days 2 and 4 p.i. for qPCR, and the results showed a decreasing trend of Z7 replication (Figure 4.1C). To measure the difference of ZIKV E protein expression of the two viruses, $5x10^5$ Vero cells were infected with the viruses of 100 FFA and 10 FFA in duplicate for 1 hour and allowed to incubate for 3 to 5 days previously determined by Focus Forming Assay staining. These results showed that Z1 had more clear foci as well as higher numbers of foci as compared to Z7, which had very faint staining but countable foci in the 10 FFA wells (Table 4.3). Z7 was incubated for 5 days whereas Z1 only needed 3 days for FFA staining, suggesting that Z7 produces a lower amount of viral proteins over a longer time than Z1, indicating Z7 has a lower replication rate compared to Z1 the WT ZIKV strain. further indicating Z7 has a lower replication rate compared to Z1 the WT ZIKV strain. 37

We next wanted to test if Z7 replicates more slowly in an animal model. Eight-week-old and four-week-old *Ifnar1*$^{-/-}$ mice were infected intraperitoneally with 300 PFU of each virus in 100 µL of DMEM. Mice were bled once for three days post-infection and the

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LIST OF ABBREVIATIONS

YFV	*Yellow Fever Virus*
WNV	*West Nile Virus*
DENV	*Dengue Virus*
ZIKV	*Zika Virus*
JEV	*Japanese Encephalitis Virus*
FDA	*Food and Drug Administration*
GBS	*Guillian-Barre Syndrome*
RNA	*Ribonucleic Acid*
UTR	*Untranslated Region*
C	*Capsid*
prM	*pre-Membrane*
E	*Envelope protein*
NS	*Non-structural protein*
RdRp	*RNA-dependent RNA polymerase*
eIF4E	*Eukaryotic translation initiation factor 4E*
SL	*Stem loop*
HP	*Hairpin*
DB	*Dumbbell*
ER	*Endoplasmic reticulum*
ATP	*Adenosine triphosphate*
CZS	*Congenital Zika Syndrome*
PNS	*Peripheral Nervous System*

Ad.	*Aedes*
DAAs	*Direct acting antivirals*
HAAs	*Host acting antivirals*
VLP	*Virus-like particles*
DNA	*Deoxyribonucleic acid*
NHP	*Non-human primate*
Ifnar1$^{-/-}$	*Interferon receptor a deficient*
USM	*University of Southern Mississippi*
IACUC	*Institutional Animal Care and Use Committee*
BSL	*Biosafety level*
UTMB	*University of Texas-Medical Branch*
ATCC	*American Type Culture Collection*
DMEM	*Dulbecco's Modified Eagle Medium*
FBS	*Fetal Bovine Serum*
P/S	*Penicillin/Streptomycin*
cDNA	*Complementary DNA*
qPCR	*Quantitative Polymerase chain reaction*
tRNA	*Transfer RNA*
MOI	*Multiplicity of infection*
PFU	*Plaque forming unit*
D.p.i	*Days post infection*
Z1	*Zika-1*
Z7	*Zika-7*

FFA	*Focus forming assay*
ELISA	*Enzyme-linked immunosorbent assay*
i.p.	*Intraperitoneal*
nt	*Nucleotide*

CHAPTER I INTRODUCTION

1.1 Flavivirus

Flaviviruses are enveloped, positive sensed, single-stranded RNA viruses that belong to the family Flaviviridae, which also includes the genera Hepacivirus, specific to Hepatitis C virus, and Pestivirus, which holds several agriculturally important viruses that infect stock animals (Yun & Lee, 2017). The first virus classified in the genus Flavivirus was the Yellow Fever virus (YFV) in 1928, but the fever associated with that virus was known in the Caribbean and Americas since the early 17th century. Its relationship to being vectored by mosquitos was established by the Yellow Fever Commission in 1897 (Holbrook, 2017). Throughout the 20th and 21st centuries, many medically important flaviviruses have been increasing in incidence and re-emerging across the tropical and subtropical areas of the world, including the emergence of West Nile virus (WNV) in the United States in 1999, Dengue virus (DENV) in the subtropics on yearly cycles, and Zika virus (ZIKV) in Brazil in 2015 (Holbrook, 2017; Musso & Gubler, 2016). WNV has spread throughout the globe except Antarctica and causes 24,000 neurological disease cases and 2,300 deaths in the U.S. (Holbrook, 2017) (Kaiser JA, Barrett ADT. 2019). DENV has four different serotypes that all can cause Dengue Fever, Dengue Hemorrhagic Fever, and Dengue Shock Syndrome through a mechanism called antibody-dependent enhancement, which has made vaccine development difficult (Barrows et al., 2018; Garcia-Blanco et al., 2016). Japanese encephalitis virus (JEV) is another genus member that causes severe neurological disease, however, there has been an effective vaccine approved by the FDA (Barrows et al., 2018; Prevention, 2019). ZIKV is a newly emerging virus in the Americas as of 2015-6 and has been implicated in causing a febrile

like illness in most patients but microcephaly in neonates and Guillain-Barré Syndrome (GBS) in some adults (Musso & Gubler, 2016; Shankar et al., 2017; Yun & Lee, 2017). Except for YFV and JEV, there are no effective and safe vaccines against most of the flaviviruses.

1.2 Genome

Flaviviruses have an 11kb long, positive sense, single-stranded RNA genome. The genome is comprised of a 5' untranslated region (UTR), an open reading frame that translates into a polyprotein, and a 3'UTR. The polyprotein is then cleaved by the cellular and viral enzymes into ten functional proteins, i.e. three structural proteins, capsid (C), pre-membrane (prM), and envelope (E) proteins that aid in the formation of the viral particles and the non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that provide crucial viral enzymes for multiplication of the viral genome (Bai et al., 2019; Barrows et al., 2018). The UTRs of the flavivirus genome have been an areas of live attenuated vaccine research in recent years due to the specific structures held within crucial for RNA-dependent RNA polymerase (RdRp) used in viral replication, with each having a slightly "sticky end" function that allows them to circularize the genome during replication (Ng et al., 2017).

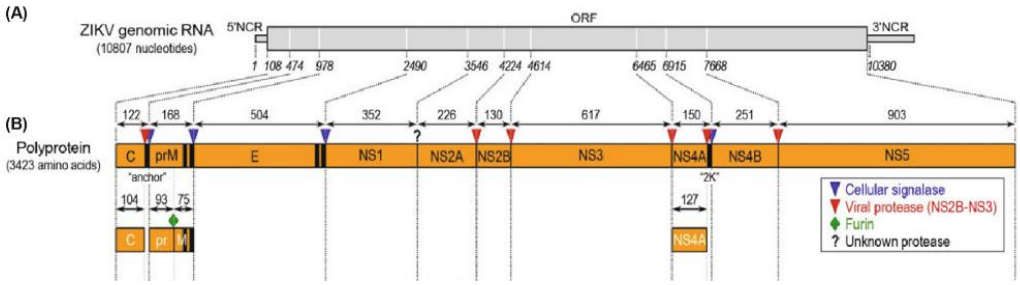


Figure 1.1 Flavivirus Genome and Polyprotein (Yun & Lee, 2017)

1.3 Flavivirus Lifecycle and Genome Replication

In general, flaviviruses enter the cell through receptor-mediated endocytosis via cell surface receptors that engage with the viral E protein. In the acidic environment of the late endosome, the membranes of the virus and endosome fuse, releasing the viral genome into the cytoplasm. The viral genome is translated within the cytosol using cellular and viral enzymes into a single polyprotein, which cleaved by viral and host proteases into 10 functional proteins needed for viral genome replication and virion assembly (Barrows et al., 2018).

To begin translation, host factor eIF4E recognizes the methyl cap and the highly structured 5'UTR. The host factor recruits ribosomes and begins the genome translation process; however, the 3'UTR is also critically important to translation as it has the downstream UAR codon that aides in proper enzyme binding after interacting with the 5' UTR AUG codon (Brinton & Basu, 2015). This is accomplished by cyclization of the genome, in which the 5' UTR secondary structures interact with the 3' UTR (Barrows et al., 2018; P. Li et al., 2018).

As the initial translation is occurring in the cytosol, the entire complex is moving toward the endoplasmic reticulum (ER) of the cell. The three viral proteins, the capsid, prM, and E proteins, are structural proteins to be built into virions. The detailed functions of NS1 during viral replication are not fully understood, but NS1 is vital to replication and localize to the sites of RNA synthesis, as does NS2. NS3, however, is highly active in genome replication as protease, an ATPase-driven helicase, and a 5' RNA triphosphatase, which is used before NS5 RNA uncapping. NS4A and B are components of the replication complex, and NS4A is thought to specifically aid in protein scaffolding and

NS4B interacts with NS3 to aid in replication. NS5 is arguably the most active viral protein, acting as the RNA dependent RNA polymerase (RdRp) and methyltransferase that caps the replicated viral genome (Barrows et al., 2018).

Once these NS proteins are processed, the virus transcribes its genome into complementary, negative-sense RNAs that serve as templates for the positive viral genome replication. Once enough genomic RNA has been replicated, the structural proteins are recruited and the virions begin to assemble and egress through the ER, taking its membrane to form the viral membrane. The virions are then fully matured as they egress from the ER to the Golgi apparatus through an acidic pH change that separates the pr- and M portions of the prM allowing for the release of pr upon entering the neutral pH of the cytoplasm. The mature virions are then secreted through exocytosis (Bai et al., 2019; Barrows et al., 2018; Yun & Lee, 2017).

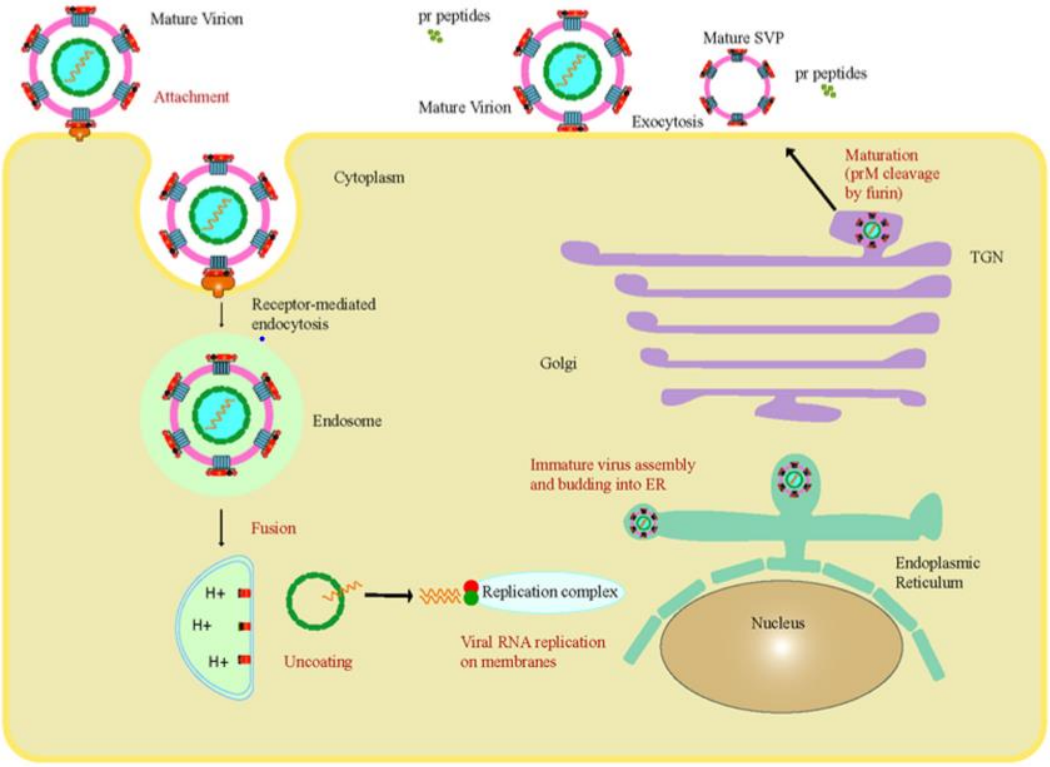


Figure 1.2 Flavivirus Life cycle of Flavivirus (Shankar et al., 2017)

1.4 Zika Virus

Zika virus (ZIKV) was first discovered in the Zika forest of Uganda in 1947 in a blood sample from a rhesus monkey (MacNamara, 1954). The first human transmission was detected in Nigeria in 1952 and isolated from *Aedes aegypti* mosquitos a year later (Musso & Gubler, 2016). During the 20th century, ZIKV spread from Africa to Asia and the oceanic states. Usually, ZIKV infection leads to either an asymptomatic or low-grade febrile illness that is described as a "summer flu" in humans (Musso & Gubler, 2016). The symptoms include fever, headache, conjunctivitis, and rash which last for about a week. However, during 2007 the Yap Island outbreak, the virus seemed to cause much

more severe symptomatic diseases, with the addition of rash and arthritis (Counotte et al., 2018; Musso & Gubler, 2016). Also, during the outbreak in French Polynesia in 2013-14, the new association of GBS in usually healthy adults developed, followed by the association of microcephaly in neonates during the 2015-2016 outbreak in Brazil (Counotte et al., 2018). In French Polynesia, the usual rate of GBS in adults was 1-3 per 100,000 population per year. During the ZIKV outbreak, the cases of GBS rose to 3-10 cases of GBS per 100,000 people between 2009 to 2012 (Musso & Gubler, 2016). GBS is an autoimmune disease in which the periphery nervous system (PNS) is attacked mononuclear and polynuclear immune cells causing acute flaccid paralysis in patients (Leonhard et al., 2019). It is caused by an instigating infection that triggers the immune system to attack the PNS. It is a serious illness that progresses rapidly, usually within two weeks, to affect the autonomic nervous system, with 20% of cases needing mechanical ventilation (Leonhard et al., 2019). The recovery period of GBS is often very long (6 months or more) and can be incredibly painful, but with only supportive treatment available (Leonhard et al., 2019).

Except for GBS in adults, ZIKV has also been documented as a causative agent of newborn microcephaly. In Brazil, the average number of microcephaly cases were 150-200 per year before 2015, but this number rose to 1,761 cases in 2015 alone (Castro et al., 2018; Musso & Gubler, 2016). At the end of the initial outbreak in January 2016, the case numbers rose to 3,893 (Musso & Gubler, 2016). According to the most recent counts from the Centers for Disease Control and Prevention (CDC), there are 2,751 cases of CZS reported to the Brazilian Ministry of Health as of 2017 (Castro et al., 2018). It has been shown that when pregnant mothers contracted the virus within the first trimester in

pregnancy, there is a higher incidence to have newborn babies with CZS (Guevara & Agarwal-Sinha, 2018; Marques et al., 2019). The CZS consists of microcephaly, neurological lesions, ophthalmological development issues, and even hearing loss in some cases (Guevara & Agarwal-Sinha, 2018; Marques et al., 2019). When children with CZS grow older, more complications such as epilepsy and sleep disturbance may arise (Marques et al., 2019).

Due to the significant health concerns that ZIKV is causing and there is no specific treatment available, the development of an effective and safe vaccine is critically needed.

1.5 Transmission of ZIKV

ZIKV is primarily transmitted by the bite of the *Aedes species* of mosquito, especially the Yellow Fever mosquito *Aedes aegypti*, but it is also transmitted by blood transfusion, via placenta from pregnant mother to fetus, as well as by sexual contact (Kauffman & Kramer, 2017; Musso & Gubler, 2016; Shankar et al., 2017; Yun & Lee, 2017). Due to the prevalence and near world-wide distribution through international travel, ZIKV has been able to flourish on nearly every continent within the tropics (Musso & Gubler, 2016). ZIKV has now become a public health threat due to the increase in international travel and development of sexual transmission, especially considering that most people are asymptomatic when infected with the virus, leading to a need for increased research and prevention measures (Shankar et al., 2017).

1.6 Current Antiviral and Vaccine Development against ZIKV

Currently, there are no approved treatments for ZIKV except supportive care in the case of symptomatic infection, which includes fever management and supplementing liquids. There are several groups currently working on antiviral development, following two main directions: direct-acting antivirals (DAAs) or host acting antivirals (HAAs) (Garcia et al., 2017; Saiz, 2019). DAAs focus on targeting the proteins and complexes of the viruses themselves, whereas HAAs target host-virus complexes that are used in the initial replication of the virus (Saiz, 2019).

For DAAs there are multiple targets used for drug development ranging from envelope binding to polymerase, methyltransferase, and protease inhibitors (Saiz, 2019). Some drugs are already well known effective drugs against RNA or DNA viruses, such as Favipiravir (a purine synthesis inhibiting NS5 polymerase) and Viperin (pyrimidine synthesis inhibitor and protease inhibitor) (Saiz, 2019). HAA development mostly focuses on the viral life cycle and the host proteins recruited for that purpose. The most relevant to this project is Silvestrol, which targets eukaryotic initiation factor-4A (eIF4A), which interacts at the 5'UTR by regulating translation through the ribosomes (Elgner et al., 2018). This drug was shown to decrease ZIKV replication *in vitro* (Elgner et al., 2018). While there is promising data for these approaches, the primary question is whether or not these drugs will be safe for pregnant women to use, meaning each must have rigorous testing *in vitro*, *in vivo*, and clinical trials. It will be a long time if and when these drugs can be on the market (Garcia et al., 2017; Saiz, 2019; Shankar et al., 2017).

There are several different approaches to vaccine development: purified inactivated vaccines, nucleic acid vaccines, virus-like particle (VLP) vaccines, subunit

vaccines, recombinant vaccines, therapeutic vaccination, live attenuated vaccines (Saiz, 2019). There are currently over 50 different vaccine candidates entering phase 1 or 2 clinical trials across the globe, but only one or two from each category of developmental strategy will be discussed here (Durbin & Wilder-Smith, 2017).

Purified inactivated vaccines are those that contain completely inactivated versions of the pathogens (Hajj Hussein et al., 2015). One of the most successful inactivated vaccines is the polio vaccine developed by Jonas Salk. In the case of ZIKV, there are several inactivated vaccines in development, with one formalin-inactivated ZIKV vaccine already in double-blind, placebo trials in the U.S. (Saiz, 2019). While inactivated vaccines are generally considered to be safe vaccines, they often need several doses of boosters to ensure total immunity, which could prove difficult in the low income, rural areas of the tropics where ZIKV has become prevalent.

Nucleic acid vaccines are originally composed of non-replicating vectors in a lipid that resulted in the generation of gene products in muscle cells (Vogel & Sarver, 1995). The immune system then creates antibodies against the proteins translated by the host. Currently, both DNA and RNA can be used to develop these vaccines, and currently are the most popular route to take for developing vaccines for ZIKV, usually using the prM/E gene as the primary target sequence in both RNA and DNA studies. PrM/E is what cells recognize when mounting the immune defense (Annamalai et al., 2019; Griffin et al., 2017; Saiz, 2019). Recent prototypes for ZIKV DNA vaccines seem to be trigger efficient cellular and humoral responses in non-human primates (NHP) (Saiz, 2019).

Other approaches to vaccine development are virus-like particle (VLP) vaccines and subunit vaccines These vaccines use the ZIKV prM/E protein to trigger an immune response through expressing the virus surface proteins instead of using the whole viral genome, allowing the immune system to mount a response without risking viral replication (Alarcon et al., 1999; Saiz, 2019; Shankar et al., 2017). This allows VLPs to be used even in pregnant women, making them very important tools for vaccination. Subunit vaccines are made by one part of a pathogen protein to trigger immunity, however, there are very few ZIKV subunit vaccines being developed, and none have made it to a clinical trial (Lin et al., 2018).

Live attenuated vaccines are vaccines that genetically altered versions of a pathogen but not completely killed. These vaccines are generally thought to be the most efficient at producing both humoral and cellular immune responses with only one dose of vaccination, making them the most highly effective vaccine. Several live attenuated vaccines are currently within phase I or II trials around the world, showing that targeting viral genes like E or NS1, deleting portions of NS genes or the 3' UTR, or a combination of the two, can effectively attenuate ZIKV (Annamalai et al., 2019; Griffin et al., 2017; Shan et al., 2017). However, the studies on ZIKV 5'UTR have primarily focused on studying the effect of editing on genome cycling and infectivity, and no studies are using the resulting decreased replication as a possible route for vaccine development. Therefore, this project aims to show if 5'UTR mutations can effectively decrease infectivity, but still induce antiviral immunity in a mouse model.

Table 1.1 Current ZIKV vaccine candidates in clinical trials (Lin et al., 2018)

Table 1. Zika virus (ZIKV) vaccines clinical trials.

Platform	Vaccine	Antigen	Adjuvant	Regimen (Dose, Route, Day/Week)	Clinical Phase	Trial Number
Inactivated	BBV121	virion	Alum	2.5 vs. 5 vs. 10 mg 2× (0, + 30)	I	CTRI/2017/05/008539
	ZPIV	virion	Alum	5 mg/IM 2× (+ 1, + 29)	I	NCT02963909
	ZPIV	virion	Alum	2.5 vs. 5 vs. 10 mg/IM 2× (0, + 29)	I	NCT02952833
	ZPIV	virion	Alum	5 mg/IM 2× (0, + 7: 0, + 14; 0, + 29)	I	NCT02937233
	ZPIV	virion	Alum	YF-VAX/IXIARO-5 mg/IM 2× (+ 1, + 29)	I	NCT03008122
	PIZV	virion	Alum	2 vs. 5 vs. 10 mg/IM 2× (0, + 30)	I	NCT03343626
	VLA1601	virion	Alum	3 vs. 6 AgU 2× (0, + 1 vs. 0, + 4)	I	NCT03425149
DNA-based	GLS-5700	pM/E	None	1 mg/ID	I	NCT02809443
	GLS-5700	pM/E	None	2 mg/ID	I	NCT02887482
	VRC5283	pM/E	None	4 mg/IM both arms, 2× (0, + 8; 0, + 12) 3× (0, + 4, + 8; 0, + 4, + 20)	I	NCT02840487
	VRC5283	pM/E	None	4 mg/IM both arms, 2× (0, + 8) (0, + 12); 3× (0, + 4, + 8) (0, + 4, + 20), needle/needle-free	I	NCT02996461
	VRC5283	pM/E	None	4 mg vs. 8 mg/IM both arms, 3× (0, + 4, + 8) needle-free	II	NCT03110770
RNA-based	mRNA-1325	pM/E	None	mRNA-1325	I	NCT03014089
Recombinant	MV	pM/E	None	low/high dose (0 vs. 0, + 30)	I	NCT02996890

1.7 The 5’ UTR and the 3’ UTR Elements

The 5’ and 3’ UTRs have several elements that are critical for virus replication, specifically so that the virus can complete the genome cycling needed for genome replication in the cytoplasm (Goertz et al., 2018; X. F. Li et al., 2010). For both UTRs, there is a cyclization sequence on each region that is complementary to the other, leading to an RNA “panhandle” structure when the genome is properly cyclized, with the functional genome of the virus forming a loop (Goertz et al., 2018). These sequences are fairly short, only ten to sixteen bases long when compared to the ~100 and ~400 bases of the entire 5’ and 3’ UTR respectively, but are highly conserved across the flavivirus genus (Goertz et al., 2018; Villordo & Gamarnik, 2009).

The other elements used by both UTRs are the self-complementary regions that form stem-loop (SL) and dumbbell structures (DB) structures that are crucial structures for the binding of host factors and viral RdRp that recruits ribosomes to the site of translation (de Borba et al., 2015; Filomatori et al., 2011; Goertz et al., 2018; X. F. Li et al., 2010). In the 3’ UTR, these secondary structures help form pseudoknots, which aid

against viral degradation by host proteases. These pseudoknots are also implicated in the formation of subgenomic flavivirus RNA (sfRNAs), which are formed when XRN1 attack the genome (Goertz et al., 2018). There have been G-quadruplexes detected in the 3' UTR on the (+) strand of the ZIKV genome. While the function of this region has not been elucidated, it is important to include in the formation of any mutant 3' UTR as it may impede the correct genome cyclization of the flavivirus(Goertz et al., 2018). The roles of 3' UTR makes a good target candidate in drug the development of attenuated ZIKV strains (Schneider & Wolfinger, 2019).

The 5'UTR, however, is much simpler in composition in comparison to the 3' UTR. ZIKV genome, specifically, has three RNA structures: two stem-loops, SLA and SLB, and the start codon and the capsid hairpin. SLA is the primary structure used for initial viral replication; however, the other two are used to bind to the 3'UTR for genome circularization (Brinton & Basu, 2015; Elgner et al., 2018; P. Li et al., 2018; Ng et al., 2017). It is also the shorter UTR, having only around 100 nucleotides per genome. However, as previously stated, it is to the 5'UTR that the host enzymes bind when initializing translation in the cytoplasm. It has also been shown that the 5'UTR of ZIKV and DENV have some Internal Ribosome Entry Site (IRES) capabilities that allow for translation control in mammalian cells, but to what functional extent these IRES regions have is still to be discovered(Song et al., 2019).

1.8 Animal Models of ZIKV Infection

Type I Interferon (IFN) receptor knockout mice (*Ifnar1*$^{-/-}$) were developed in the early 1990s to study DENV. This is because, unlike in humans, the NS5 protein of flaviviruses

do no degrade STAT-2 in mice. STAT-2 is a part of the IFN-regulated JAK-STAT pathway, so creating these knock-outs allows for similar pathway reactions to human infection (Krishnakumar et al., 2019; Paul et al., 2018). These *Ifnar1*$^{-/-}$ mice are the most commonly used animal model for ZIKV infection, but others focus on knocking out the STAT proteins or IFN Regulatory Factors (IRFs) (Krishnakumar et al., 2019). Non-human primates (NHP) are also used for ZIKV studies, considering that they are the animals of sylvatic transmission. NHP primates show similar symptoms in ZIKV to humans, including those relating to pregnancy (Pierson & Diamond, 2018).

CHAPTER II -SIGNIFICANCE, HYPOTHESIS, AND INNOVATION

2.1 Significance

Zika Virus has recently emerged as a world health concern due to its ability to cause severe neurological syndromes. In healthy adults, it can cause a slight flu-like illness or no symptoms at all. However, the virus can vertically transmit through the placenta of a pregnant woman and cause the child to be born with Congenital Zika Syndrome, which includes microcephaly, eye development, and ear development issues. In some adults, ZIKV infection can cause Guillain-Barre Syndrome, an autoimmune syndrome that attacks the peripheral nervous system. This study aims to show if editing the 5' UTR can lead to attenuation of ZIKV, it may be used as a ZIKV vaccine candidate. This research can lead to advancements in vaccine development for flaviviruses in general by showing that there are other genome targets to create effective attenuation.

2.2 Hypothesis

Vaccine development is a major task in the control of ZIKV infection. Most ZIKV vaccine development strategies are to target the prM-E, the non-structural proteins, and the 3'UTR, and there are none that focus on the 5'UTR, which is essential for both RdRp recognition as well as eIF4E to initiate ribosomal translation. In eukaryotic translation, ribosomes scan from the methyl-cap for an AUG start codon and stall at areas with stable secondary structures, such as stem-loops, as the ribosomes have to "melt" these structures to continue down the RNA strand. A previous report showed that by adding differing lengths of stem-loops in eukaryotic cells, translation was slowed considering the length

of the GC-rich structures (Babendure et al., 2006). While this 5'UTR insertion method has been used to both attenuate translation in different eukaryotic systems (Endo et al., 2013; Kozyrev et al., 2007), it has not been applied to viral research previously. Therefore, *we hypothesize that 5' UTR insertion should reduce ZIKV replication and translation without changing the viral antigenic epitopes, thus the attenuated viruses may be used as vaccine candidates.* We tested this hypothesis using the following aims.

2.2.1 Specific Aim 1: Generate Viable Attenuated ZIKV by modifying 5' UTR

ZIKV attenuation has been achieved by the deletion of nucleotides in the 3' UTR. We hypothesized that by adding nucleotides to the 5'UTR, viral replication and protein translation would be lessened. In this aim, we will generate mutant ZIKV by transfecting plasmids or PCR products containing the 5'UTR mutant ZIKV genome into Vero cells. We will measure the infectivity of the mutant ZIKVs in vitro.

2.2.2 Specific Aim 2: Assess the infectivity and immunogenicity of the mutant ZIKVs in mice

To test if the mutated viruses to replicate slower in a mouse model, we will infect *Ifnar1*$^{-/-}$ mice with wild-type and mutated virus to measure viremia after infection. We also will assess the antibody response to the mutant ZIKV infection. Finally, we will examine if the primary immunity protects a secondary high dose of ZIKV infection.

2.3 Innovation

ZIKV is a pathogen that is linked to severe birth defects leading to CZS in babies and neurological autoimmune disease (GBS) in adults, causing a significant world health concern. While there are several reports on antiviral drug and vaccine development, they are targeting replication of the virus by the prM/E dimer, the non-structural proteins, and the 3'UTR. This thesis is novel in that we are developing a new virus attenuation strategy by modifying ZIKV 5'UTR, which has not previously studied in any viral researches. In addition, this strategy may also be applied to generate other attenuated viruses, such as WNV, DENV, and SARS-Cov-2, which is currently causing the pandemic COVID-19 diseases.

CHAPTER III – Experimental Approaches

3.1 Ethics and Biosafety Statement

All animal care and experiments were conducted according to the Guide for the Care and Use of Laboratory Animals approved by The University of Southern Mississippi (USM) under the IACUC protocol # 16031002.1. All the experiments involving live ZIKV were performed by certified personnel in biosafety level 2 and 3 laboratories following standard biosafety protocols approved by the USM Institutional Biosafety Committee.

3.2 Virus Stock, Cells, Plasmid, and Animals

The original ZIKV plasmids (Cambodian strain) were generously provided by Dr. Peiyong Shi at the University of Texas Medical Branch (UTMB). The 5'UTR mutant ZIKV plasmids were developed by Dr. Faqing Huang at the University of Southern Mississippi. The rescued viruses were grown in Vero cells (ATCC CCL-81) in Dulbecco's Modified Eagles Media (DMEM, Life Technologies) supplemented by 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (P/S). Cells and viruses were grown at 37°C and 5% CO_2. Breeding pairs of type I interferon receptor-deficient (*Ifnar1*$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under standard conditions in the animal facility at USM.

Plasmid Transfection and Virus Collection

Plasmids were transfected into Vero cells using Lipofectamine 3000 reagent according to the user's manual. Briefly, Vero cells were plated at 2.5 x10^5 cells/well in a

6 well plate and allowed to incubate for 24 hours. 500 ng of the plasmid in serum- and antibiotic-free medium to 30 µl of the DNA solution, 5.5 ul of Lipofectamine 3000 was diluted into 30 µl of the same medium used in the other dilution. The DNA and Lipofectamine 3000 solutions were mixed immediately and incubated for 15 minutes at RT. The mixture was added to 1 ml of fresh media and added to the cells. The cells were then incubated for 4 to 6 hours to which another 1ml of the medium was added, and the cells were incubated for 5 days. The cell culture supernatant was then collected and stored at -70°C.

3.3 Viral Infection

Cells were infected by either direct supernatant or virus diluted in DMEM. Virus containing medium was added to cells and allowed to incubate for 1 hour at 37°C and 5% CO_2. Viral medium was then removed, and new medium was added. Cells were incubated over time in the same conditions as initial incubation.

3.4 qPCR of ZIKV detection

Cells or mouse blood was collected using the RNeasy Mini Kit (Qiagen) and converted into the first-strand cDNA using the iSCRIPT™ cDNA synthesis kit (Bio-Rad). Probe-based PCR (Bio-Rad) qPCR was performed using iTAQ polymerase™ supermix to detect ZIKV-E gene and mouse β-Actin and SYBR Green™ supermix to detect Vero cell β-Actin (Acharya et al., 2016)

3.5 qPCR of viral quantification

Viral copy numbers were determined using a modified version of the protocol previously described by the Bai lab (Acharya, Paul, Anderson, Huang, & Bai, 2015). Briefly, 300 μL of virus-containing media were treated with 75 units of RNase A for one hour at 37°C. TRI-Reagent (Molecular Research Center, Inc) was used to stop the reaction, and after 5 ug tRNA was added, RNA was extracted according to the manufacturer's instructions. cDNA and qPCR were performed as described above.

3.6 Focus Forming Assay (FFA)

FFA experiments were performed in Vero cells according to previously described protocols (Paul et al., 2015). Briefly, cells were plated at $5x10^5$ cells per well and allowed to attach overnight at 37°C with 5% CO_2. The next day, viruses were serially diluted into DMEM and allowed to infect for 1 hour at 37°C. Viral containing media was removed and FFA overlay media with 1% methylcellulose was added. Plates were incubated for several days equal to peak viral titer collection time determined by growth curves. On the day of the peak viral replication, the overlay media was removed, and the monolayers were immunologically stained using flavivirus-specific 4G2 antibody and Histomark® TrueBlue™ Peroxidase System.

3.7 Animal Studies

Ifnar1$^{-/-}$ mice were infected by intraperitoneal injection with 300 PFU (plaque-forming units) of either wild-type or mutated ZIKV. Starting day 1 post-infection (p.i.), approximately 200 μL of blood was collected for three days. Mice were then bled again

at day 24 p.i. to measure if ZIKV is still present in the blood. On day 42 p.i., the mice were challenged with a high dose of ZIKV strain PRVABC59 and bled, then euthanized to collect spleens and livers on day 2 post the secondary infection to measure *ZIKVE* RNA by qPCR.

3.8 Enzyme-Linked Immunosorbent Assay (ELISA)

ZIKV infected *Ifnar1*$^{-/-}$ mice were bled 24 d.p.i after isoflurane anesthesia. Blood was spun at 2,000 g for 10 minutes to prepare plasma. The plasma was then stored at -70°C until used. Anti-ZIKV E IgG was measured in the plasma by using the Mouse Anti-ZIKV Envelope IgG ELISA kit (Alpha Diagnostic, International).

3.9 Statistical Analysis

Data analysis was performed using Mann-Whitney U-test using GraphPad Prism software (version 6.0) with $p<0.05$ being considered as significant.

CHAPTER IV – RESULTS

4.1 Development and Structure of the Mutant 5'UTRs

To develop the new ZIKV mutants, Dr. Huang first designed new 5'UTR sequences containing 18, 38, or 50 additional GC-rich nucleotide sequences at the end of the original sequence but before the start codon (AUG) of ZIKV. He used the program SnapGene Viewer to develop the sequences, which are detailed below along with their future designations and theoretical structures (Table 4.1), using the sequence detailed in the paper "Reverse Genetics of Zika Virus" by Shan et al. Below, the bolded sequences are the insertions provided by Dr. Huang. All structural images were obtained by using the website http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form. All three of these insert sequences were added to the original sequence and theoretically folded, as shown in Table 4.1. These figures were chosen as a representative structure of the insert, however, for Z3-Z7, there were as many as four different secondary structures that have similar thermal stability as indicated by the folding energy ΔG In short, the theoretical structures showed that as the number of inserted nucleotides increases, the number of structures on 5' end of the UTR also increased. Meanwhile, the folding energy also increases, indicating higher potential to inhibit translation initiation. This is promising as cellular translation enzymes depend on stem-loop structures to begin replication and ribosomes will stall when engaged with additional stem-loop and hairpin structures. This supports the hypothesis that editing the 5' UTR will decrease the efficacy of viral translation and replication, thus attenuating the virus.

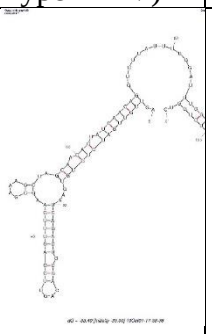
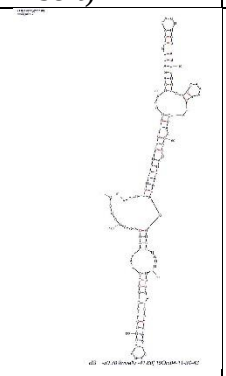
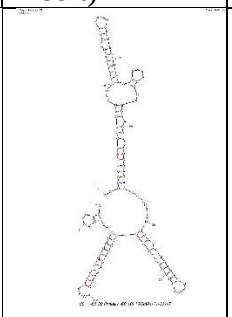
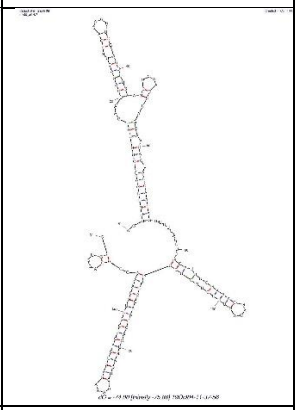
	Z1 (Wild Type ZIKV)	Z3 (18-nt insert)	Z5 (38-nt insert)	Z7 (50-nt insert)
Theoretical Structure				
Genome Sequence in DNA Format	AGTTGTTGAT CTGTGTGAAT CAGACTGCGA CAGTTCGAGT TTGAAGCGAA AGCTAGCAAC AGTATCAACA GGTTTTATTTT GGATTTGGAA ACGAGAGTTT CTGGTC	AGTTGTTGAT CTGTGTGAAT CAGACTGCGA CAGTTCGAGT TTGAAGCGAA AGCTAGCAAC AGTATCAACA GGTTTTATTTT GGATTTGGAA ACGAGAGTTT CTGGTC**CGTA CGAGCGCAG GTGC**	AGTTGTTGATC TGTGTGAATC AGACTGCGAC AGTTCGAGTTT GAAGCGAAAG CTAGCAACAG TATCAACAGG TTTTATTTTGG ATTTGGAAAC GAGAGTTTCT GGTC**CGACCA CGGCCGGAA ACGGCCGTG GTCGCGCAG GTGCC**	AGTTGTTGATCTGT GTGAATCAGACTGC GACAGTTCGAGTTT GAAGCGAAAGCTA GCAACAGTATCAAC AGGTTTTATTTTGG ATTTGGAAACGAGA GTTTCTGGTC**CGTT CCAACCACTGACT CGAAAGAGTCAGT GGTTGGAACGCGC AGGTGCC**

Table 4.1 ***Wild Type and Mutant ZIKV Theoretical Structures and Genome Sequences***

The inserted DNA sequences in the plasmids are in red. The predicted RNA structures were produced in the RNAFold website. All the plasmids were developed and provided by Dr.

4.2 Detecting ZIKV Mutants *in vitro*

To test if the transfection yielded infectious viral particles, 100 µl of supernatant from each plasmid transfection plus 900 µl of fresh medium was inoculated to $5x10^5$ Vero cells/well in a 6-well plate and incubated at 37°C with 5% CO_2 for 1 hour. The supernatant containing medium was removed and the cells were rinsed with PBS, then 2

ml of fresh DMEM supplemented with 10% FBS and 1% P/S was added to each well. After three days of incubation, the supernatant was collected and stored at -70°C, and the cells were collected in TRI-Reagent™. Total RNA was then extracted from the cells according to the manufacturer's instructions and used to make cDNA using the Bio-Rad iSCRIPT™ cDNA synthesis kit. ZIKV-E was then measured via probe-based PCR by Bio-Rad (Figure 4.1). Both Z5 and ZIKV-7 can replicate in Vero cells after five days of incubation, however, they show decreased replication compared to the wild-type virus.

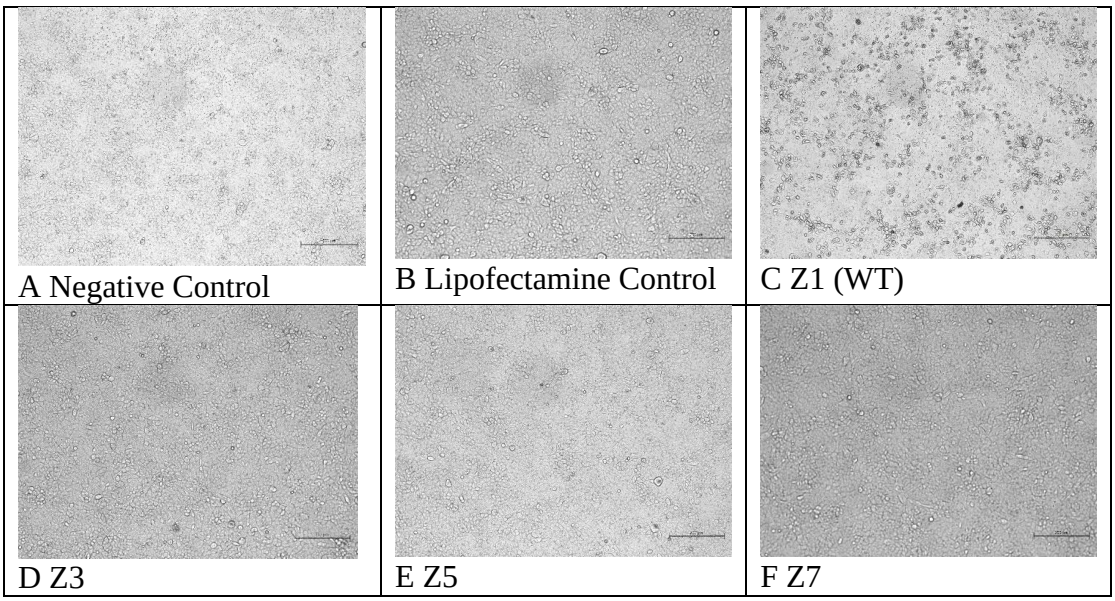


Figure 4.2 ***CPE caused by ZIKV mutants***

Images were taken at 20x magnification using a Lecia DM IL LED inverted light microscope on 4 d.p.i. *A. Negative control (medium only). B. Lipofectamine control (medium with Lipofectamine reagent). C. Z1 WT with no 5' UTR insert. D. Z3 viral mutant with an 18-nt 5' UTR. E. Z5 viral mutant with a 38-nt 5'UTR insert. F. Z7 viral mutant with a 50-nt 5' UTR insert.*

4.3 Cytopathic Effects of Mutant ZIKVs Compared to WT ZIKV

We test if these plasmids generated ZIKVs can cause a similar level of cytopathic effect (CPE) to Vero cells. The second generation of ZIKVs was incubated with Vero cells for 4 days. The images show that wild-type (WT) ZIKV caused CPE to Vero cells, while there was no sign of CPE induced by the mutant ZIKVs (Fig 4.1 A-F). This result indicates that mutant ZIKVs may replicate slower than the WT ZIKV.

4.4 Quantification of Mutant Viruses by qPCR

To quantify the amount of infections particles yielded by the transfection, we tried to use a plaque assay method. While Z1 produced plaques, Z5 and Z7 did not form plaques with Vero cells. We switched to the PFU-equivalent method, which uses qPCR to detect the copy numbers of viral genes in the viral containing medium. We also included a sample of WT ZIKV of a different strain for which we had a PFU/ml and used it to back-calculate PFU-equivalent concentrations of the viruses (Acharya et al., 2015). Due to the low titers of Z5 by qPCR (Table 4.2), we decided to test our hypothesis with only Z7 in my thesis.

Sample	**Titer detected by PCR (300µL original sample)**	**Genes/ml**
ZIKV (PRVABC59)	2999	10000
Z1	4432	14777
Z5	0.2719	0.9066
Z7	896.5	2988.4

Table. 4.2 ***Quantification of the viral genomic copies by qPCR***

4.5 Z7 Mutant Has a Lower Replication Rate In Vitro

To determine the viral growth kinetics, $5x10^5$ Vero cells were plated in 6-well plates and were infected with 100 µL of virus-containing DMEM diluted with 900 µL of fresh DMEM and allowed the virus to attach for 1 hour at 37°C. Cells were collected on days 2, 3, 4, 5 and 6 post-infection. After collection and qPCR was performed to measure the expression levels of *ZIKVE*. The qPCR results showed that while Z1 peaked after longer incubation than Z7, Z1 replicated at much higher levels than Z7 consistently at all the tested time points (Figure 4.1A and B). To exclude the possibility of an effect of dose variation, $5x10^4$ cells were plated in 24-well plates and infected with MOI of 0.05 of viruses for one hour at 37°C. Cells were collected on days 2 and 4 p.i. for qPCR, and the results showed a decreasing trend of Z7 replication (Figure 4.1C). To measure the difference of ZIKV E protein expression of the two viruses, $5x10^5$ Vero cells were infected with the viruses of 100 FFA and 10 FFA in duplicate for 1 hour and allowed to incubate for 3 to 5 days previously determined by Focus Forming Assay staining. These results showed that Z1 had more clear foci as well as higher numbers of foci as compared to Z7, which had very faint staining but countable foci in the 10 FFA wells (Table 4.3). Z7 was incubated for 5 days whereas Z1 only needed 3 days for FFA staining, suggesting that Z7 produces a lower amount of viral proteins over a longer time than Z1, indicating Z7 has a lower replication rate compared to Z1 the WT ZIKV strain. further indicating Z7 has a lower replication rate compared to Z1 the WT ZIKV strain.

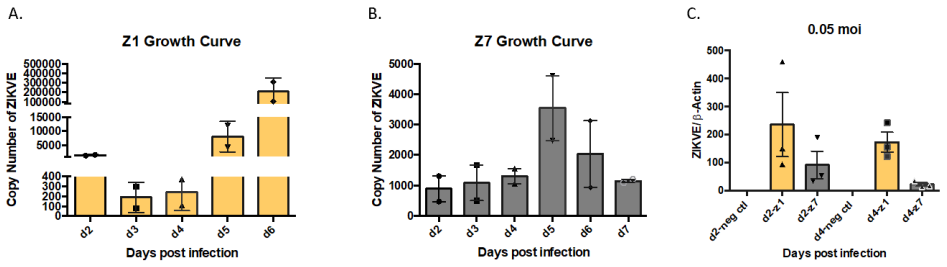


Figure 4.3 ***Z7 Mutant has a lower replication rate in vitro***

Growth curve results for Z1 wild type (A) and Z7 (B) performed on Vero cells on day 6 p.i.. (C) Vero cells were infected with plasmid derived viruses at MOI (0.05) for one hour at 37°C. *ZIKVE* gene was quantified by qPCR.

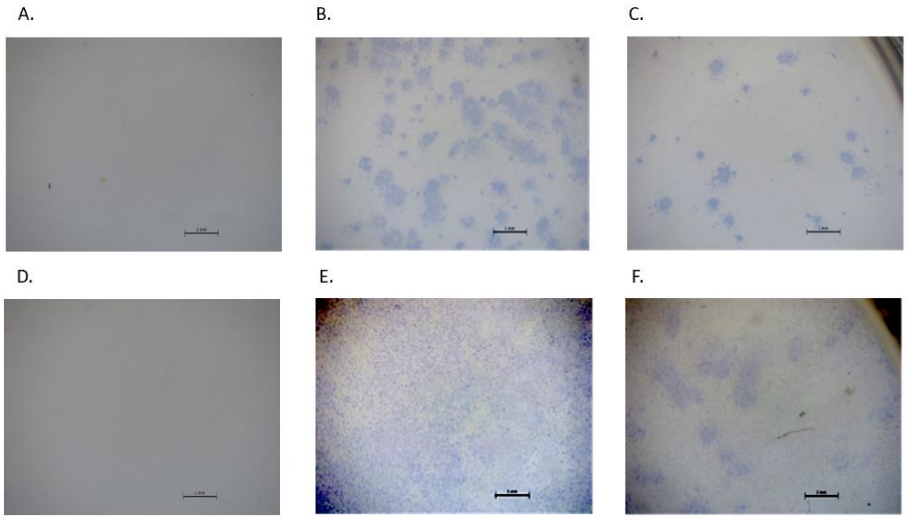


Figure 4.4 ***Immunostaining of the Z1 and Z7 infected Vero cells***

Results of 4G2/TrueBlue Peroxidase staining after a one-hour infection with Z1 or Z7. $5x10^5$ Vero cells were allowed to incubate for 3 days (Z1) or 5 days (Z7). Images were taken by a Lecia M165 FC microscope. Images A. and D. Negative control (no virus added) showing no TrueBlue peroxidase staining. Images B. and E. Cells were treated with 100 PFU-equivalent of either Z1 (B) or Z7 (E). Images C. and F. Cells were treated with 10 PFU-equivalent of either Z1 (C.) or Z7 (F).

4.6 *Ifnar1*$^{-/-}$ Mice Generates a Lower Viremia to Z7 Infection

We next wanted to test if Z7 replicates more slowly in an animal model. Eight-week-old and four-week-old *Ifnar1*$^{-/-}$ mice were infected intraperitoneally with 300 PFU of each virus in 100 µL of DMEM. Mice were bled once for three days post-infection and the viremia was measured via qPCR. Both sets of mice showed a decrease in the amount of Z7 as compared to Z1, confirming Z7 has attenuated infectivity *in vivo* as *in vitro*.

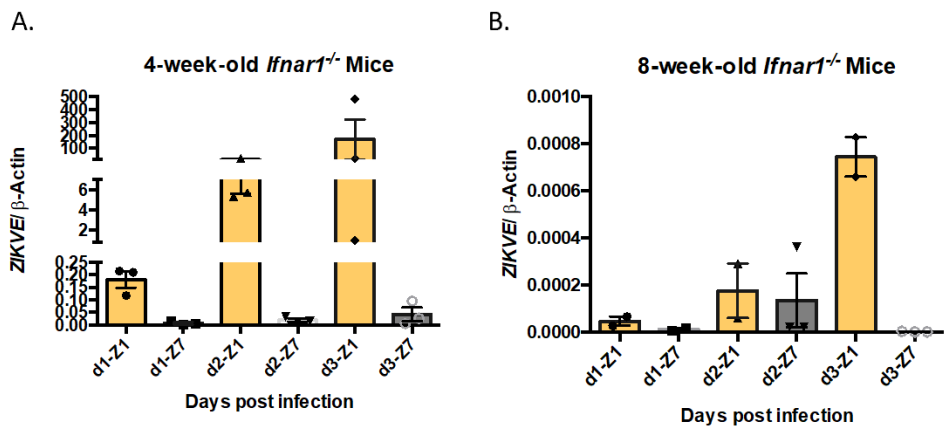


Figure 4.5 Z7 Has a Lower Viremia that Z1 in Ifnar1$^{-/-}$ mice

Four-week-old (A) or eight-week-old (B) *Ifnar1*$^{-/-}$ mice were infected with 300 PFU-equivalent either Z1 or Z7 in 100 µL of DMEM. *ZIKVE* in blood was measured via qPCR and normalized to mouse cellular beta-actin on each day. The results were compared using Mann-Whitney U-test with $p<0.05$ as significant.

4.7 Z1 But Not Z7 killed Four-week-old *Ifnar1*$^{-/-}$ Mice

To test mouse susceptibility to mutant ZIKV (Z7) infection, we measured the survival in the 4-week old mice. Mice were observed every day and any changes in vitality were noted. While no changes were observed between Z1- and Z7-infected 8-week-old *Ifnar1*$^{-/-}$ mice, 2 of 3 of Z1 (n=3) 4-week-old mice died, while all of the Z7 infected mice (n=3) survived during the observation period (Figure 4.5).

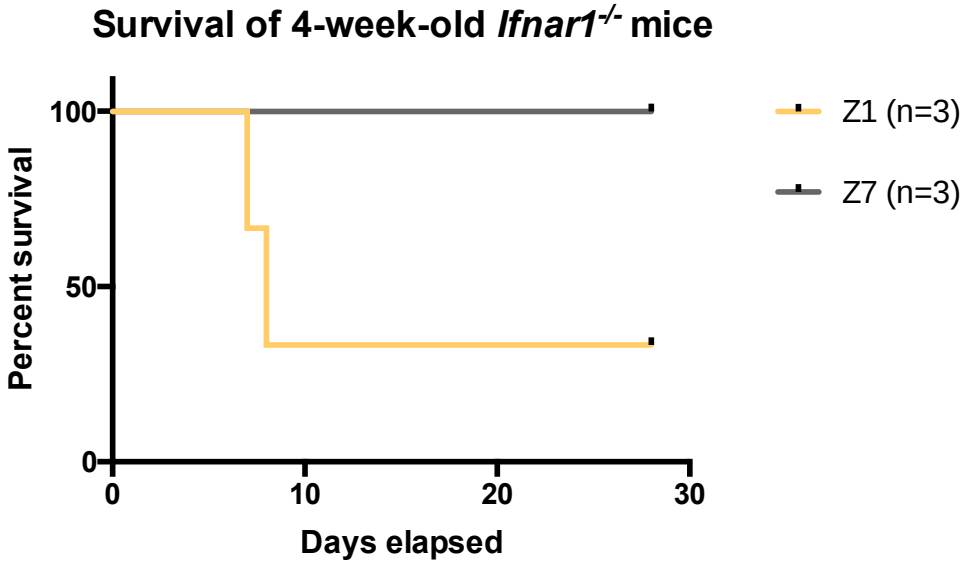


Figure 4.6 Four-week-old Ifnar1-/- Mice Survived Z7 Infection

Mice were observed daily for survival for 28 d.p.i. Z1 group (n=3), and Z7 (n=3). Percent survival was compared using log-rank test, $p > 0.05$.

4.8 Z7 Induces anti-ZIKV IgG Response

To test if Z7 could induce a comparable immune response in *Ifnar1*$^{-/-}$ mice, we measured the anti-ZIKV IgGs in the plasma from both sets of infected mice by ELISA.

We found that Z7 infection induced a similar level of IgG with Z1. These results suggested that although Z7 attenuated in replication, it still triggers anti-ZIKV IgG production (Fig. 4.7).

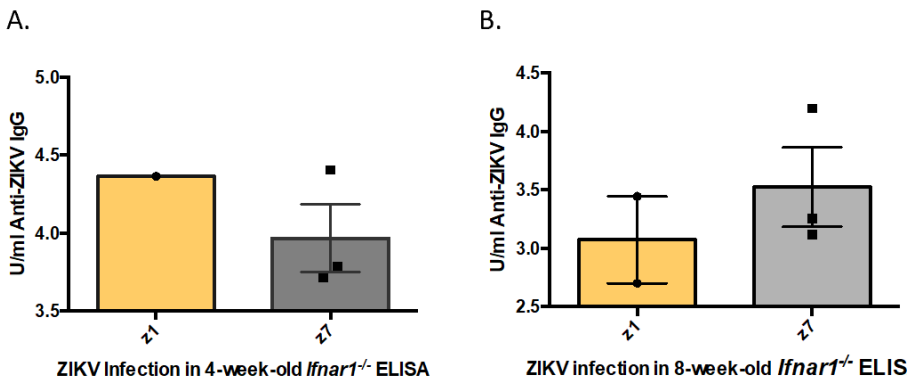


Figure 4.7 ELISA Results of anti-ZIKV IgG

Mice were infected with 300 PFU i.p. with either Z1 or Z7 and allowed to overcome infection. Mice were bled at either 24 (A) or 25(B) d.p.i to collect plasma from each experimental group. U/ml calculated by comparison to a standard curve of anti-ZIKV E IgG provided in the ELISA kit.

4.9 Immunity to Z7 Protects Against Secondary high dose of ZIKV Infection

To test whether the immunity to Z7 inoculation could provide sufficient protection against secondary high dose of ZIKV infection, we challenged the previously infected mice with a high dose ($1x10^5$ PFU/ml) of ZIKV strain PRVABC59 and sacrificed the mice on day 2 post-infection. The blood, liver, and spleen samples were collected, and qPCR was performed to detect *ZIKVE* genes. However, all samples were below the detection threshold for *ZIKVE*, showing that the both Z1 and Z7 infected mice

developed sterile immunity that sufficiently protects the mice from a high dose of ZIKV infection, suggesting that Z7 may be used a vaccine candidate for further tests.

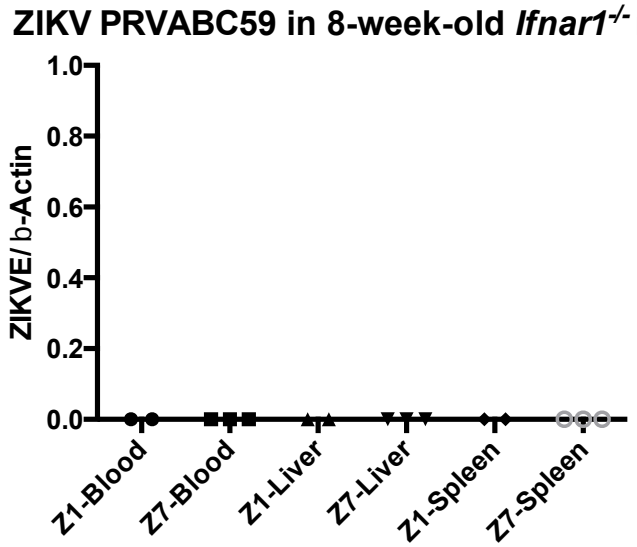


Figure 4.8 ***Lack of Detection of ZIKVE after the Secondary Infection***

Mice were injected i.p. with $1x10^5$ ZIKV strain PRVABC59. On 2 d.p.i., blood, liver, and spleen were collected for qPCR detection of *ZIKVE*. All samples were below the detection threshold of ZIKV by qPCR.

CHAPTER V -DISCUSSION

ZIKV was once considered to be a non-important human pathogen until the recent outbreaks in the twenty-first century (Yun & Lee, 2017). However, as these outbreaks revealed that ZIKV can induce GBS and the CZS, the need for effective vaccination and anti-viral medication has been on the forefront of ZIKV research since 2016 (Durbin & Wilder-Smith, 2017; Lin et al., 2018). However, development for vaccines is challenging due to the multitude of requirements to be considered for safety and efficacy and must be able to protect both mothers and fetuses (Durbin & Wilder-Smith, 2017; Lin et al., 2018; Saiz, 2019).

Due to the rapid advancements of plasmid and genome editing technology, scientists are now able to target not only the most immunogenic protein of ZIKV (prM-E), but also the non-structural proteins that are crucial to viral replication and assembly (Mazeaud, Freppel, & Chatel-Chaix, 2018; Shan et al., 2017). These proteins are also the targets of anti-viral drug development, which also goes through the rigorous testing of the FDA's clinical trials. Even with the testing of previously approved drugs against ZIKV, such as ribavirin and suramin, there are no currently approved drugs or vaccines for ZIKV (Saiz, 2019).

The flavivirus 5' and 3' UTR are both critical for viral genome cyclization after eIF4E engages the 5' end (Ng et al., 2017). This cyclization then move the genome to the ER, where ribosomes translate the initial positive sense genome into the polyprotein that is cleaved by cellular and viral enzymes, starting the active replication of the viral genome (Bidet & Garcia-Blanco, 2018). There have been DENV studies early in the 2000s in which deletions and substitutions have been made in both UTRs showing

variability in replication, however, the current focus of research on the UTRs of flavivirus is on the function of the regions in the creation of subgenomic flavivirus RNAs (sfRNA) (Goertz et al., 2018; Mazeaud et al., 2018; Sirigulpanit, Kinney, & Leardkamolkarn, 2007).

In this study, we found that by inserting nucleotides to the 5' UTR (Z7), ZIKV replication was reduced *in vitro* when compared to wild type ZIKV (Z1), following the trend established by the previously mentioned DENV UTR studies (Sirigulpanit et al., 2007). We then transfected four plasmids, one wild type (Z1) and three mutants of differing 5' UTR inserts (Z3, Z5, and Z7) into Vero cells and allowed to grow until CPE was observed, usually around four days. We found that one generation after initial transfection, Z3 did not replicate in Vero cells after 5 days as Z5 and Z7, and so was disregarded for further tests.

After the initial transfection, we quantified the growth kinetics of the two mutants Z5 and Z7 *in vitro.* However, we were unable to propagate Z5 after the initial transfection experiment, so we continued the project focusing on Z7. After infecting Vero cells with similar the same amount of virus-containing supernatant, we found that Z7 replicated much slower and reached its peak earlier compared to Z1 (Fig 4.3 A and B). This was confirmed by other in vitro studies (Fig 4.3 C) as well as qPCR viral genome quantification, which confirmed the consistent decreased trend of growth of Z7. (Table 4.2). These results show that Z7 has a lower replication rate in Vero cells than Z1 by qPCR. Next confirmed this conclusion at the protein levels. Using serial dilution to infect with the same number (PFU-equivalent) of infectious particles, we were able to see stronger peroxidase staining in the Z1 as compared to Z7, with Z1 being incubated for 3

days and Z7 being incubated for 5 days before the infected cell foci could be detected. The FFA results suggest that it takes longer for Z7 to produce detectable cell foci than Z1, and the *ZIKVE* protein detected is at lower concentrations than Z1. These data further demonstrate Z7 is attenuated in viral genome replication and protein translation in cell culture.

Next, we predicted that Z7 would also have slower replication in an animal model. We used *Infar1*$^{-/-}$ mice, in which type I interferon signaling is deficient, to test our hypothesis (Grant et al., 2016). Age did show a difference in the amount of virus detected, however, we noticed that Z7 induced decreased viral burden in both 4-week old mice and 8-week old mice. This is a promising phenotype, considering in vivo test vaccinations using the DENV 5' UTR studies also showed a lower viremia in human patients (Sirigulpanit et al., 2007). Another promising phenotype we observed during the in vivo experiments was the death of two of the Z1 infected 4-week-old mice as compared to the Z7 mice on days 7 and 8. The survival results further confirm that Z7 has an attenuated infectivity in animals.

We were able to conduct a few long-term immunological studies with the *Ifna1r*$^{-/-}$ mice groups. Approximately three weeks after primary infection, we bled the mice to measure their IgG antibody production by ELISA. Anti-ZIKV antibodies most commonly reactive against the envelope dimers of the virus, however, they can be made against the NS1 protein as well (Rey, Stiasny, Vaney, Dellarole, & Heinz, 2018; Vidarsson, Dekkers, & Rispens, 2014). The assay we used showed that Z7 induced the mouse anti-ZIKV IgG immune response against the envelope protein in both young and older mice like Z1. These results indicate that Z7 is immunogenic and can induce anti-

ZIKV immunity. This was confirmed by the results showing Z7-infected mice are completed resistant to a high dose of ZIKV strain PRVABC59 challenge. Besides the antibody response, Z7 is expected to induce effector and memory T cell responses as well since the viruses still can infect and replicate in cells. Therefore, the T cell responses to Z7 should be examined in the continuation of this project.

Our data showed that the addition of GC-rich sequences before the AUG codon was able to reduce viral production both *in vitro* and *in vivo*. It has been shown that the addition of secondary structures near the start codon of eukaryotic cells can inhibit the translation of mRNA by ribosomes, specifically thermodynamically stable secondary structures with $\Delta G \geq -50$ kcal/mol (Babendure et al., 2006). It was also shown that location in relation to the methyl cap of the mRNA had an inhibitory effect on translation with structurally stable inserts. And the opposite was also true for unstable inserts, and the further away from the methyl cap and closer to the start codon, the less translation occurred. Using the same concept, we developed three inserts for the 5'UTR whose first 5' nucleotide were at the same distance from the methyl cap of ZIKV genome. We predicted that the length of the insert itself would negatively corelate with the efficiency of viral translation and replication. However, our results showed that Z3 with the shortest (18-nt insert) didn't produce viable viral particles in two independent experiments. This could due to the 18-nt insertion dramatically changes the folding of Z3 5'UTR. According to the theoretical structures by RNAFold, the 5'UTR of Z3 exhibits an almost liner structure made of two long stem loops compared with that of Z1 (WT), which has a Y-shaped stem loop (SLA) and a small stem loop (SLB) (Figure 4.1). The major structural change in 5'UTR may block its binding to 3'UTR and abolish the cyclization

of the viral genome, which are essential to the viral genome replication by the RdRp. In contrast, Z3 and Z7 that have a 38- or 50-nt insert only slightly alter the structures of 5'UTR by adding an extra stem loop, which may only slowdown the ribosome binding to the start codon rather than entirely blocking the genome cyclization and replication. Consistent to the structure predication, our experiment results indicated that the 5'UTR modifications of Z5 and Z7 produced viable but attenuated viral particles. These results support our hypothesis *that 5'UTR insertion reduces ZIKV translation and replication, thus produces attenuated viral particles.*

In conclusion, the addition of GC-rich nucleotides to the 5' UTR can inhibit replication on both RNA and protein levels of ZIKV *in vitro* and *in vivo*. Importantly, Z7 can induce efficient protective immunity against a high dose of ZIKV infection with a current circulating strain in humans. Collectively, my results show that targeting the 5' UTR by adding ribosome-stalling secondary structures may be a new strategy to develop attenuated ZIKV strains and Z7 could be used as a vaccine candidate for further testing. This new strategy may also be applied to generate other attenuated viral vaccine candidates.

CHAPTER VI -CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusions

In this study, we showed that the mutant Z7 ZIKV virus has lowered amplification *in vitro* and *in vivo*. This phenotype was measured at both RNA and protein levels, and both in vitro and in vivo. It also induced anti-ZIKV-E IgG antibodies in comparable amounts to the WT virus, as well as protected *Ifnar*$^{-/-}$ immunocompromised mice against secondary infection of ZIKV. Therefore, Z7 may be a viable candidate for further testing and development.

6.2 Future Directions

Further study into the immunological response of *Ifnar1*$^{-/-}$ mice to infection with Z7 is needed. The *in vitro* studies should be repeated at higher MOIs to test the growth kinetics at higher infectious doses, as well as more protein studies such as Western Blot. The *in vivo* studies should be repeated with larger mice numbers to determine if the differences are statistically significant.

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