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Contributions of Structural and Non-structural Genes to the Attenuation of the 17D Vaccine

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Contributions of Structural and Non-structural Genes to the Attenuation of the 17D Vaccine

by

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Dedication

This is dedicated to my loving husband, Lance Gregory Collins, and my beautiful daughters, Makayla and Ryleigh. Lance, "our" time in graduate school has brought us closer together as a family and taught me how to appreciate all that you do for our family and me. There is no degree or military rank that completes my life like you and the girls do.

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Contributions of Structural and Non-structural Genes to the Attenuation of the 17D Vaccine

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Live-attenuated 17D vaccine, developed to control Yellow Fever virus (YFV), was derived by serial passage of wild-type Asibi strain 176 times in mouse and chicken tissues. Despite the derivation of 17D over 75 years ago, the mechanism of attenuation is still poorly understood. The genomes of wild-type Asibi virus and the 17D vaccine substrains 17D-204, 17D-213 and 17DD were sequenced using Sanger sequencing and shown to differ from wild-type Asibi virus by 9 common amino acid substitutions in the structural protein genes, 11 common amino acid substitutions in the non-structural (NS) protein genes, and four nucleotide changes in the 3' non-coding region. The NS proteins are required for viral replication and modulate the host immune response. The hypothesis to be tested in this dissertation is to determine whether or not structural and non-structural (NS) protein genes encode major genetic determinants of attenuation and contribute to decreased virulence of the 17D vaccines. RNA viruses are known to exist as populations of genetically related virions, known as quasispecies population, which contributes to the fitness and virulence of a virus. Infectious clone (IC)-derived chimeric viruses will be used to investigate contributions of NS protein genes to the generation of quasispecies population by assessing Shannon entropy of nucleotide diversity and frequency of RNA subpopulation in Specific Aim 1. Specific Aim 2, determines if the genetic determinants in NS protein genes that influence quasispecies, also influence multiplication kinetics. Finally, induced cytokines and gene expression of IC-derived viruses will be determined to assess whether or not attenuating mutations in NS proteins abate immune regulation typical of wild-type infections in Specific Aim 3. The results of this study showed that the attenuated genotype and phenotype of 17D vaccine is multi-genic, requiring both structural and NS protein genes.

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List of Abbreviations

°C	Degree Celsius
A	Adenine
Ae	*Aedes* spp.
Ala (A)	Alanine
ANOVA	Analysis of variance
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartate
BAM	Binary alignment/map
bp	Base-pair
C protein	Capsid protein
C	Cytosine
cDNA	complimentary DNA
CHIKV	Chikungunya virus
ClustalW	Clustal Omega
CO_2	Carbon dioxide
CPE	Cytopathic effects
Cys (C)	Cysteine
DENV	Dengue virus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dpi	Days post-infection
dsRNA	double-stranded RNA
DTT	Dithiothreitol
DRC	Democratic Republic of Congo
E protein	Envelope protein
EDIII	E protein domain III
FBS	Fetal bovine serum
G	Guanine
G-CSF	Granulocyte colony-stimulating factor
Gln (Q)	Glutamine
Glu (E)	Glutamate
Gly (G)	Glycine
His (H)	Histidine
hpi	Hours post infection/inoculation
IC	Infectious clone
i.c.	Intracranial
i.p.	Intraperitoneal
IFN-(α/β/γ)	Interferon-α/β/γ
IL	Interleukin
Ile (I)	Isoleucine

IP-10	Interferon gamma-induced protein 10
JEV	Japanese encephalitis virus
kb	kilobase
Leu (L)	Leucine
LP	Large plaque morphology
Lys (K)	Lysine
M protein	Membrane protein
MAVS	Mitochondrial antiviral signaling protein
MCP-1	Monocyte chemotactic protein 1
MEM	Minimal essential media
Met (M)	Methionine
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
mL	milliliter
mm	Millimeter
mM	Millimolar
MOI	Multiplicity of infection
MP	Medium plaque morphology
N	Ambiguous base
NEAA	Non-essential amino acids
ng	nanogram
NGS	Next generation sequencing
nm	nanometer
nM	Nanomolar
NOD	Nuclear organization domain
NS1 protein	Non-structural protein 1
NS2A protein	Nonstructural protein 2A
NS2B protein	Nonstructural protein 2B
NS3 protein	Nonstructural protein 3
NS4A protein	Nonstructural protein 4A
NS4B protein	Nonstructural protein 4B
NS5 protein	Nonstructural protein 5
nt	Nucleotide(s)
N-terminus	Amino (NH_3^+)-terminus or terminal domain
ORF	Open reading frame
p	*p*-value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
pg	picogram
Phe (F)	Phenylalanine
prM protein	Pre-membrane protein
Pro (P)	Proline
RNA	Ribonucleic Acid
RNaseA	Ribonuclease L
SAMtools	Sequence alignment map toolbox

Ser (S)	Serine
SLEV	St. Louis encephalitis virus
SNV	Single nucleotide variant
SP	Small plaque morphology
ssRNA	Single-stranded RNA
STAT1/2	Signal transducers and activators of transcription 1 and 2
T cell	T lymphocyte
T	Thymine
TBEV	Tick-borne encephalitis virus
TE	Tris-HCl EDTA buffer
Thr (T)	Threonine
TLR	Toll-like receptor
TMD	Transmembrane domain
TNF-α	Tumor necrosis factor alpha
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
RdRp	RNA-dependent RNA polymerase
U	Uracil
UTMB	University of Texas Medical Branch
UTR	Untranslated region (either 5' and 3')
Val (V)	Valine
WNV	West Nile virus
YFV	Yellow fever virus
µg	Microgram
µL	Microliter
ZIKV	Zika virus

CHAPTER 1: INTRODUCTION

1.1 YELLOW FEVER

1.1.1 Flavivirus genus

Flaviviruses are a group of arthropod-borne viruses that can cause a range of clinical symptoms, including hemorrhagic and neurological manifestations. The *Flavivirus* genus (named after *flavus*, the Latin for yellow) contains numerous pathogens of public health importance, including mosquito-borne yellow fever (YF), dengue (DEN), Japanese encephalitis (JE), West Nile (WN) and Zika (ZIK) viruses, and tick-borne encephalitis (TBE) virus. YF virus (YFV), the prototype flavivirus, targets the liver causing severe liver damage and jaundice, hence the "yellow" in YFV. Once in the liver, YFV spreads to the kidneys and heart, causing severe and sometimes fatal visceral disease (Monath & Vasconcelos, 2015). In comparison, JE, TBE, and WN viruses are associated with neurologic disease while DEN and ZIK viruses cause a febrile infection with a rash, although both can lead to severe disease with hemorrhagic fever and congenital Zika syndrome, respectively. Although there is a safe and effective vaccine that has been available since the late 1930's, YFV remains a public health problem in South America and sub-Saharan Africa; where it causes an estimated 130,000 cases, including 78,000 deaths annually (Garske et al., 2014). More recently, the 2016 outbreak in Angola and Democratic Republic of Congo (DRC) that began in December of 2015 and continued through September 2016 (see below), fueled concern of the potential for outbreaks outside of endemic areas to areas primed for YFV transmission due to the presence of appropriate mosquito vectors. Given the lethality of YF disease and the

numerous countries that harbor the vectors, YFV is by far the most important hemorrhagic flavivirus.

1.1.2 Genome

The YFV genome is a single-stranded, positive-sense strand of RNA with one open reading frame (ORF) encoding three structural proteins: C (capsid), prM/M (pre-membrane/membrane), E (envelope) in the 5' one-third and seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 in the C-terminal two-thirds; the ORF is flanked by 5' and 3' non-coding regions (NCRs).

1.1.2.1 Yellow fever virus structural proteins

The C protein encapsulates the single stranded RNA and this is surrounded by two structural proteins (M and E) and contains a critical cleavage site called the capsid dibasic-site, which is essential for virion release (Oliveira et al., 2017).

The prM/M protein interacts with the E and acts a chaperone for E during assembly (Konishi & Mason, 1993). Prior to virion release, prM/M is cleaved by host furin to the active M; incomplete cleavage results in the prM being incorporated into what is termed immature virions and affects structure and antigenicity (Zhang et al., 2003).

The E protein is critical to viral assembly, stability and maturation (Blazevic et al., 2016). The prM/M protein and the E protein associate to form the characteristic icosahedral lattices of the virion, with the mature virion containing 90 dimers of E protein (Blazevic et al., 2016). The stem-anchor region and ectodomain comprise the E. The ectodomain is further comprised of three domains: domain I, domain II (responsible for

dimerization and fusion of the virion to host cells), and domain III (putative receptor binding domain) (Mukhopadhyay et al., 2005). Neutralizing antibodies are directed towards all three domains, however, antibodies that recognize domain III are immunodominate (Lobigs et al., 1987; Ryman et al., 1997). Additionally, cytotoxic T cell epitopes are located within domain III of the E protein (Co et al., 2002; van der Most et al., 2002).

1.1.2.2 Yellow fever virus non-structural proteins

The seven non-structural (NS) proteins (NS1, NS2A/B, NS3, NS4A/B, and NS5) of flaviviruses are multifunctional; they comprise the replication complex (RC) required for viral replication, are implicated in modulating the immune response, and aid in immune invasion (**Table 1.1**) (Green et al., 2014; Jones et al., 2005; Shi, 2014; Xie et al., 2015; Ye et al., 2013). All seven NS proteins interact directly and indirectly as part of the RC (Shi, 2014). In addition to host antibodies directed against NS1, NS3, and NS5; cell mediated immunity is ilicited by NS1, NS2B, and NS3 (Co et al., 2002; Akey et al., 2014; Akey et al., 2015; Heinz & Stiasny, 2012; Monath, 2013; Zou et al., 2015).

Table 1.1 Functions of Non-structural Proteins		
Protein	Protein Function	
	Replication	**Innate Immunity**
NS1	Virus particle morphogenesis	Attenuation of TLR3 signaling; suppress complement response; complement lysis of infected cells; cytotoxic T-cell epitope
NS2A	Virus particle morphogenesis	Reduction of IFNβ gene transcription; inhibition STAT1 phosphorylation; inhibition STAT2 phosphorylation; cytotoxic T-cell epitopes; viroporin
NS2B	Co-factor for NS3 protease activity; induces membrane rearrangement	Inhibition STAT2 phosphorylation; virus specific T-cell epitopes; cytotoxic T-cell epitopes; viroporin
NS3	Serine protease and helicase	Inhibition STAT2 phosphorylation; cytotoxic T-cell epitopes
NS4A	Co-factor for NS3 helicase activity; induces membrane rearrangement	Inhibition STAT1 phosphorylation; inhibition STAT2 phosphorylation; viroporin
NS4B	Scaffold for replication complex	Inhibition STAT1 phosphorylation; inhibition STAT2 phosphorylation; viroporin
NS5	RNA-dependent RNA polymerase; methyltransferase; RNA capping	Inhibition of Tyk2 and Jak1 phosphorylation; reduction in STAT2 gene transcription

The NS1 protein exists as cell membrane-bound and intracellular forms (Muller & Young, 2013). The exact function of the NS1 protein in the replication complex is not fully understood; however, there is evidence that suggest the NS1 regulates NS4B during replication (Youn et al., 2012). The NS1 protein contains both non-neutralizing antibody and cytotoxic T cell epitopes, but its primary immune function in immunity is antibody dependent cellular cytotoxity and modulating the complement system (Avirutnan et al., 2011; Muller & Young, 2013).

The NS2A protein is a trans-membrane protein that has roles in both viral replication and virion assembly. Two types of NS2A protein are produced based on post-translational cleavages. NS2A interacts with NS2B and NS4A in the RC (Voßmann et al., 2015; Xie et al., 2015). Although the exact roles are not understood, it is thought that the NS2A protein contains a sequence specific cleavage site necssary for processing of the NS1-NS2A proteins (Xie et al., 2015). The NS2A protein antagonizes the IFNα/β response by blocking phosporhlylation of both STAT1 and STAT2 and acts as a viroporin (Chang et al., 1999; Liu et al., 2006; Ye et al., 2013)

The NS2B protein is a trans-membrane protein that functions as the co-factor for NS3 protease activity (Li et al., 2016; Huang et al., 2011; Yu et al., 2013). There is also evidence that the NS2B protein may function as viroporin and aid in membrane rearrangement (Chang et al., 1999; León-Juárez et al., 2016).

The NS3 protein is a non-membrane associated protein that has multiple functions. The N-terminal 180 amino acids functions as the serine protease responsible for cleavage of the polyprotein and requires NS2B as a cofactor. The C-terminal remainder of the protein incorporates a helicase that requires NS4A as a cofactor, plus

nucleoside 5' triphosphatase and 5' RNA triphosphatase activities (Apte-Sengupta et al., 2014). Within the RC, NS3 has been shown to interact with NS2B, NS4B and NS5 (Yu et al., 2013; Zou, Lee, et al., 2015). Additionally, NS3 blocks phosphorylation of STAT2 and contains both flavivirus common and virus-type specific cytotoxic T cell epitopes (Co et al., 2002; James et al., 2013; Kurane et al., 1991; van der Most et al., 2002; Ye et al., 2013).

The NS4A protein is a trans-membrane protein. In addition to functioning as the cofactor for the NS3 helicase activity, the NS4A protein interacts directly with the NS1, NS2B and NS4B (Li et al., 2015; Lindenbach & Rice, 1999; Yu et al., 2013; Zou, Xie, et al., 2015). NS4A is thought to modulate autophagy formation, is a viroporin, and has been shown to block phosphorylation of both STAT1 and STAT2 (Chang et al., 1999; McLean et al., 2011; Ye et al., 2013).

The NS4B protein is also a trans-membrane protein, thought to function as the scaffold for the RC; the NS4B has been shown to interactive directly with NS1, NS2A, NS3, and NS4A proteins (Li et al., 2015; Youn et al., 2012; Zou, Lee, et al., 2015; Zou, Xie, et al., 2015). The NS4B protein has been shown to be a viroporin and blocks STAT1 phosphorylation, thereby, interfering with IFNα/β signaling (Ye et al., 2013).

The NS5 protein is a non-membrane associated protein and multiple functions. The N-terminal region encodes a methyl-transferase and the C-terminal region a RNA-dependent RNA polymerase (RdRp), interacting directly with only NS3 protein in the RC (Li et al., 2014; Yu et al., 2013). The NS5 protein antagonizes the innate immune response at multiple points during signaling, it blocks phosphorylation of Tyk2, degrades

STAT2 gene products, and blocks phosphorylation of both STAT1 and STAT2 (Ye et al., 2013).

1.1.3 Ecology and epidemiology

YFV is a mosquito-borne virus, transmitted by *Aedes* spp. in Africa and *Haemagogus* and *Sabethes* spp. in South America. The only vertebrate hosts for YFV in nature are primates where the virus causes an acute disease with high viremia, followed by death or recovery. The virus is maintained in nature by a transmission cycle between mosquitoes and non-human primates in what is referred to as the "jungle" or sylvatic cycle. The particular non-human primate host species vary by geographic location. Humans either become infected after entering jungle areas where there are YFV-infected sylvan *Aedes* species mosquitoes ("intermediate cycle") or during the "urban" cycle. In the urban cycle, the transmission cycle is between humans and *Aedes aegypti,* and non-human primates are not involved.

Given the widespread distribution of *Ae. aegypti* in tropical climates, it is unclear why YFV has not spread to Asia (Barrett & Higgs, 2007). *Ae. aegypti* mosquitoes in Asia transmit other related flaviviruses, such as DEN (Lai et al., 2015) , and laboratory studies have shown them to be a competent vector for YFV. Since YFV originated in Africa and was introduced to the Americas during the slave trade (Beck et al., 2013; Bryant et al., 2007), concerns of spread outside the current endemic areas are warranted.

Genetic studies have shown that there are at least seven genotypes of wild-type YFV. Five are found in Africa (two in West Africa, one in East Africa, one in East/Central Africa, and one in Angola) and two in South America (South America I and II). Interestingly, the 2016 Angola outbreak was caused by a strain indistinguishable

genetically from the 1971 Angola outbreak, and these two outbreaks have been the only times when this genotype has been identified. This raises important questions about the ecology and epidemiology of YFV, namely where did the Angola strain come from and where is the virus maintained over time? Our knowledge of the molecular epidemiology of YFV is limited and is an area where additional research would likely improve our abilities to understand the epidemiology and control of YF.

Until 2016, the number of clinical cases of YFV was on the decline most likely due to improved vaccine coverage in Africa and South America. Based on data from African countries in 2013, there is a burden of 84,000 – 170,000 severe cases and 29,000 – 60,000 deaths due to YF (World Health Organization, 2016). In contrast, there were an estimated 73,000-530,000 severe cases and 27,000-250,000 deaths in 2005 (Garske et al., 2014). The vast majority of reported cases and deaths (>90%) occur in sub-Saharan Africa.

1.1.4 Vaccination

There are no antivirals to treat YF infections and the only course is supportive therapy. However, there is an excellent vaccine, which has been critical for public health measures to control YF disease. Concurrently, two live attenuated YF vaccines were developed in the 1930's. Theiler and colleagues derived 17D by 176 passages of wild-type strain Asibi initially in mouse brain, followed by minced chicken embryos, and finally in minced chicken embryos without nervous tissue (Theiler & Smith, 1937), while the French neurotropic vaccine (FNV) was derived by serial passaging of wild-type French viscerotropic virus (FVV) 128 times in mouse brain (Barrett, 1997). Further passaging of wild-type Asibi yield the sub-strains 17D-204, 17DD, and 17D-213, derived

at passage 204, 286, and 213, respectively; the original 17D is no longer available (Barrett & Teuwen, 2009; Monath & Vasconcelos, 2015). FNV was used as a vaccine at mouse brain passage 260. Although FNV was more immunogenic than the 17D vaccine, presumably due to the protein in mouse brain providing more stability in tropical temperatures, its use was associated with encephalitic reactions and was eventually discontinued in the early 1980s (Wang et al., 1995).

Since the original 17D is not available, genomic comparison of the three 17D substrains to wild-type Asibi revealed that the substrains differed from wild-type Asibi by 20 common amino acid substitutions (Hahn et al., 1987; dos Santos et al., 1995), while FNV differed from FVV by 35 amino acid substitutions (Wang et al., 1995). Of the 20 amino acid substitutions that differ between Asibi and 17D viruses, nine are in the structural protein genes (none in C; one in the M and eight in the E) and 11 are in the NS genes (one each in NS1, NS2B, NS3, NS4A, NS4B; four in the NS2B; and two in the NS5) (**Table 1.2**). There are no nucleotide changes in the 5'NCR and four nucleotide changes in the 3'NCR. However, which of the amino acid substitutions were responsible for the attenuated phenotype of either 17D or FNV are unknown. Nonetheless, despite the different passage histories of the 17D vaccines (chicken tissue) and FNV (mouse brain), M-36 and NS4B-95 amino acid substitutions were present in both vaccines. Interestingly, the M-36 and NS4B-95 amino acid substitutions are also present after passaging wild-type Asibi virus six times in HeLa cells and HeLa p6 virus has the same attenuated phenotype (loss of viscerotropism, loss of neurotropism and non-mosquito competent) as 17D vaccine (Dunster et al., 1999). It is speculated that either one or both are attenuating substitutions.

Table 2.2 The 20 common amino acid substitutions that differentiate wild-type Asibi virus and the live-attenuated 17D vaccines. The four nucleotide changes in the 3'NCR are not shown.

Nucleotide	Gene	Amino Acid	Asibi	17D-204 and 17DD
854	M	36	Lue	Phe
1127	E	52	Gly	Arg
1482		170	Ala	Val
1491		173	Thr	Ile
1572		200	Lys	Thr
1870		299	Met	Ile
1887		305	Ser	Phe
2112		380	Thr	Arg
2193		407	Ala	Val
3371	NS1	307	Ile	Val
3860	NS2A	118	Met	Val
4007		167	Thr	Ala
4022		172	Thr	Ala
4056		183	Ser	Phe
4505	NS2B	109	Ile	Leu
6023	NS3	485	Asp	Asn
6876	NS4A	146	Val	Ala
7171	NS4B	95	Ile	Met
10142	NS5	836	Glu	Lys
10338		900	Pro	Leu

The 17D sub-strains are produced by six manufacturers. 17DD is manufactured in Brazil, 17D-213 manufactured in Russia, and 17D-204 manufactured in China, France, Senegal, and the USA. Four of the vaccines (Brazil, France, Russia, and Senegal) are prequalified by the World Health Organization (WHO) and used internationally for WHO/United Nations Children Fund (UNICEF) vaccination campaigns. Since its establishment in 2000, the Global Alliance for Vaccines and Immunization (GAVI) alliance, in collaboration with WHO and UNICEF, has supported vaccination in African countries at risk from YF. Over 150 million of the approximate 700 million individuals in 17 GAVI-eligible endemic countries have received vaccine. Notably routine immunization has been introduced into 17 African countries and vaccine campaigns undertaken in 14 countries. In addition, there is an ambitious plan to immunize a further 1.3 billion individuals in the next 5 years as part of the "EYE" (eliminating yellow fever epidemics) plan (World Health Organization, 2016). This program has been very successful at preventing YF in West Africa with reduction of YF cases each year such that no outbreaks of YF were reported in West African countries during 2015.

1.1.5 Immune response to vaccination and long-term immunity

A single dose of 17D vaccine confers protection in greater than 95% of recipients within 30 days following vaccination (Barrett & Teuwen, 2009; Bonaldo et al., 2014). The effectiveness of the 17D vaccine strain is attributed to induction of both innate and adaptive immunity that leads to the induction of neutralizing antibodies directed predominately against the envelope (E) protein (Gaucher et al., 2008; Luiza-Silva et al., 2011; Quaresma et al., 2013; Silva et al., 2011). Moreover, innate immune cells secrete mixed and balanced anti-inflammatory and pro-inflammatory cytokines that modulate

other immune cells and elicit a broad adaptive response. As with natural infection with wild-type YF, immunization with 17D is thought to give protective immunity for at least 10 years, and probably induce lifelong immunity (Collaborative group for sudies on yellow fever vaccines, 2014; Staples et al., 2010). High levels of neutralizing antibodies and memory T cells have been detected in vaccinees 10-60 years following vaccination (Gotuzzo et al., 2013). The exact mechanism for this long-term immunity is still being elucidated. However, there is evidence that suggests that in conjunction with humoral immunity, YFV specific $CD4^+$ T cells are preferentially activated following vaccination (Blom et al., 2013; Watson et al., 2016); these activated $CD4^+$ T cells recognize both structural and non-structural proteins and are detectable years later (James et al., 2013). Conversely, YFV specific $CD8^+$ T cells are also detectable decades after vaccination (Fuertes Marraco et al., 2015; Wieten et al., 2016) and correlate to initial viral loads following vaccination (Akondy et al., 2015). T cells may contribute to long-term immunity; however, it is secondary to the induction of neutralizing antibodies.

Until recently, booster doses of 17D vaccine were given every 10 years. In 2013 the WHO Strategic Advisory Group of Experts on Immunization recommended an amendment to the International Health Regulations to reflect the long-term protection from YF after a single vaccination with 17D vaccine, namely booster doses are not needed except for special populations such as pregnant women, HIV infected individuals, hematopoietic stem cell transplant recipients, and persons in higher-risk setting for exposure to YF virus; this change took effect in June 2016 (Collaborative group for sudies on yellow fever vaccines, 2014; Staples et al., 2010). This was supported, in part, by very limited evidence for vaccine failures (Gotuzzo et al., 2013). However, some

countries, such as Brazil, have elected to retain the 10-year booster based on several studies from Brazil that suggest significant drops in immunity, as defined by reduction in neutralizing antibody titers over time (Campi-Azevedo et al., 2016; Collaborative group for sudies on yellow fever vaccines, 2014; de Melo et al., 2011). This has not been observed in other countries but this could be due to a lack of studies. The United States Army Medical Research Institute screened over 1029 laboratory personnel for neutralizing antibodies by 80% plaque reduction neutralization test ($PRNT_{80}$) a more stringent assay than the traditional $PRNT_{50}$ used for international tests) to evaluate the immune activation post 17D booster and determined titers dropped below 1:40 after 3 years, suggesting that a 10-year booster or earlier may be required to maintain high levels of neutralizing antibodies in high risk groups (Hepburn et al., 2006), and would exceed the neutralization titer needed for protective immunity.

Additional studies have also suggested waning immunity over time. de Melo and colleagues (de Melo et al., 2011) found that 35% of vaccinees had neutralizing antibody titers below 1:100 (although this exceeds the seroprotective neutralizing antibody titer) at 10 years following vaccination with 17DD vaccine, while others showed that cellular and humoral immunity decreased by 4 years post immunization and only 85% of vaccinees were seropositive 12 years post vaccination (Campi-Azevedo et al., 2016; Collaborative group for sudies on yellow fever vaccines, 2014; de Melo et al., 2011). A number of hypotheses have been proposed to explain these results, including variation in immune response to specific vaccine strains, pre-existing immunity and immune stimulation in endemic areas compared to non-endemic areas (Muyanja et al., 2014). A meta-analysis study of 17D vaccination efficacy, totaling 4,686 vaccinations, found that vaccine

efficacy was lower in vaccinees from endemic areas, supporting immune activation differs in endemic and non-endemic vaccinees (Jean et al., 2016). Conversely, there is evidence of waning immunity in vaccinees in non-endemic areas, independent of exposure to related flaviviruses (Collaborative group for sudies on yellow fever vaccines, 2014). Additionally, it has been suggested that vaccinees who clear the virus without prolonged presentation of antigen to T cells and B cells may not mount a strong immune response, thereby failing to induce lifelong immunity (Amanna & Slifka, 2016) .

1.1.6 Lessons learned from concurrent outbreaks in Africa: fractional dosing in emergency scenarios

Immunization strategies involve delivery of 17D vaccine in endemic settings via routine immunization and as a "travel" vaccine for those who visit endemic areas. Mass vaccination campaigns are used in endemic areas to catch-up on immunization of unvaccinated cohorts not eligible for routine immunization during outbreaks. Despite the successes of vaccination strategies in Africa, particularly West Africa, it became evident in 2016 that strategies to immunize individuals in countries where YF outbreaks are sporadic, often decades apart, needs to be re-evaluated.

The Angola outbreak began in December of 2015 and peaked during February 2016, but cases continued until June 23, 2016. All 18 provinces in Angola reported cases and deaths due to YF. After multiple vaccination campaigns, requiring 20 million doses of 17D vaccine, the outbreak in Angola was under control (World Health Organization, 2016a) and was the largest outbreak in Angola since 1971 (Grobbelaar et al., 2016), with 4,347 suspected cases and 377 deaths (World Health Organization, 2016b). There have been examples of introduction of YFV to other locales by virus-infected humans during

outbreaks; this became very evident during the Angola outbreak. Unvaccinated travelers led to cases in DRC (2800), Mauritania (1), Kenya (2), and China (11) (Barrett, 2016; Chan, 2016; Green, 2016; World Health Organization, 2016b). Of the 2800 suspected cases in DRC, at least 57 were imported Angola and 13 were autochthonous, requiring control of the outbreak with 9.4 million doses of the 17D vaccine (World Health Organization, 2016b). The cases in China were the most worrying. There were over 250,000 Chinese workers in Angola during the outbreak in 2016 and their YF vaccination status is unclear. Eleven clinical cases of YF were reported in different areas of China in travelers returning from Angola, representing the first cases of YF in Asia. This outbreak has been a wake-up call for the potential spread of YFV to Asia, presumably by flights taken by non-vaccinated humans infected in Angola. There have been no reports of YF transmission in China but the question of pre-emptive vaccination of individuals in Asia or stockpiling vaccine for the potential control of YFV in Asia have been discussed. However, enforcement of the International Health Regulations, such that all individuals traveling to YF endemic areas are vaccinated and/or evidence of those returning from YF endemic areas have been vaccinated, are more practical measures given the huge population in *Ae. aegypti* infested areas of Asia.

Concurrently, outbreaks or sporadic cases not related to the ongoing outbreak in Angola were reported in Uganda, Brazil, Colombia, Chad, Ghana, and Peru (World Health Organization, 2016) and approximately 800,000 doses of 17D vaccine were distributed in Uganda (World Health Organization, 2016a). Thus, there is a need for continual vigilance of potential YF activity and the need for vaccine to be available during emergencies.

UNICEF maintains a reserve stockpile of 6 million doses of vaccine for control of outbreaks. The outbreak in Angola, DRC, and Uganda diminished the stockpile of 17D vaccine, and in Angola specifically, the reserve was exhausted twice in 2016. There are only four WHO prequalified manufacturers who can produce 80 million doses annually between them (World Health Organization, 2016c). By the end of August 2016, the projected doses needed to control the outbreak would exceeded the supply. Discussions turned to alternative approaches that could used in emergency scenarios in which demand outstrips supply, such as administering the vaccine as a "fractional dose".

Although the minimum amount of virus is 1000 international units (IU) per dose, all producers manufacture vaccine containing an excess of virus, often 10,000 IU or higher; there are no regulations regarding the maximum amount of virus in a dose (Barrett, 2016). Consequently, there was the possibility to give vaccinees less than one dose and still administer at least 1,000 IU. Each dose of vaccine is reconstituted in a volume of 0.5ml. Thus, a strategy was investigated to give vaccinees 0.1ml via a tuberculin syringe such that there was more than 1,000 IU per 0.1 ml. Review of the literature showed that of the four prequalified vaccines, only the 17DD vaccine manufactured in Brazil had publications investigating dose sparing; according to two studies with 17DD vaccine, doses as low as 587 IU achieved equivalent seroconversion, immune activation, and neutralizing antibodies as a full dose (Campi-Azevedo et al., 2014; Martins et al., 2013); but only subdoses as low as 3,013 IU mimicked viremia kinetics of the full dose (Campi-Azevedo et al., 2014) . The WHO recommended that a subdose of >3,000 IU per 0.1ml of the 17DD vaccine be used for immunizations in

Angola and DRC on an emergency basis only in August/September 2016 (World Health Organization Secretariat information paper, 2016).

Since a full-dose of vaccine was not being administered, vaccinees would not receive an international YF vaccination certificate for the factional dose immunization. Adults were the only participants in both sub-dose studies, therefore, it was decided that fractional doses could only be given to those over two years of age. Thirty-two health zones in Kinshasa, DRC with a population of over 7 million plus 15 health zones on the border between DRC and Angola were at risk for YF (Wolrd Health Organization, 2016). Therefore, an emergency campaign was initiated in August 2016 using a fractional dose approach. One-fifth of a full dose was administered subcutaneously to everyone over 2 years, while children aged 9-23 months and pregnant women received a full dose (World Health Organization Secretariat information paper, 2016); studies are on-going to determine the immunogenicity and immune longevity to fractional dosing, providing the additional data on the immune response in children and women, which was not evaluated in the previous subdosing studies.

1.2 GENETIC DIVERSITY OF RNA VIRUSES AND FLAVIVIRUSES

1.2.1 Genetic Diversity and Viral Populations

RNA viruses have high mutation rates; rapid viral kinetics/lysis time, possessing an error-prone RdRp due to lack of error-proofing replication, replication machinery, and elements within the genomic sequence all contribute to high mutation rate (Borderia et al., 2016; Lauring & Andino, 2010; Sanjuán & Domingo-Calap, 2016). Therefore, it was hypothesized that nucleotide mis-incorporation gives rise to sub-populations of genetically related viruses, commonly referred to as single nucleotide variants (SNV).

Studies of wild-type poliovirus and RdRp fidelity mutants of poliovirus confirmed *in vitro* and *in vivo* that RNA viruses exist cooperative viral populations that contribute to immunogenicity and virulence, where virulence is proportional to genetic diversity (Pfeiffer & Kirkegaard, 2003, 2005; Vignuzzi et al., 2005; Vignuzzi et al., 2006). Additional studies, primarily focused on RdRp fidelity mutants, bolstered that mutations could be introduced into the RdRp of RNA viruses that would alter genetic diversity and viral fitness (Korboukh et al., 2014; Coffey & Vignuzzi, 2011; Zeng et al., 2014; Coffey et al., 2011; Van Slyke et al., 2015; Rozen-Gagnon et al., 2014; Stapleford et al., 2015; Zeng et al., 2013). Few studies have sought to evaluate other mechanism of viral mutation and whether or not mutations outside of the RdRp affect replication fidelity.

1.3 MOLECULAR BASIS OF ATTNENUATION OF THE 17D-204 VACCINE

The 17D vaccine strain is considered to be one of the most effective and safe, live attenuated viruses developed to date. However, despite 17D vaccine being derived in the 1930s from wild-type strain Asibi by passage in chicken and mouse tissue, the mechanism of attenuation is poorly understood. With the advent of advanced sequencing technology, some light has been shed on the mechanism of attenuation.

1.3.1 Cellular response to wild-type Asibi virus and 17D-204 vaccine

Wild-type Asibi and 17D vaccine differentially induce immune activation and it appears to be cell type dependent. Infection of human monocyte derived macrophages (MDM) with 17D vaccine induced high levels of INFα, TNFα, and IL-1RA that steadily decreased after infection, while wild-type Asibi virus initially down-regulated immune activation (Cong et al., 2016). However, in human Kupffer cells wild-type Asibi virus

did not appear to down-regulate immune activation, inducing a more prominent immune response than 17D vaccine, which was evident by higher levels of TNFα, IL-8, RANTES, and IL-10 (Woodson et al., 2011). Infection of human monocyte derived dendritic (MoDC) cells with 17D vaccine and wild-type Asibi virus infection led to minimal induction and only MoDC cells infected with 17D vaccine were able to stimulate IL-2 and IFNγ producing CD4+ T cells, suggesting that wild-type Asibi virus inhibits this response (Cong et al., 2016; Watson et al., 2016). Interestingly, wild-type Asibi virus down-regulates the immune response in human hepatocytes cells, while 17D-204 induces a strong pro-inflammatory response.

Given the role of NS proteins in replication and the innate immune response, substitutions in NS proteins may contribute to the attenuated phenotype of 17D, and the differential regulation of wild-type YF and 17D vaccine.

1.3.3 Genetic diversity of wild-type Asibi virus and 17D-204 vaccine

Like most RNA viruses, wild-type YFV replication is error-prone due to the lack of proof-reading by the virus-encoded RdRp. However, there is evidence that replication of 17D is not as error-prone as wild-type RNA viruses (Pugachev et al., 2004). Recent studies have used Next Generation Sequencing to compare the RNA populations in wild-type Asibi and 17D vaccine virus. Wild-type Asibi virus was found to have the typical heterogeneous population of an RNA virus while the 17D vaccine population was relatively homogeneous and there is limited intra- and inter variability of 17D vaccines (Beck et al., 2014; Salmona et al., 2015; Tangy & Desprès, 2014). It is hypothesized that the limited genetic diversity of the 17D vaccine virus attributes to vaccine attenuation and safety.

1.4 SPECIFIC AIMS AND RATIONALE

1.4.1 Specific Aim 1: Determine the genetic diversity of infectious clone (IC)-derived viruses using Next Generation Sequencing (NGS): wild-type Asibi strain, 17D-204 vaccine strain, and chimeric Asibi/17D viruses.

Hypothesis**:** One or more substitution in the RC is/are responsible for the lack of quasispecies population of 17D vaccine strains.

Rationale: Multiple studies have shown that a combination of lack of error-reading by the RdRp, rapid virus multiplication kinetics, and host selection pressures all participate in the generation of RNA quasispecies (Gregori et al., 2014; Grubaugh et al., 2015; Korboukh et al., 2014; Lauring & Andino, 2010; Vignuzzi et al., 2006). Most of these studies have been performed utilizing poliovirus as the prototype, where chemical mutagenesis was used to generate a G64S substitution in the RdRp to yield a high fidelity mutant (Korboukh et al., 2014; Vignuzzi et al., 2006). Interestingly, this mutation is not present in any of the Sabin vaccines derived from wild-type polioviruses (Bandyopadhyay et al., 2015; Stanway et al., 1984).

Pugachev and colleagues found, unlike typical flaviviruses (e.g., wild-type Asibi strain), 17D-204 vaccine had a theoretical error rate of 2×10^{-7} substitutions per site based on examining errors in transcription of a 17D IC cDNA template, which is not the same as a RNA genome template. Nonetheless, this intriguing finding suggested that 17D-204 vaccine should have limited viral population structure. Beck et al. (2014) confirmed this hypothesis by using NGS to compare 17D-204 vaccine strain to wild-type Asibi virus; showing that 17D-204 vaccine is less variable than the wild-type Asibi virus and quasispecies population is fixed in the vaccine, whereas Asibi virus appears as a

typical RNA virus. The relationship of wild-type Asibi virus to the 17D-204 vaccine presents a unique opportunity to study genetic diversity of a naturally high fidelity vaccine, as well as other substitutions that differ in a low diversity vaccine when compared to the wild-type virus from which it was derived.

The RdRp of Flaviviruses are encoded by the NS5 protein, which contains two (NS5-836 and NS5-900) of the 20 common amino acid substitutions that differentiate wild-type Asibi virus and the 17D vaccine. The RdRp is complexed with NS1, NS2A, NS2B, NS3, NS4A, and NS4B to form the RC (Selisko et al., 2014; Shi, 2014). Formation of the RC, along with formation of viral membranous vesicle packets, is required for viral replication. Specifically, the NS4B protein is thought to be the scaffold for the RC and mutations in NS4B protein genes adversely affect replication (Paul et al., 2011; Youn et al., 2012; Zou et al., 2014; Zou, Lee, et al., 2015; Zou, Xie, et al., 2015).

1.4.2 Specific Aim 2: Determine the multiplication kinetics in A549 cells of IC-derived Asibi strain, IC-derived 17D-204 vaccine strain and IC-derived Asibi/17D chimeric viruses.

Hypothesis: Attenuating substitutions in NS protein genes effect viral replication, evident by altered multiplication kinetics in A549 cells.

Rationale: Immunization with 17D vaccine leads to a transient viremia that rarely exceeds 10^2 pfu/ml, contrasting drastically with the viremia seen following infection with wild-type infection, which can exceed 10^8 pfu/ml (Macnamara, 1957). Studies with other flaviviruses demonstrated that mutations in NS protein genes decreased virus production and replication in host cells (Audsley et al., 2011; Grant et al., 2011; Xie et al., 2011). Therefore, attenuating substitutions in the genome of 17D vaccine that effect replication

efficiency may also affect multiplication in host cells, which may ultimately relate to differences in viremia and dissemination. Additionally, the lack of genetic diversity of 17D vaccine decreases the generation of a population with beneficial mutations that may overcome this barrier.

In vitro studies have shown that various host cells are permissive to YFV infection, yet none have conclusively differentiated between the multiplication kinetics of wild-type Asibi virus and the 17D vaccine (Woodson & Holbrook, 2011; Woodson et al., 2011). Sometimes, the multiplication kinetics of wild-type Asibi and 17D viruses differ at limited time points only prior to reaching peak titer. The lack of a suitable cell line that differentiates multiplication kinetics of Asibi and 17D viruses impedes the evaluation of attenuating substitutions on multiplication kinetics, which may contribute to decreased viremia and dissemination seen following vaccination with 17D virus.

1.4.3 Specific Aim 3: Determine induced pro- and anti-inflammatory cytokines/chemokines and expressed genes in A549 cells following infection with IC-derived Asibi strain, IC-derived 17D-204 vaccine strain and IC-derived Asibi/17D chimeric viruses.

Hypothesis*:* Attenuating substitutions will negate innate immune regulation typical of wild-type infection.

Rationale: During natural infection with wild-type YFV, IL-6, MCP-1, IP-10, TNF-α, and IL-1RA have been associated with disease severity (ter Meulen et al., 2004). The most predominant finding in YFV infection is severe liver damage characterized by apoptosis of hepatocytes with a disproportion infiltration of immune cells to the amount

of damage; induction of TNF-α and TGF-β, have been associated with liver damage (Quaresma et al., 2006; Querec & Pulendran, 2007). Interestingly, immunization with 17D-204 vaccine strain induced similar cytokines/chemokines and expressed genes as wild-type infection, but neither productive infection nor severe liver pathology are evident in healthy vaccinees (Campi-Azevedo et al., 2012; Gaucher et al., 2008). The adaptive immune response is the most critical component in viral clearance and disease recovery, but it has also been shown that the innate immune response is also important.

NS proteins of flaviviruses have been implicated in modulating the innate immune response and evading the immune system; specifically, the NS4B protein is a known interferon antagonist (Muñoz-Jordán et al., 2005; Xie et al., 2011; Zmurko et al., 2015). The interferon response is a critical component of the antiviral immune response (Aguirre et al., 2012; Ashhurst et al., 2013; Jones et al., 2005; Muñoz-Jordán et al., 2005; Ye et al., 2013). The importance of innate immunity during YFV infection and vaccination with 17D has been implicated in multiple studies, including those where vaccine associated adverse events were attributed to defects in innate immunity (Belsher et al., 2007; Gaucher et al., 2008; Pulendran, 2009; Pulendran et al., 2008, 2013; Querec & Pulendran, 2007; T. Querec et al., 2006). Attenuating substitutions in NS proteins may affect protein function; negate virus-induced regulation of innate immunity that supports viral survival and replication, thereby decreasing virulence.

Studies in host cells where differences in multiplication kinetics of wild-type Asibi virus and 17D vaccine were identified generally corresponded to differences in induced cytokines/chemokines (Woodson & Holbrook, 2011; Woodson et al., 2011). Since multiplication kinetics in A549 cells of IC-derived Asibi and IC-derived 17D

viruses differ dramatically, there should be a corresponding difference in pro- and anti-inflammatory cytokine/chemokine. This allows for the investigation of the effects of attenuating substitutions on innate immunity.

CHAPTER 2: MATERIALS AND METHODS

2.1. GENERATION OF IC cDNA PLASMIDS

Yellow fever virus (YFV) infectious clones (IC) that were not previously generated in the laboratory were generated by PCR based site-directed mutagenesis (SDM). NS2B-109 and M-36 single mutant YFV ICs were generated utilizing either pANCR 17D IC or pANCR Asibi IC (Bredenbeek et al., 2003; McElroy et al., 2005), while structural genes containing pre-membrane and envelope (prME) substitutions, pANCR 17D/Asibi prME IC or pANCR Asibi/17D prME, were utilized to generate prME + NS2B-109 ICs.

QuickChange XLII SDM kit (Strategene) along with mutagenic primer sets (**Table 2.1**) were used to generate ICs as per the manufacturer's protocol. Briefly, 10 ng of template plasmid and 125 ng of the sense and anti-sense mutagenic primers were used per reaction and subjected to PCR under standard conditions and three stage PCR reaction (**Table 2.2**). Following PCR, the reaction mixture was digested with 1 ul of *Dpn*-I for 1 hour at 37ºC. Digested cDNA was transformed in XL-1 Gold supercompetent cells (Strategene), then plated on LB agar plates (Sigma) supplemented with 0.5% sodium chloride overnight at 37ºC. Bacterial culture plates were stored at 4 ºC for future use.

Table 2.1 Mutagenesis Primer Sets (written 5' to 3')

pANCR IC	Primer Sequence
17D*M-F36L substitution	
	Sense AAAAGATTGAGAGATGGCTAGTGAGGAACCCCTTTTTTG
	Anti-sense CAAAAAAGGGGTTCCTCACTAGCCATCTCTCAATCTTTT
17D*NS2B-L109I substitution	
	Sense GCCTTGGTTGGGGCTGCCATTCATCCATTTGCTCTTCTG
	Anti-sense CAGAAGAGCAAATGGATGAATGGCAGCCCCAACCAAGGC
Asibi*NS2B-I109L	
	Sense GCCTTGGTTGGGGCTGCCCTCCATCCATTTGCTCTTCTG
	Anti-sense CAGAAGAGCAAATGGATGAATGGCAGCCCCAACCAAGGC

Table 2.2 PCR Based Mutagenesis Reaction Protocol

Stage	**Stage 1 x 1**	**Stage 2 x 20**			**Stage 3 x 1**	
Temperature	95.0ºC	95.0ºC	60.0ºC	68.0ºC	68.0ºC	4.0ºC
Time	1:00	0:50	0:50	13:00	7:00	∞

cDNA YF IC plasmids were harvested by large-scale extraction. Briefly, 200 ml cultures of a single colony of the stored bacterial plate were incubated at 37ºC, 200 rpm for 12-14 hours. The bacterial culture was then pelleted at 4ºC by centrifugation and resuspended in cold glucose-Tris-EDTA. 0.2N-NaOH/1%-SDS solution was used for cell lysis, followed by 3M KOAc (pH 4.8) to neutralize the reaction. The plasmid was pelleted by isopropanol precipitation and then resuspended in TE buffer (10 mM Tris-HCL, pH 8.0; 0.1 mM EDTA) (Corning), followed by treatment with 50 ul of RNAse A (10 mg/ml) (Amresco) for 30 minutes at 37ºC, and purification by phenol:chloroform extraction. The purified plasmid was further concentrated using Clean and Concentrate kit according to the manufacturer's instructions (Zymo). Sanger sequencing was then used to verify the engineered substitution was present. Stock cDNA YF IC were amplified in *E. coli* (strain MC1061) and plasmid harvested by large-scale extraction as described previously.

2.2 PREPARATION OF IC-DERIVED VIRUSES

Four ug/ml of cDNA YF ICs were linearized by digestion with *XhoI* (10,000 units/ml) (New England BioLabs) for 17D backbone viruses and *NruI* (10,000 units/ml) (New England BioLabs) for Asibi backbone viruses at 37ºC for 2 hours. Linearized cDNA was further digested with 50 ug/ml proteinase K, purified twice by phenol:chloroform and once by chloroform extraction, followed by ethanol precipitation. Full-length YF RNA was generated by *in vitro* transcription of purified cDNA using SP6-Scribe standard RNA in vitro transcription kit (Cellscript) according to the manufacturer's protocol; the reaction was incubated at 37ºC for 2.5 hours. The entire reaction was electroporated into $6.7x10^6$ monkey kidney Vero cells in a pre-chilled 2 mm

cuvette using a Bio-Rad Gene Pulser set at 1.5kV, infinite Ohms, and 25μF. Electroporated cells were allowed to recover for 10 minutes at room temperature and then transferred into 15 ml of pre-warmed MEM supplemented with 8% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate, and penicillin/streptomycin in a T-75 culture flask. Cell supernatant was collected when infected cells showed 80% cytopathic effect (CPE). RNA from the YF IC-derived viruses were extracted using QIAamp Viral RNA mini kit (Qiagen), following the manufacturer's guidelines.

Infectious virus was titrated in duplicate by immuno focus-forming assay (IFA) in monkey kidney Vero cells. Prior to titration, monkey kidney Vero cells were allowed to adhere to 12 well plates at 37ºC under 5% CO_2 for at least 18 hours. Adherent cells were incubated with 10-fold serially diluted virus at room temperature for 30 minutes, then overlaid with 2 ml of 0.8% carboxymethyl cellulose made with minimal essential media (MEM) supplemented with 8% fetal bovine serum (FBS), 1% L-glutamine, 1% sodium bicarbonate, and 1 % penicillin/streptomycin. IFAs were fixed with a mixture of 1 part acetone to 1 part methanol at 4 dpi. IFAs were stained using YFV Asibi mouse immune ascitic fluid polyclonal antibody (World Reference Center for Emerging Viruses and Arboviruses, UTMB, Galveston, TX) as the primary antibody (1:2500), goat anti-mouse (Sigma) as the secondary antibody (1:1000), NeutrAvidin (Sigma-Aldrich) as the tertiary stain (1:1000). Foci were visualized with 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich) following manufactures guidelines.

2.3 DATA ANALYSIS OF NEXT GENERATION SEQUENCING (NGS) DATASETS

RNA was extracted from IC-derived viruses processed according to an established and validated in house pipeline (**Figure 2.1**). NGS libraries were constructed using TruSeq RNA v2 kit as recommended by the manufacturer, then sequenced on an Illumina HiSeq1500 instrument by the UTMB Next Generation Sequencing Core. Paired-end reads were quality trimmed to minimum 35 bases and a minimum quality score of 35 using trimmomatic (version 0.22), then realigned to a reference genome for wild-type Asibi virus (AY640589) using Bowtie2 local alignment mode and very sensitive setting (version 2.2.4). PCR duplicates were removed using Picard-tools (version 1.120) using default settings and an optical duplicate distance of 0. Qualimap (version 2.2) was used to assess the quality and determine the mean read coverage of NGS datasets. The NGS datasets were matched by random down-sampling using Picard-tools (version 1.120) to the virus with the lowest mean coverage. Vphaser2 (version 2.0) was used to determine variant population; all single nucleotide variants (SNVs) controlled for false discovery and strand-bias were considered in the analysis (Yang et al., 2013) .

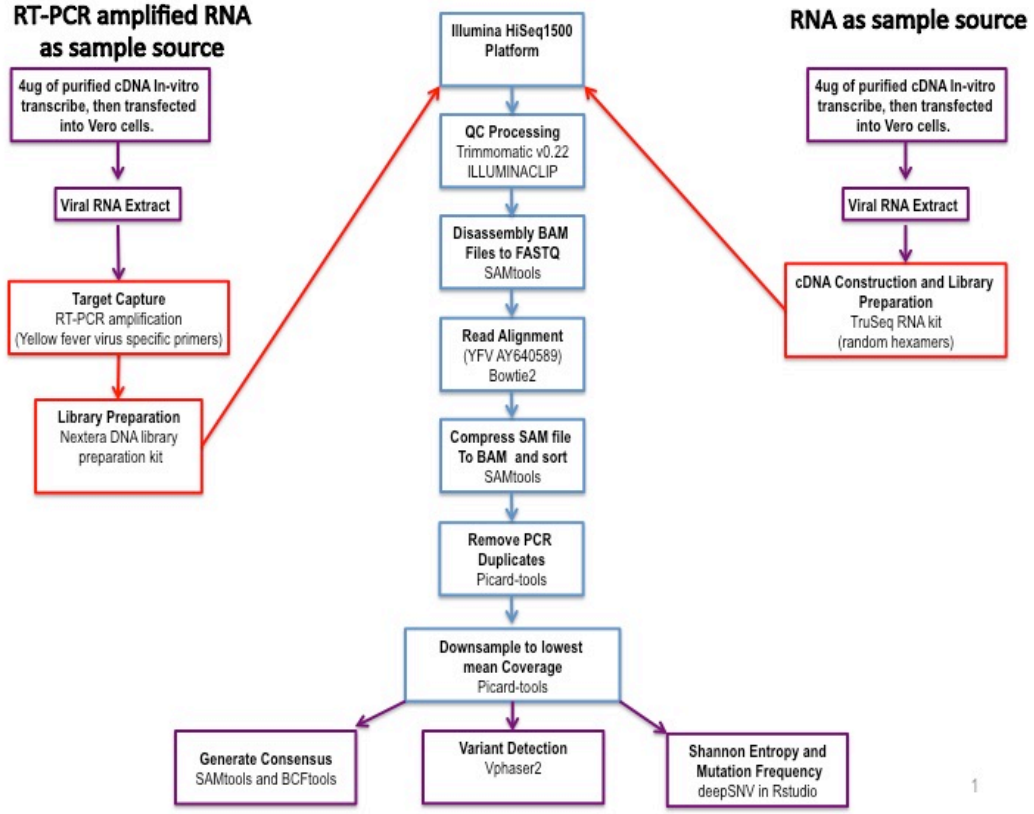

Figure 2 .1. Experimental design and Next Generation Sequencing Pipeline. Two different sample sources were used to generate the NGS libraries; red denotes where the procedures differ between the two methods, purple denotes experimental outputs needed for analysis, and blue denotes the pipeline used to process the dataset.

Diversity indices utilizing the NGS datasets were determined for the ORF by using deepSNV (version 1.16.0) and local R scripts (version 3.2.4). Briefly, nucleotide counts for genomic positions were determined and then mutation frequencies were calculated for each genomic position; the total number of mutant bases called for a genomic position was divided by total number of bases called at the genomic position, as previously described (Acevedo et al., 2014). Nucleotide counts were then converted to relative nucleotides frequency. Nucleotide diversity by gene regions was determined by calculating Shannon's entropy using the nucleotide frequencies as previously described (Nishijima et al., 2012). IC-derived Asibi and 17D-204 viruses were first compared using Mann-Whitney, before comparing the IC-derived chimeric and mutant viruses using Kruskal Wallis and Dunn's multiple comparison test unless otherwise stated; p-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***) (GraphPad Prism, version 7.0a).

2.4 INFECTION OF HUMAN ALVEOLAR A549 CELLS

A549 cells were purchased from ATCC and maintained according to ATCC guidelines in MEM supplemented with 8% fetal bovine serum, L-glutamine, sodium bicarbonate, and penicillin/streptomycin. Prior to infection, A549 cells were allowed to adhere to plates at 37ºC under 5% CO_2 for at least 18 hours. Adherent cells were infected at an MOI of 0.1 in triplicate and incubated at room temperature for 30 minutes. After the inoculum was removed, cells were washed three times with PBS then incubated with 2 ml of MEM supplemented with 2% FBS, L-glutamine, sodium bicarbonate, and penicillin/streptomycin at 37 ºC under 5% CO_2; cell supernatant collected at 0, 12, 24,

36, and 48 hours post infection (hpi). Cell supernatants were stored at -80ºC until assayed.

Infectious virus was titrated in duplicate by IFA in Vero cells as described in section 2.2. Multiplication kinetics of IC-derived Asibi and 17D-204 viruses were first compared using student's t-test, before comparing the IC-derived chimeric and mutant viruses using one-way ANOVA and Tukey's multiple comparison test; p-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***) (GraphPad Prism, version 7.0a).

Bio-Plex Pro Human Chemokine 40-Plex Assay was used to evaluate cytokine and chemokine induction at 48 hpi on cell supernatant according to the manufacturer's guidelines. Briefly, undiluted cell supernatant was thawed on ice and placed at room temperature for 15 minutes prior to assaying and 50 ul of cell supernatant was loaded in duplicate. First, coupled magnetic beads and cell supernatant were incubated for one hour and then washed three times; second, detection antibodies were added and incubated for 30 minutes and then washed three times; finally, streptavidin-PE was added and incubated for 10 minutes and washed three times. All washes were performed with a magnetic washer (Bio-Rad) and incubations were carried out at room temperature on a shaker at 850 rpm. Data was acquired on Bio-Plex 200 system (Bio-Rad). Cytokine and chemokine were reported as fold-change from mock infected; IC-derived Asibi and 17D-204 viruses were first compared using student's t-test, before comparing the IC-derived chimeric and mutant viruses using one-way ANOVA and Tukey's multiple comparison test; p-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***) (GraphPad Prism, version 7.0a).

CHAPTER 3: GENETIC DIVERSITY OF IC-DERIVED ASIBI VIRUS AND 17D-204 VACCINE

3.1 INTRODUCTION

RNA viruses exist as a collection of genetically related viral RNA population that contributes to pathogenicity and virulence (Bordería et al., 2016; Borderia et al., 2011; Vignuzzi et al., 2006; Vignuzzi et al., 2005). It has been show that wild-type flaviviruses exhibit high degree of genetic diversity, including YFV, WNV, and dengue (Beck et al., 2014; Grubaugh et al., 2016; Sessions et al., 2015; Van Slyke et al., 2015). However, it has been shown that the 17D-204 vaccine strain is relatively homogeneous with decreased genetic diversity compared to wild-type parental strain Asibi (Beck et al., 2014; Salmona et al., 2015). The mutation rate, determined by phylogenetic analysis, for wild-type Asibi virus is approximately 2.1 x 10^{-4} substitutions/site/year, consistent with what is expected for RNA viruses (Sall et al., 2010). However, an *in vitro* study determined IC-derived 17D-204 virus has an error rate of 1.9 x 10^{-7} to 2.3 x 10^{-7} errors/replication (Pugachev et al., 2004), suggesting that the higher fidelity of the replication machinery of 17D-204 vaccine may attribute to the limited diversity of the vaccine.

The differences in genetic diversity of wild-type Asibi virus and 17D-204 vaccine provides an unique opportunity to investigate RNA population theory in the context of a well described live attenuated vaccine. Utilizing infectious clone (IC) derived viruses recovered in a controlled experiment, establishes that the genetic diversity differences that exist between wild-type Asibi and 17D-204 vaccine viruses are not influenced by

either different passage history or source of samples, but rather resulted from the attenuation of wild-type Asibi virus to yield the 17D vaccine sub-strains. This dissertation chapter provides a framework to investigate whether or not one or more of the 20 amino acid substitutions that differentiates wild-type Asibi virus and 17D vaccine sub-strains also contributes to genetic diversity.

NGS datasets are largely composed of a predominant genotype with small single nucleotide variants (SNVs) that contribute to the viral population and genetic diversity of viruses. The number and percentage of SNVs identified, nucleotide diversity, and mutation frequency were used to assess the complexity of the viral population and overall genetic diversity.

3.2 RESULTS

Target capture is a technique employed to isolate viral RNA from samples and enhance NGS runs. NGS runs have either 160-180 or 250-300 million reads/lane depending on the size of the flow cell. Samples with high concentrations of host RNA detract the available read coverage for the viral RNA sample. Therefore, the more host RNA present in the sample, the less read coverage available for viral RNA. Target capture by means of full genome RT-PCR amplification of RNA samples ensures adequate read coverage for proper analysis and was the approach utilized in previous genetic diversity studies of wild-type Asibi virus and 17D-204 vaccine (Beck et al., 2014). However, target capture by RT-PCR has the potential to introduce additional sequencing bias. Therefore, two methods were employed in evaluating the genetic diversity of IC-derived Asibi and 17D-204 viruses. First, genome amplification of the IC-derived viruses from cell supernatant utilizing the same six overlapping YFV specific

primers to generate six amplicons as previously described were used for NGS libraries with the Nextera DNA library kit (Illumina), such that only the IC-derived viral DNA would be sequenced (Beck et al., 2014). Second, total RNA of the cell supernatant was used to generate cDNA and NGS libraries utilizing the Truseq RNA library preparation kit (Illumina) (**Figure 3.1**). The generation of cDNA with Truseq does not amplify the RNA with YFV specific primers as seen with target capture by RT-PCR but utilizes random hexamers for first and second strand synthesis, therefore, all RNA present in the sample was sequenced.

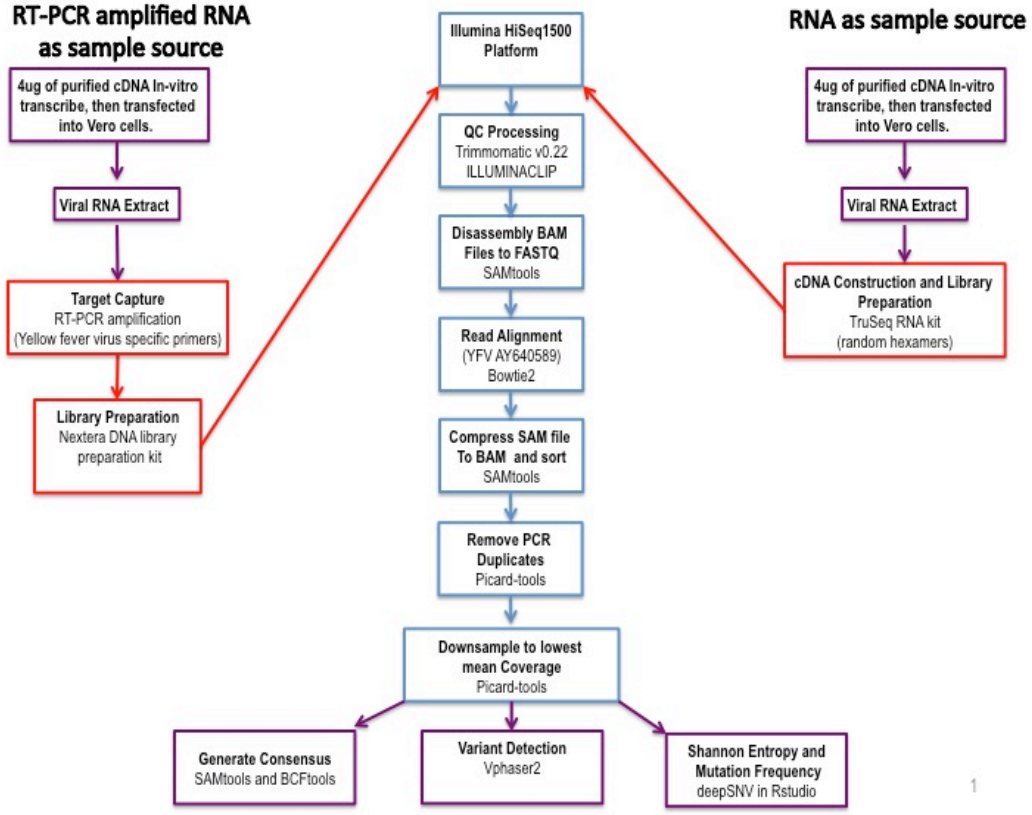

Figure 3.1. Experimental design and Next Generation Sequencing Pipeline. Two different sample sources were used to generate the NGS libraries; red denotes where the procedures differ between the two methods, purple denotes experimental outputs needed for analysis, and blue denotes the pipeline used to process the dataset.

3.2.1 Generation of IC-derived Asibi and 17D-204 viruses

Although the viruses discussed in this dissertation were rescued in green monkey kidney Vero cells, the mean infectivity titers were consistent with previous published studies that utilized baby hamster kidney (BHK)-21 cells (Bredenbeek et al., 2003; Lee & Lobigs, 2008; McElroy et al., 2006). Each IC-derived virus was recovered on at least two separate occasions to generate data that was reproducible and subject to statistical analysis.

The mean infectivity titer for IC-derived Asibi virus was 10^4 ffu/ml, while the mean infectivity titer for IC-derived 17D-204 virus was 10^6 ffu/ml (**Table 3.1**). Interestingly, the mean diameter of foci of IC-derived Asibi virus differed from that of IC-derived 17D-204 virus; the foci of IC-derived Asibi virus was variable, measuring small and medium size, while the mean foci of IC-derived 17D-204 virus was uniform and small in size (**Table 3.1**). Differences in mean foci size of the IC-derived viruses were not noted in previous studies (McElroy et al., 2006a, 2006b); however, this is likely associated with either recovery of the viruses in Vero cells rather than BHK-21 cells or harvesting the viruses later during the infection process.

The read coverage for YFV primer RT-PCR amplified IC-derived Asibi and 17D-204 viruses were 12,179 and 13,343, respectively (**Table 3.1**). For the analysis, RT-PCR amplified IC-derived 17D-204 virus was down-sampled to 12,179 (the read coverage for IC-derived Asibi virus). Conversely, the average read coverage for IC-derived Asibi and 17D-204 viruses sequenced from total RNA were 6,891 and 4,008, respectively. For the analysis, the IC-derived viruses sequenced from total RNA were down-sampled to 2026

(the lowest mean coverage for all RNA sourced IC-derived viruses used in the dissertation).

There were no differences identified between the *de novo* consensus sequences after further processing of the datasets, including removing PCR duplicates and down-sampling. The generated consensus sequences were compared to wild-type Asibi (KF769016) and 17D-204 vaccine (KF769015) reference strains. IC-derived Asibi virus had four non-coding nucleotide changes in the capsid and pre-membrane genes at genome positions 304, 370, and 493 that were not present in wild-type Asibi virus. IC-derived 17D-204 virus had one coding nucleotide change in the NS2A gene at position 4025 that was not present in the 17D-204 vaccine, which encoded for NS2A-V173M substitution, which was previously identified and shown to not affect the expected phenotype in *Ae. aegypti* mosquitoes (McElroy et al., 2006).

Table 3.1. Infectious Clone (IC)-derived viruses descriptive characteristics for IC-derived Asibi and 17D-204 viruses.
SF: small foci, < 1 mm; MF: medium foci, 1-2 mm; LF: large foci, ≥ 2 mm

IC	Coverage by Replicate R1;R2	Mean Coverage	Titer by Replicate R1;R2	Mean Titer of recovered virus (ffu/ml)	Mean Foci Size
17D	5034; 2982	4008	2.2×10^6; 3.2×10^6	6.1×10^6	Uniform; MF
Asibi	8547; 5234	6891	4.9×10^4; 7.2×10^4	6.1×10^4	Mixed; SF and MF

3.2.2 RT-PCR amplification with YFV specific primers of IC-derived Asibi and 17D-204 confirms previous studies on genetic diversity of wild-type Asibi and 17D-204 viruses

3.2.2.1 Single nucleotide variant diversity of RT-PCR amplified IC-derived Asibi and 17D-204 viruses

Vphaser2 accurately identifies low percentage SNVs (Yang, 2012) and was used to identify differences in the variant population generated by IC-derived Asibi and 17D-204 viruses. All variants were classified as coding or non-coding mutations, and those that exceeded 1% of the viral population were further evaluated.

Six hundred and fifty-nine SNVs were identified for IC-derived Asibi virus, including 395 coding SNVs and 30 SNVs in the 3' NCR (**Figure 3.2**). Seven coding SNVs and 4 SNVs in the 3' NCR exceeded 1% of the viral population (**Table 3.2**). Three additional non-coding SNVs were identified at genomic positions 6445, 7099, and 10285 that exceeded 1% of the viral population (**Table 3.2**).

Interestingly, 824 SNVs were identified for IC-derived 17D-204 virus, including 492 coding SNVs, four SNVs in the 5' NCR, and 32 SNVs in the 3' NCR (**Figure 3.2**). Only one coding SNV at genomic position 5760 and one SNV in the 3' NCR exceeded 1% of the viral population (**Table 3.2**).

There was considerable evidence of shared SNVs between IC-derived Asibi and 17D-204 viruses. Specifically, 265 SNVs were shared between the two viruses, including genomic position 10800 (**Figure 3.3**). This was most evident in IC-derived 17D-204 virus, since the majority of SNVs identified for this virus were less than 0.5% of the viral population. The SNV at genomic position 10800 represented 1.23% and 1.36% of the variant population for IC-derived Asibi and 17D-204 viruses, respectively, and was

one of only two SNVs that exceeded 1% of the population for IC-derived 17D-204 virus (**Table 3.2**).

The results show that variant population of IC-derived viruses resemble that of non-IC derived wild-type Asibi and 17D-204 vaccine viruses (Beck et al., 2014; Salmona et al., 2015) and demonstrates that IC-derived Asibi generates a more complex variant population than that of IC-derived 17D-204 virus.

Table 3.2. Single nucleotide variants greater than 1% of the population for RT-PCR amplified IC-derived Asibi and 17D-204 viruses.

Reference Base	Variant Base	Genomic Position	Gene Region	Coding/ Noncoding	Variant Frequency
IC-derived Asibi virus					
C	A	277	C	Yes; F53L	1.01%
G	T	2920	NS1	Yes; E934D	1.70%
T	A	3825	NS2A	Yes; L1236Q	1.38%
T	A	6445	NS4A	No	1.72%
G	A	7099	NS4B	No	1.28%
A	G	9605	NS5	Yes; N3163D	1.23%
G	A	10070	NS5	Yes: D3318N	5.46%
G	A	10142	NS5	Yes; E3342K	1.25%
T	C	10285	NS5	No	1.63%
T	C	10338	NS5	Yes; P3407L	1.46%
G	A	10459	3' NCR	Silent	2.04%
A	C	10466	3' NCR	Silent	1.09%
T	C	10550	3' NCR	Silent	1.62%
G	A	10800	3' NCR	Silent	1.24%
IC-derived 17D-204 virus					
G	A	5760	NS3	Yes; R1881K	1.16%
A	G	10800	3' NCR	Silent	1.24%

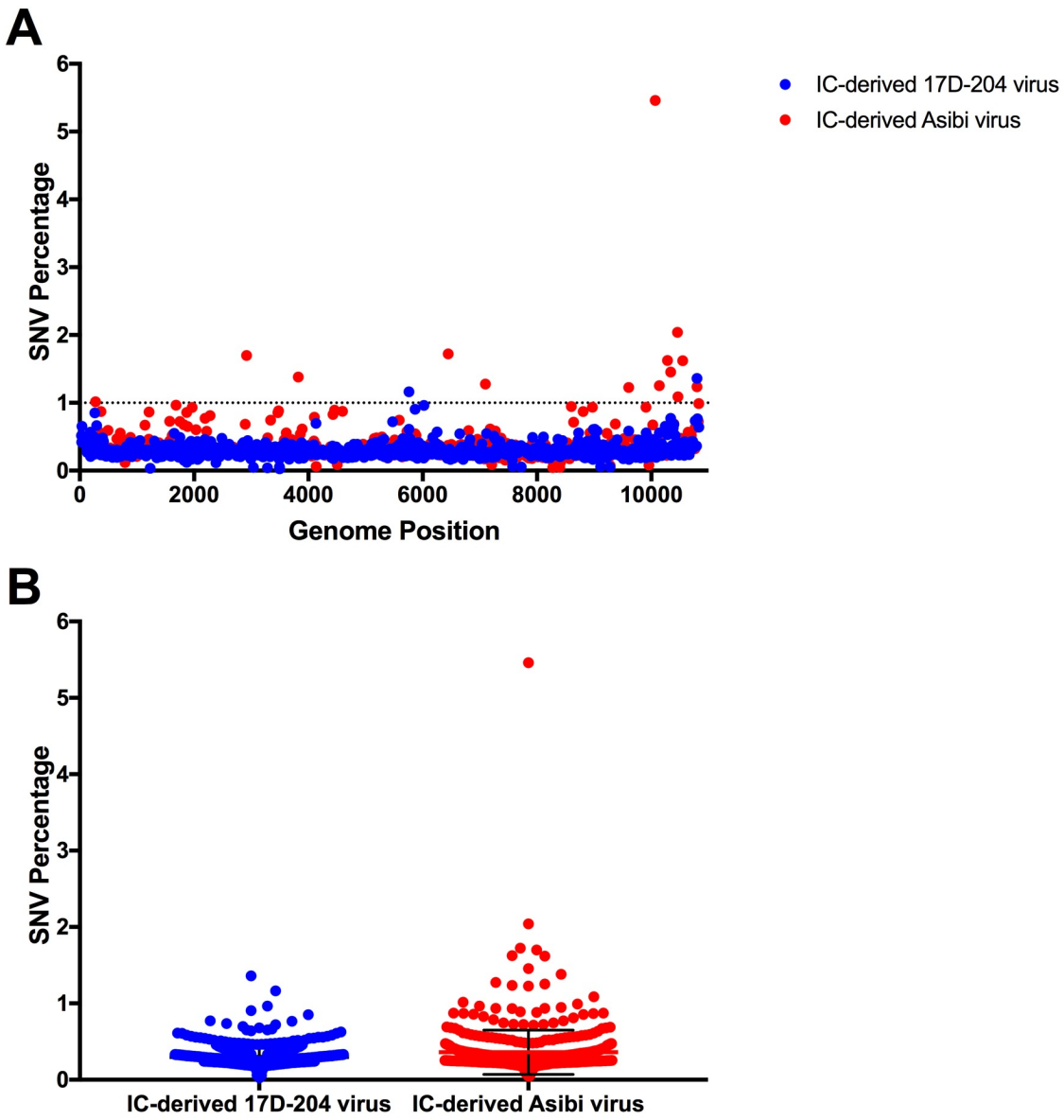

Figure 3.2. Single nucleotide variants identified for RT-PCR amplified IC-derived Asibi and 17D-204 viruses. a. SNVs across the genome, b. total number of SNVs. IC-derived Asibi virus generates a more diverse variant population than IC-derived Asibi virus.

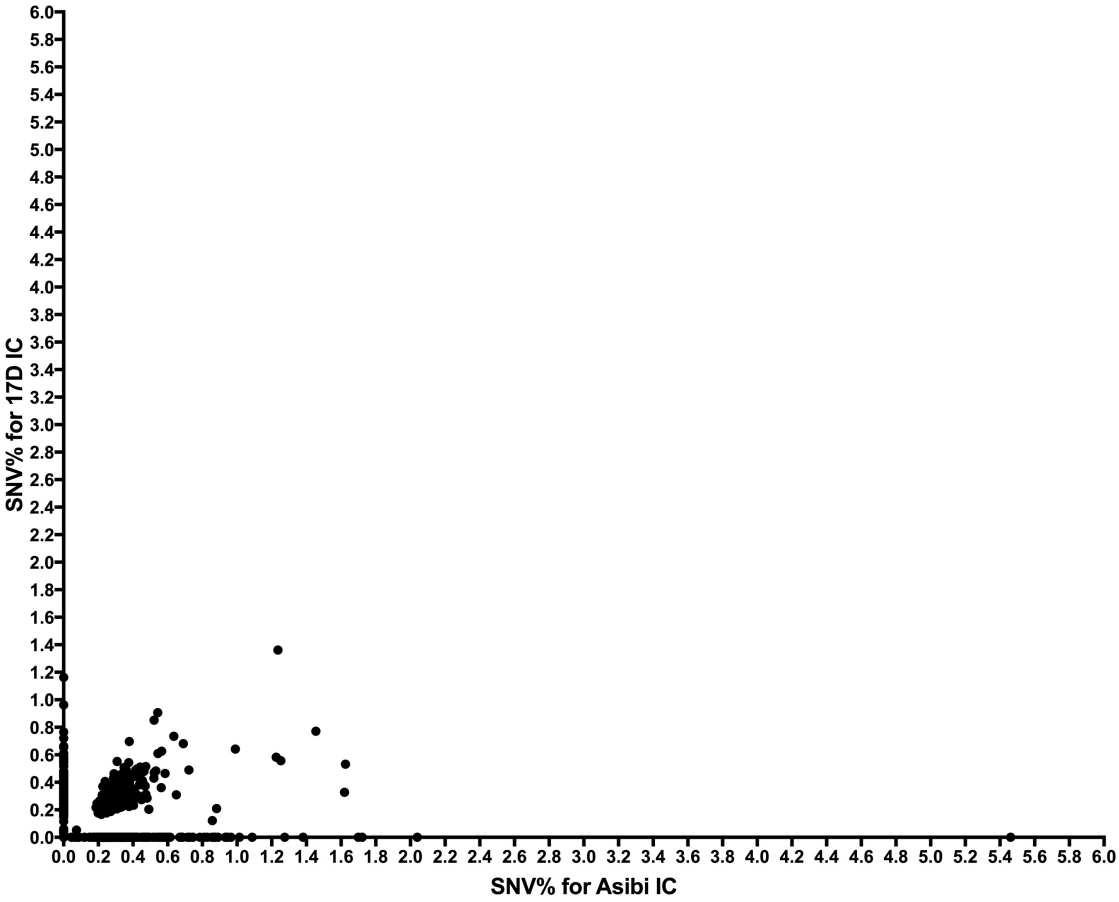

Figure 3.3. Comparison of the single nucleotide variant identified for RT-PCR amplified IC-derived Asibi and 17D-204 viruses. There were 265 SNVs shared between the two IC-derived viruses, greater than 30% were < 0.3% of the population.

3.2.2.2 Diversity indices of random hexamer RT-PCR amplified IC-derived Asibi and 17D-204 viruses

Shannon's entropy and mutational frequency were used to further evaluate genetic diversity. Both diversity indices utilize the nucleotides represented at a given genomic position. Shannon's entropy is sensitive to small deviations in nucleotide incorporation by considering all nucleotide possibilities (A, C, G, U and gap) at a given genomic position to determine nucleotide variation, while mutation frequency evaluates the total number of nucleotides in a population at a particular position that differ from the dominant nucleotide.

Similar to previous findings, the nucleotide diversity in the C, prM/M, E, NS1, NS2A/B, NS3, NS4A/B, and NS5 of IC-derived Asibi and 17D-204 viruses differed statistically (p-value < 0.001) (**Figure 3.4**). Furthermore, the mutation frequency of IC-derived Asibi virus was higher than that of IC-derived 17D-204 virus (**Figure 3.4**). Diversity indices correlated with identified SNVs for IC-derived 17D-204 virus, the Pearson r values exceed 0.5 when either Shannon's entropy or mutation frequency was compared to identified SNVs (**Figure 3.5**). However, the diversity indices did not correlate with identified SNVs for IC-derived Asibi virus, the Pearson r values were below 0.5 when either Shannon's entropy or mutation frequency was compared to identified SNVs. The correlation between diversity indices and identified SNVs for IC-derived 17D-204 virus is most likely associated with the high degree of low percentage variants observed in the dataset for IC-derived 17D-204 virus. The diversity indices for both IC-derived Asibi and 17D-204 viruses showed a greater degree of nucleotide variation than what was observed when evaluating the SNV population.

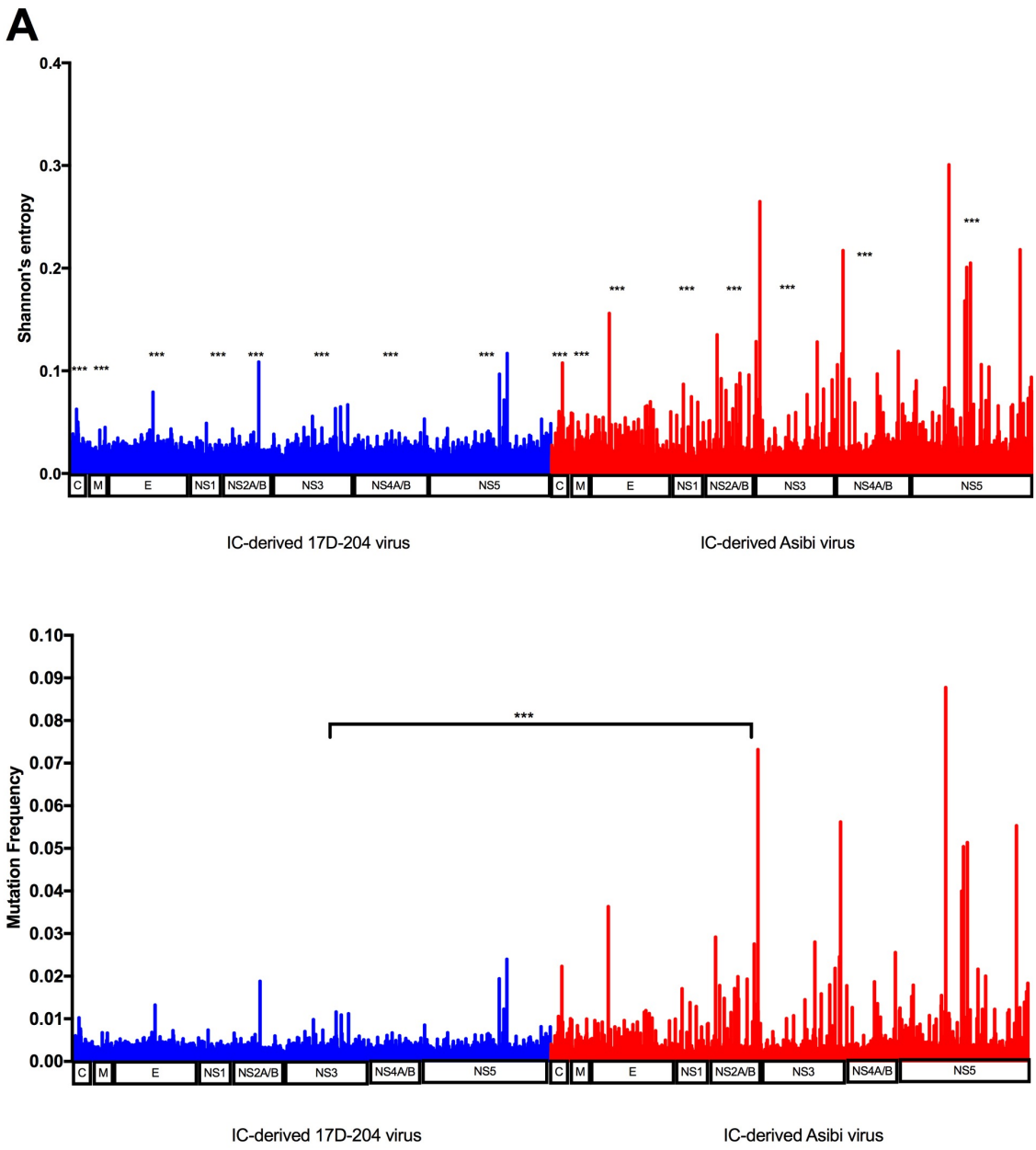

Figure 3.4. **Diversity indices for RT-PCR amplified IC-derived Asibi and 17D-204 viruses.** a, nucleotide diversity determined by Shannon's entropy. b, mutation frequency. The nucleotide diversity in all genes and mutation frequency for IC-derived 17D-204 virus were statistically less than those of IC-derived Asibi virus. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

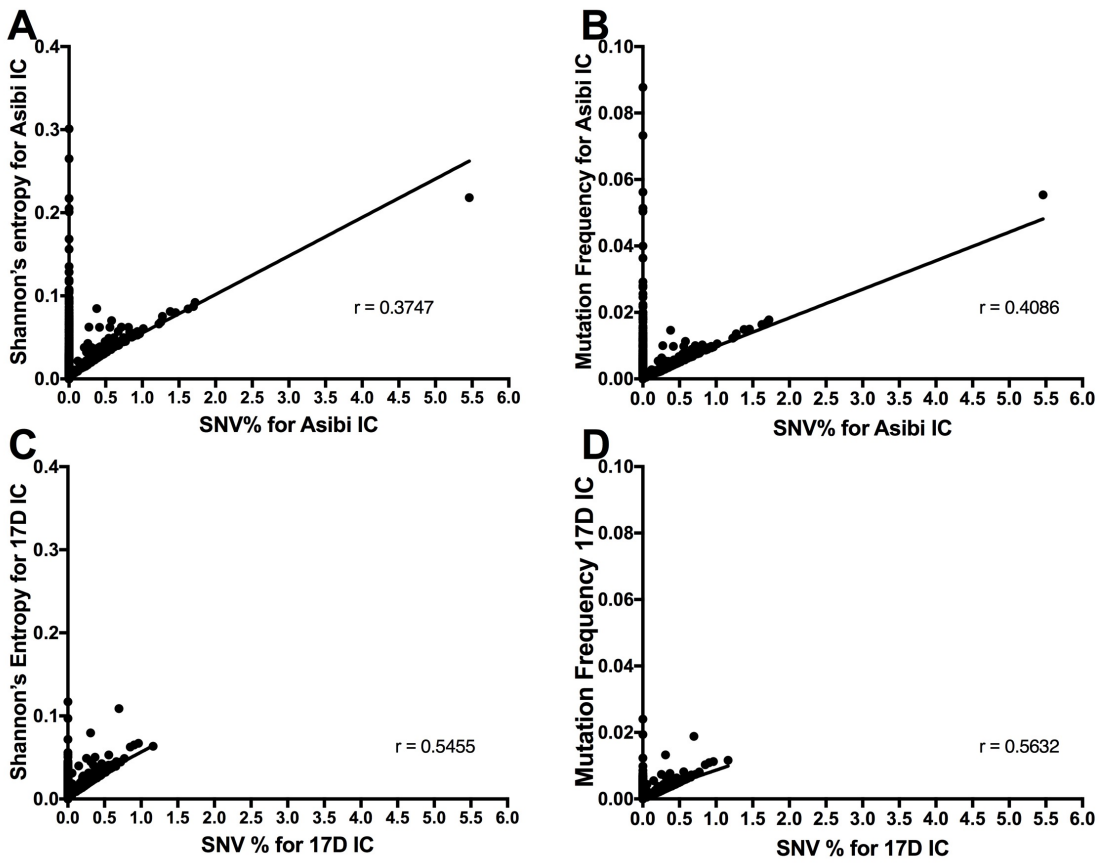

Figure 3.5. Comparison between diversity indices and SNVs for RT-PCR amplified IC-derived Asibi and 17D-204 viruses. a, b, RT-PCR amplified IC-derived Asibi virus. c, d, RT-PCR amplified IC-derived 17D-204. Diversity indices and identified SNVs were poorly correlated. All identified SNVs were represented in the diversity indices, while there were genomic positions with nucleotide variation that did not correspond to an identified SNV.

3.2.3 Trends in genetic diversity seen with YFV primer-amplified viral RNA were confirmed with sequencing of total RNA from cell supernatant following infection with IC-derived Asibi and 17D-204 samples

3.2.3.1 Single nucleotide variant population of IC-derived Asibi and 17D-204 viruses

Seventy-eight SNVs were identified for IC-derived Asibi virus, including 51 coding SNVs and 9 SNVs in the 3' NCR (**Figure 3.6**), of which 9 coding SNVs exceeded 1% of the viral population (**Table 3.3**). Three of the nine SNVs that exceeded 1% of the viral population were present in both dataset replicates at genomic positions 4505, 4517, and 6818; all of which were the highest percentages identified for the SNVs at 2.60%, 2.56%, and 3.04% of the viral population, respectively (**Table 3.3**). Significantly, five of the 9 coding SNVs were found in the NS2B gene, including genomic positions 4505 and 4517. Interestingly, the SNV at genomic position 4505 encoded for the NS2B-I109L substitution that is known to differ between wild-type Asibi virus and 17D-204 vaccine virus.

Conversely, 23 SNVs were identified for IC-derived 17D-204 virus, including 9 coding SNVs and 9 SNVs in the 3' NCR; all of the SNVs were less than 1% of the population. There were three SNV at genomic positions 2704, 10384, and 10389 that were present in both datasets. Interestingly, the SNV at genomic position 10384 was a thymine in one dataset and an adenosine in the other dataset.

There were only 6 SNVs shared between IC-derived Asibi and 17D-204 virus at genomic positions 3423, 7442, 7451, 10384, 10432, 10493; all 6 SNVs were under 1% of the population (**Figure 3.7**).

The results of the variant analysis were consistent with the variant analysis for RT-PCR amplified IC-derived Asibi and 17D-204 viruses in **section 3.2.2.1** of this

chapter; provided the coverage of the viruses is at least greater than 2026. As shown in this analysis, the variant population of IC-derived Asibi is more diverse than that of IC-derived 17D-204 virus regardless of whether or not the NGS libraries were created following target capture with RT-PCR amplification of viral RNA or prepared from total RNA.

3.2.3.2 Diversity indices for IC-derived Asibi and 17D-204 virus

The nucleotide diversity in the capsid and pre-membrane genes of IC-derived Asibi and 17D-204 viruses were not statistically different, while, the envelope and NS genes (NS1, NS2A/B, NS3, NS4A/B, NS5) of IC-derived Asibi virus were statistically more diverse than those of IC-derived 17D-204 virus (**Figure 3.8**). Similar to the clustering of SNVs in the NS2B protein gene described in **section 3.2.3.3** of this chapter, nucleotide diversity of IC-derived Asibi virus was highest in the NS2B protein gene. The results for the mutation frequency of IC-derived Asibi virus and IC-derived 17D-204 viruses were consistent with those for the nucleotide diversity (**Figure 3.8**). Similar to the comparison of the diversity indices for the RT-PCR amplified IC-derived Asibi virus, SNVs identified for both IC-derived viruses did not correlate to either nucleotide diversity or mutation frequency, the Pearson r values for all comparisons were below 0.2 (**Figure 3.9**).

Collectively, the results of the nucleotide diversity and mutation frequency analysis support that IC-derived Asibi virus is more diverse than IC-derived 17D-204

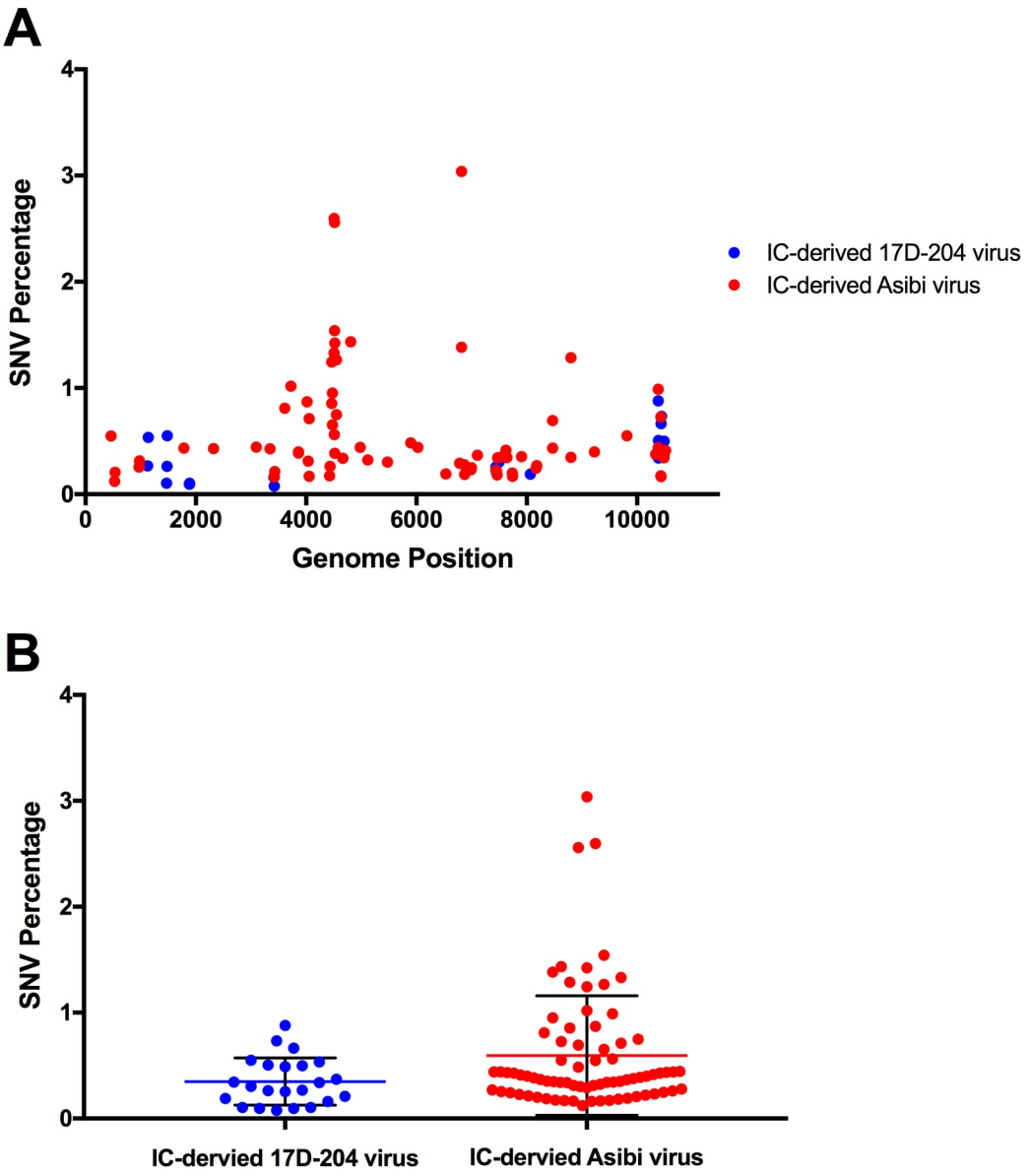

Figure 3.6. Single nucleotide variants identified for IC-derived Asibi and 17D-204 viruses. a. SNVs across the genome, b. total SNVs. IC-derived Asibi virus generates a more diverse variant population than IC-derived Asibi virus.

Table 3 3. Single nucleotide variants that exceeded 1% of the viral population for IC-derived Asibi virus.

Reference Base	Variant Base	Genomic Position	Gene Region	Coding/ Noncoding	Variant Frequency
G	A	3724	NS2A	Yes; M1202I	1.02%
G	A	4463	NS2B	Yes; D1449N	1.25%
A	C	4505	NS2B	Yes; I1465L	*2.60%
G	A	4517	NS2B	Yes; A1467T	*2.56%
C	T	4520	NS2B	Yes; L1468F	1.42%
A	C	4551	NS2B	Yes; H1478P	1.27%
G	T	4808	NS3	Yes; A1564S	1.44%
T	A	6818	NS4A	Yes; S2234T	*3.04%
G	A	8801	NS5	Yes; E2895K	1.29%

* Highest percentage is reported for SNV identified in both replicates

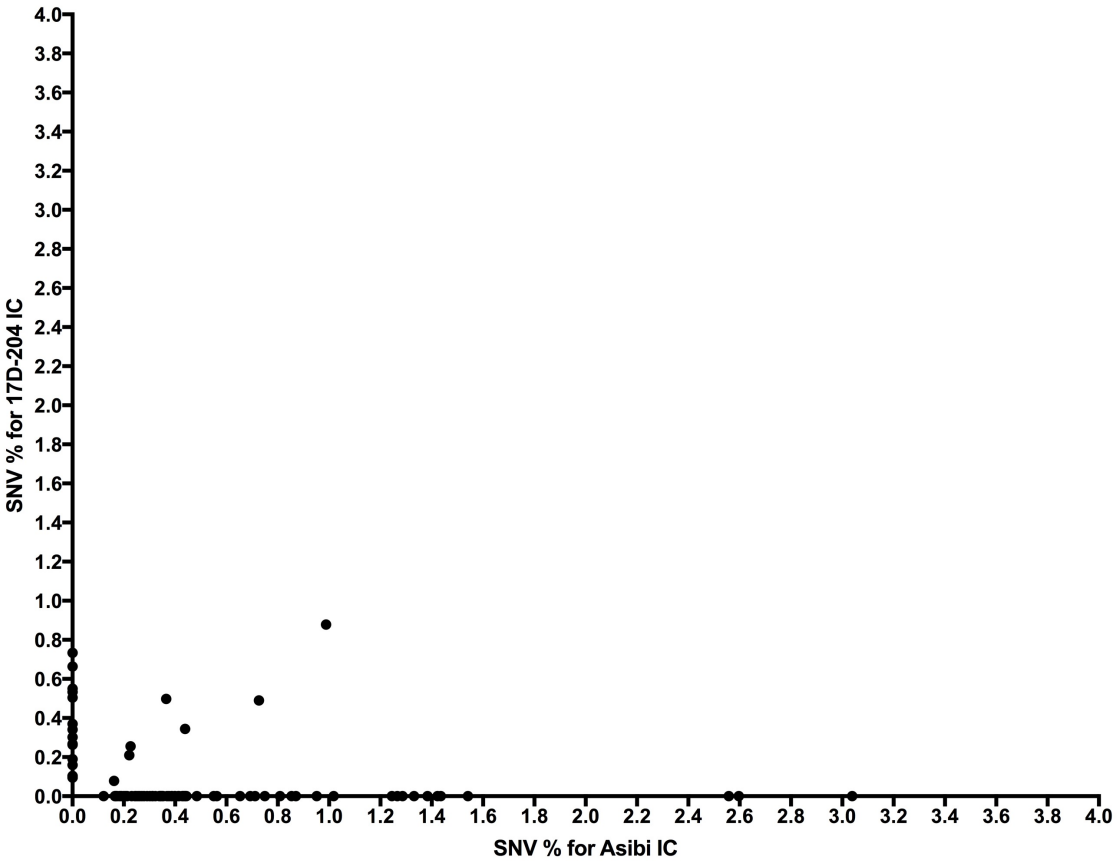

Figure 3.7. Comparison of the single nucleotide variant identified for IC-derived Asibi and 17D-204 viruses. There was limited evidence of shared SNVs between the two IC-derived viruses.

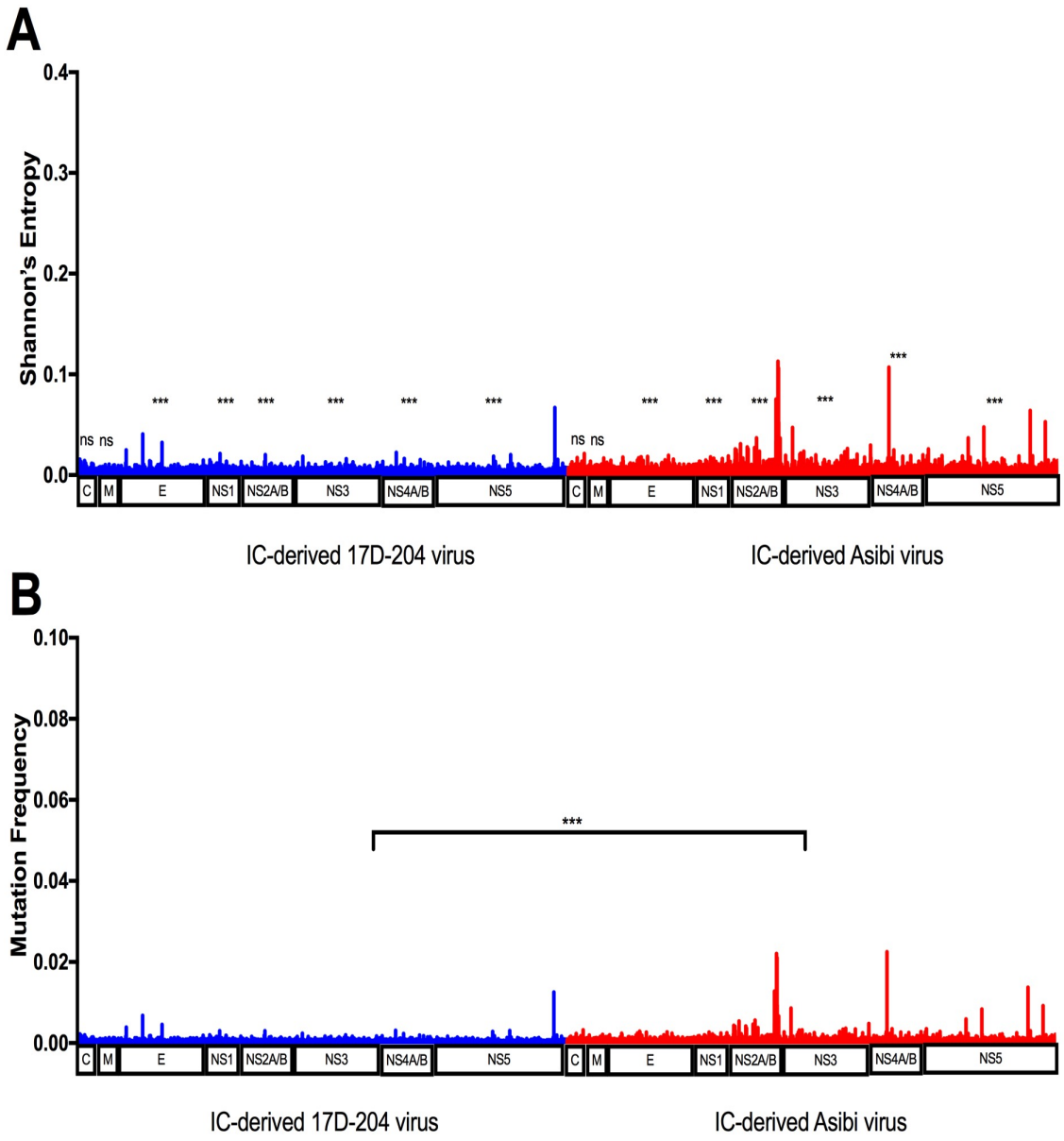

Figure 3.8. Diversity indices for RT-PCR amplified IC-derived Asibi and 17D-204 viruses. a, nucleotide diversity determined by Shannon's entropy. b, mutation frequency. The nucleotide diversity in the envelope and NS genes (NS1, NS2A/B, NS3, NS4A/B, and NS5) and mutation frequency for IC-derived 17D-204 virus were statistically less than those of IC-derived Asibi virus. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

3.3 DISCUSSION

The attenuation of wild-type Asibi virus to generate 17D vaccine virus yielded a virus with an apparently high fidelity RNA-dependent RNA-polymerase, and a distinctly different phenotype. This chapter demonstrates that the genetic diversity of IC-derived 17D-204 vaccine is less diverse than IC-derived Asibi virus, supporting previous reports based on wild-type Asibi virus and commercial 17D-204 vaccine virus (YF VAX™; Sanofi Pasteur, USA) (Beck et al., 2014; Salmona et al., 2015). Utilizing IC-derived viruses limited the potential bias associated with passage history and sample source, demonstrating that one or more of the 20 shared amino acid substitutions that distinguish wild-type Asibi virus from the 17D vaccine sub-strains contribute to the differences in genetic diversity of the two viruses.

Although creating NGS libraries using two approaches, either following target capture by RT-PCR amplification or total RNA, yielded similar conclusions, differences were noted. The SNVs for both methods did not exceed 6% of the viral population. However, the RT-PCR amplified datasets possessed a higher propensity for minority (<0.5% of the population) SNVs than the total RNA datasets; many of which were shared between RT-PCR amplified IC-derived Asibi and 17D-204 viruses. Additionally, greater than 95% of the SNVs identified for IC-derived 17D-204 virus were minority SNVs, suggesting that the sequencing bias introduced by the RT-PCR amplification step contributed to the higher number of SNVs that were identified for RT-PCR amplified IC-derived 17D-204 when compared to RT-PCR amplified IC-derived Asibi virus. Beck and colleagues (2014) utilized Varscan v.2.3.5 for variant detection, which uses heuristic criteria for identifying variants and is not sensitive enough to identify minority variants;

therefore, it is not possible to determine whether or not Beck *et al*. observed the same bias for minority variants for RT-PCR amplified datasets as observed here. Other studies with flaviviruses have not shown significant sequencing bias from target capture techniques (Grubaugh, Weger-Lucarelli, et al., 2016; Sessions et al., 2015; Van Slyke et al., 2015). However, either different target capture platforms designed for use with NGS or target amplification of specific gene regions of interest were employed in those studies. Therefore, based on the results obtained in this chapter, it was decided that NGS libraries generated from total RNA using random hexamer primers would be used for evaluating genetic diversity as described in the remainder of this dissertation.

The results of both the nucleotide diversity and mutation frequency analyses supports that IC-derived 17D-204 virus is less diverse than IC-derived Asibi virus. However, the correlation between the SNVs identified and diversity indices were poor for both IC-derived Asibi and 17D-204 viruses (**Figure 3.9**). There are genomic positions in the datasets that showed increased variation that did not correspond to SNVs. Clearly, the diversity indices and SNV variant detection evaluate different characteristics of genetic diversity. Diversity indices described in this dissertation were used to evaluate nucleotide variation at a genomic level and use the raw nucleotide counts at a given positions for calculations. Conversely, Vphaser2 uses a phasing algorithm, controlling for false detection and stand-bias to decrease erroneous variant identification. Consequently, observing increased genomic variation would be expected as the methods used to determine them are not as stringent as Vphaser2.

Recent genetic diversity studies for other flaviviruses obtained from replication in various cell cultures characterizing either the intra- and inter- host diversity or the effects

of bottlenecks on genetic diversity have all shown that wild-type flaviviruses are highly diverse (Dridi et al., 2015; Ehrbar et al., 2017; Grubaugh et al., 2015; Grubaugh, Rückert, et al., 2016; Grubaugh, Weger-Lucarelli, et al., 2016; Sessions et al., 2015; Van Slyke et al., 2015). The results in this chapter support previous reports that wild-type Asibi virus has a relatively high level of diversity typical of a flavivirus while the 17D-204 vaccine does not exhibit the high diversity that is seen with wild-type flaviviruses. The 17D-204 vaccine is apparently a virus with a high fidelity RNA-dependent RNA-polymerase that has an attenuated phenotype in both experimental studies and in humans, and this will be explored in subsequent chapters.

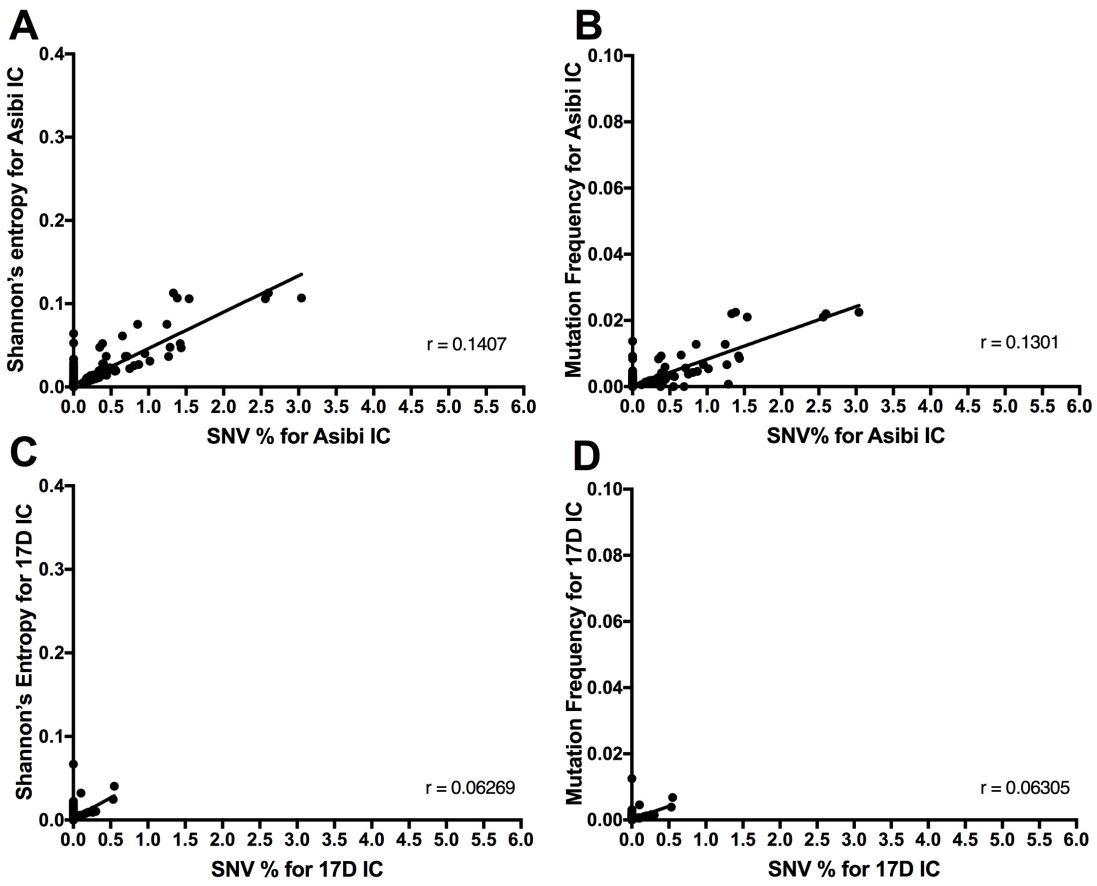

Figure 3.9. Comparison between diversity indices and SNVs for IC-derived Asibi and 17D-204 viruses. a, b, IC-derived Asibi virus. c, d, IC-derived 17D-204. Diversity indices and identified SNVs were poorly correlated. All identified SNVs were represented in the nucleotide diversity, but not in mutation frequency. Additionally, there were genomic positions with nucleotide variation that did not correspond to an identified SNV.

CHAPTER 4: CONTRIBUTIONS OF STRUCTURAL GENES TO GENETIC DIVERSITY OF 17D-204 VACCINE

4.1 INTRODUCTION

Wild-type Asibi and 17D vaccine viruses differ by 20 amino acid substitutions; 9 in the structural genes, 11 in non-structural genes, and 3 as silent nucleotide changes in the 3' NCR (dos Santos et al., 1995; Hahn, Dalrymple, Strauss, & Rice, 1987). Eight of the 9 amino acids that differentiate wild-type Asibi and 17D vaccine viruses in the structural proteins genes reside in the in the envelope (E) and one resides in the membrane. Interestingly, the membrane (M)-36 amino acid substitution is present in YF French Neurotropic vaccine derived from French Viscerotropic virus by passage in mouse brain via conditions that differed considerable from those used to derived the 17D vaccine (Wang et al., 1995). To date, amino acid substitutions in the structural genes have been studied only for their contributions to the phenotypes of vector infectivity in *Ae. aegypti* and various combinations of E protein residues in viral dissemination in the AG129 mouse model (McElroy et al., 2006; Lee & Lobigs, 2008).

Genetic diversity of RNA viruses has been exclusively studied in the context of polymerase fidelity. Specifically, the paradigm that mis-incorporation of nucleotides by the error-prone RdRp drives genetic diversity (Borderia et al., 2016; Lauring & Andino, 2010; Sanjuán & Domingo-Calap, 2016). However, it has been speculated that in addition to polymerase fidelity, multiple virus and host-dependent processes contribute to genetic diversity (Sanjuán & Domingo-Calap, 2016). Notably, sequence context, secondary structure, and replication mechanisms may also contribute to genetic diversity.

Therefore, IC-derived Asibi/17D chimeric viruses with pre-membrane and envelope (prME) structural genes "swapped" (viruses retained the capsid of the backbone virus as there are no amino acid substitutions that differ between wild-type Asibi virus and 17D-204 vaccine) were utilized to investigate the contribution of replication mechanism of YFV on genetic diversity. Since the chimeric viruses retained the non-structural genes of the backbone viruses, the contributions of the replication machinery to genetic diversity could be studied. Thereby, allowing for focused investigation of specific mutations in the NS genes in subsequent studies. Additionally, Asibi/17D mutant viruses containing the M-36 amino acid substitution were used to parse any unexpected results obtained with the prME chimeric viruses.

4.2 RESULTS

IC-derived prME chimeric and M-36 mutant viruses were named based on the backbone (dominant viral template), followed by the amino acid substitution (e.g. 17D/Asibi M-F36L denotes a 17D vaccine virus backbone with a 17D phenylalanine to Asibi leucine substitution at position 36 in the M protein) (**Figure 4.1**).

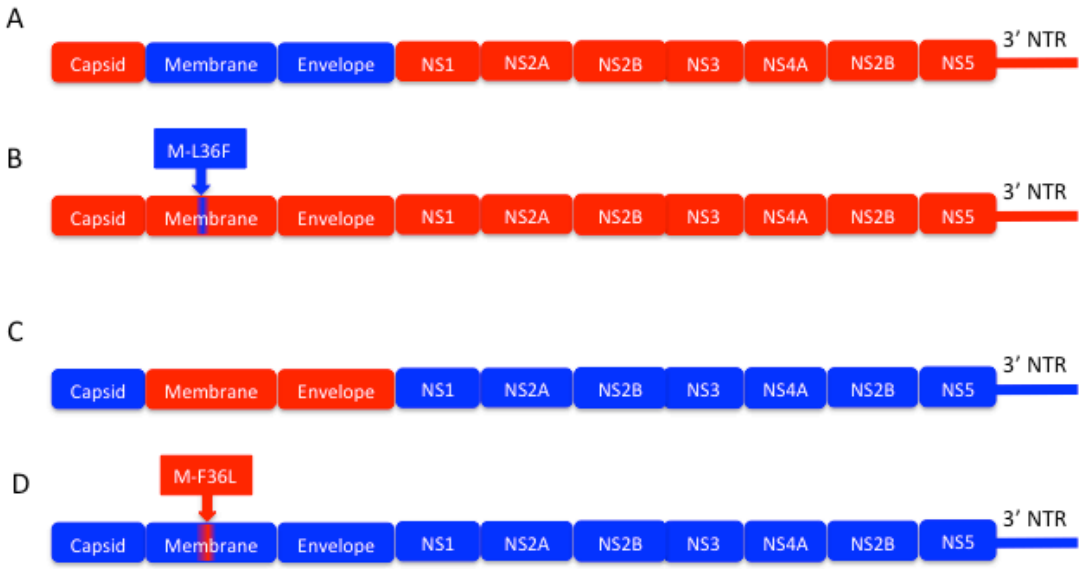

Figure 4.1. prME chimeric and M-36 Constructs. a and b are the Asibi backbone IC, while c and d are the 17D backbone viruses. Red denotes Asibi genes and blue denotes 17D-204 genes.

4.2.1 Generation of prME Chimeric and M-36 mutant viruses

Chimeric viruses were recovered by transfection of Vero cells with viral RNA transcribed from cDNA templates. Following electroporation of viral RNA in to cells, cell culture supernatants were harvested following development of cytopathic effect in the cultures. Infectivity titers for chimeras were assayed by focus forming assay in Vero cells. The consensus genomic sequences of the prME chimeric viruses were compared to both IC-derived Asibi and 17D-204 viruses, and were confirmed to be prME chimeric viruses. The 17D/Asibi prME chimeric virus did not contain any unexpected nucleotide changes. However, the Asibi/17D prME chimeric virus differed from IC-derived Asibi virus at nucleotide 2687, which encoded for NS1-L80F substitution. The genomes of the M-36 mutant viruses were compared to their respective backbone viruses; neither Asibi/17D M-L36F nor 17D/Asibi M-F36L virus contained any unexpected nucleotide changes.

The mean infectivity titer for both Asibi/17D prME and Asibi/17D M-L36F were 10^5 ffu/ml, 10-fold higher than that of IC-derived Asibi virus, while the mean infectivity titer for both 17D/Asibi prME and 17D/Asibi M-F36L were consistent with IC-derived 17D-204 virus (**Table 4.1**). Interestingly, the mean diameter of foci for both Asibi/17D prME and Asibi/17D prME chimeric viruses differed from both IC-derived Asibi and 17D-204 viruses; the foci of Asibi/17D prME virus were uniform and small in size, while the foci of 17D/Asibi prME virus were uniform and large in size (**Table 4.1**). Conversely, the mean foci size for both Asibi/17D M-L36F and 17D/Asibi M-F36L were consistent with their respective parental virus.

The mean read coverage that spanned the genome of the viruses described in this chapter ranged from approximately 700 to 8000 (**Table 4.1**). Multiple attempts to recover a virus for Asibi/17D prME with a mean coverage above 1000 were unsuccessful, while the viruses recovered for 17D/Asibi prME exceeded 6000 reads (**Table 4.1**), suggesting that wild-type viruses are more affected by disruptions in the interactions between structural (prM and E) and NS genes required for replication. Since the mean coverage for Asibi/17D prME was considerably lower than all of the IC-derived viruses used in research, it was not down-sampled.

Table 4.1 Infectious Clone (IC)-derived viruses descriptive characteristics for prME chimeric and M-36 mutant viruses. (Parental is data presented in chapter 3 of this dissertation.)

SF: small foci, < 1 mm; MF: medium foci, 1-2 mm; LF: large foci, ≥ 2 mm

IC	Coverage by Replicate R1;R2	Mean Coverage	Titer by Replicate R1;R2	Mean Titer of recovered virus (ffu/ml)	Mean Foci Size
17D	5034; 2982	4008	2.2×10^6; 3.2×10^6	6.1×10^6	Uniform; MF
17D/Asibi prME	7256; 8720	7988	1.0×10^6; 2.1×10^6	4.1×10^6	Uniform; LF
17D/Asibi M-F36L	2584; 2965	2774	6.8×10^6; 7.7×10^5	3.8×10^6	Uniform; MF
Asibi/17D M-L36F	4031; 3195	3613	6.0×10^5; 4.8×10^5	5.3×10^5	Mixed; SF and MF
Asibi/17D prME	907; 678	793	3.3×10^5; 4.8×10^5	4.1×10^5	Uniform; SF
Asibi	8547; 5234	6891	4.9×10^4; 7.2×10^4	6.1×10^4	Mixed; SF and MF

4.2.2 Pre-membrane chimeric viruses alter genetic diversity

Nineteen SNVs were identified for Asibi/17D prME virus, a four-fold reduction when compared to IC-derived Asibi virus; 8 SNVs were coding, two SNVs were non-coding, and 9 SNVs were identified in the 3' NCR. Five SNVs exceeded 1% of the population, one was a coding nucleotide change at genomic position 4517 in the NS2B present in both replicates and three SNVs were in the 3' NCR (genomic positions 10384, 10394, and 10652) (**Figure 4.2 and Table 4.2**). Additionally, 8 SNVs for Asibi/17D prME virus, including genomic positions 4505, 4517, 4551, and 8801, were exclusively shared with IC-derived Asibi; all of which exceeded 1% of the population for IC-derived Asibi virus (**Figure 4.2**). However, only 2 SNVs for Asibi/17D prME virus were exclusively shared with 17D-204 virus (Figure 4.2).

Conversely, the number of SNVs identified for 17D/Asibi prME virus was consistent with IC-derived 17D-204 virus. 32 SNVs were identified for 17D/Asibi prME virus; 12 SNV were coding, 8 SNVs were non-coding, and the remaining 12 SNVs were identified in the 3' NCR. Only two SNVs in the 3' NCR (genomic positions 10384 and 10550) exceeded 1% (**Figure 4.2 and Table 4.2**). 17D/Asibi prME virus shared 2 SNVs with IC-derived Asibi virus, while no SNVs were shared with IC-derived 17D-204 virus (**Figure 4.2**).

The nucleotide diversity of both Asibi/17D prME and 17D/Asibi prME viruses differed statistically from their respective backbone virus; the nucleotide diversity of Asibi/17D prME virus was statistically less diverse in all genes when compared to IC-derived Asibi virus, while 17D/Asibi prME virus had two genes (E and NS5) that were statistically more diverse than IC-derived 17D-204 virus (**Figure 4.3 and Table 4.3**).

The mutation frequency of Asibi/17D prME virus was statistically lower when compared to IC-derived Asibi virus, while the mutation frequency of 17D/Asibi prME virus was statistically higher when compared to IC-derived 17D-204 (**Figure 4.4**). Interestingly, three of the four positions (genomic positions 4463, 4505, and 4517) in the coding region of Asibi/17D prME that exceed a mutation frequency of 0.01 corresponded to the same position as those for IC-derived Asibi virus. Additionally, genomic positions 4505 and 4517 correspond to SNVs that were greater than 1% of the population for IC-derived Asibi virus.

Overall, the results of the prME chimeric viruses compared to their respective backbone viruses suggest that chimerization with the structural (prM and E) genes contributes to the genetic diversity.

4.2.3 M-36 substitution does not contribute to the genetic diversity profile of 17D vaccine

A two-fold reduction in SNVs was observed for Asibi/17D M-L36F virus when compared to IC-derived Asibi virus. Thirty-six SNVs were identified for Asibi/17D M-L36F virus; 25 SNVs were coding, 7 were non-coding, and four nucleotide changes were identified in the 3' NCR (**Figure 4.2**). One SNV at genomic position 6818 in the NS4A gene, present in both replicates, exceeded 1% of the viral population (**Table 4.2**). Interestingly, the SNV at genomic position 6818 was also identified for IC-derived Asibi virus and also exceeded 1% of the viral population. Additionally, 12 SNVs were identified for Asibi/17D M-F36L virus, of which greater than 30% of identified SNVs were exclusively shared with IC-derived Asibi virus (**Figure 4.2**).

Surprisingly, there was an approximate 2-fold reduction in SNVs identified for 17D/Asibi M-F36L virus when compared to IC-derived 17D-204 virus. Fourteen SNVs were identified for 17D/Asibi M-F36L virus, none of which exceeded 1% of the viral population; 7 SNVs were coding, two SNVs were non-coding, and five SNVs were identified in the 3' NCR (**Figure 4.2**). There was little evidence of SNVs shared exclusively with either IC-derived Asibi or 17D-204 viruses (**Figure 4.2**).

The results for the diversity indices trends were inconsistent for both the M-36 mutant viruses. The nucleotide diversity in the NS2B and NS4A genes of Asibi/17D M-L36F virus was statistically less diverse than IC-derived Asibi (**Figure 4.3, Table 4.3**). Interestingly, the nucleotide diversity in all genes of 17D/Asibi M-F36L virus were consistent with IC-derived 17D-204 virus, with the exception of the NS4B gene, which displayed decreased nucleotide diversity when compared to IC-derived 17D-204 virus (**Figure 4.3 and Table 4.3**). The mutation frequency of Asibi/17D M-L36F virus was statistically less diverse than IC-derived Asibi virus, while the mutation frequency of 17D/Asibi M-F36L virus was consistent with IC-derived 17D-204 virus (**Figure 4.4**). Similar to the results for Asibi/17D prME virus (Section 4.2.2 of this dissertation) and SNV analysis for Asibi/17D M-L36F virus, there were few genomic positions with increased mutation frequency when compared to IC-derived Asibi virus; only the mutation frequency for genomic positions 6818 and 10082 exceeded a mutation frequency of 0.01. However, genomic position 10082 displayed high levels of mutation for all of the IC-derived viruses, including IC-derived 17D-204 virus.

The results of the M-36 mutant viruses were unexpected, especially for 17D/Asibi M-F36L virus. 17D/Asibi M-F36L virus displayed a decrease in the number of identified

SNVs and decreased nucleotide diversity in the NS4B gene. The nucleotide diversity in all other genes of this mutant were consistent with IC-derived 17D-204 virus. Additionally, the mutation frequency of 17D/Asibi M-F36L did not differ when compared to IC-derived 17D-204 virus. Indeed, 17D/Asibi M-F36L shared more SNVs with IC-derived 17D-204 virus, while 17D/Asibi prME did not share any SNVs with IC-derived 17D-204 virus. Taken together, the M-36 amino acid substitution does not appear to contribute to the limited genetic diversity of the 17D vaccine virus. The results of Asibi/17D M-L36F supports this conclusion, since alterations in the genetic diversity is limited when compared to the prME chimeric virus.

Table 4.2. Single nucleotide variants SNVs greater than 1% of the population.

Reference Base	Variant Base	Genomic Position	Gene Region	Coding/ Noncoding	Variant Frequency
Asibi/17D prME					
G	A	4517	NS2B	Yes; A1467T	*4.12%
C	T	10384	3' NCR	Silent	1.39%
G	A	10394	3' NCR	Silent	1.10%
G	T	10652	3' NCR	Silent	1.55%
Asibi/17D M-L36F					
T	A	6818	NS4A	Yes; S2234T	*1.09%
17D/Asibi prME					
C	T	10384	3' NCR	Silent	*1.22%
C	T	10550	3' NCR	Silent	1.14%

* Highest percentage is reported for SNV identified in both replicates

Table 4.3. Nucleotide diversity comparison between prME chimeric and M-36 mutant viruses and parental IC-derived Asibi and 17D-204 viruses.

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi prME	17D IC	ns	ns	**	ns	ns	ns	ns	ns	ns	*
	Asibi IC	ns	ns	**	***	***	***	***	***	***	***
Asibi/17D prME	17D IC	**	***	***	***	*	ns	***	***	***	***
	Asibi IC	***	***	***	***	***	***	***	***	***	***

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi M-F36L	17D IC	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
	Asibi IC	ns	ns	***	***	***	***	***	***	***	***
Asibi/17D M-L36F	17D IC	ns	ns	***	***	***	**	***	ns	***	***
	Asibi IC	ns	ns	ns	ns	ns	**	ns	*	ns	ns

P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

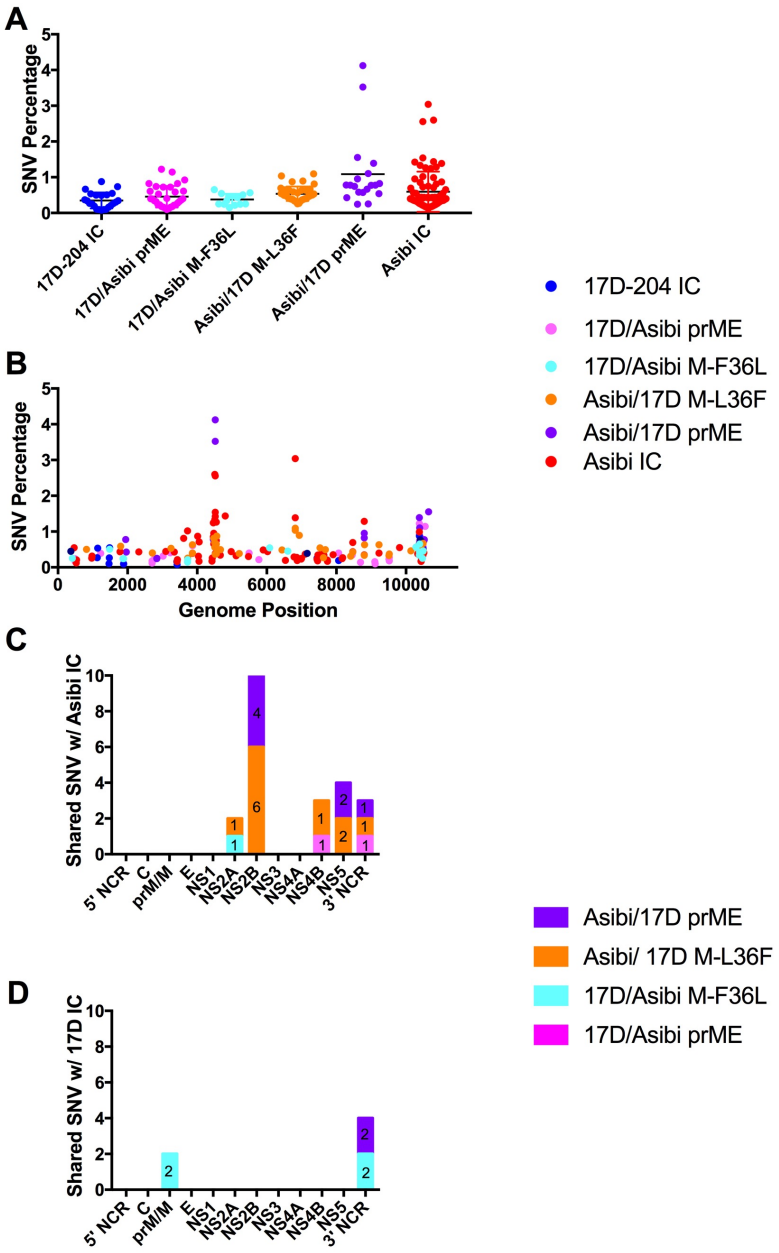

Figure 4.2. Single nucleotide variants identified for prME chimeric and M-36 mutant viruses. a. SNVs across the genome, b. total SNVs. c. number of SNVs shared between IC-derived Asibi virus and prME chimeric and M-36 mutant viruses. The number of SNVs shared between prME chimeric and M-36 and IC-derived Asibi (c) and 17D-204 (d) viruses. prME chimeric viruses display altered number and percentages of identified SNVs when compared to respective backbone virus. 17D/Asibi prME shares no SNVs with IC-derived 17D-204 virus.

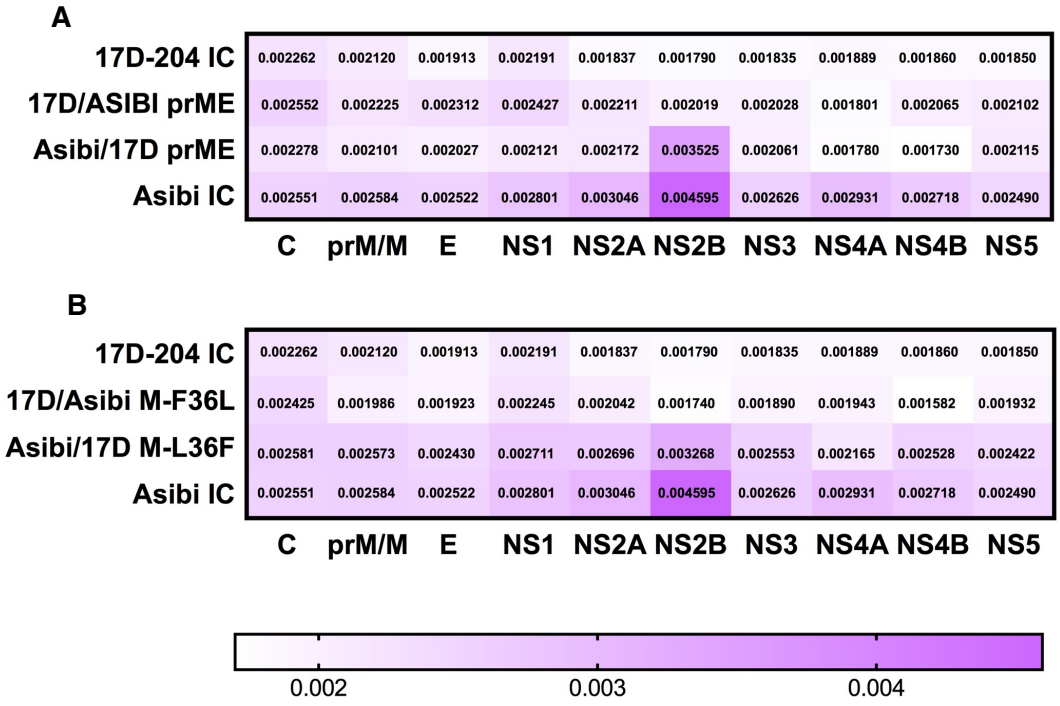

Figure 4.3. Nucleotide diversity for prME chimeric and M-36 mutant viruses shown as a heatmap. Nucleotide diversity determined by Shannon's entropy for prME chimeric viruses (a) and M-36 mutant viruses (b). b, mutation frequency. Both Asibi/17D prME and 17D/Asibi prME alter the nucleotide diversity in multiple structural and non-structural genes when compared to their respective backbone virus, whereas, the M-36 substitution has a limited effect on nucleotide diversity. Diversity is shown as a heatmap with the darker the color the more diversity.

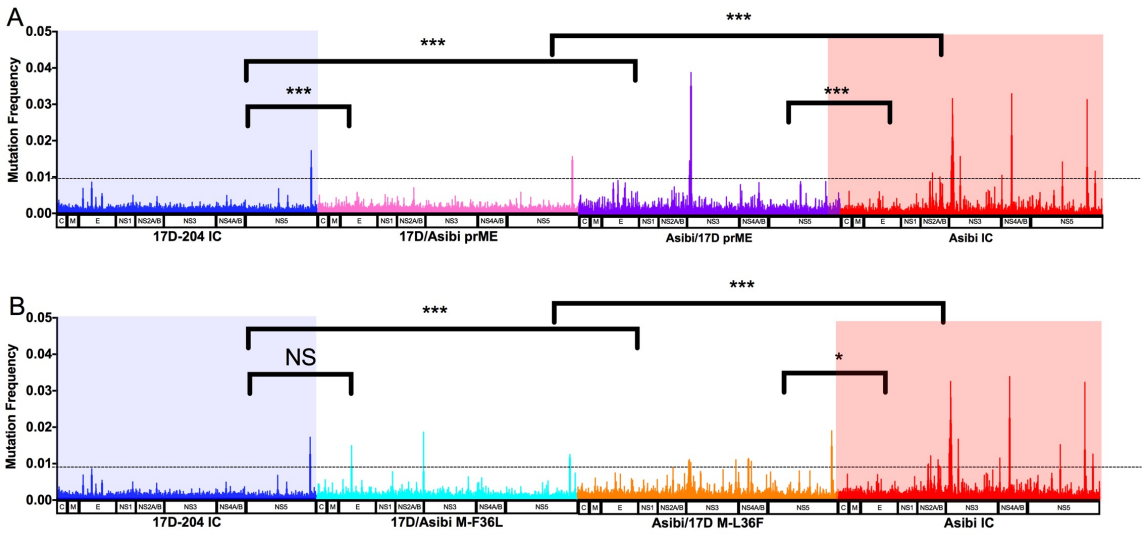

Figure 4.4. Mutation frequency for prME chimeric and M-36 mutant viruses. a. prME chimeric viruses and b. M-36 mutant viruses. Both Asibi/17D prME and 17D/Asibi prME displayed altered mutation frequency across the genome when compared to their respective parental virus, whereas, only Asibi/17D M-L36F displayed decreased mutation frequency when compared to IC-derived Asibi virus. P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

4.3 DISCUSSION

The M-36 mutant viruses were used to sparse the result of the prME chimeric viruses. The prME chimeric viruses displayed altered genetic diversity in multiple genes, including structural and non-structural genes, i.e., outside of the region of the genome "swapped" to generate the chimeric virus. In comparison, the M-36 mutant viruses displayed altered genetic diversity in limited genes, which did not correspond to those seen with the prME chimeric viruses. Specifically, introduction of the M-36 amino acid substitution into the 17D-204 backbone led to a decrease in genetic diversity evident in decreased number of identified SNVs and decreased nucleotide diversity in the NS4B gene. Conversely, introduction of Asibi prME genes into the 17D backbone lead to an increased in identified SNVs and increased nucleotide diversity in the E and NS5 genes as well as increased mutation frequency. Additionally, 17D/Asibi prME did not share any SNVs with IC-derived 17D-204 virus. The genetic diversity of the M-36 mutant viruses suggest that M-36 substitution is unlikely to be involved in genetic diversity. However, the results of the prME chimeric virus suggest that one or more substitutions in the E protein gene of the 17D vaccine contributes to the low diversity profile of 17D-204 vaccine.

The shared amino acid substitutions that differentiate between wild-type Asibi virus and 17D-204 vaccine in the structural protein region are predominately in the envelope (n=8) , with only a single amino acid substitution in the pre-membrane. The M-36 substitution is present in FNV (Wang et al., 1995) and has been shown to attenuate wild-type Japanese encephalitis virus (de Wispelaere et al., 2015). Previous studies of the M-36 substitution on vector dissemination only included the Asibi/17D M-L36F virus

and not the reciprocal 17D backbone virus, therefore the role of this substitution in the attenuation of vector competence is restricted to the effects of the mutation on wild-type YFV. Studies of the M-36 substitution in IC-derived Asibi and JEV (de Wispelaere et al., 2015; McElroy et al., 2006) , in addition to the results in this chapter, suggest that the M-36 substitution yields an attenuated phenotype for wild-type viruses. However, there was limited data that would support that the M-36, alone, contributes to genetic diversity of the vaccine.

A majority of studies on the genetic diversity of RNA viruses have focused the role of the RdRp; consistent in those studies is the use of ribavirin to generate resistant mutants and identify of specific mutations in the RdRp that are resistant to lethal mutagenesis that may effect replication fidelity, thereby contributing to genetic diversity (Borderia et al., 2015; Coffey, Beeharry et al., 2011; Coffey & Vignuzzi, 2011; Rozen-Gagnon et al., 2014; Stapleford et al., 2015; Van Slyke et al., 2015; Xie et al., 2014; Zeng et al., 2013, 2014). However, Geller and colleagues (2015) investigated the mutation rate in the gp120 envelope protein region of human immunodeficiency virus-1 and found that mutation rate was increased in highly structured gene regions, which supports the theory that sequence structure also contributes to mutation rate. Additionally, lysis time and host factors have also been shown to drive mutation rate (Sanjuán & Domingo-Calap, 2016).

Cell lysis time is influenced by multiple factors, including multiplicity of infection and the efficiency of viruses to replicate in host cells. Additionally, delayed lysis of host cells favors increase mutation rate (Sanjuán & Domingo-Calap, 2016). Although there have been limited studies investigating the contributions of structural

genes to genetic diversity, structural genes are known to contribute to entry, uncoating, and packaging and release of viral progeny; therefore, contributing to replication efficiency. The envelope protein, specifically the transmembrane region of the envelope protein, has been shown to have a critical role in viral assembly, stability and maturation (Blazevic et al, 2016).

Changes in mean foci size of the prME chimeric viruses was observed, supporting the hypothesis that the contributions of structural genes in the genetic diversity of 17D-204 vaccine is associated with replication efficiency and subsequently phenotypic changes in the virus. A previous study using the prME chimeric viruses to study viral dissemination in mosquito vectors suggested that synergism of structural and NS proteins may be required for viral escape from the midgut of mosquitoes (McElroy et al., 2006a, 2006b), which may also be the case in mammalian cells. It is possible that the amino acid substitutions in the E gene of 17D vaccine disrupt this role. Since viral diversity of Asibi/17D prME was significantly lower than both IC-derived Asibi and 17D viruses, the synergistic role of structural and NS genes to overcome host barriers is critical to viral production of wild-type viruses.

The results of this chapter showed for the first time that structural genes contribute to genetic diversity. Clearly, the role of structural genes in genetic diversity is not related to fidelity, but rather other factors that influence replication and virus production.

CHAPTER 5: CONTRIBUTIONS OF NON-STRUCTURAL GENES TO THE GENETIC DIVERSITY OF 17D-204 VACCINE

5.1. INTRODUCTION

The current paradigm for quasispecies population theory of RNA viruses is that RNA viruses generate populations of genetically related variants due to the high error rate of the RNA dependent RNA polymerase (RdRp). This theory is based on studies performed with poliovirus (Bordería et al., 2016; Vignuzzi et al., 2005, 2006); where a chemical mutagen was used to generate a high-fidelity poliovirus with a mutation in the RdRp. The high fidelity mutant poliovirus displayed decreased nucleotide mis-incorporation when compared to wild-type poliovirus, ultimately yielding an attenuated phenotype *in vivo* due to the inability of the variant to generate diverse viral populations. The study, although informative and groundbreaking, was limited by what viable mutations were induced by the mutagen. Unpublished studies preformed in our laboratory of an NS4B-P38G mutant virus of West Nile virus suggested that mutations in proteins of the replication complex of flaviviruses might also contribute to genetic diversity. Nonstructural (NS) mutant viruses utilizing either IC-derived Asibi or 17D-204 viruses were generated to test this hypothesis.

Of the 20 amino acid substitutions that distinguish between wild-type Asibi strain and 17D vaccine, 11 reside in the NS protein genes (Hahn et al,, 1987; dos Santos et al., 1995). Two of the 11 shared amino acid substitutions are found in the NS5 protein (NS5-836 and NS5-900), which encodes the RdRp. The RdRp is complexed with NS1, NS2A,

NS2B, NS3, NS4A, and NS4B to form the virus replication complex (RC), which is required for efficient viral replication (Selisko et al., 2014; Shi, 2014).

Studies of the IC-derived Asibi virus discussed in Chapter 3 of this dissertation, showed that NS2B gene was highly diverse, evident by clustering of single nucleotide variants (SNVs) and high nucleotide diversity in the NS2B gene. However, studies of the flavivirus NS2B are limited to the role of the NS2B protein as the cofactor for the NS3 protein, the protease required for processing of the polyprotein (Bollati et al., 2010; Khromykh et al., 2000; Li et al., 2014; Li et al., 2016).

The NS4B protein has been extensively studied and has roles in both replication and immunity. It is thought to be the scaffold for the RC (Li et al., 2015; Shi, 2014; Tajima et al., 2011; Nemésio et al., 2012; Youn et al., 2012; Zou et al., 2015; Zou et al., 2015) and has been shown to antagonize the host innate immune response *in vitro* and in small animal models (Zou et al., 2014; Zou et al., 2015; Zmurko et al., 2015; Muñoz-Jordán et al., 2005; Jones et al., 2005; Ye et al., 2013; Green et al., 2014).

Given the role of the NS2B and NS4B proteins in the replication complex, it was hypothesized that the NS2B-L109I and NS4B-M95I amino acid substitutions that distinguish between wild-type Asibi and the 17D vaccine contribute to the limited viral population of the 17D vaccine.

5.2. RESULTS

The same naming conventions described in chapter 4 section 4.2 of this dissertation were applied in this chapter for NS2B-109 and NS4B-95 (**Figure 5.1**). Similar to generation of the M-36 mutant virus, the NS mutant viruses were generated by

site-directed mutagenesis utilizing either IC-derived Asibi or 17D-204 viruses as templates.

5.2.1 Generation of non-structural (NS2B, NS4B, and NS5) and prME with NS2B mutant and chimeric viruses

NS mutant viruses were recovered by transfection of Vero cells with viral RNA transcribed from cDNA templates. Following electroporation of viral RNA in to cells, cell culture supernatants were harvested following development of cytopathic effect in the cultures. Infectivity titers for the viruses were assayed by focus forming assay in Vero cells. The consensus genomic sequences of the NS mutant viruses were compared with either IC-derived Asibi or IC-derived 17D-204 viruses, depending on the backbone virus. There were no consensus sequence differences noted in neither the Asibi backbone NS mutant viruses nor the 17D backbone NS backbone viruses when compared to their respective backbone virus.

The mean infectivity titer for Asibi/17D NS4B-I95M virus was 3.3×10^5 ffu/ml, however, the mean infectivity titer for Asibi/17D NS2B-I109L was 2.7×10^6 ffu/ml, 100-fold higher than that of IC-derived Asibi (**Table 5.1**). Conversely, the mean infectivity titers for 17D/Asibi NS2B-L109I and 17D/Asibi NS4B-M95I viruses were consistent with IC-derived 17D-204 virus (**Table 5.1**). The mean diameter of foci for all mutant viruses was consistent with their backbone virus.

The mean read coverage for all viruses discussed in this chapter exceeded 2000 reads (**Table 5.1**).

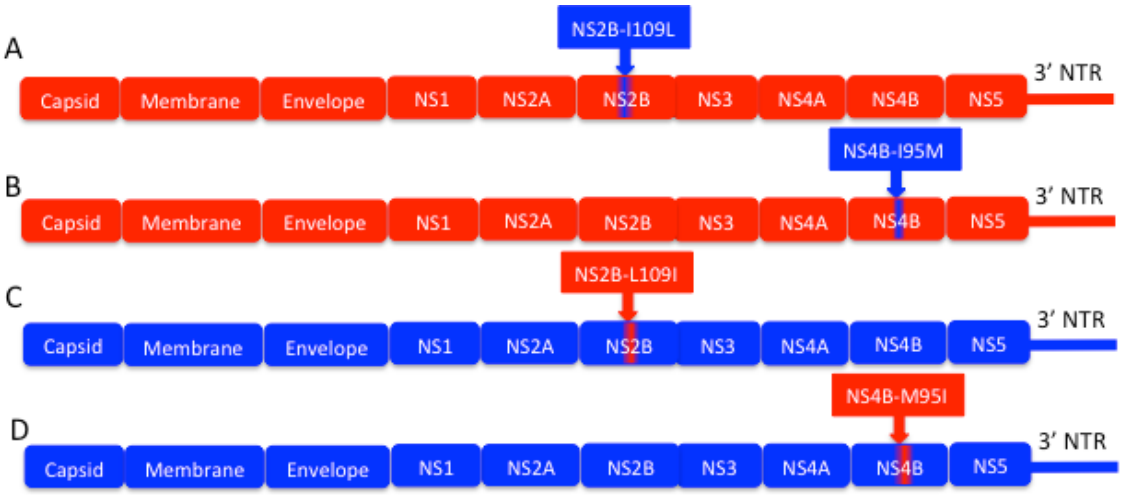

Figure 5.1. NS2B-109 and NS4B-95 Constructs. a and b are the Asibi backbone IC, while c and d are the 17D backbone viruses. Red denotes Asibi genes and blue denotes 17D-204 genes.

Table 5.1. Infectious Clone (IC)-derived viruses descriptive characteristics for NS2B-109 and NS4B-95 mutant viruses. (Parental data is presented in chapter 3 of this dissertation.)

SF: small foci, < 1 mm; MF: medium foci, 1-2 mm; LF: large foci, ≥ 2 mm

IC	Coverage by Replicate R1;R2	Mean Coverage	Titer by Replicate R1;R2	Mean Titer of recovered virus (ffu/ml)	Mean Foci Size
17D	5034; 2982	4008	2.2×10^6; 3.2×10^6	6.1×10^6	Uniform; MF
17D/Asibi NS2B-L109I	1692; 2366	2029	7.6×10^5; 4.4×10^6	2.6×10^6	Uniform; MF
17D/Asibi NS4B-M95I	5954; 2775	4364	5.7×10^6; 6.3×10^6	6.0×10^6	Uniform; MF
Asibi/17D NS4B-I95M	6903; 2304	4306	1.4×10^5; 5.2×10^5	3.3×10^5	Mixed; SF and MF
Asibi/17D NS2B-I109L	5498; 3553	7160	7.0×10^6; 3.8×10^6	2.7×10^6	Mixed; SF and MF
Asibi	8547; 5234	6891	4.9×10^4; 7.2×10^4	6.1×10^4	Mixed; SF and MF

5.2.2 NS protein genes outside of the NS5 RdRp contribute to the viral population and genetic diversity of 17D vaccine

5.2.2.1 Contribution of the NS2B-109 amino acid substitution

Thirteen SNVs were identified for Asibi/17D NS2B-I109L, approximately a six-fold reduction in identified SNVs when compared to IC-derived Asibi virus; five of the SNVs were coding, two SNVs were non-coding, and 6 SNVs were identified in the 3' NCR (**Figure 5.2**). Only two SNVs at genomic position 4550 and 7529 were exclusively shared between Asibi/17D NS2B-I109L and IC-derived Asibi virus, while none were exclusively shared with IC-derived 17D-204 virus (**Figure 5.2**).

Nineteen SNVs were identified for 17D/Asibi NS2B-L109I; 8 SNVs were coding, four SNVs were non-coding, and 6 SNVs were identified in the 3' NCR (**Figure 5.2**). Two SNVs for 17D/Asibi NS2B-L109I were exclusively shared with either IC-derived Asibi (genomic positions 4505 and 4507) or 17D-204 (genomic positions 10389 and 10437) (**Figure 5.2**).

None of the SNVs were identified for either Asibi/17D NS2B-I109L or 17D/Asibi NS2B-L109I that exceeded 1% of the population.

Interestingly, the diversity indices for Asibi/17D NS2B-I109L and were not consistent with the significant decrease in identified SNVs. The nucleotide diversity in all genes, except the NS2B gene, for Asibi/17D NS2B-I109L were consistent with that of IC-derived Asibi virus (Figure 5.3 and Table 5.3). However, the nucleotide diversity of 8 genes (C, E, NS1, NS2A, NS2B, NS3, NS4B, and NS5) of 17D/Asibi NS2B-L109I displayed increased diversity when compared to IC-derived 17D-204 virus (**Figure 5.3 and Table 5.3**). Additionally, the mutation frequency of Asibi/17D NS2B-I109L was consistent with that of IC-derived Asibi virus (Figure 5.4). However, only four genomic

positions for Asibi/17D NS2B-I109L exceeded a mutation frequency of 0.01, suggesting there were genomic regions with decreased diversity. Conversely, the mutation frequency for 17D/Asibi NS2B-L109I was statistically higher when compared to IC-derived 17D-204 virus, and 7 genomic positions exceeded a mutation frequency of 0.01 (**Figure 5.4**).

5.2.2.2 Contribution of the NS4B-95 amino acid substitution

Forty-four SNVs were identified for Asibi/17D NS4B-I95M, a 1.7 fold decrease when compared to IC-derived Asibi virus; 25 SNVs were coding, 10 SNVs were non-coding, and 9 SNVs were identified in the 3' NCR (**Figure 5.2**). One coding SNV at genomic position 6818 in the NS4A gene, present in only one replicate, exceeded 1% of the viral population (**Table 5.2**). Seven SNVs, including genomic position 6818, were exclusively shared with IC-derived Asibi virus (**Figure 5.2**). None of the SNVs were exclusively shared between Asibi/17D NS4B-I95M and IC-derived 17D-204 virus (Figure 5.2).

Forty-four SNVs were identified for 17D/Asibi NS4B-M95I virus, approximately two-fold higher than IC-derived 17D-204 virus; 18 SNVs were coding, 9 SNVs were non-coding, and 17 SNVs were identified in the 3' NCR (**Figure 5.2**). Two variants in the 3' NCR (genomic positions 10389 and 10550) for 17D/Asibi NS4B-M95I exceeded 1% of the population (**Table 5.2**). Seven SNVs were exclusively shared between 17D/Asibi NS4B-M95I virus and IC-derived 17D-204 virus, including genomic position 10389, while only four SNVs were shared with IC-derived Asibi virus (Figure 5.2).

The diversity indices for Asibi/17D NS4B-I95M and 17D/Asibi NS4B-M95I viruses displayed a similar trend to that of the NS2B-109 mutant viruses; the diversity

indices for Asibi/17D NS4B-I95M virus were consistent with IC-derived Asibi virus, while the diversity indices for 17D/Asibi NS4B-M95I virus differed when compared to IC-derived 17D/Asibi NS4B-M95I virus. Although the nucleotide diversity of Asibi/17D NS4B-I95M virus was consistent with IC-derived Asibi virus, multiple genes displayed decreased diversity; including the NS4A gene, which was similar in diversity to that of IC-derived 17D-204 virus (**Figure 5.3 and Table 5.3**). Conversely, the nucleotide diversity in E, NS3, and NS5 of 17D/Asibi NS4B-M95I virus displayed a higher degree of diversity when compared to IC-derived 17D-204 virus, and were consistent with IC-derived Asibi virus (**Figure 5.3 and Table 5.3**). The mutation frequency of Asibi/17D NS4B-I95M virus was consistent with IC-derived Asibi virus, however, the mutation frequency of 17D/Asibi NS4B-M95I was increased when compared to IC-derived 17D-204 virus (**Figure 5.4**). Interestingly, there were no genomic positions for Asibi/17D NS4B-I95M that exceeded a mutation frequency of 0.01, which as consistent with IC-derived Asibi virus, suggesting that there were regions displaying decreased mutation frequency when compared to IC-derived Asibi virus.

Overall, the results for the NS2B-109 and NS4B-95 mutant viruses suggest that these mutations contribute to the genetic diversity of the 17D vaccine and supports the hypothesis that mutations outside of the NS5 RdRp contribute to genetic diversity.

Table 5.2. Single nucleotide variant greater than 1% of the population.

	Reference Base	Variant Base	Genomic Position	Gene Region	Coding/ Noncoding	Variant Frequency
Asibi/17D NS4B-I95M						
	T	A	6818	NS4A	Yes; S2234	1.50%
17D/Asibi NS4B-M95I						
	C	T	10389	3' NCR	N/A	1.08%
	C	T	10550	3' NCR	N/A	1.09%

Table 5.3. Nucleotide diversity comparison between NS2B-109 and NS4B-95 mutant viruses and parental IC-derived Asibi and 17D-204 viruses.

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi NS2B-I109L	17D IC	*	ns	***	***	***	***	***	ns	**	***
	Asibi IC	ns	ns	ns	ns	ns	ns	ns	*	ns	*
Asibi/17D NS2B- L109I	17D IC	ns	ns	***	***	***	**	***	ns	**	***
	Asibi IC	ns	ns	ns	ns	ns	**	ns	ns	ns	ns
		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi NS4B-M95I	17D IC	ns	ns	***	ns	ns	ns	***	ns	ns	***
	Asibi IC	ns	ns	ns	***	***	***	**	*	**	ns
Asibi/17D NS4B- I95M	17D IC	ns	ns	***	***	***	***	***	ns	**	***
	Asibi IC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

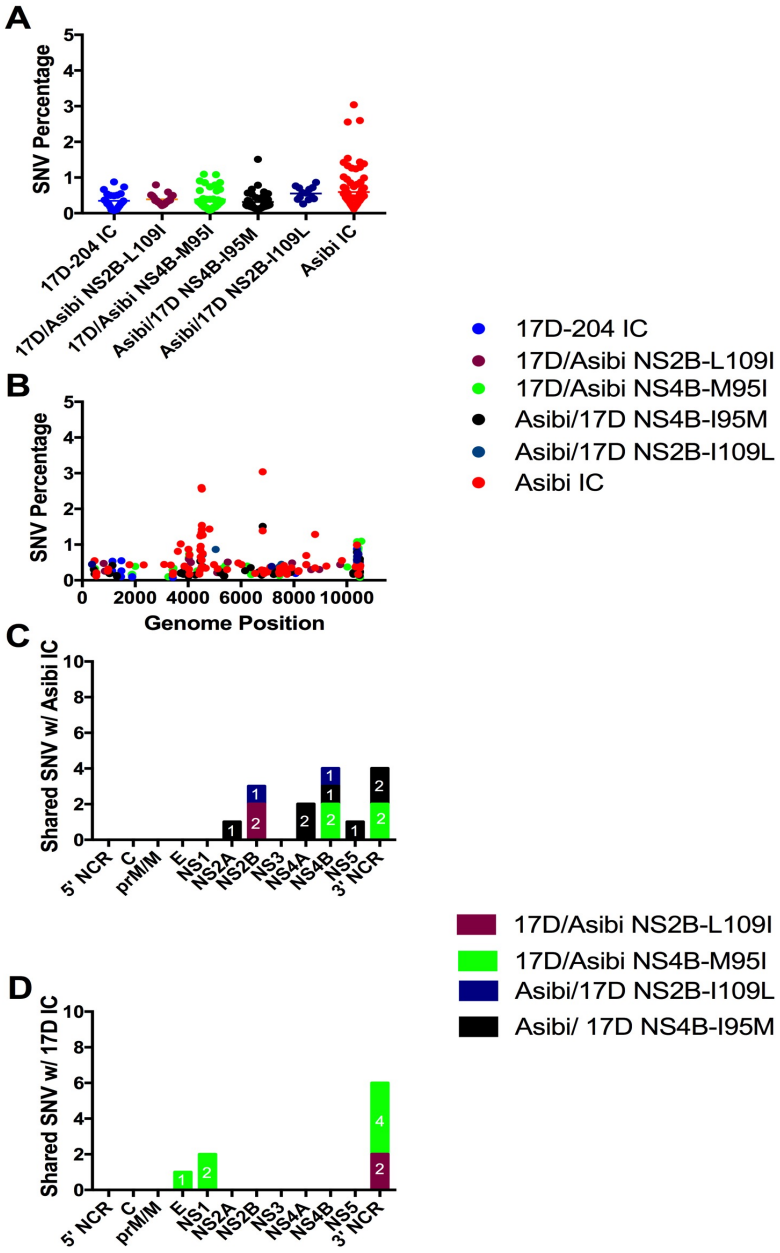

Figure 5. 2. **Single nucleotide variants identified for NS2B-109 and NS4B-95 mutant viruses.** a. SNVs across the genome, b. total SNVs. The number of SNVs shared with either IC-derived Asibi (c) or 17D-204 (d) viruses. The SNV profile for the Asibi backbone mutant viruses was altered considerable when compared to IC-derived Asibi virus, while there was limited evidence of altered SNV profile for the 17D backbone mutant viruses. There was no evidence of shared SNVs for the Asibi backbone viruses with IC-derived 17D-204 virus; however, the 17D backbone mutant viruses had SNVs present in the population of IC-derived Asibi virus.

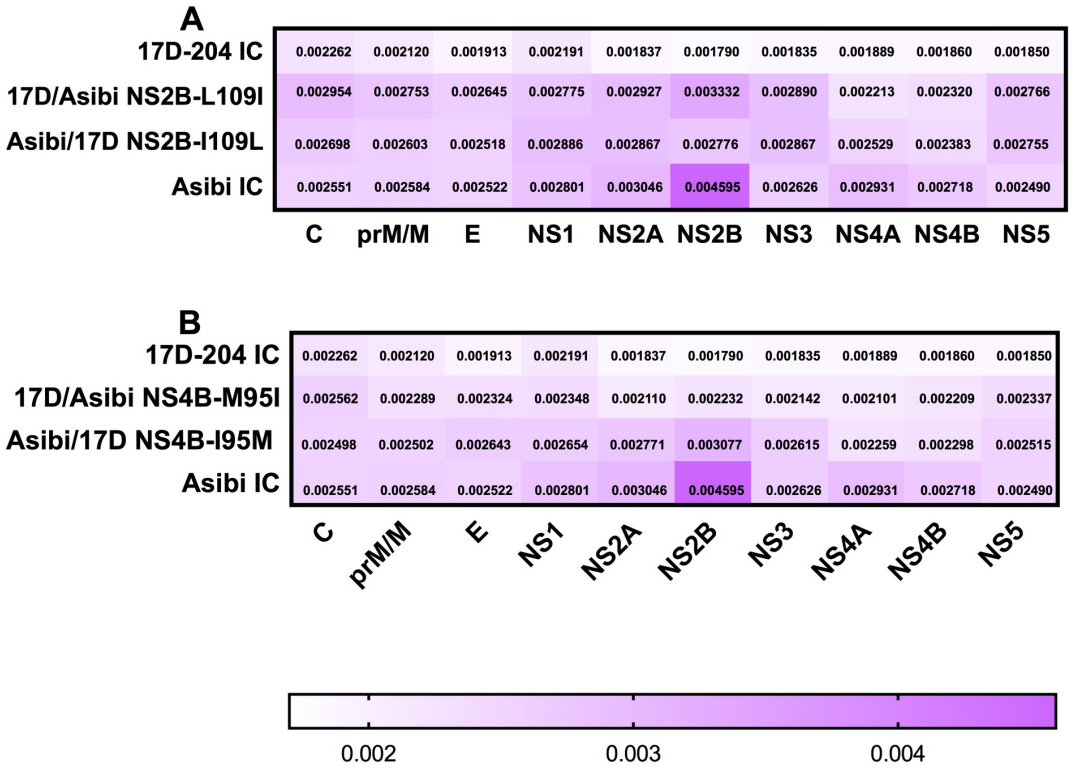

Figure 5.3. Nucleotide diversity for NS2B-109 and NS4B-95 mutant viruses. Nucleotide diversity determined by Shannon's entropy for NS2B-109 (a) and NS4B-95 (b) mutant viruses. Both 17D/Asibi NS2B-L109I and 17D/Asibi NS4B-95 alter the nucleotide diversity in multiple structural and non-structural genes when compared to IC-derived Asibi virus, whereas, mutation of the Asibi backbone has a limited effect on nucleotide diversity. Diversity is shown as a heatmap with the darker the color the more diversity.

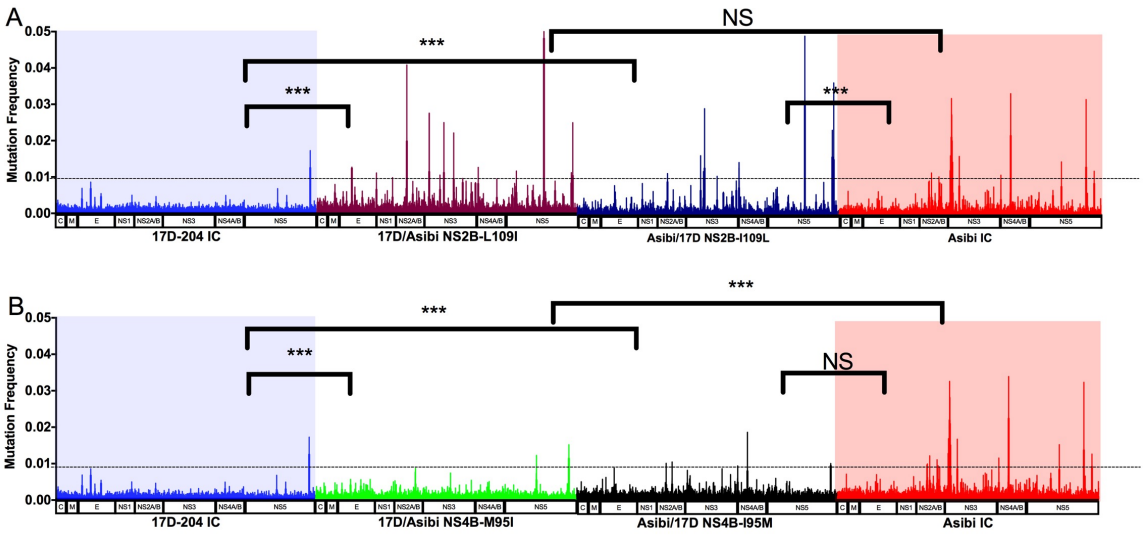

Figure 5. 4. Mutation frequency for NS2B-109 and NS4B-95 mutant viruses. a. NS2B-109 viruses and b. NS4B-95 mutant viruses. Neither Asibi/17D NS2B-I109L nor Asibi/17D NS4B-I95M displayed altered mutation frequency when compared to IC-derived Asibi virus, whereas, both 17D/Asibi NS2B-L109I and 17D/Asibi NS4B-M95I displayed increased mutation frequency when compared to IC-derived 17D-204 virus. P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

5.3. DISCUSSION

The results of this Chapter shows that NS2B and NS4B protein genes contribute to the limited genetic diversity of 17D vaccine and contradicts previous assertion that only the RdRp drives genetic diversity of RNA viruses. NS2B and NS4B proteins have critical roles in the RC of Flaviviruses (Selisko et al., 2014; Youn et al., 2012; Zou, Lee, et al., 2015; Zou, Xie, et al., 2015); therefore, the observations made in this chapter are hypothesized to contribute to these roles.

Introduction of either of the Asibi NS2B-L109I or NS4B-M95I mutations into the 17D backbone altered genetic diversity, such that multiple genes displayed increased diversity and the mutation frequency was increased when compared to IC-derived 17D-204 virus. Interestingly, only the NS4B-M95I substitution increased the number of SNVs identified and yielded SNVs in the 3' NCR at genomic positions 10389 and 10550 (Table 5.2) that exceeded 1% of the variant population. The SNV at genomic position 10389 was exclusively shared with IC-derived 17D-204 virus, while the SNV at genomic position 10550 was unique to 17D/Asibi NS4B-M95I. Although 17D/Asibi NS2B-L109I has less SNVs than both 17D/Asibi NS4B-M95I and IC-derived 17D-204 virus, changes in nucleotide diversity and mutation frequency was more evident than that of 17D/Asibi NS4B-M95I, suggesting that decrease in the number of SNVs is more likely associated with generation of additional SNVs that were not viable, and therefore not identified in the virus-containing cell culture supernatant used in these studies (i.e., intracellular viral RNA was not studied).

Conversely, the introduction of both NS2B-I109L and NS4B-I95M into the Asibi backbone altered the SNV profile only. Although there was a considerable decrease in

the number of SNVs identified and only one SNV for Asibi/17D NS4B-I95M exceeded 1% of the population, there was still evidence of SNV seen for IC-derived Asibi virus for both Asibi/17D NS2B-I109L and Asibi/17D NS4B-I95M. Additionally, the diversity indices for the Asibi backbone viruses were consistent with IC-derived Asibi, however, there were gene regions that showed decreased nucleotide diversity and limited genomic positions with high mutation frequency. Taken together, both NS2B-109 and NS4B-95 amino acid substitutions contribute to genetic diversity, however, the effect is limited. The single amino acid substitutions alone will not revert the genotype of the 17D vaccine and suggest that the genetic diversity of the vaccine is multi-genic.

Initial studies investigating whether or not RNA viruses exist as a single virion or a collection of genetically related virions used poliovirus as a model. Two independent laboratories isolated a high fidelity RdRp G64S mutant of poliovirus with an attenuated phenotype *in vitro* and *in vivo*. Additionally, the viral population of G64S mutant virus was less diverse than the viral population of wild-type poliovirus (Pfeiffer & Kirkegaard, 2003, 2005; Vignuzzi et al., 2006). It was concluded that genetic diversity is proportional to pathogenicity and that the RdRp of RNA viruses controls genetic diversity (Lauring & Andino, 2010; Vignuzzi et al., 2005; Vignuzzi et al., 2006). Subsequently, a low fidelity mutant of poliovirus was generated with increased genetic diversity when compared to poliovirus and the RdRp G64S mutant poliovirus, further supporting the hypothesis that only the RdRp contributes to replication fidelity and drives genetic diversity (Korboukh et al., 2014). The contribution of mutations in the RdRp was further supported by studies utilizing other RNA viruses, all of which used the same protocol of isolating fidelity mutants utilizing passaging of parental viruses in the presence of ribavirin, a guanosine

analog and antiviral agent (Coffey et al., 2011; Coffey & Vignuzzi, 2011; Gnädig et al., 2012; Rozen-Gagnon et al., 2014; Van Slyke et al., 2015; Xie et al., 2014; Zeng et al., 2013, 2014).

Interestingly, in attempts to generate a high fidelity mutant of chikungunya virus (CHIKV), both a RdRp and a helicase mutant were isolated; both mutations yielded viruses that were low in diversity when compared to wild-type CHIKV, suggesting that viral proteins other than those that encode the RdRp contribute to replication fidelity (Coffey & Vignuzzi, 2011; Stapleford et al., 2015). Additionally, a methyltransferase mutant of West Nile virus (WNV) was shown to also alter genetic diversity (Van Slyke et al., 2015). The results of this chapter further demonstrate that mutations in components of the replication complex also contribute to genetic diversity of RNA viruses.

Taken together with the results of the prME chimeric viruses discussed in Chapter 4 of this dissertation and the single amino acid substitutions in NS2B and NS4B in sections of this Chapter support the hypothesis that the genetic diversity of the 17D-204 vaccine is multi-genic, and that both structural and NS genes together are required to observe genetic diversity consistent with wild-type levels.

CHAPTER 6: THE GENETIC DIVERSITY OF 17D-204 VACCINE IS MULTI-GENIC

6.1. INTRODUCTION

The 17D-204 vaccine was shown to be less diverse than wild-type Asibi (Beck et al., 2014; Salmona et al., 2015) and was confirmed utilizing IC-derived Asibi and 17D-204 viruses in a control experiment in **Chapter 3** of this dissertation. Investigation into the contributions of prME, NS2B-109 and NS4B-95 that differentiate between wild-type Asibi and the 17D vaccine to genetic diversity indicated that the limited diversity of the vaccine may be multi-genic, involving structural and non-structural genes (see **Chapters 4 and 5** of this dissertation). The multi-genic nature of the attenuated phenotype of the vaccine has long been hypothesized (McElroy et al., 2006a, 2006b); however, it has not been definitively demonstrated utilizing IC-derived viruses containing both structural and NS gene swaps.

Additionally, studies of genetic diversity in RNA viruses have predominately focused on the role of single mutations in individual protein genes involved in RNA synthesis and viral processing; none have investigated whether or not genetic diversity is multi-genic. Given the results of **Chapters 4 and 5** of this dissertation, at least in the case of 17D vaccine, it is possible that genetic diversity is more complicated than the control of single mutations and more likely due to the interplay of multiple genes and/or protein products during viral replication.

Introduction of the NS2B-109 amino acid substitution into the backbones of IC-derived Asibi and 17D-204 viruses provided the most persuasive data in support of the

hypothesis that mutations outside of the RdRp contribute to replication fidelity and genetic diversity, while the prME chimeric viruses showed that structural genes also contribute to genetic diversity, probably by a different mechanism to that of NS genes. Therefore, prME chimeric viruses incorporating the NS2B-109 amino acid substitution were generated to test the hypothesis that both structural and NS genes are functioning to synergistically increase the genetic diversity of 17D vaccine to wild-type levels.

6.2. RESULTS

The same naming conventions described in **chapters 4 and 5** of this dissertation were applied in this chapter for prME + NS2B-109 chimeric viruses (**Figure 6.1**). The prME + NS2B-109 chimeric viruses were generated by site-directed mutagenesis utilizing either Asibi/17D prME or 17D/Asibi prME viruses as templates and the primers used to generate the NS2B-109 mutant viruses to ensure results would be comparable. Therefore, the prME + NS2B-109 chimeric viruses were compared to IC-derived Asibi and 17D-204 viruses, first, then to the respective prME and NS2B chimeric and mutant viruses discussed in **Chapters 4 and 5** of this dissertation.

6.2.1 Generation and NGS of NS5-386 and prME with NS2B mutant and chimeric viruses

PrME + NS2B-109 chimeric viruses were recovered by transfection of Vero cells with viral RNA transcribed from cDNA templates. Following electroporation of viral RNA in to cells, cell culture supernatants were harvested following development of cytopathic effect in the cultures. Infectivity titers for the viruses were assayed by focus forming assay in Vero cells. The consensus genomic sequences of the prME + NS2B-109 chimeric viruses were compared to either Asibi/17D prME or 17D/Asibi prME viruses,

respectively, depending on the backbone virus. No differences were identified in the consensus sequences, other than Asibi/17D prME + NS2B-I109L, which still contained the NS1-L80F substitution that differed between IC-derived Asibi virus and Asibi/17D prME identified in **Chapter 4** of this dissertation.

The mean infectivity titer for Asibi/17D prME + NS2B-I109L differed by 100-fold from the Asibi parental virus and was more consistent with the 17D parental virus, while the 17D/Asibi prME + NS2B-L109I was 10-fold lower than IC-derived 17D-204 virus. The mean infectivity titer for Asibi/17D NS2B-I109L was 4.1 x 10^6 ffu/ml, while the mean infectivity titer for 17D/Asibi prME + NS2B-L109I was 2.5 x 10^5 ffu/ml (**Table 6.1**). The mean diameter of foci for Asibi/17D prME + NS2B-L109I were less than 1 mm, noticeably smaller than those of IC-derived Asibi virus, while the foci for 17D/Asibi prME + NS2B-L109I were greater than 2 mm, which was noticeable larger than IC-derived 17D-204 virus; the foci for both Asibi/17D prME + NS2B-I109L and 17D/Asibi prME + NS2B-L109I viruses were consistent with the respective prME chimeric virus discussed in **Chapter 4** of this dissertation, suggesting that the alterations in foci size is associated with the substitutions in the structural genes (see **Table 6.1**).

The mean read coverage for 17D/Asibi prME + NS2B-L109I was 4190 reads (**Table 6.1**). Interestingly, the mean read coverage for Asibi/17D prME + NS2B-I109L virus was 1992 reads, considerable higher than the mean coverage of 793 reads achieved following NGS of Asibi/17D prME virus (**Table 6.1**). Taken together with the descriptive data for both Asibi/17D NS2B-L109I virus discussed in **Chapter 5** of this dissertation and Asibi/17D prME + NS2B-I109L virus, suggest that the addition of the NS2B-I109L substitution improved, to some degree, the replicative ability of Asibi prME

chimeric virus. Since the mean coverage for Asibi/17D prME + NS2B-L109I was considerable lower than all of the IC-derived viruses used in this dissertation, it was not down-sampled.

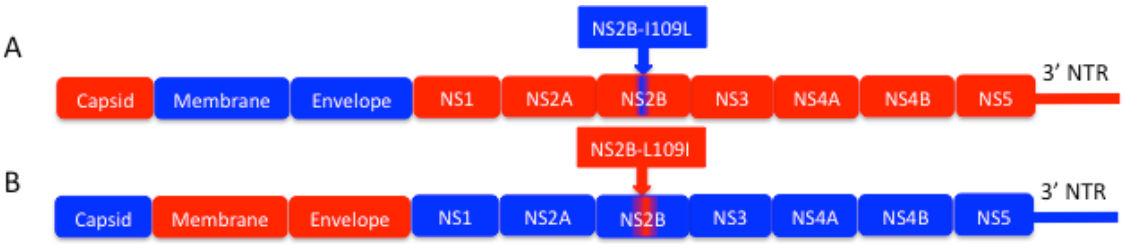

Figure 6.1. Chimeric prME + NS2B-109 Constructs. a is the Asibi backbone IC, while b is the 17D backbone viruses. Red denotes Asibi genes and blue denotes 17D-204 genes.

Table 6.1. Infectious Clone (IC)-derived viruses descriptive characteristics for prME + NS2B-109 chimeric viruses. (Parental, prME, and NS2B-109 are data presented in chapters 3, 4, and 5 of this dissertation.)

SF: small foci, < 1 mm; MF: medium foci, 1-2 mm; LF: large foci, ≥ 2 mm

IC	Coverage by Replicate R1;R2	Mean Coverage	Titer by Replicate R1;R2	Mean Titer of recovered virus (ffu/ml)	Mean Foci Size
17D	5034; 2982	4008	2.2×10^6; 3.2×10^6	6.1×10^6	Uniform; MF
17D/Asibi prME	7256; 8720	7988	1.0×10^6; 2.1×10^6	4.1×10^6	Uniform; LF
17D/Asibi NS2B-L109I	1692; 2366	2029	7.6×10^5; 4.4×10^6	2.6×10^6	Uniform; MF
17D/Asibi prME + NS2B-L109I	2701; 5679	4190	9.7×10^4; 2.0×10^5	1.5×10^5	Uniform; LF
Asibi/17D prME + NS2B-I109L	1824; 2160	1992	2.1×10^6; 6.0×10^6	4.1×10^6	Uniform; SF
Asibi/17D NS2B-I109L	5498; 3553	7160	7.0×10^6; 3.8×10^6	2.7×10^6	Mixed; SF and MF
Asibi/17D prME	907; 678	793	3.3×10^5; 4.8×10^5	4.1×10^5	Uniform; SF
Asibi	8547; 5234	6891	4.9×10^4; 7.2×10^4	6.1×10^4	Mixed; SF and MF

6.2.2 Multi-gene mutations contribute to the genetic diversity of 17D vaccine protein to wild-type levels

6.2.2.1 Comparison of prME + NS2B-109 chimeric viruses with parental virus

Seventy-two SNVs were identified for Asibi/17D prME + NS2B-I109L virus, considerable higher than other Asibi backbone viruses investigated in the dissertation; 33 of the SNVs were coding, 30 SNVs were non-coding, and 9 SNVs were identified in the 3' NCR (**Figure 6.2**). Interestingly, the distribution of SNVs identified in the two replicates differed greatly; five SNVs were identified in one replicate with higher read coverage, while 67 SNVs were identified in the other replicate with lower read coverage. Four coding SNVs (genomic positions 6452, 6599, 4557, and 2112) and one non-coding SNV at genomic position 739 exceeded 1% of the viral population, all 5 were present in the same replicate (**Figure 6.2** and **Table 6.2**). Unlike Asibi/17D prME and other Asibi backbone viruses, none of the SNVs that exceeded 1% of the population for Asibi/17D prME + NS2B-I109L virus were observed in the variant population of IC-derived Asibi viruses. These five SNVs were unique to Asibi/17D prME + NS2B-I109L virus. Given the number of SNVs identified for Asibi/17D prME + NS2B-I109L virus, it was anticipated that there would be a considerable number of shared SNVs. However, only two and three SNVs for Asibi/17D prME + NS2B-I109L virus were exclusively shared with IC-derived Asibi and 17D-204 viruses, respectively.

Thirty SNVs were identified for 17D/Asibi prME + NS2B-L109I virus; 14 SNVs were coding, 10 SNVs were non-coding, and 6 SNVs were identified in the 3' NCR (**Figure 6.2**). Thirteen SNVs for 17D/Asibi prME + NS2B-L109I virus exceeded 1% of the population, greater than 50% of the SNVs were in the NS5 gene and many exceeded 5% of the variant population (**Figure 6.2** and **Table 6.2**). Two SNVs for 17D/Asibi

prME + NS2B-L109I virus were shared with either IC-derived Asibi or 17D-204 virus (**Figure 6.2**).

The diversity indices for prME + NS2B-109 chimeric virus correlated with the SNV profile. The nucleotide diversity in seven genes (C, prM/M, E, NS1, NS2B, NS4A and NS4B) for Asibi/17D prME + NS2B-I109L chimeric virus were consistent with that of IC-derived Asibi virus (**Figure 6.3** and **Table 6.3**). Interestingly, the diversity indices of Asibi/17D prME chimeric virus were decreased when compared to IC-derived Asibi virus.

The nucleotide diversity of six genes (E, NS2A, NS2B, NS3, NS4B, and NS5) of 17D/Asibi prME + NS2B-L109I chimeric virus displayed increased diversity when compared to IC-derived 17D-204 virus; five of the six genes (E, NS2A, NS3, NS4B, and NS5) were consistent with IC-derived Asibi virus (**Figure 5.3** and **Table 5.3**). Additionally, the mutation frequency of both Asibi/17D prME + NS2B-I109L and 17D/Asibi prME + NS2B-L109I chimeric viruses were increased when compared to their respective parental virus (**Figure 5.4**). Twenty-six and 34 genomic positions for Asibi/17D prME + NS2B-I109L and 17D/Asibi prME + NS2B-L109I chimeric viruses, respectively, exceeded a mutation frequency of 0.01, which are significantly higher mutation frequencies than both parental viruses.

6.2.2.2 Comparison of prME + NS2B-109 chimeric viruses with prME chimeric viruses

As discussed in **Chapter 4** of this dissertation, 37% of the SNVs identified for Asibi/17D prME virus were shared with IC-derived Asibi. However, only 3% of the SNVs identified for Asibi/17D prME + NS2B-109 virus were shared with IC-derived Asibi (**Figure 6.2**). Interestingly, only one SNV at genomic position 10394 for

Asibi/17D prME + NS2B-I109L virus was shared with Asibi/17D prME virus (**Figure 6.2**). The diversity indices for Asibi/17D prME + NS2B-I109L virus were also significantly higher compared to Asibi/17D prME (**Figures 6.3 and 6.4**, and **Table 6.3**).

Conversely, both 17D/prME and 17D/prME + NS2B-L109I viruses shared little identity with 17D-204 virus. No SNVs for 17D/prME + NS2B-L109I were shared exclusively with 17D/prME (**Figure 6.2**). The nucleotide diversity of six genes (NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and the mutation frequency for 17D/Asibi prME + NS2B-L109I virus were significantly higher when compared to 17D/Asibi prME virus. In comparison, only the nucleotide diversity in the E and NS5 genes for 17D/Asibi prME were significantly higher when compared to IC-derived 17D-204 virus.

6.2.2.3 Comparison of prME + NS2B-109 chimeric viruses with NS2B-109 mutant viruses

Comparison of the prME + NS2B-109 to the NS2B-109 mutant virus were similar to that of the comparison with the prME chimeric viruses. Asibi/17D prME + NS2B-I109L shared no SNVs with Asibi/17D NS2B-I109L virus (**Figure 6.2**). The nucleotide diversity for Asibi/17D prME + NS2B-I109L virus varied when compared to Asibi/17D NS2B-I109L virus; the nucleotide diversity in the E, NS2A and NS5 genes were statistically higher while the nucleotide diversity in the NS2B gene was statistically lower (**Figure 6.3** and **Table 6.3**). Additionally, the mutation frequency for Asibi/17D prME + NS2B-109 virus was higher than that of Asibi/17D NS2B-I109L virus (**Figure 6.4**).

Similarly, 17D/Asibi prME + NS2B-L109I shared one SNV with 17D/Asibi NS2B-L109I virus (**Figure 6.2**). The nucleotide diversity for 17D/Asibi prME + NS2B-L109I virus also varied when compared to 17D/Asibi NS2B-L109I virus; the nucleotide

diversity in the NS3 gene was statistically higher while the nucleotide diversity in the C, prM and NS1 genes were statistically lower (**Figure 6.3** and **Table 6.3**). The mutation frequency for 17D/Asibi prME + NS2B-L109I virus was higher than that of 17D/Asibi NS2B-L109I virus (**Figure 6.4**).

Table 6.2. Single nucleotide variant greater than 1% of the population.

	Reference Base	Variant Base	Genomic Position	Gene Region	Coding/ Noncoding	Variant Frequency
Asibi/17D prME + NS2B-I109L						
	G	A	739	prM	No	1.64%
	G	A	2112	E	Yes; R665K	1.53%
	G	A	4557	NS2B	Yes; R1480K	1.40%
	G	A	6452	NS4A	Yes; V6452M	1.02%
	G	A	6599	NS4B	Yes; V2161I	1.21%
17D/Asibi prME + NS2B-L109I						
	G	A	1814	E	Yes; V566I	1.69%
	C	A	2383	E	Yes; N755K	2.68%
	C	A	3769	NS2A	No	26.67
	G	A	3773	NS2A	Yes; G1219S	9.04%
	C	A	3850	NS2A	No	3.89%
	G	A	6239	NS3	Yes; A2041T	8.55%
	G	A	6439	NS4A	No	15.37%
	G	T	7636	NS2B	No	2.56%
	G	A	8462	NS5	Yes; E2782K	20.14%
	C	T	8563	NS5	No	1.14%
	G	C	8948	NS5	Yes; E2944Q	3.06%
	A	T	9167	NS5	Yes; I3017F	14.71%
	C	A	9317	NS5	Yes; H3067N	2.43%

Table 6.3. Nucleotide diversity comparisons for prME + NS2B-109 chimeric viruses.

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi prME + NS2B-L109I	17D IC	ns	ns	***	ns	***	***	***	ns	***	***
	Asibi IC	ns	ns	ns	**	ns	*	ns	ns	ns	ns
Asibi/17D prME + NS2B-I109L	17D IC	*	**	***	***	***	***	***	*	***	***
	Asibi IC	ns	ns	ns	ns	*	ns	*	ns	ns	***

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
17D/Asibi prME + NS2B-L109I	17D/ Asibi PrME	ns	ns	ns	ns	*	**	***	*	**	***
	17D/Asibi NS2B-L109I	*	*	ns	**	ns	ns	*	ns	ns	ns

		C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Asibi/17D prME + NS2B- I109L	Asibi/17D prME	***	***	***	***	***	***	***	***	***	***
	Asibi/17D NS2B-I109L	ns	ns	*	ns	*	*	ns	ns	ns	**

P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

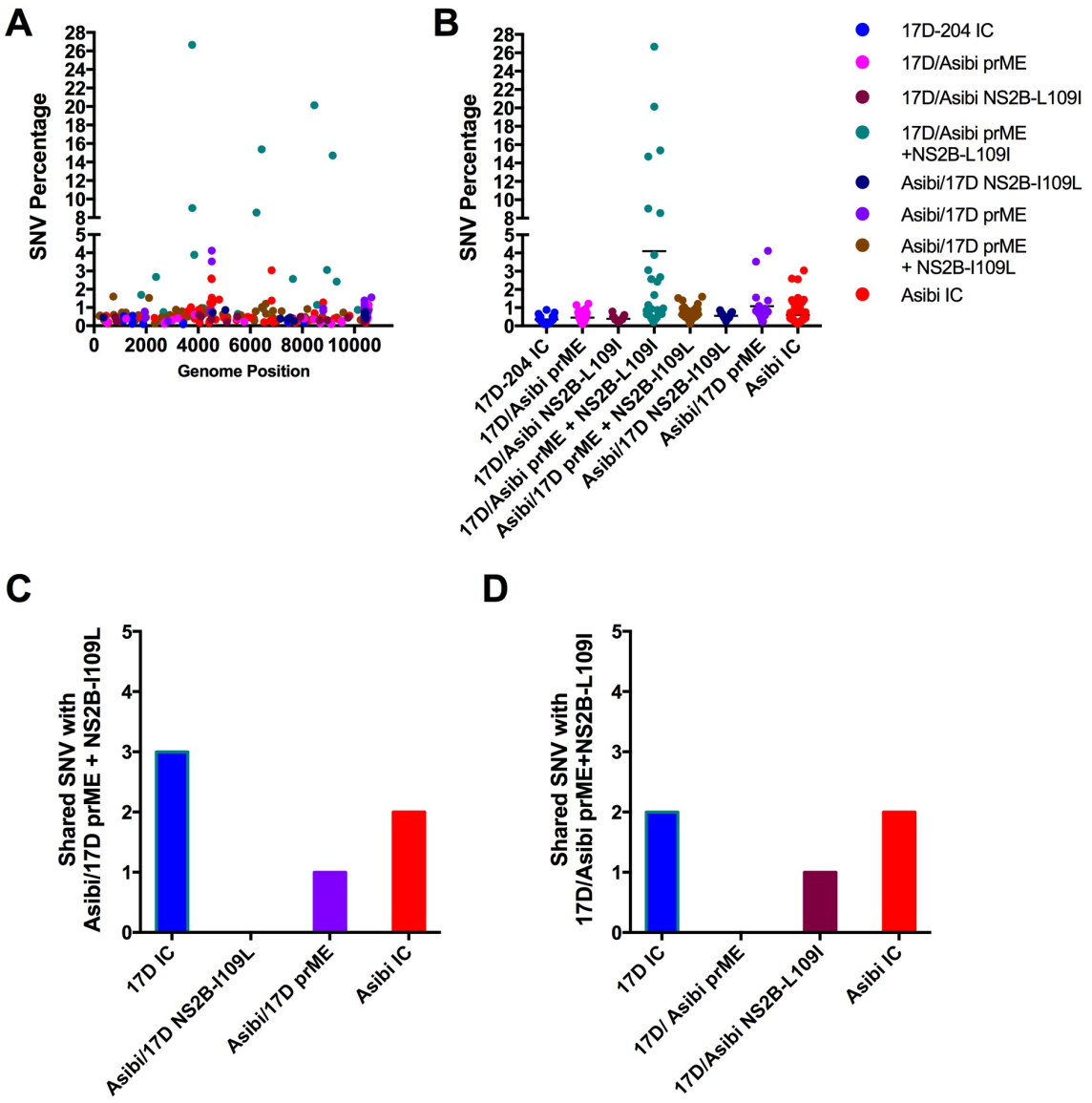

Figure 6.2. Single nucleotide variants (SNVs) identified for prME + NS2B-109 chimeric viruses. a. SNVs across the genome, b. total SNVs. b. Asibi/17D prME + NS2B-I109L and c. 17D/Asibi prME + NS2B-L109I shared SNVs. The SNV profile for17D/Asibi prME + NS2B-L109I was altered considerable when compared to all other viruses investigated, while there was limited evidence of altered SNV profile for the Asibi/17D prME + NS2B-I109L when compared to IC-derived Asibi and other Asibi backbone viruses. There was limited evidence of shared SNVs for either Asibi/17D prME + NS2B-I109L or 17D/Asibi prME + NS2B-L109I with either IC-derived Asibi or 17D-204 viruses.

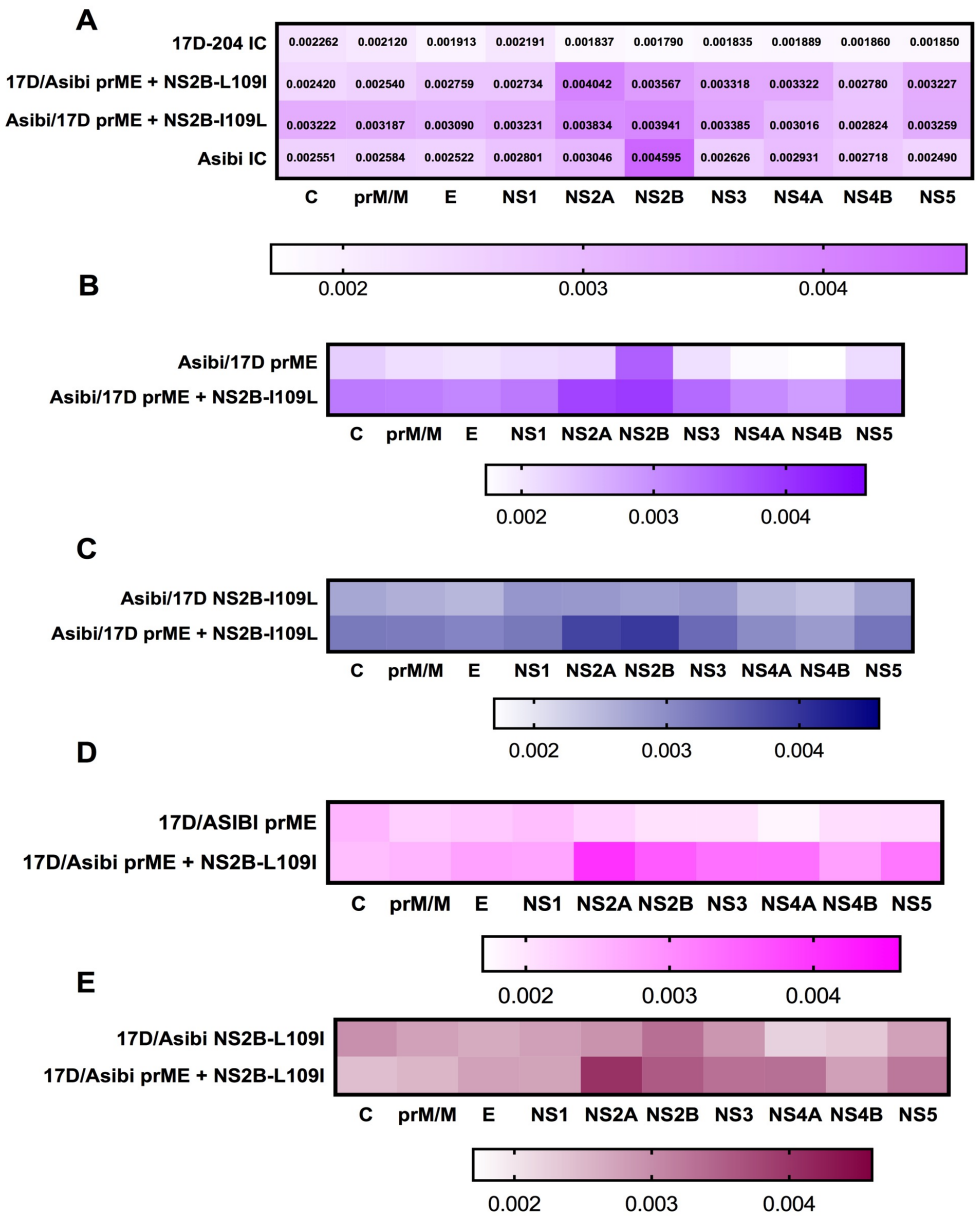

Figure 6.3. Comparison of nucleotide diversity for prME + NS2B-109 chimeric viruses. Nucleotide diversity determined by Shannon's entropy. a. parental virus, b Asibi/17D prME, c. Asibi/17D NS2B-I109L, d 17D/Asibi prME and e. 17D/Asibi NS2B-L109I. 17D/Asibi prME + NS2B-L109I alter the nucleotide diversity in multiple structural and non-structural genes, whereas, mutation of the Asibi backbone has a limited effect on nucleotide diversity. Diversity is shown as a heatmap with the darker the color the more diversity.

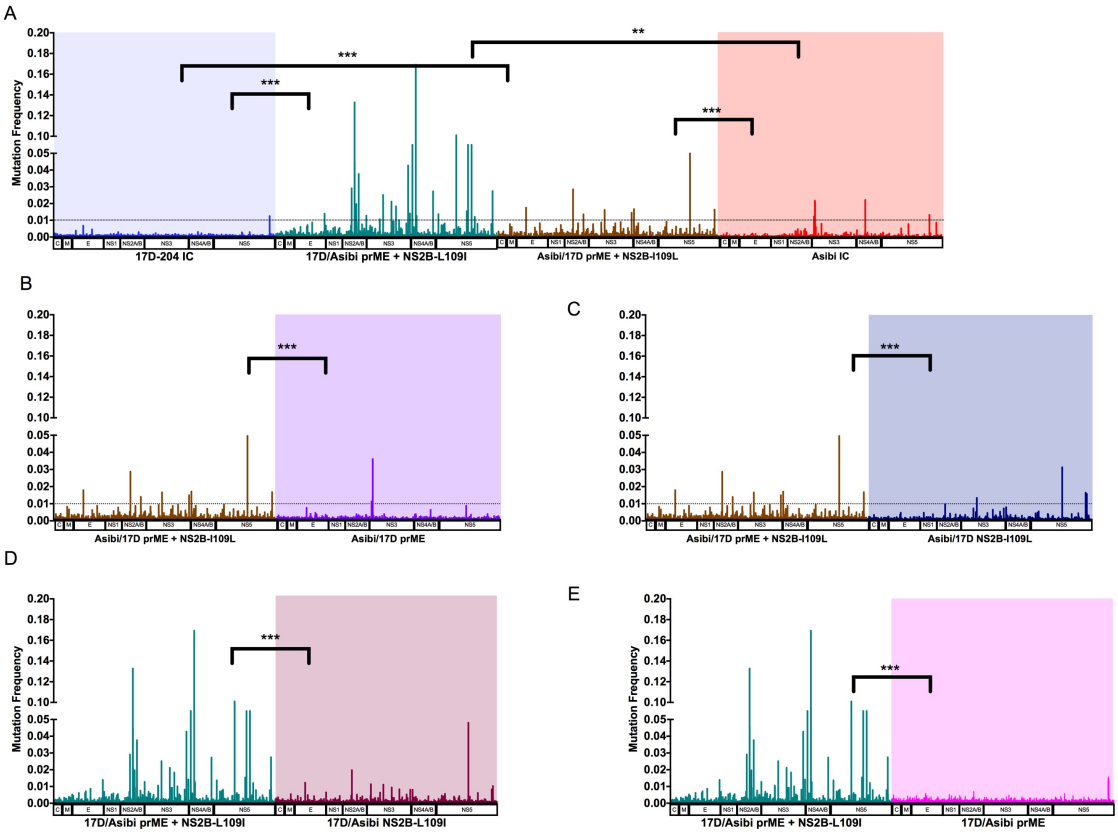

Figure 6.4. Comparison of mutation frequency for prME + NS2B-109. a. parental virus, b Asibi/17D prME, c. Asibi/17D NS2B-I109L, d 17D/Asibi prME and e. 17D/Asibi NS2B-L109I. The mutation frequency for both Asibi/17D prME + NS2B-I109L and 17D/Asibi prME + NS2B-L109I displayed increased mutation frequency when compared to either parental, prME chimeric, or NS2B-109 viruses. P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

6.3. DISCUSSION

The results of this Chapter demonstrated that structural and NS genes, together, contribute to the genetic diversity of 17D vaccine and highlights that combining these mutations effect the overall genetic stability of YFV. **Chapters 4 and 5** showed that the prME and NS2B-109 Asibi backbone viruses alone had decreased diversity when compared to IC-derived Asibi virus, while prME and NS2B-109 17D backbone viruses displayed increased diversity when compared to IC-derived 17D-204 virus. However, in this Chapter it was found that incorporation of prME + NS2B-109 into the either Asibi or 17D backbone increased diversity when compared to both parental viruses. Clearly, the results of the prME + NS2B-109 chimeric viruses demonstrated that genetic diversity can be multi-genic. In general, genetic diversity for both Asibi/17D prME + NS2B-I109L and 17D/Asibi prME + NS2B-L109I viruses were increased when compared to either parental, prME chimeric, or NS2B-109 mutant viruses. Furthermore, combination of the prME with NS2B-209 substitution yielded viruses with an intermediate nucleotide diversity by gene when compared to the NS2B-109 mutant virus, as shown by genes with both increased and decreased nucleotide diversity. Additionally, alterations in SNV percentages for the 17D backbone viruses were only observed with the 17D/Asibi prME + NS2B-L109I virus. However, while these studies indicate that prME and NS2B mutations act synergistically, it is not known if this is a general phenomenon for all the Asibi to 17D mutations and whether or not any combination of single NS substitutions (e.g., NS4B-95) in conjunction with prME genes will yield the same results. Given the 176 passages of Asibi to generate 17D, it is unlikely that every mutation contributes to the attenuated phenotype. This warrants additional studies.

The 17D/Asibi prME + NS2B-L109I chimeric virus showed that combining structural and NS genes of Asibi into the backbone resulted in a synergistic effect on the genetic diversity of the vaccine; the diversity indices display genes that were either consistent, increased, or decreased when compared to either 17D/Asibi prME or 17D/Asibi NS2B-L109I and the mutation frequency for 17D/Asibi prME + NS2B-L109I chimeric virus was increased when compared to both 17D/Asibi prME or 17D/Asibi NS2B-L109I, however, no other 17D backbone virus studied in this dissertation yielded multiple SNVs outside of the 3' NCR with percentages greater than 1%. Additionally, the nucleotide diversity in multiple genes of 17D/Asibi prME + NS2B-L109I chimeric virus was consistent with IC-derived Asibi virus, while the mutation frequency was significantly increased when compared to both parental IC-derived Asibi and 17D-204 viruses.

Although there were a considerable number of SNVs for 17D/Asibi prME + NS2B-L109I chimeric virus with percentages that exceeded those seen with IC-derived Asibi virus, the combination of structural and NS genes drives mutation rate such that the variant profile was consistent with other wild-type and IC-derived flaviviruses (Metsky et al., 2017; Faria et al., 2017; Van Slyke et al., 2015; Sessions et al., 2015) Additionally, the SNVs that exceeded 1% of the population clustered in the NS5 gene (notably18 SNVs in one replicate and 15 in the other replicate with clustering in the NS5 for both replicates), which was not observed in the population of any other virus investigated in this dissertation. The clustering of high percentage SNVs in the NS5 suggests that combining the prME and NS2-L109I substitution of Asibi into the backbone of the

vaccine acts on the NS5 gene selectively, similar to what is seen when wild-type viruses are passaged in the presence of nucleoside analogs, such as ribavirin.

Importantly, the genetic diversity of Asibi/17D prME + NS2B-I109L chimeric virus was significantly higher when compared to both parental IC-derived Asibi and 17D-204 viruses; despite the increased number of SNVs identified for Asibi/17D prME + NS2B-I109L chimeric virus, few were shared with either IC-derived Asibi or 17D-204 virus, and the diversity indices were higher when compared to both parental viruses. In addition, Asibi/17D prME + NS2B-I109L chimeric virus was more diverse than both Asibi/17D prME and Asibi/17D NS2B-I109L; however, the NS1, NS4A, and NS4B genes were consistent with Asibi/17D NS2B-I109L. Taken together, combining the prME and NS2B-I109L of 17D into the Asibi backbone led synergistic effect of the mutations and increased the genetic diversity.

Low fidelity mutants have been generated and investigated for RNA viruses; however, the majority of these studies have focused on mutation of the RdRp (Gnädig et al., 2012; Korboukh et al., 2014; Rozen-Gagnon et al., 2014; Van Slyke et al., 2015; Xie et al., 2014). These studies were suggestive of a significant increase nucleotide mis-incorporation when compared to either wild-type or parental IC-derived viruses. Interestingly, the accepted paradigm that high fidelity correlated with decreased fitness was challenged with the identification of low fidelity mutants that also appeared attenuated *in vivo* (Gnädig et al., 2012; Xie et al., 2014). This discrepancy was attributed to the catastrophe theory of RNA viruses, which states RNA viruses exist within a mutational or diversity threshold; significantly high mutation rate drives the virus to extinction due to an increase in deleterious mutations, while a low mutation rate

decreases the generation of beneficial mutations, thus decreasing fitness (Crotty et al., 2001). Error catastrophe may explain the results of the prME + NS2B-109 chimeric viruses. Interestingly, infectivity titers for the recovered viruses were consistent with the source of the parental virus for the mutation. Therefore, it is possible that the results of Asibi/17D prME + NS2B-I109L indicate attenuation of virulence, while the results of 17D/Asibi prME + NS2B-L109I indicate increased virulence phenotype. This needs to be tested.

Several studies have speculated that the attenuated phenotype of 17D vaccine is multi-genic (McElroy et al., 2006, 2006) and the previous Chapters in this dissertation support this hypothesis. Although the diversity indices for prME, NS2B-L109I, and NS4B-M95I were increased when compared to IC-derived 17D-204 virus, significant alterations in the SNV profile was not observed until both the prME and NS2B-L109I of Asibi were introduced into the 17D backbone. Additionally, the mean infectivity titer was consistent with other Asibi backbone viruses, suggesting that that prME and NS2B-109 together may lead to phenotypic changes.

CHAPTER 7: CONTRIBUTIONS OF NON-STRUCTURAL GENES TO MULTIPLICATION KINETICS AND INNATE IMMUNITY

7.1 INTRODUCTION

Immunization with 17D vaccine leads to a transient viremia that rarely exceeds 10^2 pfu/ml, contrasting drastically with the viremia seen following infection with wild-type infection, which can exceed 10^8 pfu/ml in humans (Macnamara, 1957). Studies with other flaviviruses demonstrated that mutations in NS protein genes decreased virus production and replication in host cells (Audsley et al., 2011; Grant et al., 2011; Xie et al., 2011). Therefore, attenuating mutations in the genome of 17D vaccine that effect replication efficiency in humans, may also effect multiplication in host cells, ultimately effecting viremia and dissemination in natural hosts.

In vitro studies have shown that various cell types are permissive to YFV infection, yet have not conclusively differentiated between the multiplication kinetics of wild-type Asibi virus and the 17D vaccine (Woodson & Holbrook, 2011; S. Woodson et al., 2011). In most cell types multiplication kinetics of wild-type Asibi and 17D viruses are indistinguishable, or the multiplication kinetics of wild-type Asibi and 17D viruses differ at limited time points only prior to reaching peak titer. The lack of a suitable cell line that differentiates multiplication kinetics of Asibi and 17D viruses has impeded the evaluation of attenuating mutations on multiplication kinetics, which may have implications to decreased viremia and dissemination seen following vaccination with 17D vaccine.

The adaptive immune response is the most critical component in viral clearance and disease recovery following wild-type YFV infection, but it has also been shown that the innate immune response is also important. There are a number of cytokine and chemokine changes during natural human infection with wild-type YFV, including IL-6, MCP-1, IP-10, TNF-α, and IL-1RA, which have been associated with disease severity (ter Meulen et al., 2004). Interestingly, immunization with 17D-204 vaccine strain induced similar production of cytokines/chemokines and gene expression as wild-type infection, but neither productive infection nor severe liver pathology are evident in healthy vaccinees (Gaucher et al., 2008; Campi-Azevedo et al., 2012).

NS proteins of flaviviruses have been implicated in modulating the innate immune response and evasion of the immune system; specifically, the NS4B protein is a known interferon antagonist (Aguirre et al., 2012; Niewold et al., 2013; Jones et al., 2005; Muñoz-Jordán et al., 2005; Ye et al., 2013). The importance of innate immunity during YFV infection and vaccination with 17D vaccine has been implicated in multiple studies, including those where vaccine associated adverse events were attributed to defects in innate immunity (Belsher et al., 2007; Gaucher et al., 2008; Pulendran, 2009; Pulendran et al., 2008; Pulendran et al., 2013; Querec & Pulendran, 2007; Querec et al., 2006). Attenuating mutations in NS proteins may affect protein function, abating virus-induced regulation of innate immunity that supports viral survival and replication, thereby decreasing virulence.

7.2 RESULTS

7.2.1. Multiplication kinetics as a phenotypic marker of virulence

Earlier chapters in this dissertation have identified genetic differences between wild-type Asibi and 17D vaccine virus. Such studies do not inform about phenotypic differences that may be associated with an attenuated phenotype. Therefore, to investigate whether or not the substitutions that differ between wild-type Asibi and 17D vaccine translated to phenotypic differences, multiplication kinetics of parental IC-derived Asibi and 17D viruses were compared to the chimeric and mutant viruses in human alveolar A549 cells. These cells were selected because they have a functional interferon-α/β receptor, which was considered important for two reasons. First, all cell types used so far to investigate YFV multiplication lacked a functional interferon receptor. Second, as stated above, the innate immune response plays an important role in YFV infection and so a cell type with a functional interferon system was considered important.

7.2.1.1 Multiplication kinetics of parental IC-derived Asibi and 17D-204 viruses

Comparison of multiplication kinetics of wild-type IC-derived Asibi to IC-derived 17D-204 vaccine virus demonstrated IC-derived 17D-204 virus multiplies more efficiently than IC-derived Asibi virus following infection of A549 cells at a MOI of 0.1, with 17D virus having significantly higher infectivity titers at 24 (p-value = 0.007), 36 (p-value <0.001), and 48 (p-value < 0.001) hours post infection (hpi) (**Figure 7.1)**; infectious virus was below the limit of detection at 12 hpi.

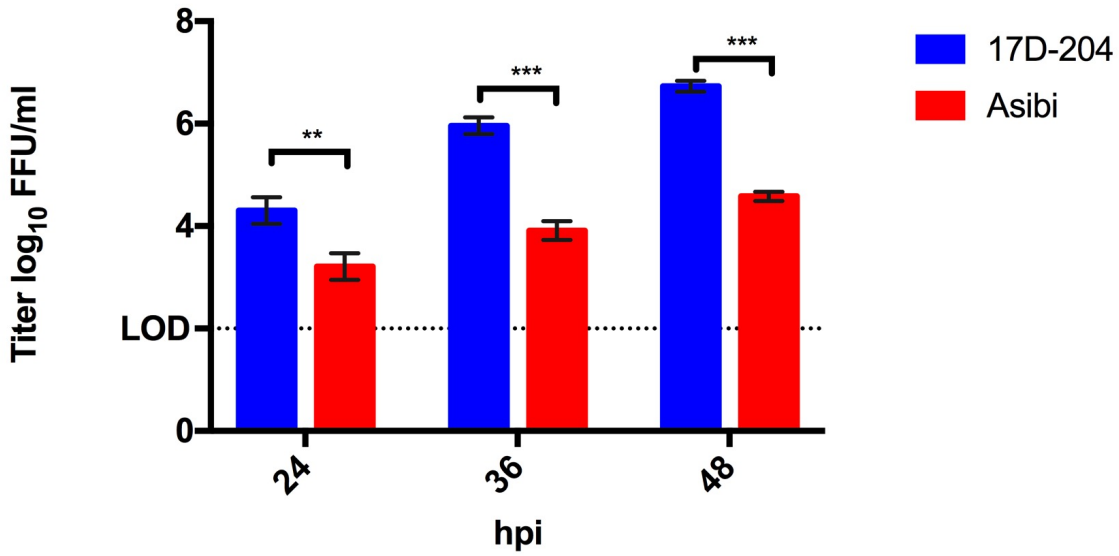

Figure 7.1. Multiplication kinetics of IC-derived Asibi and 17D-204 virus at a MOI of 0.1 in A549 cells. Multiplication kinetics of IC-derived 17D-204 virus were undertaken in triplicate and were found to multiply to statistically higher titers than Asibi virus at 24, 36, and 48 hpi. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

7.2.1.2 Multiplication kinetics of prME and M-36 chimeric and mutant viruses

The multiplication kinetics of the prME chimeric viruses at a MOI of 0.1 displayed an intermediate phenotype when compared to IC-derived Asibi and 17D-204 viruses, such that the infectivity titers of both Asibi/17DprME and 17D/Asibi prME viruses were statistically different from their respective parental virus at 36 and 48 hpi (**Figure 7.2**). Importantly, the infectivity titers of Asibi/17D prME and 17D/Asibi prME viruses were statistically higher (p-value = 0.001 and p-value = 0.003, respectively) when compared to IC-derived Asibi virus and statistically lower at 36 hpi (p-value = 0.002 and p-value < 0.001, respectively) when compared to IC-derived 17D-204 virus.

In comparison, the infectivity titers of neither 17D/Asibi M-F36L nor Asibi/17D M-L36F differed when compared to their respective backbone virus, suggesting this substitution did not contribute to multiplication kinetics.

7.2.1.3 Multiplication kinetics of NS2B-109 and NS4B-95 mutant viruses

The infectivity titers of NS2B-109 and NS4B-95 mutants were the most intriguing of the findings when compared to backbone viruses (**Figure 7.3**). 17D/Asibi N2B-L109I and 17D/Asibi NS4B-M95I multiplied just as efficiently as did IC-derived 17D-204 virus in A549 cells. However, the infectivity titers of Asibi/17D NS2B-I109L and Asibi/17D NS4B-I95M displayed an intermediate phenotype, differing from both IC-derived 17D virus and IC-derived Asibi virus at 36 and 48 hpi. Asibi/17D NS2B-I109L and Asibi/17D NS4B-I95M multiplied to significantly higher titers at 36 hpi (p-value of 0.004 and p-value of 0.003, respectively) and 48 hpi (p-value = 0.035 and p-value <

0.001, respectively) when compared to IC-derived Asibi virus. Conversely, Asibi/17D NS2B-I109L and Asibi/17D NS4B-I95M replicated to significantly lower titers at 36 hpi (p-value = 0.004 and p-value < 0.001, respectively) and 48 hpi (p-value = 0.035 and p-value <0.001 respectively) when compared to IC-derived 17D virus.

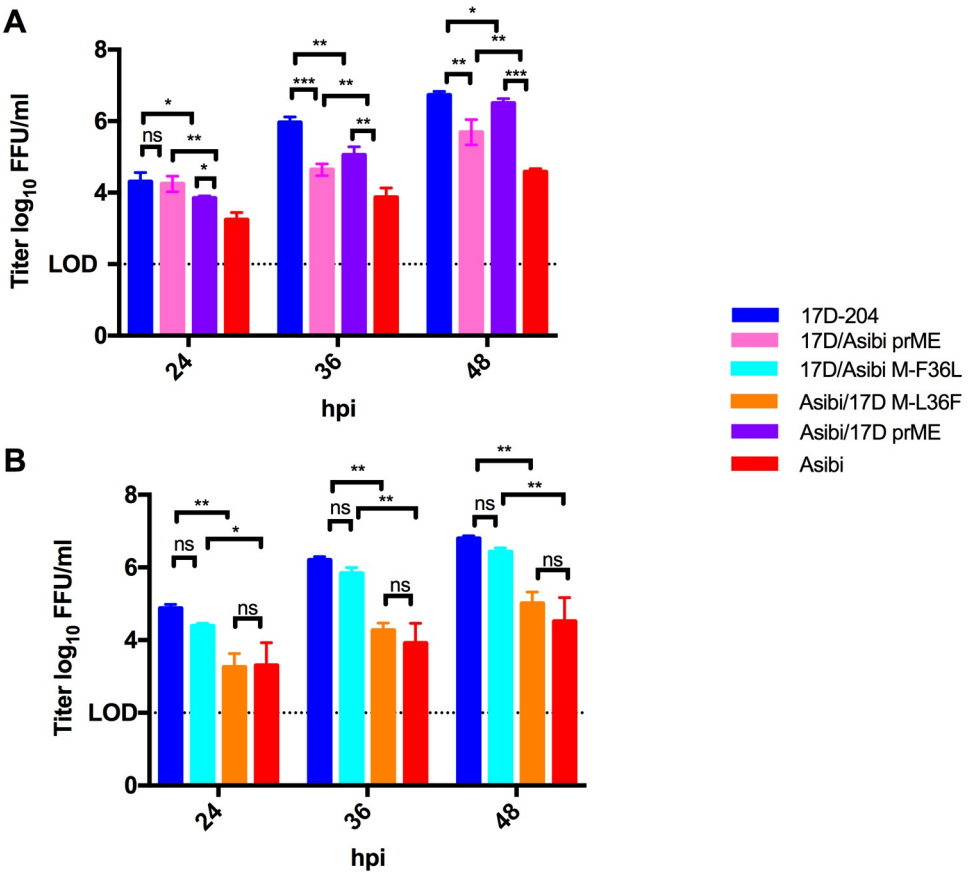

Figure 7.2. Multiplication kinetics of prME and M-36 chimeric and mutant viruses at a MOI of 0.1 in A549 cells. a. prME chimeric viruses and b. M-36 mutant viruses. Multiplication kinetics were undertaken in triplicate. Multiplication of the prME chimeric viruses differed significantly when compared to their respective parental viruses, while the M-36 mutant viruses did not. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

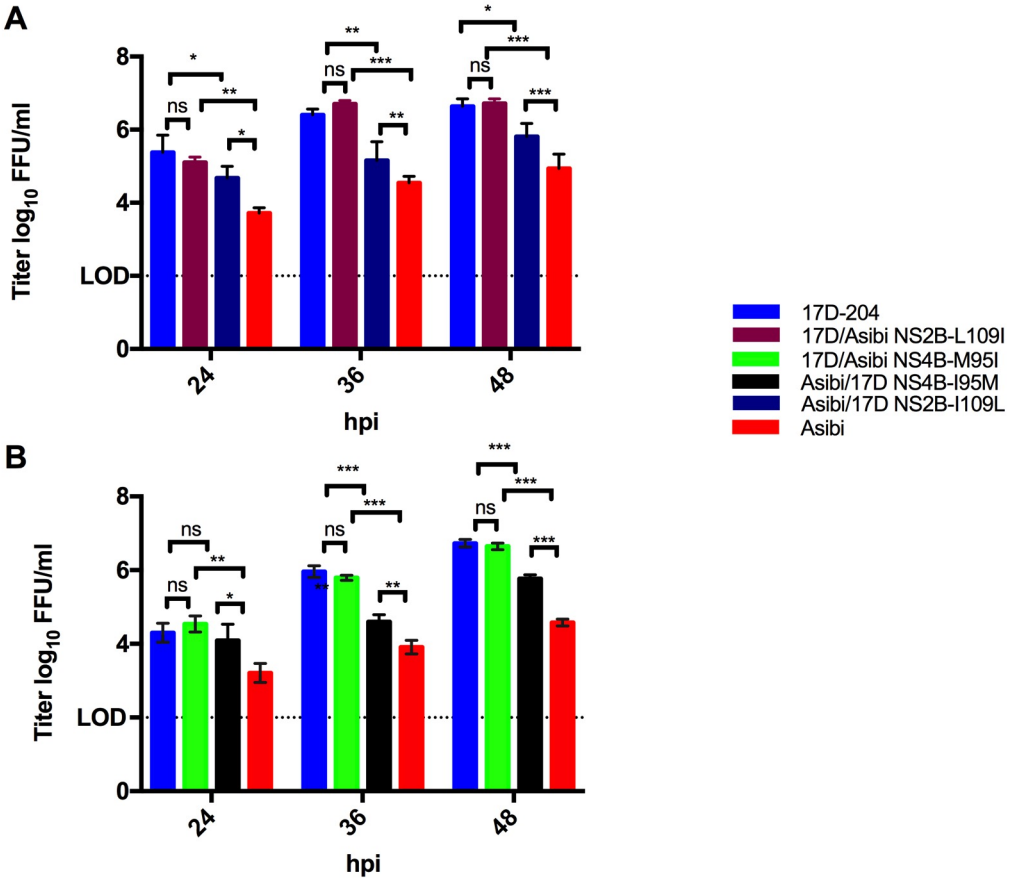

Figure 7. 3. Multiplication kinetics of NS2B-109 and NS4B-95 mutant viruses at a MOI of 0.1 in A549 cells. a. NS2B-109 mutant viruses and b. NS4B-95 mutant viruses. Multiplication kinetics were undertaken in triplicate. The introduction of neither NS2B-L109I nor NS4B-M95I into the 17D-204 backbone altered the multiplication kinetics, while introduction of either NS2B-I109L or NS4B-I95M into the Asibi backbone did alter the multiplication kinetics. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

7.2.1.4 Multiplication kinetics of prME + NS2B-109 viruses

Similar to the infectivity titers of the prME chimeric viruses, the prME + NS2B-109 chimeric viruses were statistically different from their respective parental virus at 36 and 48 hpi (**Figure 7.4**). Interestingly, the infectivity titers of Asibi/17D prME + NS2B-I109L were statistically higher at 36 and 48 (p-value = 0.001 and p-value = 0.003, respectively) when compared to IC-derived Asibi virus and consistent with IC-derived 17D-204 virus; whereas, the Asibi backbone virus with only 17D prME differed from both parental virus at 36 and 48 hpi (discussed in section 7.2.1.2 of this dissertation). However, the infectivity titer of 17D/Asibi prME + NS2B-L109I was statistically higher when compared IC-derived Asibi and statistically lower when compared to IC-derived 17D-204 viruses at 36 hpi. Interestingly, the infectivity titer at 48 hpi for 17D/Asibi prME + NS2B-L109I was statistically lower when compared to IC-derived 17D-204 virus and consistent that of IC-derived Asibi virus.

The multiplication kinetics of prME + NS2B-109 chimeric viruses were compared to their respective prME and NS2B-109 chimeric and mutant viruses. The infectivity titers of Asibi/17D prME + NS2B-I109L chimeric virus were significantly higher than both Asibi/17D prME and Asibi/17D NS2B-I109L viruses at 24 hpi (p-values = <0.001 and p-value = 0.033, respectively) and 36 hpi (p-values = <0.003 and p-value = 0.034, respectively). Interestingly, the infectivity titers at 48 hpi of Asibi/17D prME + NS2B-I109L were consistent with Asibi/17D prME chimeric virus and significantly higher than Asibi/17D NS2B-I109L viruses (p-value = 0.034).

The infectivity titers of 17D/Asibi prME + NS2B-L109I were significantly higher than 17D/Asibi prME at 24 hpi (p-value = 0.043) and 36 hpi (p-value = 0.017), and

consistent with 17D/Asibi prME at 48 hpi. Conversely, the infectivity titers of 17D/Asibi prME + NS2B-L109I were significantly lower than 17D/Asibi NS2B-L109I at 24 hpi (p-value = 0.003) and 36 hpi (p-value = 0.023), and consistent with 17D/Asibi NS2B-L109I at 24 hpi.

Taken together, the prME chimeric, and NS2B-109, and NS4B-95 single-site mutant contributed to phenotypic differences in terms of multiplication kinetics of the Asibi backbone viruses. However, the viral yield of the NS2B-109 and NS4B-95 backbone viruses suggests that these amino acid substitutions in the 17D backbone do not contribute to multiplication kinetics of the 17D vaccine. The prME+NS2B-109 chimeric viruses differ from both the parental and the NS2B-109 mutant viruses. However, the results are generally consistent with the prME mutant viruses, which was most evident at 48 hpi when the viruses reach peak infectivity titer; this indicates that the E protein gene contributes significantly to viral multiplication.

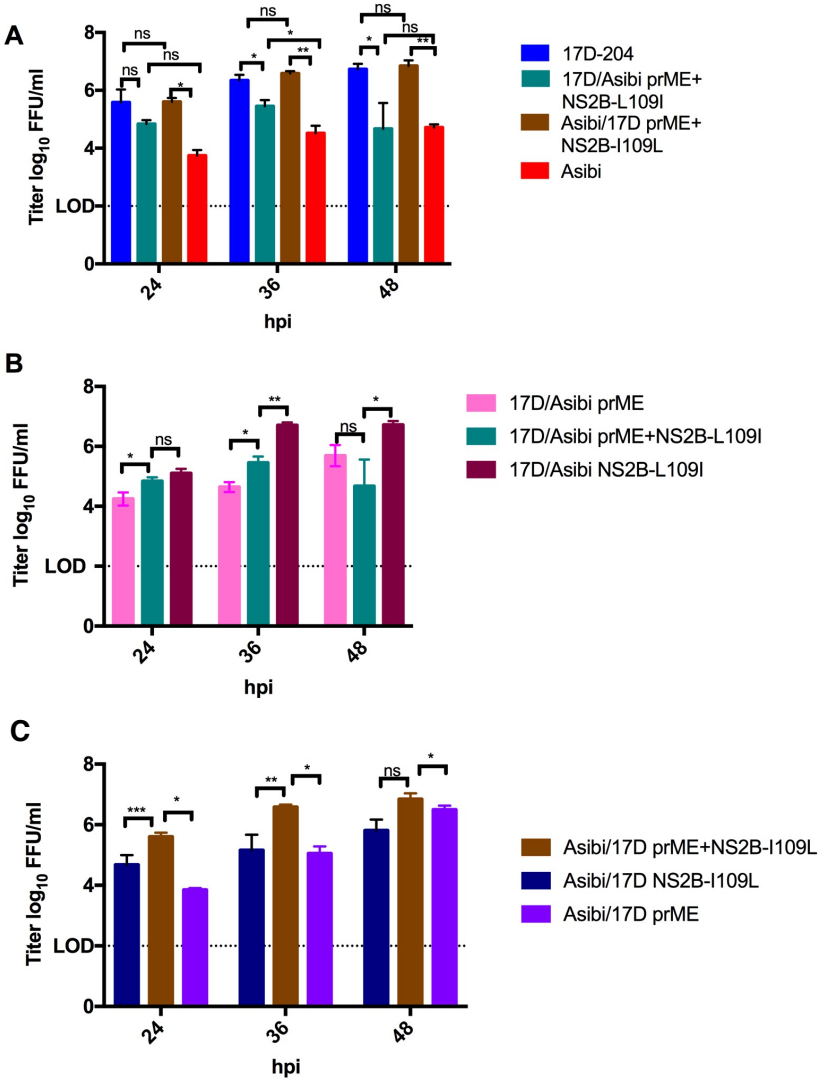

Figure 7. 4. Multiplication kinetics of prME + NS2B-109 compared to both prME and NS2B-1009 chimeric and mutant viruses at a MOI of 0.1 in A549 cells. a. prME + NS2B-109 chimeric viruses, b. comparison of 17D backbone viruses, and c. comparison of Asibi backbone viruses. Multiplication kinetics were undertaken in triplicate. The multiplication kinetics of prME + NS2B-109 chimeric viruses differed significantly from their respective parent virus and NS2B-109 mutant virus. The multiplication kinetics of Asibi/17D prME + NS2B-I109L differed significantly at all time points measured when compared to Asibi prME chimeric virus, while 17D/Asibi prME + NS2B-L109I was consistent with 17D/Asibi prME at 48 hpi. P-value = .12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

7.2.2. Innate immunity as a phenotypic marker of virulence

In addition to evaluating multiplication kinetics, cell culture supernatants were investigated for production of 40 cytokines and chemokines from A549 cells using the BioPlex system (**Table 7.1**). YFV-infection induced 27 of the 40 chemokines and cytokines, however, 11 chemokine and cytokines (CCL21, CCL27, CCL11, CCL26, GM-CSF, CCL1, IL-2, IL-4, IL-16, CCL22, CCL25) did not differ from mock-infected supernatants and were not evaluated. Therefore, innate immunity was evaluated by examining production of innate response chemokines and cytokines at 48 hpi, including: IFNγ, IL-1β, IL-6, IL-8, IL-10, IP-9, IP-10, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1A, MIP-1D, MIP3A, MIP3B, and TNF-α. This section includes evaluation of parental Asibi and 17D, prME chimeric, M-36 mutant, and NS4B-95 mutant viruses only.

7.2.2.1 Innate immunity of IC-derived Asibi and 17D-204 viruses

IC-derived Asibi virus displayed a lower level of cytokine and chemokine induction than IC-derived 17D-204 virus (**Figure 7.5**). IC-derived 17D-204 virus induced significantly higher levels of IFNγ (p-value = 0.035), IL-1β (p-value = 0.045), IL-6 (p-value = 0.006), IL-8 (p-value = 0.014), IL-10 (p-value = 0.005), IP-9 (p-value = 0.014), MCP-4 (p-value = 0.007), MIP-1A (p-value = 0.001), and TNF-α (p-value = 0.006), while IP-10, MCP-1, MCP-2, MCP-3, MIP-1D, MIP-3A, and MIP-3B did not differ between Asibi and 17D virus infected supernatants. Interestingly, IL-6, a potent pro-inflammatory cytokine, was produced at higher levels following exposure to both IC-derived Asibi and 17D-204 viruses.

Table 7.1. Bio-plex cytokines and chemokines panel.

CCL21	CXCL6	CCL8	IL-4	CCL20
CXCL13	GM-CSF	CCL7	IL-6	CCL19
CCL27	CXCL1	CCL13	CXCL8	CCL23
CXCL5	CXCL2	CCL22	IL-10	CXCL16
CCL11	CCL1	MIF	IL-16	CXCL12
CCL24	IFNγ	CXCL9	CXCL10	CCL17
CCL26	IL1β	CCL3	CXCL11	CCL25
CX3CL1	IL-2	CCL15	CCL2	TNF-α

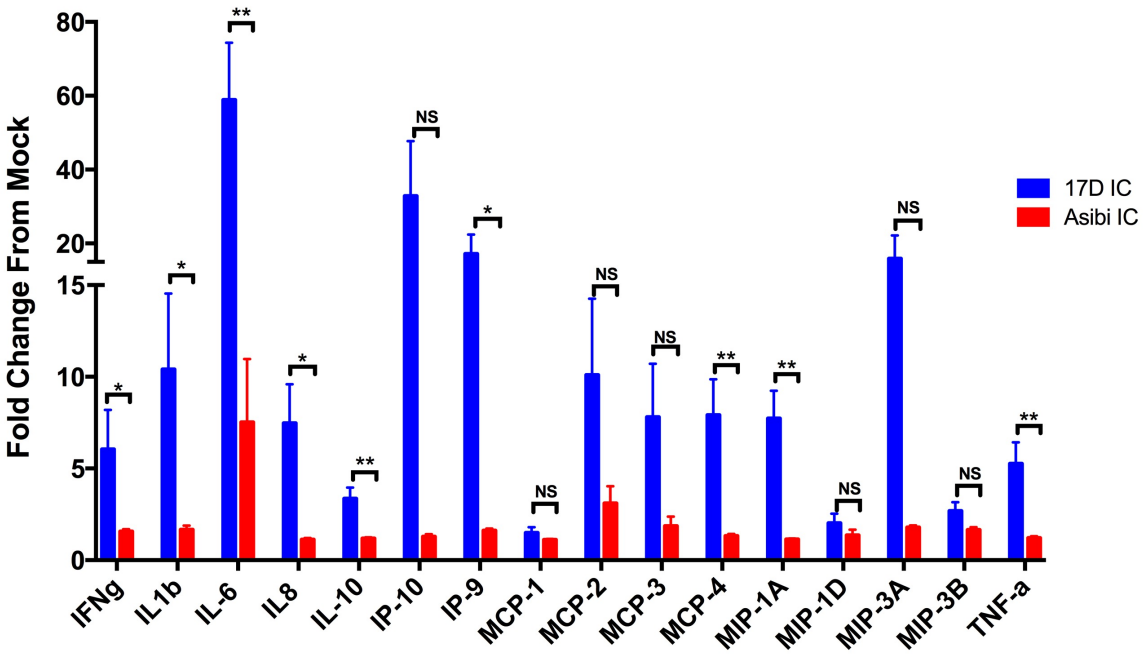

Figure 7. 5. Cytokine and chemokine production for parental viruses at a MOI of 0.1 in A549 cells. Innate immune exposure was measured and reported as mean of triplicate cultures. IC-derived 17D-204 virus produced higher levels of chemokine and cytokines than IC-derived Asibi virus.

7.2.2.2 Innate immunity of Asibi backbone viruses

In general, the cytokine and chemokine profiles for the Asibi backbone chimeric and mutant viruses were consistent with IC-derived Asibi virus, and were significantly lower when compared to those induced by IC-derived 17D-204 virus (**Figures 7.6, 7.7, and 7.8**). Specifically, Asibi/17D prME, M-L36F, and Asibi/17 NS4B-I95M induced significantly lower levels of IFNγ (p-value = 0.020, p-value = 0.027, and p-value = 0.046, respectively), IL-6 (p-value = 0.001, p-value = 0.001, and p-value = 0.002, respectively), IL-8 (p-value = 0.005, p-value = 0.005, and p-value = 0.01, respectively), IL-10 (p-value = <0.001, p-value = 0.001, and p-value = 0.003, respectively), IP-9 (p-value = 0.006, p-value = 0.005, and p-value = 0.008, respectively), MCP-4 (p-value = 0.002, p-value = 0.002, and p-value = 0.003, respectively), MIP-1A (p-value = <0.001, p-value = <0.001, and p-value = <0.001, respectively), and TNF-α (p-value = 0.001, p-value = 0.002, and p-value = 0.003, respectively) when compared to IC-derived 17D-204 virus.

7.2.2.2 Innate immunity of 17D backbone viruses

Conversely, exposure to both 17D/Asibi prME virus and 17D/Asibi NS4B-M-95I virus resulted in production of significantly lower levels of chemokine and cytokine induction when compared to IC-derived 17D-204 virus (**Figures 7.6 and 7.8**). 17D/Asibi prME induced lower levels of IL-1β (p-value = 0.048), IL-6 (p-value = 0.014), IL-8 (p-value = 0.005), IL-10 (p-value = 0.023), IP-9 (p-value = 0.030, MCP-4 (p-value = 0.018), MIP-1A (p-value = < 0.001), and TNF-α (p-value = 0.001) when compared to IC-derived 17D-204 virus. 17D/Asibi NS4B-M95I induced lower levels of IL-6 (p-value = 0.034), IL-10 (p-value = <0.027), IP-9 (p-value = 0.027, MCP-4 (p-value = 0.022), MIP-1A (p-

value = <0.002), and TNF-α (p-value = 0.021) when compared to IC-derived 17D-204 virus. The cytokines and chemokines for both 17D/Asibi prME and 17D/Asibi NS4B-M95I differed significantly from IC-derived 17D-204 virus but were consistent with IC-derived Asibi virus.

Interestingly, 17D/Asibi M-F36L displayed an intermediate phenotype (Figure 7.7). 17D/Asibi M-F36L exposure resulted in production of similar levels of IFNγ, IL-1β, IL-6, IL-8, IL-10, IP-9, MCP-4 and TNF-α as both IC-derived Asibi and 17D-204 viruses.

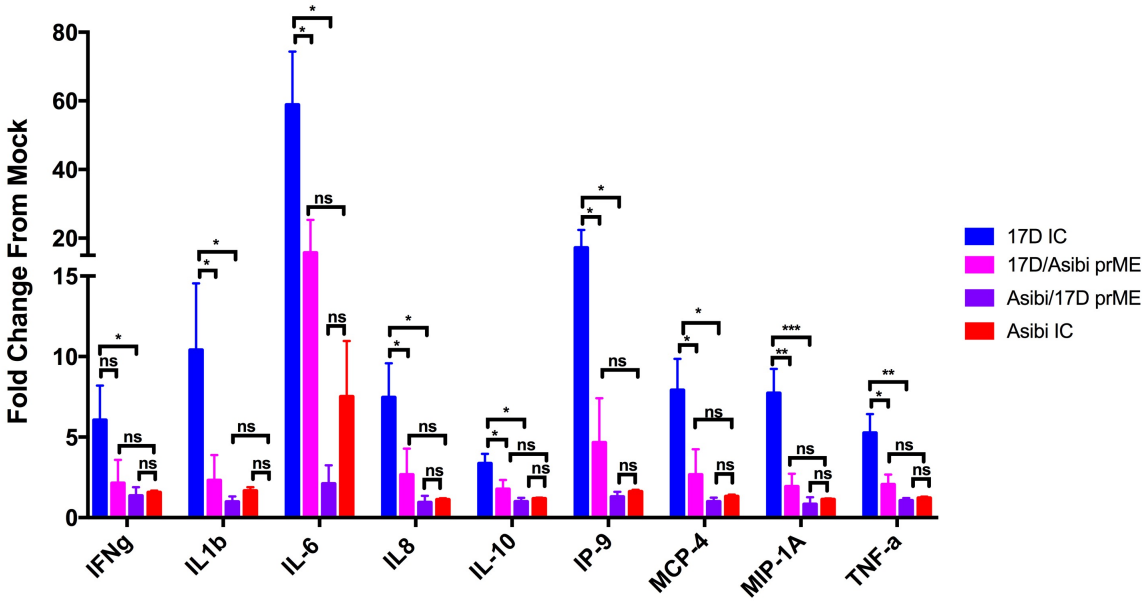

Figure 7. 6. Cytokine and chemokine production for prME chimeric viruses at a MOI of 0.1 in A549 cells. Innate immune exposure was measured and reported as mean of triplicate cultures. Introduction of prME genes of Asibi into the 17D backbone altered the immune signature of IC-derived 17D-204 virus, while introduction of prME genes of 17D into the Asibi backbone did not. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

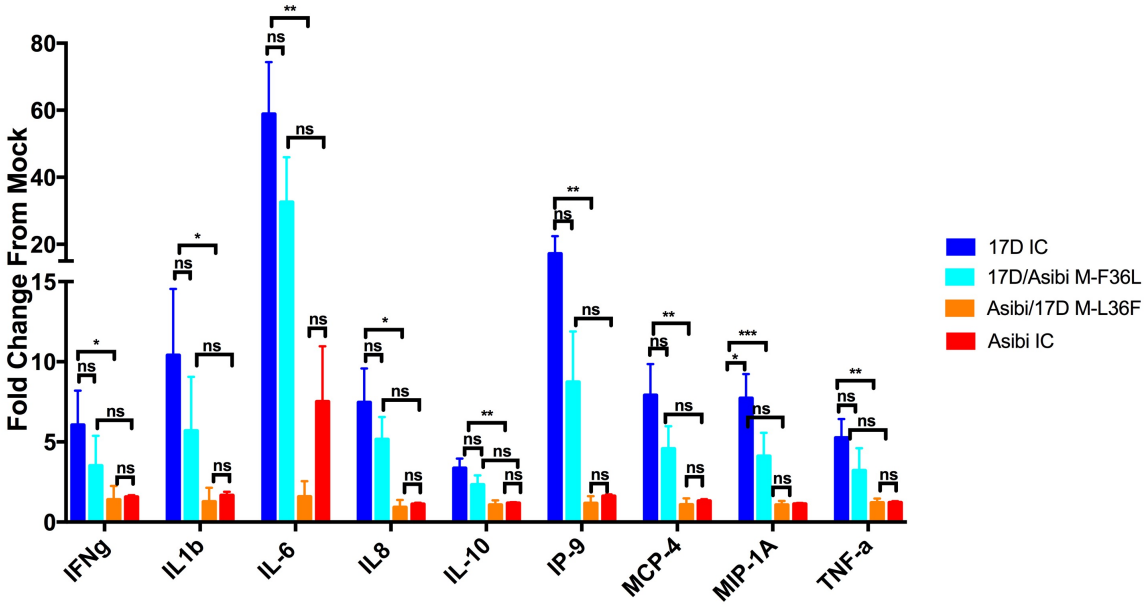

Figure 7. 7. Cytokine and chemokine production for M-36 mutant viruses at a MOI of 0.1 in A549 cells. Innate immune exposure was measured and reported as mean of triplicate cultures. Introduction of M-36 substitution into either the 17D backbone or Asibi backbone did not alter the immune signature. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

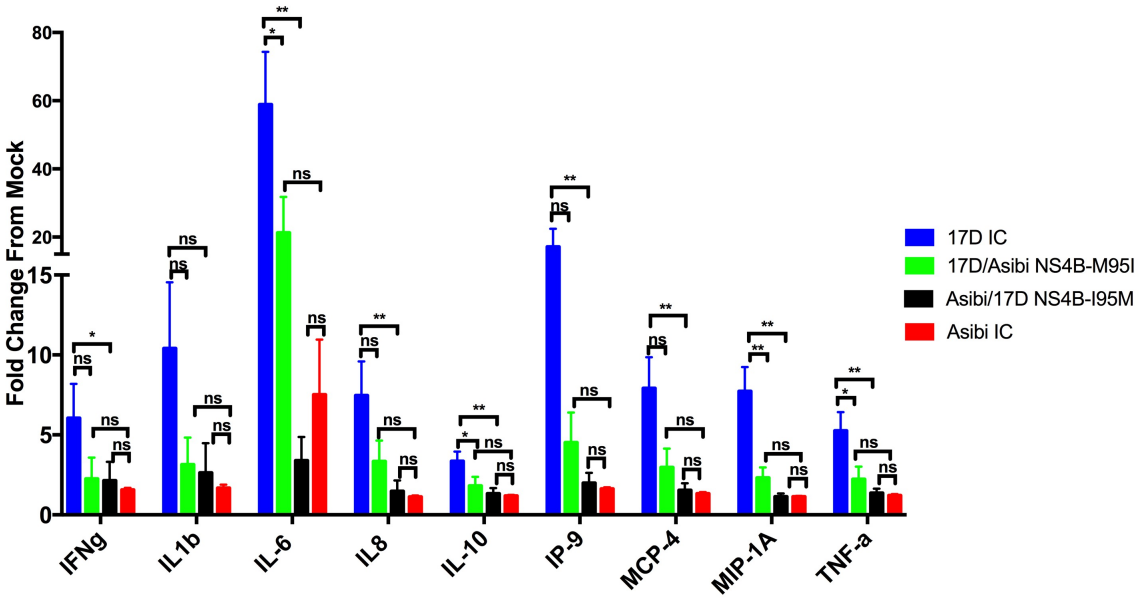

Figure 7. 8. Cytokine and chemokine induction for NS4B-95 mutant viruses at a MOI of 0.1 in A549 cells. Cytokines and chemokines were measured were in triplicate. Introduction of NS4B-M95I substitution into the 17D backbone altered the immune signature of IC-derived 17D-204 virus, while introduction of NS4B-I95M into the Asibi backbone did not. P-value = 0.12 (ns), 0.033 (*), 0.002 (**), and < 0.001 (***).

7.3 DISCUSSION

Multiplication kinetics and cytokine and chemokine production were used as phenotypic markers to evaluate the contributions of structural and NS genes to the phenotypic differences observed between wild-type Asibi and 17D vaccine viruses. As anticipated, the phenotypes of IC-derived Asibi and 17D-204 viruses differed significantly in A549 cells. IC-derived 17D-204 virus multiplied to significantly higher titers throughout the course of infection and induced higher pro- and anti-inflammatory cytokines than IC-derived Asibi virus. The results of the chimeric and mutant viruses demonstrated that prME and NS genes contribute differently to the phenotypes of the 17D vaccine and did not correlate; thus, multiplication kinetics (**Figure 7.9**) and cytokine and chemokine production are not concordant.

Introduction of either prME genes of 17D into the Asibi backbone or prME genes of Asibi into the 17D backbone altered the multiplication kinetics of the viruses when compared to their respective parental viruses (**Figure 7.9**). The NS2B-109 and NS4B-95 amino acid substitutions had differing effects depending on the backbone virus; the NS2B-109 and NS4B-95 substitutions did not alter the multiplication kinetics of IC-derived 17D virus, while the same substitutions would alter the multiplication kinetics of IC-derived Asibi virus. However, differences in multiplication kinetics of other wild-type flaviviruses demonstrated single mutation in either the NS2B or NS4B adversely effected viral replication (Grant et al., 2011; Li et al., 2016; Wicker et al., 2012; Xie et al., 2011). Taken together, it would appear that wild-type viruses are more sensitive to mutation in critical NS residues and there is limited capability within the genome to maintain replication efficiency. However, the genome of 17D vaccine appears to have

multiple substitutions that maintain efficient multiplication. Introduction of the M-36 substitution into the genome of either the Asibi or 17D-204 backbone viruses did not alter the multiplication kinetics, therefore, one or more substitutions in the E protein gene contribute to the multiplication kinetics of the prM/E chimeric viruses. Since the E protein has critical roles in viral attachment, assembly, stability and maturation (Blazeic, 2016), the contributions of prME are independent of those for NS proteins and probably contributes to the different results observed between the prME and NS proteins (**Figure 7.9**). Furthermore, it has been reported that wild-type Asibi and 17D-204 viruses utilize different entry mechanisms and 17D-204 virus entered host cells more efficiently (Fernandez-Garcia, 2016); replacing the E protein gene of IC-derived Asibi and 17D-204 viruses with the opposite E protein gene likely disrupted the entry mechanisms employed by the two viruses. Interestingly, the prME + NS2B-109 mutant viruses contribution to multiplication kinetics differed and further demonstrated the multi-genic nature of the vaccine virus.

Combining the prME and NS2B-I109L and introducing them into the Asibi backbone yield multiplication kinetics that were significantly higher than both prME and NS2B-I109L, alone, and were consistent with IC-derived 17D-204 virus, suggesting an additive effect on multiplication kinetics. Conversely, combining the prME and NS2B-I109L and introducing them into the 17D backbone seemed to adversely affect the contribution of the structural genes that was observed with just 17D/Asibi prME alone.

Evaluating cytokine and chemokine production in A549 cells for IC-derived Asibi and 17D-204 viruses provided a signature to which the prME chimeric, M-36, and NS4B-95 mutant viruses were compared. It has been shown that the 17D vaccine virus induces

production of higher levels of cytokine and chemokine from host cells in the cell culture supernatant than wild-type Asibi virus, including hepatocytes (Fernandez-Garcia et al., 2016; Woodson & Holbrook, 2011); this was confirmed by infection of A549 cells with IC-derived Asibi and 17D-204 viruses. Clearly, the E and NS4B protein genes contribute to the innate immune response of the vaccine virus. Introduction of prME and NS4B-M95I of Asibi into the backbone of 17D-204 altered the immune signature when compared to IC-derived 17D-204 virus, while incorporation of the same mutations into the Asibi backbone viruses were consistent with IC-derived Asibi virus. One or more substitutions in the E protein and the NS4B-95 substitution is critical for maintaining the innate immune response phenotype of the vaccine virus, however, wild-type Asibi virus employs multiple mechanisms for down-regulating the immune response. Additionally, neither Asibi/17D M-L36F nor 17D/Asibi M-F36L differed from their respective parental viruses, suggesting this substitution may not be critical to the immune regulation and that not all 20 of the 17D vaccine amino acid substitutions contribute to the innate immune response.

Overall, the results decribed in this Chapter demonstrate the importance of the E protein genes and NS4B-95 substitution to the phenotype of the vaccine and that these mutations contribute differently to the phenotype.

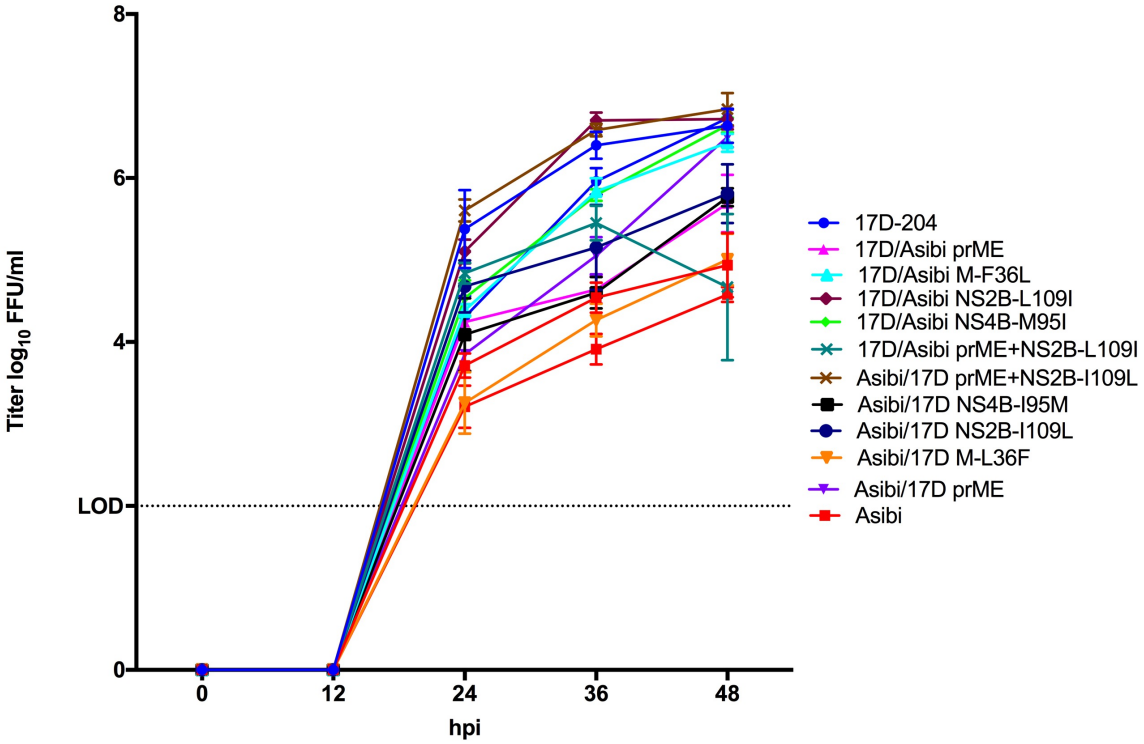

Figure 7. 9. Multiplication kinetics of parental, chimeric, and mutant viruses at a MOI of 0.1 in A549 cells. Data were transcribed from previous figures in Chapter 7 and displayed as a line graph.

CHAPTER 8: DISCUSSION

Serial passage of wild-type Asibi strain 176 times in mouse and chicken tissues yielded live attenuated vaccine strain 17D, one of the most effective and safest live attenuated vaccines developed to date (Lefeuvre, Marianneau, & Deubel, 2004; Norrby, 2007; Theiler & Smith, 1937). **Despite the isolation of 17D over 75 years ago, the mechanism of attenuation is still poorly understood.** Comparative genomic sequencing of wild-type Asibi strain versus 17D vaccine substrains showed that the vaccine strains differed from wild-type Asibi strain by 20 common amino acid substitutions: 9 amino acids substitutions in the structural protein genes and 11 in the NS protein genes, plus four nucleotide changes in the 3' non-coding region (dos Santos et al., 1995).

17D vaccine strain differs phenotypically from wild-type Asibi strain, as it is neither viscerotropic, nor neurotropic, and cannot be transmitted by mosquito vectors. Additionally, wild-type Asibi virus possesses a viral population typical of RNA viruses, while 17D vaccine strain is atypical and is relatively homogeneous (Beck et al., 2014; Salmona et al., 2015). This suggests that the atypical quasispecies population of 17D vaccines is a genotypic difference between wild-type Asibi and the 17D vaccine strains. The objective of this dissertation was to utilize structural and non-structural IC-derived chimeric and mutant viruses to determine the contributions of specific substitutions that contribute to the genotype and phenotype of 17D vaccine and generate information that would contribute to elucidation of the possible mechanism of attenuation.

This dissertation is the first to report contributions of structural and NS gene regions, and individual amino acid substitutions that contribute to both genotypic and phenotypic differences that exist between wild-type Asibi and 17D vaccine. While attenuation of virulence was not investigated directly, the results of this dissertation suggest that the attenuation of wild-type Asibi to yield 17D vaccine is due to multiple substitutions, involving both structural (prM and E) and NS genes (at least NS2B and NS4B). Additionally, genotypic and phenotypic (multiplication in A549 cells) studies were correlated, suggesting that the genotype of the vaccine may be critical to understanding the mechanism of attenuation.

8.1 GENETIC DIVERSITY AS A BIOMARKER OF ATTENUATION

Few studies have investigated whether or not mutations outside the RNA dependent RNA polymerase (RdRp) drive genetic diversity; none have investigated whether or not structural genes contribute to genetic diversity. However, studies that have investigated genetic diversity of RNA viruses have been limited to what mutant viruses were isolated following cell culture passages of wild-type or IC-derived viruses in the presence of nucleoside analogs (Coffey, Beeharry, Bordería, Blanc, & Vignuzzi, 2011; Coffey & Vignuzzi, 2011; Gnädig et al., 2012; Korboukh et al., 2014; Van Slyke et al., 2015; Stapleford et al., 2015; Vignuzzi, Stone, & Andino, 2005; Vignuzzi, Stone, Arnold, Cameron, & Andino, 2006; Xie et al., 2014; Zeng et al., 2014, 2013). Live attenuated 17D vaccine has proven to be safe and attenuated in vaccinees; this providing a unique opportunity to study the relationship between genetic diversity and attenuation. Of the 20 amino acid substitutions that differ between wild-type Asibi and 17D vaccine, this dissertation investigated the contributions of structural (prME and M-36) and NS (all

NS genes, NS2B-109 and NS4B-95) genes to the limited diversity of the vaccine **(Chapters 4, 5, and 6)**.

It was shown that introductions of either prME of Asibi, NS2B-L109I or NS4B-M95I into the 17D backbone altered the diversity indices of the vaccine. However, no changes were noted in the viral population until substitutions in both structural (prME) and NS (NS2B-L109I) genes were combined. Conversely, alterations in the variant population of Asibi backbone viruses were observed, while diversity indices remained consistent with IC-derived Asibi virus. Interestingly, combining prME and NS2B-I109L yielded a unique virus that appeared to be just as diverse, if not more, than IC-derived Asibi virus. However, evaluation of the prME + NS2B-I109L chimeric virus is complicated by the inconsistent data between the two replicates. In addition, the results for both M-36 Asibi and 17D-204 backbone viruses suggested that the M-36 alone marginally effects genetic diversity and is unlikely to be a critical substitution. This finding suggests that generating chimeric viruses with multiple 17D substitutions can be used as a tool to investigate diversity and hypothesize which of the 20 amino acid substitutions are attenuating mutations that can be investigated for phenotype using animal models. In this way, numbers of animals can be limited.

Despite the unexpected results of the prME + NS2B-I109l Asibi backbone virus, the collective results for the chimeric and mutant viruses compared to parental virus clearly demonstrates that the limited diversity of the vaccine is multi-genic and requires the contributions of both structural and non-structural genes where reversions in multiple genes would be necessary to return to a diversity profile consistent with wild-type Asibi virus. Whether or not all 20 amino acids contribute to the attenuated phenotype is not

known but given there are 176 passages it would be surprising if all contributed to the attenuated phenotype.

8.2 GENETIC DIVERSITY CORRELATES TO PHENOTYPIC CHANGES

The genetic results correlated to phenotypic changes in terms of multiplication in A549 cells **(Chapter 7)** and further supported that multiple substitutions contributed to the attenuated phenotype of the vaccine. The diversity indices of the NS2B-L109I and NS4B-M95I 17D backbone viruses differed, while the multiplication kinetics did not when compared 17D-204 virus, supporting the hypothesis that these two substitutions likely contribute to replication fidelity. Surprisingly, the introduction of the prME of Asibi into the 17D backbone also altered diversity indices as well as multiplication kinetics, suggesting that the contributions of the structural genes in not related to fidelity and more likely associated with factors that influence multiplication of YFV. Additionally, combining prME + NS2B-L109I of Asibi into the 17D backbone clearly altered the variant population evident as variants that exceeded 1% of the population, while multiplication kinetics displayed an intermediate phenotype resembling both IC-derived Asibi and prME 17D backbone virus at 48 hours post infection and showed the synergistic interaction of the multiple attenuating substitutions in the vaccine virus. Introduction of prME, NS2B-I109L, NS4B-I95M 17D genes into the Asibi backbone increased multiplication kinetics when compared to IC-derived Asibi, however, only the variant population were altered. Although the genetic diversity of the prME + NS2B-I109L Asibi backbone was similar to IC-derived Asibi, the multiplication kinetics showed the virus was most consistent with IC-derived 17D-204 virus and significantly higher than all other Asibi backbone viruses. This supports the hypothesis that the

genetic diversity prME + NS2B-I109L Asibi backbone virus may have indicted that the virus was displaying a more attenuated phenotype.

Generally, the multiplication kinetics of fidelity mutants did not differ when compared to parental viruses in interferon incompetent cell lines, demonstrating that the alterations in the diversity phenotype were solely related to fidelity and not the multiplication kinetics of the virus (Pfeiffer & Kirkegaard, 2005; Coffey et al., 2011; Korboukh et al., 2014; Vignuzzi et al., 2006). However, studies of WNV fidelity mutants in C6/36 mosquito cells showed that the multiplication kinetics of the fidelity mutants did differ from parental virus (Van Slyke et al., 2015), suggesting that the cell line influences evaluation of multiplication kinetics of fidelity mutants. This, coupled with multi-genic nature of the 17D vaccine may explain the unexpected results for the NS2B-I109L and NS4B-I95M Asibi backbone viruses. Determination of differences in the multiplication kinetics of NS2B-I109L and NS4B-I95M Asibi backbone viruses in this dissertation was likely attributed at least in part to the utilization of an interferon competent cell line (A549). Additionally, it is likely that the multiple attenuating NS substitutions present in the genome of 17D vaccine maintained the 17D phenotype.

The results for innate immune induction in A549 cells in comparison with the genotypic and phenotypic studies, suggested the immune regulation was independent of both genetic diversity and multiplication kinetics. The substitutions in the prME, M-36, and NS4B-95 in both the Asibi and 17D-204 backbones were evaluated and also demonstrated the vaccine is multi-genic. Additionally, the same substitutions (one or more in the envelope and NS4B-M95I) that contributed to the genetic diversity of the vaccine also contribute to innate immune induction. However, introduction of either

prME or NS4B-M95I of Asibi into the 17D-204 backbone did not yield a genetic diversity profile consistent with IC-derived Asibi virus, while the innate immune profile of the vaccine in A549 cells was consistent with IC-derived Asibi virus when either prME or NS4B-M95I of Asibi were introduced into the 17D-204 backbone. Conversely, introduction of either prME or NS4B-I95M of 17D into the Asibi backbone had no effect on immune induction. It appears that genetic diversity and multiplication kinetics tolerate reversion in critical substitutions, whereas innate immune induction by the 17D vaccine does not. Thus, it is hypothesized that multiple attenuating substitutions of the vaccine are required for immune modulation. This should be investigated in the future.

Similar to the minimal contribution of the M-36 substitution to genetic diversity, the M-36 substitution alone does not appear to be critical for to either multiplication kinetics or innate immune induction.

8.3 CONCLUSION

It has been suggested that the limited genetic diversity might in part explain the superior safety record for 17D vaccine (Beck et al., 2014; Tangy & Desprès, 2014), indeed the multiple attenuating mutations in the both the envelope and NS regions of the genome may maintain the limited diversity of the vaccine virus and consequently the attenuated phenotype. Support for this conclusion has been shown in vaccinees, where the attenuated phenotype was maintained despite minimal mutation of 17D vaccine in vaccinees (Coffey et al., 2011; Coffey & Vignuzzi, 2011; Gnädig et al., 2012; Korboukh et al., 2014; Van Slyke et al., 2015; Stapleford et al., 2015; Vignuzzi et al., 2005; Vignuzzi et al., 2006; Xie et al., 2014; Zeng et al., 2014, 2013). Interestingly, it has been speculated that attenuation of live attenuated SA14-14-2 used to control Japanese

encephalitis virus (JEV) is multi-genic; substitutions in both the envelope and NS genes contributed to virulence in weanling mice (Gromowski et al., 2015). Additionally, studies on the envelope gene that differ between poorly immunogenic JEV substrains SA14-5-3 and SA14-9-7 and the highly immunogenic JEV SA14-14-2 demonstrated that the phenotype of JEV SA14-14-2 was not recovered from just the envelope genes, suggesting substitutions in the NS genes also contribute to protection.

Overall, the phenotypic studies support the genetic analysis of the viruses, namely, substitutions in the structural (one ore more in the envelope), NS2B-109, and NS4B-95 genes contribute to the attenuated phenotype. Additionally, it is speculated that the contribution of the NS2B-109 and NS4B-95 mutations to the genetic diversity profile is likely associated with the roles in the RC (Selisko et al., 2014; Zou, Xie, et al., 2015; Youn et al., 2012; Zou, Lee, et al., 2015). This dissertation showed that mutations outside of the NS5 that encodes the RdRp, contribute to both genetic diversity and phenotypic changes; thus, the limited diversity of the 17D vaccine could be, in part, the mechanism of attenuation. The multiple attenuating substitutions in the 17D vaccine renders the vaccine unable to generate a diverse viral population to combat host selection pressures, resulting in attenuation of virulence.

Finally, while this dissertation has identified variability in population structure and diversity, relatively little has been undertaken on phenotypic studies. This dissertation project has opened the door to allow for rational studies using diversity as a model to hypothesize potential attenuating mutations, thereby, allowing for investigating with a variety of phenotypic tools to look at neurotropic, viscerotropic and mosquito competence to elucidate the molecular basis of attenuation of 17D vaccine virus.

References

Acevedo, A., Brodsky, L., & Andino, R. (2014). Mutational and fitness landscapes of an RNA virus revealed through population sequencing. *Nature*, *505*(7485), 686–90.

Aguirre, S., Maestre, A. M., Pagni, S., Patel, J. R., Savage, T., Gutman, D., Maringer, K., et al. (2012). DENV inhibits type I IFN production in infected cells by cleaving human STING. *PLoS pathogens*, *8*(10), e1002934.

Akey, D. L., Brown, W. C., Dutta, S., Konwerski, J., Jose, J., Jurkiw, T. J., DelProposto, J., et al. (2014). Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. *Science (New York, N.Y.)*, *343*(6173), 881–5.

Akey, D. L., Brown, W. C., Jose, J., Kuhn, R. J., & Smith, J. L. (2015). Structure-guided insights on the role of NS1 in flavivirus infection. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *37*(5), 489–94.

Akondy, R. S., Johnson, P. L., Nakaya, H. I., Edupuganti, S., Mulligan, M. J., Lawson, B., Miller, J. D., et al. (2015). Initial viral load determines the magnitude of the human CD8 T cell response to yellow fever vaccination. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(10), 3050–5.

Amanna, I. J., & Slifka, M. K. (2016). Questions regarding the safety and duration of immunity following live yellow fever vaccination. *Expert review of vaccines*, *15*(12), 1519–1533.

Ashhurst, T. M., Vreden, C. v, Munoz-Erazo, L., Niewold, P., Watabe, K., Terry, R. L., Deffrasnes, C., et al. (2013). Antiviral macrophage responses in flavivirus encephalitis. *The Indian journal of medical research*, *138*(5), 632–47.

Audsley, M., Edmonds, J., Liu, W., Mokhonov, V., Mokhonova, E., Melian, E. B., Prow, N., et al. (2011). Virulence determinants between New York 99 and Kunjin strains of West Nile virus. *Virology*, *414*(1), 63–73.

Audsley, M., Edmonds, J., Liu, W., Mokhonov, V., Mokhonova, E., Melian, E. B., Prow, N., et al. (2011). Virulence determinants between New York 99 and Kunjin strains of West Nile virus. *Virology*, *414*(1), 63–73.

Bandyopadhyay, A. S., Garon, J., Seib, K., & Orenstein, W. A. (2015). Polio vaccination: past, present and future. *Future microbiology*, *10*(5), 791–808.

Barrett, A. D. (1997). Yellow fever vaccines. *Biologicals : journal of the International Association of Biological Standardization*, *25*(1), 17–25.

Barrett, A. D. (2016). Yellow Fever in Angola and Beyond--The Problem of Vaccine Supply and Demand. *The New England Journal of medicine*, *375*(4), 301–3.

Barrett, A. D., & Higgs, S. (2007). Yellow fever: a disease that has yet to be conquered. *Annual review of entomology*, *52*, 209–29.

Barrett, A. D., & Teuwen, D. E. (2009). Yellow fever vaccine - how does it work and why do rare cases of serious adverse events take place? *Current opinion in immunology*, *21*(3), 308–13.

Beck, A., Guzman, H., Li, L., Ellis, B., Tesh, R. B., & Barrett, A. D. (2013). Phylogeographic reconstruction of African yellow fever virus isolates indicates recent simultaneous dispersal into east and west Africa. *PLoS neglected tropical diseases*, *7*(3), e1910.

Beck, A., Tesh, R. B., Wood, T. G., Widen, S. G., Ryman, K. D., & Barrett, A. D. (2014). Comparison of the live attenuated yellow fever vaccine 17D-204 strain to its virulent parental strain Asibi by deep sequencing. *The Journal of infectious diseases*, *209*(3), 334–44.

Belsher, J. L., Gay, P., Brinton, M., DellaValla, J., Ridenour, R., Lanciotti, R., Perelygin, A., et al. (2007). Fatal multiorgan failure due to yellow fever vaccine-associated viscerotropic disease. *Vaccine*, *25*(50), 8480–7.

Blazevic, J., Rouha, H., Bradt, V., Heinz, F. X., & Stiasny, K. (2016). Membrane Anchors of the Structural Flavivirus Proteins and Their Role in Virus Assembly. *Journal of virology*, *90*(14), 6365–78. doi:10.1128/JVI.00447-16

Blom, K., Braun, M., Ivarsson, M. A., Gonzalez, V. D., Falconer, K., Moll, M., Ljunggren, H.-G. G., et al. (2013). Temporal dynamics of the primary human T cell response to yellow fever virus 17D as it matures from an effector- to a memory-type response. *Journal of immunology (Baltimore, Md. : 1950)*, *190*(5), 2150–8.

Bollati, M., Alvarez, K., Assenberg, R., Baronti, C., Canard, B., Cook, S., Coutard, B., et al. (2010). Structure and functionality in flavivirus NS-proteins: perspectives for drug design. *Antiviral research*, *87*(2), 125–48.

Bonaldo, M. C., Sequeira, P. C. C., & Galler, R. (2014). The yellow fever 17D virus as a platform for new live attenuated vaccines. *Human vaccines & immunotherapeutics*, *10*(5), 1256–65.

Bordería, A. V., Rozen-Gagnon, K., & Vignuzzi, M. (2016). Fidelity Variants and RNA Quasispecies. *Current topics in microbiology and immunology*, *392*, 303–22.

Bordería, A. V., Stapleford, K. A., & Vignuzzi, M. (2011). RNA virus population diversity: implications for inter-species transmission. *Current opinion in virology*, *1*(6), 643–8. doi:10.1016/j.coviro.2011.09.012

Bredenbeek, P. J., Kooi, E. A., Lindenbach, B., Huijkman, N., Rice, C. M., & Spaan, W. J. (2003). A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication. *The Journal of general virology*, *84*(Pt 5), 1261–8.

Bryant, J. E., Holmes, E. C., & Barrett, A. D. (2007). Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS pathogens*, *3*(5), e75.

Campi-Azevedo, A. C., Costa-Pereira, C., Antonelli, L. R., Fonseca, C. T., Teixeira-Carvalho, A., Villela-Rezende, G., Santos, R. A., et al. (2016). Booster dose after 10 years is recommended following 17DD-YF primary vaccination. *Human vaccines & immunotherapeutics*, *12*(2), 491–502.

Campi-Azevedo, A. C., de Araújo-Porto, L. P., Luiza-Silva, M., Batista, M. A. A., Martins, M. A., Sathler-Avelar, R., da Silveira-Lemos, D., et al. (2012). 17DD and 17D-213/77 yellow fever substrains trigger a balanced cytokine profile in primary vaccinated children. *PloS one*, *7*(12), e49828.

Chan, M. (2016). Yellow fever: the resurgence of a forgotten disease. *Lancet (London, England)*, *387*(10034), 2165–6.

Coffey, L. L., & Vignuzzi, M. (2011). Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. *Journal of virology*, *85*(2), 1025–35.

Coffey, L. L., Beeharry, Y., Bordería, A. V., Blanc, H., & Vignuzzi, M. (2011). Arbovirus high fidelity variant loses fitness in mosquitoes and mice. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16038–43.

Collins, N. D., & Barrett, A. D. (2017). Live Attenuated Yellow Fever 17D Vaccine: A Legacy Vaccine Still Controlling Outbreaks In Modern Day. *Current infectious disease reports*, *19*(3), 14.

Combe, M., Garijo, R., Geller, R., Cuevas, J. M. M., & Sanjuán, R. (2015). Single-Cell Analysis of RNA Virus Infection Identifies Multiple Genetically Diverse Viral Genomes within Single Infectious Units. *Cell host & microbe*, *18*(4), 424–32. doi:10.1016/j.chom.2015.09.009

Cong, Y., McArthur, M. A., Cohen, M., Jahrling, P. B., Janosko, K. B., Josleyn, N., Kang, K., et al. (2016). Characterization of Yellow Fever Virus Infection of

Human and Non-human Primate Antigen Presenting Cells and Their Interaction with CD4+ T Cells. *PLoS neglected tropical diseases*, *10*(5), e0004709.

Crotty, S., Cameron, C. E., & Andino, R. (2001). RNA virus error catastrophe: direct molecular test by using ribavirin. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(12), 6895–900.

Luiza-Silva, M., Campi-Azevedo, A. C., Batista, M. A. A., Martins, M. A., Avelar, R. S., da Silveira Lemos, D., Bastos Camacho, L. A., et al (2011). Cytokine signatures of innate and adaptive immunity in 17DD yellow fever vaccinated children and its association with the level of neutralizing antibody. *The Journal of infectious diseases*, *204*(6), 873–83.

De Melo, AB, da Paz, M. C., & Magalhães, M. (2011). Description of a prospective 17DD yellow fever vaccine cohort in Recife, Brazil. *The American journal of tropical medicine and hygiene*, 85(4), 739-747.

De Wispelaere, M., Khou, C., Frenkiel, M.-P. P., Desprès, P., & Pardigon, N. (2015). A Single Amino Acid Substitution in the M Protein Attenuates Japanese Encephalitis Virus in Mammalian Hosts. *Journal of virology*, *90*(5), 2676–89. doi:10.1128/JVI.01176-15

Dos Santos, C. N., Post, P. R., Carvalho, R., Ferreira, I. I., Rice, C. M., & Galler, R. (1995). Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D-213. *Virus research*, *35*(1), 35–41.

Dos Santos, C. N., Post, P. R., Carvalho, R., Ferreira, I. I., Rice, C. M., & Galler, R. (1995). Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D-213. *Virus research*, *35*(1), 35–41.

Dridi, M., Rosseel, T., Orton, R., Johnson, P., Lecollinet, S., Muylkens, B., … Van Borm, S. (2015). Next-generation sequencing shows West Nile virus quasispecies diversification after a single passage in a carrion crow (Corvus corone) in vivo infection model. *The Journal of general virology*, *96*(10), 2999–3009. doi:10.1099/jgv.0.000231

Dunster, L. M., Wang, H., Ryman, K. D., Miller, B. R., Watowich, S. J., Minor, P. D., & Barrett, A. D. (1999). Molecular and biological changes associated with HeLa cell attenuation of wild-type yellow fever virus. *Virology*, *261*(2), 309–18.

Collaborative group for studies on yellow fever vaccines (2014). Duration of post-vaccination immunity against yellow fever in adults. *Vaccine*, *32*(39), 4977–84.

Ehrbar, D. J., Ngo, K. A., Campbell, S. R., Kramer, L. D., & Ciota, A. T. (2017). High levels of local inter- and intra-host genetic variation of West Nile virus and evidence of fine-scale evolutionary pressures. *Infection, genetics and evolution :*

journal of molecular epidemiology and evolutionary genetics in infectious diseases. doi:10.1016/j.meegid.2017.04.010

Faria, N. R., Quick, J., Claro, I. M., Thézé, J., de Jesus, J. G., Giovanetti, M., Kraemer, M. U. G. U., et al. (2017). Establishment and cryptic transmission of Zika virus in Brazil and the Americas. *Nature*.

Fernandez-Garcia, M. D., Meertens, L., Chazal, M., Hafirassou, M. L., Dejarnac, O., Zamborlini, A., Despres, P., et al. (2016). Vaccine and Wild-Type Strains of Yellow Fever Virus Engage Distinct Entry Mechanisms and Differentially Stimulate Antiviral Immune Responses. *mBio*, *7*(1), e01956–17.

Fuertes Marraco, S. A., Soneson, C., Cagnon, L., Gannon, P. O., Allard, M., Abed Maillard, S., Montandon, N., et al. (2015). Long-lasting stem cell-like memory CD8+ T cells with a naïve-like profile upon yellow fever vaccination. *Science translational medicine*, *7*(282), 282ra48.

Garske, T., Van Kerkhove, M. D., Yactayo, S., Ronveaux, O., Lewis, R. F., Staples, J. E., Perea, W., et al. (2014). Yellow Fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and serological data. *PLoS medicine*, *11*(5), e1001638.

Gaucher, D., Therrien, R., Kettaf, N., Angermann, B. R., Boucher, G., Filali-Mouhim, A., Moser, J. M., et al. (2008). Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *The Journal of experimental medicine*, *205*(13), 3119–31.

Gnädig, N. F., Beaucourt, S., Campagnola, G., Bordería, A. V., Sanz-Ramos, M., Gong, P., Blanc, H., et al. (2012). Coxsackievirus B3 mutator strains are attenuated in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), E2294–303.

Gotuzzo, E., Yactayo, S., & Córdova, E. (2013). Efficacy and duration of immunity after yellow fever vaccination: systematic review on the need for a booster every 10 years. *The American journal of tropical medicine and hygiene*, *89*(3), 434–44.

Grant, D., Tan, G. K., Qing, M., Ng, J. K., Yip, A., Zou, G., Xie, X., et al. (2011). A single amino acid in nonstructural protein NS4B confers virulence to dengue virus in AG129 mice through enhancement of viral RNA synthesis. *Journal of virology*, *85*(15), 7775–87.

Green, A. (2016). Yellow fever continues to spread in Angola. *Lancet (London, England)*, *387*(10037), 2493.

Green, A. M., Beatty, P. R., Hadjilaou, A., & Harris, E. (2014). Innate immunity to dengue virus infection and subversion of antiviral responses. *Journal of molecular biology*, *426*(6), 1148–60.

Gregori, J., Salicrú, M., Domingo, E., Sanchez, A., Esteban, J. I., Rodríguez-Frías, F., & Quer, J. (2014). Inference with viral quasispecies diversity indices: clonal and NGS approaches. *Bioinformatics (Oxford, England)*.

Grobbelaar, A. A., Weyer, J., Moolla, N., Jansen van Vuren, P., Moises, F., & Paweska, J. T. (2016). Resurgence of Yellow Fever in Angola, 2015-2016. *Emerging infectious diseases*, *22*(10), 1854–5.

Gromowski, G. D., Firestone, C.-Y. Y., & Whitehead, S. S. (2015). Genetic Determinants of Japanese Encephalitis Virus Vaccine Strain SA14-14-2 That Govern Attenuation of Virulence in Mice. *Journal of virology*, *89*(12), 6328–37.

Grubaugh, N. D., Rückert, C., Armstrong, P. M., Bransfield, A., Anderson, J. F., Ebel, G. D., & Brackney, D. E. (2016). Transmission bottlenecks and RNAi collectively influence tick-borne flavivirus evolution. *Virus evolution*, *2*(2), vew033. doi:10.1093/ve/vew033

Grubaugh, N. D., Smith, D. R., Brackney, D. E., Bosco-Lauth, A. M., Fauver, J. R., Campbell, C. L., … Ebel, G. D. (2015). Experimental evolution of an RNA virus in wild birds: evidence for host-dependent impacts on population structure and competitive fitness. *PLoS pathogens*, *11*(5), e1004874. doi:10.1371/journal.ppat.1004874

Grubaugh, N. D., Weger-Lucarelli, J., Murrieta, R. A., Fauver, J. R., Garcia-Luna, S. M., Prasad, A. N., … 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), 481–92. doi:10.1016/j.chom.2016.03.002

Hahn, C. S., Dalrymple, J. M., Strauss, J. H., & Rice, C. M. (1987). Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(7), 2019–23.

Heinz, F. X., & Stiasny, K. (2012). Flaviviruses and flavivirus vaccines. *Vaccine*, *30*(29), 4301–6.

Hepburn, M. J., Kortepeter, M. G., Pittman, P. R., Boudreau, E. F., Mangiafico, J. A., Buck, P. A., Norris, S. L., et al. (2006). Neutralizing antibody response to booster vaccination with the 17d yellow fever vaccine. *Vaccine*, *24*(15), 2843–9.

Huang, Q., Chen, A. S., Li, Q., & Kang, C. (2011). Expression, purification, and initial structural characterization of nonstructural protein 2B, an integral membrane

protein of Dengue-2 virus, in detergent micelles. *Protein expression and purification*, *80*(2), 169–75.

James, E. A., LaFond, R. E., Gates, T. J., Mai, D. T., Malhotra, U., & Kwok, W. W. (2013). Yellow fever vaccination elicits broad functional CD4+ T cell responses that recognize structural and nonstructural proteins. *Journal of virology*, *87*(23), 12794–804.

Jean, K., Donnelly, C., Ferguson, N., & Garske, T. (2016). A Meta-Analysis of Serological Response Associated with Yellow Fever Vaccination. *American Journal of Tropical Medicine and Hygiene*.

Jones, M., Davidson, A., Hibbert, L., Gruenwald, P., Schlaak, J., Ball, S., Foster, G. R., et al. (2005). Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. *Journal of virology*, *79*(9), 5414–20.

Khromykh, A. A., Sedlak, P. L., & Westaway, E. G. (2000). cis- and trans-acting elements in flavivirus RNA replication. *Journal of virology*, *74*(7), 3253–63.

Korboukh, V. K., Lee, C. A., Acevedo, A., Vignuzzi, M., Xiao, Y., Arnold, J. J., Hemperly, S., et al. (2014). RNA virus population diversity, an optimum for maximal fitness and virulence. *The Journal of biological chemistry*, *289*(43), 29531–44.

Korboukh, V. K., Lee, C. A., Acevedo, A., Vignuzzi, M., Xiao, Y., Arnold, J. J., Hemperly, S., et al. (2014). RNA virus population diversity, an optimum for maximal fitness and virulence. *The Journal of biological chemistry*, *289*(43), 29531–44.

Lai, S., Huang, Z., Zhou, H., Anders, K. L., Perkins, T. A., Yin, W., Li, Y., et al. (2015). The changing epidemiology of dengue in China, 1990-2014: a descriptive analysis of 25 years of nationwide surveillance data. *BMC medicine*, *13*, 100.

Lauring, A. S., & Andino, R. (2010). Quasispecies theory and the behavior of RNA viruses. *PLoS pathogens*, *6*(7), e1001005.

Lee, E., & Lobigs, M. (2008). E protein domain III determinants of yellow fever virus 17D vaccine strain enhance binding to glycosaminoglycans, impede virus spread, and attenuate virulence. *Journal of virology*, *82*(12), 6024–33. doi:10.1128/JVI.02509-07

Lefeuvre, A., Marianneau, P., & Deubel, V. (2004). Current Assessment of Yellow Fever and Yellow Fever Vaccine. *Current infectious disease reports*, *6*(2), 96–104.

León-Juárez, M., Martínez-Castillo, M., Shrivastava, G., García-Cordero, J., Villegas-Sepulveda, N., Mondragón-Castelán, M., Mondragón-Flores, R., et al. (2016).

Recombinant Dengue virus protein NS2B alters membrane permeability in different membrane models. *Virology journal*, *13*, 1.

Leyssen, P., De Clercq, E., & Neyts, J. (2006). The anti-yellow fever virus activity of ribavirin is independent of error-prone replication. *Molecular pharmacology*, *69*(4), 1461–7.

Li, K., Phoo, W. W., & Luo, D. (2014). Functional interplay among the flavivirus NS3 protease, helicase, and cofactors. *Virologica Sinica*, *29*(2), 74–85.

Li, X.-D. D., Deng, C.-L. L., Ye, H.-Q. Q., Zhang, H.-L. L., Zhang, Q.-Y. Y., Chen, D.-D. D., Zhang, P.-T. T., et al. (2016). Transmembrane Domains of NS2B Contribute to both Viral RNA Replication and Particle Formation in Japanese Encephalitis Virus. *Journal of virology*, *90*(12), 5735–49.

Li, X.-D. D., Ye, H.-Q. Q., Deng, C.-L. L., Liu, S.-Q. Q., Zhang, H.-L. L., Shang, B.-D. D., Shi, P.-Y. Y., et al. (2015). Genetic interaction between NS4A and NS4B for replication of Japanese encephalitis virus. *The Journal of general virology*, *96*(Pt 6), 1264–75.

Luiza-Silva, M., Campi-Azevedo, A. C., Batista, M. A. A., Martins, M. A., Avelar, R. S., da Silveira Lemos, D., Bastos Camacho, L. A., et al. (2011). Cytokine signatures of innate and adaptive immunity in 17DD yellow fever vaccinated children and its association with the level of neutralizing antibody. *The Journal of infectious diseases*, *204*(6), 873–83.

Macnamara, F. N. (1957). A clinico-pathological study of yellow fever in Nigeria. *The West African medical journal*, *6*(4), 137–46.

Martins, R. M., Maia, M. de L., Farias, R. H., Camacho, L. A., Freire, M. S., Galler, R., Yamamura, A. M., et al. (2013). 17DD yellow fever vaccine: a double blind, randomized clinical trial of immunogenicity and safety on a dose-response study. *Human vaccines & immunotherapeutics*, *9*(4), 879–88.

McElroy, K., Tsetsarkin, K., Vanlandingham, D., & Higgs, S. (2006). Characterization of an infectious clone of the wild-type yellow fever virus Asibi strain that is able to infect and disseminate in mosquitoes. *Journal of General Virology*, *86*(Pt 6), 1747–1751.

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L., & Higgs, S. (2006a). Manipulation of the yellow fever virus non-structural genes 2A and 4B and the 3'non-coding region to evaluate genetic determinants of viral dissemination from the Aedes aegypti midgut. *The American journal of tropical medicine and hygiene*, *75*(6), 1158–64.

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L., & Higgs, S. (2006b). Role of the yellow fever virus structural protein genes in viral dissemination from the Aedes aegypti mosquito midgut. *The Journal of general virology*, *87*(Pt 10), 2993–3001. doi:10.1099/vir.0.82023-0

Metsky, H. C., Matranga, C. B., Wohl, S., Schaffner, S. F., Freije, C. A., Winnicki, S. M., West, K., et al. (2017). Zika virus evolution and spread in the Americas. *Nature*. doi: 10.1038/nature22402

Monath, T. P. (2013). 17D Yellow Fever Virus Vaccine. *The American journal of tropical medicine and hygiene*, *89*(6), 1225.

Monath, T. P., & Vasconcelos, P. F. (2015). Yellow fever. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology*, *64*, 160–73.

Muñoz-Jordán, J. L., Laurent-Rolle, M., Ashour, J., Martínez-Sobrido, L., Ashok, M., Lipkin, W. I., & García-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *Journal of virology*, *79*(13), 8004–13.

Muyanja, E., Ssemaganda, A., Ngauv, P., Cubas, R., Perrin, H., Srinivasan, D., Canderan, G., et al. (2014). Immune activation alters cellular and humoral responses to yellow fever 17D vaccine. *The Journal of clinical investigation*, *124*(7), 3147–58.

Nemésio, H., Palomares-Jerez, F., & Villalaín, J. (2012). NS4A and NS4B proteins from dengue virus: membranotropic regions. *Biochimica et biophysica acta*, *1818*(11), 2818–30.

Nishijima, N., Marusawa, H., Ueda, Y., Takahashi, K., Nasu, A., Osaki, Y., Kou, T., et al. (2012). Dynamics of hepatitis B virus quasispecies in association with nucleos(t)ide analogue treatment determined by ultra-deep sequencing. *PloS one*, *7*(4), e35052.

Norrby, E. (2007). Yellow fever and Max Theiler: the only Nobel Prize for a virus vaccine. *The Journal of experimental medicine*, *204*(12), 2779–84.

Paul, D., Romero-Brey, I., Gouttenoire, J., Stoitsova, S., Krijnse-Locker, J., Moradpour, D., & Bartenschlager, R. (2011). NS4B self-interaction through conserved C-terminal elements is required for the establishment of functional hepatitis C virus replication complexes. *Journal of virology*, *85*(14), 6963–76.

Pfeiffer, J. K., & Kirkegaard, K. (2003). A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(12), 7289–94.

Pfeiffer, J. K., & Kirkegaard, K. (2005). Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. *PLoS pathogens*, *1*(2), e11.

Pugachev, K. V., Guirakhoo, F., Ocran, S. W., Mitchell, F., Parsons, M., Penal, C., … Monath, T. P. (2004). High fidelity of yellow fever virus RNA polymerase. *Journal of virology*, *78*(2), 1032–8. doi:10.1128/JVI.78.2.1032-1038.2004

Pulendran, B. (2009). Learning immunology from the yellow fever vaccine: innate immunity to systems vaccinology. *Nature reviews. Immunology*, *9*(10), 741–7.

Pulendran, B., Miller, J., Querec, T. D., Akondy, R., Moseley, N., Laur, O., Glidewell, J., et al. (2008). Case of yellow fever vaccine--associated viscerotropic disease with prolonged viremia, robust adaptive immune responses, and polymorphisms in CCR5 and RANTES genes. *The Journal of infectious diseases*, *198*(4), 500–7.

Pulendran, B., Oh, J. Z., Nakaya, H. I., Ravindran, R., & Kazmin, D. A. (2013). Immunity to viruses: learning from successful human vaccines. *Immunological reviews*, *255*(1), 243–55.

Quaresma, J. A., Barros, V. L., Pagliari, C., Fernandes, E. R., Guedes, F., Takakura, C. F., Andrade, H. F., et al. (2006). Revisiting the liver in human yellow fever: virus-induced apoptosis in hepatocytes associated with TGF-beta, TNF-alpha and NK cells activity. *Virology*, *345*(1), 22–30.

Quaresma, J. A., Pagliari, C., Medeiros, D. B., Duarte, M. I., & Vasconcelos, P. F. (2013). Immunity and immune response, pathology and pathologic changes: progress and challenges in the immunopathology of yellow fever. *Reviews in medical virology*, *23*(5), 305–18.

Querec, T. D., & Pulendran, B. (2007). Understanding the role of innate immunity in the mechanism of action of the live attenuated Yellow Fever Vaccine 17D. *Advances in experimental medicine and biology*, *590*, 43–53.

Querec, T., Bennouna, S., Alkan, S., Laouar, Y., Gorden, K., Flavell, R., Akira, S., et al. (2006). Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *The Journal of experimental medicine*, *203*(2), 413–24.

Rozen-Gagnon, K., Stapleford, K. A., Mongelli, V., Blanc, H., Failloux, A.-B. B., Saleh, M.-C. C., & Vignuzzi, M. (2014). Alphavirus mutator variants present host-specific defects and attenuation in mammalian and insect models. *PLoS pathogens*, *10*(1), e1003877. doi:10.1371/journal.ppat.1003877

Sall, A. A., Faye, O., Diallo, M., Firth, C., Kitchen, A., & Holmes, E. C. (2010). Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. *Journal of virology*, *84*(2), 765–72. doi:10.1128/JVI.01738-09

Salmona, M., Gazaigne, S., Mercier-Delarue, S., Garnier, F., Korimbocus, J., Colin de Verdière, N., LeGoff, J., et al. (2015). Molecular characterization of the 17D-204 yellow fever vaccine. *Vaccine*, *33*(41), 5432–6.

Sanjuán, R., & Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and molecular life sciences : CMLS*. doi:10.1007/s00018-016-2299-6

Selisko, B., Wang, C., Harris, E., & Canard, B. (2014). Regulation of Flavivirus RNA synthesis and replication. *Current opinion in virology*, *9*, 74–83.

Sessions, O. M., Wilm, A., Kamaraj, U. S., Choy, M. M., Chow, A., Chong, Y., … Ooi, E. E. (2015). Analysis of Dengue Virus Genetic Diversity during Human and Mosquito Infection Reveals Genetic Constraints. *PLoS neglected tropical diseases*, *9*(9), e0004044. doi:10.1371/journal.pntd.0004044

Sessions, O. M., Wilm, A., Kamaraj, U. S., Choy, M. M., Chow, A., Chong, Y., Ong, X. M., et al. (2015). Analysis of Dengue Virus Genetic Diversity during Human and Mosquito Infection Reveals Genetic Constraints. *PLoS neglected tropical diseases*, *9*(9), e0004044.

Shi, P.-Y. Y. (2014). Structural biology. Unraveling a flavivirus enigma. *Science (New York, N.Y.)*, *343*(6173), 849–50.

Silva, M. L., Martins, M. A., Espírito-Santo, L. R. R., Campi-Azevedo, A. C., Silveira-Lemos, D., Ribeiro, J. G. G., Homma, A., et al. (2011). Characterization of main cytokine sources from the innate and adaptive immune responses following primary 17DD yellow fever vaccination in adults. *Vaccine*, *29*(3), 583–92.

Stanway, G., Hughes, P. J., Mountford, R. C., Reeve, P., Minor, P. D., Schild, G. C., & Almond, J. W. (1984). Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon 12a1b. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(5), 1539–43.

Stapleford, K. A., Moratorio, G., Henningsson, R., Chen, R., Matheus, S., Enfissi, A., … Vignuzzi, M. (2016). Whole-Genome Sequencing Analysis from the Chikungunya Virus Caribbean Outbreak Reveals Novel Evolutionary Genomic Elements. *PLoS neglected tropical diseases*, *10*(1), e0004402. doi:10.1371/journal.pntd.0004402

Stapleford, K. A., Rozen-Gagnon, K., Das, P. K., Saul, S., Poirier, E. Z., Blanc, H., … Vignuzzi, M. (2015). Viral Polymerase-Helicase Complexes Regulate Replication Fidelity To Overcome Intracellular Nucleotide Depletion. *Journal of virology*,

89(22), 11233–44. doi:10.1128/JVI.01553-15

Stapleford, K. A., Rozen-Gagnon, K., Das, P. K., Saul, S., Poirier, E. Z., Blanc, H., Vidalain, P.-O. O., et al. (2015). Viral Polymerase-Helicase Complexes Regulate Replication Fidelity To Overcome Intracellular Nucleotide Depletion. *Journal of virology*, *89*(22), 11233–44.

Staples, J. E., Gershman, M., & Fischer, M. (2010). Yellow fever vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports*, *59*(RR-7), 1–27.

Tajima, S., Takasaki, T., & Kurane, I. (2011). Restoration of replication-defective dengue type 1 virus bearing mutations in the N-terminal cytoplasmic portion of NS4A by additional mutations in NS4B. *Archives of virology*, *156*(1), 63–9.

Tangy, F., & Desprès, P. (2014). Yellow fever vaccine attenuation revealed: loss of diversity. *The Journal of infectious diseases*, *209*(3), 318–20.

Ter Meulen, J., Sakho, M., Koulemou, K., Magassouba, N., Bah, A., Preiser, W., Daffis, S., et al. (2004). Activation of the cytokine network and unfavorable outcome in patients with yellow fever. *The Journal of infectious diseases*, *190*(10), 1821–7.

Theiler, M., & Smith, H. H. (1937). The use of yellow fever virus modified by in vitro cultivation for human immunization. *The Journal of experimental medicine*, *65*(6), 787–800.

Van Slyke, G. A., Arnold, J. J., Lugo, A. J., Griesemer, S. B., Moustafa, I. M., Kramer, L. D., Cameron, C. E., et al. (2015). Sequence-Specific Fidelity Alterations Associated with West Nile Virus Attenuation in Mosquitoes. *PLoS pathogens*, *11*(6), e1005009.

Vignuzzi, M., Stone, J. K., & Andino, R. (2005). Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. *Virus research*, *107*(2), 173–81.

Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., & Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature*, *439*(7074), 344–8.

Vignuzzi, M., Stone, J. K., Arnold, J. J., Cameron, C. E., & Andino, R. (2006). Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature*, *439*(7074), 344–8.

Wang, E., Ryman, K. D., Jennings, A. D., Wood, D. J., Taffs, F., Minor, P. D., Sanders, P. G., et al. (1995). Comparison of the genomes of the wild-type French

viscerotropic strain of yellow fever virus with its vaccine derivative French neurotropic vaccine. *The Journal of general virology*, *76 (Pt 11)*, 2749–55.

Watson, A. M., Lam, L. K., Klimstra, W. B., & Ryman, K. D. (2016). The 17D-204 Vaccine Strain-Induced Protection against Virulent Yellow Fever Virus Is Mediated by Humoral Immunity and CD4+ but not CD8+ T Cells. *PLoS pathogens*, *12*(7), e1005786.

Wicker, J. A., Whiteman, M. C., Beasley, D. W., Davis, C. T., McGee, C. E., Lee, J. C., Higgs, S., et al. (2012). Mutational analysis of the West Nile virus NS4B protein. *Virology*, *426*(1), 22–33.

Wieten, R. W., Goorhuis, A., Jonker, E. F. F., de Bree, G. J., de Visser, A. W., van Genderen, P. J. J., Remmerswaal, E. B., et al. (2016). 17D yellow fever vaccine elicits comparable long-term immune responses in healthy individuals and immune-compromised patients. *The Journal of infection*, *72*(6), 713–22.

Woodson, S. E., & Holbrook, M. R. (2011). Infection of hepatocytes with 17-D vaccine-strain yellow fever virus induces a strong pro-inflammatory host response. *The Journal of general virology*, *92*(Pt 10), 2262–71.

Woodson, S., Freiberg, A., & Holbrook, M. (2011). Differential cytokine responses from primary human Kupffer cells following infection with wild-type or vaccine strain yellow fever virus. *Virology*, *412*(1), 188–195.

World Health Organization Secretariat information paper. (2016, July 3). Fractional dose yellow fever vaccine as a dose-sparing option for outbreak response.

World Health Organization. (2016a, May). Yellow Fever.

World Health Organization. (2016b, August 5). World Yellow Fever Situation Report.

World Health Organization. (2016c, May 4). WHO Prequalified Vaccines.

Xie, H., Cass, A. R., & Barrett, A. D. (1998). Yellow fever 17D vaccine virus isolated from healthy vaccinees accumulates very few mutations. *Virus research*, *55*(1), 93–9.

Xie, X., Wang, H., Zeng, J., Li, C., Zhou, G., Yang, D., & Yu, L. (2014). Foot-and-mouth disease virus low-fidelity polymerase mutants are attenuated. *Archives of virology*, *159*(10), 2641–50.

Xie, X., Wang, Q.-Y. Y., Xu, H. Y., Qing, M., Kramer, L., Yuan, Z., & Shi, P.-Y. Y. (2011). Inhibition of dengue virus by targeting viral NS4B protein. *Journal of virology*, *85*(21), 11183–97.

Xie, X., Zou, J., Puttikhunt, C., Yuan, Z., & Shi, P.-Y. Y. (2015). Two distinct sets of NS2A molecules are responsible for dengue virus RNA synthesis and virion assembly. *Journal of virology*, *89*(2), 1298–313.

Yang, X., Charlebois, P., Macalalad, A., Henn, M. R., & Zody, M. C. (2013). V-Phaser 2: variant inference for viral populations. *BMC genomics*, *14*, 674.

Ye, J., Zhu, B., Fu, Z. F., Chen, H., & Cao, S. (2013). Immune evasion strategies of flaviviruses. *Vaccine*, *31*(3), 461–71.

Youn, S., Li, T., McCune, B., Edeling, M., Fremont, D., Cristea, I., & Diamond, M. (2012). Evidence for a genetic and physical interaction between nonstructural proteins NS1 and NS4B that modulates replication of West Nile virus. *Journal of virology*, *86*(13), 7360–71.

Yu, L., Takeda, K., & Markoff, L. (2013). Protein-protein interactions among West Nile non-structural proteins and transmembrane complex formation in mammalian cells. *Virology*, *446*(1-2), 365–77.

Zeng, J., Wang, H., Xie, X., Li, C., Zhou, G., Yang, D., & Yu, L. (2014). Ribavirin-resistant variants of foot-and-mouth disease virus: the effect of restricted quasispecies diversity on viral virulence. *Journal of virology*, *88*(8), 4008–20.

Zeng, J., Wang, H., Xie, X., Yang, D., Zhou, G., & Yu, L. (2013). An increased replication fidelity mutant of foot-and-mouth disease virus retains fitness in vitro and virulence in vivo. *Antiviral research*, *100*(1), 1–7.

Zmurko, J., Neyts, J., & Dallmeier, K. (2015). Flaviviral NS4b, chameleon and jack-in-the-box roles in viral replication and pathogenesis, and a molecular target for antiviral intervention. *Reviews in medical virology*, *25*(4), 205–23.

Zou, J., Lee, L. T. e T., Wang, Q. Y., Xie, X., Lu, S., Yau, Y. H., Yuan, Z., et al. (2015). Mapping the Interactions between the NS4B and NS3 proteins of dengue virus. *Journal of virology*, *89*(7), 3471–83.

Vita

Natalie Collins was born Natalie White in Leavenworth, KS, February 2nd, 1982 to Mitchell and Carolyn Tatum. Natalie Collins obtained her Bachelors of Science in the area of biology with a concentration in microbiology from Southern University (SU) and A&M College (2000-2004), while attending SU she was selected for an internship at Weill Cornell Graduate School of Medical Sciences (summer, 2002). Natalie Collins was commission into the United States Army (Active Duty) in 2004 as a 1st Lieutenant and continues to serve as a MAJ in the United States Army. She married her college sweetheart, Lance Collins, shortly after graduating college and being commissioned in 2005. She earned a Medical Laboratory Scientist (MLS) certification from American Society for Clinical Pathology in (2007-2008). Subsequently, she worked as a supervisor of specimen processing (2007-2009), and then supervisor of anatomical pathology at Brooke Army Medical Center (2009-2011). She then obtained her Masters in Public Health with an emphasis in epidemiology from Kaplan University (2011-2013). Natalie is currently a graduate student in the Microbiology and Immunology graduate program at the University of Texas-Medical Branch, Galveston, TX. She is investigating the molecular basis of attenuation of yellow fever 17D vaccine using classical virology and molecular techniques. She developed and validated a nucleotide sequence data pipeline for next generation sequencing to investigate viral diversity.

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This dissertation was typed by Natalie Collins

Curriculum Vitae

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EDUCATION

Civilian

University of Texas-Medical Branch, Galveston, TX
Ph.D. in Microbiology and Immunology, 2013-Curr

Kaplan University, Davenport, IA
Master's of Public Health w/ an emphasis in Epidemiology, 2011-2013

Southern University and A&M College, Baton Rouge, LA
Bachelor's of Science (*summa cum laude*), 2000-2004
Honors Thesis: "Research Study into Siderophores: The Cloning, Purification, and Expression of Mycobactin T."

Military

Brooke Army Medical Center (AMEDD) Officer Advance Course, AMEDD Center and School
Fort Sam Houston, TX
Certificate, 2010

AMEDD Officer Basic Course, AMEDD Center and School
Fort Sam Houston, TX
Certificate, 2004

Clinical Laboratory Officer Course, Walter Reed Army Medical Center
Washington, DC
Certification as Medical Laboratory Scientist (ASCP), 2007-2008

RESEARCH EXPERIENCE

University of Texas-Medical Branch, Galveston, TX
Graduate Assistant, Barrett Laboratory, Department of Pathology, 2014-Curr
Conduct research on yellow fever virus using classical virology and molecular techniques. Develop and validate data pipeline to investigate viral diversity using Next Generation Sequencing as a platform. Supervised and mentored two graduate rotation students.

Southern University and A&M College, Baton Rouge, LA
Undergraduate Honor Student, Lewis Laboratory, Department of Microbiology, 2001-2004
In corroboration with and extension of a summer undergraduate research internship Quadri Laboratory, Weill College of Cornell University Graduate School, New York, NY, conducted

research on siderophores of Mycobacterium tuberculosis using molecular and analytical/preparative purification methods to study iron acquisition.

PUBLICATION

Collins, N. D., & Barrett, A. D. (2017). Live Attenuated Yellow Fever 17D Vaccine: A Legacy Vaccine Still Controlling Outbreaks In Modern Day. *Current infectious disease reports*, *19*(3), 14.

SUBMITTED MANUSCRIPTS

Collins, N.D., Beck, A.S., Widen, S.G., Wood, T.G., Higgs, S, & Barrett, AD. Structural and non-structural genes contribute to the genetic diversity of RNA viruses.

Collins, N.D., Widen, S.G., Tesh, R.B., Li, L., Shi, P.Y., Wood, T.G., & Sarathy, V.V. Inter and intra-lineage genetic diversity of Zika virus differs for American isolates.

PRESENTATIONS/POSTERS

"Role of nonstructural protein genes in attenuated phenotype of yellow fever live attenuated 17D vaccine." Andrew Beck[1], Stephen Higgs[2], Alan Barrett[1], and Natalie Collins[1]; [1]University of Texas Medical Branch; and [2]Kansas State University. Presented at the 34th Annual American Society of Virology Meeting. London, Ontario, Canada, July 2015.

"Viral quasispecies profile of structural and non-structural Asibi/17D chimeric yellow fever virus." Andrew Beck[1], Stephen Higgs[2], Alan Barrett[1], and Natalie Collins[1]; [1]University of Texas Medical Branch; and [2]Kansas State University. Presented at the 19th Annual Conference on Vaccine Research by the National Foundation for Infectious Diseases. Baltimore, Maryland, April 2016.

"Viral diversity profile and *In vitro* characterization of structural and non-structural Asibi/17D chimeric yellow fever virus." Andrew Beck[1], Stephen Higgs[2], Alan Barrett[1], and Natalie Collins[1]; [1]University of Texas Medical Branch; and [2]Kansas State University. Posters at the triennial Positive-Strand RNA Viruses by Keystone Symposia. Austin, Texas, May 2016.

The replication complex of yellow fever virus contributes to the limited genetic diversity of 17D vaccine. Stephen Higgs[2], Alan Barrett[1], and Natalie Collins[1]; [1]University of Texas Medical Branch; and [2]Kansas State University. Poster at 2017 Tropical Infectious Diseases, Gordon Research Conference. Galveston, Texas, 2017.

"Genetic Diversity of Nonstructural Asibi/17D Mutant Yellow Fever Viruses. " Natalie Collins[2], Stephen Higgs[3], and Alan Barrett[1,2]; [1]Sealy Center for Vaccine Development and [2]Department of Microbiology and Immunology , University of Texas Medical Branch; and [3]Biosecurity Research Institute, Kansas State University. Presentation at the 2017 Annual Conference on Vaccine Research. Bethesda, Maryland, 2017.

PROFESSIONAL WORK EXPERIENCE

Company Commander, US Army Institute of Surgical Research, Fort Sam Houston, TX, 2011-2013

Commands United States Army Institute of Surgical Research (USAISR) Company, comprised of over 232 Soldiers serving 5 divisions, responsible for battlefield advances in combat casualty care through research and clinically responsible for the DOD's only Burn Center and Burn Flight Team. USAISR Institute Adjutant for a joint service facility comprised of over 800 Soldiers, Airmen, Sailors, DA civilians, and contractors.

Anatomic Pathology Officer in Charge, Brooke Army Medical Center, Fort Sam Houston, TX, 2009-2011
Provide pathology support and complex laboratory testing for 36 DoD medical facilities worldwide. Directly supervise the technical knowledge and performance of three civilian supervisors and one senior non-commissioned officer. Oversee the technical knowledge and performance of 25 civilian and military personnel.

Specimen Processing and Ancillary Services Officer in Charge, Brooke Army Medical Center, Fort Sam Houston, TX, 2008-2009
Provide Ancillary Laboratory support for trainees and military dependents. Directly supervise the technical knowledge and performance of 20 military, DA, and contract civilian professionals.

Assistant Operations Officer, **14th Combat Support Hospital,** Fort Benning, GA, 2007-2007
Provide Operational support to echelons above Corp Level III Combat Health Support unit. Responsible for the operational readiness and training of all personnel within the CSH, supporting 210 assigned personnel and 150 PROFIS.

Platoon Leader, 690th Medical Company, Fort Benning, GA, 2004-2007
Provide ground evacuation support to the Infantry Training Brigade, Ranger Training Brigade and Airborne School. While deployed, was responsible for the completion of the Platoon mission of providing medical support to logistical convoys.

MILITARY AWARDS AND RECOGNITION

Meritorious Service Medal w/3 olc
Army Commendation Medal w/ 2 olc
Army Achievement Medal
National Defense Service Medal
Iraq Campaign Medal
Army Service Ribbon
Overseas Service Ribbon

CIVILIAN AWARDS AND RECOGNITION

Keystone Symposia Future of Science Recipient, 2016
McLaughlin Symposium Poster Award, 2016
Gordon Research Fellowship, 2017