Development, Characterization, and Use of Molecular Tools to Study Immune-Driven Zika Virus Evolution

Jeffrey Matthew Marano

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Translational Biology, Medicine, and Health

James Weger-Lucarelli - Chair
Sally Paulson
X.J Meng
Frank Aylward

January 30, 2023
Blacksburg, VA

Keywords: flavivirus, immune-driven evolution, dengue virus, Zika virus, reverse genetics, bacterial-free cloning

Development, Characterization, and Use of Molecular Tools to Study Immune-Driven Zika Virus Evolution

Jeffrey Matthew Marano

ACADEMIC ABSTRACT

Emerging viruses represent a significant threat to human health. Understanding the drivers of emergence, such as viral evolution, is a critical avenue to combat these pathogens. One specific group of emerging pathogens of interest is flaviviruses. Flaviviruses are arthropod-borne viruses (arbovirus) in the family Flaviviridae. The medically relevant flaviviruses can be divided into two groups – tick-borne and mosquito-borne. Included within the mosquito-borne flaviviruses group are dengue viruses 1-4 (DENV 1-4), which causes 400 million infections annually, and Zika virus (ZIKV), which caused over 128 million infections from 2013-2018. These viruses, which are cocirculating, share high sequence similarity in key antigenic regions. Because of these similarities, pre-existing immunity to DENV has been correlated with altered pathogenesis of subsequent ZIKV infections. Despite this, there has been little analysis of the effects of pre-existing DENV immunity on the evolution of subsequent flavivirus infection, despite being characterized for many other viruses. Given that mutation that could arise from cross-reactive immune selection could alter pathology or transmissibility, it is critical to assess the role of cross-reactive immune selection as an evolutionary driver. However, this line of research has historically been difficult due to the inherent toxicity of flavivirus infectious clones in bacteria. To mitigate the toxic nature of flavivirus clones, we developed several entirely in vitro workflows using a combination of rolling circle amplification (RCA) and replication cycle reaction (RCR). We demonstrated that RCA was a comparable substitute to traditional plasmid propagation using an alphavirus infection clone. We further demonstrated that RCR could be used to generate infectious clones by producing infectious clones of DENV2 and SARS-CoV-2, as well as demonstrating it could be used to introduce mutations into infectious clones by producing a D614G SARS-CoV-2 mutations. With this technology in place, we used in vitro directed evolution system, where we passaged ZIKV in convalescent patient serum to assess the role of cross-reactive immune selection as an evolutionary driver. After passaging, we performed next-generation sequencing to assess the impacts of cross-reactive immune selection on the viral populations and to identify mutations that arose post-passaging. We observed that ZIKV passaged in convalescent DENV serum had reduced diversity and divergence in the premembrane region. Within the convalescent DENV passaged population, we identified two mutations of interest with the dominant antibody binding region – E-V335I and NS1-T139A. These mutations were then introduced using our in vitro workflows. The resulting mutant viruses were then assessed for their replicative fitness in mammalian cell culture and mosquito models and their sensitivity to neutralization. We observed that while both E-V355I and NS1-T139A have increased fitness in mammalian cells, they had reduced fitness in mosquitoes. These results align with the trade-off hypothesis, which states that in a multi-host system, adaptation to one host reduces fitness in the other hosts. When we assessed the neutralization sensitivity of the mutants, we observed that while NS1-T193A was resistant to neutralization, E-V355I was more sensitive to neutralization. These results indicate that neutralization escape is not necessary for enhanced post-passaging in convalescent DENV serum. Our findings demonstrate that cross-reactive immune selection can generate several mutations with altered fitness in mammalian cells and mosquitos. This research is significant for both highlighting novel technologies to facilitate molecular virology and demonstrating that cross-reactive immune selection has the potential to alter the evolutionary trajectory of flaviviruses. This work provides critical information to understand how flaviviruses are evolving and emerging, and therefore critical information to address their threat to human health.

Development, Characterization, and Use of Molecular Tools to Study Immune-Driven Zika Virus Evolution

Jeffrey Matthew Marano

GENERAL AUDIENCE ABSTRACT

Emerging viruses represent a significant threat to human health. We must understand what drives these viruses to adapt and evolve to respond to these threats. One virus family of extreme importance is the genera flavivirus. Flaviviruses are arthropod-borne viruses (arbovirus) that can be spread by the bites of ticks and mosquitoes. Included in the mosquito-borne flavivirus are dengue virus 1-4 (DENV1-4), which accounts for 400 million new infections annually, and Zika virus (ZIKV), which caused more than 128 million infection from 2013-2018. In addition to co-circulating, DENV 1-4 and ZIKV share several key similarities in their protein structures, which results in pre-existing DENV immunity effect how subsequent infections behave. The effect of pre-existing immunity on the evolution of these viruses has not been well established, despite similar studies being performed for other viruses. Given that the mutations that could arise from immune-driven evolution could alter disease severity or transmissibility, the impacts of immune-driven evolution must be characterized. However, the current tools available to perform this research are suboptimal, as the toxicity of flavivirus genomes hampers out ability to perform bacterial cloning, which has historically been necessary to develop and modify infectious clones. To mitigate the toxicity, we developed a "bacteria-free" workflow using emerging technologies like rolling circle amplification (RCA) and replication cycle reaction (RCR). With the technology in place, we propagated several generations of ZIKV or DENV in the presence of serum from human patients with a history of DENV infections. We then sequenced the viruses and identified mutations that arose during passaging. The mutations were then inserted using our bacteria-free workflow into infectious clones. The resulting viruses were assessed for their ability to replicate in mammalian cells, their ability to infect mosquitos, and their sensitivity to patient serum. We found that exposing ZIKV to serum from patients with pre-existing immunity to DENV can result in ZIKV developing several mutations. These mutations make the virus more effective at infecting mammalian cells and less effective at infecting mosquitos. This research is significant as it highlights novel technologies to aid researchers, and it demonstrates that pre-existing immunity has the potential to alter the evolutionary trajectory of flaviviruses. This information is critical in understanding flavivirus evolution and their emergence and therefore is critical to addressing their threat to human healt

Acknowledgments

The start of my research journey involved many stumbles off the block. I knew that I wanted to be a scientist, however, coming from a small town meant I didn't have any experience when I arrived at Boston University. However, I was able to connect with Dr. Wilson Wong and his student Dr. Benjamin Weinberg. They gave me a chance to work in their lab in genetic engineering. I spent three years working with that group developing genetic logic computers, competing with my work, and ultimately publishing it. Without them, I can safely say that I would not be defending my dissertation in molecular virology.

I want to thank my committee members, Drs. Sally Paulson, X.J Meng, and Frank Aylward. The Book of Proverbs says, "Where there is no guidance, a people will fall, but in the abundance of wise counselors there is victory" (Proverbs 11:14). Meeting with my committee was a consistent joy and pleasure because they sought to make me the best scientist that I could be. Long after I leave Tech, I hope that I can still look to them as my teachers and guides.

One guide that needs specific thanks and praise is Dr. James Weger-Lucarelli. My start to graduate school went about as well as the start of my research career regarding finding a lab home. Coming from engineering, I found it difficult at times to explain why I decided to become a biologist. As such, I finished my first year of graduate school with a passed qualifying exam and no lab. Then I met Dr. Weger-Lucarelli, and he took me into his lab. I am not sure what he saw in me then, but he pushed me to become that person and that scientist. Not only is he a scientific mentor for me, but a personal one as well. As an autistic scientist, I have struggled with feelings that I was far too alien and abhorrent to fit into any community. Dr. Weger-Lucarelli helped me see that my mind, my way of thinking, and myself had that value, and for that, I see him as not only a mentor but a consummate friend.

I want to thank my parents, Judy and Mark Marano, and my twin brother Jason. Somehow you put up with my wild ideas, half-brained hypothesis, and long lectures about topics you did not sign up for. Thank you for your enthusiasm about my work and my dreams.

Finally, I would like to dedicate my dissertation to three people, my great uncle, Dr. Jan Kurdwanowski, my grandfather, Benjamin Marano, and my wife Kelsey Marano. My great uncle and grandfather, while unable to see this dissertation come to fruition, were brilliant men who made me believe that I could be a scientist just like them, even in the face of adversity. And my wife, Kelsey, no words will ever describe how this dissertation and victory belongs to you as much as it does me. You were with me when I got into graduate school, on the phone with me through every disappointing experimental result and reading my papers (even when you were busy writing your own). Thank you for helping me grow and be the best man I can be.

So, to all those mentioned and those whom I haven't, thank you.

TABLE OF CONTENTS

	Page
Academic Abstract	
Public Abstract	
Acknowledgments	*i*
Table of Contents	*iii*
Chapter 1: Introduction and Literature Review	
1.1 Emerging Arboviruses	1
1.2 Flaviviruses	2
1.3 Dengue virus	4
1.4 Zika virus	5
1.5 Immune-driven ecology and pathology	6
1.6 Immune-drive evolution	7
1.7 Technical hurdles to studying evolution	8
1.8 Summary	11
1.9 Supplementary Data	12
1.10 References	12
Chapter 2: Rolling circle amplification: A high fidelity and efficient alternative to plasmid preparation for the rescue of infectious clones	
2.1 Abstract	33
2.2 Introduction	34
2.3 Materials and methods	36
2.4 Results and discussion	38
2.5 Conclusions	44
2.6 Author Contributions	45
2.7 References	46
Chapter 3: An in vitro workflow to create and modify novel infectious clones using replication cycle reaction	
3.1. Abstract	50
3.2. Introduction	51
3.3. Materials and methods	54
3.4. Results	57
3.5. Discussion	64
3.6. Author Contributions	66
3.7. References	66
Chapter 4: Replication in the presence of dengue convalescent serum impacts Zika virus neutralization sensitivity and fitness	
4.1. Abstract	78
4.2. Introduction	79
4.3. Materials and methods	80
4.4. Results	85
4.5. Discussion	93
4.6. Supplemental information	97
4.7. Author Contributions	100
4.8. References	100
Chapter 5: Conclusion	
5.1 Conclusion	131
5.2 References	135

Chapter One: Introduction and Literature Review

1.1 Emerging arboviruses

Emerging infectious diseases represent one of the most significant threats to human health. Within the last 20 years, there have been at least 52 different viral emergence events globally (Keusch et al., 2022), and evidence has indicated that the frequency of these events will increase in the next several decades (Marani et al., 2021). The drivers of continual emergence can be stratified into socio-ecological and molecular factors.

At an ecological level, viral emergence is generally associated with regions of high biodiversity whose natural habitats are depleted by rising human populations (Bogich et al., 2012). Furthermore, the expanding animal-human interface through bushmeat consumption and farming increases the probability of zoonosis (Bogich et al., 2012). Finally, as global temperatures rise, geographic ranges of animal reservoirs are predicted to shift, promoting novel cross-species viral transmission that previously was unlikely (Carlson et al., 2022).

At a molecular level, the most significant driver for disease emergence is the ability of RNA viruses to exist as complex swarms of diverse genotypes, referred to as quasispecies (Domingo and Perales, 2019). One driver of quasispecies generation is the high mutation rate of many RNA viruses (Sanjuán and Domingo-Calap, 2016). Due to a lack of proofreading capacity in many RNA-dependent RNA polymerases, the average mutation rate of many RNA viruses equates to roughly one mutation per round of genomic replication (Sanjuán and Domingo-Calap, 2016). Other critical mechanisms that promote the development of quasispecies include recombination (Goldstein et al., 2022) and reassortment (Postnikova et al., 2021). This complex swarm of diverse genotypes has been correlated with enhanced fitness in multi-host systems, such as in arthropod-borne viruses (Jerzak et al., 2008; Coffey and Vignuzzi, 2011; Ciota et al., 2012).

Arthropod-borne viruses, or arboviruses, are spread by biting arthropods (mosquitoes, ticks, and other hematophagous insects and arachnids) (Coffey et al., 2013). These viruses can exist in enzootic/sylvatic (animals), epidemic/endemic (humans), and zoonotic (animal/human) cycles (Coffey et al., 2013). In the last 20 years, at least 17 arbovirus outbreaks globally (Keusch et al., 2022). Within arboviruses, there are several genera of extreme importance: *Phleboviruses*, *Orthabunyaviruses*, *Alphaviruses*, and *Flaviviruses* (Ciota, 2019).

1.2 Flaviviruses

Flaviviruses, of the family *Flaviviridae*, are positive-sense single-stranded RNA viruses (Pierson and Diamond, 2020). The viral genomes are roughly 11kb and encode a single open reading frame (ORF) flanked by two highly structured untranslated regions (Ng et al., 2017) (Figure 1). The ORF encodes 11 proteins – 3 structural (capsid, envelope, and premembrane) and 8 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5) (Pierson and Diamond, 2020). Viral entry is mediated by binding of the envelope protein to one of the several putative viral receptors (Chen et al., 1997; Tassaneetrithep et al., 2003; Meertens et al., 2012), followed by clathrin-mediated endocytosis (Hackett et al., 2015; Perreira et al., 2015; Hackett and Cherry, 2018). Through a pH-dependent fusion event, the virion releases its capsid into the cytoplasm (Chao et al., 2014). Replication, translation, assembly, and budding occur within the endoplasmic reticulum (Reid et al., 2018), while maturation of the virus occurs in the trans-Golgi apparatus (Yu et al., 2008; Yu et al., 2009; Renner et al., 2021). The virus is released from infected cells via exocytosis (Yu et al., 2008; Yu et al., 2009). During natural infection, the mammalian hosts will generate both a humoral and cellular response to clear the virus (Vaughan et al., 2010). The dominant epitopes for the humoral response are found in the prM, E, and NS1 proteins (Vaughan et al., 2010), while the dominant epitopes for the cellular response are found in the E and NS3 proteins (Lobigs et al., 1994; Vaughan et al., 2010).

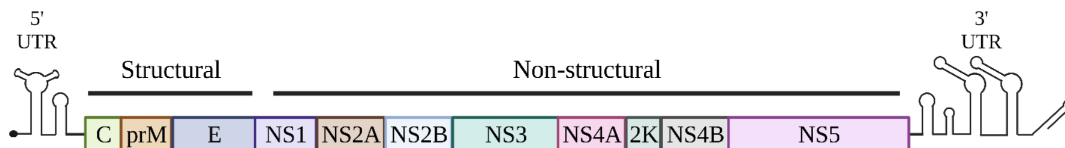


Figure 1: Flavivirus Genome Structure. Diagrammatic overview of a standard flavivirus genome. The genome consists of capped RNA, a 5’ untranslated region (UTR), a single open reading frame consisting of three structural proteins (capsid, premembrane, and envelope), eight nonstructural proteins (indicated by an NS or 2K), and a 3’ UTR.

Over 50% of the world population is at risk of contracting a flavivirus infection, with 400 million cases being detected in over 128 countries annually (Brady et al., 2012; Bhatt et al., 2013). In addition to the large case count, there are also many species of flaviviruses, with over 50 species identified (Moureau et al., 2015). These viruses can be sorted based on similar characteristics.

One method is to stratify by vector type. These include insect-specific flavivirus (ISFVs), flavivirus with no known vector (NKVs), tick-borne flaviviruses (TBFVs), and mosquito-borne flaviviruses (MBFVs) (Moureau et al., 2015) (Figure 2 and Supplemental Table 1). MBFVs can be further stratified based on the mosquito species and feeding habits of those mosquitoes, such as *Culex* mosquitoes, which are ornithophilic (bird feeders) (Moureau et al., 2015). Viruses primarily vectored by *Culex* include Japanese encephalitis virus (JEV), West Nile virus (WNV), Usutu virus (USUV), Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV) (Khare and Kuhn, 2022). The second group of MBFVs is those vectored by *Aedes* mosquitoes, which are mammalophillic (mammal feeders) (Moureau et al., 2015). Two significant viruses within this group are dengue virus (DENV) (Pierson and Diamond, 2020) and Zika virus (ZIKV).

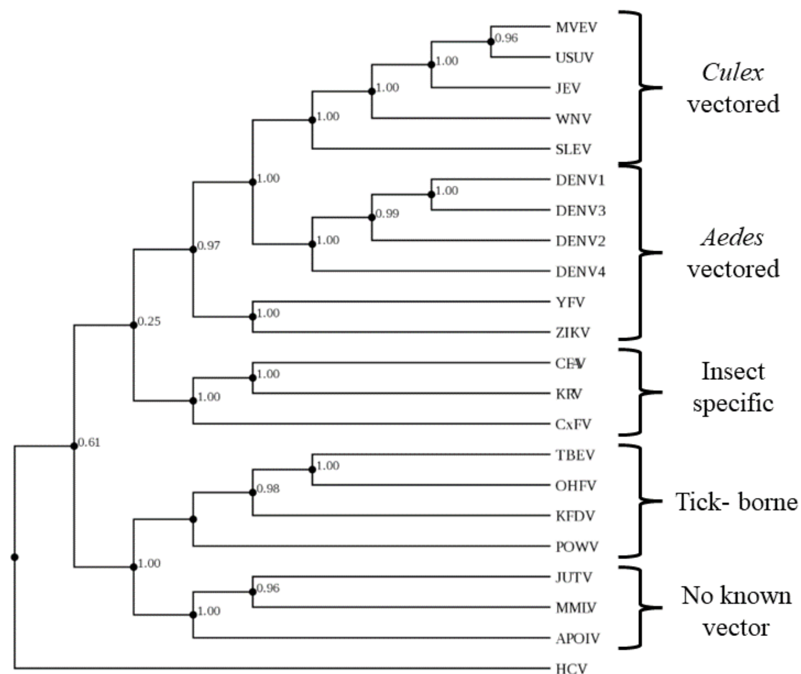


Figure 2: Phylogenetic Analysis of Flaviviruses Stratified by Vector. Maximum likelihood phylogenetic tree constructed using 1000 bootstraps. Hepatitis C virus (HCV) was used as an outgroup for this analysis. MVEV – Murray Valley encephalitis virus, USUV – Usutu virus, JEV – Japanese encephalitis virus, WNV – West Nile virus, SLEV – St. Louis encephalitis virus, DENV1 – dengue virus serotype 1, DENV2 – dengue virus serotype 2, DENV3 – dengue virus serotype 3, DENV4 – dengue virus serotype 4, YFV – yellow fever virus, ZIKV – Zika virus, CFAV – cell fusing agent virus, KRV – Kamiti River virus, CxFV – Culex flavivirus, TBEV – tick-borne encephalitis, OHFV – Omsk hemorrhagic fever virus, KFDV – Kyasanur Forest disease virus, POWV – Powassan virus, JUTV - Jutiapa virus, MMLV – Montana myotis leukoencephalitis virus, APOIV – Apoi virus.

1.3 Dengue virus

The first reports of a DENV-like infection trace back to an encyclopedia of diseases during the Qin Dynasty in China (265 to 420 C.E) (Gubler, 1998). The next major historical reports of dengue occurred between 1779 and 1780, with outbreaks across Asia, Africa, and North America (Gubler, 1998). This spread was likely facilitated by the trans-Atlantic slave trade (Simmons et al., 2012). DENV was first isolated by Ren Kimura and Susumu Hotta in Japan in 1943 (Hotta, 1952). Post-World War II, epidemics of DENV increased in frequency and range (Gubler, 1998), resulting in DENV becoming the most significant contributor to arboviral disease (Bhatt et al., 2013).

Interestingly, DENV is a slight misnomer, as DENV is not a single virus but four serologically related viruses (referred to as serotypes) (Simmons et al., 2012). Regardless of serotype, DENV is the causative agent for three progressively worsening diseases – dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Simmons et al., 2012). In cases of DF, infections are characterized by fevers, headaches, malaise, myalgia, and mild hemorrhagic symptoms (petechia and bruising) (Simmons et al., 2012). In DHF, patients can develop a systemic vascular leakage, which results in increased hematocrit, pleural effusions, ascites, skin and mucosal hemorrhaging, and neurological dysfunction (Simmons et al., 2012; Calderón-Peláez et al., 2019). Unmanaged DHF can result in the patient developing DSS, which requires careful administration of supportive fluids (crystalloid therapies, isotonic colloids, and blood transfusions) (Simmons et al., 2012) and has a fatality rate of 10% in adults (Moulton et al., 2016) and 12-44% in children (Alejandria, 2015). Currently, no specific antivirals are approved for DENV, and only a single DENV vaccine has been approved for regulated usage in some countries (Deng et al., 2020).

1.4 Zika Virus

Similar to DENV, ZIKV was identified in the mid-20th century. ZIKV was first isolated from a rhesus monkey in 1947 in the Ziika Forest near Entebbe, Uganda, during a YFV survey (Dick et al., 1952). The first confirmed human case of ZIKV occurred in Nigeria in 1953 (MacNamara, 1954). Over the next six decades, despite its emergence in Asia, less than 20 human confirmed cases were reported (Musso et al., 2019), though this is likely an underestimation due to the misdiagnosis of ZIKV as DENV or other arboviruses (Braga et al., 2017). The first significant confirmed outbreak of ZIKV occurred in the State of Yap within the Federated States of Micronesia, where 73% of the population was infected (approximately 5400 people) in 2007 (Duffy et al., 2009).

The next major outbreak occurred in French Polynesia from 2013-2014, with over 20,000 cases suspected (Cao-Lormeau et al., 2014). In French Polynesia, the first associations of Guillain-Barré (GBS) with ZIKV infection were reported (Oehler et al., 2014). After spreading through several countries in Oceania (Petersen et al., 2016), ZIKV was first identified in Brazil in 2015 (Campos et al., 2015), though ZIKV likely arrived in the Americas in 2013 (Faria et al., 2016). Within a year of its identification in Brazil, ZIKV had spread to at least 33 countries and territories in the Americas (Petersen et al., 2016). Importantly, during the outbreak in the Americas, microcephaly was first identified as a pathology associated with maternal infection by ZIKV (Brasil et al., 2016; de Araújo et al., 2016; Rasmussen et al., 2016). Retrospective analysis has indicated that from the initial emergence until 2018, over 123 million people were infected in the Americas (Moore et al., 2020). Currently, localized ZIKV outbreaks are reported globally (Grubaugh et al., 2019; Yadav et al., 2019; Yakob, 2022).

Like the other flaviviruses spread by *Aedes* ssp. mosquitoes, ZIKV can cause visceral and febrile disease (Musso et al., 2019; Pierson and Diamond, 2020). The symptoms of ZIKV infection include macular or papular rashes, fever, myalgia, conjunctivitis, and headaches (Petersen et al., 2016). ZIKV can also manifest in severe neurological disease. As previously mentioned, ZIKV is associated with GBS in adult patients (Cao-Lormeau et al., 2014; Parra et al., 2016), specifically the acute inflammatory demyelinating polyneuropathy subtype (Parra et al., 2016). This condition causes progressive and ascending limb weakness and possibly acute flaccid paralysis (Shahrizaila et al., 2021). ZIKV, unlike DENV and YFV, can also cause significant tissue

damage within the male and female reproductive tracts (Govero et al., 2016; Murray et al., 2017; Tobar et al., 2018). ZIKV can persist in human semen for months (Vogt et al., 2022), not only affecting fertility but allowing for sexual transmission of the virus (Joguet et al., 2017).

What further separates ZIKV from the other flaviviruses spread by *Aedes* ssp. is its ability to manifest adverse fetal outcomes (Petersen et al., 2016). When a pregnant mother, generally one in their first or second trimester (Reefhuis et al., 2016), becomes infected with ZIKV, they can vertically transmit the virus to the gestating fetus via the placenta (Bhatnagar et al., 2017), where it infects the neural progenitor cells of the fetus (Ferraris et al., 2019). The resulting fetal infection can result in spontaneous abortion or developmental abnormalities, the latter of which is referred to as congenital Zika syndrome (CZS) (Freitas et al., 2020). These developmental abnormalities are primarily neurological (microcephaly, cerebral calcification, cerebral atrophy, cerebral cysts, hypertonia, seizures); however, abnormalities could be detected in other systems (Freitas et al., 2020). For all manifestations of ZIKV, there are no antivirals or a vaccine.

1.5 Immune-driven ecology and pathology

In addition to sharing a vector, ZIKV and DENV share several other vital characteristics critical to their epidemiology. The global distribution of ZIKV and DENV show significant overlaps (Rückert et al., 2017). At a molecular level, ZIKV and DENV show high amino acid similarities in key antibody targets (Barba-Spaeth et al., 2016; Chang et al., 2017; Andrade et al., 2019). Because of this, antibodies generated against one virus can interact with the remaining four viruses (Katzelnick et al., 2021).

This pre-existing immunity to ZIKV or DENV can protect against subsequent infections (Reich et al., 2013; Andrade et al., 2019; Rodriguez-Barraquer et al., 2019; Andrade et al., 2020; Katzelnick et al., 2021). It is proposed that immediately after infection, the patient has broad protection against the other four viruses, but over time this protection wanes (Sabin, 1950; 1952; Toh et al., 2014). This dynamic of broad and waning protection post-DENV and ZIKV infections has been postulated as the driver for the cyclical nature of DENV epidemics (Adams et al., 2006; Wearing and Rohani, 2006; Aguas et al., 2019; Perez et al., 2019; Brito et al., 2021) and why certain regions of Brazil had lower rates of CZS during the 2015-2016 epidemic (Pedroso et al., 2019).

Waning immunity has also been proposed as a driver of severe disease. This theory postulates that waning immunity promotes antibody-dependent enhancement (ADE), which involves sub-neutralizing antibodies binding to a pathogen and allowing for previously uncommon uptake by leukocytes via the Fcγ receptor (Dejnirattisai et al., 2016), resulting in enhanced replication and more severe pathologies (Flipse et al., 2016; Katzelnick et al., 2017). In DENV, ADE is a proposed driver of DHF and DSS (Guzmán et al., 2002; Halstead et al., 2002; Katzelnick et al., 2017; Fowler et al., 2018; Katzelnick et al., 2020). In ZIKV, ADE has been posited to be a driver of CZS (Castanha et al., 2016; Zimmerman et al., 2018; Rathore et al., 2019; Carvalho et al., 2020), where pre-existing DENV antibodies can promote the infection of placental macrophage (Zimmerman et al., 2018; Rathore et al., 2019), resulting in fetal infection and microcephaly (Rathore et al., 2019). The balance between protection against and enhancement of CZS appears to have a temporal component, similar to the cyclical nature of DENV (Carvalho et al., 2020). Given that pre-existing immunity plays a significant role in the ecology and pathology of flaviviruses, its impact on evolution warrants further examination.

1.6 Immune-driven evolution

Immune-driven evolution functions similarly to other forms of natural selection, where an external pressure (in this case, host immunity) drives a virus to alter its population to best suit that environment. In the case of immune-driven evolution, pre-existing host immunity will only neutralize a subpopulation of the virus (Marchi et al., 2021). The remaining virus, which likely was resistant to neutralization due to changes in available epitopes, becomes the “founder” or progenitor for subsequent transmitted generations of virus (Coffey et al., 2013; Morris et al., 2020) before it is neutralized by specific antibodies (Tan et al., 2020). The immunological driver for this behavior is a phenomenon called original antigenic sin, where the body does not initially generate novel antibodies against a similar pathogen but instead favors potent antigens from prior infections (Vatti et al., 2017).

Immune-driven evolution has been assessed for several viruses, including chikungunya (Jin et al., 2015), WNV (Sapkal et al., 2011), influenza (Lambkin et al., 1994; Cleveland et al., 1997; Ferguson et al., 2003; Doud et al., 2018; Lee et al., 2019), severe acute respiratory syndrome coronavirus 1 (Rockx et al., 2010), and severe acute respiratory syndrome coronavirus 2. These prior studies have highlighted that immune-driven evolution can render previous vaccines and

monoclonal antibody treatments ineffective (Boni, 2008; Willett et al., 2022; Xiong et al., 2022) and can lead to more infectious and virulent variants becoming the dominant species (Chen et al., 2022; Saito et al., 2022).

In the case of ZIKV, the theorized targets for immune-driven evolution are prM, E, NS1, and NS3. As mentioned above, these proteins contain many major epitopes for the host adaptive immune system (Lobigs et al., 1994; Vaughan et al., 2010) and play critical roles during the viral life cycle. For instance, prM and E form the external structure of the virion and thus play a significant role in cellular and tissue tropism (Chen et al., 1997; Lee and Lobigs, 2000; Tassaneetrithep et al., 2003; Meertens et al., 2012). NS1, which exists as an intracellular monomer, a membrane-bound dimer, and an extracellular hexamer (Rastogi et al., 2016), plays an important role in priming specific tissues for infection by increasing endothelial permeability (Puerta-Guardo et al., 2019; Puerta-Guardo et al., 2022). Finally, NS3, specifically the serine protease motif, is critical for processing the viral polyprotein (Falgout et al., 1991). Mutations within prM, E, NS1, and NS3 have all been associated with enhanced disease (Yuan et al., 2017; Xia et al., 2018; Collette et al., 2020; Shan et al., 2020; Liu et al., 2021) and transmission (Liu et al., 2017; Kuo et al., 2020; Liu et al., 2021). Despite the risk associated with mutations within these proteins, there have only been limited studies assessing the possible role of immune-driven evolution on ZIKV (Keeffe et al., 2018; Bailey et al., 2019; Dussupt et al., 2020; Regla-Nava et al., 2022). Several of these studies only use monoclonal antibodies as their immune pressure, which are not broadly representative of the complex epitope selection environment in human subjects (Keeffe et al., 2018; Bailey et al., 2019; Dussupt et al., 2020). Additionally, some studies did not draw direct conclusions from mutations associated with immune escape (Keeffe et al., 2018), or researchers studied mutations that were not exclusive to their immune pressure (Regla-Nava et al., 2022). Finally, only a subset compared the growth of the identified mutants to the wildtype virus (Dussupt et al., 2020; Regla-Nava et al., 2022), though none performed direct competitions between the mutants and the relevant wildtype virus. Therefore, there still exists a significant gap in understanding regarding immune-driven evolution of ZIKV.

1.7 Technical hurdles to studying evolution

One of the most fundamental tools of molecular virology necessary to study immune-driven evolution is the infectious clone. An infectious clone is a plasmid that contains the full-length

viral genome, which can be expressed, allowing for infectious virus to be rescued. (Aubry et al., 2015). Once developed, these clones can be manipulated for many functions, such as studying viral kinetics (Chuong et al., 2019; Bates et al., 2020), tropism (Kümmerer et al., 2012; Ayers et al., 2021; Kuchinsky et al., 2021), vaccinology (Mire et al., 2012; Plennevaux et al., 2017; Biswal et al., 2019; Furuyama et al., 2022), and, most critically, in our case, evolution (Atieh et al., 2018; Roesch et al., 2022). The traditional approach (Figure 3) to developing and manipulating infectious clones involves amplifying the viral genome by RT-PCR (RNA genomes) or PCR (DNA genome) and inserting the fragments into a plasmid which is then transformed into bacteria. This approach, however, can prove problematic due to the genomic instability of viruses within living hosts. This problem commonly arises in several medically relevant virus genera, such as *Flaviviruses* (Pu et al., 2011b), *Alphaviruses* (Steel et al., 2011), and *Betacoronaviruses* (Scobey et al., 2013). The mechanism that drives this behavior remains unclear; however, it is hypothesized that cryptic bacterial promoters exist within the viral genome, which drive the expression of toxic viral genes, play a significant role (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Li et al., 2011; Pu et al., 2011a; Pu et al., 2011b; Mudaliar and Sreekumar, 2016; Weger-Lucarelli et al., 2017). Because of this, when these clones are transformed into bacteria, a selective pressure is placed on the bacteria to favor plasmids containing deletions, mutations, or recombination that reduce the toxicity (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Li et al., 2011; Pu et al., 2011a; Pu et al., 2011b; Mudaliar and Sreekumar, 2016; Weger-Lucarelli et al., 2017), potentially altering the sequence of the clone and/or rendering a non-infectious clone.

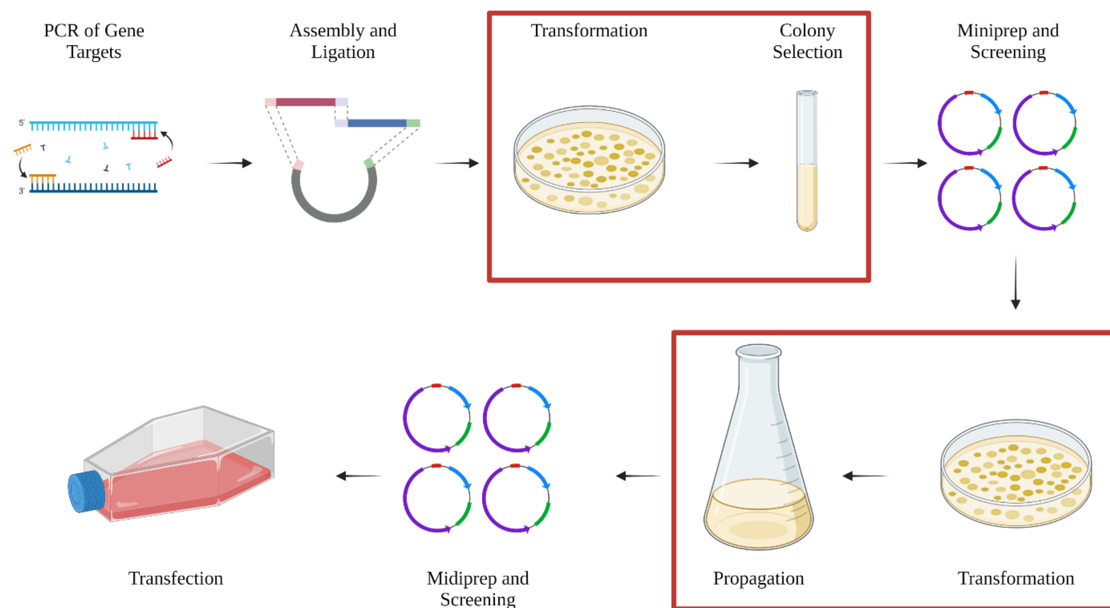


Figure 3: Genomic Instability in the Standard Cloning Workflow. Diagrammatic overview outlining the generation of a novel infectious clone. First, viral genes are amplified by either RT-PCR or PCR, depending on the genome type. Next, the products are assembled into plasmids which are then transformed into bacteria. After transformation, individual colonies are selected and screened. After initial screening, the plasmids are retransformed for the propagation of large-scale plasmid extractions, the products of which will be used in transfections. Red boxes highlight the stages of the workflow that are highly susceptible to the toxicity of infectious clones, resulting in alterations in the construct.

Previously, the main approaches to address these concerns have been ameliorating or mitigating the toxicity. To ameliorate the toxicity, introns (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Yamshchikov et al., 2001; Ulper et al., 2008; Tuo et al., 2015) or synonymous mutations (Pu et al., 2011b) can be introduced into the ORF. The goal of these methods is to disrupt transcription by the cryptic bacterial promoters, thus preventing toxicity. Modification can also be made to the backbone of the infectious clones to reduce its stress on the host (van der Most et al., 1999; Mishin et al., 2001; Yun et al., 2003; Cai et al., 2005; Pierro et al., 2006; Suzuki et al., 2007; Usme-Ciro et al., 2014). Additionally, non-bacterial hosts have been used (Polo et al., 1997; Puri et al., 2000; Kelly et al., 2010; Santos et al., 2013; Rihn et al., 2021). Regardless of technique, these systems have struggled to entirely remove all viral genome toxicities. To mitigate toxicity is maintaining the viral genome across several plasmids, combining fragments from these plasmids *in vitro,* and then using the product for downstream transfection (Rice et al., 1989; Messer et al., 2012; Scobey et al., 2013; Gallichotte et al., 2015;

Messer et al., 2016; Cockrell et al., 2017; Weger-Lucarelli et al., 2017; Ayers et al., 2021). An alternative mitigation approach is to remove the bacteria and directly use PCR products from viral genomes or synthesized fragments in Gibson assembly, infectious subgenomic amplicons, or circular polymerase extension cloning/reaction (CPEC/CPER) (Edmonds et al., 2013; Siridechadilok et al., 2013; Aubry et al., 2014a; Aubry et al., 2014b; Aubry et al., 2015; Mohamed Ali et al., 2018; Torii et al., 2021). The products of these reactions can then be directly transfected into cells (Edmonds et al., 2013; Siridechadilok et al., 2013; Aubry et al., 2014a; Aubry et al., 2014b; Aubry et al., 2015; Mohamed Ali et al., 2018; Torii et al., 2021). While these attempts to mitigate toxicity will produce infectious virus, they often fail to produce a full-length cDNA clone that can be manipulated and shared.

Due to the emergence of novel *in vitro* technologies, there have been reinvigorated attempts to streamline the development and manipulation of infectious clones. Previous evidence has shown that rolling circle amplification (RCA) can rapidly generate large quantities of transfection-grade DNA from infectious clones (Weger-Lucarelli et al., 2018; Bates et al., 2020; Marano et al., 2020; Kang et al., 2021), effectively replacing the need for large-scale bacterial plasmid extractions. Furthermore, the development of replication cycle reaction (RCR) technologies (Su'Etsugu et al., 2017; Hasebe et al., 2018; Nara and Su'Etsugu, 2021; Ueno et al., 2021) has offered a new avenue for developing novel clones and manipulating pre-existing clones. The RCR system, which reconstitutes the bacterial replication machinery *in vitro* (Su'Etsugu et al., 2017), allows for high-fidelity replication (Su'Etsugu et al., 2017) without a host. Leveraging this technology in molecular virology will help other researchers perform the work they see as critical and provide appropriate tools to understand the impacts of pre-existing host immunity more fully on ZIKV evolution.

1.8 Summary

The purpose of this dissertation is twofold. First, we set out to address the genomic instability of infectious clones in bacteria, reducing the barrier to entry for other researchers. To do this, we demonstrated that *in vitro* RCA can replace traditional large-scale plasmid isolations as a template for transfections of infectious clones. We then improved on a previous RCA-driven pipeline to manipulate infectious clones (Weger-Lucarelli et al., 2018) by using RCR, which produces a supercoiled product with fewer steps. Furthermore, we demonstrated that the RCR

platform could also be used to generate novel infectious clones, therefore removing the need for bacteria completely.

With a more efficient and robust method for making and manipulating infectious clones, we could then utilize these tools to study immune-driven evolution of ZIKV. We observed that ZIKV, after passaging in the presence of convalescent DENV serum, became significantly more fit in mammalian hosts at the expense of its fitness in mosquitoes, aligning with the trade-off hypothesis (Wilson and Yoshimura, 1994; Kassen, 2002). Further, we determined that the enhanced fitness in mammalian cells was not necessarily due to neutralization escape. This work will aid in combating emerging pathogens in the future by providing the necessary tools to study viral evolution and demonstrating the critical role that pre-existing immunity has on flavivirus evolution.

1.9 Supplementary Data

Virus	NCBI Accession Number	Virus	NCBI Accession Number
Hepatitis C virus	EU781801.1	Zika virus	KX377337.1
Murray Valley encephalitis virus	NC_000943.1	Cell fusing agent virus	MZ972994.1
Usutu virus	KF573410.1	Kamiti River virus	NC_005064.1
Japanese encephalitis virus	MK558811.1	Culex flavivirus	OK413947.1
West Nile virus	KF647251.1	Tick-borne encephalitis virus	ON228434.1
St. Louis encephalitis virus	NC_007580.2	Omsk hemorrhagic fever virus	MW847419.1
Dengue virus serotype 1	ON123667.1	Kyasanur Forest disease virus	MH013227.1
Dengue virus serotype 2	ON398847.1	Powassan virus	MZ576219.1
Dengue virus serotype 3	AB189128.1	Jutiapa virus	NC_026620.1
Dengue virus serotype 4	ON799403.1	Montana myotis leukoencephalitis virus	NC_004119.1
Yellow fever virus	NC_002031.1	Apoi virus	NC_003676.1

Supplemental Table 1: Reference for Flavivirus Phylogenetic Tree: Virus names and NCBI accession numbers of the sequences used to construct the phylogenetic tree.

1.10 References

Adams, B., Holmes, E.C., Zhang, C., Mammen, M.P., Nimmannitya, S., Kalayanarooj, S., et al. (2006). Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. *Proceedings of the National Academy of Sciences* 103(38), 14234-14239. doi: 10.1073/pnas.0602768103.

Aguas, R., Dorigatti, I., Coudeville, L., Luxemburger, C., and Ferguson, N.M. (2019). Cross-serotype interactions and disease outcome prediction of dengue infections in Vietnam. *Scientific Reports* 9(1). doi: 10.1038/s41598-019-45816-6.

Alejandria, M.M. (2015). Dengue haemorrhagic fever or dengue shock syndrome in children. *BMJ Clin Evid* 2015.

Andrade, P., Gimblet-Ochieng, C., Modirian, F., Collins, M., Cárdenas, M., Katzelnick, L.C., et al. (2019). Impact of pre-existing dengue immunity on human antibody and memory B cell responses to Zika. *Nature Communications* 10(1). doi: 10.1038/s41467-019-08845-3.

Andrade, P., Narvekar, P., Montoya, M., Michlmayr, D., Balmaseda, A., Coloma, J., et al. (2020). Primary and secondary dengue virus infections elicit similar memory B cell responses but breadth to other serotypes and cross-reactivity to Zika virus is higher in secondary dengue. *The Journal of Infectious Diseases*. doi: 10.1093/infdis/jiaa120.

Atieh, T., El Ayoubi, M.D., Aubry, F., Priet, S., De Lamballerie, X., and Nougairède, A. (2018). Haiku: New paradigm for the reverse genetics of emerging RNA viruses. *PLOS ONE* 13(2)**,** e0193069. doi: 10.1371/journal.pone.0193069.

Aubry, F., Nougairede, A., De Fabritus, L., Querat, G., Gould, E.A., and De Lamballerie, X. (2014a). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. 95(Pt_11)**,** 2462-2467. doi: 10.1099/vir.0.068023-0.

Aubry, F., Nougairède, A., de Fabritus, L., Querat, G., Gould, E.A., and de Lamballerie, X. (2014b). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *J Gen Virol* 95(Pt 11)**,** 2462-2467. doi: 10.1099/vir.0.068023-0.

Aubry, F., Nougairède, A., Gould, E.A., and de Lamballerie, X. (2015). Flavivirus reverse genetic systems, construction techniques and applications: a historical perspective. *Antiviral research* 114**,** 67-85. doi: 10.1016/j.antiviral.2014.12.007.

Ayers, J.B., Xie, X., Coatsworth, H., Stephenson, C.J., Waits, C.M., Shi, P.Y., et al. (2021). Infection Kinetics and Transmissibility of a Reanimated Dengue Virus Serotype 4 Identified Originally in Wild Aedes aegypti From Florida. *Front Microbiol* 12**,** 734903. doi: 10.3389/fmicb.2021.734903.

Bailey, M.J., Broecker, F., Freyn, A.W., Choi, A., Brown, J.A., Fedorova, N., et al. (2019). Human Monoclonal Antibodies Potently Neutralize Zika Virus and Select for Escape

Mutations on the Lateral Ridge of the Envelope Protein. *Journal of Virology* 93(14). doi: 10.1128/jvi.00405-19.

Barba-Spaeth, G., Dejnirattisai, W., Rouvinski, A., Vaney, M.-C., Medits, I., Sharma, A., et al. (2016). Structural basis of potent Zika–dengue virus antibody cross-neutralization. *Nature* 536(7614)**,** 48-53. doi: 10.1038/nature18938.

Bates, T., Chuong, C., Hawks, S.A., Rai, P., Salgado, R.M., Duggal, N.K., et al. (2020). "Development and characterization of infectious clones of two strains of Usutu virus". Cold Spring Harbor Laboratory).

Bhatnagar, J., Rabeneck, D.B., Martines, R.B., Reagan-Steiner, S., Ermias, Y., Estetter, L.B., et al. (2017). Zika Virus RNA Replication and Persistence in Brain and Placental Tissue. *Emerg Infect Dis* 23(3)**,** 405-414. doi: 10.3201/eid2303.161499.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., et al. (2013). The global distribution and burden of dengue. *Nature* 496(7446)**,** 504-507. doi: 10.1038/nature12060.

Biswal, S., Reynales, H., Saez-Llorens, X., Lopez, P., Borja-Tabora, C., Kosalaraksa, P., et al. (2019). Efficacy of a Tetravalent Dengue Vaccine in Healthy Children and Adolescents. *New England Journal of Medicine* 381(21)**,** 2009-2019. doi: 10.1056/nejmoa1903869.

Bogich, T.L., Chunara, R., Scales, D., Chan, E., Pinheiro, L.C., Chmura, A.A., et al. (2012). Preventing Pandemics Via International Development: A Systems Approach. *PLoS Medicine* 9(12)**,** e1001354. doi: 10.1371/journal.pmed.1001354.

Boni, M.F. (2008). Vaccination and antigenic drift in influenza. *Vaccine* 26**,** C8-C14. doi: https://doi.org/10.1016/j.vaccine.2008.04.011.

Brady, O.J., Gething, P.W., Bhatt, S., Messina, J.P., Brownstein, J.S., Hoen, A.G., et al. (2012). Refining the Global Spatial Limits of Dengue Virus Transmission by Evidence-Based Consensus. *PLoS Neglected Tropical Diseases* 6(8)**,** e1760. doi: 10.1371/journal.pntd.0001760.

Braga, J.U., Bressan, C., Dalvi, A.P.R., Calvet, G.A., Daumas, R.P., Rodrigues, N., et al. (2017). Accuracy of Zika virus disease case definition during simultaneous Dengue and Chikungunya epidemics. *PLOS ONE* 12(6)**,** e0179725. doi: 10.1371/journal.pone.0179725.

Brasil, P., Pereira, J.P., Jr., Moreira, M.E., Ribeiro Nogueira, R.M., Damasceno, L., Wakimoto, M., et al. (2016). Zika Virus Infection in Pregnant Women in Rio de Janeiro. *N Engl J Med* 375(24)**,** 2321-2334. doi: 10.1056/NEJMoa1602412.

Brito, A.F., Machado, L.C., Oidtman, R.J., Siconelli, M.J.L., Tran, Q.M., Fauver, J.R., et al. (2021). Lying in wait: the resurgence of dengue virus after the Zika epidemic in Brazil. *Nature Communications* 12(1)**,** 2619. doi: 10.1038/s41467-021-22921-7.

Cai, Z., Zhang, C., Chang, K.-S., Jiang, J., Ahn, B.-C., Wakita, T., et al. (2005). Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. *Journal of virology* 79(22)**,** 13963-13973.

Calderón-Peláez, M.A., Velandia-Romero, M.L., Bastidas-Legarda, L.Y., Beltrán, E.O., Camacho-Ortega, S.J., and Castellanos, J.E. (2019). Dengue Virus Infection of Blood-Brain Barrier Cells: Consequences of Severe Disease. *Front Microbiol* 10**,** 1435. doi: 10.3389/fmicb.2019.01435.

Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika Virus Outbreak, Bahia, Brazil. *Emerg Infect Dis* 21(10)**,** 1885-1886. doi: 10.3201/eid2110.150847.

Cao-Lormeau, V.M., Roche, C., Teissier, A., Robin, E., Berry, A.L., Mallet, H.P., et al. (2014). Zika virus, French polynesia, South pacific, 2013. *Emerg Infect Dis* 20(6)**,** 1085-1086. doi: 10.3201/eid2006.140138.

Carlson, C.J., Albery, G.F., Merow, C., Trisos, C.H., Zipfel, C.M., Eskew, E.A., et al. (2022). Climate change increases cross-species viral transmission risk. *Nature* 607(7919)**,** 555-562. doi: 10.1038/s41586-022-04788-w.

Carvalho, M.S., Freitas, L.P., Cruz, O.G., Brasil, P., and Bastos, L.S. (2020). Association of past dengue fever epidemics with the risk of Zika microcephaly at the population level in Brazil. *Scientific Reports* 10(1). doi: 10.1038/s41598-020-58407-7.

Castanha, P.M., Nascimento, E.J.M., Cynthia, B., Cordeiro, M.T., De Carvalho, O.V., De Mendonça, L.R., et al. (2016). Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection. *Journal of Infectious Diseases***,** jiw638. doi: 10.1093/infdis/jiw638.

Chang, H.H., Huber, R.G., Bond, P.J., Grad, Y.H., Camerini, D., Maurer-Stroh, S., et al. (2017). Systematic analysis of protein identity between Zika virus and other arthropod-borne viruses. *Bull World Health Organ* 95(7)**,** 517-525i. doi: 10.2471/blt.16.182105.

Chao, L.H., Klein, D.E., Schmidt, A.G., Peña, J.M., and Harrison, S.C. (2014). Sequential conformational rearrangements in flavivirus membrane fusion. *Elife* 3**,** e04389. doi: 10.7554/eLife.04389.

Chen, J., Wang, R., Gilby, N.B., and Wei, G.-W. (2022). Omicron Variant (B.1.1.529): Infectivity, Vaccine Breakthrough, and Antibody Resistance. *Journal of Chemical Information and Modeling* 62(2)**,** 412-422. doi: 10.1021/acs.jcim.1c01451.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., et al. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3(8)**,** 866-871. doi: 10.1038/nm0897-866.

Chuong, C., Bates, T.A., and Weger-Lucarelli, J. (2019). Infectious cDNA clones of two strains of Mayaro virus for studies on viral pathogenesis and vaccine development. *Virology* 535**,** 227-231. doi: https://doi.org/10.1016/j.virol.2019.07.013.

Ciota, A.T. (2019). The role of co-infection and swarm dynamics in arbovirus transmission. *Virus Research* 265**,** 88-93. doi: https://doi.org/10.1016/j.virusres.2019.03.010.

Ciota, A.T., Ehrbar, D.J., Van Slyke, G.A., Willsey, G.G., and Kramer, L.D. (2012). Cooperative interactions in the West Nile virus mutant swarm. *BMC Evol Biol* 12**,** 58. doi: 10.1186/1471-2148-12-58.

Cleveland, S.M., Taylor, H.P., and Dimmock, N.J. (1997). Selection of neutralizing antibody escape mutants with type A influenza virus HA-specific polyclonal antisera: possible significance for antigenic drift. *Epidemiology and Infection* 118(2)**,** 149-154. doi: 10.1017/s0950268896007303.

Cockrell, A.S., Beall, A., Yount, B., and Baric, R. (2017). "Efficient Reverse Genetic Systems for Rapid Genetic Manipulation of Emergent and Preemergent Infectious Coronaviruses." Springer New York), 59-81.

Coffey, L.L., Forrester, N., Tsetsarkin, K., Vasilakis, N., and Weaver, S.C. (2013). Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. 8(2)**,** 155-176. doi: 10.2217/fmb.12.139.

Coffey, L.L., and Vignuzzi, M. (2011). Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. *J Virol* 85(2)**,** 1025-1035. doi: 10.1128/jvi.01918-10.

Collette, N.M., Lao, V.H.I., Weilhammer, D.R., Zingg, B., Cohen, S.D., Hwang, M., et al. (2020). Single Amino Acid Mutations Affect Zika Virus Replication In Vitro and Virulence In Vivo. *Viruses* 12(11). doi: 10.3390/v12111295.

de Araújo, T.V.B., Rodrigues, L.C., de Alencar Ximenes, R.A., de Barros Miranda-Filho, D., Montarroyos, U.R., de Melo, A.P.L., et al. (2016). Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. *Lancet Infect Dis* 16(12)**,** 1356-1363. doi: 10.1016/s1473-3099(16)30318-8.

Dejnirattisai, W., Supasa, P., Wongwiwat, W., Rouvinski, A., Barba-Spaeth, G., Duangchinda, T., et al. (2016). Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nature Immunology* 17(9)**,** 1102-1108. doi: 10.1038/ni.3515.

Deng, S.Q., Yang, X., Wei, Y., Chen, J.T., Wang, X.J., and Peng, H.J. (2020). A Review on Dengue Vaccine Development. *Vaccines (Basel)* 8(1). doi: 10.3390/vaccines8010063.

Dick, G.W., Kitchen, S.F., and Haddow, A.J. (1952). Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 46(5)**,** 509-520. doi: 10.1016/0035-9203(52)90042-4.

Domingo, E., and Perales, C. (2019). Viral quasispecies. *PLOS Genetics* 15(10)**,** e1008271. doi: 10.1371/journal.pgen.1008271.

Doud, M.B., Lee, J.M., and Bloom, J.D. (2018). How single mutations affect viral escape from broad and narrow antibodies to H1 influenza hemagglutinin. *Nature Communications* 9(1). doi: 10.1038/s41467-018-03665-3.

Duffy, M.R., Chen, T.-H., Hancock, W.T., Powers, A.M., Kool, J.L., Lanciotti, R.S., et al. (2009). Zika Virus Outbreak on Yap Island, Federated States of Micronesia. *New England Journal of Medicine* 360(24)**,** 2536-2543. doi: 10.1056/NEJMoa0805715.

Dussupt, V., Sankhala, R.S., Gromowski, G.D., Donofrio, G., De La Barrera, R.A., Larocca, R.A., et al. (2020). Potent Zika and dengue cross-neutralizing antibodies induced by Zika vaccination in a dengue-experienced donor. *Nature Medicine* 26(2)**,** 228-235. doi: 10.1038/s41591-019-0746-2.

Edmonds, J., Van Grinsven, E., Prow, N., Bosco-Lauth, A., Brault, A.C., Bowen, R.A., et al. (2013). A Novel Bacterium-Free Method for Generation of Flavivirus Infectious DNA by

Circular Polymerase Extension Reaction Allows Accurate Recapitulation of Viral Heterogeneity. *Journal of Virology* 87(4)**,** 2367-2372. doi: 10.1128/jvi.03162-12.

Falgout, B., Pethel, M., Zhang, Y.M., and Lai, C.J. (1991). Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol* 65(5)**,** 2467-2475. doi: 10.1128/jvi.65.5.2467-2475.1991.

Faria, N.R., Azevedo, R., Kraemer, M.U.G., Souza, R., Cunha, M.S., Hill, S.C., et al. (2016). Zika virus in the Americas: Early epidemiological and genetic findings. *Science* 352(6283)**,** 345-349. doi: 10.1126/science.aaf5036.

Ferguson, N.M., Galvani, A.P., and Bush, R.M. (2003). Ecological and immunological determinants of influenza evolution. *Nature* 422(6930)**,** 428-433. doi: 10.1038/nature01509.

Ferraris, P., Cochet, M., Hamel, R., Gladwyn-Ng, I., Alfano, C., Diop, F., et al. (2019). Zika virus differentially infects human neural progenitor cells according to their state of differentiation and dysregulates neurogenesis through the Notch pathway. *Emerg Microbes Infect* 8(1)**,** 1003-1016. doi: 10.1080/22221751.2019.1637283.

Flipse, J., Diosa-Toro, M.A., Hoornweg, T.E., van de Pol, D.P., Urcuqui-Inchima, S., and Smit, J.M. (2016). Antibody-Dependent Enhancement of Dengue Virus Infection in Primary Human Macrophages; Balancing Higher Fusion against Antiviral Responses. *Sci Rep* 6**,** 29201. doi: 10.1038/srep29201.

Fowler, A.M., Tang, W.W., Young, M.P., Mamidi, A., Viramontes, K.M., McCauley, M.D., et al. (2018). Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. *Cell Host & Microbe* 24(5)**,** 743-750.e745. doi: 10.1016/j.chom.2018.09.015.

Freitas, D.A., Souza-Santos, R., Carvalho, L.M.A., Barros, W.B., Neves, L.M., Brasil, P., et al. (2020). Congenital Zika syndrome: A systematic review. *PLOS ONE* 15(12)**,** e0242367. doi: 10.1371/journal.pone.0242367.

Furuyama, W., Shifflett, K., Pinski, A.N., Griffin, A.J., Feldmann, F., Okumura, A., et al. (2022). Rapid Protection from COVID-19 in Nonhuman Primates Vaccinated Intramuscularly but Not Intranasally with a Single Dose of a Vesicular Stomatitis Virus-Based Vaccine. *mBio* 13(1)**,** e0337921-e0337921. doi: 10.1128/mbio.03379-21.

Gallichotte, E.N., Widman, D.G., Yount, B.L., Wahala, W.M., Durbin, A., Whitehead, S., et al. (2015). A New Quaternary Structure Epitope on Dengue Virus Serotype 2 Is the Target

of Durable Type-Specific Neutralizing Antibodies. *mBio* 6(5), e01461-01415. doi: 10.1128/mbio.01461-15.

Goldstein, S.A., Brown, J., Pedersen, B.S., Quinlan, A.R., and Elde, N.C. (2022). Extensive Recombination-driven Coronavirus Diversification Expands the Pool of Potential Pandemic Pathogens. *Genome Biol Evol* 14(12). doi: 10.1093/gbe/evac161.

Govero, J., Esakky, P., Scheaffer, S.M., Fernandez, E., Drury, A., Platt, D.J., et al. (2016). Zika virus infection damages the testes in mice. *Nature* 540(7633), 438-442. doi: 10.1038/nature20556.

Grubaugh, N.D., Saraf, S., Gangavarapu, K., Watts, A., Tan, A.L., Oidtman, R.J., et al. (2019). Travel Surveillance and Genomics Uncover a Hidden Zika Outbreak during the Waning Epidemic. *Cell* 178(5), 1057-1071.e1011. doi: https://doi.org/10.1016/j.cell.2019.07.018.

Gubler, D.J. (1998). Dengue and Dengue Hemorrhagic Fever. *Clinical Microbiology Reviews* 11(3), 480-496. doi: doi:10.1128/CMR.11.3.480.

Guzmán, M.G., Kourí, G., Valdés, L., Bravo, J., Vázquez, S., and Halstead, S.B. (2002). Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica* 11(4), 223-227. doi: 10.1590/s1020-49892002000400003.

Hackett, B.A., and Cherry, S. (2018). Flavivirus internalization is regulated by a size-dependent endocytic pathway. *Proc Natl Acad Sci U S A* 115(16), 4246-4251. doi: 10.1073/pnas.1720032115.

Hackett, B.A., Yasunaga, A., Panda, D., Tartell, M.A., Hopkins, K.C., Hensley, S.E., et al. (2015). RNASEK is required for internalization of diverse acid-dependent viruses. *Proc Natl Acad Sci U S A* 112(25), 7797-7802. doi: 10.1073/pnas.1424098112.

Halstead, S.B., Lan, N.T., Myint, T.T., Shwe, T.N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic fever in infants: research opportunities ignored. *Emerg Infect Dis* 8(12), 1474-1479. doi: 10.3201/eid0812.020170.

Hasebe, T., Narita, K., Hidaka, S., and Su'etsugu, M. (2018). Efficient Arrangement of the Replication Fork Trap for In Vitro Propagation of Monomeric Circular DNA in the Chromosome-Replication Cycle Reaction. *Life (Basel)* 8(4). doi: 10.3390/life8040043.

Hotta, S. (1952). Experimental studies on dengue. I. Isolation, identification and modification of the virus. *J Infect Dis* 90(1), 1-9. doi: 10.1093/infdis/90.1.1.

Jerzak, G.V., Brown, I., Shi, P.Y., Kramer, L.D., and Ebel, G.D. (2008). Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. *Virology* 374(2)**,** 256-260. doi: 10.1016/j.virol.2008.02.032.

Jin, J., Nathan, Chen, D.-H., Liao, M., Julie, Raeann, et al. (2015). Neutralizing Monoclonal Antibodies Block Chikungunya Virus Entry and Release by Targeting an Epitope Critical to Viral Pathogenesis. *Cell Reports* 13(11)**,** 2553-2564. doi: 10.1016/j.celrep.2015.11.043.

Joguet, G., Mansuy, J.M., Matusali, G., Hamdi, S., Walschaerts, M., Pavili, L., et al. (2017). Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis* 17(11)**,** 1200-1208. doi: 10.1016/s1473-3099(17)30444-9.

Johansen, I.E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 93(22)**,** 12400-12405. doi: 10.1073/pnas.93.22.12400.

Kang, L., He, G., Sharp, A.K., Wang, X., Brown, A.M., Michalak, P., et al. (2021). A selective sweep in the Spike gene has driven SARS-CoV-2 human adaptation. *bioRxiv***,** 2021.2002.2013.431090. doi: 10.1101/2021.02.13.431090.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* 15(2)**,** 173-190. doi: https://doi.org/10.1046/j.1420-9101.2002.00377.x.

Katzelnick, L.C., Gresh, L., Halloran, M.E., Mercado, J.C., Kuan, G., Gordon, A., et al. (2017). Antibody-dependent enhancement of severe dengue disease in humans. *Science* 358(6365)**,** 929-932. doi: doi:10.1126/science.aan6836.

Katzelnick, L.C., Narvaez, C., Arguello, S., Lopez Mercado, B., Collado, D., Ampie, O., et al. (2020). Zika virus infection enhances future risk of severe dengue disease. *Science* 369(6507)**,** 1123-1128. doi: 10.1126/science.abb6143.

Katzelnick, L.C., Zambrana, J.V., Elizondo, D., Collado, D., Garcia, N., Arguello, S., et al. (2021). Dengue and Zika virus infections in children elicit cross-reactive protective and enhancing antibodies that persist long term. *Sci Transl Med* 13(614)**,** eabg9478. doi: 10.1126/scitranslmed.abg9478.

Keeffe, J.R., Van Rompay, K.K.A., Olsen, P.C., Wang, Q., Gazumyan, A., Azzopardi, S.A., et al. (2018). A Combination of Two Human Monoclonal Antibodies Prevents Zika Virus Escape Mutations in Non-human Primates. *Cell Reports* 25(6)**,** 1385-1394.e1387. doi: 10.1016/j.celrep.2018.10.031.

Kelly, E.P., Puri, B., Sun, W., and Falgout, B. (2010). Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate. *Vaccine* 28(17)**,** 3030-3037.

Keusch, G.T., Amuasi, J.H., Anderson, D.E., Daszak, P., Eckerle, I., Field, H., et al. (2022). Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. *Proceedings of the National Academy of Sciences* 119(42)**,** e2202871119. doi: doi:10.1073/pnas.2202871119.

Khare, B., and Kuhn, R.J. (2022). The Japanese Encephalitis Antigenic Complex Viruses: From Structure to Immunity. *Viruses* 14(10). doi: 10.3390/v14102213.

Kuchinsky, S.C., Frere, F., Heitzman-Breen, N., Golden, J., Vázquez, A., Honaker, C.F., et al. (2021). Pathogenesis and shedding of Usutu virus in juvenile chickens. *Emerging Microbes & Infections* 10(1)**,** 725-738. doi: 10.1080/22221751.2021.1908850.

Kümmerer, B.M., Grywna, K., Gläsker, S., Wieseler, J., and Drosten, C. (2012). Construction of an infectious Chikungunya virus cDNA clone and stable insertion of mCherry reporter genes at two different sites. *Journal of General Virology* 93(9)**,** 1991-1995. doi: https://doi.org/10.1099/vir.0.043752-0.

Kuo, L., Jaeger, A.S., Banker, E.M., Bialosuknia, S.M., Mathias, N., Payne, A.F., et al. (2020). Reversion to ancestral Zika virus NS1 residues increases competence of Aedes albopictus. *PLoS Pathog* 16(10)**,** e1008951. doi: 10.1371/journal.ppat.1008951.

Lambkin, R., McLain, L., Jones, S.E., Aldridge, S.L., and Dimmock, N.J. (1994). Neutralization escape mutants of type A influenza virus are readily selected by antisera from mice immunized with whole virus: a possible mechanism for antigenic drift. *Journal of General Virology* 75(12)**,** 3493-3502. doi: 10.1099/0022-1317-75-12-3493.

Lee, E., and Lobigs, M. (2000). Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. *J Virol* 74(19)**,** 8867-8875. doi: 10.1128/jvi.74.19.8867-8875.2000.

Lee, J.M., Eguia, R., Zost, S.J., Choudhary, S., Wilson, P.C., Bedford, T., et al. (2019). Mapping person-to-person variation in viral mutations that escape polyclonal serum targeting influenza hemagglutinin. *eLife* 8. doi: 10.7554/elife.49324.

Li, D., Aaskov, J., and Lott, W.B. (2011). Identification of a Cryptic Prokaryotic Promoter within the cDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLoS ONE* 6(3)**,** e18197. doi: 10.1371/journal.pone.0018197.

Liu, J., Liu, Y., Shan, C., Nunes, B.T.D., Yun, R., Haller, S.L., et al. (2021). Role of mutational reversions and fitness restoration in Zika virus spread to the Americas. *Nature Communications* 12(1). doi: 10.1038/s41467-020-20747-3.

Liu, Y., Liu, J., Du, S., Shan, C., Nie, K., Zhang, R., et al. (2017). Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. *Nature* 545(7655)**,** 482-486. doi: 10.1038/nature22365.

Lobigs, M., Arthur, C.E., Müllbacher, A., and Blanden, R.V. (1994). The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. *Virology* 202(1)**,** 195-201. doi: 10.1006/viro.1994.1335.

López-Moya, J.J., and García, J.A. (2000). Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res* 68(2)**,** 99-107. doi: 10.1016/s0168-1702(00)00161-1.

MacNamara, F.N. (1954). Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 48(2)**,** 139-145. doi: https://doi.org/10.1016/0035-9203(54)90006-1.

Marani, M., Katul, G.G., Pan, W.K., and Parolari, A.J. (2021). Intensity and frequency of extreme novel epidemics. *Proceedings of the National Academy of Sciences* 118(35)**,** e2105482118. doi: doi:10.1073/pnas.2105482118.

Marano, J.M., Chuong, C., and Weger-Lucarelli, J. (2020). Rolling circle amplification: A high fidelity and efficient alternative to plasmid preparation for the rescue of infectious clones. *Virology* 551**,** 58-63. doi: 10.1016/j.virol.2020.08.016.

Marchi, J., Lässig, M., Walczak, A.M., and Mora, T. (2021). Antigenic waves of virus–immune coevolution. *Proceedings of the National Academy of Sciences* 118(27)**,** e2103398118. doi: 10.1073/pnas.2103398118.

Meertens, L., Carnec, X., Lecoin, M.P., Ramdasi, R., Guivel-Benhassine, F., Lew, E., et al. (2012). The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. *Cell Host Microbe* 12(4)**,** 544-557. doi: 10.1016/j.chom.2012.08.009.

Messer, W.B., Yount, B., Hacker, K.E., Donaldson, E.F., Huynh, J.P., de Silva, A.M., et al. (2012). Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS neglected tropical diseases* 6(2)**,** e1486-e1486. doi: 10.1371/journal.pntd.0001486.

Messer, W.B., Yount, B.L., Royal, S.R., De Alwis, R., Widman, D.G., Smith, S.A., et al. (2016). Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *Journal of Virology* 90(10)**,** 5090-5097. doi: 10.1128/jvi.00155-16.

Mire, C.E., Miller, A.D., Carville, A., Westmoreland, S.V., Geisbert, J.B., Mansfield, K.G., et al. (2012). Recombinant vesicular stomatitis virus vaccine vectors expressing filovirus glycoproteins lack neurovirulence in nonhuman primates. *PLoS Negl Trop Dis* 6(3)**,** e1567. doi: 10.1371/journal.pntd.0001567.

Mishin, V.P., Cominelli, F., and Yamshchikov, V.F. (2001). A 'minimal' approach in design of flavivirus infectious DNA. *Virus Research* 81(1)**,** 113-123. doi: https://doi.org/10.1016/S0168-1702(01)00371-9.

Mohamed Ali, S., Vega-Rúa, A., Driouich, J.-S., de Lamballerie, X., Failloux, A.-B., and Nougairède, A. (2018). Comparison of chikungunya viruses generated using infectious clone or the Infectious Subgenomic Amplicons (ISA) method in Aedes mosquitoes. *PLOS ONE* 13(6)**,** e0199494. doi: 10.1371/journal.pone.0199494.

Moore, S.M., Oidtman, R.J., Soda, K.J., Siraj, A.S., Reiner, R.C., Jr., Barker, C.M., et al. (2020). Leveraging multiple data types to estimate the size of the Zika epidemic in the Americas. *PLOS Neglected Tropical Diseases* 14(9)**,** e0008640. doi: 10.1371/journal.pntd.0008640.

Morris, D.H., Petrova, V.N., Rossine, F.W., Parker, E., Grenfell, B.T., Neher, R.A., et al. (2020). Asynchrony between virus diversity and antibody selection limits influenza virus evolution. *eLife* 9**,** e62105. doi: 10.7554/eLife.62105.

Moulton, S.L., Mulligan, J., Srikiatkhachorn, A., Kalayanarooj, S., Grudic, G.Z., Green, S., et al. (2016). State-of-the-art monitoring in treatment of dengue shock syndrome: a case series. *J Med Case Rep* 10(1)**,** 233. doi: 10.1186/s13256-016-1019-z.

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N.L., Khasnatinov, M., et al. (2015). New insights into flavivirus evolution, taxonomy and biogeographic history, extended by analysis of canonical and alternative coding sequences. *PLoS One* 10(2)**,** e0117849. doi: 10.1371/journal.pone.0117849.

Mudaliar, P.P., and Sreekumar, E. (2016). Exploring the instability of reporters expressed under the subgenomic promoter in Chikungunya virus infectious cDNA clones. 45**,** 448. doi: 10.1016/j.ijid.2016.02.950.

Murray, K.O., Gorchakov, R., Carlson, A.R., Berry, R., Lai, L., Natrajan, M., et al. (2017). Prolonged Detection of Zika Virus in Vaginal Secretions and Whole Blood. *Emerg Infect Dis* 23(1)**,** 99-101. doi: 10.3201/eid2301.161394.

Musso, D., Ko, A.I., and Baud, D. (2019). Zika Virus Infection — After the Pandemic. *New England Journal of Medicine* 381(15)**,** 1444-1457. doi: 10.1056/NEJMra1808246.

Nara, S., and Su'Etsugu, M. (2021). In vitro amplification of whole large plasmids via transposon-mediated oriC insertion. *BioTechniques* 71(4)**,** 528-533. doi: 10.2144/btn-2021-0068.

Ng, W.C., Soto-Acosta, R., Bradrick, S.S., Garcia-Blanco, M.A., and Ooi, E.E. (2017). The 5' and 3' Untranslated Regions of the Flaviviral Genome. *Viruses* 9(6). doi: 10.3390/v9060137.

Oehler, E., Watrin, L., Larre, P., Leparc-Goffart, I., Lastère, S., Valour, F., et al. (2014). Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. *Eurosurveillance* 19(9)**,** 20720. doi: doi:https://doi.org/10.2807/1560-7917.ES2014.19.9.20720.

Olsen, B.S., and Johansen, I.E. (2001). Nucleotide sequence and infectious cDNA clone of the L1 isolate of Pea seed-borne mosaic potyvirus. *Arch Virol* 146(1)**,** 15-25. doi: 10.1007/s007050170187.

Parra, B., Lizarazo, J., Jiménez-Arango, J.A., Zea-Vera, A.F., González-Manrique, G., Vargas, J., et al. (2016). Guillain–Barré Syndrome Associated with Zika Virus Infection in Colombia. *New England Journal of Medicine* 375(16)**,** 1513-1523. doi: 10.1056/NEJMoa1605564.

Pedroso, C., Fischer, C., Feldmann, M., Sarno, M., Luz, E., Moreira-Soto, A., et al. (2019). Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil. *Emerg Infect Dis* 25(8)**,** 1485-1493. doi: 10.3201/eid2508.190113.

Perez, F., Llau, A., Gutierrez, G., Bezerra, H., Coelho, G., Ault, S., et al. (2019). The decline of dengue in the Americas in 2017: discussion of multiple hypotheses. *Trop Med Int Health* 24(4)**,** 442-453. doi: 10.1111/tmi.13200.

Perreira, J.M., Aker, A.M., Savidis, G., Chin, C.R., McDougall, W.M., Portmann, J.M., et al. (2015). RNASEK Is a V-ATPase-Associated Factor Required for Endocytosis and the Replication of Rhinovirus, Influenza A Virus, and Dengue Virus. *Cell Rep* 12(5)**,** 850-863. doi: 10.1016/j.celrep.2015.06.076.

Petersen, L.R., Jamieson, D.J., Powers, A.M., and Honein, M.A. (2016). Zika Virus. *New England Journal of Medicine* 374(16)**,** 1552-1563. doi: 10.1056/NEJMra1602113.

Pierro, D.J., Salazar, M.I., Beaty, B.J., and Olson, K.E. (2006). Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation. *Journal of general virology* 87(8)**,** 2263-2268.

Pierson, T.C., and Diamond, M.S. (2020). The continued threat of emerging flaviviruses. *Nature Microbiology* 5(6)**,** 796-812. doi: 10.1038/s41564-020-0714-0.

Plennevaux, E., Moureau, A., Arredondo-García, J.L., Villar, L., Pitisuttithum, P., Tran, N.H., et al. (2017). Impact of Dengue Vaccination on Serological Diagnosis: Insights From Phase III Dengue Vaccine Efficacy Trials. *Clinical Infectious Diseases*. doi: 10.1093/cid/cix966.

Polo, S., Ketner, G., Levis, R., and Falgout, B. (1997). Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *Journal of virology* 71(7)**,** 5366-5374.

Postnikova, Y., Treshchalina, A., Boravleva, E., Gambaryan, A., Ishmukhametov, A., Matrosovich, M., et al. (2021). Diversity and Reassortment Rate of Influenza A Viruses in Wild Ducks and Gulls. *Viruses* 13(6). doi: 10.3390/v13061010.

Pu, S.-Y., Wu, R.-H., Yang, C.-C., Jao, T.-M., Tsai, M.-H., Wang, J.-C., et al. (2011a). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *Journal of Virology* 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., et al. (2011b). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Puerta-Guardo, H., Biering, S.B., de Sousa, F.T.G., Shu, J., Glasner, D.R., Li, J., et al. (2022). Flavivirus NS1 Triggers Tissue-Specific Disassembly of Intercellular Junctions Leading to Barrier Dysfunction and Vascular Leak in a GSK-3β-Dependent Manner. *Pathogens* 11(6). doi: 10.3390/pathogens11060615.

Puerta-Guardo, H., Glasner, D.R., Espinosa, D.A., Biering, S.B., Patana, M., Ratnasiri, K., et al. (2019). Flavivirus NS1 Triggers Tissue-Specific Vascular Endothelial Dysfunction Reflecting Disease Tropism. *Cell Reports* 26(6)**,** 1598-1613.e1598. doi: 10.1016/j.celrep.2019.01.036.

Puri, B., Polo, S., Hayes, C.G., and Falgout, B. (2000). Construction of a full length infectious clone for dengue-1 virus Western Pacific, 74 strain. *Virus genes* 20(1)**,** 57-63.

Rasmussen, S.A., Jamieson, D.J., Honein, M.A., and Petersen, L.R. (2016). Zika virus and birth defects—reviewing the evidence for causality. *New England Journal of Medicine* 374(20)**,** 1981-1987.

Rastogi, M., Sharma, N., and Singh, S.K. (2016). Flavivirus NS1: a multifaceted enigmatic viral protein. *Virol J* 13**,** 131. doi: 10.1186/s12985-016-0590-7.

Rathore, A.P.S., Saron, W.A.A., Lim, T., Jahan, N., and St. John, A.L. (2019). Maternal immunity and antibodies to dengue virus promote infection and Zika virus–induced microcephaly in fetuses. *Science Advances* 5(2)**,** eaav3208. doi: 10.1126/sciadv.aav3208.

Reefhuis, J., Gilboa, S.M., Johansson, M.A., Valencia, D., Simeone, R.M., Hills, S.L., et al. (2016). Projecting Month of Birth for At-Risk Infants after Zika Virus Disease Outbreaks. *Emerg Infect Dis* 22(5)**,** 828-832. doi: 10.3201/eid2205.160290.

Regla-Nava, J.A., Wang, Y.-T., Fontes-Garfias, C.R., Liu, Y., Syed, T., Susantono, M., et al. (2022). A Zika virus mutation enhances transmission potential and confers escape from protective dengue virus immunity. *Cell Reports* 39(2)**,** 110655. doi: 10.1016/j.celrep.2022.110655.

Reich, N.G., Shrestha, S., King, A.A., Rohani, P., Lessler, J., Kalayanarooj, S., et al. (2013). Interactions between serotypes of dengue highlight epidemiological impact of cross-immunity. *J R Soc Interface* 10(86)**,** 20130414. doi: 10.1098/rsif.2013.0414.

Reid, D.W., Campos, R.K., Child, J.R., Zheng, T., Chan, K.W.K., Bradrick, S.S., et al. (2018). Dengue Virus Selectively Annexes Endoplasmic Reticulum-Associated Translation Machinery as a Strategy for Co-opting Host Cell Protein Synthesis. *J Virol* 92(7). doi: 10.1128/jvi.01766-17.

Renner, M., Dejnirattisai, W., Carrique, L., Martin, I.S., Karia, D., Ilca, S.L., et al. (2021). Flavivirus maturation leads to the formation of an occupied lipid pocket in the surface glycoproteins. *Nature Communications* 12(1)**,** 1238. doi: 10.1038/s41467-021-21505-9.

Rice, C.M., Grakoui, A., Galler, R., and Chambers, T.J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biol* 1(3)**,** 285-296.

Rihn, S.J., Merits, A., Bakshi, S., Turnbull, M.L., Wickenhagen, A., Alexander, A.J.T., et al. (2021). A plasmid DNA-launched SARS-CoV-2 reverse genetics system and coronavirus toolkit for COVID-19 research. *PLOS Biology* 19(2)**,** e3001091. doi: 10.1371/journal.pbio.3001091.

Rockx, B., Donaldson, E., Frieman, M., Sheahan, T., Corti, D., Lanzavecchia, A., et al. (2010). Escape from human monoclonal antibody neutralization affects in vitro and in vivo fitness of severe acute respiratory syndrome coronavirus. *J Infect Dis* 201(6)**,** 946-955. doi: 10.1086/651022.

Rodriguez-Barraquer, I., Costa, F., Nascimento, E.J.M., Nery, N.J., Castanha, P.M.S., Sacramento, G.A., et al. (2019). Impact of preexisting dengue immunity on Zika virus emergence in a dengue endemic region. *Science* 363(6427)**,** 607-610. doi: 10.1126/science.aav6618.

Roesch, F., Cereghino, C., Carrau, L., Hardy, A., Ribeiro-Filho, H., Lacritick, A.H., et al. (2022). The E2 glycoprotein holds key residues for Mayaro virus adaptation to the urban *Aedes aegypti* mosquito. *bioRxiv***,** 2022.2004.2005.487100. doi: 10.1101/2022.04.05.487100.

Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S.M., Young, M.C., Byas, A.D., Murrieta, R.A., et al. (2017). Impact of simultaneous exposure to arboviruses on infection and

transmission by Aedes aegypti mosquitoes. *Nature Communications* 8(1), 15412. doi: 10.1038/ncomms15412.

Sabin, A.B. (1950). The dengue group of viruses and its family relationships. *Bacteriol Rev* 14(3), 225-232.

Sabin, A.B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg* 1(1), 30-50. doi: 10.4269/ajtmh.1952.1.30.

Saito, A., Irie, T., Suzuki, R., Maemura, T., Nasser, H., Uriu, K., et al. (2022). Enhanced fusogenicity and pathogenicity of SARS-CoV-2 Delta P681R mutation. *Nature* 602(7896), 300-306. doi: 10.1038/s41586-021-04266-9.

Sanjuán, R., and Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences* 73(23), 4433-4448. doi: 10.1007/s00018-016-2299-6.

Santos, J.J.d.S., Cordeiro, M.T., Bertani, G.R., Marques, E.T.d.A., and Gil, L.H.V.G. (2013). Construction and characterisation of a complete reverse genetics system of dengue virus type 3. *Memórias do Instituto Oswaldo Cruz* 108, 983-991.

Sapkal, G.N., Harini, S., Ayachit, V.M., Fulmali, P.V., Mahamuni, S.A., Bondre, V.P., et al. (2011). Neutralization escape variant of West Nile virus associated with altered peripheral pathogenicity and differential cytokine profile. *Virus Res* 158(1-2), 130-139. doi: 10.1016/j.virusres.2011.03.023.

Scobey, T., Yount, B.L., Sims, A.C., Donaldson, E.F., Agnihothram, S.S., Menachery, V.D., et al. (2013). Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences* 110(40), 16157-16162. doi: 10.1073/pnas.1311542110.

Shahrizaila, N., Lehmann, H.C., and Kuwabara, S. (2021). Guillain-Barré syndrome. *The Lancet* 397(10280), 1214-1228. doi: https://doi.org/10.1016/S0140-6736(21)00517-1.

Shan, C., Xia, H., Haller, S.L., Azar, S.R., Liu, Y., Liu, J., et al. (2020). A Zika virus envelope mutation preceding the 2015 epidemic enhances virulence and fitness for transmission. *Proc Natl Acad Sci U S A* 117(33), 20190-20197. doi: 10.1073/pnas.2005722117.

Simmons, C.P., Farrar, J.J., van Vinh Chau, N., and Wills, B. (2012). Dengue. *New England Journal of Medicine* 366(15), 1423-1432. doi: 10.1056/NEJMra1110265.

Siridechadilok, B., Gomutsukhavadee, M., Sawaengpol, T., Sangiambut, S., Puttikhunt, C., Chin-inmanu, K., et al. (2013). A Simplified Positive-Sense-RNA Virus Construction

Approach That Enhances Analysis Throughput. *Journal of Virology* 87(23)**,** 12667-12674. doi: doi:10.1128/JVI.02261-13.

Steel, J.J., Henderson, B.R., Lama, S.B., Olson, K.E., and Geiss, B.J. (2011). Infectious alphavirus production from a simple plasmid transfection+. 8(1)**,** 356. doi: 10.1186/1743-422x-8-356.

Su'Etsugu, M., Takada, H., Katayama, T., and Tsujimoto, H. (2017). Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. *Nucleic Acids Research* 45(20)**,** 11525-11534. doi: 10.1093/nar/gkx822.

Suzuki, R., de Borba, L., Duarte dos Santos, C.N., and Mason, P.W. (2007). Construction of an infectious cDNA clone for a Brazilian prototype strain of dengue virus type 1: Characterization of a temperature-sensitive mutation in NS1. *Virology* 362(2)**,** 374-383. doi: https://doi.org/10.1016/j.virol.2006.11.026.

Tan, W., Lu, Y., Zhang, J., Wang, J., Dan, Y., Tan, Z., et al. (2020). Viral kinetics and antibody responses in patients with COVID-19. *MedRxiv*.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., et al. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* 197(7)**,** 823-829. doi: 10.1084/jem.20021840.

Tobar, P., Vega, M., Ordoñez, C., Rivera, L., Landivar, J., and Zambrano, H. (2018). Detection of Zika Virus and Human Papilloma Virus in Cervical Cytology Samples using Two Real Time PCR Based Techniques in Ecuadorian Women diagnosed with ASCUS. *P R Health Sci J* 37(Spec Issue)**,** S96-s98.

Toh, Y.X., Gan, V., Balakrishnan, T., Zuest, R., Poidinger, M., Wilson, S., et al. (2014). Dengue serotype cross-reactive, anti-e protein antibodies confound specific immune memory for 1 year after infection. *Front Immunol* 5**,** 388. doi: 10.3389/fimmu.2014.00388.

Torii, S., Ono, C., Suzuki, R., Morioka, Y., Anzai, I., Fauzyah, Y., et al. (2021). Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Reports* 35(3)**,** 109014. doi: https://doi.org/10.1016/j.celrep.2021.109014.

Tuo, D., Shen, W., Yan, P., Li, X., and Zhou, P. (2015). Rapid Construction of Stable Infectious Full-Length cDNA Clone of Papaya Leaf Distortion Mosaic Virus Using In-Fusion Cloning. *Viruses* 7(12)**,** 6241-6250. doi: 10.3390/v7122935.

Ueno, H., Sawada, H., Soga, N., Sano, M., Nara, S., Tabata, K.V., et al. (2021). Amplification of over 100 kbp DNA from Single Template Molecules in Femtoliter Droplets. *ACS Synthetic Biology* 10(9)**,** 2179-2186. doi: 10.1021/acssynbio.0c00584.

Ulper, L., Sarand, I., Rausalu, K., and Merits, A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of virological methods* 148(1-2)**,** 265-270. doi: 10.1016/j.jviromet.2007.10.007.

Usme-Ciro, J.A., Lopera, J.A., Enjuanes, L., Almazán, F., and Gallego-Gomez, J.C. (2014). Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome. *Virus Research* 180**,** 12-22. doi: https://doi.org/10.1016/j.virusres.2013.12.001.

van der Most, R.G., Corver, J., and Strauss, J.H. (1999). Mutagenesis of the RGD Motif in the Yellow Fever Virus 17D Envelope Protein. *Virology* 265(1)**,** 83-95. doi: https://doi.org/10.1006/viro.1999.0026.

Vatti, A., Monsalve, D.M., Pacheco, Y., Chang, C., Anaya, J.M., and Gershwin, M.E. (2017). Original antigenic sin: A comprehensive review. *J Autoimmun* 83**,** 12-21. doi: 10.1016/j.jaut.2017.04.008.

Vaughan, K., Greenbaum, J., Blythe, M., Peters, B., and Sette, A. (2010). Meta-analysis of All Immune Epitope Data in theFlavivirusGenus: Inventory of Current Immune Epitope Data Status in the Context of Virus Immunity and Immunopathology. *Viral Immunology* 23(3)**,** 259-284. doi: 10.1089/vim.2010.0006.

Vogt, M.B., McDonald, E.M., Delorey, M., Mead, P.S., Hook, S.A., Hinckley, A.F., et al. (2022). Prolonged shedding of Zika virus in human semen is associated with male reproductive tract inflammation. *J Infect Dis*. doi: 10.1093/infdis/jiac329.

Wearing, H.J., and Rohani, P. (2006). Ecological and immunological determinants of dengue epidemics. *Proceedings of the National Academy of Sciences* 103(31)**,** 11802-11807. doi: 10.1073/pnas.0602960103.

Weger-Lucarelli, J., Duggal, N.K., Bullard-Feibelman, K., Veselinovic, M., Romo, H., Nguyen, C., et al. (2017). Development and Characterization of Recombinant Virus Generated from a New World Zika Virus Infectious Clone. *Journal of Virology* 91(1)**,** JVI.01765-01716. doi: 10.1128/jvi.01765-16.

Weger-Lucarelli, J., Garcia, S.M., Rückert, C., Byas, A., O'Connor, S.L., Aliota, M.T., et al. (2018). Using barcoded Zika virus to assess virus population structure in vitro and in Aedes aegypti mosquitoes. *Virology* 521**,** 138-148. doi: 10.1016/j.virol.2018.06.004.

Willett, B.J., Grove, J., MacLean, O.A., Wilkie, C., De Lorenzo, G., Furnon, W., et al. (2022). SARS-CoV-2 Omicron is an immune escape variant with an altered cell entry pathway. *Nature Microbiology* 7(8)**,** 1161-1179. doi: 10.1038/s41564-022-01143-7.

Wilson, D.S., and Yoshimura, J. (1994). On the Coexistence of Specialists and Generalists. *The American Naturalist* 144(4)**,** 692-707. doi: 10.1086/285702.

Xia, H., Luo, H., Shan, C., Muruato, A.E., Nunes, B.T.D., Medeiros, D.B.A., et al. (2018). An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. *Nat Commun* 9(1)**,** 414. doi: 10.1038/s41467-017-02816-2.

Xiong, D., Zhao, X., Luo, S., Cong, Y., Zhang, J.Z.H., and Duan, L. (2022). Immune Escape Mechanisms of SARS-CoV-2 Delta and Omicron Variants against Two Monoclonal Antibodies That Received Emergency Use Authorization. *The Journal of Physical Chemistry Letters* 13(26)**,** 6064-6073. doi: 10.1021/acs.jpclett.2c00912.

Yadav, P.D., Malhotra, B., Sapkal, G., Nyayanit, D.A., Deshpande, G., Gupta, N., et al. (2019). Zika virus outbreak in Rajasthan, India in 2018 was caused by a virus endemic to Asia. *Infect Genet Evol* 69**,** 199-202. doi: 10.1016/j.meegid.2019.01.026.

Yakob, L. (2022). Zika Virus after the Public Health Emergency of International Concern Period, Brazil. *Emerg Infect Dis* 28(4)**,** 837-840. doi: 10.3201/eid2804.211949.

Yamshchikov, V., Mishin, V., and Cominelli, F. (2001). A New Strategy in Design of (+)RNA Virus Infectious Clones Enabling Their Stable Propagation in E. coli. *Virology* 281(2)**,** 272-280. doi: 10.1006/viro.2000.0793.

Yu, I.-M., Zhang, W., Holdaway, H.A., Li, L., Kostyuchenko, V.A., Chipman, P.R., et al. (2008). Structure of the Immature Dengue Virus at Low pH Primes Proteolytic Maturation. *Science* 319(5871)**,** 1834-1837. doi: doi:10.1126/science.1153264.

Yu, I.M., Holdaway, H.A., Chipman, P.R., Kuhn, R.J., Rossmann, M.G., and Chen, J. (2009). Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. *J Virol* 83(23)**,** 12101-12107. doi: 10.1128/jvi.01637-09.

Yuan, L., Huang, X.-Y., Liu, Z.-Y., Zhang, F., Zhu, X.-L., Yu, J.-Y., et al. (2017). A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science* 358(6365)**,** 933-936. doi: 10.1126/science.aam7120.

Yun, S.-I., Kim, S.-Y., Rice, C.M., and Lee, Y.-M. (2003). Development and application of a reverse genetics system for Japanese encephalitis virus. *Journal of virology* 77(11)**,** 6450-6465.

Zimmerman, M.G., Quicke, K.M., O'Neal, J.T., Arora, N., Machiah, D., Priyamvada, L., et al. (2018). Cross-Reactive Dengue Virus Antibodies Augment Zika Virus Infection of Human Placental Macrophages. *Cell Host & Microbe* 24(5)**,** 731-742.e736. doi: 10.1016/j.chom.2018.10.008.

Chapter Two: Rolling Circle Amplification: A high fidelity and efficient alternative to plasmid preparation for the rescue of infectious clones.

Jeffrey M. Marano[1,2], Christina Chuong[1], James Weger-Lucarelli[1]

1. Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, USA
2. Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Blacksburg, VA, United States

2.1 Abstract

Alphaviruses (genus *Alphavirus*; family *Togaviridae*) are a medically relevant family of viruses that include chikungunya virus and Mayaro virus. Infectious cDNA clones of these viruses are necessary molecular tools to understand viral biology. Traditionally, rescuing virus from an infectious cDNA clone requires propagating plasmids in bacteria, which can result in mutations in the viral genome due to bacterial toxicity or recombination and requires specialized equipment and knowledge to propagate the bacteria. Here, we present an alternative- rolling circle amplification (RCA), an *in vitro* technology. We demonstrate that the viral yield of transfected RCA product is comparable to midiprepped plasmid, albeit with a slight delay in kinetics. RCA, however, is cheaper and less time-consuming. Further, sequential RCA did not introduce mutations into the viral genome, subverting the need for glycerol stocks and retransformation. These results indicate that RCA is a viable alternative to traditional plasmid-based approaches to viral rescue.

Importance

The development of infectious cDNA clones is critical to studying viral pathogenesis and for developing vaccines. The current method for propagating clones in bacteria is limited by the toxicity of the viral genome within the bacterial host, resulting in deleterious mutations in the viral genome, which can only be detected through whole-genome sequencing. These mutations can cause unexpected and unwanted results leading to wasted time and resources. To that end, we have developed an alternative method of preparing large quantities of DNA that can be directly transfected to recover infectious virus without the need for bacteria by amplifying the infectious cDNA clone plasmid using rolling circle amplification (RCA). Our results indicate that viral rescue from an RCA product produces a viral yield equal to bacterial-derived plasmid

DNA, albeit with a slight delay in replication kinetics. The RCA platform, however, is significantly more cost and time-efficient compared to traditional approaches. When the simplicity and costs of RCA are combined, we propose that a shift to an RCA platform will benefit the field of molecular virology and could have significant advantages for recombinant vaccine production.

2.2. Introduction

RNA viruses produce significant disease in humans and animals, highlighted by the current outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Ahlquist et al., 2003). Infectious cDNA clones of these viruses are necessary molecular tools to understand viral biology because they facilitate the study of single-nucleotide polymorphisms (Atieh et al., 2018) and enable the insertion of reporter proteins to study virus replication or cell tropism (Kümmerer et al., 2012). cDNA clones have also been instrumental in developing vaccines for RNA viruses, notably CYD-TDV (Dengvaxia) (Plennevaux et al., 2017) and TAK-003 (Takeda) (Biswal et al., 2019), both of which are tetravalent chimeric vaccines against dengue virus.

Typically, the propagation of infectious cDNA clones before viral rescue requires the generation of high concentration plasmid stocks from bacteria, which is not only cumbersome and time-consuming but also presents an opportunity for the introduction of unwanted mutations during amplification in bacteria and toxic bacterial byproducts. Bacterial instability of viral genomes has been reported for flaviviruses (Pu et al., 2011b), alphaviruses (Steel et al., 2011), and coronaviruses (Scobey et al., 2013). The cause of this is likely cryptic prokaryotic promoters, which results in the expression of viral proteins inside of the bacteria, which due to their toxicity, can lead to the selection of plasmids with deletions, mutations, or recombination with reduced bacterial toxicity (Li et al., 2011; Mudaliar and Sreekumar, 2016; Weger-Lucarelli et al., 2017).

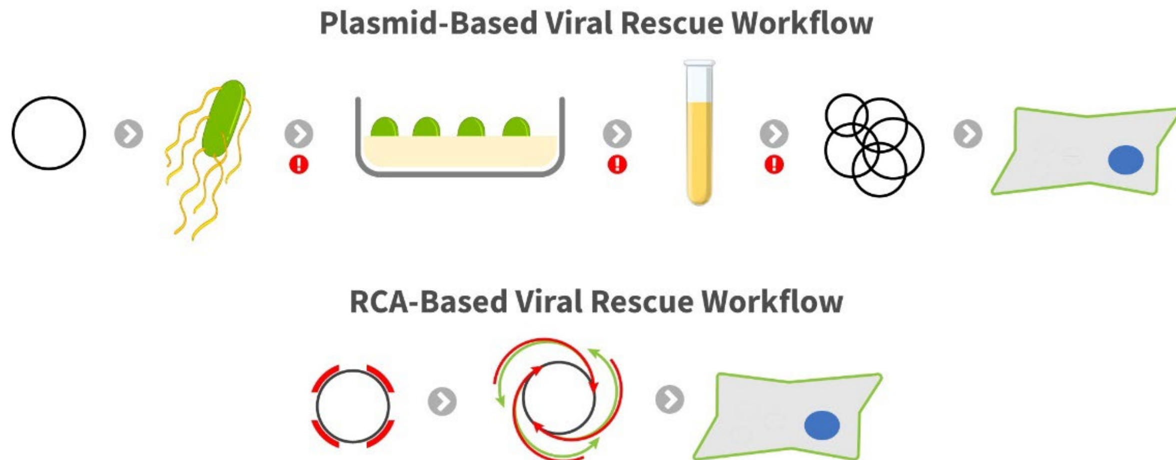


Fig. 1: Comparison of plasmid- and RCA-based workflows for viral rescue. The plasmid-based system involves the transformation of a plasmid into bacteria. The bacteria are then selected and propagated using antibiotic enriched media, and the plasmid is purified from the bacteria and transfected into the cell type of interest. The red exclamation points indicate points during the workflow where mutations and error can be introduced or enhanced. The RCA-based system involves amplification of the plasmid using random hexamers to produce the hyperbranched product. This product can then be directly transfected into cells.

There are several critical points within the plasmid-based rescue workflow where deletion or mutations can occur (Fig. 1). These include the transformation of the plasmid into the bacteria, the selection of colonies from the agar plate, and the propagation of the colony in liquid culture. While deletions are easy to identify in plasmids by restriction enzyme digestion, mutations can only be determined by whole-genome sequencing, which is costly and laborious. Furthermore, even synonymous changes can have profound impacts on viral replication (Cuevas et al., 2011; Nougairede et al., 2013; Canale et al., 2018) and should be avoided in cDNA clones. These unwanted changes to the viral genome can confound experimental results and, therefore, necessitate sequencing of the full viral genome every time new plasmid stocks are generated, a time-consuming and expensive task. Thus, removing the need for the bacterial host to maintain and propagate infectious cDNA clone plasmids would simplify the process of viral rescue and remove the possibility of deleterious bacterial-derived mutations

In this report, we describe a simple alternative to bacterial-based growth of infectious cDNA clones—*in vitro* amplification using rolling circle amplification (RCA) and direct transfection. RCA is an isothermal, high yield method of DNA amplification (Mohsen and Kool, 2016) that

uses a highly processive polymerase that can amplify DNA over 70kb (Blanco et al., 1989). Importantly, the enzyme replicates DNA with high fidelity due to its 3'—5'exonuclease—or proofreading—activity (Garmendia et al., 1992). We have previously used RCA to rescue infectious cDNA clones; however, in these studies, the RCA product was linearized with endonucleases, column purified, and then transfected (Aliota et al., 2018; Weger-Lucarelli et al., 2018b). In this study, using a clone driven by a cytomegalovirus (CMV) promoter, we show that peak virus yields were similar following the direct transfection of RCA- and plasmid-derived cDNA clones in several cell lines and that small amounts of the RCA product could be used to rescue virus successfully. Finally, we showed that we could further amplify an RCA product through additional rounds of RCA without the introduction of unwanted mutations, thereby allowing a simple, cheap, and high-fidelity means to propagate infectious cDNA clone plasmids. RCA-launched infectious cDNA clones represent a technical improvement in rescuing viruses without the need for bacteria.

2.3 Materials and Methods

Cell Culture.

Vero (*Cercopithecus aethiops* kidney epithelial cells, ATCC® CCL-81™), BHK-21 clone 13 (baby hamster kidney fibroblasts, ATCC® CCL-10™), and HEK293T (human embryonic kidney cells, ATCC® CRL-11268™) cell lines were maintained at 37°C in 5% CO2 using Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin. Plaque assays were performed as previously described [17] except that plates were either fixed for two days with tragacanth gum overlay (MP BIOMEDICALS catalog 0210479280) or three days with methylcellulose overlay (Spectrum Chemical catalog ME136-100GM) post-infection.

Rescue of infectious cDNA clones.

We performed transfections in 24 well plates at 60-80% confluency using the JetOptimus (Polyplus) DNA transfection reagent per manufacturer's instructions. Briefly, we mixed 200 µl of JetOptimus buffer with the DNA concentration of interest. JetOptimus reagent was then added at a ratio of 1 µl per 1 µg of DNA and incubated at room temperature for 10 minutes. The transfection mix was then added dropwise into the wells containing cells. We collected the

supernatant at different time points based on the experiment: each day for three days for the growth curves in multiple cell lines, each day for two days for the RCA input comparison and the kit comparison, and two days post-transfection for the sequential RCA experiment. Viral titer was then determined using plaque assays on Vero cells.

Plasmid Preparation

We used an infectious cDNA clone of Mayaro virus strain TRVL 4675, which has previously been described (Chuong et al., 2019), for our plasmid control. The plasmid was initially transformed into NEBstable electrocompetent cells. Cells were incubated for 16 hours at 30°C and then 24 hours at room temperature. Colonies were picked and incubated in Lennox Broth (LB) supplemented with 25 µg/ml of carbenicillin for 16 hours. We extracted DNA using both Promega PureYield miniprep kit, for verification, and Zymo Midiprep Kit, for transfection. We verified the plasmids using endonuclease digestion and gel electrophoresis and transfected the samples to ensure the infectivity of the clone. DNA concentration was determined using Invitrogen's Qubit 1x dsDNA HS kit.

RCA Protocols

For the SuperPhi RCA Premix Kit with Random Primers (Evomics catalog number PM100), 1 µl of 1 ng/µl of plasmid DNA was mixed with 4 µl of sample buffer while the thermocycler was preheated to 95°C. The mixture was incubated at 95°C for 1 minute and then rapidly cooled to 4°C. 5 µl of 2x SuperPhi Master Mix was then mixed with the sample, which was then incubated for 16 hours at 30°C before polymerase inactivation at 65°C for 10 minutes. For the GenomiPhi V3 DNA Amplification Kit (GE Healthcare), 1 µl of 10 ng/µl of plasmid DNA was mixed with 9 µl of molecular grade water and 10 µl of denaturation buffer. At the same time, the thermocycler was preheated to 95°C. The mixture was incubated for one minute at 95°C and then rapidly cooled to 4°C. 20 µl of the denatured template was then added to the lyophilized reaction cake containing enzymes, dNTPs, and buffers and thoroughly mixed by pipetting. Samples were incubated at 30°C for 90 minutes, and then the enzyme was inactivated at 65°C for 10 minutes. RCA product concentration was determined using Qubit after a 200-fold dilution in molecular grade water. Amplification of the plasmid was confirmed using endonuclease digestion and gel electrophoresis. Dilutions were performed using molecular grade water. To generate the RCA

passages, an initial RCA was performed using the SuperPhi protocol as described above and validated using gel electrophoresis. 1 µl of RCA product was then used as the template for a subsequent 10 µl RCA reaction. We then repeated the process of RCA for a total of three passages.

Sequencing Protocol

RCA products were Sanger sequenced at the Genomics Sequencing Center at Virginia Tech. RCA products were diluted to 100 ng/µl to prepare them for sequencing. 1 µl of diluted RCA product was mixed with 3 µl of 1 µM primer and 9 µl of molecular grade water. Resulting reads were aligned using SnapGene® 5.0.7 software (GSL Biotech).

Statistical Analysis

Statistics were performed using GraphPad Prism 8 (San Diego, CA). Two-way ANOVA tests were performed using Sidak's corrections for multiple comparisons for the comparison of titers in different cell lines. For the comparison of RCA kits, two-way ANOVA tests were performed using Dunnett's correction for multiple comparisons against the plasmid control. A one-way ANOVA was performed using Sidak's correction for multiple comparisons against the plasmid control for the sequential RCA test.

2.4 Results and Discussion

Peak virus yields are similar for RCA- and plasmid-derived virus in different cell lines

We sought to determine the efficiency of virus recovery following the direct transfection of an RCA product in several commonly used cell lines, including Vero, HEK293T, and BHK21 (Clone 13) cells (Ozaki et al., 2004; Atieh et al., 2017). For these experiments, we used a CMV-driven Mayaro virus (MAYV; Genus *Alphavirus*, Family *Togaviridae*) infectious cDNA clone as both the template for our RCA reactions and as our plasmid control for the transfections (Chuong et al., 2019). Alphaviruses are a group of small, enveloped, medically relevant positive-sense RNA viruses with genomes of 11-12 kilobases in length (Leung et al., 2011). Our rationale for using an alphavirus clone for these studies was their ease of use and that they have been used as expression vectors for foreign proteins extensively (Lundstrom, 2016; Singh et al., 2019). Following transfection, we collected virus each day until 90% of the cells showed cytopathic

effect (CPE), which occurred by day three in all cases. On the first day post-transfection, the viral titer in the plasmid transfection was significantly higher compared to the RCA product in both BHK21 and HEK293T cells (Fig. 2; p=0.0002 and p= 0.0007, respectively). No difference was observed in Vero cells (p=0.0961). There was no significant difference in any cell line (Vero p= 0.9445, BHK21 p=0.2937, HEK293T p=0.0599) two days post-transfection, the peak of virus replication for all cell lines. At three days post-transfection, there was no difference between viral titers produced by RCA and plasmid in Vero and BHK21 cells (p=0.1736 and p=0.6140, respectively). However, the titer of RCA product transfection was significantly higher than the plasmid titer in HEK293T cells (p=0.0027).

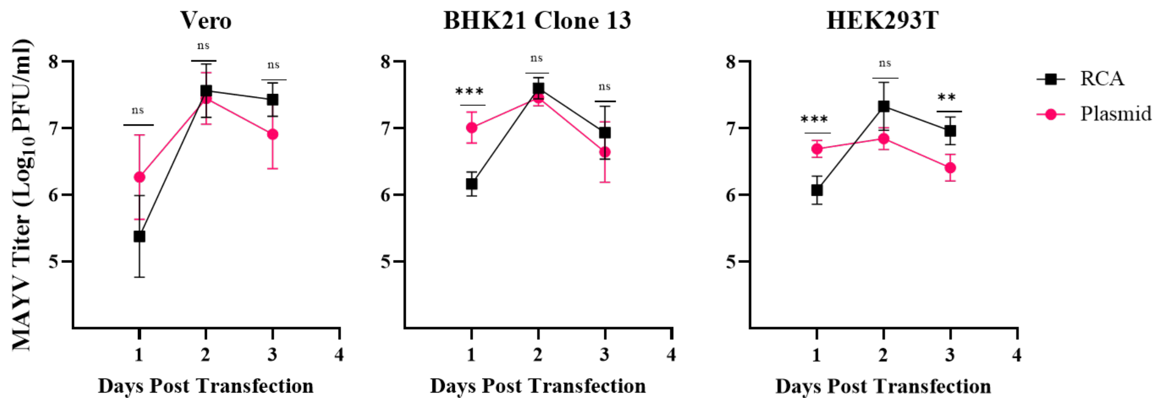


Fig. 2: Comparison of viral titers produced by either plasmid or RCA in various cell lines. The kinetics of plasmid and RCA techniques to produce virus were assessed in Vero, BHK21 Clone 13, and HEK293T cells. Cells were transfected in triplicate with either 500 ng of Evomics SuperPhi RCA or plasmid DNA in triplicate. The experiment was done in two independent biological replicates. The supernatant was collected each day post-transfection until cells reached 90% CPE for plaque assay. Error bars represent the standard deviation from the mean. Statistical analysis was performed using two-way ANOVA with *ad hoc* Sidak's correction for multiple comparisons (ns P > 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

The above results demonstrate two critical features of using RCA for viral rescue: similar replication kinetics—albeit with a slight delay—and identical peak yield. In all the cell lines, the peak viral titer occurred on the second-day post-transfection for both plasmid and RCA product transfection. These data demonstrate that RCA-rescue is equivalent to plasmid-rescue in its ability to rescue virus using standard transfection conditions in terms of viral yield. A hypothesis for the delay in viral production seen in BHK and HEK293T cells is that the complex structure

of RCA products (i.e., branched RCA molecules) compared to plasmid DNA may produce steric inhibition and delay transcription from the CMV promoter by RNA polymerase II (Mohsen and Kool, 2016).

Peak viral titer is not dependent on DNA input or RCA kit

Since the kinetics of virus recovery were similar for all cell lines, and since peak titers were observed two days post-transfection, we only used Vero cells and only sampled on the first- and second days following transfection for all future studies. Next, we sought to determine whether transfections with different RCA concentrations would result in efficient virus rescue. To that end, we transfected Vero cells with a range of RCA inputs produced using the Evomics SuperPhi kit (Fig. 3). One-day post-transfection, 100 ng of RCA resulted in a decreased viral titer compared to a plasmid input of 500 ng ($p = 0.0002$). We observed no differences in any of the other input concentrations. As in the above experiment, by two days post-transfection, RCA and plasmid titers were the same (100 ng SuperPhi $p= .9275$, 250 ng SuperPhi $p=.9991$, 500 ng $p>.9999$, 1000 ng $p=.9952$). These results indicate that the transfection of RCA product is robust and can tolerate a wide variety of input concentrations without altering peak viral yield.

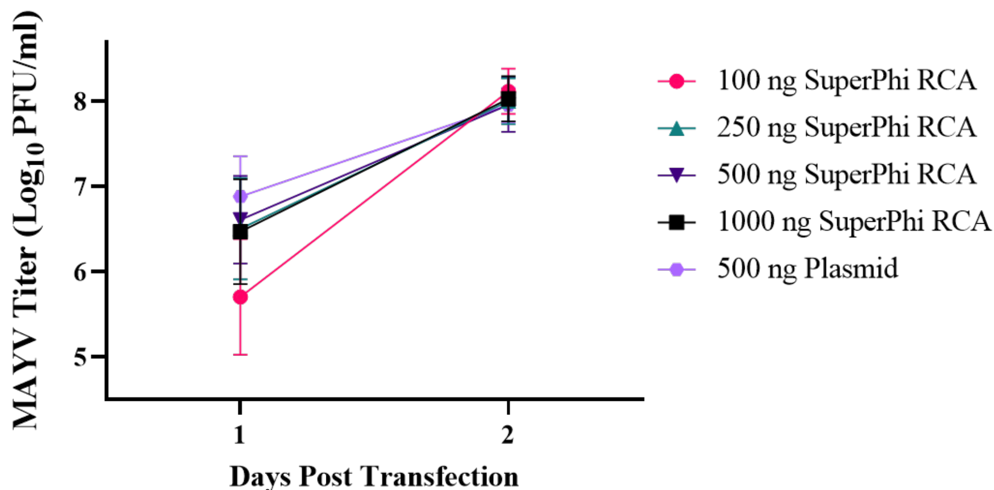


Fig. 3: Assessing the effects of RCA input on the resulting viral titer. The effect of RCA input on viral production kinetics was examined using Vero cells. Cells were transfected in triplicate with 100 ng, 250 ng, 500 ng, or 1000 ng of Evomics SuperPhi RCA or 500 ng of the plasmid. The supernatant was collected at one and 2-days post-transfection for plaque assay. Error bars represent the standard deviation from the mean. Statistical analysis was performed using two-way ANOVA with *ad hoc* Dunnett's correction for multiple comparisons.

To ensure that the above results were not restricted to a specific RCA kit, Vero cells were transfected in triplicate in two independent replicates with RCA product produced using both the Evomics SuperPhi Kit and the GE GenomiPhi Kit or plasmid DNA (Fig. 4). One day post-transfection, the viral titers produced from both 250 ng and 500 ng of GenomiPhi RCA products were lower than the titers produced by plasmid ($p = 0.0008$ and $p = 0.0005$, respectively). There was no significant difference between the SuperPhi samples and the plasmid samples one-day post-transfection (250 ng p=.4915, 500 ng p=.4490). All titers were the same two days post-transfection compared to plasmid rescue (250 ng SuperPhi p=.9997, 500 ng SuperPhi p=.9748, 250 ng GenomiPhi p=.9966, 500 ng GenomiPhi p=.9927.) Thus, peak viral yields or recovery kinetics of RCA products are not dependent on the RCA kit.

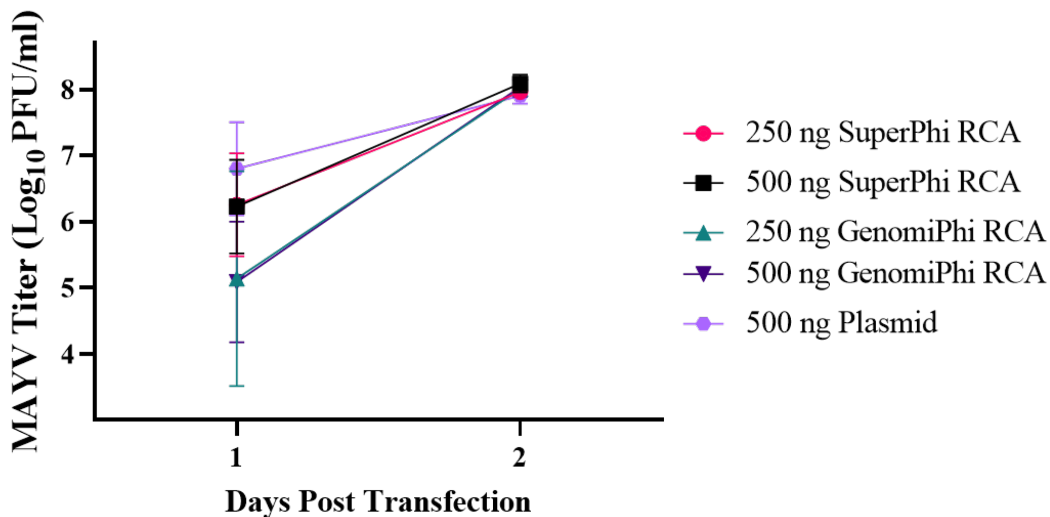


Fig. 4: Assessing the effects of RCA kits on resulting viral titer. The effect of RCA kits on viral production kinetics was examined using Vero cells. Cells were transfected with 250 ng or 500 ng of either Evomics SuperPhi or GE GenomiPhi RCA or 500 ng of plasmid DNA. The supernatant was collected at one and 2-days post-transfection for plaque assay. Error bars represent the standard deviation from the mean. Statistical analysis was performed using two-way ANOVA with ad hoc Dunnett's correction for multiple comparisons against a plasmid control.

Sequential RCA allows for simple propagation of an infectious cDNA clone without introducing errors in the viral genome

To determine if RCA can further amplify an RCA product without introducing unwanted mutations, an initial RCA was performed using plasmid DNA as a template (subsequently referred to as Passage 0) and amplified three more times. Following the transfection of the different "passages," we found no significant differences between the viral titer produced in

passage 0 RCA DNA and plasmid DNA (p= 0.2518) (Fig. 5). However, we did note a difference between the titers of later RCA passages and plasmid DNA (p = 0.0008, p = 0.0007, and p = 0.0016, respectively).

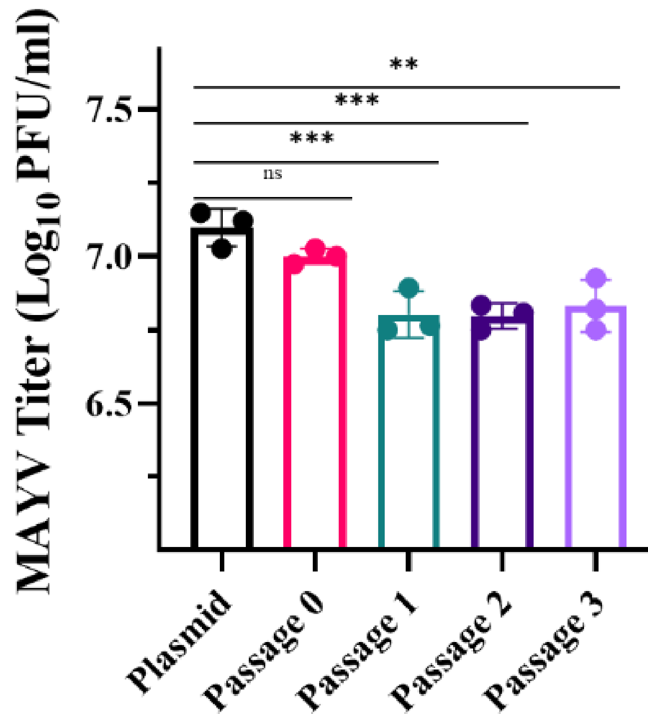


Fig. 5: Assessing the effects of repetitive RCA on resulting viral titer. We examined the impact of sequential RCA on the ability to rescue virus in Vero cells. We used the Evomics SuperPhi RCA kit and a sample of midiprepped DNA as a template to generate RCA products (Passage 0). We then used the RCA product as the template for subsequent RCA reactions (Passage 1-3). We transfected the cells using 500 ng of RCA product or 500 ng of plasmid DNA in triplicate. The supernatant was collected 2-days post-transfection for plaque assay. Error bars represent the standard deviation from the mean. Statistical analysis was performed using one-way ANOVA with ad hoc Dunnett's correction for multiple comparisons against the plasmid control (ns $P > 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

To determine if these differences were due to either mutations or artifacts of repeated amplification, RCA DNA from passage 0 and 3 was sequenced using Sanger Sequencing. The sequences for passage 0, passage 3, and the original plasmid were identical, indicating that no mutations were introduced in the viral genome during repeated RCAs. Repeated RCAs may have amplified both specific and non-specific DNA leading to the decreased viral yield. Amplification of non-specific DNA, caused by the concatemerization of the random hexamer primers (Inoue et al., 2006), alters the ratio of specific to non-specific DNA, resulting in a reduced amount of

target DNA for transfection. To mitigate the effect of moderate titer reduction with sequential RCA reaction, harvesting virus at a slightly later time or increasing DNA input may be effective. However, these results indicate that RCA products can effectively act as a template for subsequent RCA reactions without introducing unwanted mutations. RCA products can, therefore, substitute the standard glycerol bacterial stock protocol, or repeated bacterial transformations to generate midi- or maxi-prepped DNA. In both bacterial methods, mutations can be introduced during growth and, thus, require sequencing.

Cost analysis and time comparisons show that the RCA platform is both less expensive and rapidly deployable, compared to the traditional bacterial plasmid platform.

The evidence above demonstrates that the RCA platform is equivalent to the plasmid-based platform for generating virus from cDNA infectious clones. However, when considering the time and cost to perform these two processes, it is apparent that RCA offers many advantages (Table 1). For the SuperPhi kit, one RCA reaction costs $3.79 and can produce 10 µg of DNA in 16 hours. When using the plasmid approach, colonies would need to be selected and screened using endonuclease digestion. An average screening of ten colonies using the Promega PureYield Plasmid Miniprep System will cost approximately $15.70 in total. Positive colonies would then be used to inoculate liquid cultures for midiprep. Minimally, one to four midiprepped colonies, using the ZymoPURE II Plasmid Midiprep Kit, costs between $9.16 and $36.64. The extracted midiprep DNA would need to be confirmed by Sanger sequencing, which can cost roughly $150 per genome. Therefore, the final cost of the plasmid workflow ranges from $174.86 to $652.34, with a final DNA yield of 80 µg.

Stepwise Cost and Time Analysis of RCA and Plasmid Systems					
Plasmid			RCA System		
Protocol	**Cost**	**Time**	**Protocol**	**Cost**	**Time**
Transformation and Miniprep	$15.70	3 days	RCA Amplification	$3.79	16 hours
Midiprep	$9.16 - $36.64	1 day	DNA Yield	10 µg	
Sequencing	$150 - $600	2 days	Cost per µg	$.38/µg	
Total	$174.86 - $652.34	6 days			
DNA Yield	80 µg				
Cost per µg	$2.19 - $8.15/µg				

Table 1: Comparison of the cost and time requirements for the plasmid and RCA systems. The cost calculations in the table are based on the following kits: Promega PureYield™ Plasmid Miniprep System (catalog A1222), ZymoPURE™ II Plasmid Midiprep Kit (catalog D4201), and Evomics SuperPhi RCA Premix Kit with Random Primers (catalog number PM100). In constructing the cost estimate, it was assumed that ten colonies were selected for screening, and one to four of those colonies were then midiprepped and sequenced.

When comparing cost per µg of DNA, the RCA system is superior to the plasmid approach. An RCA reaction costs $0.38/µg, while the plasmid approach costs between $2.19-$8.15/µg. This difference is further emphasized when considering the time to complete the two workflows. An RCA reaction takes 16 hours or less and can be transfected directly while the bacterial approach takes several days and requires sequencing. Given these analyses, the RCA platform is a more time and cost-efficient method for rescuing viruses and produces similar results, indicating that a shift to the RCA-based approach would simplify viral rescue while saving time and money.

2.5 Conclusion

Here, we report a simple method to recover infectious virus from a cDNA clone using RCA to amplify a plasmid. We observed that both RCA and plasmid-based transfection produced similar peak viral titers following transfection for several cell lines, using several RCA kits, and when transfecting variable input DNA amounts. Importantly, RCA products can be reamplified by RCA to maintain a DNA record without generating mutations in the viral genome. Finally, the use of an RCA platform will reduce both the cost and time required to amplify a plasmid stock and rescue virus.

The use of RCA-launched expression from RNA polymerase II promoter containing constructs has several potential commercial applications as well, including DNA and recombinant live-

attenuated vaccines. Bacterial-derived plasmids have several safety concerns, including endotoxins, transposition of pathogenic elements, and the introduction of antibiotic resistance into the environment (Glenting and Wessels, 2005). RCA mitigates many of these concerns since the antibiotic resistance marker is not required, and RCA products are free from endotoxin. This study has two limitations: first, we only used a single MAYV infectious cDNA clone to characterize the RCA rescue system, and second, we only tested viral rescue from a clone driven by a cytomegalovirus (CMV) promoter. However, we have successfully used RCA to amplify and rescue infectious virus from several infectious cDNA clones. These include emerging zoonotic viruses (Usutu virus; Genus *Flavivirus*, Family *Flaviviridae*) (Bates et al., 2020), and other medically relevant viruses including, Zika virus (Genus *Flavivirus*, Family *Flaviviridae*) (Weger-Lucarelli et al., 2018b), West Nile virus (Genus *Flavivirus*, Family *Flaviviridae*) (Grubaugh et al., 2016; Grubaugh et al., 2017), chikungunya virus (unpublished data; Genus *Alphavirus*, Family *Togaviridae*), and SARS-CoV-2 (unpublished data; Genus *Betacoronavirus*, Family *Coronaviridae*). We anticipate that this system can be used for other positive-sense RNA virus cDNA clones and likely negative-strand viruses as well. Moreover, using a CMV promoter allows for simple transfection of small amounts of plasmid DNA without the need for extra reagents to produce viral RNA or potential issues with unwanted mutations derived from the error-prone bacteriophage promoters (Steel et al., 2011). However, we have also used a linearized and purified RCA product to generate full-length infectious RNA transcripts from bacteriophage-driven clones, indicating the versatility of this system (Grubaugh et al., 2016; Grubaugh et al., 2017). Taken together, RCA represents a simple, high-fidelity, and cost-effective means to produce large amounts of plasmid DNA that can be repeatedly propagated and used to rescue infectious virus directly.

2.6 Author Contributions:

Conceptualization, J.W.L; methodology, J.M.M, C.C and J.W.L; experimentation and formal analysis, J.M.M; validation, J.M.M, C.C, and J.W.L; writing—original draft preparation, J.M.M; writing—review and editing, J.M.M, C.C, and J.W.L; visualization, J.M.M, and J.W.L. All authors have read and agreed to the published version of the manuscript.

Copyright: As the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required.

2.7 References

Ahlquist, P., Noueiry, A. O., Lee, W.-M., Kushner, D. B., & Dye, B. T. (2003). Host factors in positive-strand RNA virus genome replication. *Journal of Virology*, *77*(15), 8181-8186. https://doi.org/10.1128/jvi.77.15.8181-8186.2003

Aliota, M. T., Dudley, D. M., Newman, C. M., Weger-Lucarelli, J., Stewart, L. M., Koenig, M. R., Breitbach, M. E., Weiler, A. M., Semler, M. R., Barry, G. L., Zarbock, K. R., Haj, A. K., Moriarty, R. V., Mohns, M. S., Mohr, E. L., Venturi, V., Schultz-Darken, N., Peterson, E., Newton, W., . . . O'Connor, D. H. (2018). Molecularly barcoded Zika virus libraries to probe in vivo evolutionary dynamics. *PLoS Pathog*, *14*(3), e1006964. https://doi.org/10.1371/journal.ppat.1006964

Atieh, T., El Ayoubi, M. D., Aubry, F., Priet, S., De Lamballerie, X., & Nougairède, A. (2018). Haiku: New paradigm for the reverse genetics of emerging RNA viruses. *PLOS ONE*, *13*(2), e0193069. https://doi.org/10.1371/journal.pone.0193069

Atieh, T., Nougairède, A., Klitting, R., Aubry, F., Failloux, A.-B., de Lamballerie, X., & Priet, S. (2017). New reverse genetics and transfection methods to rescue arboviruses in mosquito cells. *Scientific Reports*, *7*(1), 13983. https://doi.org/10.1038/s41598-017-14522-6

Bates, T., Chuong, C., Hawks, S. A., Rai, P., Salgado, R. M., Duggal, N. K., & Weger-Lucarelli, J. A. (2020). *Development and characterization of infectious clones of two strains of Usutu virus*. Cold Spring Harbor Laboratory. https://dx.doi.org/10.1101/2020.08.05.238543

Biswal, S., Reynales, H., Saez-Llorens, X., Lopez, P., Borja-Tabora, C., Kosalaraksa, P., Sirivichayakul, C., Watanaveeradej, V., Rivera, L., Espinoza, F., Fernando, L., Dietze, R., Luz, K., Venâncio Da Cunha, R., Jimeno, J., López-Medina, E., Borkowski, A., Brose, M., Rauscher, M., . . . Wallace, D. (2019). Efficacy of a Tetravalent Dengue Vaccine in Healthy Children and Adolescents. *New England Journal of Medicine*, *381*(21), 2009-2019. https://doi.org/10.1056/nejmoa1903869

Blanco, L., Bernad, A., Lázaro, J. M., Martin, G., Garmendia, C., & Salas, M. (1989). Highly efficient DNA synthesis by the phage Φ29 DNA polymerase. Symmetrical mode of DNA replication. *The Journal of biological chemistry*, *264*, 8935-8940.

Canale, A. S., Venev, S. V., Whitfield, T. W., Caffrey, D. R., Marasco, W. A., Schiffer, C. A., Kowalik, T. F., Jensen, J. D., Finberg, R. W., Zeldovich, K. B., Wang, J. P., & Bolon, D. N. A. (2018). Synonymous mutations at the beginning of the influenza A virus hemagglutinin gene impact experimental fitness. *Journal of Molecular Biology*. https://doi.org/10.1016/j.jmb.2018.02.009

Chuong, C., Bates, T. A., & Weger-Lucarelli, J. (2019). Infectious cDNA clones of two strains of Mayaro virus for studies on viral pathogenesis and vaccine development. *Virology*, *535*, 227-231. https://doi.org/https://doi.org/10.1016/j.virol.2019.07.013

Cuevas, J. M., Domingo-Calap, P., & Sanjuán, R. (2011). The Fitness Effects of Synonymous Mutations in DNA and RNA Viruses. *Molecular Biology and Evolution*, *29*(1), 17-20. https://doi.org/10.1093/molbev/msr179

Garmendia, C., Bernad, A., Esteban, J. A., Blanco, L., & Salas, M. (1992). The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. *Journal of Biological Chemistry*, *267*(4), 2594-2599. http://www.jbc.org/content/267/4/2594.abstract

Glenting, J., & Wessels, S. (2005). Ensuring safety of DNA vaccines. *Microbial cell factories*, *4*, 26-26. https://doi.org/10.1186/1475-2859-4-26

Grubaugh, N. D., Fauver, J. R., Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S., Murrieta, R. A., Gendernalik, A., Smith, D. R., Brackney, D. E., & Ebel, G. D. (2017). Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. *Cell Rep*, *19*(4), 709-718. https://doi.org/10.1016/j.celrep.2017.03.076

Grubaugh, N. D., Weger-Lucarelli, J., Murrieta, R. A., Fauver, J. R., Garcia-Luna, S. M., Prasad, A. N., Black, W. C. t., & Ebel, G. D. (2016). Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. *Cell Host Microbe*, *19*(4), 481-492. https://doi.org/10.1016/j.chom.2016.03.002

Inoue, J., Shigemori, Y., & Mikawa, T. (2006). Improvements of rolling circle amplification (RCA) efficiency and accuracy using Thermus thermophilus SSB mutant protein. *34*(9), e69-e69. https://doi.org/10.1093/nar/gkl350

Kümmerer, B. M., Grywna, K., Gläsker, S., Wieseler, J., & Drosten, C. (2012). Construction of an infectious Chikungunya virus cDNA clone and stable insertion of mCherry reporter

genes at two different sites. *Journal of General Virology*, *93*(9), 1991-1995. https://doi.org/https://doi.org/10.1099/vir.0.043752-0

Leung, J. Y.-S., Ng, M. M.-L., & Chu, J. J. H. (2011). Replication of alphaviruses: a review on the entry process of alphaviruses into cells. *Advances in virology*, *2011*, 249640-249640. https://doi.org/10.1155/2011/249640

Li, D., Aaskov, J., & Lott, W. B. (2011). Identification of a Cryptic Prokaryotic Promoter within the cDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLOS ONE*, *6*(3), e18197. https://doi.org/10.1371/journal.pone.0018197

Lundstrom, K. (2016). Alphavirus vectors as tools in neuroscience and gene therapy. *Virus Research*, *216*, 16-25. https://doi.org/https://doi.org/10.1016/j.virusres.2015.08.015

Mohsen, M. G., & Kool, E. T. (2016). The Discovery of Rolling Circle Amplification and Rolling Circle Transcription. *Accounts of Chemical Research*, *49*(11), 2540-2550. https://doi.org/10.1021/acs.accounts.6b00417

Mudaliar, P. P., & Sreekumar, E. (2016). Exploring the instability of reporters expressed under the subgenomic promoter in Chikungunya virus infectious cDNA clones. *45*, 448. https://doi.org/10.1016/j.ijid.2016.02.950

Nougairede, A., De Fabritus, L., Aubry, F., Gould, E. A., Holmes, E. C., & De Lamballerie, X. (2013). Random Codon Re-encoding Induces Stable Reduction of Replicative Fitness of Chikungunya Virus in Primate and Mosquito Cells. *9*(2), e1003172. https://doi.org/10.1371/journal.ppat.1003172

Ozaki, H., Govorkova, E. A., Li, C., Xiong, X., Webster, R. G., & Webby, R. J. (2004). Generation of High-Yielding Influenza A Viruses in African Green Monkey Kidney (Vero) Cells by Reverse Genetics. *Journal of Virology*, *78*(4), 1851-1857. https://doi.org/10.1128/jvi.78.4.1851-1857.2004

Plennevaux, E., Moureau, A., Arredondo-García, J. L., Villar, L., Pitisuttithum, P., Tran, N. H., Bonaparte, M., Chansinghakul, D., Coronel, D. L., L'Azou, M., Leon Ochiai, R., Toh, M.-L., Noriega, F., & Bouckenooghe, A. (2017). Impact of Dengue Vaccination on Serological Diagnosis: Insights From Phase III Dengue Vaccine Efficacy Trials. *Clinical Infectious Diseases*. https://doi.org/10.1093/cid/cix966

Pu, S. Y., Wu, R. H., Yang, C. C., Jao, T. M., Tsai, M. H., Wang, J. C., Lin, H. M., Chao, Y. S., & Yueh, A. (2011). Successful Propagation of Flavivirus Infectious cDNAs by a Novel

Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *85*(6), 2927-2941. https://doi.org/10.1128/jvi.01986-10

Scobey, T., Yount, B. L., Sims, A. C., Donaldson, E. F., Agnihothram, S. S., Menachery, V. D., Graham, R. L., Swanstrom, J., Bove, P. F., Kim, J. D., Grego, S., Randell, S. H., & Baric, R. S. (2013). Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences*, *110*(40), 16157-16162. https://doi.org/10.1073/pnas.1311542110

Singh, A., Koutsoumpli, G., van de Wall, S., & Daemen, T. (2019). An alphavirus-based therapeutic cancer vaccine: from design to clinical trial. *Cancer Immunology, Immunotherapy*, *68*(5), 849-859. https://doi.org/10.1007/s00262-018-2276-z

Steel, J. J., Henderson, B. R., Lama, S. B., Olson, K. E., & Geiss, B. J. (2011). Infectious alphavirus production from a simple plasmid transfection+. *8*(1), 356. https://doi.org/10.1186/1743-422x-8-356

Weger-Lucarelli, J., Duggal, N. K., Bullard-Feibelman, K., Veselinovic, M., Romo, H., Nguyen, C., Rückert, C., Brault, A. C., Bowen, R. A., Stenglein, M., Geiss, B. J., & Ebel, G. D. (2017). Development and Characterization of Recombinant Virus Generated from a New World Zika Virus Infectious Clone. *Journal of Virology*, *91*(1), JVI.01765-01716. https://doi.org/10.1128/jvi.01765-16

Weger-Lucarelli, J., Garcia, S. M., Rückert, C., Byas, A., O'Connor, S. L., Aliota, M. T., Friedrich, T. C., O'Connor, D. H., & Ebel, G. D. (2018). Using barcoded Zika virus to assess virus population structure in vitro and in Aedes aegypti mosquitoes. *Virology*, *521*, 138-148. https://doi.org/10.1016/j.virol.2018.06.004

Chapter Three: An *in vitro* workflow to create and modify novel infectious clones using replication cycle reaction

Jeffrey M. Marano [1,2], Chelsea Cereghino [2], Carla V. Finkielstein[3,4,5], and James Weger-Lucarelli [2,*]

[1] Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Roanoke, VA, USA; jmarano@vt.edu
[2] Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, United States.
[3] Molecular Diagnostics Laboratory, Fralin Biomedical Research Institute, Virginia Tech, Roanoke, VA, USA
[4] Integrated Cellular Responses Laboratory, Fralin Biomedical Research Institute at VTC, Roanoke, VA, USA
[5] Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA
+ J.M.M and C.C contributed equally to this work.
* Correspondence: weger@vt.edu

3.1 Abstract

Reverse genetics systems are a critical tool in combating emerging viruses by enabling a better understanding of the genetic mechanisms by which they cause disease. Traditional cloning approaches using bacteria are fraught with difficulties due to the bacterial toxicity of many viral sequences, resulting in low sequence integrity and difficulties with manipulation. Systems involving *in vitro* assembly and eukaryotic amplification, or assembly within the eukaryotic hosts, struggle less with sequence integrity. However, the infectious clone is not recoverable; thus, the ease of manipulation and distribution is low. Many attempts have been made to develop a simplified workflow to produce easily distributable, simple-to-use infectious clones without the concerns of host toxicity; however, no system addresses all three facets to date. This paper delivers a novel *in vitro* workflow that leverages gene synthesis and replication cycle reaction—an innovative technology that reconstitutes the bacterial replication machinery in a tube—to develop a supercoiled infectious clone plasmid that is easy to distribute and manipulate. As a

proof of concept, we developed two infectious clones, one of a low passage dengue virus serotype 2 isolate (PUO-218) and the Washington strain of SARS-CoV-2, which behaved similarly to their respective parental viruses. Furthermore, we generated a medically relevant mutant of SARS-CoV-2, Spike D614G. Results indicate that our novel *in vitro* workflow is a viable method to generate and manipulate infectious clones, specifically for viruses that are notoriously difficult for traditional bacterial-based cloning methods, while still producing shareable plasmid products.

3.2 Introduction

The development of reverse genetics systems for emerging viruses is critical in responding to global health threats as it enables studies to dissect the mechanisms by which these viruses cause disease (Aubry et al., 2015). The infectious cDNA clone allows researchers to explore many different aspects of virology, including viral kinetics (Chuong et al., 2019; Bates et al., 2020), evolution (Atieh et al., 2018; Roesch et al., 2022), tropism (Kümmerer et al., 2012; Ayers et al., 2021; Kuchinsky et al., 2021), and vaccinology (Mire et al., 2012; Plennevaux et al., 2017; Biswal et al., 2019; Furuyama et al., 2022). The traditional approach to produce infectious clones involves generating fragments from viral RNA by RT-PCR and then cloning these fragments into bacterial plasmids (Marano et al., 2020). However, traditional cloning approaches can be difficult due to viral genomic instability in living hosts, which often arises when the genomes of flaviviruses (Pu et al., 2011b), alphaviruses (Steel et al., 2011), and coronaviruses (Scobey et al., 2013) are introduced into bacteria. The proposed mechanism for this instability is cryptic bacterial promoters in the viral genome, driving the production of toxic viral genes (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Li et al., 2011; Pu et al., 2011a; Pu et al., 2011b; Mudaliar and Sreekumar, 2016; Weger-Lucarelli et al., 2017). In response to the plasmids' toxicity, a selective pressure is imposed on the bacteria to propagate plasmids containing viral genomes with deletions, mutations, and recombination events that mitigate the toxicity (Li et al., 2011; Mudaliar and Sreekumar, 2016; Weger-Lucarelli et al., 2017), making mechanistic studies extremely challenging.

Several approaches have been published to mitigate the effects of host-specific toxicity on infectious clone stability. One approach involves maintaining the viral genome across several plasmids, combining the genomic fragments *in vitro,* and then using that product for direct

transfection for CMV-based clones or *in vitro* transcription followed by transfection (Rice et al., 1989; Messer et al., 2012; Scobey et al., 2013; Gallichotte et al., 2015; Messer et al., 2016; Cockrell et al., 2017; Weger-Lucarelli et al., 2017; Ayers et al., 2021). This approach is effective but often requires maintaining up to 7 plasmids, making these systems cumbersome. Several groups have developed reverse genetics systems in which the products of circular polymerase extension cloning/reaction (CPEC/CPER), Gibson assembly, or infectious subgenomic amplicons (ISAs) are directly transfected into permissive cells (Edmonds et al., 2013; Siridechadilok et al., 2013; Aubry et al., 2014a; Aubry et al., 2014b; Aubry et al., 2015; Mohamed Ali et al., 2018; Torii et al., 2021). While these approaches remove the need for bacteria because the assembly occurs molecularly or within permissive eukaryotic cells, the full-length cDNA clone cannot be re-used for manipulation, like generating mutant viruses, or easily shared.

Other attempts have been made to reduce the toxicity of the full-length clones in bacteria. One approach was to insert introns into toxic transcripts to disrupt the open reading frame (ORF) (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Yamshchikov et al., 2001; Ulper et al., 2008; Tuo et al., 2015). Another approach is introducing synonymous mutations into sequences believed to contain cryptic bacterial promoters to prevent transcription by bacterial polymerases (Pu et al., 2011b). Others involved altering the plasmid architecture, like using low copy number bacterial artificial chromosomes (van der Most et al., 1999; Yun et al., 2003; Pierro et al., 2006; Suzuki et al., 2007; Usme-Ciro et al., 2014), heavily repressed promoters (Mishin et al., 2001; Cai et al., 2005), or using yeast-based systems (Polo et al., 1997; Puri et al., 2000; Kelly et al., 2010; Santos et al., 2013; Rihn et al., 2021). Nonetheless, these systems still struggle to remove the toxicity of viral genomes (Aubry et al., 2015).

In summary, there have been many attempts to develop a workflow that can ameliorate the inherent toxicity of the viral genomes in bacteria while also producing a recoverable full-length cDNA clone that is straightforward to work with; unfortunately, these attempts have only modest success. However, an alternative and emerging idea is to shift from ameliorating the host issue to removing the host from the system entirely.

Having previously demonstrated the ability to rescue and manipulate infectious clones using rolling circle amplification (RCA), an *in vitro* process (Weger-Lucarelli et al., 2018b; Bates et al., 2020; Marano et al., 2020; Kang et al., 2021a), we set out to use an emerging technology

called replication-cycle reaction (RCR) (Su'Etsugu et al., 2017; Hasebe et al., 2018; Nara and Su'Etsugu, 2021; Ueno et al., 2021) to develop a pipeline to produce novel infectious clones. The RCR system works by reconstituting 14 proteins and 25 polypeptides critical for chromosomal replication in *E.coli* bacteria (Su'Etsugu et al., 2017). This process produces supercoiled DNA and acts with high fidelity, with an error rate of ~10^{-8} per base per replication cycle (Su'Etsugu et al., 2017). This approach removes the need for a living host and the risk of selection due to sequence-specific toxicity (Pu et al., 2011b; Steel et al., 2011; Scobey et al., 2013). Leveraging RCR, we developed a novel workflow to generate and manipulate viral infectious clones (Figure 1). Briefly, this process uses the high efficiency and rapidly decreasing costs of chemical gene synthesis (Scobey et al., 2013; Cockrell et al., 2017; Rihn et al., 2021), high fidelity PCR, *in vitro* assembly, and *in vitro* amplification. The resulting clonal supercoiled plasmid population can then be rescued and manipulated identically to a bacterial-derived plasmid.

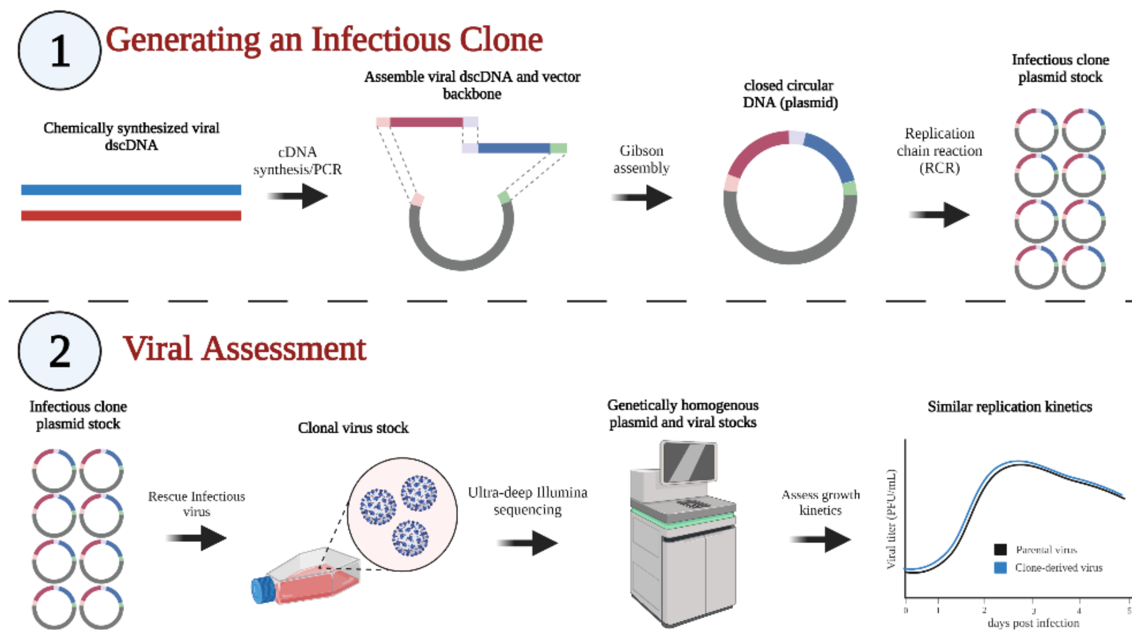


Figure 1. Novel in vitro infectious clone generation workflow. To produce a new clone, chemical gene synthesis is performed, followed by PCR, Gibson assembly, and replication cycle reaction (RCR) to produce a supercoiled infectious clone. Infectious virus is then recovered by transfection into susceptible cells. The clone and/or viral stock is then assessed by deep sequencing and growth kinetics to ensure matching sequences and behaviors with the parental virus.

In this paper, we demonstrate that the above-described workflow could be used to generate infectious clones of medically relevant viruses. For this work, we selected dengue virus serotype

2 (DENV), strain PUO-218, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), strain USA-WA1/2020. Based on sequencing and viral kinetics, we concluded that our innovative workflow could effectively generate new infectious clones. Furthermore, we demonstrate that the infectious clones, specifically the SARS-CoV-2 clone, can be used as a template to generate medically relevant mutant viruses. This workflow represents a paradigm shift in developing and manipulating infectious clones for emerging pathogens.

3.3 Materials and Methods

Cells and viruses

Vero (CCL-81), BHK-21 clone 13 (CCL-10), and Calu-3 (HTB-55) cells were obtained from American Type Culture Collection. Vero E6 cells expressing transmembrane protease, serine 2, and human angiotensin-converting enzyme 2 (VeroE6 hACE2-TMPRSS2) cells were obtained from BEI (Catalog No. NR-54970). HEK293A cells were kindly provided by Dr. Jamie Smyth from the Fralin Biomedical Research Institute. Vero and BHK-21 clone 13 cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin. For VeroE6 hACE2-TMPRSS2 cells, the media described above was supplemented with puromycin to a 0.01 mg/mL final concentration. Dengue virus serotype 2 (DENV2) strain PUO-218 (GenBank: ON398847) was obtained from the Center for Disease Control and Prevention (Lot: TC00838). SARS-CoV-2 USA-WA1/2020 strain (GenBank: MN985325.1) was acquired from BEI resources (NR-52281; Lot: 70034262). Before testing, both parental viruses were passaged once in Vero (DENV2) and VeroE6 hACE2-TMPRSS2 (SARS-CoV-2).

Construction of novel infectious clones

The infectious clone was designed *in silico* using SnapGene 6.0.2 software (GSL Biotech). When designing the infectious clones, one (DENV2) or two (SARS-CoV-2) restriction enzyme sites were ablated by synonymous mutation to allow downstream differentiation between the parental and clone-derived virus (Wang et al., 2016; Xie et al., 2020). Fragments with 40 bp overlaps were designed for clonal gene synthesis either by Twist Bioscience (DENV2) or Bio Basic Inc. (SARS-CoV-2). These plasmids were then used as templates for PCRs using the SuperFi II Master Mix (Catalog number: 12368010). The bands were then extracted and purified using the

Machary-Nagel NucleoSpin Gel and PCR Clean-up kit (Item Number 740609). Fragments were quantified by Qubit prior to assembly and then mixed in equimolar concentrations to a final concentration of 8 ng/µL, which was then assembled using the OriCiro 2x Recombination Assembly (RA) mix and incubated for 30 minutes at 42°C, followed by a 65°C step for 2 minutes to eliminate misassembled products. The amplification mixture was prepared according to the kit's protocol during the assembly step. The amplification mixture was primed by incubating at 33 °C for 15 minutes. After priming, the assembly reaction was mixed into the amplification mix and incubated for 6 hours at 33°C and then held at 12°C. The next day, the product was supercoiled by diluting the product two-fold in 1x Amplification Buffer and incubating at 33 °C for 30 minutes. The supercoiled product was then tested by restriction digestion to confirm proper assembly. The remaining supercoiled product was diluted to a final concentration of 20 mM EDTA.

Rescue of infectious clones

For the DENV2 infectious clone, viral rescue was performed similarly as previously described (Marano et al., 2020). Briefly, we used the EquiPhi29™ DNA Polymerase (Catalog number: A39390) to amplify the RCR product by RCA and then used HEK293A cells for transfection. The virus, representing the passage zero (p0) stock, was harvested seven days post-transfection and titered by plaque assay on Vero cells (Marano et al., 2020). The p0 stock was then used to passage the virus once in Vero cells at an MOI of .01 to produce the p1 stock, which was used for downstream testing. For the SARS-CoV-2 infectious clone, supercoiled plasmid obtained by RCR was co-transfected with pUC19 (Søndergaard et al., 2020) into BHK-21 clone 13 cells. The supernatant was collected two- and three days post-transfection, pooled, and used to blindly infect VeroE6 hACE2-TMPRSS2 (Rihn et al., 2021) to produce a p1 stock used for downstream testing.

Library Preparation and NGS Sequencing

For the DENV2 infectious clone, libraries were prepared from the RCA product using the sparQ DNA Frag & Library Prep Kit from Quantabio (Cat. 95194-024). After assessing the library size by Tapestation, the samples were sequenced using 150 bp paired-end reads on the Illumina Novaseq 6000. To analyze the resulting data, a previously described workflow was used (Roesch

et al., 2022). Briefly, paired-end reads were trimmed to a quality score of 30 using BBDuk (Bushnell, 2014) before being mapped to the parental sequence using the Burrows-Wheeler aligner (BWA) (Rodgers et al., 2017), and variants were identified using LoFreq (Li and Durbin, 2009). The consensus sequence of the clone was then produced using Genome Analysis Toolkit (GATK) (McKenna et al., 2010) and then aligned to the *in silico* to confirm the correct sequence. For SARS-CoV-2, clone-derived viral RNA was sequenced using the plexWell ARTIC protocol V3 and the plexWell 384 Library Prep Kit protocol. Briefly, cDNA was prepared from RNA samples using SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA). PCR amplification was performed using Q5 Polymerase (NEB, Ipswitch, MA) and primers from ARTIC nCoV-2019 Amplicon Panel, V4.1 (Integrated DNA Technologies, Coralville, IA). Amplicons were quantitatively assessed using a Qubit 2.0 fluorometer (Thermo/Fisher) and then barcoded and pooled using the plexWell™ 384 Library Preparation Kit (seqWell, Beverly, MA). Pooled libraries were amplified using a Kappa HiFi Hot Start Ready Mix kit (Roche, CA). Libraries were sequenced using the 300-cycle Miseq Sequencing Kit (Illumina, San Diego, CA). Bioinformatic analysis was performed identically to the DENV samples. To determine if the minority variants in the viral stocks were present in the plasmid stocks, the NSP3 region of the plasmid was amplified by PCR and purified. Samples were submitted for Sanger sequencing to the Virginia Tech Genomic Sequencing Center. Sanger sequencing reads were aligned to the reference sequence using SnapGene.

Comparing viral kinetics of clone-derived and parental viruses

Vero (DENV2) or VeroE6 hACE2-TMPRSS2 (SARS-CoV-2) cells were plated to an 80-90% confluency in 24-well plates. Viral stocks were diluted in Roswell Park Memorial Institute medium (RPMI 1640) with 25 mM HEPES and 1% FBS. Cells were infected at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU) per cell and incubated at 37°C for one hour. After the adsorption period, the cells were washed in phosphate-buffered saline (PBS), and fresh media was added. The supernatant was harvested every 24 hours until 50-75% CPE was observed. Harvested samples were stored at -80°C until they were tested by plaque assay. Viral RNA was used to generate cDNA for both viruses using the Maxima H- RT kit (Catalog number: EP0752). The resulting cDNA was used to amplify the region of the genome containing the ablated restriction site. The amplicons were then digested with either EcoRI (DENV2) or HindIII

(SARS-CoV-2) and tested by gel electrophoresis to observe the banding differences between parental- and clone-derived viruses.

Generating mutant viruses and comparing fitness by competition assay.

To generate the mutant SARS-CoV-2 Spike D614G virus, mutagenic primers were designed bearing the desired point mutation. The SARS-CoV-2 infectious clone served as a template for PCRs and was serially diluted to the lowest concentration that still resulted in amplification. The mutants were then constructed and rescued identically to the infectious clones via a bacteria-free cloning strategy (Weger-Lucarelli et al., 2018b; Bates et al., 2020; Marano et al., 2020; Kang et al., 2021a). To confirm that the new viral stocks contained the appropriate mutation, the viral stocks were first confirmed by Sanger sequencing, followed by whole genome next generation sequencing (NGS) as described above. After confirmation, the mutant virus was mixed with the wild-type virus at a 1:1 PFU ratio. The virus mix was then used to infect VeroE6 hACE2-TMPRSS2 cells at an MOI of 0.01. Viral RNA was extracted from the inoculum and viral supernatant at 1 day post infection (dpi), which was used to generate cDNA using the Maxima H- RT kit. The resulting cDNA was used to amplify the region of the genome containing the mutation of interest. The amplicon was then submitted for Sanger sequencing, and the relative ratios of wild-type and mutant virus were determined using EditR (Kluesner et al., 2018). Relative fitness of the mutant compared to the wild-type virus was defined as $W = F(t)/F(0)$, where $F(x)$ represents the ratio of mutant virus (either at baseline or 1 dpi) (Liu et al., 2021a).

Statistical Analysis

Statistical analysis was performed in Prism 8 (GraphPad, San Diego, CA, USA). A 2-way ANOVA test was performed with a Šidák correction for multiple comparisons for the viral kinetics assays. A one-sample t-test was used for the competition assays after a Shapiro-Wilk test confirmed normality.

3.4 Results

Generating a Medically Relevant DENV2 Infectious Clone

DENV represents one of the most significant global health threats, infecting 400 million people yearly (roughly 5% of the global population) (Weger-Lucarelli et al., 2018a). Unfortunately, the

high concentration of cryptic bacterial promoters within the DENV genome makes the development of infectious clones extremely challenging (Li et al., 2011; Pu et al., 2011a). Here, we sought to develop a new, recoverable, full-length DENV serotype 2 infectious clone using an *in vitro* approach, which has yet to be done. We selected DENV2 strain PUO-218, a low passage virus isolated in Thailand in 1980 (Gruenberg et al., 1988) that is also a component of Sanofi's Dengvaxia vaccine (Thomas and Yoon, 2019; Tully and Griffiths, 2021; Torres-Flores et al., 2022). To generate the clone, the viral genome was synthesized in four clonal fragments (Figure 2). We created a synonymous mutation that ablated an EcoRI site within the NS3 sequence to allow for differentiation between the clone and the parental virus. The four synthesized fragments and a donor plasmid containing the CMV promoter, hepatitis delta virus ribozyme (HDVr) sequence, and the OriC sequence were used as templates for high fidelity PCR. The CMV promoter allows for direct recovery of infectious virus through cellular RNA polymerase II while the HDVr removes extra nucleotides at the 3' end of the genome, ensuring an authentic 3' UTR sequence; the OriC sequence is required for RCR amplification. The amplicons were then gel purified and assembled into a circular plasmid using Gibson assembly. We then amplified the circular molecule containing the OriC sequence by RCR to produce a supercoiled plasmid containing the full viral genome and expression machinery (CMV and HDVr).

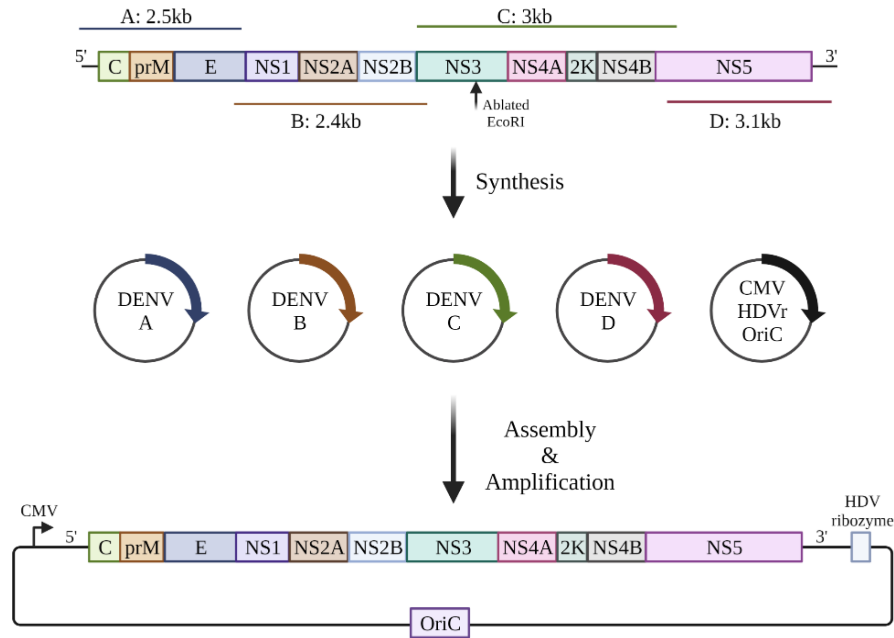


Figure 2. Schematic for Producing DENV2 Infectious Clone. To produce the DENV2 PUO-218 infectious clone, the viral genome was synthesized in four clonal fragments. The four resulting plasmids and a donor plasmid containing the necessary components for expression and replication were used as templates for PCRs. The amplicons from these reactions were then assembled and amplified by RCR to produce a supercoiled infectious clone.

Comparison of DENV2 Infectious Clone to the Parental Virus

We compared the growth of the parental DENV2 PUO-218 isolate to the infectious clone-derived virus in Vero cells to ensure similar growth kinetics. Vero cells were selected as they are highly susceptible to DENV (MartíNez-BarragáN and Del Angel, 2001). The cells were infected at an MOI of 0.01 PFU/cell, allowing for multiple rounds of replication. Supernatants from the infected cells were collected until 50-75% of cells showed cytopathic effect (CPE). We found no significant differences in viral titer between the parental and the clone on any day (Figure 3A). To confirm the identity of each virus, we performed RT-PCR on the viral stocks and digested the amplicons with EcoRI. We expected two bands at 400 bp and 732 bp for the parental virus and only a single band in the clone-derived virus at roughly 1.1 kb, which we observed (Figure 3B). Finally, next-generation sequencing revealed no variants above consensus level within the clone, indicating no unwanted mutations were generated during the cloning or viral rescue process (data not shown). These data indicate that the DENV2 clone's sequence and behavior match the parental virus.

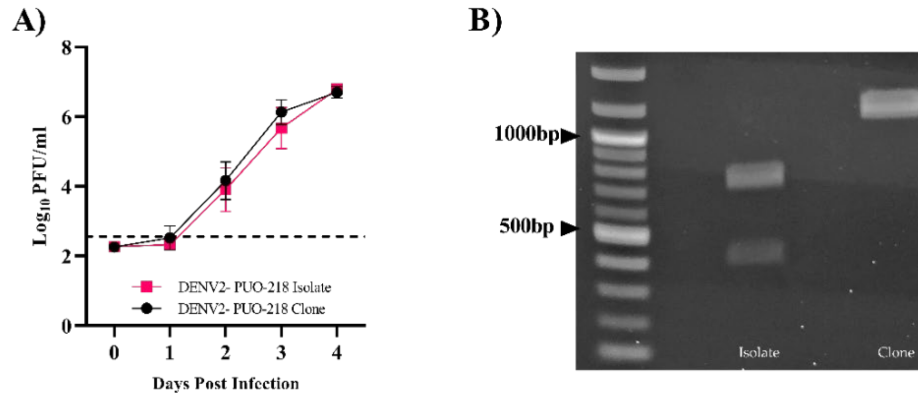


Figure 3. Comparison of DENV2 Infectious Clone to the Parental Virus. (A) Growth curve of DENV2 PUO-218 isolate and infectious clone in Vero cells. Data represent two biological replicates, each containing three technical replicates, and the error bars represent the standard deviation. No significant difference was detected at any time by a 2-way ANOVA with a Šidák correction for multiple comparisons. (B) A representative gel image of an EcoRI digest of the genetically marked region of the infectious clone. The 100bp DNA ladder from NEB (#N3231) was used for reference, with the first sample lane containing the viral isolate and the second containing the clone.

Generating a SARS-CoV-2 Infectious Clone

From its emergence in late 2019 to June 2022, SARS-CoV-2 has infected over 500 million people, and at least 6 million have died due to the virus or associated conditions (Organization, 2020). During the early stages of the pandemic, there was a significant push to develop an infectious clone of SARS-CoV-2. However, the difficulty of manipulating the SARS-CoV-2 genome in bacteria—and coronaviruses as a whole (Scobey et al., 2013; Cockrell et al., 2017)—led to a near six-month lag between the virus's first identification (Wu et al., 2020) and the first clone being reported (Xie et al., 2020), which limited our ability to understand the emergence of SARS-CoV-2 and to develop countermeasures. Towards generating a workflow to develop infectious clones more quickly, we sought to produce an infectious clone of an early isolate of SARS-CoV-2, USA-WA1/2020 strain. The SARS-CoV-2 genome was synthesized as four clonal fragments (Figure 4). Two HindIII sites within Orf1a and the Spike protein were ablated during the design phase to distinguish between parental- and clone-derived viruses. As with the DENV2 clone, the clonal fragments and a donor plasmid were used as templates for PCR, and those amplicons were used in an RCR to produce a supercoiled infectious clone plasmid.

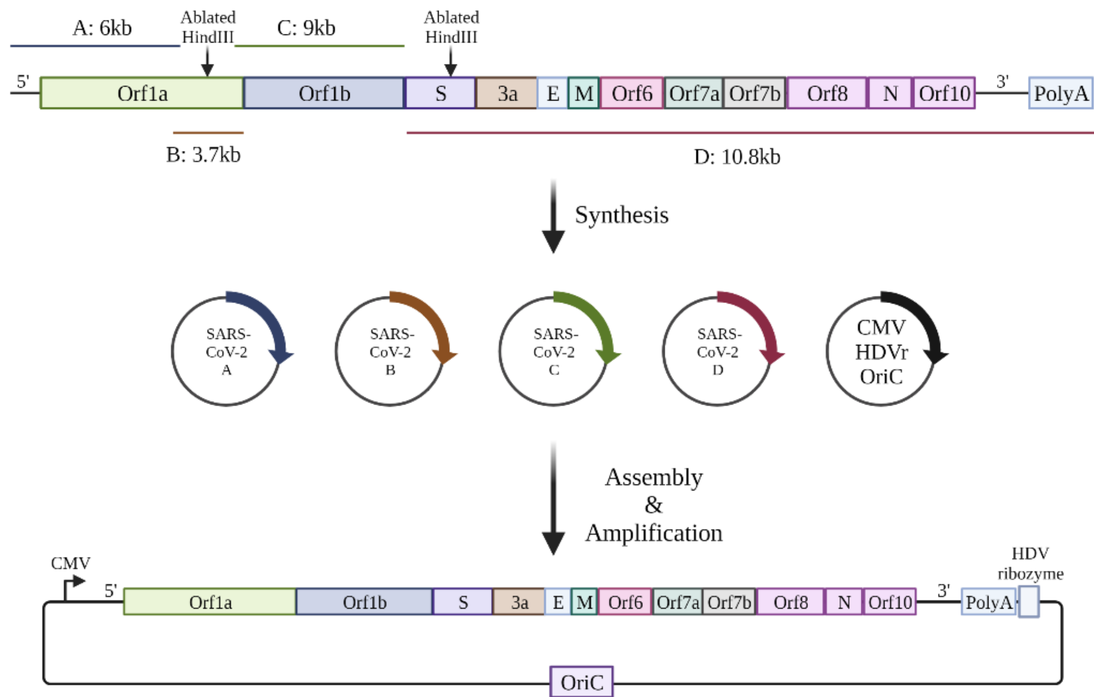


Figure 4. Schematic for Producing SARS-CoV-2 Infectious Clone. To produce the SARS-CoV-2 USA-WA1/2020 infectious clone, the viral genome was synthesized in four clonal fragments. The four resulting plasmids, along with a donor plasmid that contained the necessary components for expression and replication, were used as templates for PCRs. The amplicons from these reactions were then assembled and amplified by RCR to produce a supercoiled infectious clone.

Comparison of SARS-CoV-2 Clone-derived virus to the Parental Virus

We tested the growth of the parental and clone-derived virus in VeroE6 hACE2-TMPRSS2 since they are highly susceptible to SARS-CoV-2 (Matsuyama et al., 2020). When quantifying the viral kinetics by plaque assay (Figure 5A), we found the clone-derived virus was attenuated on day 1 (P = .0005) and day 2 (P = .003). RT-PCR confirmation was performed on the stocks, and the amplicons were digested with HindIII. We expected to see a band at 1.1kb (representing two overlapping bands of equal size) for the parental virus and 2.2 kb for the clonal virus, which we observed (Figure 5B).

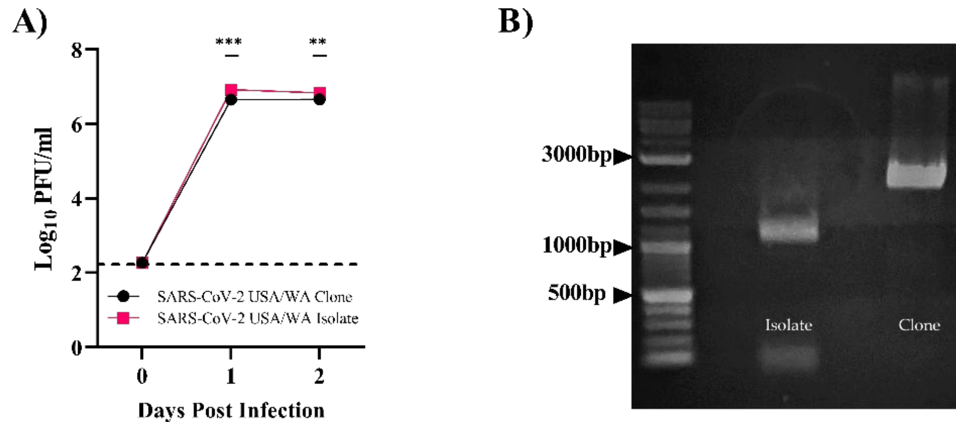


Figure 5. Comparison of SARS-CoV-2 Infectious Clone to the Parental Virus. (A) Growth curve of SARS-CoV-2 USA-WA1/2020 strain isolate and the infectious clone in VeroE6 hACE2-TMPRSS2 cells. Data represent two biological replicates, each consisting of three technical replicates. Statistical analysis was performed using a 2-way ANOVA test with a Šidák correction for multiple comparisons (** P = .003 *** P = .0005). (B) A representative gel image of a HindIII digest of the genetically marked region of the infectious clone. The 1kb Plus DNA Ladder from NEB (#N3200) was used for reference, with the first sample lane containing the viral isolate and the second containing the clone.

Next-generation sequencing was first performed on the viral RNA to compare the clone-derived virus sequence to the parental virus. The consensus sequences of both viruses were identical. A minority variant at genome position 13,169 was identified in the clone-derived virus and was detected at a percentage of 45.9%, resulting in a mutation at NSP3-P125Q. We also sequenced the infectious clone plasmid and could not detect the mutation, suggesting that this mutation was generated during the viral rescue process. These data indicate that the clone sequence matches the parental isolate.

Generation and characterization of medically relevant early pandemic mutant using site-directed mutagenesis

Having demonstrated that the *in vitro* clone development workflow produces a full-length, recoverable product, the next critical step was demonstrating that the new clones could serve as templates for further manipulation. One of the advantages of using infectious clones is that they allow connecting viral genotypes and phenotypes through the generation of viral mutants (Kümmerer et al., 2012; Atieh et al., 2018; Chuong et al., 2019; Bates et al., 2020; Ayers et al., 2021; Kuchinsky et al., 2021; Roesch et al., 2022). To that end, using the SARS-CoV-2 infectious clone, we engineered a virus bearing a medically significant mutation, Spike D614G,

hereafter called D614G, an early variant of SARS-CoV-2 (Isabel et al., 2020). D614G quickly became the dominant genotype globally (Isabel et al., 2020; Korber et al., 2020; Pandey et al., 2020; Chakraborty et al., 2021) and has been shown to have increased infectivity (Zhang et al., 2020; Daniloski et al., 2021) and fitness (Plante et al., 2021; Yang et al., 2021) when compared to earlier genotypes.

The mutant virus was generated by site-directed mutagenesis and assembled similarly to the parental infectious clone. Specifically, the mutagenic primers were designed to replace the A with a G at position 23,403 and the T with a C at position 23,404, which resulted in the D614G mutation. After confirming that the mutant virus bore the mutation and titration, the mutant was prepared for a competition assay (Figure 6).

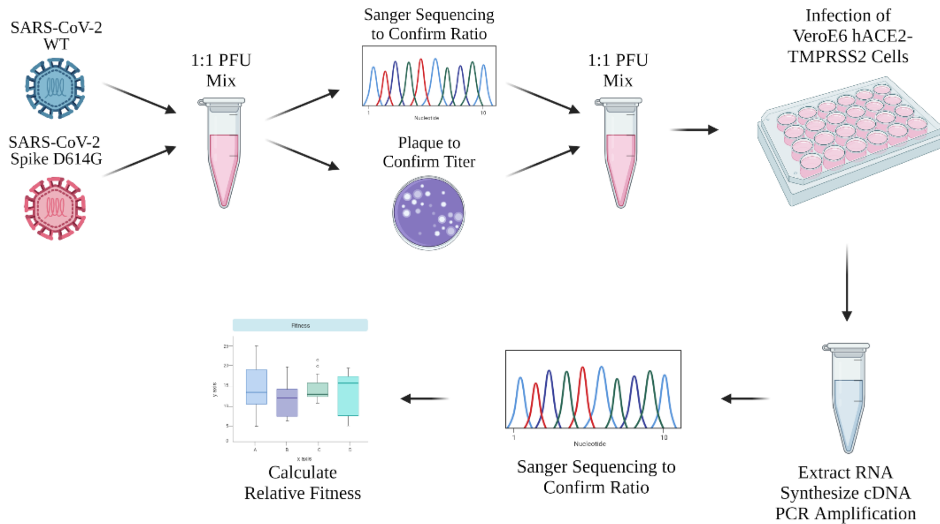


Figure 6. SARS-CoV-2 Mutant Competition Assay Workflow. To assess the fitness of Spike D614G, wild-type (WT) and D614G were mixed at a 1:1 PFU ratio. After confirming the mix composition by Sanger sequencing and plaque assay, the mix was used to infect VeroE6 hACE2-TMPRSS2 cells. RNA was extracted from viral supernatant at 1 dpi and used to synthesize cDNA for downstream.

Briefly, the mutant was mixed at a 1:1 PFU ratio with the wild-type SARS-CoV-2 infectious clone-derived virus. These mixes were then used to infect VeroE6 hACE2-TMPRSS2, using the same methods as the infectious clone validation. Viral RNA was extracted from the inoculum and the viral supernatant at 1 dpi and was used for reverse transcription. The viral cDNA was amplified by PCR to capture the regions of the genome bearing the mutations. The amplicons were submitted for Sanger sequencing, and relative fitness was assessed by comparing the starting and ending proportions for each virus (Figure 7). The D614G mutant generated using the *in vitro* workflow has significantly increased fitness ($P = .0005$), which aligns with previous

findings regarding this mutation (Plante et al., 2021; Yang et al., 2021). These data demonstrate that the *in vitro* workflow can effectively be used to engineer mutations into infectious clones.

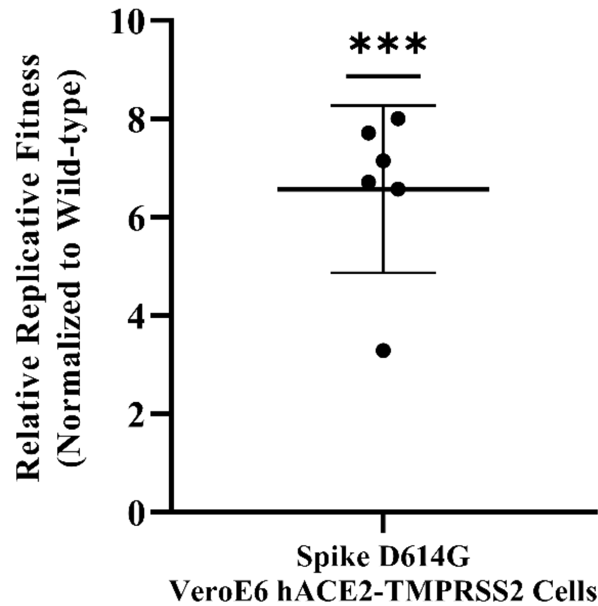


Figure 7. Comparing Fitness of Early Pandemic SARS-COV-2 Mutants Using a Novel Infectious Clone. Competition assays using SARS-CoV-2 USA-WA1/2020 wild-type infectious clone and mutants bearing Spike-D614G in VeroE6 hACE2-TMPRSS2 cells. Data represent two biological replicates, each consisting of three technical replicates, and the error bars represent the standard deviation from the mean. Statistical analysis was performed using a one-sample t-test with a null value of 1 (*** P = .0005).

3.5 Discussion

Infectious clones enable mechanistic studies to understand the impacts of mutations and studies to generate vaccines and diagnostic tools. However, the currently available systems struggle to mitigate host toxicity, making generating infectious clones for many viruses extremely cumbersome [14, 17-22, 37-48]. To address these issues, we leveraged several emerging *in vitro* technologies, including gene synthesis and RCR, to develop a novel workflow to develop infectious clones (Fig 1). Using this system, we successfully produced infectious clones for DENV2 and SARS-CoV-2, two viruses of great medical health importance.

An important consideration when producing an infectious clone is that clone-derived and parental viruses replicate similarly and have the same amino acid sequence. For the DENV clone, the parental- and clone-derived sequence and growth kinetics were identical. For SARS-CoV-2, the sequence of the infectious clone plasmid matched the parental virus; however, we detected a significant minority variant in the viral stock (nsp3 P125Q). Since the mutation was

not detected in the infectious clone plasmid, we hypothesize that the mutation is a byproduct of the low-efficiency transfection of the clone, a known problem with large plasmid transfections, which caused a bottleneck, resulting in changes in the variant frequencies in the stock (Kreiss et al., 1999; Campeau et al., 2001; Søndergaard et al., 2020). We view this as a limitation of the transfection system we use and not the cloning workflow. Electroporation, which has been shown to have higher efficiency for large plasmids (Lesueur et al., 2016; Søndergaard et al., 2020), represents a viable alternative to the current transfection approach used in this paper and will be explored in the future for rescuing large infectious clones. The difference in kinetics that we observe with the SARS-CoV-2 infectious clone could be due to the mutation mentioned above; however, a reduction in fitness has also been shown in several other clones with correct consensus sequences, though the cause has not been experimentally defined (Thi Nhu Thao et al., 2020; Xie et al., 2020). We further demonstrated that the infectious clone could be easily manipulated, as we were able to generate a medically relevant mutant variant of SARS-CoV-2, Spike D614G (Isabel et al., 2020; Korber et al., 2020; Pandey et al., 2020; Zhang et al., 2020; Chakraborty et al., 2021; Daniloski et al., 2021; Plante et al., 2021; Yang et al., 2021), and characterize it using a competition assay. In its totality, this report represents a significant shift in developing infectious clones. The ease of access to the technologies that underpin this workflow provides ample ability for other researchers to use this system, thus lowering the barrier of entry for researchers to develop and manipulate infectious clones for emerging viruses.

Limitations of this work include our use of only two viruses from two viral families: *Flaviviridae* and *Coronaviridae*. However, these families represent the most challenging positive-sense RNA viruses to manipulate in bacteria; thus, we are confident that the workflow could be easily transferred to other positive-sense RNA viruses. Future studies involving negative-sense RNA viruses, multipartite viruses, and DNA viruses should be performed to expand the usage of this technology. Another limitation is the genome size that was explored in this report. While coronaviruses have the largest RNA viral genomes (Almazán et al., 2000; Ogando et al., 2019), they are by no means the largest viral genomes, particularly when compared to the giant aquatic viruses, like Megavirus (Legendre et al., 2012) and Mimivirus (Colson et al., 2017). Given that the largest reported genome amplified with the OriC system was 1 Mb (Nara and Su'Etsugu, 2021), slightly below the genome size of some large aquatic viruses (Legendre et al., 2012; Colson et al., 2017), further characterizations may be needed for these giant viruses.

3.6 Author Contributions:

Conceptualization, J.W.L; methodology, J.M.M, C.C and J.W.L; cloning, J.M.M, C.C, and J.W.L; characterization, J.M.M., C.C, C.V.F; formal analysis, J.M.M, C.C, C.V.F, and J.W.L; validation, J.M.M, C.C, and J.W.L; writing—original draft preparation, J.M.M; writing—review and editing, J.M.M, C.C, and J.W.L; visualization, J.M.M, and J.W.L. All authors have read and agreed to the published version of the manuscript.

Copyright: This work is licensed under a CC BY 4.0 License – Open Use

3.7 References

Almazán, F., González, J. M., Pénzes, Z., Izeta, A., Calvo, E., Plana-Durán, J., & Enjuanes, L. (2000). Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proceedings of the National Academy of Sciences*, *97*(10), 5516-5521. https://doi.org/doi:10.1073/pnas.97.10.5516

Atieh, T., El Ayoubi, M. D., Aubry, F., Priet, S., De Lamballerie, X., & Nougairède, A. (2018). Haiku: New paradigm for the reverse genetics of emerging RNA viruses. *PLOS ONE*, *13*(2), e0193069. https://doi.org/10.1371/journal.pone.0193069

Aubry, F., Nougairede, A., De Fabritus, L., Querat, G., Gould, E. A., & De Lamballerie, X. (2014). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *95*(Pt_11), 2462-2467. https://doi.org/10.1099/vir.0.068023-0

Aubry, F., Nougairède, A., de Fabritus, L., Querat, G., Gould, E. A., & de Lamballerie, X. (2014). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *J Gen Virol*, *95*(Pt 11), 2462-2467. https://doi.org/10.1099/vir.0.068023-0

Aubry, F., Nougairède, A., Gould, E. A., & de Lamballerie, X. (2015). Flavivirus reverse genetic systems, construction techniques and applications: a historical perspective. *Antiviral research*, *114*, 67-85. https://doi.org/10.1016/j.antiviral.2014.12.007

Ayers, J. B., Xie, X., Coatsworth, H., Stephenson, C. J., Waits, C. M., Shi, P. Y., & Dinglasan, R. R. (2021). Infection Kinetics and Transmissibility of a Reanimated Dengue Virus

Serotype 4 Identified Originally in Wild Aedes aegypti From Florida. *Front Microbiol*, *12*, 734903. https://doi.org/10.3389/fmicb.2021.734903

Bates, T., Chuong, C., Hawks, S. A., Rai, P., Salgado, R. M., Duggal, N. K., & Weger-Lucarelli, J. A. (2020). *Development and characterization of infectious clones of two strains of Usutu virus*. Cold Spring Harbor Laboratory. https://dx.doi.org/10.1101/2020.08.05.238543

Biswal, S., Reynales, H., Saez-Llorens, X., Lopez, P., Borja-Tabora, C., Kosalaraksa, P., Sirivichayakul, C., Watanaveeradej, V., Rivera, L., Espinoza, F., Fernando, L., Dietze, R., Luz, K., Venâncio Da Cunha, R., Jimeno, J., López-Medina, E., Borkowski, A., Brose, M., Rauscher, M., . . . Wallace, D. (2019). Efficacy of a Tetravalent Dengue Vaccine in Healthy Children and Adolescents. *New England Journal of Medicine*, *381*(21), 2009-2019. https://doi.org/10.1056/nejmoa1903869

Bushnell, B. (2014). *BBMap: A Fast, Accurate, Splice-Aware Aligner*. ; Lawrence Berkeley National Lab. (LBNL), Berkeley, CA (United States). https://www.osti.gov/servlets/purl/1241166

Cai, Z., Zhang, C., Chang, K.-S., Jiang, J., Ahn, B.-C., Wakita, T., Liang, T. J., & Luo, G. (2005). Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. *Journal of Virology*, *79*(22), 13963-13973.

Campeau, P., Chapdelaine, P., Seigneurin-Venin, S., Massie, B., & Tremblay, J. P. (2001). Transfection of large plasmids in primary human myoblasts. *Gene Ther*, *8*(18), 1387-1394. https://doi.org/10.1038/sj.gt.3301532

Chakraborty, C., Saha, A., Sharma, A. R., Bhattacharya, M., Lee, S.-S., & Agoramoorthy, G. (2021). D614G mutation eventuates in all VOI and VOC in SARS-CoV-2: Is it part of the positive selection pioneered by Darwin? *Molecular Therapy - Nucleic Acids*, *26*, 237-241. https://doi.org/10.1016/j.omtn.2021.07.011

Chuong, C., Bates, T. A., & Weger-Lucarelli, J. (2019). Infectious cDNA clones of two strains of Mayaro virus for studies on viral pathogenesis and vaccine development. *Virology*, *535*, 227-231. https://doi.org/https://doi.org/10.1016/j.virol.2019.07.013

Cockrell, A. S., Beall, A., Yount, B., & Baric, R. (2017). Efficient Reverse Genetic Systems for Rapid Genetic Manipulation of Emergent and Preemergent Infectious Coronaviruses. In (pp. 59-81). Springer New York. https://doi.org/10.1007/978-1-4939-6964-7_5

Colson, P., La Scola, B., Levasseur, A., Caetano-Anollés, G., & Raoult, D. (2017). Mimivirus: leading the way in the discovery of giant viruses of amoebae. *Nature Reviews Microbiology*, *15*(4), 243-254. https://doi.org/10.1038/nrmicro.2016.197

Daniloski, Z., Jordan, T. X., Ilmain, J. K., Guo, X., Bhabha, G., Tenoever, B. R., & Sanjana, N. E. (2021). The Spike D614G mutation increases SARS-CoV-2 infection of multiple human cell types. *eLife*, *10*. https://doi.org/10.7554/elife.65365

Edmonds, J., Van Grinsven, E., Prow, N., Bosco-Lauth, A., Brault, A. C., Bowen, R. A., Hall, R. A., & Khromykh, A. A. (2013). A Novel Bacterium-Free Method for Generation of Flavivirus Infectious DNA by Circular Polymerase Extension Reaction Allows Accurate Recapitulation of Viral Heterogeneity. *Journal of Virology*, *87*(4), 2367-2372. https://doi.org/10.1128/jvi.03162-12

Furuyama, W., Shifflett, K., Pinski, A. N., Griffin, A. J., Feldmann, F., Okumura, A., Gourdine, T., Jankeel, A., Lovaglio, J., Hanley, P. W., Thomas, T., Clancy, C. S., Messaoudi, I., O'Donnell, K. L., & Marzi, A. (2022). Rapid Protection from COVID-19 in Nonhuman Primates Vaccinated Intramuscularly but Not Intranasally with a Single Dose of a Vesicular Stomatitis Virus-Based Vaccine. *mBio*, *13*(1), e0337921-e0337921. https://doi.org/10.1128/mbio.03379-21

Gallichotte, E. N., Widman, D. G., Yount, B. L., Wahala, W. M., Durbin, A., Whitehead, S., Sariol, C. A., Crowe, J. E., De Silva, A. M., & Baric, R. S. (2015). A New Quaternary Structure Epitope on Dengue Virus Serotype 2 Is the Target of Durable Type-Specific Neutralizing Antibodies. *mBio*, *6*(5), e01461-01415. https://doi.org/10.1128/mbio.01461-15

Gruenberg, A., Woo, W. S., Biedrzycka, A., & Wright, P. J. (1988). Partial Nucleotide Sequence and Deduced Amino Acid Sequence of the Structural Proteins of Dengue Virus Type 2, New Guinea C and PUO-218 Strains. *Journal of General Virology*, *69*(6), 1391-1398. https://doi.org/10.1099/0022-1317-69-6-1391

Hasebe, T., Narita, K., Hidaka, S., & Su'etsugu, M. (2018). Efficient Arrangement of the Replication Fork Trap for In Vitro Propagation of Monomeric Circular DNA in the Chromosome-Replication Cycle Reaction. *Life (Basel)*, *8*(4). https://doi.org/10.3390/life8040043

Isabel, S., Graña-Miraglia, L., Gutierrez, J. M., Bundalovic-Torma, C., Groves, H. E., Isabel, M. R., Eshaghi, A., Patel, S. N., Gubbay, J. B., Poutanen, T., Guttman, D. S., & Poutanen, S. M. (2020). Evolutionary and structural analyses of SARS-CoV-2 D614G spike protein mutation now documented worldwide. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-70827-z

Johansen, I. E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(22), 12400-12405. https://doi.org/10.1073/pnas.93.22.12400

Kang, L., He, G., Sharp, A. K., Wang, X., Brown, A. M., Michalak, P., & Weger-Lucarelli, J. (2021). A selective sweep in the Spike gene has driven SARS-CoV-2 human adaptation. *bioRxiv*, 2021.2002.2013.431090. https://doi.org/10.1101/2021.02.13.431090

Kelly, E. P., Puri, B., Sun, W., & Falgout, B. (2010). Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate. *Vaccine*, *28*(17), 3030-3037.

Kluesner, M. G., Nedveck, D. A., Lahr, W. S., Garbe, J. R., Abrahante, J. E., Webber, B. R., & Moriarity, B. S. (2018). EditR: A Method to Quantify Base Editing from Sanger Sequencing. *The CRISPR Journal*, *1*(3), 239-250. https://doi.org/10.1089/crispr.2018.0014

Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E. E., Bhattacharya, T., Foley, B., Hastie, K. M., Parker, M. D., Partridge, D. G., Evans, C. M., Freeman, T. M., de Silva, T. I., Sheffield, C.-G. G., McDanal, C., Perez, L. G., . . . Montefiori, D. C. (2020). Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. *Cell*, *182*(4), 812-827.e819. https://doi.org/10.1016/j.cell.2020.06.043

Kreiss, P., Cameron, B., Rangara, R., Mailhe, P., Aguerre-Charriol, O., Airiau, M., Scherman, D., Crouzet, J., & Pitard, B. (1999). Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic Acids Res*, *27*(19), 3792-3798. https://doi.org/10.1093/nar/27.19.3792

Kuchinsky, S. C., Frere, F., Heitzman-Breen, N., Golden, J., Vázquez, A., Honaker, C. F., Siegel, P. B., Ciupe, S. M., Leroith, T., & Duggal, N. K. (2021). Pathogenesis and

shedding of Usutu virus in juvenile chickens. *Emerging Microbes & Infections*, *10*(1), 725-738. https://doi.org/10.1080/22221751.2021.1908850

Kümmerer, B. M., Grywna, K., Gläsker, S., Wieseler, J., & Drosten, C. (2012). Construction of an infectious Chikungunya virus cDNA clone and stable insertion of mCherry reporter genes at two different sites. *Journal of General Virology*, *93*(9), 1991-1995. https://doi.org/https://doi.org/10.1099/vir.0.043752-0

Legendre, M., Arslan, D., Abergel, C., & Claverie, J.-M. (2012). Genomics of Megavirus and the elusive fourth domain of Life. *Communicative & Integrative Biology*, *5*(1), 102-106. https://doi.org/10.4161/cib.18624

Lesueur, L. L., Mir, L. M., & André, F. M. (2016). Overcoming the Specific Toxicity of Large Plasmids Electrotransfer in Primary Cells In Vitro. *Molecular Therapy - Nucleic Acids*, *5*, e291. https://doi.org/https://doi.org/10.1038/mtna.2016.4

Li, D., Aaskov, J., & Lott, W. B. (2011). Identification of a Cryptic Prokaryotic Promoter within the cDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLOS ONE*, *6*(3), e18197. https://doi.org/10.1371/journal.pone.0018197

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, *25*(14), 1754-1760. https://doi.org/10.1093/bioinformatics/btp324

Liu, J., Liu, Y., Shan, C., Nunes, B. T. D., Yun, R., Haller, S. L., Rafael, G. H., Azar, S. R., Andersen, C. R., Plante, K., Vasilakis, N., Shi, P.-Y., & Weaver, S. C. (2021). Role of mutational reversions and fitness restoration in Zika virus spread to the Americas. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-020-20747-3

López-Moya, J. J., & García, J. A. (2000). Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res*, *68*(2), 99-107. https://doi.org/10.1016/s0168-1702(00)00161-1

Marano, J. M., Chuong, C., & Weger-Lucarelli, J. (2020). Rolling circle amplification: A high fidelity and efficient alternative to plasmid preparation for the rescue of infectious clones. *Virology*, *551*, 58-63. https://doi.org/10.1016/j.virol.2020.08.016

MartíNez-BarragáN, J. D. J. S., & Del Angel, R. M. (2001). Identification of a Putative Coreceptor on Vero Cells That Participates in Dengue 4 Virus Infection. *Journal of Virology*, *75*(17), 7818-7827. https://doi.org/10.1128/jvi.75.17.7818-7827.2001

Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., Nagata, N., Sekizuka, T., Katoh, H., Kato, F., Sakata, M., Tahara, M., Kutsuna, S., Ohmagari, N., Kuroda, M., Suzuki, T., Kageyama, T., & Takeda, M. (2020). Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proceedings of the National Academy of Sciences*, *117*(13), 7001-7003. https://doi.org/doi:10.1073/pnas.2002589117

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., & Depristo, M. A. (2010). The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, *20*(9), 1297-1303. https://doi.org/10.1101/gr.107524.110

Messer, W. B., Yount, B., Hacker, K. E., Donaldson, E. F., Huynh, J. P., de Silva, A. M., & Baric, R. S. (2012). Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLOS Neglected Tropical Diseases*, *6*(2), e1486-e1486. https://doi.org/10.1371/journal.pntd.0001486

Messer, W. B., Yount, B. L., Royal, S. R., De Alwis, R., Widman, D. G., Smith, S. A., Crowe, J. E., Pfaff, J. M., Kahle, K. M., Doranz, B. J., Ibarra, K. D., Harris, E., De Silva, A. M., & Baric, R. S. (2016). Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *Journal of Virology*, *90*(10), 5090-5097. https://doi.org/10.1128/jvi.00155-16

Mire, C. E., Miller, A. D., Carville, A., Westmoreland, S. V., Geisbert, J. B., Mansfield, K. G., Feldmann, H., Hensley, L. E., & Geisbert, T. W. (2012). Recombinant vesicular stomatitis virus vaccine vectors expressing filovirus glycoproteins lack neurovirulence in nonhuman primates. *PLoS Negl Trop Dis*, *6*(3), e1567. https://doi.org/10.1371/journal.pntd.0001567

Mishin, V. P., Cominelli, F., & Yamshchikov, V. F. (2001). A ‘minimal’ approach in design of flavivirus infectious DNA. *Virus Research*, *81*(1), 113-123. https://doi.org/https://doi.org/10.1016/S0168-1702(01)00371-9

Mohamed Ali, S., Vega-Rúa, A., Driouich, J.-S., de Lamballerie, X., Failloux, A.-B., & Nougairède, A. (2018). Comparison of chikungunya viruses generated using infectious clone or the Infectious Subgenomic Amplicons (ISA) method in Aedes mosquitoes. *PLOS ONE*, *13*(6), e0199494. https://doi.org/10.1371/journal.pone.0199494

Mudaliar, P. P., & Sreekumar, E. (2016). Exploring the instability of reporters expressed under the subgenomic promoter in Chikungunya virus infectious cDNA clones. *45*, 448. https://doi.org/10.1016/j.ijid.2016.02.950

Nara, S., & Su'Etsugu, M. (2021). In vitro amplification of whole large plasmids via transposon-mediated oriC insertion. *BioTechniques*, *71*(4), 528-533. https://doi.org/10.2144/btn-2021-0068

Ogando, N. S., Ferron, F., Decroly, E., Canard, B., Posthuma, C. C., & Snijder, E. J. (2019). The Curious Case of the Nidovirus Exoribonuclease: Its Role in RNA Synthesis and Replication Fidelity [Review]. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01813

Olsen, B. S., & Johansen, I. E. (2001). Nucleotide sequence and infectious cDNA clone of the L1 isolate of Pea seed-borne mosaic potyvirus. *Arch Virol*, *146*(1), 15-25. https://doi.org/10.1007/s007050170187

Organization, W. H. (2020). *WHO COVID-19 Dashboard*. World Health Organization. Retrieved 04/07/2022 from https://covid19.who.int/

Pandey, U., Yee, R., Shen, L., Judkins, A. R., Bootwalla, M., Ryutov, A., Maglinte, D. T., Ostrow, D., Precit, M., Biegel, J. A., Bender, J. M., Gai, X., & Bard, J. D. (2020). High Prevalence of SARS-CoV-2 Genetic Variation and D614G Mutation in Pediatric Patients with COVID-19. *Open Forum Infectious Diseases*. https://doi.org/10.1093/ofid/ofaa551

Pierro, D. J., Salazar, M. I., Beaty, B. J., & Olson, K. E. (2006). Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation. *Journal of General Virology*, *87*(8), 2263-2268.

Plante, J. A., Liu, Y., Liu, J., Xia, H., Johnson, B. A., Lokugamage, K. G., Zhang, X., Muruato, A. E., Zou, J., Fontes-Garfias, C. R., Mirchandani, D., Scharton, D., Bilello, J. P., Ku, Z., An, Z., Kalveram, B., Freiberg, A. N., Menachery, V. D., Xie, X., . . . Shi, P.-Y. (2021). Spike mutation D614G alters SARS-CoV-2 fitness. *Nature*, *592*(7852), 116-121. https://doi.org/10.1038/s41586-020-2895-3

Plennevaux, E., Moureau, A., Arredondo-García, J. L., Villar, L., Pitisuttithum, P., Tran, N. H., Bonaparte, M., Chansinghakul, D., Coronel, D. L., L'Azou, M., Leon Ochiai, R., Toh, M.-L., Noriega, F., & Bouckenooghe, A. (2017). Impact of Dengue Vaccination on

Serological Diagnosis: Insights From Phase III Dengue Vaccine Efficacy Trials. *Clinical Infectious Diseases*. https://doi.org/10.1093/cid/cix966

Polo, S., Ketner, G., Levis, R., & Falgout, B. (1997). Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *Journal of Virology*, *71*(7), 5366-5374.

Pu, S.-Y., Wu, R.-H., Yang, C.-C., Jao, T.-M., Tsai, M.-H., Wang, J.-C., Lin, H.-M., Chao, Y.-S., & Yueh, A. (2011). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *Journal of Virology*, *85*(6), 2927-2941. https://doi.org/10.1128/jvi.01986-10

Pu, S. Y., Wu, R. H., Yang, C. C., Jao, T. M., Tsai, M. H., Wang, J. C., Lin, H. M., Chao, Y. S., & Yueh, A. (2011). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *85*(6), 2927-2941. https://doi.org/10.1128/jvi.01986-10

Puri, B., Polo, S., Hayes, C. G., & Falgout, B. (2000). Construction of a full length infectious clone for dengue-1 virus Western Pacific, 74 strain. *Virus genes*, *20*(1), 57-63.

Rice, C. M., Grakoui, A., Galler, R., & Chambers, T. J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biol*, *1*(3), 285-296.

Rihn, S. J., Merits, A., Bakshi, S., Turnbull, M. L., Wickenhagen, A., Alexander, A. J. T., Baillie, C., Brennan, B., Brown, F., Brunker, K., Bryden, S. R., Burness, K. A., Carmichael, S., Cole, S. J., Cowton, V. M., Davies, P., Davis, C., De Lorenzo, G., Donald, C. L., . . . Mahalingam, S. (2021). A plasmid DNA-launched SARS-CoV-2 reverse genetics system and coronavirus toolkit for COVID-19 research. *PLOS Biology*, *19*(2), e3001091. https://doi.org/10.1371/journal.pbio.3001091

Rodgers, M. A., Wilkerson, E., Vallari, A., McArthur, C., Sthreshley, L., Brennan, C. A., Cloherty, G., & De Oliveira, T. (2017). Sensitive next generation sequencing method reveals deep genetic diversity of HIV-1 in the Democratic Republic of the Congo. JVI.01841-01816. https://doi.org/10.1128/jvi.01841-16

Roesch, F., Cereghino, C., Carrau, L., Hardy, A., Ribeiro-Filho, H., Lacritick, A. H., Koh, C., Marano, J., Bates, T., Rai, P., Chuong, C., Akter, S., Vallet, T., Blanc, H., Elliot, T., Brown, A. M., Michalak, P., LeRoith, T., Bloom, J., . . . Weger-Lucarelli, J. (2022). The E2 glycoprotein holds key residues for Mayaro virus adaptation to the urban *Aedes*

aegypti mosquito. *bioRxiv*, 2022.2004.2005.487100. https://doi.org/10.1101/2022.04.05.487100

Santos, J. J. d. S., Cordeiro, M. T., Bertani, G. R., Marques, E. T. d. A., & Gil, L. H. V. G. (2013). Construction and characterisation of a complete reverse genetics system of dengue virus type 3. *Memórias do Instituto Oswaldo Cruz*, *108*, 983-991.

Scobey, T., Yount, B. L., Sims, A. C., Donaldson, E. F., Agnihothram, S. S., Menachery, V. D., Graham, R. L., Swanstrom, J., Bove, P. F., Kim, J. D., Grego, S., Randell, S. H., & Baric, R. S. (2013). Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences*, *110*(40), 16157-16162. https://doi.org/10.1073/pnas.1311542110

Siridechadilok, B., Gomutsukhavadee, M., Sawaengpol, T., Sangiambut, S., Puttikhunt, C., Chin-inmanu, K., Suriyaphol, P., Malasit, P., Screaton, G., & Mongkolsapaya, J. (2013). A Simplified Positive-Sense-RNA Virus Construction Approach That Enhances Analysis Throughput. *Journal of Virology*, *87*(23), 12667-12674. https://doi.org/doi:10.1128/JVI.02261-13

Søndergaard, J. N., Geng, K., Sommerauer, C., Atanasoai, I., Yin, X., & Kutter, C. (2020). Successful delivery of large-size CRISPR/Cas9 vectors in hard-to-transfect human cells using small plasmids. *Communications Biology*, *3*(1). https://doi.org/10.1038/s42003-020-1045-7

Steel, J. J., Henderson, B. R., Lama, S. B., Olson, K. E., & Geiss, B. J. (2011). Infectious alphavirus production from a simple plasmid transfection+. *8*(1), 356. https://doi.org/10.1186/1743-422x-8-356

Su'Etsugu, M., Takada, H., Katayama, T., & Tsujimoto, H. (2017). Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. *Nucleic Acids Research*, *45*(20), 11525-11534. https://doi.org/10.1093/nar/gkx822

Suzuki, R., de Borba, L., Duarte dos Santos, C. N., & Mason, P. W. (2007). Construction of an infectious cDNA clone for a Brazilian prototype strain of dengue virus type 1: Characterization of a temperature-sensitive mutation in NS1. *Virology*, *362*(2), 374-383. https://doi.org/https://doi.org/10.1016/j.virol.2006.11.026

Thi Nhu Thao, T., Labroussaa, F., Ebert, N., V'Kovski, P., Stalder, H., Portmann, J., Kelly, J., Steiner, S., Holwerda, M., Kratzel, A., Gultom, M., Schmied, K., Laloli, L., Hüsser, L.,

Wider, M., Pfaender, S., Hirt, D., Cippà, V., Crespo-Pomar, S., . . . Thiel, V. (2020). Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform. *Nature*, *582*(7813), 561-565. https://doi.org/10.1038/s41586-020-2294-9

Thomas, S. J., & Yoon, I.-K. (2019). A review of Dengvaxia®: development to deployment. *Human Vaccines & Immunotherapeutics*, *15*(10), 2295-2314. https://doi.org/10.1080/21645515.2019.1658503

Torii, S., Ono, C., Suzuki, R., Morioka, Y., Anzai, I., Fauzyah, Y., Maeda, Y., Kamitani, W., Fukuhara, T., & Matsuura, Y. (2021). Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Reports*, *35*(3), 109014. https://doi.org/https://doi.org/10.1016/j.celrep.2021.109014

Torres-Flores, J. M., Reyes-Sandoval, A., & Salazar, M. I. (2022). Dengue Vaccines: An Update. *BioDrugs*, *36*(3), 325-336. https://doi.org/10.1007/s40259-022-00531-z

Tully, D., & Griffiths, C. L. (2021). Dengvaxia: the world's first vaccine for prevention of secondary dengue. *Therapeutic Advances in Vaccines and Immunotherapy*, *9*, 251513552110158. https://doi.org/10.1177/25151355211015839

Tuo, D., Shen, W., Yan, P., Li, X., & Zhou, P. (2015). Rapid Construction of Stable Infectious Full-Length cDNA Clone of Papaya Leaf Distortion Mosaic Virus Using In-Fusion Cloning. *Viruses*, *7*(12), 6241-6250. https://doi.org/10.3390/v7122935

Ueno, H., Sawada, H., Soga, N., Sano, M., Nara, S., Tabata, K. V., Su'Etsugu, M., & Noji, H. (2021). Amplification of over 100 kbp DNA from Single Template Molecules in Femtoliter Droplets. *ACS Synthetic Biology*, *10*(9), 2179-2186. https://doi.org/10.1021/acssynbio.0c00584

Ulper, L., Sarand, I., Rausalu, K., & Merits, A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of Virological Methods*, *148*(1-2), 265-270. https://doi.org/10.1016/j.jviromet.2007.10.007

Usme-Ciro, J. A., Lopera, J. A., Enjuanes, L., Almazán, F., & Gallego-Gomez, J. C. (2014). Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome. *Virus Research*, *180*, 12-22. https://doi.org/https://doi.org/10.1016/j.virusres.2013.12.001

van der Most, R. G., Corver, J., & Strauss, J. H. (1999). Mutagenesis of the RGD Motif in the Yellow Fever Virus 17D Envelope Protein. *Virology*, *265*(1), 83-95. https://doi.org/https://doi.org/10.1006/viro.1999.0026

Wang, J., Huang, Y., Zhou, M., Hardwidge, P. R., & Zhu, G. (2016). Construction and sequencing of an infectious clone of the goose embryo-adapted Muscovy duck parvovirus vaccine strain FZ91-30. *Virology Journal*, *13*(1). https://doi.org/10.1186/s12985-016-0564-9

Weger-Lucarelli, J., Auerswald, H., Vignuzzi, M., Dussart, P., & Karlsson, E. A. (2018). Taking a bite out of nutrition and arbovirus infection. *PLOS Neglected Tropical Diseases*, *12*(3), e0006247. https://doi.org/10.1371/journal.pntd.0006247

Weger-Lucarelli, J., Duggal, N. K., Bullard-Feibelman, K., Veselinovic, M., Romo, H., Nguyen, C., Rückert, C., Brault, A. C., Bowen, R. A., Stenglein, M., Geiss, B. J., & Ebel, G. D. (2017). Development and Characterization of Recombinant Virus Generated from a New World Zika Virus Infectious Clone. *Journal of Virology*, *91*(1), JVI.01765-01716. https://doi.org/10.1128/jvi.01765-16

Weger-Lucarelli, J., Garcia, S. M., Rückert, C., Byas, A., O'Connor, S. L., Aliota, M. T., Friedrich, T. C., O'Connor, D. H., & Ebel, G. D. (2018). Using barcoded Zika virus to assess virus population structure in vitro and in Aedes aegypti mosquitoes. *Virology*, *521*, 138-148. https://doi.org/10.1016/j.virol.2018.06.004

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., Yuan, M.-L., Zhang, Y.-L., Dai, F.-H., Liu, Y., Wang, Q.-M., Zheng, J.-J., Xu, L., Holmes, E. C., & Zhang, Y.-Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature*, *579*(7798), 265-269. https://doi.org/10.1038/s41586-020-2008-3

Xie, X., Muruato, A., Lokugamage, K. G., Narayanan, K., Zhang, X., Zou, J., Liu, J., Schindewolf, C., Bopp, N. E., Aguilar, P. V., Plante, K. S., Weaver, S. C., Makino, S., Leduc, J. W., Menachery, V. D., & Shi, P.-Y. (2020). An Infectious cDNA Clone of SARS-CoV-2. *Cell Host & Microbe*, *27*(5), 841-848.e843. https://doi.org/10.1016/j.chom.2020.04.004

Yamshchikov, V., Mishin, V., & Cominelli, F. (2001). A New Strategy in Design of (+)RNA Virus Infectious Clones Enabling Their Stable Propagation in E. coli. *Virology*, *281*(2), 272-280. https://doi.org/10.1006/viro.2000.0793

Yang, T.-J., Yu, P.-Y., Chang, Y.-C., & Hsu, S.-T. D. (2021). D614G mutation in the SARS-CoV-2 spike protein enhances viral fitness by desensitizing it to temperature-dependent denaturation. *Journal of Biological Chemistry*, *297*(4), 101238. https://doi.org/10.1016/j.jbc.2021.101238

Yun, S.-I., Kim, S.-Y., Rice, C. M., & Lee, Y.-M. (2003). Development and application of a reverse genetics system for Japanese encephalitis virus. *Journal of Virology*, *77*(11), 6450-6465.

Zhang, L., Jackson, C. B., Mou, H., Ojha, A., Peng, H., Quinlan, B. D., Rangarajan, E. S., Pan, A., Vanderheiden, A., Suthar, M. S., Li, W., Izard, T., Rader, C., Farzan, M., & Choe, H. (2020). SARS-CoV-2 spike-protein D614G mutation increases virion spike density and infectivity. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-19808-4

Chapter Four: Replication in the presence of dengue convalescent serum impacts Zika virus neutralization sensitivity and fitness

Jeffrey M. Marano[1-3] and James Weger-Lucarelli[2,3]*

[1] Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Roanoke, VA, United States.

[2] Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, United States.

[3] Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Tech, Blacksburg, VA, United States

*** Correspondence:** James Weger-Lucarelli - weger@vt.edu

4.1 Abstract

Flaviviruses like dengue virus (DENV) and Zika virus (ZIKV) are mosquito-borne viruses that cause febrile, hemorrhagic, and neurological diseases in humans, resulting in 400 million infections annually. Due to their co-circulation in many parts of the world, flaviviruses must replicate in the presence of pre-existing adaptive immune responses targeted at serologically closely related pathogens, which can provide protection or enhance disease. However, the impact of pre-existing cross-reactive immunity as a driver of flavivirus evolution, and subsequently the implications on the emergence of immune escape variants, is poorly understood. Therefore, we investigated how replication in the presence of convalescent dengue serum drives ZIKV evolution. We used an *in vitro* directed evolution system, passaging ZIKV in the presence of serum from humans previously infected with DENV (anti-DENV) or serum from DENV-naïve patients (control serum). Following five passages in the presence of serum, we performed next-generation sequencing to identify mutations that arose during passaging. We studied two non-synonymous mutations found in the anti-DENV passaged population (E-V355I and NS1-T139A) by generating individual ZIKV mutants and assessing fitness in mammalian cells and live mosquitoes, as well as their sensitivity to antibody neutralization. Both viruses had increased fitness in Vero cells with and without the addition of anti-DENV serum and in human lung epithelial and monocyte cells. In Aedes aegypti mosquitoes—using blood meals with and

without anti-DENV serum—the mutant viruses had significantly reduced fitness compared to wild-type ZIKV. These results align with the trade-off hypothesis of constrained mosquito-borne virus evolution. Notably, only the NS1-T139A mutation escaped neutralization, while E-V335I demonstrated enhanced neutralization sensitivity to neutralization by anti-DENV serum, indicating that neutralization escape is not necessary for viruses passaged under cross-reactive immune pressures. Future studies are needed to assess cross-reactive immune selection in humans and relevant animal models or with different flaviviruses.

4.2 Introduction

Dengue virus (DENV; Genus *Flavivirus;* Family *Flaviviridae)* infects roughly five percent of the global population annually (Bhatt et al., 2013a). Zika virus (ZIKV; Genus *Flavivirus;* Family *Flaviviridae)* emerged in the western hemisphere in 2013 (Faria et al., 2016) and is estimated to have caused over 100 million infections by 2018 (Moore et al., 2020). ZIKV causes severe pathologies, including microcephaly and seizures (reviewed in (Freitas et al., 2020)). One proposed driver of severe disease is pre-existing immunity against DENV (Castanha et al., 2016; Fowler et al., 2018; Zimmerman et al., 2018; Rathore et al., 2019; Carvalho et al., 2020; Castanha et al., 2020; Katzelnick et al., 2021), four genetically and serologically closely related viruses (Barba-Spaeth et al., 2016). While pre-existing cross-reactive DENV immunity from antibodies can be protective (Pedroso et al., 2019; Carvalho et al., 2020; Katzelnick et al., 2021), it can also enhance disease (Castanha et al., 2016; Fowler et al., 2018; Zimmerman et al., 2018; Rathore et al., 2019; Carvalho et al., 2020; Castanha et al., 2020; Katzelnick et al., 2021) upon ZIKV infection. Given that these cross-reactive humoral responses play a significant role in disease, their role in ZIKV evolution should also be examined to more fully understand flavivirus evolution.

Immune-driven evolution occurs when the host immune response neutralizes only a subset of viruses, placing selective pressure on the virus population (Marchi et al., 2021); the surviving viruses—which likely have some resistance to the immune pressure—become founders for a subsequent generation (Coffey et al., 2013; Morris et al., 2020). Evolution driven by antibodies has been described for several viruses, including West Nile virus (Sapkal et al., 2011), Nipah virus (Borisevich et al., 2016), chikungunya virus (Jin et al., 2015), influenza (Lambkin et al., 1994; Cleveland et al., 1997; Ferguson et al., 2003; Doud et al., 2018; Lee et al., 2019), SARS-

CoV-2 (Sui et al., 2008; Baum et al., 2020; Haslwanter et al., 2021), and many others (Zhao et al., 2004; Zhao et al., 2006; Gal-Tanamy et al., 2008; Rockx et al., 2010; Anthony et al., 2017; Mishra et al., 2020). While attempts have been made to study the impact of cross-reactive immune-driven evolution in ZIKV, these previous studies use monoclonal antibodies (Keeffe et al., 2018), which are a simplistic model for the complex polyclonal human antibody response or study mutations that were not specific to immune selection(Regla-Nava et al., 2022). It is critical that cross-reactive immune-driven evolution be studied since mutations that arise may have implications for transmission (Liu et al., 2017; Liu et al., 2021a) or disease severity (Yuan et al., 2017; Xia et al., 2018; Shan et al., 2020; Liu et al., 2021a).

To address this gap, we examined the effects of cross-reactive antibody selection by passaging ZIKV in the presence of serum from convalescent dengue patients from the Dominican Republic or control serum from dengue-naïve donors from the United States. After passaging, we sequenced the viral populations using next-generation sequencing (NGS). Compared to the virus passaged in the control serum, the premembrane (prM) region of the anti-DENV serum passaged virus was less divergent from the starting virus and had lower non-synonymous diversity. We then examined the anti-DENV serum passaged virus for enriched mutations and engineered two unique mutations using a reverse genetics system. We assessed the impact of these two mutations, E-V355I and NS1-T139A, on fitness in various mammalian cell lines and *Aedes aegypti* mosquitoes and their sensitivity to neutralization by anti-DENV immune serum. Notably, both mutations had increased fitness in mammalian cell culture and reduced fitness in live mosquitoes. These results align with the trade-off hypothesis, which states that multi-host viruses that adapt to one host lose fitness in the other hosts (Wilson and Yoshimura, 1994; Kassen, 2002). When their neutralization sensitivity was assessed, NS1-T139A escaped neutralization, but E-V355I was more sensitive to neutralization. These results demonstrate that neutralization escape is not necessary for viruses that have been passaged in cross-reactive immune environments. As a whole, these results suggest that pre-existing immunity may play a significant role in ZIKV evolution.

4.3 Methods

Cells and Viruses

We obtained Vero cells (CCL-81) and U937-DC-SIGN cells (CRL-3253) from the American Type Culture Collection (ATCC). HEK293A cells were kindly provided by Dr. Jamie Smyth from the Fralin Biomedical Research Institute. A549 cells were kindly provided by Dr. Nisha Duggal from the Virginia-Maryland College of Veterinary Medicine. All cells were maintained at 37°C with 5% CO_2. Vero, HEK293A, and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin. We cultured U937-DC-SIGN cells in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 2 mM L-glutamine, 5% FBS, 1% nonessential amino acids, 0.1% gentamicin, and 0.05 mM 2-mercaptoethanol. The ZIKV strain used was derived from an infectious clone of strain PRVABC59 (Weger-Lucarelli et al., 2017) and was rescued and passaged once in Vero cells (p1). DENV1 strain R99142 and DENV2 strain PUO-218 were obtained from the CDC. DENV3 strain BC188/97 (NR-3801) and strain DENV4 703-4 (NR-48801) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). West Nile virus (WNV) Kunjin strain SW28919 and yellow fever virus (YFV) strain 17-D were obtained from the University of Texas Medical Branch World Reference Center for Emerging Viruses and Arboviruses.

Serum Sources

All human samples were de-identified by their respective sources prior to purchase. We obtained serum samples from convalescent dengue patients from the Dominican Republic who tested positive for DENV and negative for ZIKV by ELISA via Boca Biolistics, LLC. These samples were referred to as anti-DENV patients A-D. The serum from the four anti-DENV patients was subsequently pooled at equal volumes (hereafter called the anti-DENV pool). Convalescent Zika serum was obtained from BEI Resources (NR-50867). Control serum, derived from blood donors from Kentucky, USA, was from Valley Biomedical Products and Services, INC.

Plaque Reduction Neutralization Test (PRNT)

Serum samples were serially diluted in RPMI-1640 with 10 mM HEPES and 2% FetalPure bovine serum (Genesee Scientific 25-525H), hereafter referred to as viral diluent. Serum samples were mixed with 800 plaque-forming units per mL (PFU/mL) of the virus of interest. The mixture was then incubated at 37°C for one hour, and the virus-serum mixture was used to

inoculate wells in a confluent 24-well plate of Vero cells. After a one-hour adsorption period, plaque assay overlay media was added to each well, as previously described (Liu et al., 2021b). We fixed the plates four to seven days later, depending on the virus. We defined the $PRNT_{50}$ as the highest reciprocal dilution that neutralized the virus by at least 50%.

Viral Passaging

Vero cells were plated to an 80-90% confluency in 24-well plates. On the day of the infection, we mixed ZIKV with an equal volume of the appropriate serum. The virus-serum mixtures were incubated for one hour at 37°C and then used to inoculate the Vero cells. After the one-hour adsorption period, the cells were washed in phosphate-buffered saline (PBS); we then added fresh media supplemented with the appropriate human serum. Cells were monitored every 12 hours, and the supernatant was harvested once the cells demonstrated 50-75% cytopathic effect (CPE). Harvested supernatant was stored at -80°C until they were titered plaque assay. This process was repeated for a total of five passages, where the multiplicity of infection (MOI) for each passage was maximized based on the available titer (.01-1). These experiments were performed in triplicate, producing three unique lineages for each serum condition.

Library Preparation and Next-Generation Sequencing (NGS) Analysis

We prepared libraries of the unpassaged virus, virus passaged in the anti-DENV pool, and virus passaged in the control serum. To enrich for encapsidated virus and to remove nucleic acids, 120 µL of viral supernatant was mixed with 15 µL of 250 units/mL of Benzonase (Millipore Sigma E1014-5KU) diluted in 10x Benzonase Buffer (200 mM Tris-Cl [pH 7.5], 100 mM NaCl, 20 mM MgCl2) (Depew et al., 2013; Berg et al., 2016; Rodgers et al., 2017) and 15 µL of 250 units/mL of RNAse A (Millipore Sigma 10109142001) (Aryani and Denecke, 2015). Samples were then incubated at 37°C for three hours. RNA was extracted from the samples using the Zymo Quick-RNA Viral Kit (R1035). After extraction, we purified the samples using a 0.8x bead selection with the sparQ PureMag magnetic beads (95196-005) to remove small RNA fragments. First-strand cDNA synthesis was performed using random nonamers and the Maxima H- Reverse Transcriptase kit (EP0751). We synthesized second-strand cDNA using the Q5 High-Fidelity 2X Master Mix (M0492S) (Bates et al., 2021). NGS Libraries were then produced using the sparQ DNA Frag & Library Prep Kit from Quantabio (Cat. 95194-024). Samples were sequenced using

150 bp paired-end reads on the Illumina Novaseq 6000 (Novogene Co., Ltd.). Bioinformatic analysis was performed as previously described (Marano et al., 2022; Roesch et al., 2022). Briefly, adapters and bases with <Q30 were trimmed using Bbduk (Bushnell, 2014); the remaining reads were mapped to the ZIKV reference using the Burrows-Wheeler Aligner (BWA)(Li and Durbin, 2009). Variants were called with LoFreq (Wilm et al., 2012), and new consensus sequences were produced using the Genome Analysis Toolkit (GATK) (McKenna et al., 2010). To perform diversity and selection analysis, we used SNPGenie (Nelson et al., 2015) to calculate the non-synonymous (π_N) and synonymous (π_S) diversity. To determine the divergence of passaged samples, we generated an all-site VCF file using bcftools (Danecek et al., 2021) and calculated D_{xy} using pixy (Korunes and Samuk, 2021).

Generation and Rescue of Single Mutant Viruses by Bacteria-Free Cloning

Mutagenic PCR primers were designed *in silico* to introduce the identified mutations using SnapGene 6.0.2 software (GSL Biotech). We performed PCRs using the SuperFi II Master Mix (Thermo Fisher 12368010), and the products were gel purified using the Machary-Nagel NucleoSpin Gel and PCR Clean-up kit (740609). We assembled the products using the NEB Builder HiFi DNA Assembly Master Mix (E2621L). The assemblies were digested with DpnI (R0176S), Lambda exonuclease (M0262S), and Exonuclease I (M0293S) and amplified using SuperPhi RCA Premix Kit with Random Primers (Evomics catalog number PM100) (Bates et al., 2020). As previously described, RCA products were then transfected into HEK293A cells to produce p0 stocks of the virus (Marano et al., 2020; Marano et al., 2022). The p0 stocks were then used to infect Vero cells at an MOI of 0.01 to produce p1 stocks, which were used for all downstream tests. The p1 stocks were verified by Sanger sequencing to confirm that the mutant of interest was introduced.

In vitro Competition Assays

Competition assays were performed similarly to previously described methods (Marano et al., 2022). Briefly, the mutant and wild-type viruses were mixed at a 1:1 PFU ratio to prepare the competition mixes. This mix was then confirmed by plaque assay, and we amplified a PCR amplicon around the mutation site using extraction-free one-step RT-PCR (Genoud et al., 2021) with qScript XLT One-Step RT-PCR Kit (95143-200). We then purified the amplicon and

submitted it for Sanger sequencing, and then assessed the ratio of each virus from the resulting chromatograms using QSVanalyzer (Carr et al., 2009). After confirmation, we used the mixes to infect cells at an MOI of 0.01 for each virus. For Vero and A549 cells, virus mixes were added to the cells, and after one-hour adsorption, cells were washed with PBS, and fresh media was added. For the Vero cells with serum supplementation experiments, the mixes were treated identically to the passaging experiments described above. For U937-DC-SIGN cells, we first centrifuged the cells, removed the media, and the cells were resuspended in the diluted virus mix (Fowler et al., 2018). After a two-hour adsorption period, we washed cells with PBS before adding fresh growth media (Fowler et al., 2018). Virus was harvested at 2 (U937-DC-SIGN), 3 (Vero and A549), or 4 (Vero with convalescent DENV serum) days post-infection (dpi). Extraction-free RT-PCR and Sanger sequencing were performed on all samples, and the data was analyzed using QSVanalyzer (Liu et al., 2021a). Relative fitness was calculated as $W(t) = F(t)/F(0)$, where $F(t)$ is defined as the ratio of the mutant virus following the competition and $F(0)$ is defined as the ratio of the mutant virus at baseline (Liu et al., 2021a). We considered the mutant to have increased fitness in the tested environment if $W>1$. In contrast, we considered the mutant virus to have reduced or no fitness change if $W<1$ or $W=0$, respectively.

Mosquito Rearing and In vivo Competition Assays

We reared *Aedes aegypti* Poza Rica mosquitoes using previously published methods (Weger-Lucarelli et al., 2016). Briefly, mosquitos were maintained at 28°C with a relative humidity of 75% and a 12:12 (light/dark) photoperiod. During larval stages, mosquitoes were maintained on ground Nishikoi fish food. Adult mosquitoes were given 10% sucrose solution *ad libitum* via cotton balls. Mosquitoes were separated from the colony 6 – 8 days post eclosion at a 5:1 ratio of females to males into disposable 16 oz containers. Mosquitoes were starved of glucose and water for 24 hours before the infectious blood meal of defibrinated sheep's blood. Mosquitoes were fed for one hour using the Hemotek membrane feeder system (SP4W1-3). After feeding, mosquitoes were anesthetized at 4°C, and females fed to repletion were separated into a new container. We maintained these mosquitoes for ten days under rearing conditions with sucrose *ad libitum*. Mosquitoes were then anesthetized using triethylamine, and whole bodies were collected in viral diluent supplemented with 50 μg/mL gentamicin and 2.5 μg/mL of amphotericin B. Samples were processed by adding a single sterile metallic bead per tube, homogenizing the samples at 30 freq/s for 2 min using the Qiagen TissueLyser II (85300), and diluting the samples 1:5 in viral

diluent. We used the extraction-free RT-PCR, Sanger sequencing, and QSVanalyzer workflow described above to analyze the samples. The "naïve" blood meal consisted of defibrinated sheep's blood, 10^6 PFU/mL of the competition mix, and 0.5 μM ATP. For the "immune" blood meal, we pretreated the virus for one hour by incubating it with anti-DENV serum, but other conditions were unchanged.

Statistical Analysis

Statistical analysis was performed in Prism 9 (GraphPad, San Diego, CA, USA). A two-way ANOVA with Šídák's correction for multiple comparisons was used to compare the divergence and non-synonymous diversity (π_n) within the antibody binding regions of the passaged populations. A two-way ANOVA with Dunnett's correction for multiple comparisons was used to compare the neutralization of the mutant viruses to the wild type. For *in vitro* competition assays, a Shapiro-Wilk test for normality was performed to ensure normality, and then a one-sample t-test was performed. For the *in vivo* competition assays, a Fisher's exact test was used to determine which species (mutant or WT) would become dominant in a population of mosquitoes.

4.4 Results

Donor Serum Characterization

We first evaluated the serostatus of the donors from the Dominican Republic (referred to as anti-DENV patients A-D) and the US (Kentucky; hereafter referred to as control serum) by performing plaque reduction neutralization tests (PRNTs) against DENV and ZIKV. Data are presented as the reciprocal serum dilution which resulted in a 50% reduction in plaques ($PRNT_{50}$) (Table 1). Since closely related flaviviruses are known to be cross-neutralized by convalescent serum, we defined the infecting virus as having a $PRNT_{50} \geq$ 4-fold higher than the other viruses within the group (Sasmono et al., 2018; Sharp et al., 2019). Based on these parameters, we concluded that all anti-DENV donors had a history of DENV infection without prior ZIKV infection. Furthermore, we concluded that the control serum donors had no history of infection with either DENV or ZIKV.

Virus	anti-DENV Patient A	anti-DENV Patient B	anti-DENV Patient C	anti-DENV Patient D	Control Serum
DENV1 R99142	640	1280	160	10240	<20
DENV2 PUO218	640	10240	80	2560	<20
DENV3 BC188/97	1280	320	640	5120	<20
DENV4 703-4	640	640	360	1280	<20
ZIKV PRVABC59	20	<20	<20	80	<20

Table 1: Serological Characterization of Donor Blood. Donor blood was serially diluted and mixed with dengue virus (DENV) serotypes 1-4 or Zika virus (ZIKV). The reciprocal of the highest serum dilution that neutralized 50% of the challenge virus is reported as the PRNT50 value. These data represent two biological replicates, each with three technical replicates.

To increase antibody diversity, we pooled the four anti-DENV donors (hereafter referred to as the anti-DENV pool) at equal volumes. We then tested the pool's neutralization of DENV1-4 and ZIKV (Figure 1), as well as yellow fever virus (YFV) and West Nile virus (WNV) (Supplemental Table 1). The cross-reactive $PRNT_{50}$ of the anti-DENV pool against ZIKV was 40, which represents an intermediate between the specific neutralization from the convalescent ZIKV patient ($PRNT_{50} = 320$) and the control serum ($PRNT_{50} < 20$). Thus, we generated an anti-DENV pool with a history of DENV infection but no previous exposure to ZIKV, WNV, or YFV.

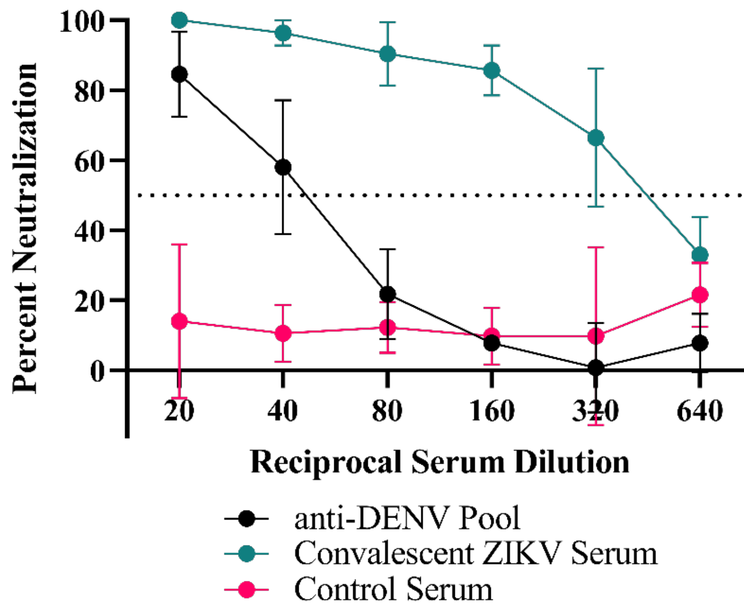


Figure 1: Zika virus (ZIKV) neutralization by the anti-dengue virus (DENV) serum pool. The anti-DENV pool, control serum and convalescent ZIKV serum were serially diluted and mixed with 800 PFU/ml of ZIKV. The reciprocal of the highest serum dilution that neutralized 50% (presented as a dotted line) of the challenging virus is reported as the PRNT50 value. These data represent two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean.

Passaging Virus in the Presence of anti-DENV Serum

To assess the impact of convalescent dengue serum on ZIKV evolution, we adapted previously reported immune-driven evolution protocols (Borisevich et al., 2016; Lee et al., 2019). Specifically, we mixed ZIKV at a 1:1 ratio by volume with either the anti-DENV pool at the $PRNT_{50}$ concentration (1:40 dilution) or control serum at the same dilution (Figure 2A). After one hour of incubation, we infected Vero cells with the virus-serum mixtures. After adsorption, we washed the cells and maintained them with media supplemented with anti-DENV pool or control serum. We monitored the cells and harvested the supernatant when we observed >75% cytopathic effect (CPE). We then titered the harvested virus (Figure 2B) and used it for a subsequent passage. By the fifth passage, we observed that the time to produce CPE in the anti-DENV pool passaged virus increased by 1.5 days from the initial passage, while the control serum passaged virus decreased by 0.5 days (Supplemental Figure 1). Given this phenotypic change, we next sequenced the virus populations.

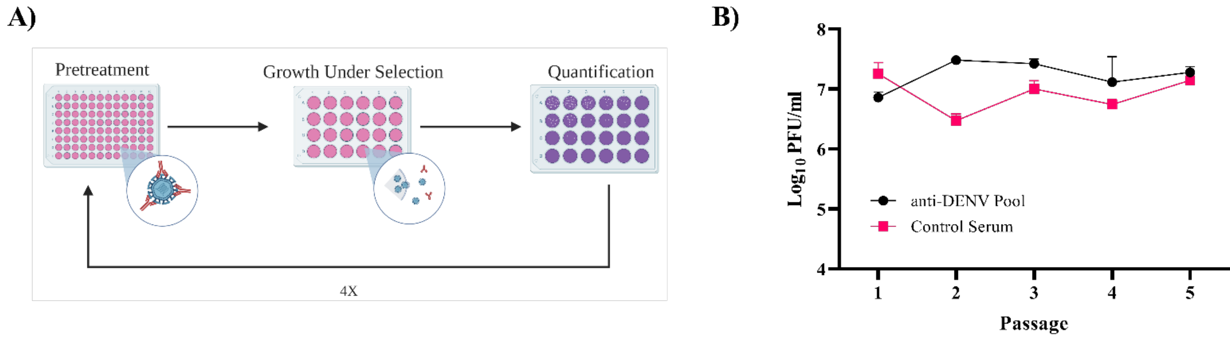


Figure 2: Zika virus (ZIKV) Passaging Workflow. A) Virus was mixed with either anti-dengue virus (DENV) or control serum that had been diluted 40-fold and then incubated for one hour at 37°C. The mix was then used to infect Vero cells. After adsorption and washing, we added fresh media that contained either anti-DENV serum or control serum. Supernatant was harvested when the cells showed at least 75% cytopathic effects. After clarifying and freezing the supernatant, it was assessed by plaque assay to quantify infectious virus. This process was repeated a total of five times. B) The post-passage titers of the anti-DENV pool and control serum passaged virus, as measured by plaque assay.

Population Divergence, Diversity, and Mutation Selection

We next sought to determine the evolutionary impact of passaging ZIKV in convalescent dengue serum, hypothesizing that the viral populations would differ significantly from the virus passaged in control serum. To this end, we performed Illumina next-generation sequencing (NGS) on viral RNA from the unpassaged virus, the control serum passaged virus, and the anti-DENV pool passaged virus. We included the unpassaged virus to establish an accurate starting consensus sequence. By sequencing the virus passaged in the control serum and the anti-DENV pool, we could differentiate between mutants derived from passaging ZIKV in Vero cells with human sera (control) and the impact of passage in the presence of dengue convalescent serum. For our analysis, we focused on the regions of known antibody binding: premembrane (prM), envelope (E), and nonstructural protein 1 (NS1) (Vaughan et al., 2010). We examined the average nucleotide distance (divergence) between our unpassaged and passaged viruses (Figure 3A) and the non-synonymous diversity, π_n (Figure 3B), within the antibody binding regions. We observed significantly lower divergence ($p = 0.043$) and non-synonymous diversity ($p = 0.001$) in the prM region of the anti-DENV pool passaged virus compared to the control serum passaged virus. These results suggest that selection by anti-DENV serum limited the divergence and diversification of the prM protein.

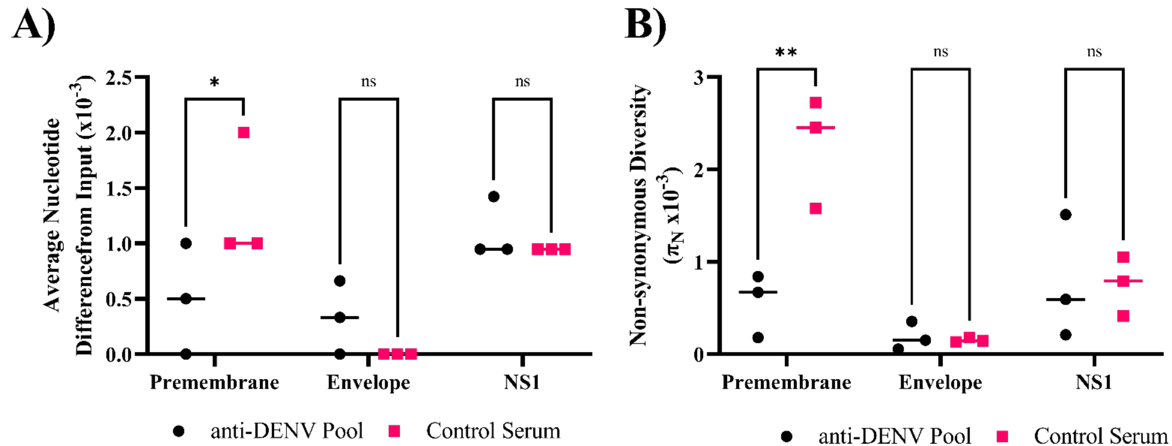


Figure 3: Anti-dengue virus (DENV) Immune Selection Limits Zika virus (ZIKV) Divergence and Nonsynonymous Diversity in the Premembrane Protein. A) Divergence within the antibody binding region of passaged samples compared to the input virus. B) Comparing the πn values at each protein with the antibody binding region of the passaged populations. Statistics were performed using a two-way ANOVA with a Šídák's correction for multiple comparisons (* $p = 0.043$ ** $p = 0.001$). NS – nonstructural protein

We next examined the antibody binding region for single nucleotide variants enriched after passaging in the presence of convalescent dengue serum. To prioritize variants, we used an allelic frequency cutoff of 0.1 as our focus was on higher frequency mutations, which we expected would be more likely to have a phenotypic impact on the virus. Of the 43 mutations identified across the input virus and the passaged populations at an allelic frequency ≥ 0.1 (Supp. Table 2-4), five non-synonymous mutations were found exclusively in the anti-DENV pool passaged virus within the antibody binding region that increased in frequency (Table 2). We selected two mutations to construct within the envelope (E), E-V355I, and NS1, NS1-T139A (Figure 4A), regions which have been shown to mediate cell entry (Fontes-Garfias et al., 2017) and alter infectivity in mosquitoes (Liu et al., 2017; Kuo et al., 2020), respectively. Interestingly, ZIKV E-355I has been previously isolated from a human in Brazil (NCBI Accession OL423668) (Hadfield et al., 2018). We used a bacteria-free cloning approach to generate mutant viruses (Weger-Lucarelli et al., 2018b; Kang et al., 2021b) and a previously characterized ZIKV infectious clone as a template (Weger-Lucarelli et al., 2017).

nt Position	Protein	Reference	Alternative	AA Change	Allelic Freq. Rep. 1	Depth Rep. 1	Allelic Freq. Rep. 2	Depth Rep. 2	Allelic Freq. Rep. 3	Depth Rep. 3
721	Premembrane	A	G	[H/R]	0.997832	13838	0.073241 *	3823	0.148524	2168
1435	Envelope	T	C	[V/A]					0.736453	2030
2040	**Envelope**	**G**	**A**	**[V/I]**			0.920766	3395		
2904	**NS1**	**A**	**G**	**[T/A]**	0.998622	13788			0.887506	2249
3222	NS1	A	G	[K/E]					0.103163	3222

Table 2: Variants Identified in Passaged Zika virus (ZIKV) Populations. Non-synonymous mutations within the key antibody binding domains of ZIKV (prM, E, and NS1) with an allelic frequency ≥ 0.1. If a mutation was not found in a replicate, the box is grayed out. The mutations that were selected for downstream use are bolded. NS1 refers to nonstructural protein 1.

Mutants identified during passage in convalescent dengue serum have increased fitness in mammalian cell culture

To assess the impact of the mutations on replicative fitness, we performed competition assays comparing the mutants to WT ZIKV (Figure 4B). We mixed mutant and wild-type viruses at a 1:1 PFU ratio and then used the mixes to infect several cell lines. To calculate the fitness of the mutant virus in each environment, we compared the relative proportion of the mutant virus pre- and post-infection (Liu et al., 2021a). If the proportion increased during infection, we concluded the virus had increased fitness; if the proportion remained the same or decreased, we concluded the virus had neutral or decreased fitness, respectively. We tested four cell culture environments: Vero cells without supplementation of anti-DENV serum, representing an immune naïve model (Immune Naïve); Vero cells supplemented with the anti-DENV pool, representing a dengue immune model (Immune); A549 cells representing a human epithelial cell model (Human Epithelial); U937-DC-SIGN cells, representing a human monocyte model (Human Monocyte). These cell lines were selected as they previously were shown to be susceptible to ZIKV infection and represent cell types critical to pathogenesis (Hamel et al., 2015; Khaiboullina et al., 2017; Fowler et al., 2018). The NS1-T139A (Figure 4C) and the E-V355I mutant (Figure 4D) had increased fitness compared to WT ZIKV in all environments tested. These data demonstrate that the ZIKV mutants derived from passaging in the presence of anti-DENV serum have increased fitness in mammalian cells.

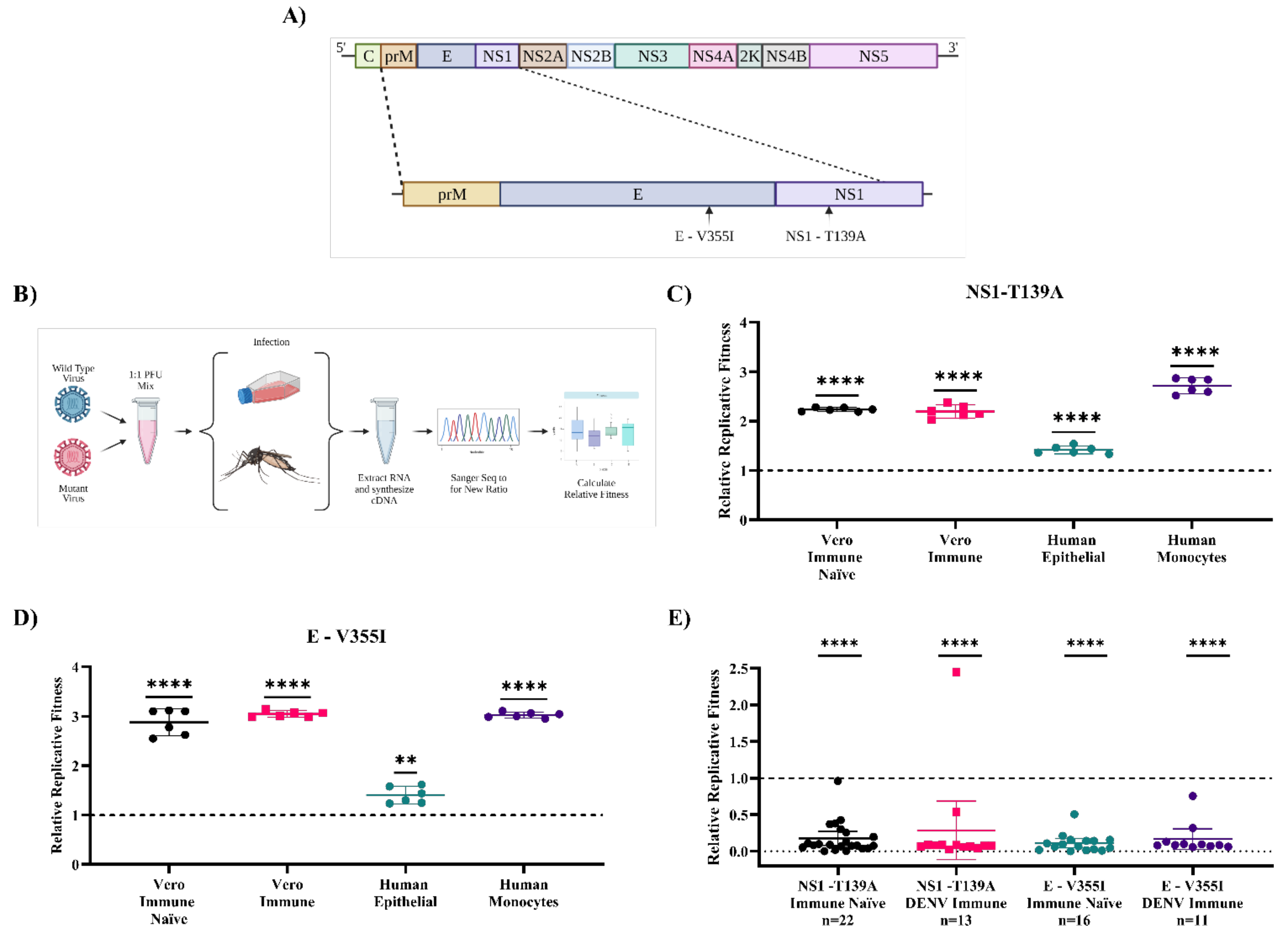


Figure 4: Zika virus (ZIKV) mutants have enhanced fitness in mammalian cells and reduced fitness in mosquitoes. A) Within the ZIKV genome, antibody selection primarily occurs against the M, E, and NS1 proteins. Within those regions, based on several criteria, we selected to generate mutations at E-V355I and NS1-T139A. B) To assess replicative fitness, WT virus was mixed with a given mutant virus at a 1:1 PFU ratio. The mix was then used to infect cell lines (MOI = 0.01) or live mosquitoes (10^6 pfu/ml). After infection, virus was harvested, RNA extracted, and RT-PCR was performed. The resulting amplicon was sequenced to calculate the relative fitness of each mutant. (C-E) In vitro competition assays. NS1- T139A (C) and E- V355I (D) in Vero cells without supplementation of anti-DENV serum (Immune Naïve), Vero cells with supplementation of anti-DENV serum (Immune), A549 cells (Human Epithelial), and U937-DC-SIGN cells (Human Monocytes) were performed. These data represent two biological replicates and three technical replicates, and the error bars represent standard deviation from the mean. Statistical analysis was performed using a one sample t-test (**** $p< .0001$ and ** $p = .0021$) with a null value of 1. E) Competition assays in mosquitoes were performed by feeding Aedes aegypti Poza Rica mosquitoes with either untreated virus (Immune Naïve) or virus pre-treated with anti-DENV serum (DENV Immune). Each data point represents a single mosquito, and the error bars represent the standard deviation from the mean of pooled fitness across all mosquitoes. The pooled group sizes from two independent experiments are reported below the group titles on the x-axis. Statistical analysis was performed using a Fisher's exact test (**** $p < .0001$).

Mutants identified during passage in convalescent dengue serum have reduced fitness in mosquitoes

Because ZIKV is transmitted between mammalian and insect hosts, it is critical to test the fitness of these immune-selected mutants in mosquitoes to assess the likelihood that these mutations would be maintained in nature. To that end, we fed *Aedes aegypti* an artificial blood meal containing the WT/mutant competition mixes used in the previous studies. To mimic the impact of a prior DENV infection, we also performed an experiment where we pretreated the virus with the anti-DENV serum pool before the blood meal. We then collected mosquitoes at ten days post-feeding, and whole-body homogenates were tested to determine the proportion of each virus compared to the original blood meal (Figure 4E). Regardless of host immune status, the mutant viruses had significantly reduced fitness compared to WT ZIKV in mosquitoes ($p<.0001$). These data suggest that the ZIKV mutants derived from cross-reactive immune selection have significantly reduced fitness in the mosquito host. These results, taken with those from the *in vitro* competitions assays, indicate that our findings align with the trade-off hypothesis (Wilson and Yoshimura, 1994; Kassen, 2002).

Neutralization escape is not required for fitness enhancement during cross-reactive immune selection.

To understand the impact of immune selection on neutralization sensitivity, we performed PRNTs on the ZIKV mutants using the anti-DENV pool (Figure 5). We hypothesized that the mutants would escape neutralization because they were generated from passaging in anti-DENV serum. The anti-DENV pool neutralized wild-type ZIKV at a $PRNT_{50}$ of 40, similar to what we found previously (Figure 1). The mutants showed polarized neutralization susceptibilities; specifically, the anti-DENV pool no longer neutralized NS1-T139A ($PRNT_{50}$ <20), indicative of escape, while E-V355I was slightly more sensitive to neutralization than the WT ($PRNT_{50}$ =80 $p < .0001$). Interestingly, when we examined the neutralization sensitivity of the populations from which these mutants were derived, we saw neither escape nor sensitization phenotypes (Supplemental Figure 2). These results suggest that cross-reactive antibody selection can generate mutants with enhanced sensitivity (E-V355I) and reduced sensitivity (NS1-T139A) to antibody selection and that neutralization escape is not necessary for fitness enhancement during cross-reactive immune selection. To further contextualize the results, we generated two mutants from mutations identified in the control population (Supplemental Table 4): prM-S109P and prM-M159V. These mutations were selected because we observed higher genetic divergence and diversity in the prM protein of the control population compared to the anti-DENV pool

population (Figure 3A-B). Both mutants from the control serum passaged population showed greatly enhanced sensitivity to neutralization by the anti-DENV pool ($PRNT_{50} > 640$). This behavior was also observed in the passaged populations that these mutants were derived from (Supplemental Figure 2). These results indicate that passaging in anti-DENV serum protected ZIKV from taking on these extreme neutralization phenotypes. When we assessed the replication of the passaged populations in Vero cells, we observed that the control serum passaged virus had increased replicative fitness (Supplemental Figure 3A). In contrast, when we tested the passaged populations in Vero cells supplemented with the anti-DENV pool, we observed that the control serum passaged virus did not replicate in this environment (Supplemental Figure 3B). These results indicate that cross-reactive immunity constrains the fitness of the population, similar to the constraint in diversificiation and divergence we observed in the NGS data (Figure 2), but also protect the virus from developing extreme neutralization phenotypes.

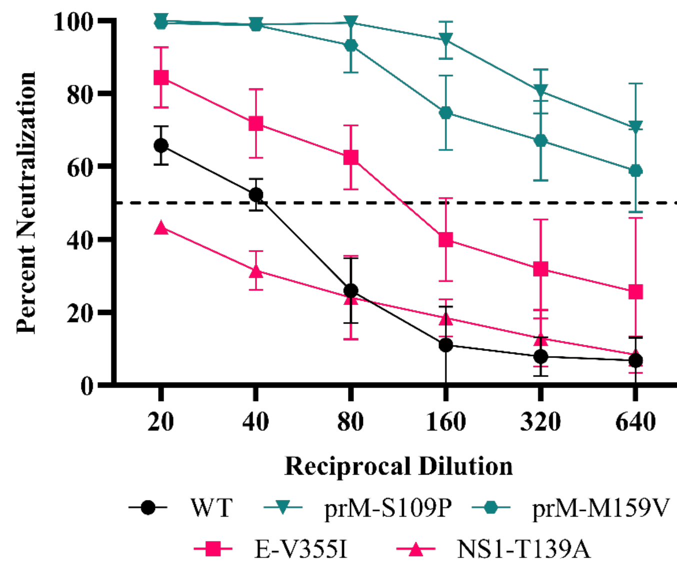


Figure 5: ZIKV Mutant Neutralization Sensitivity. anti-DENV pool were serially diluted and mixed with WT ZIKV (black line), mutants from the anti-DENV pool population (red lines), or mutants identified from the Control Serum-passaged population (green lines). Data are from two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean.

4.6 Discussion

The recent ZIKV outbreak in the Americas was associated with several adaptive mutations that potentially facilitated its rapid emergence (Liu et al., 2017; Yuan et al., 2017; Xia et al., 2018; Shan et al., 2020; Liu et al., 2021a), though the driver of these mutations is unknown. One

potential driver of ZIKV evolution is selection mediated by pre-existing adaptive immune responses to closely related flaviviruses such as DENV. While immune-driven evolution has been well-characterized for other viruses (Lambkin et al., 1994; Cleveland et al., 1997; Ferguson et al., 2003; Zhao et al., 2004; Zhao et al., 2006; Gal-Tanamy et al., 2008; Sui et al., 2008; Jin et al., 2015; Borisevich et al., 2016; Anthony et al., 2017; Doud et al., 2018; Lee et al., 2019; Mishra et al., 2020), previous studies with ZIKV have some limitations. Regla-Nava et al. sought to examine how pre-existing immunity to DENV affected the evolution and pathology of ZIKV (Regla-Nava et al., 2022). Using a passaging system in which they oscillated ZIKV infection between mosquito cells and dengue-naïve or dengue-immune mice, they identified a mutant, NS2B I39V, that appeared to escape pre-existing dengue immunity, and increased viral loads, adult and fetal mortality, and mosquito infection (Regla-Nava et al., 2022). However, NS2B I39V occurred in both the experimental and control populations; therefore, the mutation was likely not driven by immune pressure. Interestingly, we also found NS2B I39V in our anti-DENV passaged population (Supplemental Table 3). Thus, there remained a gap in understanding the specific impact of cross-reactive immune selection on ZIKV evolution. To fill this gap, we passaged ZIKV in the presence of serum from convalescent dengue patients or dengue naïve serum and then studied the evolutionary and fitness consequences on the virus. Within the population passaged in the anti-DENV pool, we identified two mutations, NS1-T139A and E-V355I, that had altered sensitivity to antibody neutralization, increased fitness in mammalian cells, and reduced fitness in *Aedes aegypti* mosquitoes.

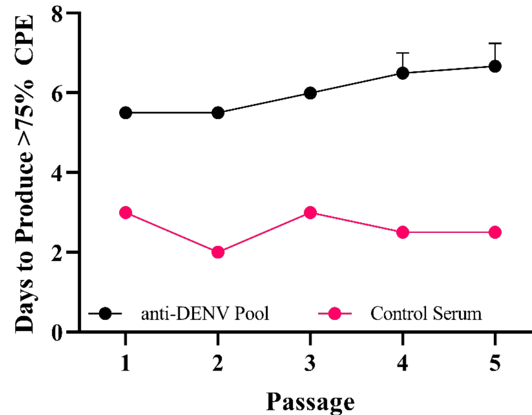
Immune selection provides a complex pressure as there is both the enrichment for escape mutations (diversifying selection) within a population resistant to neutralization (Lambkin et al., 1994; Cleveland et al., 1997; Ferguson et al., 2003; Zhao et al., 2004; Zhao et al., 2006; Gal-Tanamy et al., 2008; Rockx et al., 2010; Sapkal et al., 2011; Jin et al., 2015; Borisevich et al., 2016; Anthony et al., 2017; Doud et al., 2018; Lee et al., 2019) and the removal of genotypes sensitive to neutralization (purifying selection) (Hu et al., 2021). Two critical aspects of this selection are genetic diversity (Lee et al., 2015) and divergence (MacLean et al., 2021): we observed higher genetic divergence and diversity within the prM protein in the control serum-passaged population compared to the virus passaged in the anti-DENV pool. Thus, we concluded that anti-DENV serum likely tempered the ability of ZIKV to diverge during passaging. When we generated two mutants found in the control population, prM-S109P and prM-M159V, we

observed that both were highly sensitive to neutralization by the anti-DENV pool, suggesting that the prM protein may be important for ZIKV neutralization by dengue convalescent serum. Given previous results, we hypothesized that immune-driven evolution would generate neutralization escape mutants (Zhao et al., 2004; Borisevich et al., 2016; Lee et al., 2019), as we observed with NS1-T139A; in contrast, E-V355I was more sensitive to neutralization by the anti-DENV pool than WT ZIKV. This possible paradox may be explained by the overall fitness benefit conferred by the E-V355I mutation in all mammalian environments tested here (Fig. 5) (Blish et al., 2008). The escape phenotype produced by a nonstructural protein mutant, NS1-T139A, may also appear unexpected since nonstructural proteins are generally not found extracellularly. NS1, however, exists as an intracellular monomer, a membrane-bound dimer, and an *extracellular* hexamer (Rastogi et al., 2016). During infection, secreted NS1 disrupts endothelial junctions, resulting in tissue permeabilization and vascular leakage (Puerta-Guardo et al., 2019; Puerta-Guardo et al., 2022), so neutralizing NS1 may be an important protective strategy. Indeed, passive transfer studies using serum from mice vaccinated with NS1 protected against a lethal ZIKV challenge (Bailey et al., 2019a). These results further demonstrate NS1's importance as a target for antibody selection. NS1-T139A also increased fitness *in vitro* in mammalian cells, possibly due to its ability to "prime" cells for infection, resulting in increased viral replication (Alcon-LePoder et al., 2005; Avirutnan et al., 2007; Crook et al., 2014).
While the mutations identified following passage in the presence of anti-DENV serum enhanced fitness in mammalian cell culture, they significantly reduced fitness in live mosquitoes. These results align with the trade-off hypothesis (Wilson and Yoshimura, 1994; Kassen, 2002), which states that as an arthropod-borne virus, or any virus in a multi-host system, adapts to one of its hosts, it may lose fitness in the other host(s) (Wilson and Yoshimura, 1994; Kassen, 2002). These data suggest that while mutants arise following exposure to cross-reactive antibodies in a DENV-immune person, if wild-type virus remains within the population, they might be removed from the population upon mosquito infection, thereby limiting the mutant's spread.
Limitations of the study: We used only a single post-epidemic strain of ZIKV and convalescent serum from the Dominican Republic. We may have observed different results using different viruses or serum samples. We focused only on humoral immunity generated from natural infection. However, cross-reactive T cells, which have been shown to play a role in protection against ZIKV (Regla-Nava et al., 2018) and to drive the evolution of other viruses (Ciurea et al.,

2001; Cao et al., 2003; Bowen and Walker, 2005; Zompi et al., 2012; Agerer et al., 2020), should also be examined as a driver of ZIKV evolution. Our passaging approach involved only mammalian cells, representing the multiple rounds of replication within a single host; however, an alternating passaging system between mosquito and mammalian cells could also have been used to mimic the natural transmission cycle (Deardorff et al., 2011; Talavera-Aguilar et al., 2021; Regla-Nava et al., 2022). Finally, we generated only single mutants; however, we could have engineered mutant viruses with multiple mutations to assess potential epistatic interactions which would explain the differences observed between the mutant viruses and the populations (Ferreira et al., 2021).

Conclusions: We demonstrated that cross-reactive selection in the presence of anti-DENV antibodies alters ZIKV evolution and fitness. Specifically, we found that passaging ZIKV in mammalian cells with anti-DENV antibodies results in the generation of mutants with altered sensitivity to neutralization. Notably, passaging ZIKV in the presence of anti-DENV serum constrained the evolution of the virus population compared to control populations, which became highly sensitive to neutralization and more fit in Vero cells. However, the mutants from populations passaged in the presence of anti-DENV serum had increased fitness in mammalian cells. This is possibly due to epsistatic interactions reducing fitness. We observed a significant reduction in fitness for both mutants in mosquitoes, consistent with the trade-off hypothesis. These results improve our understanding of the drivers of ZIKV evolution and suggest that future work is needed to more fully dissect the evolutionary implications of inter-flavivirus immune interactions, including inter-serotype interactions with DENV and viruses within the Japanese encephalitis complex (As reviewed (Rathore and St. John, 2020)).

4.7 Supplementary Data



Supplemental Figure 1: Cytopathic Effect Production Post Passaging. During passaging, viral supernatant was harvested when >75% of cells demonstrated cytopathic effects (CPE). The days post-infection where this occurred were recorded for each passage. Data represent the three independent lineages within each passaging condition (anti-dengue virus (DENV) Pool or Control Serum).

Virus	**$PRNT_{50}$ Reciprocal Dilution**
DENV1 R99142	10240
DENV2 PUO218	2560
DENV3 BC188/97	81920
DENV4 703-4	5120
West Nile Virus (WNV) Kunjin	80
Yellow Fever Virus (YFV) 17D	<20

Supplemental Table 1: Serological Characterization of the anti-dengue virus (DENV) Pool. The anti-DENV pool was serially diluted and mixed with 800 PFU/mL of DENV1-4, West Nile virus (WNV), or yellow fever virus (YFV). The reciprocal of the highest serum dilution that neutralized 50% of the challenge virus is reported as the PRNT50. These data represent two biological replicates, each with three technical replicates.

nt Position	Protein	Reference	Alternative	AA Change	Allelic Freq. Rep. 1	Depth Rep. 1	Allelic Freq. Rep. 2	Depth Rep. 2	Allelic Freq. Rep 3	Depth Rep. 3
1436	Envelope	T	C	[V/V]	0.089398*	1745	0.102114	1939	0.114118	2550
2904	NS1	A	G	[T/A]	0.363902	2050	0.345592	1985	0.344643	2800
2905	NS1	C	T	[T/I]	0.09196*	1990	0.083739*	2054	0.10833	2797
2922	NS1	C	A	[L/I]	0.40373	1984	0.415464	1940	0.402186	2745
10685	3’ UTR	G	C	-	0.148893	497	0.169043	491	0.136192	793
10702	3’ UTR	A	C	-					0.128049	164

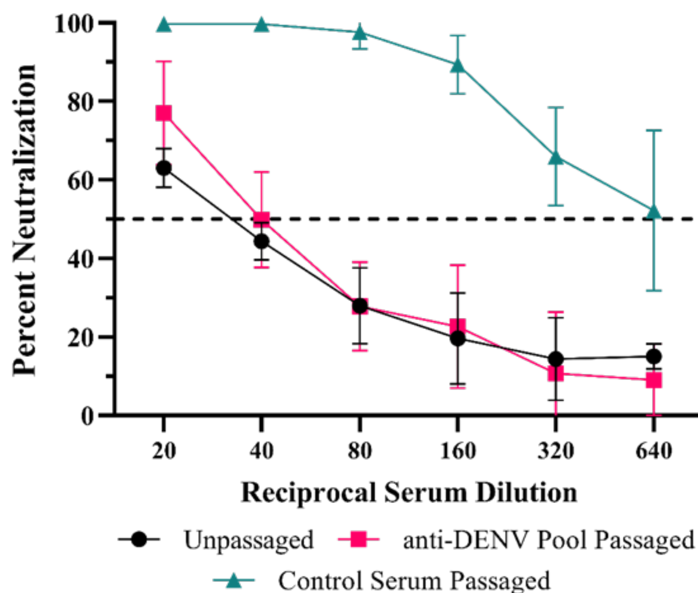
Supplemental Table 2: Identified Variants in the Unpassaged Zika virus (ZIKV) Populations. Mutations within the unpassaged ZIKV population with at least one variant with an allelic frequency ≥ 0.1. If a mutation was observed to occur at an allelic frequency < .1, it is marked with a *. If a mutation was not observed at the limit of detection for LoFreq, the box is grayed out. NS refers to nonstructural proteins. UTR refers to the untranslated region.

nt Position	Protein	Reference	Alternative	AA Change	Allelic Freq. Rep. 1	Depth Rep. 1	Allelic Freq. Rep. 2	Depth Rep. 2	Allelic Freq. Rep. 3	Depth Rep. 3
324	Capsid	G	A	[G/R]	0.368546	6333				
721	Premembrane	A	G	[H/R]	0.997832	13838	0.073241*	3823	0.148524	2168
1435	Envelope	T	C	[V/A]					0.736453	2030
2040	Envelope	G	A	[V/I]			0.920766	3395		
2904	NS1	A	G	[T/A]	0.998622	13788			0.887506	2249
3050	NS1	T	C	[A/A]	0.478473	14563				
3222	NS1	A	G	[K/E]					0.103163	3222
3674	NS2a	T	C	[A/A]			0.926575	3936		
4338	NS2b	A	G	[I/V]	0.4646	13870				
4502	NS2b	C	T	[P/P]	0.454186	13915				
5480	NS3	C	T	[F/F]					0.712617	1712
5680	NS3	C	T	[S/F]					0.108287	1810
6336	NS3	A	G	[S/G]			0.933468	3457		
6373	NS3	A	T	[K/I]			0.916324	4135		
6903	Peptide 2K	T	C	[L/L]			0.919512	4510		
6954	NS4b	A	G	[S/G]	0.998938	18839				
7026	NS4b	T	C	[S/P]	0.470057	23144				
7055	NS4b	A	G	[T/T]					0.146478	3748
7463	NS4b	C	T	[A/A]					0.141935	1550
7754	NS5	G	A	[K/K]					0.31018	2505
7887	NS5	T	C	[Y/H]					0.737383	2239
8897	NS5	A	G	[L/L]	0.564385	11276				
9227	NS5	A	G	[L/L]			0.929367	4219		
10390	3’ UTR	T	C	-					0.745545	1010
10680	3' UTR	C	T	-			0.268034	1414		
10685	3’ UTR	G	C	-	0.113841	2890	0.066581 *	781	0.131336	434
10702	3’ UTR	A	C	-	0.134301	551	0.116564	163	0.122449	98
10703	3’ UTR	T	C	-	0.095794*	428			0.113924	79
10766	3’ UTR	T	C	-	0.163636	55				

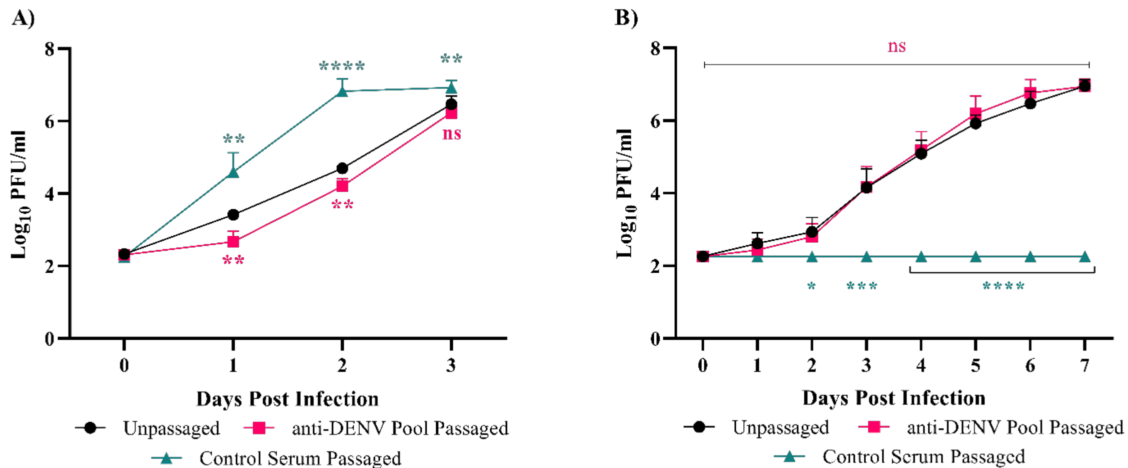
Supplemental Table 3: Identified Variants in the anti-dengue virus (DENV) Pool Passaged Zika virus (ZIKV) Populations. Mutations within the anti-DENV pool passaged ZIKV with at least one variant with an allelic frequency ≥ 0.1. If a mutation was observed to occur at an allelic frequency < .1, it is marked with a *. If a mutation was not observed at the limit of detection for LoFreq, the box is grayed out. NS refers to nonstructural proteins. UTR refers to the untranslated region.

nt Position	Protein	Reference	Alternative	AA Change	Allelic Freq. Rep. 1	Depth Rep. 1	Allelic Freq. Rep. 2	Depth Rep. 2	Allelic Freq. Rep. 3	Depth Rep. 3
674	Premembrane	T	C	[C/C]	0.545297	9813	0.708497	30082	0.856781	30003
798	Premembrane	T	C	[S/P]	0.121016	7404	0.141045	23255	0.085191 *	23277
948	Premembrane	A	G	[M/V]	0.64813	7085	0.787246	23177	0.868867	23724
950	Premembrane	G	T	[M/I]	0.109198	7143	0.035087 *	23342	0.026538 *	23815
2922	NS1	C	A	[L/I]	0.222485	8041	0.082942 *	29201	0.049856 *	30107
3019	NS1	T	C	[L/S]	0.100242	9078	0.130526	32936	0.080527 *	33877
3674	NS2a	T	C	[A/A]	0.546211	9013	0.716048	32759	0.85645	34448
6336	NS3	A	G	[S/G]	0.540782	7062	0.698219	27848	0.854323	27863
6373	NS3	A	T	[K/I]	0.508521	10269	0.684632	38853	0.840522	37140
10702	3' UTR	A	C	-	0.084746 *	708	0.123345	1435	0.125207	1206
10703	3' UTR	T	C	-	0.047934 *	605			0.108559	958
10749	3' UTR	T	C	-	0.119658	117	0.050157 *	319	0.058036 *	224
10766	3' UTR	T	C	-	0.1	60	0.154762	168	0.053435 *	131
10771	3' UTR	G	C	-			0.168539	89	0.191781	73
10773	3' UTR	A	C	-			0.333333	39		

Supplemental Table 4: Identified Variants in the Control Serum Passaged Zika virus (ZIKV) Populations. Mutations within the control serum passaged ZIKV population with at least one variant with an allelic frequency ≥ .1. If a mutation was observed to occur at an allelic frequency < .1, it is marked with a *. If a mutation was not observed at the limit of detection for LoFreq, the box is grayed out. NS refers to nonstructural proteins. UTR refers to the untranslated region.



Supplemental Figure 2: Neutralization Dynamics of Passaged Populations. The anti-dengue virus (DENV) serum pool was serially diluted and mixed with 800 PFU/mL unpassaged WT ZIKV (black line), passage 5 of the anti-DENV pool population (red lines), or passage 5 of the Control Serum-passaged population (green lines). Data are from two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean.



Supplemental Figure 3: Replicative Fitness of Passaged Populations. A) Vero cells were infected at an MOI of 0.01 with unpassaged WT Zika virus (ZIKV; black line), passage 5 of the anti-dengue virus (DENV) pool population (red lines), or passage 5 of the Control Serum-passaged population (green lines). B) Vero cells were infected at an MOI of 0.01 with unpassaged WT Zika virus (ZIKV; black line), passage 5 of the anti-dengue virus (DENV) pool population (red lines), or passage 5 of the Control Serum-passaged population (green lines) after the virus was incubated with the anti-DENV pool for 1 hour. Data are from two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean. Statistics were performed using a two-way ANOVA with a Dunnett's correction for multiple comparisons comparing the passaged viruses to the unpassaged virus (ns - nonsignificant, * - $p = 0.0139$, ** - $p = 0.001$, *** - $p = 0.005$, **** - $p < 0.0001$).

4.8 Author Contributions

Conceptualization, J.W.L; methodology, J.M.M, and J.W.L; cloning, J.M.M; characterization, J.M.M; formal analysis, J.M.M and J.W.L; validation, J.M.M; writing—original draft preparation, J.M.M; writing—review and editing, J.M.M and J.W.L; visualization, J.M.M, and J.W.L. All authors have read and agreed to the published version of the manuscript.

Copyright: The copyright holder for this preprint is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license.

4.9 References

Adams, B., Holmes, E.C., Zhang, C., Mammen, M.P., Nimmannitya, S., Kalayanarooj, S., et al. (2006). Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. *Proceedings of the National Academy of Sciences* 103(38)**,** 14234-14239. doi: 10.1073/pnas.0602768103.

Agerer, B., Koblischke, M., Gudipati, V., Smyth, M., Popa, A., Genger, J.-W., et al. (2020). "SARS-CoV-2 escapes CD8 T cell surveillance via mutations in MHC-I restricted epitopes". Cold Spring Harbor Laboratory).

Aguas, R., Dorigatti, I., Coudeville, L., Luxemburger, C., and Ferguson, N.M. (2019). Cross-serotype interactions and disease outcome prediction of dengue infections in Vietnam. *Scientific Reports* 9(1). doi: 10.1038/s41598-019-45816-6.

Ahlquist, P., Noueiry, A.O., Lee, W.-M., Kushner, D.B., and Dye, B.T. (2003). Host factors in positive-strand RNA virus genome replication. *Journal of virology* 77(15)**,** 8181-8186. doi: 10.1128/jvi.77.15.8181-8186.2003.

Alcon-LePoder, S., Drouet, M.-T., Roux, P., Frenkiel, M.-P., Arborio, M., Durand-Schneider, A.-M., et al. (2005). The Secreted Form of Dengue Virus Nonstructural Protein NS1 Is Endocytosed by Hepatocytes and Accumulates in Late Endosomes: Implications for Viral Infectivity. *Journal of Virology* 79(17)**,** 11403-11411. doi: doi:10.1128/JVI.79.17.11403-11411.2005.

Alejandria, M.M. (2015). Dengue haemorrhagic fever or dengue shock syndrome in children. *BMJ Clin Evid* 2015.

Aliota, M.T., Dudley, D.M., Newman, C.M., Weger-Lucarelli, J., Stewart, L.M., Koenig, M.R., et al. (2018). Molecularly barcoded Zika virus libraries to probe in vivo evolutionary dynamics. *PLoS Pathog* 14(3)**,** e1006964. doi: 10.1371/journal.ppat.1006964.

Almazán, F., González, J.M., Pénzes, Z., Izeta, A., Calvo, E., Plana-Durán, J., et al. (2000). Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proceedings of the National Academy of Sciences* 97(10)**,** 5516-5521. doi: doi:10.1073/pnas.97.10.5516.

Andrade, P., Gimblet-Ochieng, C., Modirian, F., Collins, M., Cárdenas, M., Katzelnick, L.C., et al. (2019). Impact of pre-existing dengue immunity on human antibody and memory B cell responses to Zika. *Nature Communications* 10(1). doi: 10.1038/s41467-019-08845-3.

Andrade, P., Narvekar, P., Montoya, M., Michlmayr, D., Balmaseda, A., Coloma, J., et al. (2020). Primary and secondary dengue virus infections elicit similar memory B cell responses but breadth to other serotypes and cross-reactivity to Zika virus is higher in secondary dengue. *The Journal of Infectious Diseases*. doi: 10.1093/infdis/jiaa120.

Anthony, C., York, T., Bekker, V., Matten, D., Selhorst, P., Ferreria, R.-C., et al. (2017). Cooperation between Strain-Specific and Broadly Neutralizing Responses Limited Viral Escape and Prolonged the Exposure of the Broadly Neutralizing Epitope. *Journal of Virology* 91(18)**,** e00828-00817. doi: 10.1128/jvi.00828-17.

Aryani, A., and Denecke, B. (2015). In vitro application of ribonucleases: comparison of the effects on mRNA and miRNA stability. 8(1). doi: 10.1186/s13104-015-1114-z.

Atieh, T., El Ayoubi, M.D., Aubry, F., Priet, S., De Lamballerie, X., and Nougairède, A. (2018). Haiku: New paradigm for the reverse genetics of emerging RNA viruses. *PLOS ONE* 13(2)**,** e0193069. doi: 10.1371/journal.pone.0193069.

Atieh, T., Nougairède, A., Klitting, R., Aubry, F., Failloux, A.-B., de Lamballerie, X., et al. (2017). New reverse genetics and transfection methods to rescue arboviruses in mosquito cells. *Scientific Reports* 7(1)**,** 13983. doi: 10.1038/s41598-017-14522-6.

Aubry, F., Nougairede, A., De Fabritus, L., Querat, G., Gould, E.A., and De Lamballerie, X. (2014a). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. 95(Pt_11)**,** 2462-2467. doi: 10.1099/vir.0.068023-0.

Aubry, F., Nougairède, A., de Fabritus, L., Querat, G., Gould, E.A., and de Lamballerie, X. (2014b). Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *J Gen Virol* 95(Pt 11)**,** 2462-2467. doi: 10.1099/vir.0.068023-0.

Aubry, F., Nougairède, A., Gould, E.A., and de Lamballerie, X. (2015). Flavivirus reverse genetic systems, construction techniques and applications: a historical perspective. *Antiviral research* 114**,** 67-85. doi: 10.1016/j.antiviral.2014.12.007.

Avirutnan, P., Zhang, L., Punyadee, N., Manuyakorn, A., Puttikhunt, C., Kasinrerk, W., et al. (2007). Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. *PLoS Pathog* 3(11)**,** e183. doi: 10.1371/journal.ppat.0030183.

Ayers, J.B., Xie, X., Coatsworth, H., Stephenson, C.J., Waits, C.M., Shi, P.Y., et al. (2021). Infection Kinetics and Transmissibility of a Reanimated Dengue Virus Serotype 4 Identified Originally in Wild Aedes aegypti From Florida. *Front Microbiol* 12**,** 734903. doi: 10.3389/fmicb.2021.734903.

Bailey, M.J., Broecker, F., Duehr, J., Arumemi, F., Krammer, F., Palese, P., et al. (2019a). Antibodies Elicited by an NS1-Based Vaccine Protect Mice against Zika Virus. *mBio* 10(2). doi: 10.1128/mBio.02861-18.

Bailey, M.J., Broecker, F., Freyn, A.W., Choi, A., Brown, J.A., Fedorova, N., et al. (2019b). Human Monoclonal Antibodies Potently Neutralize Zika Virus and Select for Escape Mutations on the Lateral Ridge of the Envelope Protein. *Journal of Virology* 93(14). doi: 10.1128/jvi.00405-19.

Barba-Spaeth, G., Dejnirattisai, W., Rouvinski, A., Vaney, M.-C., Medits, I., Sharma, A., et al. (2016). Structural basis of potent Zika–dengue virus antibody cross-neutralization. *Nature* 536(7614)**,** 48-53. doi: 10.1038/nature18938.

Bates, T., Chuong, C., Hawks, S.A., Rai, P., Salgado, R.M., Duggal, N.K., et al. (2020). "Development and characterization of infectious clones of two strains of Usutu virus". Cold Spring Harbor Laboratory).

Bates, T.A., Chuong, C., Hawks, S.A., Rai, P., Duggal, N.K., and Weger-Lucarelli, J. (2021). Development and characterization of infectious clones of two strains of Usutu virus. *Virology* 554**,** 28-36. doi: 10.1016/j.virol.2020.12.004.

Baum, A., Fulton, B.O., Wloga, E., Copin, R., Pascal, K.E., Russo, V., et al. (2020). Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science* 369(6506)**,** 1014-1018. doi: doi:10.1126/science.abd0831.

Berg, M.G., Yamaguchi, J., Alessandri-Gradt, E., Tell, R.W., Plantier, J.-C., and Brennan, C.A. (2016). A Pan-HIV Strategy for Complete Genome Sequencing. *Journal of Clinical Microbiology* 54(4)**,** 868-882. doi: 10.1128/jcm.02479-15.

Bhatnagar, J., Rabeneck, D.B., Martines, R.B., Reagan-Steiner, S., Ermias, Y., Estetter, L.B., et al. (2017). Zika Virus RNA Replication and Persistence in Brain and Placental Tissue. *Emerg Infect Dis* 23(3)**,** 405-414. doi: 10.3201/eid2303.161499.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., et al. (2013a). The global distribution and burden of dengue. *Nature* 496(7446)**,** 504-507. doi: 10.1038/nature12060.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., et al. (2013b). The global distribution and burden of dengue. *Nature* 496(7446)**,** 504-507. doi: 10.1038/nature12060.

Biswal, S., Reynales, H., Saez-Llorens, X., Lopez, P., Borja-Tabora, C., Kosalaraksa, P., et al. (2019). Efficacy of a Tetravalent Dengue Vaccine in Healthy Children and Adolescents. *New England Journal of Medicine* 381(21)**,** 2009-2019. doi: 10.1056/nejmoa1903869.

Blanco, L., Bernad, A., Lázaro, J.M., Martin, G., Garmendia, C., and Salas, M. (1989). Highly efficient DNA synthesis by the phage Φ29 DNA polymerase. Symmetrical mode of DNA replication. *The Journal of biological chemistry* 264**,** 8935-8940.

Blish, C.A., Nguyen, M.-A., and Overbaugh, J. (2008). Enhancing Exposure of HIV-1 Neutralization Epitopes through Mutations in gp41. *PLoS Medicine* 5(1)**,** e9. doi: 10.1371/journal.pmed.0050009.

Bogich, T.L., Chunara, R., Scales, D., Chan, E., Pinheiro, L.C., Chmura, A.A., et al. (2012). Preventing Pandemics Via International Development: A Systems Approach. *PLoS Medicine* 9(12)**,** e1001354. doi: 10.1371/journal.pmed.1001354.

Boni, M.F. (2008). Vaccination and antigenic drift in influenza. *Vaccine* 26**,** C8-C14. doi: https://doi.org/10.1016/j.vaccine.2008.04.011.

Borisevich, V., Lee, B., Hickey, A., Debuysscher, B., Broder, C.C., Feldmann, H., et al. (2016). Escape From Monoclonal Antibody Neutralization Affects Henipavirus Fitness In Vitro and In Vivo. 213(3)**,** 448-455. doi: 10.1093/infdis/jiv449.

Bowen, D.G., and Walker, C.M. (2005). Mutational escape from CD8+ T cell immunity. *Journal of Experimental Medicine* 201(11)**,** 1709-1714. doi: 10.1084/jem.20050808.

Brady, O.J., Gething, P.W., Bhatt, S., Messina, J.P., Brownstein, J.S., Hoen, A.G., et al. (2012). Refining the Global Spatial Limits of Dengue Virus Transmission by Evidence-Based Consensus. *PLoS Neglected Tropical Diseases* 6(8)**,** e1760. doi: 10.1371/journal.pntd.0001760.

Braga, J.U., Bressan, C., Dalvi, A.P.R., Calvet, G.A., Daumas, R.P., Rodrigues, N., et al. (2017). Accuracy of Zika virus disease case definition during simultaneous Dengue and Chikungunya epidemics. *PLOS ONE* 12(6)**,** e0179725. doi: 10.1371/journal.pone.0179725.

Brasil, P., Pereira, J.P., Jr., Moreira, M.E., Ribeiro Nogueira, R.M., Damasceno, L., Wakimoto, M., et al. (2016). Zika Virus Infection in Pregnant Women in Rio de Janeiro. *N Engl J Med* 375(24)**,** 2321-2334. doi: 10.1056/NEJMoa1602412.

Brito, A.F., Machado, L.C., Oidtman, R.J., Siconelli, M.J.L., Tran, Q.M., Fauver, J.R., et al. (2021). Lying in wait: the resurgence of dengue virus after the Zika epidemic in Brazil. *Nature Communications* 12(1)**,** 2619. doi: 10.1038/s41467-021-22921-7.

Bushnell, B. (2014). *BBMap: A Fast, Accurate, Splice-Aware Aligner.* ; Lawrence Berkeley National Lab. (LBNL), Berkeley, CA (United States).

Cai, Z., Zhang, C., Chang, K.-S., Jiang, J., Ahn, B.-C., Wakita, T., et al. (2005). Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. *Journal of virology* 79(22)**,** 13963-13973.

Calderón-Peláez, M.A., Velandia-Romero, M.L., Bastidas-Legarda, L.Y., Beltrán, E.O., Camacho-Ortega, S.J., and Castellanos, J.E. (2019). Dengue Virus Infection of Blood-Brain Barrier Cells: Consequences of Severe Disease. *Front Microbiol* 10**,** 1435. doi: 10.3389/fmicb.2019.01435.

Campeau, P., Chapdelaine, P., Seigneurin-Venin, S., Massie, B., and Tremblay, J.P. (2001). Transfection of large plasmids in primary human myoblasts. *Gene Ther* 8(18)**,** 1387-1394. doi: 10.1038/sj.gt.3301532.

Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika Virus Outbreak, Bahia, Brazil. *Emerg Infect Dis* 21(10)**,** 1885-1886. doi: 10.3201/eid2110.150847.

Canale, A.S., Venev, S.V., Whitfield, T.W., Caffrey, D.R., Marasco, W.A., Schiffer, C.A., et al. (2018). Synonymous mutations at the beginning of the influenza A virus hemagglutinin gene impact experimental fitness. *Journal of Molecular Biology*. doi: 10.1016/j.jmb.2018.02.009.

Cao-Lormeau, V.M., Roche, C., Teissier, A., Robin, E., Berry, A.L., Mallet, H.P., et al. (2014). Zika virus, French polynesia, South pacific, 2013. *Emerg Infect Dis* 20(6)**,** 1085-1086. doi: 10.3201/eid2006.140138.

Cao, J., McNevin, J., Malhotra, U., and McElrath, M.J. (2003). Evolution of CD8+T Cell Immunity and Viral Escape Following Acute HIV-1 Infection. *The Journal of Immunology* 171(7)**,** 3837-3846. doi: 10.4049/jimmunol.171.7.3837.

Carlson, C.J., Albery, G.F., Merow, C., Trisos, C.H., Zipfel, C.M., Eskew, E.A., et al. (2022). Climate change increases cross-species viral transmission risk. *Nature* 607(7919)**,** 555-562. doi: 10.1038/s41586-022-04788-w.

Carr, I.M., Robinson, J.I., Dimitriou, R., Markham, A.F., Morgan, A.W., and Bonthron, D.T. (2009). Inferring relative proportions of DNA variants from sequencing electropherograms. *Bioinformatics* 25(24)**,** 3244-3250. doi: 10.1093/bioinformatics/btp583.

Carvalho, M.S., Freitas, L.P., Cruz, O.G., Brasil, P., and Bastos, L.S. (2020). Association of past dengue fever epidemics with the risk of Zika microcephaly at the population level in Brazil. *Scientific Reports* 10(1). doi: 10.1038/s41598-020-58407-7.

Castanha, P.M., Nascimento, E.J.M., Cynthia, B., Cordeiro, M.T., De Carvalho, O.V., De Mendonça, L.R., et al. (2016). Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection. *Journal of Infectious Diseases***,** jiw638. doi: 10.1093/infdis/jiw638.

Castanha, P.M.S., Erdos, G., Watkins, S.C., Falo, L.D., Jr., Marques, E.T.A., and Barratt-Boyes, S.M. (2020). Reciprocal immune enhancement of dengue and Zika virus infection in human skin. *JCI Insight* 5(3). doi: 10.1172/jci.insight.133653.

Chakraborty, C., Saha, A., Sharma, A.R., Bhattacharya, M., Lee, S.-S., and Agoramoorthy, G. (2021). D614G mutation eventuates in all VOI and VOC in SARS-CoV-2: Is it part of the positive selection pioneered by Darwin? *Molecular Therapy - Nucleic Acids* 26**,** 237-241. doi: 10.1016/j.omtn.2021.07.011.

Chang, H.H., Huber, R.G., Bond, P.J., Grad, Y.H., Camerini, D., Maurer-Stroh, S., et al. (2017). Systematic analysis of protein identity between Zika virus and other arthropod-borne viruses. *Bull World Health Organ* 95(7)**,** 517-525i. doi: 10.2471/blt.16.182105.

Chao, L.H., Klein, D.E., Schmidt, A.G., Peña, J.M., and Harrison, S.C. (2014). Sequential conformational rearrangements in flavivirus membrane fusion. *Elife* 3**,** e04389. doi: 10.7554/eLife.04389.

Chen, J., Wang, R., Gilby, N.B., and Wei, G.-W. (2022). Omicron Variant (B.1.1.529): Infectivity, Vaccine Breakthrough, and Antibody Resistance. *Journal of Chemical Information and Modeling* 62(2)**,** 412-422. doi: 10.1021/acs.jcim.1c01451.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., et al. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3(8)**,** 866-871. doi: 10.1038/nm0897-866.

Chuong, C., Bates, T.A., and Weger-Lucarelli, J. (2019). Infectious cDNA clones of two strains of Mayaro virus for studies on viral pathogenesis and vaccine development. *Virology* 535**,** 227-231. doi: https://doi.org/10.1016/j.virol.2019.07.013.

Ciota, A.T. (2019). The role of co-infection and swarm dynamics in arbovirus transmission. *Virus Research* 265**,** 88-93. doi: https://doi.org/10.1016/j.virusres.2019.03.010.

Ciota, A.T., Ehrbar, D.J., Van Slyke, G.A., Willsey, G.G., and Kramer, L.D. (2012). Cooperative interactions in the West Nile virus mutant swarm. *BMC Evol Biol* 12**,** 58. doi: 10.1186/1471-2148-12-58.

Ciurea, A., Hunziker, L., Martinic, M.M.A., Oxenius, A., Hengartner, H., and Zinkernagel, R.M. (2001). CD4+ T-cell–epitope escape mutant virus selected in vivo. *Nature Medicine* 7(7)**,** 795-800. doi: 10.1038/89915.

Cleveland, S.M., Taylor, H.P., and Dimmock, N.J. (1997). Selection of neutralizing antibody escape mutants with type A influenza virus HA-specific polyclonal antisera: possible significance for antigenic drift. *Epidemiology and Infection* 118(2)**,** 149-154. doi: 10.1017/s0950268896007303.

Cockrell, A.S., Beall, A., Yount, B., and Baric, R. (2017). "Efficient Reverse Genetic Systems for Rapid Genetic Manipulation of Emergent and Preemergent Infectious Coronaviruses." Springer New York), 59-81.

Coffey, L.L., Forrester, N., Tsetsarkin, K., Vasilakis, N., and Weaver, S.C. (2013). Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. 8(2)**,** 155-176. doi: 10.2217/fmb.12.139.

Coffey, L.L., and Vignuzzi, M. (2011). Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. *J Virol* 85(2)**,** 1025-1035. doi: 10.1128/jvi.01918-10.

Collette, N.M., Lao, V.H.I., Weilhammer, D.R., Zingg, B., Cohen, S.D., Hwang, M., et al. (2020). Single Amino Acid Mutations Affect Zika Virus Replication In Vitro and Virulence In Vivo. *Viruses* 12(11). doi: 10.3390/v12111295.

Colson, P., La Scola, B., Levasseur, A., Caetano-Anollés, G., and Raoult, D. (2017). Mimivirus: leading the way in the discovery of giant viruses of amoebae. *Nature Reviews Microbiology* 15(4)**,** 243-254. doi: 10.1038/nrmicro.2016.197.

Crook, K.R., Miller-Kittrell, M., Morrison, C.R., and Scholle, F. (2014). Modulation of innate immune signaling by the secreted form of the West Nile virus NS1 glycoprotein. *Virology* 458-459**,** 172-182. doi: https://doi.org/10.1016/j.virol.2014.04.036.

Cuevas, J.M., Domingo-Calap, P., and Sanjuán, R. (2011). The Fitness Effects of Synonymous Mutations in DNA and RNA Viruses. *Molecular Biology and Evolution* 29(1)**,** 17-20. doi: 10.1093/molbev/msr179.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., et al. (2021). Twelve years of SAMtools and BCFtools. *Gigascience* 10(2). doi: 10.1093/gigascience/giab008.

Daniloski, Z., Jordan, T.X., Ilmain, J.K., Guo, X., Bhabha, G., Tenoever, B.R., et al. (2021). The Spike D614G mutation increases SARS-CoV-2 infection of multiple human cell types. *eLife* 10. doi: 10.7554/elife.65365.

de Araújo, T.V.B., Rodrigues, L.C., de Alencar Ximenes, R.A., de Barros Miranda-Filho, D., Montarroyos, U.R., de Melo, A.P.L., et al. (2016). Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. *Lancet Infect Dis* 16(12)**,** 1356-1363. doi: 10.1016/s1473-3099(16)30318-8.

Deardorff, E.R., Fitzpatrick, K.A., Jerzak, G.V.S., Shi, P.-Y., Kramer, L.D., and Ebel, G.D. (2011). West Nile Virus Experimental Evolution in vivo and the Trade-off Hypothesis. *PLoS Pathogens* 7(11)**,** e1002335. doi: 10.1371/journal.ppat.1002335.

Dejnirattisai, W., Supasa, P., Wongwiwat, W., Rouvinski, A., Barba-Spaeth, G., Duangchinda, T., et al. (2016). Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nature Immunology* 17(9)**,** 1102-1108. doi: 10.1038/ni.3515.

Deng, S.Q., Yang, X., Wei, Y., Chen, J.T., Wang, X.J., and Peng, H.J. (2020). A Review on Dengue Vaccine Development. *Vaccines (Basel)* 8(1). doi: 10.3390/vaccines8010063.

Depew, J., Zhou, B., McCorrison, J.M., Wentworth, D.E., Purushe, J., Koroleva, G., et al. (2013). Sequencing viral genomes from a single isolated plaque. 10(1)**,** 181. doi: 10.1186/1743-422x-10-181.

Dick, G.W., Kitchen, S.F., and Haddow, A.J. (1952). Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 46(5)**,** 509-520. doi: 10.1016/0035-9203(52)90042-4.

Domingo, E., and Perales, C. (2019). Viral quasispecies. *PLOS Genetics* 15(10)**,** e1008271. doi: 10.1371/journal.pgen.1008271.

Doud, M.B., Lee, J.M., and Bloom, J.D. (2018). How single mutations affect viral escape from broad and narrow antibodies to H1 influenza hemagglutinin. *Nature Communications* 9(1). doi: 10.1038/s41467-018-03665-3.

Duffy, M.R., Chen, T.-H., Hancock, W.T., Powers, A.M., Kool, J.L., Lanciotti, R.S., et al. (2009). Zika Virus Outbreak on Yap Island, Federated States of Micronesia. *New England Journal of Medicine* 360(24)**,** 2536-2543. doi: 10.1056/NEJMoa0805715.

Dussupt, V., Sankhala, R.S., Gromowski, G.D., Donofrio, G., De La Barrera, R.A., Larocca, R.A., et al. (2020). Potent Zika and dengue cross-neutralizing antibodies induced by Zika vaccination in a dengue-experienced donor. *Nature Medicine* 26(2)**,** 228-235. doi: 10.1038/s41591-019-0746-2.

Edmonds, J., Van Grinsven, E., Prow, N., Bosco-Lauth, A., Brault, A.C., Bowen, R.A., et al. (2013). A Novel Bacterium-Free Method for Generation of Flavivirus Infectious DNA by Circular Polymerase Extension Reaction Allows Accurate Recapitulation of Viral Heterogeneity. *Journal of Virology* 87(4)**,** 2367-2372. doi: 10.1128/jvi.03162-12.

Falgout, B., Pethel, M., Zhang, Y.M., and Lai, C.J. (1991). Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol* 65(5)**,** 2467-2475. doi: 10.1128/jvi.65.5.2467-2475.1991.

Faria, N.R., Azevedo, R., Kraemer, M.U.G., Souza, R., Cunha, M.S., Hill, S.C., et al. (2016). Zika virus in the Americas: Early epidemiological and genetic findings. *Science* 352(6283)**,** 345-349. doi: 10.1126/science.aaf5036.

Ferguson, N.M., Galvani, A.P., and Bush, R.M. (2003). Ecological and immunological determinants of influenza evolution. *Nature* 422(6930)**,** 428-433. doi: 10.1038/nature01509.

Ferraris, P., Cochet, M., Hamel, R., Gladwyn-Ng, I., Alfano, C., Diop, F., et al. (2019). Zika virus differentially infects human neural progenitor cells according to their state of

differentiation and dysregulates neurogenesis through the Notch pathway. *Emerg Microbes Infect* 8(1), 1003-1016. doi: 10.1080/22221751.2019.1637283.

Ferreira, I.A.T.M., Kemp, S.A., Datir, R., Saito, A., Meng, B., Rakshit, P., et al. (2021). SARS-CoV-2 B.1.617 Mutations L452R and E484Q Are Not Synergistic for Antibody Evasion. *The Journal of Infectious Diseases* 224(6), 989-994. doi: 10.1093/infdis/jiab368.

Flipse, J., Diosa-Toro, M.A., Hoornweg, T.E., van de Pol, D.P., Urcuqui-Inchima, S., and Smit, J.M. (2016). Antibody-Dependent Enhancement of Dengue Virus Infection in Primary Human Macrophages; Balancing Higher Fusion against Antiviral Responses. *Sci Rep* 6, 29201. doi: 10.1038/srep29201.

Fontes-Garfias, C.R., Shan, C., Luo, H., Muruato, A.E., Medeiros, D.B.A., Mays, E., et al. (2017). Functional Analysis of Glycosylation of Zika Virus Envelope Protein. *Cell Rep* 21(5), 1180-1190. doi: 10.1016/j.celrep.2017.10.016.

Fowler, A.M., Tang, W.W., Young, M.P., Mamidi, A., Viramontes, K.M., McCauley, M.D., et al. (2018). Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. *Cell Host & Microbe* 24(5), 743-750.e745. doi: 10.1016/j.chom.2018.09.015.

Freitas, D.A., Souza-Santos, R., Carvalho, L.M.A., Barros, W.B., Neves, L.M., Brasil, P., et al. (2020). Congenital Zika syndrome: A systematic review. *PLOS ONE* 15(12), e0242367. doi: 10.1371/journal.pone.0242367.

Furuyama, W., Shifflett, K., Pinski, A.N., Griffin, A.J., Feldmann, F., Okumura, A., et al. (2022). Rapid Protection from COVID-19 in Nonhuman Primates Vaccinated Intramuscularly but Not Intranasally with a Single Dose of a Vesicular Stomatitis Virus-Based Vaccine. *mBio* 13(1), e0337921-e0337921. doi: 10.1128/mbio.03379-21.

Gal-Tanamy, M., Keck, Z.-Y., Yi, M., McKeating, J.A., Patel, A.H., Foung, S.K.H., et al. (2008). In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. *Proceedings of the National Academy of Sciences* 105(49), 19450-19455. doi: 10.1073/pnas.0809879105.

Gallichotte, E.N., Widman, D.G., Yount, B.L., Wahala, W.M., Durbin, A., Whitehead, S., et al. (2015). A New Quaternary Structure Epitope on Dengue Virus Serotype 2 Is the Target of Durable Type-Specific Neutralizing Antibodies. *mBio* 6(5), e01461-01415. doi: 10.1128/mbio.01461-15.

Garmendia, C., Bernad, A., Esteban, J.A., Blanco, L., and Salas, M. (1992). The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. *Journal of Biological Chemistry* 267(4)**,** 2594-2599.

Genoud, V., Stortz, M., Waisman, A., Berardino, B.G., Verneri, P., Dansey, V., et al. (2021). Extraction-free protocol combining proteinase K and heat inactivation for detection of SARS-CoV-2 by RT-qPCR. *PLOS ONE* 16(2)**,** e0247792. doi: 10.1371/journal.pone.0247792.

Glenting, J., and Wessels, S. (2005). Ensuring safety of DNA vaccines. *Microbial cell factories* 4**,** 26-26. doi: 10.1186/1475-2859-4-26.

Goldstein, S.A., Brown, J., Pedersen, B.S., Quinlan, A.R., and Elde, N.C. (2022). Extensive Recombination-driven Coronavirus Diversification Expands the Pool of Potential Pandemic Pathogens. *Genome Biol Evol* 14(12). doi: 10.1093/gbe/evac161.

Govero, J., Esakky, P., Scheaffer, S.M., Fernandez, E., Drury, A., Platt, D.J., et al. (2016). Zika virus infection damages the testes in mice. *Nature* 540(7633)**,** 438-442. doi: 10.1038/nature20556.

Grubaugh, N.D., Fauver, J.R., Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S., Murrieta, R.A., et al. (2017). Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. *Cell Rep* 19(4)**,** 709-718. doi: 10.1016/j.celrep.2017.03.076.

Grubaugh, N.D., Saraf, S., Gangavarapu, K., Watts, A., Tan, A.L., Oidtman, R.J., et al. (2019). Travel Surveillance and Genomics Uncover a Hidden Zika Outbreak during the Waning Epidemic. *Cell* 178(5)**,** 1057-1071.e1011. doi: https://doi.org/10.1016/j.cell.2019.07.018.

Grubaugh, N.D., Weger-Lucarelli, J., Murrieta, R.A., Fauver, J.R., Garcia-Luna, S.M., Prasad, A.N., et al. (2016). Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. *Cell Host Microbe* 19(4)**,** 481-492. doi: 10.1016/j.chom.2016.03.002.

Gruenberg, A., Woo, W.S., Biedrzycka, A., and Wright, P.J. (1988). Partial Nucleotide Sequence and Deduced Amino Acid Sequence of the Structural Proteins of Dengue Virus Type 2, New Guinea C and PUO-218 Strains. *Journal of General Virology* 69(6)**,** 1391-1398. doi: 10.1099/0022-1317-69-6-1391.

Gubler, D.J. (1998). Dengue and Dengue Hemorrhagic Fever. *Clinical Microbiology Reviews* 11(3)**,** 480-496. doi: doi:10.1128/CMR.11.3.480.

Guzmán, M.G., Kourí, G., Valdés, L., Bravo, J., Vázquez, S., and Halstead, S.B. (2002). Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica* 11(4)**,** 223-227. doi: 10.1590/s1020-49892002000400003.

Hackett, B.A., and Cherry, S. (2018). Flavivirus internalization is regulated by a size-dependent endocytic pathway. *Proc Natl Acad Sci U S A* 115(16)**,** 4246-4251. doi: 10.1073/pnas.1720032115.

Hackett, B.A., Yasunaga, A., Panda, D., Tartell, M.A., Hopkins, K.C., Hensley, S.E., et al. (2015). RNASEK is required for internalization of diverse acid-dependent viruses. *Proc Natl Acad Sci U S A* 112(25)**,** 7797-7802. doi: 10.1073/pnas.1424098112.

Hadfield, J., Megill, C., Bell, S.M., Huddleston, J., Potter, B., Callender, C., et al. (2018). Nextstrain: real-time tracking of pathogen evolution. *Bioinformatics* 34(23)**,** 4121-4123. doi: 10.1093/bioinformatics/bty407.

Halstead, S.B., Lan, N.T., Myint, T.T., Shwe, T.N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic fever in infants: research opportunities ignored. *Emerg Infect Dis* 8(12)**,** 1474-1479. doi: 10.3201/eid0812.020170.

Hamel, R., Dejarnac, O., Wichit, S., Ekchariyawat, P., Neyret, A., Luplertlop, N., et al. (2015). Biology of Zika Virus Infection in Human Skin Cells. *Journal of Virology* 89(17)**,** 8880-8896. doi: 10.1128/jvi.00354-15.

Hasebe, T., Narita, K., Hidaka, S., and Su'etsugu, M. (2018). Efficient Arrangement of the Replication Fork Trap for In Vitro Propagation of Monomeric Circular DNA in the Chromosome-Replication Cycle Reaction. *Life (Basel)* 8(4). doi: 10.3390/life8040043.

Haslwanter, D., Dieterle, M.E., Wec, A.Z., O'Brien, C.M., Sakharkar, M., Florez, C., et al. (2021). A Combination of Receptor-Binding Domain and N-Terminal Domain Neutralizing Antibodies Limits the Generation of SARS-CoV-2 Spike Neutralization-Escape Mutants. *mBio* 12(5)**,** e02473-02421. doi: doi:10.1128/mBio.02473-21.

Hotta, S. (1952). Experimental studies on dengue. I. Isolation, identification and modification of the virus. *J Infect Dis* 90(1)**,** 1-9. doi: 10.1093/infdis/90.1.1.

Hu, Y., Zou, S., Wang, Z., Liu, Y., Ren, L., Hao, Y., et al. (2021). Virus Evolution and Neutralization Sensitivity in an HIV-1 Subtype B' Infected Plasma Donor with Broadly Neutralizing Activity. *Vaccines (Basel)* 9(4). doi: 10.3390/vaccines9040311.

Inoue, J., Shigemori, Y., and Mikawa, T. (2006). Improvements of rolling circle amplification (RCA) efficiency and accuracy using Thermus thermophilus SSB mutant protein. 34(9), e69-e69. doi: 10.1093/nar/gkl350.

Isabel, S., Graña-Miraglia, L., Gutierrez, J.M., Bundalovic-Torma, C., Groves, H.E., Isabel, M.R., et al. (2020). Evolutionary and structural analyses of SARS-CoV-2 D614G spike protein mutation now documented worldwide. *Scientific Reports* 10(1). doi: 10.1038/s41598-020-70827-z.

Jerzak, G.V., Brown, I., Shi, P.Y., Kramer, L.D., and Ebel, G.D. (2008). Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. *Virology* 374(2), 256-260. doi: 10.1016/j.virol.2008.02.032.

Jin, J., Nathan, Chen, D.-H., Liao, M., Julie, Raeann, et al. (2015). Neutralizing Monoclonal Antibodies Block Chikungunya Virus Entry and Release by Targeting an Epitope Critical to Viral Pathogenesis. *Cell Reports* 13(11), 2553-2564. doi: 10.1016/j.celrep.2015.11.043.

Joguet, G., Mansuy, J.M., Matusali, G., Hamdi, S., Walschaerts, M., Pavili, L., et al. (2017). Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis* 17(11), 1200-1208. doi: 10.1016/s1473-3099(17)30444-9.

Johansen, I.E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 93(22), 12400-12405. doi: 10.1073/pnas.93.22.12400.

Kang, L., He, G., Sharp, A.K., Wang, X., Brown, A.M., Michalak, P., et al. (2021a). A selective sweep in the Spike gene has driven SARS-CoV-2 human adaptation. *bioRxiv*, 2021.2002.2013.431090. doi: 10.1101/2021.02.13.431090.

Kang, L., He, G., Sharp, A.K., Wang, X., Brown, A.M., Michalak, P., et al. (2021b). A selective sweep in the Spike gene has driven SARS-CoV-2 human adaptation. *Cell* 184(17), 4392-4400.e4394. doi: 10.1016/j.cell.2021.07.007.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* 15(2), 173-190. doi: https://doi.org/10.1046/j.1420-9101.2002.00377.x.

Katzelnick, L.C., Gresh, L., Halloran, M.E., Mercado, J.C., Kuan, G., Gordon, A., et al. (2017). Antibody-dependent enhancement of severe dengue disease in humans. *Science* 358(6365)**,** 929-932. doi: doi:10.1126/science.aan6836.

Katzelnick, L.C., Narvaez, C., Arguello, S., Lopez Mercado, B., Collado, D., Ampie, O., et al. (2020). Zika virus infection enhances future risk of severe dengue disease. *Science* 369(6507)**,** 1123-1128. doi: 10.1126/science.abb6143.

Katzelnick, L.C., Zambrana, J.V., Elizondo, D., Collado, D., Garcia, N., Arguello, S., et al. (2021). Dengue and Zika virus infections in children elicit cross-reactive protective and enhancing antibodies that persist long term. *Sci Transl Med* 13(614)**,** eabg9478. doi: 10.1126/scitranslmed.abg9478.

Keeffe, J.R., Van Rompay, K.K.A., Olsen, P.C., Wang, Q., Gazumyan, A., Azzopardi, S.A., et al. (2018). A Combination of Two Human Monoclonal Antibodies Prevents Zika Virus Escape Mutations in Non-human Primates. *Cell Reports* 25(6)**,** 1385-1394.e1387. doi: 10.1016/j.celrep.2018.10.031.

Kelly, E.P., Puri, B., Sun, W., and Falgout, B. (2010). Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate. *Vaccine* 28(17)**,** 3030-3037.

Keusch, G.T., Amuasi, J.H., Anderson, D.E., Daszak, P., Eckerle, I., Field, H., et al. (2022). Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. *Proceedings of the National Academy of Sciences* 119(42)**,** e2202871119. doi: doi:10.1073/pnas.2202871119.

Khaiboullina, S.F., Uppal, T., Sarkar, R., Gorzalski, A., St Jeor, S., and Verma, S.C. (2017). ZIKV infection regulates inflammasomes pathway for replication in monocytes. *Scientific Reports* 7(1). doi: 10.1038/s41598-017-16072-3.

Khare, B., and Kuhn, R.J. (2022). The Japanese Encephalitis Antigenic Complex Viruses: From Structure to Immunity. *Viruses* 14(10). doi: 10.3390/v14102213.

Kluesner, M.G., Nedveck, D.A., Lahr, W.S., Garbe, J.R., Abrahante, J.E., Webber, B.R., et al. (2018). EditR: A Method to Quantify Base Editing from Sanger Sequencing. *The CRISPR Journal* 1(3)**,** 239-250. doi: 10.1089/crispr.2018.0014.

Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., et al. (2020). Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. *Cell* 182(4), 812-827.e819. doi: 10.1016/j.cell.2020.06.043.

Korunes, K.L., and Samuk, K. (2021). pixy: Unbiased estimation of nucleotide diversity and divergence in the presence of missing data. *Molecular Ecology Resources* 21(4), 1359-1368. doi: https://doi.org/10.1111/1755-0998.13326.

Kreiss, P., Cameron, B., Rangara, R., Mailhe, P., Aguerre-Charriol, O., Airiau, M., et al. (1999). Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic Acids Res* 27(19), 3792-3798. doi: 10.1093/nar/27.19.3792.

Kuchinsky, S.C., Frere, F., Heitzman-Breen, N., Golden, J., Vázquez, A., Honaker, C.F., et al. (2021). Pathogenesis and shedding of Usutu virus in juvenile chickens. *Emerging Microbes & Infections* 10(1), 725-738. doi: 10.1080/22221751.2021.1908850.

Kümmerer, B.M., Grywna, K., Gläsker, S., Wieseler, J., and Drosten, C. (2012). Construction of an infectious Chikungunya virus cDNA clone and stable insertion of mCherry reporter genes at two different sites. *Journal of General Virology* 93(9), 1991-1995. doi: https://doi.org/10.1099/vir.0.043752-0.

Kuo, L., Jaeger, A.S., Banker, E.M., Bialosuknia, S.M., Mathias, N., Payne, A.F., et al. (2020). Reversion to ancestral Zika virus NS1 residues increases competence of Aedes albopictus. *PLoS Pathog* 16(10), e1008951. doi: 10.1371/journal.ppat.1008951.

Lambkin, R., McLain, L., Jones, S.E., Aldridge, S.L., and Dimmock, N.J. (1994). Neutralization escape mutants of type A influenza virus are readily selected by antisera from mice immunized with whole virus: a possible mechanism for antigenic drift. *Journal of General Virology* 75(12), 3493-3502. doi: 10.1099/0022-1317-75-12-3493.

Lee, A.J., Das, S.R., Wang, W., Fitzgerald, T., Pickett, B.E., Aevermann, B.D., et al. (2015). Diversifying Selection Analysis Predicts Antigenic Evolution of 2009 Pandemic H1N1 Influenza A Virus in Humans. *J Virol* 89(10), 5427-5440. doi: 10.1128/jvi.03636-14.

Lee, E., and Lobigs, M. (2000). Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. *J Virol* 74(19), 8867-8875. doi: 10.1128/jvi.74.19.8867-8875.2000.

Lee, J.M., Eguia, R., Zost, S.J., Choudhary, S., Wilson, P.C., Bedford, T., et al. (2019). Mapping person-to-person variation in viral mutations that escape polyclonal serum targeting influenza hemagglutinin. *eLife* 8. doi: 10.7554/elife.49324.

Legendre, M., Arslan, D., Abergel, C., and Claverie, J.-M. (2012). Genomics of Megavirus and the elusive fourth domain of Life. *Communicative & Integrative Biology* 5(1)**,** 102-106. doi: 10.4161/cib.18624.

Lesueur, L.L., Mir, L.M., and André, F.M. (2016). Overcoming the Specific Toxicity of Large Plasmids Electrotransfer in Primary Cells In Vitro. *Molecular Therapy - Nucleic Acids* 5**,** e291. doi: https://doi.org/10.1038/mtna.2016.4.

Leung, J.Y.-S., Ng, M.M.-L., and Chu, J.J.H. (2011). Replication of alphaviruses: a review on the entry process of alphaviruses into cells. *Advances in virology* 2011**,** 249640-249640. doi: 10.1155/2011/249640.

Li, D., Aaskov, J., and Lott, W.B. (2011). Identification of a Cryptic Prokaryotic Promoter within the cDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLoS ONE* 6(3)**,** e18197. doi: 10.1371/journal.pone.0018197.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25(14)**,** 1754-1760. doi: 10.1093/bioinformatics/btp324.

Liu, J., Liu, Y., Shan, C., Nunes, B.T.D., Yun, R., Haller, S.L., et al. (2021a). Role of mutational reversions and fitness restoration in Zika virus spread to the Americas. *Nature Communications* 12(1). doi: 10.1038/s41467-020-20747-3.

Liu, J.L., Webb, E.M., Zabetakis, D., Burke, C.W., Gardner, C.L., Glass, P.J., et al. (2021b). Stabilization of a Broadly Neutralizing Anti-Chikungunya Virus Single Domain Antibody. *Front Med (Lausanne)* 8**,** 626028. doi: 10.3389/fmed.2021.626028.

Liu, Y., Liu, J., Du, S., Shan, C., Nie, K., Zhang, R., et al. (2017). Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. *Nature* 545(7655)**,** 482-486. doi: 10.1038/nature22365.

Lobigs, M., Arthur, C.E., Müllbacher, A., and Blanden, R.V. (1994). The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. *Virology* 202(1)**,** 195-201. doi: 10.1006/viro.1994.1335.

López-Moya, J.J., and García, J.A. (2000). Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res* 68(2)**,** 99-107. doi: 10.1016/s0168-1702(00)00161-1.

Lundstrom, K. (2016). Alphavirus vectors as tools in neuroscience and gene therapy. *Virus Research* 216**,** 16-25. doi: https://doi.org/10.1016/j.virusres.2015.08.015.

MacLean, O.A., Lytras, S., Weaver, S., Singer, J.B., Boni, M.F., Lemey, P., et al. (2021). Natural selection in the evolution of SARS-CoV-2 in bats created a generalist virus and highly capable human pathogen. *PLOS Biology* 19(3)**,** e3001115. doi: 10.1371/journal.pbio.3001115.

MacNamara, F.N. (1954). Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 48(2)**,** 139-145. doi: https://doi.org/10.1016/0035-9203(54)90006-1.

Marani, M., Katul, G.G., Pan, W.K., and Parolari, A.J. (2021). Intensity and frequency of extreme novel epidemics. *Proceedings of the National Academy of Sciences* 118(35)**,** e2105482118. doi: doi:10.1073/pnas.2105482118.

Marano, J., Cereghino, C., Finkielstein, C., and Weger-Lucarelli, J. (2022). "An in vitro workflow to create and modify novel infectious clones using replication cycle reaction". Research Square).

Marano, J.M., Chuong, C., and Weger-Lucarelli, J. (2020). Rolling circle amplification: A high fidelity and efficient alternative to plasmid preparation for the rescue of infectious clones. *Virology* 551**,** 58-63. doi: 10.1016/j.virol.2020.08.016.

Marchi, J., Lässig, M., Walczak, A.M., and Mora, T. (2021). Antigenic waves of virus–immune coevolution. *Proceedings of the National Academy of Sciences* 118(27)**,** e2103398118. doi: 10.1073/pnas.2103398118.

MartíNez-BarragáN, J.D.J.S., and Del Angel, R.M. (2001). Identification of a Putative Coreceptor on Vero Cells That Participates in Dengue 4 Virus Infection. *Journal of Virology* 75(17)**,** 7818-7827. doi: 10.1128/jvi.75.17.7818-7827.2001.

Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., et al. (2020). Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proceedings of the National Academy of Sciences* 117(13)**,** 7001-7003. doi: doi:10.1073/pnas.2002589117.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* 20(9)**,** 1297-1303. doi: 10.1101/gr.107524.110.

Meertens, L., Carnec, X., Lecoin, M.P., Ramdasi, R., Guivel-Benhassine, F., Lew, E., et al. (2012). The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. *Cell Host Microbe* 12(4)**,** 544-557. doi: 10.1016/j.chom.2012.08.009.

Messer, W.B., Yount, B., Hacker, K.E., Donaldson, E.F., Huynh, J.P., de Silva, A.M., et al. (2012). Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS neglected tropical diseases* 6(2)**,** e1486-e1486. doi: 10.1371/journal.pntd.0001486.

Messer, W.B., Yount, B.L., Royal, S.R., De Alwis, R., Widman, D.G., Smith, S.A., et al. (2016). Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *Journal of Virology* 90(10)**,** 5090-5097. doi: 10.1128/jvi.00155-16.

Mire, C.E., Miller, A.D., Carville, A., Westmoreland, S.V., Geisbert, J.B., Mansfield, K.G., et al. (2012). Recombinant vesicular stomatitis virus vaccine vectors expressing filovirus glycoproteins lack neurovirulence in nonhuman primates. *PLoS Negl Trop Dis* 6(3)**,** e1567. doi: 10.1371/journal.pntd.0001567.

Mishin, V.P., Cominelli, F., and Yamshchikov, V.F. (2001). A 'minimal' approach in design of flavivirus infectious DNA. *Virus Research* 81(1)**,** 113-123. doi: https://doi.org/10.1016/S0168-1702(01)00371-9.

Mishra, N., Sharma, S., Dobhal, A., Kumar, S., Chawla, H., Singh, R., et al. (2020). Broadly neutralizing plasma antibodies effective against autologous circulating viruses in infants with multivariant HIV-1 infection. *Nature Communications* 11(1). doi: 10.1038/s41467-020-18225-x.

Mohamed Ali, S., Vega-Rúa, A., Driouich, J.-S., de Lamballerie, X., Failloux, A.-B., and Nougairède, A. (2018). Comparison of chikungunya viruses generated using infectious clone or the Infectious Subgenomic Amplicons (ISA) method in Aedes mosquitoes. *PLOS ONE* 13(6)**,** e0199494. doi: 10.1371/journal.pone.0199494.

Mohsen, M.G., and Kool, E.T. (2016). The Discovery of Rolling Circle Amplification and Rolling Circle Transcription. *Accounts of Chemical Research* 49(11)**,** 2540-2550. doi: 10.1021/acs.accounts.6b00417.

Moore, S.M., Oidtman, R.J., Soda, K.J., Siraj, A.S., Reiner, R.C., Jr., Barker, C.M., et al. (2020). Leveraging multiple data types to estimate the size of the Zika epidemic in the Americas. *PLOS Neglected Tropical Diseases* 14(9)**,** e0008640. doi: 10.1371/journal.pntd.0008640.

Morris, D.H., Petrova, V.N., Rossine, F.W., Parker, E., Grenfell, B.T., Neher, R.A., et al. (2020). Asynchrony between virus diversity and antibody selection limits influenza virus evolution. *eLife* 9**,** e62105. doi: 10.7554/eLife.62105.

Moulton, S.L., Mulligan, J., Srikiatkhachorn, A., Kalayanarooj, S., Grudic, G.Z., Green, S., et al. (2016). State-of-the-art monitoring in treatment of dengue shock syndrome: a case series. *J Med Case Rep* 10(1)**,** 233. doi: 10.1186/s13256-016-1019-z.

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N.L., Khasnatinov, M., et al. (2015). New insights into flavivirus evolution, taxonomy and biogeographic history, extended by analysis of canonical and alternative coding sequences. *PLoS One* 10(2)**,** e0117849. doi: 10.1371/journal.pone.0117849.

Mudaliar, P.P., and Sreekumar, E. (2016). Exploring the instability of reporters expressed under the subgenomic promoter in Chikungunya virus infectious cDNA clones. 45**,** 448. doi: 10.1016/j.ijid.2016.02.950.

Murray, K.O., Gorchakov, R., Carlson, A.R., Berry, R., Lai, L., Natrajan, M., et al. (2017). Prolonged Detection of Zika Virus in Vaginal Secretions and Whole Blood. *Emerg Infect Dis* 23(1)**,** 99-101. doi: 10.3201/eid2301.161394.

Musso, D., Ko, A.I., and Baud, D. (2019). Zika Virus Infection — After the Pandemic. *New England Journal of Medicine* 381(15)**,** 1444-1457. doi: 10.1056/NEJMra1808246.

Nara, S., and Su'Etsugu, M. (2021). In vitro amplification of whole large plasmids via transposon-mediated oriC insertion. *BioTechniques* 71(4)**,** 528-533. doi: 10.2144/btn-2021-0068.

Nelson, C.W., Moncla, L.H., and Hughes, A.L. (2015). SNPGenie: estimating evolutionary parameters to detect natural selection using pooled next-generation sequencing data. *Bioinformatics* 31(22)**,** 3709-3711. doi: 10.1093/bioinformatics/btv449.

Ng, W.C., Soto-Acosta, R., Bradrick, S.S., Garcia-Blanco, M.A., and Ooi, E.E. (2017). The 5' and 3' Untranslated Regions of the Flaviviral Genome. *Viruses* 9(6). doi: 10.3390/v9060137.

Nougairede, A., De Fabritus, L., Aubry, F., Gould, E.A., Holmes, E.C., and De Lamballerie, X. (2013). Random Codon Re-encoding Induces Stable Reduction of Replicative Fitness of Chikungunya Virus in Primate and Mosquito Cells. 9(2)**,** e1003172. doi: 10.1371/journal.ppat.1003172.

Oehler, E., Watrin, L., Larre, P., Leparc-Goffart, I., Lastère, S., Valour, F., et al. (2014). Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. *Eurosurveillance* 19(9)**,** 20720. doi: doi:https://doi.org/10.2807/1560-7917.ES2014.19.9.20720.

Ogando, N.S., Ferron, F., Decroly, E., Canard, B., Posthuma, C.C., and Snijder, E.J. (2019). The Curious Case of the Nidovirus Exoribonuclease: Its Role in RNA Synthesis and Replication Fidelity. *Frontiers in Microbiology* 10. doi: 10.3389/fmicb.2019.01813.

Olsen, B.S., and Johansen, I.E. (2001). Nucleotide sequence and infectious cDNA clone of the L1 isolate of Pea seed-borne mosaic potyvirus. *Arch Virol* 146(1)**,** 15-25. doi: 10.1007/s007050170187.

Organization, W.H. (2020). *WHO COVID-19 Dashboard* [Online]. Geneva: World Health Organization. Available: https://covid19.who.int/ [Accessed 04/07/2022 2022].

Ozaki, H., Govorkova, E.A., Li, C., Xiong, X., Webster, R.G., and Webby, R.J. (2004). Generation of High-Yielding Influenza A Viruses in African Green Monkey Kidney (Vero) Cells by Reverse Genetics. *Journal of Virology* 78(4)**,** 1851-1857. doi: 10.1128/jvi.78.4.1851-1857.2004.

Pandey, U., Yee, R., Shen, L., Judkins, A.R., Bootwalla, M., Ryutov, A., et al. (2020). High Prevalence of SARS-CoV-2 Genetic Variation and D614G Mutation in Pediatric Patients with COVID-19. *Open Forum Infectious Diseases*. doi: 10.1093/ofid/ofaa551.

Parra, B., Lizarazo, J., Jiménez-Arango, J.A., Zea-Vera, A.F., González-Manrique, G., Vargas, J., et al. (2016). Guillain–Barré Syndrome Associated with Zika Virus Infection in Colombia. *New England Journal of Medicine* 375(16)**,** 1513-1523. doi: 10.1056/NEJMoa1605564.

Pedroso, C., Fischer, C., Feldmann, M., Sarno, M., Luz, E., Moreira-Soto, A., et al. (2019). Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil. *Emerg Infect Dis* 25(8)**,** 1485-1493. doi: 10.3201/eid2508.190113.

Perez, F., Llau, A., Gutierrez, G., Bezerra, H., Coelho, G., Ault, S., et al. (2019). The decline of dengue in the Americas in 2017: discussion of multiple hypotheses. *Trop Med Int Health* 24(4)**,** 442-453. doi: 10.1111/tmi.13200.

Perreira, J.M., Aker, A.M., Savidis, G., Chin, C.R., McDougall, W.M., Portmann, J.M., et al. (2015). RNASEK Is a V-ATPase-Associated Factor Required for Endocytosis and the Replication of Rhinovirus, Influenza A Virus, and Dengue Virus. *Cell Rep* 12(5)**,** 850-863. doi: 10.1016/j.celrep.2015.06.076.

Petersen, L.R., Jamieson, D.J., Powers, A.M., and Honein, M.A. (2016). Zika Virus. *New England Journal of Medicine* 374(16)**,** 1552-1563. doi: 10.1056/NEJMra1602113.

Pierro, D.J., Salazar, M.I., Beaty, B.J., and Olson, K.E. (2006). Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation. *Journal of general virology* 87(8)**,** 2263-2268.

Pierson, T.C., and Diamond, M.S. (2020). The continued threat of emerging flaviviruses. *Nature Microbiology* 5(6)**,** 796-812. doi: 10.1038/s41564-020-0714-0.

Plante, J.A., Liu, Y., Liu, J., Xia, H., Johnson, B.A., Lokugamage, K.G., et al. (2021). Spike mutation D614G alters SARS-CoV-2 fitness. *Nature* 592(7852)**,** 116-121. doi: 10.1038/s41586-020-2895-3.

Plennevaux, E., Moureau, A., Arredondo-García, J.L., Villar, L., Pitisuttithum, P., Tran, N.H., et al. (2017). Impact of Dengue Vaccination on Serological Diagnosis: Insights From Phase III Dengue Vaccine Efficacy Trials. *Clinical Infectious Diseases*. doi: 10.1093/cid/cix966.

Polo, S., Ketner, G., Levis, R., and Falgout, B. (1997). Infectious RNA transcripts from full-length dengue virus type 2 cDNA clones made in yeast. *Journal of virology* 71(7)**,** 5366-5374.

Postnikova, Y., Treshchalina, A., Boravleva, E., Gambaryan, A., Ishmukhametov, A., Matrosovich, M., et al. (2021). Diversity and Reassortment Rate of Influenza A Viruses in Wild Ducks and Gulls. *Viruses* 13(6). doi: 10.3390/v13061010.

Pu, S.-Y., Wu, R.-H., Yang, C.-C., Jao, T.-M., Tsai, M.-H., Wang, J.-C., et al. (2011a). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *Journal of Virology* 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., et al. (2011b). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Puerta-Guardo, H., Biering, S.B., de Sousa, F.T.G., Shu, J., Glasner, D.R., Li, J., et al. (2022). Flavivirus NS1 Triggers Tissue-Specific Disassembly of Intercellular Junctions Leading to Barrier Dysfunction and Vascular Leak in a GSK-3β-Dependent Manner. *Pathogens* 11(6). doi: 10.3390/pathogens11060615.

Puerta-Guardo, H., Glasner, D.R., Espinosa, D.A., Biering, S.B., Patana, M., Ratnasiri, K., et al. (2019). Flavivirus NS1 Triggers Tissue-Specific Vascular Endothelial Dysfunction Reflecting Disease Tropism. *Cell Reports* 26(6)**,** 1598-1613.e1598. doi: 10.1016/j.celrep.2019.01.036.

Puri, B., Polo, S., Hayes, C.G., and Falgout, B. (2000). Construction of a full length infectious clone for dengue-1 virus Western Pacific, 74 strain. *Virus genes* 20(1)**,** 57-63.

Rasmussen, S.A., Jamieson, D.J., Honein, M.A., and Petersen, L.R. (2016). Zika virus and birth defects—reviewing the evidence for causality. *New England Journal of Medicine* 374(20)**,** 1981-1987.

Rastogi, M., Sharma, N., and Singh, S.K. (2016). Flavivirus NS1: a multifaceted enigmatic viral protein. *Virol J* 13**,** 131. doi: 10.1186/s12985-016-0590-7.

Rathore, A.P.S., Saron, W.A.A., Lim, T., Jahan, N., and St. John, A.L. (2019). Maternal immunity and antibodies to dengue virus promote infection and Zika virus–induced microcephaly in fetuses. *Science Advances* 5(2)**,** eaav3208. doi: 10.1126/sciadv.aav3208.

Rathore, A.P.S., and St. John, A.L. (2020). Cross-Reactive Immunity Among Flaviviruses. *Frontiers in Immunology* 11. doi: 10.3389/fimmu.2020.00334.

Reefhuis, J., Gilboa, S.M., Johansson, M.A., Valencia, D., Simeone, R.M., Hills, S.L., et al. (2016). Projecting Month of Birth for At-Risk Infants after Zika Virus Disease Outbreaks. *Emerg Infect Dis* 22(5)**,** 828-832. doi: 10.3201/eid2205.160290.

Regla-Nava, J.A., Elong Ngono, A., Viramontes, K.M., Huynh, A.-T., Wang, Y.-T., Nguyen, A.-V.T., et al. (2018). Cross-reactive Dengue virus-specific CD8+ T cells protect against Zika virus during pregnancy. *Nature Communications* 9(1). doi: 10.1038/s41467-018-05458-0.

Regla-Nava, J.A., Wang, Y.-T., Fontes-Garfias, C.R., Liu, Y., Syed, T., Susantono, M., et al. (2022). A Zika virus mutation enhances transmission potential and confers escape from protective dengue virus immunity. *Cell Reports* 39(2)**,** 110655. doi: 10.1016/j.celrep.2022.110655.

Reich, N.G., Shrestha, S., King, A.A., Rohani, P., Lessler, J., Kalayanarooj, S., et al. (2013). Interactions between serotypes of dengue highlight epidemiological impact of cross-immunity. *J R Soc Interface* 10(86)**,** 20130414. doi: 10.1098/rsif.2013.0414.

Reid, D.W., Campos, R.K., Child, J.R., Zheng, T., Chan, K.W.K., Bradrick, S.S., et al. (2018). Dengue Virus Selectively Annexes Endoplasmic Reticulum-Associated Translation Machinery as a Strategy for Co-opting Host Cell Protein Synthesis. *J Virol* 92(7). doi: 10.1128/jvi.01766-17.

Renner, M., Dejnirattisai, W., Carrique, L., Martin, I.S., Karia, D., Ilca, S.L., et al. (2021). Flavivirus maturation leads to the formation of an occupied lipid pocket in the surface glycoproteins. *Nature Communications* 12(1)**,** 1238. doi: 10.1038/s41467-021-21505-9.

Rice, C.M., Grakoui, A., Galler, R., and Chambers, T.J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biol* 1(3)**,** 285-296.

Rihn, S.J., Merits, A., Bakshi, S., Turnbull, M.L., Wickenhagen, A., Alexander, A.J.T., et al. (2021). A plasmid DNA-launched SARS-CoV-2 reverse genetics system and coronavirus toolkit for COVID-19 research. *PLOS Biology* 19(2)**,** e3001091. doi: 10.1371/journal.pbio.3001091.

Rockx, B., Donaldson, E., Frieman, M., Sheahan, T., Corti, D., Lanzavecchia, A., et al. (2010). Escape from human monoclonal antibody neutralization affects in vitro and in vivo fitness of severe acute respiratory syndrome coronavirus. *J Infect Dis* 201(6)**,** 946-955. doi: 10.1086/651022.

Rodgers, M.A., Wilkerson, E., Vallari, A., McArthur, C., Sthreshley, L., Brennan, C.A., et al. (2017). Sensitive next generation sequencing method reveals deep genetic diversity of

HIV-1 in the Democratic Republic of the Congo. JVI.01841-01816. doi: 10.1128/jvi.01841-16.

Rodriguez-Barraquer, I., Costa, F., Nascimento, E.J.M., Nery, N.J., Castanha, P.M.S., Sacramento, G.A., et al. (2019). Impact of preexisting dengue immunity on Zika virus emergence in a dengue endemic region. *Science* 363(6427)**,** 607-610. doi: 10.1126/science.aav6618.

Roesch, F., Cereghino, C., Carrau, L., Hardy, A., Ribeiro-Filho, H., Lacritick, A.H., et al. (2022). The E2 glycoprotein holds key residues for Mayaro virus adaptation to the urban *Aedes aegypti* mosquito. *bioRxiv***,** 2022.2004.2005.487100. doi: 10.1101/2022.04.05.487100.

Rückert, C., Weger-Lucarelli, J., Garcia-Luna, S.M., Young, M.C., Byas, A.D., Murrieta, R.A., et al. (2017). Impact of simultaneous exposure to arboviruses on infection and transmission by Aedes aegypti mosquitoes. *Nature Communications* 8(1)**,** 15412. doi: 10.1038/ncomms15412.

Sabin, A.B. (1950). The dengue group of viruses and its family relationships. *Bacteriol Rev* 14(3)**,** 225-232.

Sabin, A.B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg* 1(1)**,** 30-50. doi: 10.4269/ajtmh.1952.1.30.

Saito, A., Irie, T., Suzuki, R., Maemura, T., Nasser, H., Uriu, K., et al. (2022). Enhanced fusogenicity and pathogenicity of SARS-CoV-2 Delta P681R mutation. *Nature* 602(7896)**,** 300-306. doi: 10.1038/s41586-021-04266-9.

Sanjuán, R., and Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences* 73(23)**,** 4433-4448. doi: 10.1007/s00018-016-2299-6.

Santos, J.J.d.S., Cordeiro, M.T., Bertani, G.R., Marques, E.T.d.A., and Gil, L.H.V.G. (2013). Construction and characterisation of a complete reverse genetics system of dengue virus type 3. *Memórias do Instituto Oswaldo Cruz* 108**,** 983-991.

Sapkal, G.N., Harini, S., Ayachit, V.M., Fulmali, P.V., Mahamuni, S.A., Bondre, V.P., et al. (2011). Neutralization escape variant of West Nile virus associated with altered peripheral pathogenicity and differential cytokine profile. *Virus Res* 158(1-2)**,** 130-139. doi: 10.1016/j.virusres.2011.03.023.

Sasmono, R.T., Taurel, A.-F., Prayitno, A., Sitompul, H., Yohan, B., Hayati, R.F., et al. (2018). Dengue virus serotype distribution based on serological evidence in pediatric urban

population in Indonesia. *PLOS Neglected Tropical Diseases* 12(6), e0006616. doi: 10.1371/journal.pntd.0006616.

Scobey, T., Yount, B.L., Sims, A.C., Donaldson, E.F., Agnihothram, S.S., Menachery, V.D., et al. (2013). Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proceedings of the National Academy of Sciences* 110(40), 16157-16162. doi: 10.1073/pnas.1311542110.

Shahrizaila, N., Lehmann, H.C., and Kuwabara, S. (2021). Guillain-Barré syndrome. *The Lancet* 397(10280), 1214-1228. doi: https://doi.org/10.1016/S0140-6736(21)00517-1.

Shan, C., Xia, H., Haller, S.L., Azar, S.R., Liu, Y., Liu, J., et al. (2020). A Zika virus envelope mutation preceding the 2015 epidemic enhances virulence and fitness for transmission. *Proc Natl Acad Sci U S A* 117(33), 20190-20197. doi: 10.1073/pnas.2005722117.

Sharp, T.M., Fischer, M., Muñoz-Jordán, J.L., Paz-Bailey, G., Staples, J.E., Gregory, C.J., et al. (2019). Dengue and Zika Virus Diagnostic Testing for Patients with a Clinically Compatible Illness and Risk for Infection with Both Viruses. *MMWR. Recommendations and Reports* 68(1), 1-10. doi: 10.15585/mmwr.rr6801a1.

Simmons, C.P., Farrar, J.J., van Vinh Chau, N., and Wills, B. (2012). Dengue. *New England Journal of Medicine* 366(15), 1423-1432. doi: 10.1056/NEJMra1110265.

Singh, A., Koutsoumpli, G., van de Wall, S., and Daemen, T. (2019). An alphavirus-based therapeutic cancer vaccine: from design to clinical trial. *Cancer Immunology, Immunotherapy* 68(5), 849-859. doi: 10.1007/s00262-018-2276-z.

Siridechadilok, B., Gomutsukhavadee, M., Sawaengpol, T., Sangiambut, S., Puttikhunt, C., Chin-inmanu, K., et al. (2013). A Simplified Positive-Sense-RNA Virus Construction Approach That Enhances Analysis Throughput. *Journal of Virology* 87(23), 12667-12674. doi: doi:10.1128/JVI.02261-13.

Søndergaard, J.N., Geng, K., Sommerauer, C., Atanasoai, I., Yin, X., and Kutter, C. (2020). Successful delivery of large-size CRISPR/Cas9 vectors in hard-to-transfect human cells using small plasmids. *Communications Biology* 3(1). doi: 10.1038/s42003-020-1045-7.

Steel, J.J., Henderson, B.R., Lama, S.B., Olson, K.E., and Geiss, B.J. (2011). Infectious alphavirus production from a simple plasmid transfection+. 8(1), 356. doi: 10.1186/1743-422x-8-356.

Su'Etsugu, M., Takada, H., Katayama, T., and Tsujimoto, H. (2017). Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. *Nucleic Acids Research* 45(20), 11525-11534. doi: 10.1093/nar/gkx822.

Sui, J., Aird, D.R., Tamin, A., Murakami, A., Yan, M., Yammanuru, A., et al. (2008). Broadening of Neutralization Activity to Directly Block a Dominant Antibody-Driven SARS-Coronavirus Evolution Pathway. *PLoS Pathogens* 4(11), e1000197. doi: 10.1371/journal.ppat.1000197.

Suzuki, R., de Borba, L., Duarte dos Santos, C.N., and Mason, P.W. (2007). Construction of an infectious cDNA clone for a Brazilian prototype strain of dengue virus type 1: Characterization of a temperature-sensitive mutation in NS1. *Virology* 362(2), 374-383. doi: https://doi.org/10.1016/j.virol.2006.11.026.

Talavera-Aguilar, L.G., Murrieta, R.A., Kiem, S., Cetina-Trejo, R.C., Baak-Baak, C.M., Ebel, G.D., et al. (2021). Infection, dissemination, and transmission efficiencies of Zika virus in Aedes aegypti after serial passage in mosquito or mammalian cell lines or alternating passage in both cell types. *Parasites & Vectors* 14(1). doi: 10.1186/s13071-021-04726-1.

Tan, W., Lu, Y., Zhang, J., Wang, J., Dan, Y., Tan, Z., et al. (2020). Viral kinetics and antibody responses in patients with COVID-19. *MedRxiv*.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., et al. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* 197(7), 823-829. doi: 10.1084/jem.20021840.

Thi Nhu Thao, T., Labroussaa, F., Ebert, N., V'Kovski, P., Stalder, H., Portmann, J., et al. (2020). Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform. *Nature* 582(7813), 561-565. doi: 10.1038/s41586-020-2294-9.

Thomas, S.J., and Yoon, I.-K. (2019). A review of Dengvaxia®: development to deployment. *Human Vaccines & Immunotherapeutics* 15(10), 2295-2314. doi: 10.1080/21645515.2019.1658503.

Tobar, P., Vega, M., Ordoñez, C., Rivera, L., Landivar, J., and Zambrano, H. (2018). Detection of Zika Virus and Human Papilloma Virus in Cervical Cytology Samples using Two Real Time PCR Based Techniques in Ecuadorian Women diagnosed with ASCUS. *P R Health Sci J* 37(Spec Issue), S96-s98.

Toh, Y.X., Gan, V., Balakrishnan, T., Zuest, R., Poidinger, M., Wilson, S., et al. (2014). Dengue serotype cross-reactive, anti-e protein antibodies confound specific immune memory for 1 year after infection. *Front Immunol* 5**,** 388. doi: 10.3389/fimmu.2014.00388.

Torii, S., Ono, C., Suzuki, R., Morioka, Y., Anzai, I., Fauzyah, Y., et al. (2021). Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Reports* 35(3)**,** 109014. doi: https://doi.org/10.1016/j.celrep.2021.109014.

Torres-Flores, J.M., Reyes-Sandoval, A., and Salazar, M.I. (2022). Dengue Vaccines: An Update. *BioDrugs* 36(3)**,** 325-336. doi: 10.1007/s40259-022-00531-z.

Tully, D., and Griffiths, C.L. (2021). Dengvaxia: the world's first vaccine for prevention of secondary dengue. *Therapeutic Advances in Vaccines and Immunotherapy* 9**,** 251513552110158. doi: 10.1177/25151355211015839.

Tuo, D., Shen, W., Yan, P., Li, X., and Zhou, P. (2015). Rapid Construction of Stable Infectious Full-Length cDNA Clone of Papaya Leaf Distortion Mosaic Virus Using In-Fusion Cloning. *Viruses* 7(12)**,** 6241-6250. doi: 10.3390/v7122935.

Ueno, H., Sawada, H., Soga, N., Sano, M., Nara, S., Tabata, K.V., et al. (2021). Amplification of over 100 kbp DNA from Single Template Molecules in Femtoliter Droplets. *ACS Synthetic Biology* 10(9)**,** 2179-2186. doi: 10.1021/acssynbio.0c00584.

Ulper, L., Sarand, I., Rausalu, K., and Merits, A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of virological methods* 148(1-2)**,** 265-270. doi: 10.1016/j.jviromet.2007.10.007.

Usme-Ciro, J.A., Lopera, J.A., Enjuanes, L., Almazán, F., and Gallego-Gomez, J.C. (2014). Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome. *Virus Research* 180**,** 12-22. doi: https://doi.org/10.1016/j.virusres.2013.12.001.

van der Most, R.G., Corver, J., and Strauss, J.H. (1999). Mutagenesis of the RGD Motif in the Yellow Fever Virus 17D Envelope Protein. *Virology* 265(1)**,** 83-95. doi: https://doi.org/10.1006/viro.1999.0026.

Vatti, A., Monsalve, D.M., Pacheco, Y., Chang, C., Anaya, J.M., and Gershwin, M.E. (2017). Original antigenic sin: A comprehensive review. *J Autoimmun* 83**,** 12-21. doi: 10.1016/j.jaut.2017.04.008.

Vaughan, K., Greenbaum, J., Blythe, M., Peters, B., and Sette, A. (2010). Meta-analysis of All Immune Epitope Data in theFlavivirusGenus: Inventory of Current Immune Epitope Data Status in the Context of Virus Immunity and Immunopathology. *Viral Immunology* 23(3)**,** 259-284. doi: 10.1089/vim.2010.0006.

Vogt, M.B., McDonald, E.M., Delorey, M., Mead, P.S., Hook, S.A., Hinckley, A.F., et al. (2022). Prolonged shedding of Zika virus in human semen is associated with male reproductive tract inflammation. *J Infect Dis*. doi: 10.1093/infdis/jiac329.

Wang, J., Huang, Y., Zhou, M., Hardwidge, P.R., and Zhu, G. (2016). Construction and sequencing of an infectious clone of the goose embryo-adapted Muscovy duck parvovirus vaccine strain FZ91-30. *Virology Journal* 13(1). doi: 10.1186/s12985-016-0564-9.

Wearing, H.J., and Rohani, P. (2006). Ecological and immunological determinants of dengue epidemics. *Proceedings of the National Academy of Sciences* 103(31)**,** 11802-11807. doi: 10.1073/pnas.0602960103.

Weger-Lucarelli, J., Auerswald, H., Vignuzzi, M., Dussart, P., and Karlsson, E.A. (2018a). Taking a bite out of nutrition and arbovirus infection. *PLOS Neglected Tropical Diseases* 12(3)**,** e0006247. doi: 10.1371/journal.pntd.0006247.

Weger-Lucarelli, J., Duggal, N.K., Bullard-Feibelman, K., Veselinovic, M., Romo, H., Nguyen, C., et al. (2017). Development and Characterization of Recombinant Virus Generated from a New World Zika Virus Infectious Clone. *Journal of Virology* 91(1)**,** JVI.01765-01716. doi: 10.1128/jvi.01765-16.

Weger-Lucarelli, J., Garcia, S.M., Rückert, C., Byas, A., O’Connor, S.L., Aliota, M.T., et al. (2018b). Using barcoded Zika virus to assess virus population structure in vitro and in Aedes aegypti mosquitoes. *Virology* 521**,** 138-148. doi: 10.1016/j.virol.2018.06.004.

Weger-Lucarelli, J., Rückert, C., Chotiwan, N., Nguyen, C., Garcia Luna, S.M., Fauver, J.R., et al. (2016). Vector Competence of American Mosquitoes for Three Strains of Zika Virus. *PLOS Neglected Tropical Diseases* 10(10)**,** e0005101. doi: 10.1371/journal.pntd.0005101.

Willett, B.J., Grove, J., MacLean, O.A., Wilkie, C., De Lorenzo, G., Furnon, W., et al. (2022). SARS-CoV-2 Omicron is an immune escape variant with an altered cell entry pathway. *Nature Microbiology* 7(8)**,** 1161-1179. doi: 10.1038/s41564-022-01143-7.

Wilm, A., Aw, P.P., Bertrand, D., Yeo, G.H., Ong, S.H., Wong, C.H., et al. (2012). LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. *Nucleic Acids Res* 40(22), 11189-11201. doi: 10.1093/nar/gks918.

Wilson, D.S., and Yoshimura, J. (1994). On the Coexistence of Specialists and Generalists. *The American Naturalist* 144(4), 692-707. doi: 10.1086/285702.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579(7798), 265-269. doi: 10.1038/s41586-020-2008-3.

Xia, H., Luo, H., Shan, C., Muruato, A.E., Nunes, B.T.D., Medeiros, D.B.A., et al. (2018). An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. *Nat Commun* 9(1), 414. doi: 10.1038/s41467-017-02816-2.

Xie, X., Muruato, A., Lokugamage, K.G., Narayanan, K., Zhang, X., Zou, J., et al. (2020). An Infectious cDNA Clone of SARS-CoV-2. *Cell Host & Microbe* 27(5), 841-848.e843. doi: 10.1016/j.chom.2020.04.004.

Xiong, D., Zhao, X., Luo, S., Cong, Y., Zhang, J.Z.H., and Duan, L. (2022). Immune Escape Mechanisms of SARS-CoV-2 Delta and Omicron Variants against Two Monoclonal Antibodies That Received Emergency Use Authorization. *The Journal of Physical Chemistry Letters* 13(26), 6064-6073. doi: 10.1021/acs.jpclett.2c00912.

Yadav, P.D., Malhotra, B., Sapkal, G., Nyayanit, D.A., Deshpande, G., Gupta, N., et al. (2019). Zika virus outbreak in Rajasthan, India in 2018 was caused by a virus endemic to Asia. *Infect Genet Evol* 69, 199-202. doi: 10.1016/j.meegid.2019.01.026.

Yakob, L. (2022). Zika Virus after the Public Health Emergency of International Concern Period, Brazil. *Emerg Infect Dis* 28(4), 837-840. doi: 10.3201/eid2804.211949.

Yamshchikov, V., Mishin, V., and Cominelli, F. (2001). A New Strategy in Design of (+)RNA Virus Infectious Clones Enabling Their Stable Propagation in E. coli. *Virology* 281(2), 272-280. doi: 10.1006/viro.2000.0793.

Yang, T.-J., Yu, P.-Y., Chang, Y.-C., and Hsu, S.-T.D. (2021). D614G mutation in the SARS-CoV-2 spike protein enhances viral fitness by desensitizing it to temperature-dependent denaturation. *Journal of Biological Chemistry* 297(4), 101238. doi: 10.1016/j.jbc.2021.101238.

Yu, I.-M., Zhang, W., Holdaway, H.A., Li, L., Kostyuchenko, V.A., Chipman, P.R., et al. (2008). Structure of the Immature Dengue Virus at Low pH Primes Proteolytic Maturation. *Science* 319(5871)**,** 1834-1837. doi: doi:10.1126/science.1153264.

Yu, I.M., Holdaway, H.A., Chipman, P.R., Kuhn, R.J., Rossmann, M.G., and Chen, J. (2009). Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. *J Virol* 83(23)**,** 12101-12107. doi: 10.1128/jvi.01637-09.

Yuan, L., Huang, X.-Y., Liu, Z.-Y., Zhang, F., Zhu, X.-L., Yu, J.-Y., et al. (2017). A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science* 358(6365)**,** 933-936. doi: 10.1126/science.aam7120.

Yun, S.-I., Kim, S.-Y., Rice, C.M., and Lee, Y.-M. (2003). Development and application of a reverse genetics system for Japanese encephalitis virus. *Journal of virology* 77(11)**,** 6450-6465.

Zhang, L., Jackson, C.B., Mou, H., Ojha, A., Peng, H., Quinlan, B.D., et al. (2020). SARS-CoV-2 spike-protein D614G mutation increases virion spike density and infectivity. *Nature Communications* 11(1). doi: 10.1038/s41467-020-19808-4.

Zhao, X., Chen, F.-P., and Sullender, W.M. (2004). Respiratory syncytial virus escape mutant derived in vitro resists palivizumab prophylaxis in cotton rats. *Virology* 318(2)**,** 608-612. doi: https://doi.org/10.1016/j.virol.2003.10.018.

Zhao, X., Liu, E., Chen, F.-P., and Sullender, W.M. (2006). In Vitro and In Vivo Fitness of Respiratory Syncytial Virus Monoclonal Antibody Escape Mutants. *Journal of Virology* 80(23)**,** 11651-11657. doi: 10.1128/jvi.01387-06.

Zimmerman, M.G., Quicke, K.M., O'Neal, J.T., Arora, N., Machiah, D., Priyamvada, L., et al. (2018). Cross-Reactive Dengue Virus Antibodies Augment Zika Virus Infection of Human Placental Macrophages. *Cell Host & Microbe* 24(5)**,** 731-742.e736. doi: 10.1016/j.chom.2018.10.008.

Zompi, S., Santich, B.H., Beatty, P.R., and Harris, E. (2012). Protection from Secondary Dengue Virus Infection in a Mouse Model Reveals the Role of Serotype Cross-Reactive B and T Cells. *The Journal of Immunology* 188(1)**,** 404-416. doi: 10.4049/jimmunol.1102124.

Chapter Five: Conclusion

5.1 Conclusion

Emerging arthropod-borne viruses represent a significant threat to human health. Over the last two decades, we have seen at least 17 arbovirus outbreaks (Keusch et al., 2022). One important genus of arboviruses is *flavivirus* (Ciota, 2019). Flaviviruses, of the family *Flaviviridae,* cause febrile disease and can be grouped by symptomology and vectors (Moureau et al., 2015). Viruses vectored by *Culex* ssp. mosquitoes, such as JEV, WNV, SLEV, and USUV (Moureau et al., 2015), tend to present with encephalitis (Khare and Kuhn, 2022). Viruses vectored by *Aedes* ssp. mosquitoes, such as YFV, DENV, and ZIKV (Moureau et al., 2015), generally present with hemorrhagic disease (Pierson and Diamond, 2020).

The drivers for these viruses to emerge or re-emerge in different regions can be split between socio-ecological factors, such as high biodiversity regions with depleted habitats (Keusch et al., 2022), increased human-animal interactions (Keusch et al., 2022), and global warming (Carlson et al., 2022), and molecular factors, such as a high mutation rate (Sanjuán and Domingo-Calap, 2016). One driver which has been understudied in flavivirus evolution is pre-existing cross-reactive immunity, despite it being extensively characterized for other viruses (Lambkin et al., 1994; Cleveland et al., 1997; Ferguson et al., 2003; Rockx et al., 2010; Sapkal et al., 2011; Jin et al., 2015; Doud et al., 2018; Lee et al., 2019). Cross-reactive immune selection involves a subpopulation of viruses surviving neutralization (Marchi et al., 2021) and becoming the founder for subsequent generations (Coffey et al., 2013; Morris et al., 2020).

The hypothesis that immune-driven evolution of ZIKV occurs, specifically by pre-existing DENV immunity, is well founded. First, with nearly five percent (~400 million people) of the world's population infected with DENV each year (Bhatt et al., 2013) and with reports of co-circulations between DENV and ZIKV (Patterson et al., 2016), there is ample opportunity for ZIKV to infect a DENV-immune patient. Moreover, a large body of evidence suggests that pre-existing immunity to either DENV or ZIKV can protect from (Reich et al., 2013; Andrade et al., 2019; Rodriguez-Barraquer et al., 2019; Andrade et al., 2020; Katzelnick et al., 2021) or enhance (Halstead and O'Rourke, 1977; Guzmán et al., 2002; Halstead et al., 2002; Castanha et al., 2016; Katzelnick et al., 2017; Fowler et al., 2018; Zimmerman et al., 2018; Rathore et al., 2019; Carvalho et al., 2020; Katzelnick et al., 2020) subsequent infections. While attempts have been

made to study immune-driven evolution of ZIKV, these prior studies were limited by their designs and translatability (Keeffe et al., 2018; Bailey et al., 2019; Dussupt et al., 2020; Regla-Nava et al., 2022). This gap leaves us vulnerable to novel variants selected through cross-reactive immune selection that could alter transmissibility (Liu et al., 2017; Liu et al., 2021) or disease severity (Yuan et al., 2017; Xia et al., 2018; Shan et al., 2020; Liu et al., 2021). Therefore, we set out to further characterize the role of pre-existing immunity on ZIKV evolution.

An important tool to study this phenomenon is the infectious clone; however, there are limitations with the current technologies available to molecular virologists. Traditional techniques involve generating an infectious clone through bacterial cloning; however, these approaches are hindered by the toxicity of viral genomes in bacteria (Johansen, 1996; López-Moya and García, 2000; Olsen and Johansen, 2001; Li et al., 2011; Pu et al., 2011a; Pu et al., 2011b; Mudaliar and Sreekumar, 2016). While there have been many attempts to mitigate or ameliorate the toxicity of the clones (as reviewed extensively by Aubry et al. (Aubry et al., 2015)), these techniques failed to generate a streamlined workflow to generate and manipulate infectious clones.

The first step to optimize the cloning workflow involved replacing the standard DNA extraction (e.g., miniprep or midiprep) with an *in vitro* process. To do this, we leverage the technology of rolling circle amplification (RCA). Using an infectious clone of Mayaro virus (MAYV), we compared the length of time needed to reach peak titer and the difference in peak viral titer after transfecting bacterial-derived plasmid and RCA product. In several transfectable cell lines (human embryonic kidney cells, green monkey kidney cells, and baby hamster kidney cells), we observed no significant difference between the two methods in the time to or magnitude of the peak titer, thereby suggesting that RCA product is comparable to bacterial-derived plasmid as a template for viral rescue. To further scrutinize our results, we assessed variable input amounts of DNA and different RCA kits. We observed that the RCA product behaved similarly to the bacterial-derived plasmid regardless of the amount of DNA used or RCA kit utilized. Finally, we tested the sequence fidelity of our RCA products by performing serial RCA reactions. After three serial reactions, while there was a slight reduction in peak titer, we detected no mutations in the MAYV clone using Sanger sequencing, demonstrating that RCA can maintain sequence integrity over multiple rounds of propagation. Interestingly, when we assessed the costs associated with a

bacterial pipeline and an RCA platform, we calculated that the RCA platform is roughly an order of magnitude less expensive per microgram of DNA compared to the traditional bacterial pipeline. Holistically, these results demonstrated that RCA could replace large-scale DNA extractions.

The above RCA step, while being able to produce large quantities of transfection-quality DNA, was predicated on a pre-existing, covalently sealed, infectious clone to serve as a template. The generation of novel clones with these characteristics has historically required bacteria. However, given the success of using an *in vitro* process to propagate an infectious clone, we attempted to produce a clone using novel *in vitro* technologies. We generated two infectious clones of medically relevant viruses, DENV2 strain PUO-218 and SARS-CoV-2 strain USA/WA1/2020 using replication cycle reaction (RCR). This process involved synthesizing the viral genome over several clonal fragments and utilizing a system that reconstitutes the entirety of *E. coli* replication machinery *in vitro* to assemble and amplify the fragments into a new clone (Su'Etsugu et al., 2017).

The DENV2 clone replicated with identical kinetics compared to the isolate from which it was derived. The SARS-CoV-2 clone was attenuated compared to the isolate; however, the sequence of the clone was identical to the isolate. This attenuation of clonal SARS-CoV-2 sequences has also been reported with other clones (Thi Nhu Thao et al., 2020; Xie et al., 2020). After demonstrating that this pipeline could generate novel infectious clones, we attempted to use it to generate mutant viruses. Using site-directed mutagenic PCR, we engineered the D614G mutation within the SARS-CoV-2 infection clone. This mutation had previously been reported to increase the fitness of SARS-CoV-2 (Plante et al., 2021; Yang et al., 2021). When we compared the D614G mutant virus we generated to the wild-type virus, we also observed an increase in fitness, recapitulating the previously reported results. Therefore, we concluded that RCR could be used to both generate novel infectious clones and engineer mutations within infectious clones. With both RCA and RCR characterized, we now had an entirely *in* vitro cloning pipeline.

With our new cloning pipeline established, we then designed an *in vitro*-directed evolution system to study immune-driven evolution. We did this by passaging ZIKV PRVABC59 in the presence of convalescent DENV serum (anti-DENV serum) or serum from DENV-naïve patients (control serum). By performing these passages in parallel, we could separate non-specific mutations associated with cell culture adaptations, such as those seen in the control serum and

anti-DENV passaged populations, and those specific to cross-reactive immune section. After passaging, we performed next-generation sequencing to identify changes in population divergence, population diversity, and enriched mutations associated with cross-reactive immune selection. We observed that passaging in anti-DENV serum significantly reduced the divergence and nonsynonymous diversity of the premembrane region compared to virus passaged in control serum, suggesting that passaging in anti-DENV serum limited the ability of ZIKV to adapt to cell culture. These findings were mirrored by the growth kinetics of the passaged viruses in Vero cells, as the control serum passage virus had enhanced replication compared to the unpassaged virus. When we assessed the passaged populations for enriched mutations, we observed five nonsynonymous mutations within the critical antibody binding regions, PrM-E-NS1 (Vaughan et al., 2010), of the anti-DENV passaged virus. We selected two mutations for further study, E-V355I, and NS1-T139A, as they were in regions known to mediate cell entry (Fontes-Garfias et al., 2017) and infectivity in mosquitoes (Liu et al., 2017; Kuo et al., 2020).

Site-directed mutagenesis was used to generate each mutation to evaluate their effects on the fitness of ZIKV in four cell culture systems: Vero cells without supplementation of anti-DENV serum, Vero cells supplemented with anti-DENV serum, A549 cells, and U937-DC-SIGN cells. These environments were selected to model the effect of host immune status on the fitness of the mutations (Vero models) or because they represent cell types associated with human infection (A549 and U937-DC-SIGN) (Hamel et al., 2015; Khaiboullina et al., 2017; Fowler et al., 2018). Across all four mammalian cell environments, the mutations demonstrated greater fitness than the wild-type virus. Because ZIKV is a two-host pathogen, we tested the mutants' fitness in mosquitoes using an artificial blood meal. We performed two types of mosquito feeds. In the first, we used virus mixed with defibrinated sheep's blood, representing an immune naïve patient. In the second, we treated the virus with anti-DENV serum, representing a DENV immune patient. Regardless of the immune status of the blood, the mutant viruses were significantly less fit compared to the wild-type. These results, having increased fitness in the mammalian environments and decreased fitness in the mosquitoes, align closely with the trade-off hypothesis, which states that specialization to one host reduces fitness in all other systems (Wilson and Yoshimura, 1994; Kassen, 2002). Finally, because the mutations were derived by passaging ZIKV in cross-reactive neutralizing serum, we were interested to see if these mutations affected neutralization sensitivity and could both be escape mutants. While this was

true for NS1-T139A, we observed that E-V355I was more sensitive to neutralization than the wild-type virus. This led us to conclude that while passaging ZIKV in anti-DENV serum results in mutations with altered fitness in mammalian cells and mosquitoes, these mutations do not need to escape neutralization. These results demonstrate that pre-existing cross-reactive DENV immunity can significantly affect ZIKV evolution.

Our work provides a solid foundation for understanding the role of immune-driven evolution of ZIKV and provides the tools for further study; however, significant work still needs to be done to fully understand immune-driven evolution. Additional studies should be performed examining the impacts of cross-reactive T cells, as they have been shown to impact the evolution of other viruses (Ciurea et al., 2001; Cao et al., 2003; Bowen and Walker, 2005; Zompi et al., 2012; Agerer et al., 2020). Other flavivirus immune interactions should be studied, such as those within the Japanese encephalitis complex (Rathore and St. John, 2020) or inter-serotype interactions with DENV. However, we have made a significant step toward understanding the immune-driven evolution of ZIKV and providing tools for others to continue this work. Our work provides the necessary framework for others to continue combating the evolution and emergence of flaviviruses around the globe.

5.2 References

Agerer, B., Koblischke, M., Gudipati, V., Smyth, M., Popa, A., Genger, J.-W., et al. (2020). "SARS-CoV-2 escapes CD8 T cell surveillance via mutations in MHC-I restricted epitopes". Cold Spring Harbor Laboratory).

Andrade, P., Gimblet-Ochieng, C., Modirian, F., Collins, M., Cárdenas, M., Katzelnick, L.C., et al. (2019). Impact of pre-existing dengue immunity on human antibody and memory B cell responses to Zika. *Nature Communications* 10(1). doi: 10.1038/s41467-019-08845-3.

Andrade, P., Narvekar, P., Montoya, M., Michlmayr, D., Balmaseda, A., Coloma, J., et al. (2020). Primary and secondary dengue virus infections elicit similar memory B cell responses but breadth to other serotypes and cross-reactivity to Zika virus is higher in secondary dengue. *The Journal of Infectious Diseases*. doi: 10.1093/infdis/jiaa120.

Aubry, F., Nougairède, A., Gould, E.A., and de Lamballerie, X. (2015). Flavivirus reverse genetic systems, construction techniques and applications: a historical perspective. *Antiviral research* 114**,** 67-85. doi: 10.1016/j.antiviral.2014.12.007.

Bailey, M.J., Broecker, F., Freyn, A.W., Choi, A., Brown, J.A., Fedorova, N., et al. (2019). Human Monoclonal Antibodies Potently Neutralize Zika Virus and Select for Escape Mutations on the Lateral Ridge of the Envelope Protein. *Journal of Virology* 93(14). doi: 10.1128/jvi.00405-19.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., et al. (2013). The global distribution and burden of dengue. *Nature* 496(7446)**,** 504-507. doi: 10.1038/nature12060.

Bowen, D.G., and Walker, C.M. (2005). Mutational escape from CD8+ T cell immunity. *Journal of Experimental Medicine* 201(11)**,** 1709-1714. doi: 10.1084/jem.20050808.

Cao, J., McNevin, J., Malhotra, U., and McElrath, M.J. (2003). Evolution of CD8+T Cell Immunity and Viral Escape Following Acute HIV-1 Infection. *The Journal of Immunology* 171(7)**,** 3837-3846. doi: 10.4049/jimmunol.171.7.3837.

Carlson, C.J., Albery, G.F., Merow, C., Trisos, C.H., Zipfel, C.M., Eskew, E.A., et al. (2022). Climate change increases cross-species viral transmission risk. *Nature* 607(7919)**,** 555-562. doi: 10.1038/s41586-022-04788-w.

Carvalho, M.S., Freitas, L.P., Cruz, O.G., Brasil, P., and Bastos, L.S. (2020). Association of past dengue fever epidemics with the risk of Zika microcephaly at the population level in Brazil. *Scientific Reports* 10(1). doi: 10.1038/s41598-020-58407-7.

Castanha, P.M., Nascimento, E.J.M., Cynthia, B., Cordeiro, M.T., De Carvalho, O.V., De Mendonça, L.R., et al. (2016). Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection. *Journal of Infectious Diseases***,** jiw638. doi: 10.1093/infdis/jiw638.

Ciota, A.T. (2019). The role of co-infection and swarm dynamics in arbovirus transmission. *Virus Research* 265**,** 88-93. doi: https://doi.org/10.1016/j.virusres.2019.03.010.

Ciurea, A., Hunziker, L., Martinic, M.M.A., Oxenius, A., Hengartner, H., and Zinkernagel, R.M. (2001). CD4+ T-cell–epitope escape mutant virus selected in vivo. *Nature Medicine* 7(7)**,** 795-800. doi: 10.1038/89915.

Cleveland, S.M., Taylor, H.P., and Dimmock, N.J. (1997). Selection of neutralizing antibody escape mutants with type A influenza virus HA-specific polyclonal antisera: possible significance for antigenic drift. *Epidemiology and Infection* 118(2)**,** 149-154. doi: 10.1017/s0950268896007303.

Coffey, L.L., Forrester, N., Tsetsarkin, K., Vasilakis, N., and Weaver, S.C. (2013). Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. 8(2), 155-176. doi: 10.2217/fmb.12.139.

Doud, M.B., Lee, J.M., and Bloom, J.D. (2018). How single mutations affect viral escape from broad and narrow antibodies to H1 influenza hemagglutinin. *Nature Communications* 9(1). doi: 10.1038/s41467-018-03665-3.

Dussupt, V., Sankhala, R.S., Gromowski, G.D., Donofrio, G., De La Barrera, R.A., Larocca, R.A., et al. (2020). Potent Zika and dengue cross-neutralizing antibodies induced by Zika vaccination in a dengue-experienced donor. *Nature Medicine* 26(2), 228-235. doi: 10.1038/s41591-019-0746-2.

Ferguson, N.M., Galvani, A.P., and Bush, R.M. (2003). Ecological and immunological determinants of influenza evolution. *Nature* 422(6930), 428-433. doi: 10.1038/nature01509.

Fontes-Garfias, C.R., Shan, C., Luo, H., Muruato, A.E., Medeiros, D.B.A., Mays, E., et al. (2017). Functional Analysis of Glycosylation of Zika Virus Envelope Protein. *Cell Rep* 21(5), 1180-1190. doi: 10.1016/j.celrep.2017.10.016.

Fowler, A.M., Tang, W.W., Young, M.P., Mamidi, A., Viramontes, K.M., McCauley, M.D., et al. (2018). Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. *Cell Host & Microbe* 24(5), 743-750.e745. doi: 10.1016/j.chom.2018.09.015.

Guzmán, M.G., Kourí, G., Valdés, L., Bravo, J., Vázquez, S., and Halstead, S.B. (2002). Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica* 11(4), 223-227. doi: 10.1590/s1020-49892002000400003.

Halstead, S.B., Lan, N.T., Myint, T.T., Shwe, T.N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic fever in infants: research opportunities ignored. *Emerg Infect Dis* 8(12), 1474-1479. doi: 10.3201/eid0812.020170.

Halstead, S.B., and O'Rourke, E.J. (1977). Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 265(5596), 739-741. doi: 10.1038/265739a0.

Hamel, R., Dejarnac, O., Wichit, S., Ekchariyawat, P., Neyret, A., Luplertlop, N., et al. (2015). Biology of Zika Virus Infection in Human Skin Cells. *Journal of Virology* 89(17), 8880-8896. doi: 10.1128/jvi.00354-15.

Jin, J., Nathan, Chen, D.-H., Liao, M., Julie, Raeann, et al. (2015). Neutralizing Monoclonal Antibodies Block Chikungunya Virus Entry and Release by Targeting an Epitope Critical to Viral Pathogenesis. *Cell Reports* 13(11)**,** 2553-2564. doi: 10.1016/j.celrep.2015.11.043.

Johansen, I.E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 93(22)**,** 12400-12405. doi: 10.1073/pnas.93.22.12400.

Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* 15(2)**,** 173-190. doi: https://doi.org/10.1046/j.1420-9101.2002.00377.x.

Katzelnick, L.C., Gresh, L., Halloran, M.E., Mercado, J.C., Kuan, G., Gordon, A., et al. (2017). Antibody-dependent enhancement of severe dengue disease in humans. *Science* 358(6365)**,** 929-932. doi: doi:10.1126/science.aan6836.

Katzelnick, L.C., Narvaez, C., Arguello, S., Lopez Mercado, B., Collado, D., Ampie, O., et al. (2020). Zika virus infection enhances future risk of severe dengue disease. *Science* 369(6507)**,** 1123-1128. doi: 10.1126/science.abb6143.

Katzelnick, L.C., Zambrana, J.V., Elizondo, D., Collado, D., Garcia, N., Arguello, S., et al. (2021). Dengue and Zika virus infections in children elicit cross-reactive protective and enhancing antibodies that persist long term. *Sci Transl Med* 13(614)**,** eabg9478. doi: 10.1126/scitranslmed.abg9478.

Keeffe, J.R., Van Rompay, K.K.A., Olsen, P.C., Wang, Q., Gazumyan, A., Azzopardi, S.A., et al. (2018). A Combination of Two Human Monoclonal Antibodies Prevents Zika Virus Escape Mutations in Non-human Primates. *Cell Reports* 25(6)**,** 1385-1394.e1387. doi: 10.1016/j.celrep.2018.10.031.

Keusch, G.T., Amuasi, J.H., Anderson, D.E., Daszak, P., Eckerle, I., Field, H., et al. (2022). Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. *Proceedings of the National Academy of Sciences* 119(42)**,** e2202871119. doi: doi:10.1073/pnas.2202871119.

Khaiboullina, S.F., Uppal, T., Sarkar, R., Gorzalski, A., St Jeor, S., and Verma, S.C. (2017). ZIKV infection regulates inflammasomes pathway for replication in monocytes. *Scientific Reports* 7(1). doi: 10.1038/s41598-017-16072-3.

Khare, B., and Kuhn, R.J. (2022). The Japanese Encephalitis Antigenic Complex Viruses: From Structure to Immunity. *Viruses* 14(10). doi: 10.3390/v14102213.

Kuo, L., Jaeger, A.S., Banker, E.M., Bialosuknia, S.M., Mathias, N., Payne, A.F., et al. (2020). Reversion to ancestral Zika virus NS1 residues increases competence of Aedes albopictus. *PLoS Pathog* 16(10)**,** e1008951. doi: 10.1371/journal.ppat.1008951.

Lambkin, R., McLain, L., Jones, S.E., Aldridge, S.L., and Dimmock, N.J. (1994). Neutralization escape mutants of type A influenza virus are readily selected by antisera from mice immunized with whole virus: a possible mechanism for antigenic drift. *Journal of General Virology* 75(12)**,** 3493-3502. doi: 10.1099/0022-1317-75-12-3493.

Lee, J.M., Eguia, R., Zost, S.J., Choudhary, S., Wilson, P.C., Bedford, T., et al. (2019). Mapping person-to-person variation in viral mutations that escape polyclonal serum targeting influenza hemagglutinin. *eLife* 8. doi: 10.7554/elife.49324.

Li, D., Aaskov, J., and Lott, W.B. (2011). Identification of a Cryptic Prokaryotic Promoter within the cDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLoS ONE* 6(3)**,** e18197. doi: 10.1371/journal.pone.0018197.

Liu, J., Liu, Y., Shan, C., Nunes, B.T.D., Yun, R., Haller, S.L., et al. (2021). Role of mutational reversions and fitness restoration in Zika virus spread to the Americas. *Nature Communications* 12(1). doi: 10.1038/s41467-020-20747-3.

Liu, Y., Liu, J., Du, S., Shan, C., Nie, K., Zhang, R., et al. (2017). Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. *Nature* 545(7655)**,** 482-486. doi: 10.1038/nature22365.

López-Moya, J.J., and García, J.A. (2000). Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res* 68(2)**,** 99-107. doi: 10.1016/s0168-1702(00)00161-1.

Marchi, J., Lässig, M., Walczak, A.M., and Mora, T. (2021). Antigenic waves of virus–immune coevolution. *Proceedings of the National Academy of Sciences* 118(27)**,** e2103398118. doi: 10.1073/pnas.2103398118.

Morris, D.H., Petrova, V.N., Rossine, F.W., Parker, E., Grenfell, B.T., Neher, R.A., et al. (2020). Asynchrony between virus diversity and antibody selection limits influenza virus evolution. *eLife* 9**,** e62105. doi: 10.7554/eLife.62105.

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N.L., Khasnatinov, M., et al. (2015). New insights into flavivirus evolution, taxonomy and biogeographic history, extended by analysis of canonical and alternative coding sequences. *PLoS One* 10(2)**,** e0117849. doi: 10.1371/journal.pone.0117849.

Mudaliar, P.P., and Sreekumar, E. (2016). Exploring the instability of reporters expressed under the subgenomic promoter in Chikungunya virus infectious cDNA clones. 45**,** 448. doi: 10.1016/j.ijid.2016.02.950.

Olsen, B.S., and Johansen, I.E. (2001). Nucleotide sequence and infectious cDNA clone of the L1 isolate of Pea seed-borne mosaic potyvirus. *Arch Virol* 146(1)**,** 15-25. doi: 10.1007/s007050170187.

Patterson, J., Sammon, M., and Garg, M. (2016). Dengue, Zika and Chikungunya: Emerging Arboviruses in the New World. *West J Emerg Med* 17(6)**,** 671-679. doi: 10.5811/westjem.2016.9.30904.

Pierson, T.C., and Diamond, M.S. (2020). The continued threat of emerging flaviviruses. *Nature Microbiology* 5(6)**,** 796-812. doi: 10.1038/s41564-020-0714-0.

Plante, J.A., Liu, Y., Liu, J., Xia, H., Johnson, B.A., Lokugamage, K.G., et al. (2021). Spike mutation D614G alters SARS-CoV-2 fitness. *Nature* 592(7852)**,** 116-121. doi: 10.1038/s41586-020-2895-3.

Pu, S.-Y., Wu, R.-H., Yang, C.-C., Jao, T.-M., Tsai, M.-H., Wang, J.-C., et al. (2011a). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. *Journal of Virology* 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., et al. (2011b). Successful Propagation of Flavivirus Infectious cDNAs by a Novel Method To Reduce the Cryptic Bacterial Promoter Activity of Virus Genomes. 85(6)**,** 2927-2941. doi: 10.1128/jvi.01986-10.

Rathore, A.P.S., Saron, W.A.A., Lim, T., Jahan, N., and St. John, A.L. (2019). Maternal immunity and antibodies to dengue virus promote infection and Zika virus–induced microcephaly in fetuses. *Science Advances* 5(2)**,** eaav3208. doi: 10.1126/sciadv.aav3208.

Rathore, A.P.S., and St. John, A.L. (2020). Cross-Reactive Immunity Among Flaviviruses. *Frontiers in Immunology* 11. doi: 10.3389/fimmu.2020.00334.

Regla-Nava, J.A., Wang, Y.-T., Fontes-Garfias, C.R., Liu, Y., Syed, T., Susantono, M., et al. (2022). A Zika virus mutation enhances transmission potential and confers escape from protective dengue virus immunity. *Cell Reports* 39(2)**,** 110655. doi: 10.1016/j.celrep.2022.110655.

Reich, N.G., Shrestha, S., King, A.A., Rohani, P., Lessler, J., Kalayanarooj, S., et al. (2013). Interactions between serotypes of dengue highlight epidemiological impact of cross-immunity. *J R Soc Interface* 10(86)**,** 20130414. doi: 10.1098/rsif.2013.0414.

Rockx, B., Donaldson, E., Frieman, M., Sheahan, T., Corti, D., Lanzavecchia, A., et al. (2010). Escape from human monoclonal antibody neutralization affects in vitro and in vivo fitness of severe acute respiratory syndrome coronavirus. *J Infect Dis* 201(6)**,** 946-955. doi: 10.1086/651022.

Rodriguez-Barraquer, I., Costa, F., Nascimento, E.J.M., Nery, N.J., Castanha, P.M.S., Sacramento, G.A., et al. (2019). Impact of preexisting dengue immunity on Zika virus emergence in a dengue endemic region. *Science* 363(6427)**,** 607-610. doi: 10.1126/science.aav6618.

Sanjuán, R., and Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences* 73(23)**,** 4433-4448. doi: 10.1007/s00018-016-2299-6.

Sapkal, G.N., Harini, S., Ayachit, V.M., Fulmali, P.V., Mahamuni, S.A., Bondre, V.P., et al. (2011). Neutralization escape variant of West Nile virus associated with altered peripheral pathogenicity and differential cytokine profile. *Virus Res* 158(1-2)**,** 130-139. doi: 10.1016/j.virusres.2011.03.023.

Shan, C., Xia, H., Haller, S.L., Azar, S.R., Liu, Y., Liu, J., et al. (2020). A Zika virus envelope mutation preceding the 2015 epidemic enhances virulence and fitness for transmission. *Proc Natl Acad Sci U S A* 117(33)**,** 20190-20197. doi: 10.1073/pnas.2005722117.

Su'Etsugu, M., Takada, H., Katayama, T., and Tsujimoto, H. (2017). Exponential propagation of large circular DNA by reconstitution of a chromosome-replication cycle. *Nucleic Acids Research* 45(20)**,** 11525-11534. doi: 10.1093/nar/gkx822.

Thi Nhu Thao, T., Labroussaa, F., Ebert, N., V'Kovski, P., Stalder, H., Portmann, J., et al. (2020). Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform. *Nature* 582(7813)**,** 561-565. doi: 10.1038/s41586-020-2294-9.

Vaughan, K., Greenbaum, J., Blythe, M., Peters, B., and Sette, A. (2010). Meta-analysis of All Immune Epitope Data in theFlavivirusGenus: Inventory of Current Immune Epitope Data Status in the Context of Virus Immunity and Immunopathology. *Viral Immunology* 23(3)**,** 259-284. doi: 10.1089/vim.2010.0006.

Wilson, D.S., and Yoshimura, J. (1994). On the Coexistence of Specialists and Generalists. *The American Naturalist* 144(4)**,** 692-707. doi: 10.1086/285702.

Xia, H., Luo, H., Shan, C., Muruato, A.E., Nunes, B.T.D., Medeiros, D.B.A., et al. (2018). An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. *Nat Commun* 9(1)**,** 414. doi: 10.1038/s41467-017-02816-2.

Xie, X., Muruato, A., Lokugamage, K.G., Narayanan, K., Zhang, X., Zou, J., et al. (2020). An Infectious cDNA Clone of SARS-CoV-2. *Cell Host & Microbe* 27(5)**,** 841-848.e843. doi: 10.1016/j.chom.2020.04.004.

Yang, T.-J., Yu, P.-Y., Chang, Y.-C., and Hsu, S.-T.D. (2021). D614G mutation in the SARS-CoV-2 spike protein enhances viral fitness by desensitizing it to temperature-dependent denaturation. *Journal of Biological Chemistry* 297(4)**,** 101238. doi: 10.1016/j.jbc.2021.101238.

Yuan, L., Huang, X.-Y., Liu, Z.-Y., Zhang, F., Zhu, X.-L., Yu, J.-Y., et al. (2017). A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science* 358(6365)**,** 933-936. doi: 10.1126/science.aam7120.

Zimmerman, M.G., Quicke, K.M., O'Neal, J.T., Arora, N., Machiah, D., Priyamvada, L., et al. (2018). Cross-Reactive Dengue Virus Antibodies Augment Zika Virus Infection of Human Placental Macrophages. *Cell Host & Microbe* 24(5)**,** 731-742.e736. doi: 10.1016/j.chom.2018.10.008.

Zompi, S., Santich, B.H., Beatty, P.R., and Harris, E. (2012). Protection from Secondary Dengue Virus Infection in a Mouse Model Reveals the Role of Serotype Cross-Reactive B and T Cells. *The Journal of Immunology* 188(1)**,** 404-416. doi: 10.4049/jimmunol.1102124.