ABSTRACT

STEVENSON, LINDSEY CAITLIN. The Effects of P320Sc Mutation in West Nile Non-structural Protein 1 (NS1) and the Generation of Aptamers to Detect Dengue NS1 in the Context of Infection. (Under the direction of Dr. Frank Scholle).

West Nile virus is a small RNA virus which can cause serious disease in humans. The viral genome is translated into a single polyprotein that is cleaved into three structural and seven non-structural proteins. Non-structural protein 1 (NS1) is essential for viral replication but is also instrumental in immune evasion. NS1 is able to inhibit cytokine signaling by Toll-like receptor 3 (TLR3), which detects the pathogen associated molecular pattern (PAMP) double-stranded RNA (dsRNA), which must be generated by the virus during replication. Previous work in our lab discovered a point mutation that allowed NS1 to support replication without inhibiting TLR3. This was a single nucleotide change that resulted in a change from proline to serine at the 320 position in the NS1 protein. However, this mutation was unstable and rapidly reverted to wild-type.

In Chapter 2, we stabilized the mutation by introducing a full codon change from proline to serine at NS1 320 (P320Sc). We demonstrated that the mutation was stable up to six passages by sequencing of viral supernatants. However, we showed that, when the P320S mutation is forced to remain in the virus, it confers a significant fitness disadvantage. Therefore, we conclude that the P320Sc mutant virus cannot be used to determine the effects of TLR3 signaling on viral growth.

Dengue virus is a serious human pathogen, affecting an estimated 40% of the world’s population. There are severe limitations to the usefulness of the only human vaccine. Thus, early detection and palliative care are still the best ways of increasing patient survival. The
most effective methods for early detection of dengue include reverse-transcriptase polymerase chain reaction (RT-PCR) detection of viral genomes and enzyme-linked immunosorbent assay (ELISA) detection of dengue NS1. ELISA-based detection of NS1 is considered the best test for use in low-resource settings. However, ELISA’s use antibodies which require refrigeration and are expensive to develop and produce. Enzyme-linked aptamer sorbent assays (ELASA’s) substitute aptamers for one or more antibodies in an ELISA technique. Aptamers are short, single-stranded pieces of RNA or DNA that form a three-dimensional shape that allows them to bind a target. They are relatively inexpensive to produce, and do not require refrigeration.

In Chapter 3, we select two single-stranded DNA aptamers that can detect NS1 from Dengue serotypes 2-4 and from the West Nile variant Kunjin virus in clarified viral supernatants. We also demonstrate that aptamer DV1NS1 5-4 is able to detect Dengue 2-4 and Kunjin NS1 in artificially infected human plasma. The aptamer does not significantly bind native human plasma, so can be used to detect NS1 in patient samples without the need for inactivation of complement. With more testing, aptamer DV1NS1 5-4 may be able to replace one of the antibodies used in current ELISA-based screening, allowing tests to be made more cheaply, and reducing overall cost of dengue screening.
The Effects of P320Sc Mutation in West Nile Non-structural Protein 1 (NS1) and the Generation of Aptamers to Detect Dengue NS1 in the Context of Infection

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DEDICATION

This work is dedicated to my parents, who stood by me through the good times and bad. I would not be here without their love and support. Thank you.
BIOGRAPHY

Lindsey Stevenson was born in Asheville, North Carolina, on June 25, 1989. She was homeschooled beginning in 6th grade until her graduation in 2007. She then attended Liberty University, where she achieved her Bachelor of Science in Biochemistry. During her senior year, she took a course in Immunology and fell in love with the topic. After graduation, Lindsey was accepted into the Immunology program at NC State University as a Ph.D. student in the lab of Frank Scholle.
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Chapter 1

Literature Review

Phylogeny of West Nile virus and Dengue virus

West Nile virus (WNV) and Dengue virus (DV) are members of the family *Flaviviridae* (1). Within *Flaviviridae* are three genera: *Hepadivirus, Pestivirus,* and *Flavivirus* (2). The sole member of genus *Hepadivirus* is Hepatitis C virus. Genus *Pestivirus* has several members but none of them infect humans. Genus *Flavivirus* is a large genus that includes both human and non-human pathogens. WNV and DV belong to this last genus. Other members of this genus include the less virulent Australian variant of WNV, Kunjin (3, 4), Yellow Fever, Japanese Encephalitis Virus (2), and the various subtypes of Tick-borne Encephalitis (JE) Virus (5). Within the genus *Flavivirus*, the viruses are divided into clusters, based on their method of spread. These clusters are the Non-vector cluster, the Tick-borne cluster, including Tick-borne Encephalitis Virus, and Mosquito-borne cluster. WNV, DV, YF, Kunjin, and JE all fall into this last cluster (6). Mosquito-borne cluster can be further split into serogroups of like viruses. WNV is categorized within the Japanese encephalitis group, while the four Dengue serotypes have their own Dengue serogroup (5).

Members of *Flaviviridae* are small, spherical (icosahedral) viruses, typically only 40-60 nanometers in diameter and with viral genomes between 11 and 12 kb. The viral genome is (+)-sense, single-stranded RNA with a 5’ cap and no poly-A tail (2). They transcribe this viral genome as a single polyprotein, which is later cleaved into structural and non-structural proteins. Structural proteins, along with a new viral genome, form new viral particles, while the
non-structural proteins are mainly tasked with viral replication (5), although they can have multiple functions.

**WNV and DV Virus Structure and Genome Organization**

Both WNV and DV particles are icosahedral shaped, containing a single, positive sense, single-stranded RNA genome (7, 8). The genome is enclosed in a 30 nm capsid formed exclusively of C protein. Enclosing this is cellular membrane, taken from former host cells, that has been scattered with trimers of heterodimers of the envelope (E) protein and pre-membrane (PrM) protein, which is cleaved to M protein upon maturation of the virion. The trimers of PrM and E heterodimers form the “spikey”, immature version of the capsid. Once PrM is cleaved to M by the host proteins furin during viral maturation, E and M proteins will form flat homodimers that arrange into a characteristic herringbone formation (9–11) (Figure 1).
Figure 1: Maturation of Virion. Trimers of PrM and E heterodimers form “spikey” immature virion exterior (A). Upon cleaving of Pr peptide from PrM, M homodimers and E homodimers form flat herringbone configuration (B). Image adapted from (A) Chapter 33, Field’s Virology, 2007 (5) and (B) Mukhopadhyay, et al. (12).

The viral genome consists of an m7GpppAmp-capped positive sense single-stranded piece of RNA about 11000 nucleotides long (1, 13). The genome consists of a 5’ untranslated region (UTR), followed by a single open reading frame that codes for three structural proteins: C, PrM, and E, and 7 non-structural proteins: NS1, NS2a NS2b, NS3, NS4a, NS4b, and NS5. Following the open reading frame is another UTR at the 3’ end of the genome.

Flavivirus Replication Cycle

Like all Flaviviruses, DV and WNV enter their host cells by binding a cell surface protein and stimulating clathrin-mediated endocytosis (14, 15). WNV and DV may bind to a number of cell surface markers, depending on the type of host cell to be entered, such as DC-SIGN on Dendritic cells, heparin sulfate on epithelial and endothelial cells, Integrin α,β3 on activated endothelial cells. DV can further bind mannose receptor on macrophages and can enter
through Fc mediated phagocytosis when the virus has been bound but not neutralized by antibodies in a process known as antibody dependent enhancement (ADE) (15–17).

Once internalized within the cell, the virus containing phagosome will fuse with a lysosome. ATP dependent proton pumps will then rapidly drop the pH within the phagolysosome (18), which will activate various proteases within the lysosome (19), which would ideally kill and degrade the contents of the phagosome, so it can be presented to the immune system. However, the drop in pH causes a structural change of the E protein in the viral membrane, causing the homodimers to dissociate into monomers, which uncovers their fusion loops. These fusion loops are then inserted into the membrane of the phagolysosome, allowing the viral membrane to fuse with it and releasing the viral capsid into the cell cytoplasm (20, 21). At this point, the capsid degrades by an unknown mechanism (22) and releases the viral genome into the cytoplasm.

The WNV and DV genomes can be immediately translated as mRNA by host ribosomes upon entry into the host cell cytoplasm (7, 8). The viral genome is transcribed into one single polyprotein, which is co- and post-translationally cleaved into the three structural proteins, C, PrM, and E, and the 7 non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 by several host proteases including signalases and furin (Figure 2) and by the viral protease NS3 (23). Most of the non-structural proteins assemble around the viral genome to replicate it, while the structural proteins will eventually assemble to form a new viral particle containing a single genome (24). However, occasionally the structural proteins will assemble without an RNA genome inside. This is known as a subviral particle.
Replication of the viral genome occurs on the cytoplasmic side of the ER membranes (9). NS5, NS3, and NS2b have been shown to associate with NS1 and NS4a along these membranes, while the viral genome is being replicated (25). It is believed that NS4a stabilizes the replication complex (26). Additionally, NS4a has been shown to stimulate the membrane modification characteristic of cytopathic effect associated with flaviviral infection (27, 28).

NS1 is required for replication of the viral genome, but it is not directly associated with the replication complex, existing on the lumen side of the ER membrane, instead of the cytoplasmic side (29). It is believed that, though it lacks a transmembrane domain (30), NS1 interacts with NS4b to anchor the replication complex to the membrane pockets (31) known as Vesicle Packets (23) (Figure 3).

Figure 2: Cleaving of Flavivirus Polyprotein by Host and Viral Proteases. Image taken from Acosta, et al. (9).
Non-structural protein 5 (NS5) is the RNA dependent RNA polymerase (10). It will first create a number of (-) RNA strands complementary to the (+)-strand RNA viral genome. To do this, complementary regions of the 5’ and 3’ untranslated regions (UTR’s) will interact (32), causing the genome to circularize (33). The 3’ UTR will then recruit the viral replication complex, beginning with NS3 (33) and its cofactor NS2b (10), and culminating in NS5 recruitment. NS3 acts as the combination helicase and NTPase (10), while NS5 synthesizes a (-)-stranded RNA complement to the viral genome. More (+)-stranded RNA genomes will be made from the (-)-strand genomes. However, more (+) strand genomes are made overall, as the efficiency for synthesizing (+)-strand RNA genomes is roughly 10 times more efficient than the synthesis of (-)-strand RNA (22). Newly synthesized RNA genomes can then either reenter the replication cycle or be packaged into new viral particles (24).

Following replication, a single (+)-strand viral genome will exit the neck region of the ER invagination in which it was synthesized and associate with capsid proteins in the cytoplasm,
causing them to aggregate and form the nucleocapsid (34). This is a spontaneous reaction upon 
C proteins coming into contact with nucleic acid. C protein is very positively charged, having 26 
basic amino acid and only 3 acidic ones. It is therefore believed that the neutralization effect 
that occurs when the negatively charged sugar-phosphate backbone of the RNA genome 
associates with the positively charged C protein stimulates this nucleation effect (34). At the 
same time, PrM and E proteins will be inserted into ER membranes. The nucleocapsid will 
associate with a portion of ER membrane that contains a high concentration of PrM and E (9). 
The nucleocapsid will then bud into the lumen of the ER, where it will form an immature viral 
particle with an envelope consisting of captured ER membrane and around 180 copies of PrM 
proteins and 180 copies of E protein embedded in it (9). The PrM and E proteins will organize 
into homotrimer spikes in the membrane, with each spike consisting of three PrM/E 
heterodimers (11, 35).

PrM molecules on the surface of immature viral particle will then interact with KDEL 
receptor to facilitate processing out of the ER to the Golgi apparatus (327), where the lower pH 
will cause a conformation change in the PrM proteins from homotrimer spikes of heterodimers 
to a flat, herringbone pattern of PrM/PrM and E/E homodimers (9, 10, 35). The cellular 
protease furin is then able to cleave PrM into its mature form, M. However, the pr peptide 
remains closely associated with M until the pH within the Golgi vesicle raises to neutral, so that 
M will not be able to fuse with the Golgi membrane (35). Once the pH inside the vesicle 
approaches 7, the pr peptide dissociates from M, leaving a mature viral particle that can be 
released via the secretory pathway (9).
TLR Receptors and TLR Signaling

The innate immune system is capable of detecting and responding to macromolecules which are not found in normal host tissues. Such macromolecules are known as pathogen associated molecular patterns (PAMP’s), and encompass a wide variety ranging from double stranded RNA to components of bacterial cell walls (36, 37). Receptors that detect PAMP’s are known as pattern recognition receptors (PRR’s). PRR’s can be expressed on the cell surface or internally. Different PRR’s detect PAMP’s commonly associated with different categories of invading organisms. For example, Toll-like receptor (TLR) 4 detects lipopolysaccharide, found in the cell walls of most Gram – bacteria (38), and the Nod-like receptor (NLR), NALP 3, that detects free ATP, indicating cellular destruction (39). Some PRR’s detect the effect of invading pathogens, rather than any particular part of the pathogen itself, and so can signal for a response to a wide range of insults. NALP 3, for example, detects ATP in the extracellular space. ATP should only be found outside of a cell in the event of traumatic cell death (40). Therefore, NALP 3 could be activated by any pathogen causing cell damage. Other PRR’s target specific patterns of macromolecules associated directly with pathogens. TLR4 is an example of this category. It targets LPS specifically, so it should only respond during an infection with Gram – bacteria. We will focus on PRR’s that either or indirectly detect viral infection.

RIG-I-like receptors (RLR’s), including retinoic acid inducible gene-1 (RIG-I) and Melanoma differentiation-associated protein-5 (MDA-5), detect dsRNA in the cell cytoplasm (41), which should not exist in healthy cells, except in short stem-loops, which are not recognized by RIG-I. RIG-I itself is able to detect short pieces of dsRNA with blunted ends, which keeps it from reacting to stem-loop regions of host RNA (41–44). MDA-5 detects dsRNA
longer than 1000 bp, with no end preference (41–44). RIG-I and MDA-5 each contain two caspase activation and recruitment domains (CARD) at the N-terminus, connected to a target binding helicase core and a Zn\(^{2+}\) containing regulatory domain at the C-terminus (45). When not bound to its target, RIG-I exists in an open conformation which sterically inhibits CARD binding to ubiquitin or its adaptor protein mitochondrial antiviral-signaling protein (MAVS), also known as IPS-I, VISA, and Cardif (46), thus auto-inhibiting itself in the absence of dsRNA (41, 47). Upon encountering dsRNA, the helicase core of RIG-I will wrap itself around the RNA, causing a conformational change which exposes the CARD domains (45, 48). MDA-5 does not undergo a conformational change from auto-inhibited to active upon introduction of dsRNA. Instead multiple MDA-5 proteins bind along the dsRNA ligand, oligomerizing the CARD domains (41, 49). RIG-I and MDA-5 signals are transduced by binding of MAVS protein to CARD domains (45). MAVS then activates TAK1, which leads to Type I interferon production via phosphorylated IRF3 (50–53), which dimerizes and translocates to the nucleus.

Of the TLR’s, TLR3, TLR7/8, and TLR9 are the most important for detecting viruses (54). TLR7, and human TLR8, are expressed primarily on endosomal membranes of plasmacytoid dendritic cells and detect guanosine- or uridine-rich ssRNA (55–57). TLR7 and TLR8 do not usually detect host ssRNA, though it is unclear if this is due to host cell’s ssRNA being less rich in guanosine and uridine than viral RNA, or if this is due to the TLR’s placement in the endosome, where they are unlikely to come into contact with host ssRNA (58). Unlike TLR3, TLR7 and TLR8 signal through the adaptor molecule myeloid differentiation factor-88 (MyD88) (55). Upon binding their ligands, TLR7 and TLR8 homodimerize and bind the TIR domain-containing adaptor proteins (TIRAP). The TIRAP proteins then recruit MyD88 to the TLR cytoplasmic tails. MyD88
then complexes with IRAK4, which recruits and activates IRAK1 (59). IRAK1 then autophosphorylates (60) and separates from the complex and associates with TRAF6 and the ring-shaped E3 domain of ubiquitin. This association promotes the ubiquitination and subsequent activation of TRAF6 and TAK1, which will then associate with TAB to promote NFκB dependent transcription, as mentioned above (59). TRAF6 is then left to mediate IRAK1 dependent activation of IRF7 (61), which will dimerize and translocate to the nucleus to mediate transcription of Type I interferon (62). The mechanism by which TRAF6 mediates this activation is still unclear, but it has been shown that TRAF6 is required for IRAK1 mediated activation of IRF7 (63).

Like TLR7 and TLR8, TLR9 is expressed on endosomal membranes (36). TLR9 detects unmethylated CpG (cytosine-phosphate-guanidine) motifs in DNA (58). TLR9 can therefore respond to a variety of insults, including bacterial DNA, which is naturally not phosphorylated, or to a change in the normal patterns of host DNA CpG methylation, which can indicate either viral infection or transformation into a cancer cell (64). Because TLR9 recognizes any CpG DNA that does not conform to the normal cellular pattern of methylation, it has also been implicated in autoimmune disorders, such as systemic lupus erythematosus (SLE) (65). Upon binding its PAMP, TLR9 signals using the adaptor molecule MyD88, as described above.

TLR3, like RIG-I, detects double-stranded RNA (66). However, TLR3 detects dsRNA in the endosome rather than the cytoplasm (37, 66). TLR3 is specific to long double stranded RNA (67). TLR3’s preference for long dsRNA, coupled with its placement in the endosome prevents it from detecting the short hairpin loops characteristic of many cellular RNA’s, including tRNA’s. RNA viruses, however, must generate long dsRNA molecules during genome replication,
efficiently triggering dimerization of TLR3 (68). TLR3, like all TLR’s, is comprised of an endosomal horseshoe-shaped leucine rich repeat (LRR) domain, a transmembrane domain, and a cytoplasmic signaling domain (69). TLR3 homodimerizes upon binding to dsRNA, with each TLR binding one half of the dsRNA molecule (37). Upon dimerization, the cytoplasmic tails of TLR3, which contain a Toll/Interleukin-1 Receptor (TIR) domain, are brought together, stimulating recruitment of the adaptor molecule TRIF-related adapter molecule (TRAM). TRAM then recruits the adaptor molecule TIR domain-containing adapter inducing IFNβ (TRIF) (58). TLR3, unlike other TLR’s binds TRIF exclusively (61, 63, 70). TRIF’s C terminal contains a Rip homotypic interaction motif (RHIM), which will interact with RIP-1 to induce its phosphorylation by RIP-3 (71, 72). Phosphorylated RIP-1 is then capable of inducing NFκB dependent gene expression via TAK1 activation (61, 73). At the same time, TRIF’s N terminal can initiate binding with TRAF6, inducing phosphorylation (61). Phosphorylated TRAF6 will then bind to IRAK1 to induce activation of TAK1 (73, 74). Once TAK1 is activated, it binds its regulatory subunits TAB1, TAB2, and TAB3 (59). The TAK/TAB complex can then induce NFκB dependent transcription through the classical pathway of ubiquitination of NEMO and phosphorylation of IKK (75, 76) and induce AP-1 transcription factor activity through the MAPK family of signaling molecules (59).

In parallel to these pathways, the TRIF/TRAM complex recruits TBK1, also known as NFκB-activating kinase (NAK) (77) and TRAF2-associated kinase (T2K) (78). TBK1 associates with IKKe, also known as IKKi (79), which then phosphorylates interferon regulatory factor 3 (IRF3). IRF3 then dimerizes and translocates to the nucleus to mediate transcription of the chemokine RANTES (80, 81) and Type I interferon (IFNα/β) (74, 82).
AP-1 is more important in cell survival and apoptosis signaling (83, 84), so it will not be a focus of this review. NFκB induces transcription of a host of pro-inflammatory cytokines such as IL-1β (85), IL-6 (86, 87), IFNβ (88), and TNFα (89); as well as a number of chemokines such as CCL5 (90), CCL17, CCL22 (91, 92), and CCL23 (93), for inducing trafficking of immune cells to areas of infection. NFκB can also enhance RANTES transcription in activated T cells (94).

**NS1 and Its Role in Infection**

Our lab has focused heavily on West Nile and Dengue Virus NS1 proteins and their role in modulating virus-immune system interactions. While it is found intracellularly, our lab has shown that it is secreted to high amounts by infected cells and that that secreted form of NS1 can have immunomodulatory effects on neighboring uninfected cells (95). Other labs have shown other ways in which NS1 is able to inhibit innate immune response to infection. It has also been shown that NS1 is essential for viral replication (29, 31, 96). In this section, we will explore the various properties of this protein.

1. **NS1 Processing**

   NS1 is produced, along with other viral proteins, when the polyprotein transcribed by host ribosomes is cleaved into the three structural and seven non-structural viral proteins (29). Once the E-NS1 junction of the polyprotein has been cleaved, NS1-NS2a is trafficked to the ER lumen and cleaved (23). Once in the ER lumen, DV NS1 in glycosylated at two conserved asparagine residues, Asn 130 and Asn 207, by glycosidase. WNV NS1 has one additional N-linked glycosylation site at Asn 175 (97).
2. NS1 Structure

Intracellular NS1 exists as membrane-bound homodimers. Each NS1 protein consists of three domains: a hydrophobic β-roll between amino acid residues 1 and 29, a RIG-I like α/β Wing domain between residues 38 and 151, and a central β ladder between residues 181 and 352, which extends into a “spaghetti loop” that is hydrophilic but has no discernible structure (23, 98). These major structural domains are separated by two 3-stranded β sheet subdomains (Figure 4).

Figure 4: Structure of NS1. sNS1 hexamer (A) is made up of three NS1 homodimers (B) that are connected to form a barrel-like structure. Each NS1 homodimer (C) consisting of 2 β rolls (blue), 2 central β ladders (red), and 6 β sheet subdomains (orange). Image adapted from Akey, et al. (98)

The structure is stabilized by 12 cystine residues, conserved across flavivirus NS1 proteins, which form 6 disulfide crossbridges (98, 99). The homodimer is formed when two of these proteins form a cross-like structure, with the hydrophobic elements, consisting of the β roll and the β sheet portion of the β ladder, facing toward each other in a hydrophobic-
hydrophobic attraction. The hydrophilic elements comprise the rest of the homodimer.

Intracellular NS1 is primarily associated with viral replication and has been shown to colocalize with the replication complex, albeit on the opposite side of the ER membrane (31). It is also believed to work with NS4a to modify the ER membrane into Vesicle Packets (100) and to associate with NS4b to anchor the replication complex to the ER membrane (31). However, a small portion of intracellular, dimeric NS1 is trafficked to the plasma membrane (mNS1) of infected cells on cholesterol or lipid rafts (23, 29).

Soluble secreted NS1 (sNS1) is hexameric, consisting of a trimer of homodimers in the cross-like position described above (100). The sNS1 hexamer crystal structure was solved in 2010, using cryoelectron microscopy. sNS1 was shown to form a barrel shaped lipoprotein of three twisted rods, held together by hydrophobic bonding, looping around an open center. Each rod was made from an NS1 dimer, which was capable of membrane association (101). sNS1 is secreted at high levels by infected mammalian cells (102) and is associated with inhibition of several major pathways of the innate immune system. NS1 homodimers to be secreted are processed through the Golgi apparatus, where any exposed carbohydrate moieties are glycosylated and trimmed (100). It was once thought that this glycosylation was necessary for NS1 secretion, as insect cells, which lack the ability to do so (103), were thought to be unable to secrete NS1 (29, 104). However, some more recent studies have indicated that NS1 can be secreted efficiently by mosquito cells (105–107).

Within the Japanese Encephalitis group of flaviviruses there also exists a longer alternative form of NS1, known as NS1’, that is believed to contribute to the neuroinvasiveness of these viruses (108). NS1’ is generated infrequently when a conserved heptanucleotide motif
and stable pseudoknot in the viral RNA causes a -1 ribosomal frameshift when translating NS1. This results in a 52-53 kDa protein instead of the standard 48 kDa (23). The frameshift event that causes NS1’ is quite rare, only occurring during 6-8.5% of translation events (108). NS1’ has been shown in WNV to be able to colocalize with NS1 and promote viral replication (109), but it also contributes to viral pathogenesis. In the mouse model, the presence of functional NS1’ has been linked to neuroinvasiveness, while modifications that interfere with the heptanucleotide sequence or the formation of the pseudoknot result in less neuroinvasiveness (108).

3. Innate Immune Inhibitory Properties of NS1

NS1 has been shown to associate with a number of host proteins. As far back as 1970, NS1 was found to inhibit complement, known at the time as Soluble Complement- Fixing Antigen (110–113). Soluble Complement-Fixing Antigen was however identified as soluble NS1 a year later (114). While other flaviviral non-structural proteins have been shown to be involved in viral immune evasion, such as NS5 inhibiting STAT2 mediated IFNβ production, or NS2A, NS4A, and NS4B inhibiting STAT1 (54, 115), NS1 is considered to take the most active role in inhibiting innate immune responses to flaviviruses (116). sNS1 has been shown to actively inhibit formation of the complement membrane attack complex (MAC). NS1 has been shown to bind to complement S-protein (vitronectin), preventing its dissociation from complement protein C5 (117–119). Cleaving of C5 into C5a and C5b is then prevented. C5b is then unavailable to bind to the membranes of infected cells or the membranes of viral particles (116, 120), and the MAC is unable to form C9 polymer pores in the membranes (121).
NS1 has been shown to directly bind to the C4 protein of the C3 convertase and enhance its cleavage. This prevents the C3 convertase cleaving of C3 into C3a and C3b (122). C3b is then unable to bind to the membranes of infected cells or viral particles, effectively negating complement mediated opsinization via the classical and lectin pathways (119, 122). NS1 has also been shown to interact with Factor H, of the alternative pathway, to facilitate rapid degradation of C3bBb (123). C3bBb is then unable to facilitate further C3 cleavage into C3a and C3b, again impairing complement based opsinization (124).

Our lab has also shown that NS1 is able to inhibit TLR3 mediated cytokine production (95, 125) via both NFκB and IRF3 (126). Although the exact mechanism was not elucidated, Wilson, et al. were able to show that NS1 effectively blocked NFκB and IRF3 translocation from the cytoplasm to the nucleus. Indeed, they were also able to show that constitutive expression of NS1 effectively negated TLR3 signaling. TLR3 activation can induce Type I IFN production, which has been shown to inhibit infection of new cells (54). NS1 mediated inhibition of IRF3 also enables inhibition of RIG-I and MDA-5 signaling (127). However, NS1 has also been shown to inhibit RIG-I and MDA-5 mediated IFN production by directly binding to RIG-I and MDA-5 and inhibiting K63-linked polyubiquitination, so that adaptor molecules cannot bind (127). Our lab has also shown that pre-treatment of monocyte-derived dendritic cells with secreted NS1 enhanced infection and production of pro-inflammatory cytokines (128). This last was particularly surprising, considering the previous findings that NS1 inhibits TLR3-mediated cytokine production. However, as NS1 uptake by monocytes was not found to stimulate maturation to dendritic cells, it was proposed that increased pro-inflammatory cytokine and chemokine production might result in more monocyte trafficking to the area of infection. This
seems particularly likely, as one of the chemokines upregulated was CCL2 (MCP-1), which is a chemoattractant for monocytes.

**Viral Maintenance and Transmission**

In nature, WNV cycles between its natural reservoir, birds, and its mosquito vector. While, upwards of 60 species of mosquito in North America have screened positive for WNV, the primary vector of transmission is the *Culex* mosquito, with *Culex pipiens*, *Cx. restuans*, and *Ct. salinarius* being the most prevalent (129, 130). The *Culex* mosquito makes an ideal vector for WNV, as its midgut and salivary gland cells not only support levels of viral replication that are high enough for transmission (131, 132), but it also feeds on a variety of hosts (133). These hosts include a variety of birds, such as the northern cardinals (*Cardinalis cardinalis*), bluejays (*Cyanocitta cristata*), carrion crows (*Corvus corone*), and many others (132, 133). However, *Culex* mosquitoes will feed on a variety of mammalian hosts as well, including white tailed deer, eastern gray squirrels, raccoons, horses, and humans (133).

In avian hosts, viral loads in the blood reach high enough levels to infect a naïve mosquito, as it takes a blood meal (132). In a competent vector, such as *Culex*, WNV will infect the midgut, where it will replicate. Once an appropriate number of viral particles have been reached, the virus will spread from the midgut to the salivary glands. Viral progeny are shed into the saliva, rendering the vector infectious (132). The infected mosquito then deposits viral particles into the next host it feeds on, while it probes for blood (134). Hosts that are susceptible to WNV infection but are unable to produce sufficient titers to infect new mosquito
vectors are called dead-end hosts. Such dead-end hosts for WNV include horses and humans (135).

Similarly, Dengue virus is spread by the feeding of an infected mosquito, however the primary vector for DV are *Aedes aegypti* mosquitoes (136–138). *Aedes aegypti* mosquitoes make effective human vectors because of their tendency to live near humans and to bite primarily during the day when humans are likely to be out of their houses, instead of primarily at night when people can protect themselves with mosquito netting, etc (136, 137). Additionally, unlike other mosquitoes, *Aedes aegypti* mosquitoes prefer human blood to that of other mammals and prefer to lay their eggs in standing water in artificial containers (136, 138).

**Human Disease**

Only about 1 in 5 individuals infected with WNV are symptomatic (139). Clinical symptoms begin with high fever for an extended period along with severe flu-like symptoms including fatigue, muscle pain and weakness, and rash (140), and is known as West Nile Fever. West Nile Fever is, by far, the less severe form of infection, and most people recover within a week (141, 142). The more severe form of West Nile infection occurs when the virus crosses the blood brain barrier to cause encephalitis and meningitis symptoms (141). Encephalitis symptoms include headache and stiff neck which can rapidly progress to coma and death (140, 141, 143). While it is estimated that only about 1 in 150 people infected progress to neurologic symptoms, the symptoms associated with neurologic disease can be severe and debilitating, even if they do not lead to death of the infected individual. Neurologic complications apart
from death can include flaccid paralysis, peripheral neuropathy, polyradiculopathy, and optic neuritis, with symptoms continuing to be reported months after viral clearance (144). Incidence of neurological complications is more likely in elderly patients (141, 144). Coupled with the fact that there is still no vaccine approved for human use (145–147), this makes WNV a major threat to the elderly in regions where the virus is endemic, among which is the United States of America (22).

Like West Nile, primary Dengue infection with any of the four viral serotypes results in high fever, severe muscle aches, and fatigue (148). Dengue Fever, as the milder infection is known, is generally recoverable, but so debilitating it is colloquially known as “break bone fever” (149). However, a much more severe form of disease can occur during secondary infection. Unlike WNV, there are four major serotypes of Dengue virus. These serotypes are similar enough that antibodies generated against one will cross-react with the other three. However, the four serotypes differ enough from each other that any cross-reacting antibodies are unable to neutralize the virus (150–153). These non-neutralizing cross-reacting antibodies are believed to promote Fc receptor-dependent uptake of the viral particles into antigen presenting cells like dendritic cells. This results in the much more severe forms of Dengue infection: Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). DHF is characterized by thrombocytopenia, elevated vascular permeability, and petechiae (154, 155). Severe DHF can then lead to the weak pulse and low blood pressure characteristic of DSS (154), which can result in death of the patient if not properly treated (156).

Antibodies generated during primary Dengue infection have been shown to be protective against re-infection with that same serotype (150–152, 157). This is believed to be
because the antibodies are able to bind and neutralize the viral particles (152). Among other cell types, DV has been shown to be able to infect dendritic cells (15, 17, 158). The severe illness associated with secondary infection with a different serotype is believed to be because of antibody dependent enhancement (ADE), in which antibodies are able to facilitate viral entry into dendritic cells by Fc receptor dependent internalization, while at the same time not inactivating the virus. This would allow viral particles to more readily infect their preferred cell type than if any anti-Dengue antibodies had not been present, leading to earlier, more severe infection. The cytokine storm resulting from such a severe infection would then lead to symptoms such as hemorrhagic fever and Dengue shock syndrome (150–152). Thus, the antibody-dependent enhancement of dendritic cell infection, resulting from cross-reactive but non-neutralizing antibodies, is direct precursor to more severe illness.

**Diagnosis of Infection**

Most screening methods are not used unless a patient has symptoms. However, this does not mean all screening methods apply only to those who are symptomatic. Indeed, blood donated by apparently healthy individuals living in areas where Dengue virus is endemic must still be screened, as transmission of Dengue by contaminated blood supply has happened (159, 160). These screening methods exploit a variety of targets associated with viral infection, including serological methods that detect anti-viral antibodies in patient serum or molecular methods that detect the genomes of viral particles (161).
Major serological tests used to screen for Dengue infection include the hemagglutination-inhibition (HAI) test, complement fixation (CF) test, plaque reduction neutralization test (PRNT), and enzyme linked immunosorbent assay (ELISA) (161, 162). ELISA-based screening can either target patient response to infection by using a milieu of viral antigens to immobilize anti-viral antibodies from patient serum onto the ELISA plate. Discrimination as to whether the antibodies are IgM or IgG is then performed using labeled antibodies specific to the constant/Fc region of said human antibodies (163, 164).

The hemagglutination-inhibition test similarly tests patient responses to the virus by mixing viral particles with red blood cells (RBC’s) and dilutions of patient serum (165). When virus is present, it will form a complex between the sialic acid on the surface of the RBC’s and fill the well (agglutination). If the viral particles are unable to complex, the RBC’s will fall to the bottom of the well and collect there, forming a “button” instead of a complex. When neutralizing levels of antibodies are present, the viral particles will be unable to complex, so agglutination is prevented by the neutralizing levels of antibody. The most dilute serum that is still able to inhibit hemagglutination is the patient’s HAI titer. HAI can be used to differentiate between primary and secondary infection. Convalescent titers from patients experiencing a primary Dengue infection tend to be below 1:640, while secondary titers tend to be at or above 1:1280 (161).

Similarly, the plaque reduction neutralization test can measure a patient’s neutralizing antibody titer. Virus is mixed with dilutions of patient serum before being plated on a monolayer of cells and allowed to infect. If the levels of patient antibody are sufficient to inactivate the virus, it will no longer be able to infect the cell monolayer. The patient’s titer in
PRNT$_{50}$ is the highest dilution of serum that is able to reduce the number of viral plaques by 50% when compared to the number of plaques generated by virus alone plated on the monolayer (166, 167).

ELISA-based screening is widely used to detect anti-Dengue antibodies generated by the patient. The use of ELISA’s has the advantage of being able to detect the exact virus afflicting the patient. The ELISA test is also able to separate between primary and secondary infection by comparing IgM antibody levels to those of IgG. IgG is generated later in infection than IgM, so a high IgG/IgM ratio is indicative of a secondary response, in which the major antibody production will be from memory B cells which have already undergone class switching (162). However, the main drawbacks to the tests discussed so far are the need for both an acute and convalescent blood draw and the delay in detecting infection. Additionally, both the HAI and IgG/IgM ELISA tests rely on testing for anti-Dengue antibodies within the patient’s blood. While IgG antibodies are produced at high levels and easily detected as early as the acute phase of infection during secondary infection, it is not uncommon to have to wait two weeks after onset of symptoms to be able to detect any IgG in primary infections (168). IgM detection, on the other hand is more unpredictable. There have been reports of positive anti-Dengue IgM ELISA’s beginning 3 days after onset of symptoms in the blood of infected individuals (162). However, IgM is not an optimal marker for infection before 5 days post onset of symptoms (161, 168). Additionally, during secondary infection, IgM is sometimes not produced to high enough levels to detect (168). So, these tests are not optimal for diagnosing early infection. As decreased mortality has been linked to early diagnosis (156), it is critical to positively identify WNV or DV infection as quickly as possible.
There are several tests which may be used for early detection of flavivirus infection. These include RT-PCR based amplification of viral genome from patient blood, ELISA-based detection of viral NS1 protein in patient blood, and rapid diagnostic test (RDT)- based detection of NS1, including laminar flow assays. These tests can all be used immediately upon onset of symptoms, allowing for rapid identification of the infecting agent (169–171). Detection of flavivirus infection by RT-PCR is both rapid and highly sensitive, with some of the newer protocols being able to detect as few as 50-100 plaque forming units (pfu) of virus, enabling them to positively diagnose infection even earlier in viremia (168). Additionally, universal primers targeting a conserved region of the 3’UTR can detect all four Dengue serotypes in a single reaction (168, 172). If the patient is then confirmed to have Dengue, a second round of testing can be run, using serotype specific primer sets. However, RT-PCR-based screening requires specialized equipment, temperature-controlled environments, and highly trained laboratory personnel (173, 174), making it difficult to adapt to low resource settings (175). Additionally, the very sensitivity of RT-PCR makes RT-PCR-based screens more prone to false positives caused by cross-contamination between samples (169).

RDT’s, unlike RT-PCR methods, have the added benefit of being very easy to use and not requiring special training in their use (174). RDT’s target protein, usually NS1, so they have no need for special equipment or storage conditions, as the test can be run on fresh samples in the field. However, RDT’s have historically had issues with both false positive readings and false negative readings. Indeed a 2009 study by Hunsperger, et al. found that, while RDT’s were useful in diagnosis of DV, they were not as reliable as ELISA-based techniques (176). A 2014 study by the same lab confirmed this opinion, granting that while RDT’s have become better
since 2009, there has not been adequate testing to conclude that they can replace ELISA-based tests (175). However, as the sensitivity of RDT’s continue to improve (171), this may change.

Detection of soluble NS1 in patient blood by ELISA has thus far been the best test for balancing specificity with ease of use (174, 175). NS1 is secreted to high levels during infection (95). Levels of soluble NS1 generally follow viremia kinetics in patients (156, 169, 177), making NS1-targeted screening most effective for early infection. However, a study by Pok, et al. detected NS1 in the majority of patients as soon as day 1 of symptoms and continuing until days 5-6: a full two days after PCR based techniques were losing effectiveness (170), indicating the window for detecting NS1 is somewhat broader than that for PCR-based techniques. In addition, levels of NS1 in patient blood have been directly correlated with severity of disease in Dengue infections (177–179). While most RDT’s also target NS1 for screening, they have historically been less reliable, especially during secondary infection, when binding by the patient’s own anti-NS1 antibodies can result in steric hindrance and lower levels of detectible NS1 (180, 181). ELISA tests are generally more sensitive to smaller amounts of antigen than RDT’s and are still considered to be the best laboratory standard for Dengue screening, as they combine high sensitivity with ease of handling and a minimum requirement for specialized equipment (168, 174, 175).

One final test which bears a mention is the tourniquet test. The tourniquet test does not confirm Dengue diagnosis, nor can it differentiate between Dengue serotypes, or between flaviviral infection. It is, however, still widely used in patients suspected or confirmed to have Dengue to determine whether the patient is progressing to Dengue hemorrhagic fever (156). The test is performed by simply inflating a blood pressure cuff on the individual's arm for 5
minutes. The number of petechiae left on the skin are then counted and reported per square inch of skin. 10 or more petechiae per square inch of skin constitutes hemorrhage (155).

**Vaccination and Treatment**

Dengue virus infection is broken into three stages: the febrile stage, characterized by sudden high fever, flu-like symptoms, viremia, and an expansion of total white blood cell count in circulation, sometimes accompanied by petechiae and mucous membrane bleeding; the critical phase, characterized by hemorrhage, symptoms of shock including leukopenia and drop in body temperature to or below normal body temperature, and organ impairment which varies with the degree of shock; and the recovery phase, during which the patient’s immune system contracts and any blood or fluid lost to the tissues during hemorrhage is reabsorbed (156). Treatments vary slightly depending on the stage of illness.

When first presenting with Dengue, standard practice is to perform a complete blood count on the patient. Leukopenia is a characteristic of a patient being in the critical stage of infection. This can be combined with other physical markers such as cold extremities, thready pulse, and whether there is any alteration in the patient’s mental state (is the patient alert, for example) (156). On the other hand, a high fever not accompanied by drop in blood pressure, cold extremities, and general alertness of the patient indicates febrile stage. Additionally, a positive tourniquet test can indicate that a patient is moving from febrile stage toward critical and hemorrhage.
General treatment varies depending on severity of illness. If symptoms are quite mild, the patient may even be sent home with instructions to push fluids, particularly fruit juice and other electrolyte-rich fluids, and return if symptoms worsen. In the more severe cases, patients may be hospitalized. Following hospitalization, treatment can again vary widely depending on severity of symptoms. Some treatments, such as administration of fluids with normal saline and glucose are standard for any patient hospitalized. Flow rate, however, varies to compensate for fluid loss, so patients experiencing hemorrhage will be given more fluids than those not hemorrhaging. Additionally, if hemorrhage is severe, blood transfusions will be substituted for some of the fluid volume being given. Indeed, if a patient tests positive to the tourniquet test, the hospital blood bank is notified to put blood on reserve for that patient (156).

If a patient enters shock, the primary concern is keeping blood volume up to acceptable levels. To this end, normal saline and blood transfusions are standard. However, blood hematocrit levels are carefully monitored before and after transfusions to avoid circulatory overload, iron overload, or any of the other complications associated with large volume transfusions (182). Blood volume is carefully monitored until the crisis has passed, at which point intravenous fluids are decreased to maintenance levels (156).

Thus, standard hospital procedures for treating Dengue are largely palliative. Although there have been a multitude of compounds shown to have antiviral effects against Dengue in vitro, or in animal models, none have yet been approved for human use (183, 184). Targets for antiviral therapies range from suppressing transcription of the viral genome to directly inhibiting activity of viral non-structural proteins to attempting to circumvent some of the
virus’s immune modulatory properties to simply trying to decrease severity of symptoms (183, 185, 186).

1. Antivirals Targeting Viral Replication

As flavivirus genomes are (+)-sense single stranded pieces of RNA, that effectively function as mRNA, several labs have attempted to use interfering RNA (RNAi) to target the viral genome for degradation (187–190). RNAi-mediated degradation of mRNA transcript is a common method of gene regulation within the cell itself (191) and has been exploited to create knockdown models of many genes (192, 193). When applied as an antiviral therapeutic, a short piece of RNA is generated that is complementary to some portion of the viral genome. That piece of RNA is generally delivered to the cell by viral carrier. Once inside the cell, the RNA will anneal to the viral genome, creating a region of dsRNA. The presence of dsRNA will initiate Dicer mediated degradation and generation of RNA “guides” which the RISC complex will use to degrade other viral genomes (187, 194, 195).

Other labs have attempted to target the transcription process itself. Diamond, et. al. was able to inhibit DV replication using mycophenolic acid, which depletes intracellular levels of guanosine (328). The synthetic guanosine nucleoside Ribavirin was tested in both mice and non-human primates and found to be ineffective against DV (185, 196). Another nucleoside analog, RG1626 or Balapiravir, which is effective against Hepatitis C, was tested and also unable to inhibit DV replication (197). An additional concern with nucleoside analogs is toxicity. Indeed, a clinical trial, testing the effectiveness of Balapiravir against HCV, was abandoned after
it was shown to have toxic side effects when taken for more than 2 months, in conjunction with other anti-viral therapies (197–199).

Other labs have targeted the viral RNA-dependent RNA polymerase (RdRp), NS5 (200–203). The allosteric RdRp inhibitor N-sulfonylanthanilic acid has been shown to be effective at decreasing levels of Dengue RNA in infected cells, in a recent in vitro study (203). Another lab was able to identify 7 compounds that inhibited viral replication by hindering the methyltransferase activity of NS5 (202), preventing capping of the viral RNA (204). In another in vitro study, Qing, et al. showed that WNV, DV, and Yellow Fever genome replication could be inhibited by using cyclosporin to decrease cyclophilin-mediated folding of NS5 (201). Perhaps the most surprising was the discovery of the antiviral effects of the antiparasitic medication Ivermectin (200, 205). Ivermectin has been approved for human use for treating nematode infections (206–208). Ivermectin inhibits nuclear localization of proteins not labeled by a nuclear localization tag (NLT), and has been found to inhibit replication of multiple viruses (201). Indeed, Ivermectin is being considered for testing as a broad-spectrum antiviral (205). For flaviviruses, Ivermectin inhibits replication by decreasing NS5 translocation into the nucleus (209). While it is known that NS5 accumulates in the nucleus during infection (210), the exact mechanism of how Ivermectin affects replication, which occurs in the cytoplasm (10).

2. Antiviral Drugs That Circumvent Viral Inhibition of Innate Immunity

Flaviviruses, like all successful pathogens, have ways of avoiding immune-mediated killing. The flavivirus NS1 protein in particular is able to suppress signaling of the PAMP’s TLR3
and RIG-I (95, 126, 127), limiting production of Type I interferon (75), which has been shown to be effective in preventing infection of new cells (54). Goulet, et al. were able to show that activation of RIG-I with virus derived 5’pppRNA was able to inhibit DV growth in A549 cells (211). Simultaneously, Zhang, et al. were able to show that treatment with the artificial TLR3 and RIG-I agonist PIKA, which has been shown to be better tolerated than other TLR3 agonists such as Poly I:C (212, 213), was able to inhibit growth of DV in C6/36 insect cells (214). PIKA stimulates TLR3 and RIG-I dependent Type I interferon production, and thus is being studied as a broad-range antiviral (215–218). TLR3 and RIG-I dependent Type I interferon will activate INFα/B receptor (IFNAR), which initiates both JAK/STAT and IRF9 mediated transcription of interferon stimulated genes (ISG’s) (219, 220). However, these methods focus on hyperstimulating TLR3 and RIG-I in order to overcome viral inhibition of these pathways. Therefore, several labs have experimented with bypassing inhibition of TLR3 and RIG-I by giving pegylated-recombinant IFN (185, 221). The administration of pegylated-IFNα is not a new concept. Indeed, it, in conjunction with ribavirin, is an accepted treatment for human HCV infection (221). Administration of recombinant IFNα and IFNβ has been shown to inhibit DV growth in human hepatoma cells (222), and peg-IFNα inhibits DV in the in vivo rhesus monkey model (223).

Another proposed method of treatment that bears mention is TNFα antagonists. This method of treatment targets the immune response to DV, but with the intention of inhibiting some of its deleterious effects, rather than expanding it for the purpose of viral rejection. The overstimulation of the immune system and resulting cytokine storm is widely considered to be responsible for patient symptoms moving from the relatively mild dengue fever (DF) to the
considerably more dangerous dengue hemorrhagic fever (DHF) (224). In a 2012 clinical trial, Salgado, et al. were able to decrease levels of circulating TNFα in children with DHF who were given the drug pentoxifylline. However, as there was no clinical change associated with this decrease, they admit more trials are needed (225). Treatment with pentoxifylline has decreased mortality in studies with septic neonates (225, 226). As shock is also caused by systemic overproduction of pro-inflammatory cytokines, including TNFα (227), a larger study of pentoxifylline in treatment of DHF may yield more conclusive results.

3. Vaccination

Perhaps the most effective method of treating any disease is prevention. To this end, much of the focus of flaviviral research, particularly that relating to DV has been focused on creating a safe and effective human vaccine. Antibodies generated against one Dengue serotype can crossreact with but not inactivate other serotypes. Fc-mediated endocytosis of these antibody-tagged but not inactivated viruses results in increased infection of the primary Dengue host cell, the dendritic cell. This antibody dependent enhancement (ADE) is the prevailing theory as to why secondary infections with different Dengue serotypes is more likely to result in Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (150–153). Simply tailoring which of the four strains a patient receives based place of residence is not a viable solution either, as it is common for two or more strains to circulate in the same region at any given time and this method does not account for movement of the individual to other
regions where Dengue is endemic (228). Thus, any effective vaccine for DV must target all four serotypes simultaneously.

It is commonly recognized that live attenuated vaccines confer more potent and longer lasting immunity than killed or subunit vaccines. Because of the risk of ADE if antibody levels were to fall too low, DV vaccine research has primarily focused on generating a live attenuated vaccine incorporating PrM and E surface proteins from all four serotypes (228). The two major approaches to generation of a live-attenuated tetravalent Dengue vaccine have been to inoculate with a mixture of all four serotypes that have been passage attenuated to different degrees, depending on serotype and cell line, and to create a chimeric virus, in which the PrM and E surface proteins of all four Dengue serotypes are genetically engineered onto the backbone of another flavivirus, which is then injected (228).

Halstead et al. demonstrated in 2003 that different passage numbers are required to render each of the four serotypes attenuated (229). In this study, they measured attenuation by decreased plaque size, increased temperature sensitivity, and loss of cytopathic effect (CPE) in the simian kidney cell line LLC-MK2. In order to reach equivalent attenuation, DV1 was passaged 15 times in primary dog kidney cells (PDK), DV2 and most DV4 strains were both passaged around 50 times in PDK cells, and DV3, which would not grow in PDK cells, was passaged 29 times in African green monkey kidney cells (GMK). However, the issue is further complicated by strain, as DV4 strains 1036 and H-241 required 50 passages to be fully attenuated, whereas DV4 strain 341750 Carib only required 30 passages. Additionally, fully attenuated strains may no longer be sufficiently immunogenic to confer protection, as PDK50 1036 was not able to protect mice from challenge with homologous challenge with PDK30 1036.
Even fully attenuated strains have had some safety concerns when used in vaccine trials, as evidenced by the PDK48 341750 Carib strain of DV4 used in both a monovalent vaccine trial and a tetravalent one (230, 231). In both cases, PDK48 341750 Carib immunization resulted in seroconversion and protective titers but induced viremia in over half of the subjects. Additionally, in the study by Bhamarapravati et al., subject titers were measured 1 year after vaccination and found to be less than 1:10 (231).

The problems of viremia, and related side effects, in subjects continue to persist in other clinical trials (232–235). However, progress has been made on percentages of seroconversion (232, 235), with the tetravalent vaccine tested by Saez-Llorens et al. stimulating very similar levels of seroconversion after only one dose as after two or three (235). However, seroconversion is not consistent across all four serotypes. Indeed, while the vaccine tested by Saez-Llorenz et al. was able to stimulate 96-98% seroconversion against up to three Dengue strains with a single dose (Group 2), the percentage of seroconversion against all four Dengue serotypes consistently lagged behind, even when multiple doses were given (235).

An alternative approach has been to express the PrM and E proteins of Dengue virus on the backbone of the attenuated Yellow Fever vaccine strain, YF 17D (236). This chimeric vaccine strain is called CYD-TDV (brand name Dengvaxia®) and has recently been approved for human use in 20 different countries (245). CYD-TDV was generated by substituting Dengue PrM and E for Yellow Fever PrM and E (236), creating a chimeric viral particle that would, theoretically, be safe for human use, just as YF 17D has been, but present the immune system with antigens unique to DV. CYD-TDV immunogenicity has been extensively tested, both in vitro (236, 237) and in in vivo models (238, 239), and has been shown to confer protection with minimal
adverse reactions. Additionally, it has been subjected to a variety of clinical trials and has been shown to confer protection through both antibody- and cell-mediated immunity (240–243).

However, it a recent vaccination campaign in Paraguay, naïve subjects vaccinated with Dengvaxia© were more likely to be hospitalized with severe dengue than those unvaccinated (244, 246). Immunity to dengue, stimulated by the vaccine was not complete, leaving subjects vulnerable to complications from secondary Dengue. Dengvaxia© is therefore only recommended for individuals living in endemic areas who have already been exposed to one Dengue serotype (245), as they are already at risk for the complications associated with secondary Dengue exposure, such as DHF and DSS (154, 246).

There is also no WNV vaccine approved for human use (247). This is not for lack of candidates. There are several vaccines approved for use in horses (248), and many different strategies for developing a vaccine for humans (145–147). Among these are chimeric vaccines, like those mentioned above for DV. Recombiteck® is a chimeric vaccine in which the region of the WNV genome coding for PrM and E, derived a European neuroinvasive strain, are spliced onto a canarypox backbone (249). ChimeriVax® is a chimeric vaccine in which WNV PrM and E are engineered onto the YF 17D vaccine strain, as with Dengvaxia® (250). Since Domain III of the envelope protein is most immunogenic and has been shown to protect against WNV infection (145–147, 249, 251), it has been the target for several subunit vaccines (249, 251–253) and a vaccine which forms virus like particles (VLP’s) by covalently linking recombinant Domain III of WNV E to VLP’s derived from bacteriophage QB (254).

The primary reason that none of the above-mentioned vaccine candidates, or indeed any of the myriad of others not mentioned here, have yet been approved for human use is lack
of commercial funding to finance the long-term Phase II and III clinical trials (146). While WNV is considered endemic in the US (255, 256), yearly hospitalizations and deaths associated with the disease have remained fairly low, compared to other yearly diseases (146, 147). Additionally, WNV infection is difficult to predict (147). Unlike with DV, there is not a particular population that is consistently at high risk for WNV infection, rather a generalized area that is consistently at low risk. Even when a member of said population contracts WNV, they may not become symptomatic (146). Therefore, targeting populations for clinical trials, or indeed for vaccination should a vaccine be approved for humans, becomes somewhat difficult. The prospect of vaccinating the entire population of the US against WNV has not been considered economically feasible (257). There has been interest in tailoring vaccine trials either to identified “hot spots” where the population is more likely to encounter WNV (258), or to target the aged among the population (259), as they are much more likely to develop severe symptoms with WNV infection (147).

**Selective Evolution of Ligands by Exponential Enrichment (SELEX)**

Selective evolution of Ligands by Exponential Enrichment (SELEX) is an in vitro method for selecting for short oligonucleotides that bind to a target (260, 261). These short oligonucleotides were coined as “aptamers” by Dr. Andrew Ellington of the University of Texas at Austin (262). The term is a conjunction of the Latin word *aptus*, meaning “to fit”, and the Greek word *meros*, meaning “part”. Aptamers are typically single stranded RNA or DNA oligonucleotides around 100 nucleotides in length (260). However, that number is increasing as
companies become more adept at synthesizing longer products (263, 264). They form a series of secondary structures that vary based on the aptamer length and sequence, but can include stem-loop, bugle, pseudoknot, G-quadruplex, and kissing domains (262, 265). These secondary structures combine into a tertiary structure, which enables the aptamer to bind to its target.

Actual selection of the aptamers can vary widely between protocols (260, 261, 266–271), as individual labs adapt basic SELEX to fit their individual purposes. Aptamer selection begins with synthesis of a random DNA or RNA library of desired length. Each oligonucleotide within the library must be flanked by known sequences (266) for later PCR amplification. So, the known segments need to be long enough to accommodate a primer. However, the longer they are, the less space within the rest of the aptamer can be variable, thereby decreasing diversity. The target to which aptamers will be raised is then usually immobilized on a membrane, nitrocellulose is common (266), or on beads either by antibody- or tag-mediated binding (261, 269). However, if the selection target is on the surface of a cell, binding is generally done without immobilization and bound aptamers are harvested along with the cells by centrifugation (260, 272, 273).

Once the target has been immobilized, the random library is added to it (266). The target is then washed to remove any unbound aptamers. Bound aptamers are then released from the target, usually be proteinase-mediated degradation of the target (262, 262, 266). However, methodology may vary depending on the nature of the target. Once target-specific aptamers have been released, they are purified and amplified by a low cycle PCR, to avoid PCR bias (266, 274). An error-prone Taq polymerase should be used, to introduce mutations which might improve binding. Should the aptamers be RNA oligonucleotides, reverse transcription
must be done on the recovered aptamers before PCR amplification (262). Amplified aptamers are then denatured back to single stranded form. If aptamers are RNA, this is generally done by cloning PCR products into a plasmid and transcribing them using an in vitro RNA transcription system (262). DNA aptamers can be denatured by immobilizing the complementary strand of the double stranded PCR product and adding a strong base (260, 271), such as NaOH at 1 mol/L (275). The complementary strand remains immobilized and the aptamer strand is free to be harvested from the supernatants. A full SELEX procedure generally requires 10-12 selections (266). Once multiple rounds of SELEX have enriched the aptamers that bind to the target, the sequences are cloned into a plasmid and sequenced (260, 262, 266).

After the first round of SELEX, it is recommended to perform negative- or counter-SELEX rounds before subsequent positive selections (260, 262, 266). This removes aptamers that bind to irrelevant targets. Generally, this is done by immobilizing the irrelevant protein on beads or a membrane and allowing the library of aptamers to bind, just as one would do with positive selections (260, 266, 269). However, in counter-SELEX, any aptamers bound to the irrelevant protein are discarded, and only the unbound aptamers continue to the next round of positive selection. Counter-SELEX against the surface of irrelevant cell types is performed using centrifugation, just like in positive selection, but with discard of binding aptamers (262, 272, 273). However, these techniques do not work for every type of negative selection. There is, for example, currently no standard protocol for performing counter-SELEX on a medium rich in soluble protein, such as serum, plasma, or cell media. This leaves each lab to construct and test their own methods, which may be modifications of one of the standard protocols (271), or to simply avoid running extensive counter-SELEX (270, 276, 277).
Aptamers vs Antibodies

Aptamers have been called “chemical antibodies” (262). And, in many ways, one may think of their selection process as an artificial system that seeks to mimic the natural process of antibody selection, except applied to nucleic acid rather than to protein. Both aptamers and antibodies go through a form of negative and positive selection. Antibody binding sites are identical to the binding sites of the B-cell receptor (BCR) of the B cell that makes them (278). The B cell receptor goes through both a negative and a positive selection before the B cell leaves the bone marrow.

The body generates a semi-random library of B and T cells during V(d)J recombination. The germline DNA that codes for the BCR is made up of a series of cassettes that can be spliced together to form the final sequence which will code for the BCR. However, there are multiple cassettes for each locus of the BCR. This increases diversity among the B cell population, as B cell 1 is not likely to have used the same cassettes in all loci that were recombined as B cell 2. However, there is only so much diversity that can result from variation in splicing patterns. Further diversity results from the fact that the splicing complex purposefully introduces random mutations into the joint between loci. Between the variation in patterns of cassettes “chosen” to splice together and the random nucleotides introduced at the joint where the chosen cassettes for each locus are spliced together, each BCR should be, if not unique, uncommon (278, 279).

This diversity through mutation comes at the cost of some of the recombined BCR’s being non-functional. During the Pre-B cell stage of development, the developing B cell will test
whether it has created a functional heavy chain during recombination by attempting to express it with a surrogate light chain. If the heavy chain is successful, the cell will move on to light chain recombination and testing. If the B cell fails to produce both a functional heavy and light chain, it will die. This process is called B cell “positive selection” (278).

Once the B cell has a fully functional BCR, it goes through negative selection, to ensure that it does not bind any self antigens. This process directly mirrors the negative selection process of aptamer selection. When performing counter-SELEX, any bound aptamers are removed from the pool and discarded (262, 266). Any immature B cell that recognizes self antigens is killed or inactivated, effectively removing them from the “pool” of viable B cells.

Additionally, we can loosely compare B cell stimulation to aptamer positive selection. Activation of a B cell, upon binding its antigen, results in clonal expansion, generating more of the same type of B cell. This can be compared with the PCR amplification step performed on target-binding aptamers during positive SELEX. Beyond that, a subset of activated B cells will not produce antibodies but undergo further somatic hypermutation to enrich binding to the antigen. The best binders will then become memory B cells. One can loosely compare this to using error-prone Taq polymerase during SELEX PCR to introduce random mutations which might improve binding.

For all their similarities, however there are some key differences between antibodies and aptamers. Antibodies are constructed of amino acids, therefore have greater innate variability than aptamers, which are made of nucleic acids (280). There are 20 amino acids and only 4 nucleotide bases. This results in a greater degree of diversity in the antibody binding
region—each position has the potential to be one of 20 amino acids rather than one of four nucleotides. There is also a greater variability between characteristics of amino acids, leading to a greater variability of bonds that can be formed between the binding pocket and the target (281, 282). For example, both antibodies and aptamers are capable of forming charge-charge interactions and hydrogen bonds, but only antibody binding pockets can form hydrophobic-hydrophobic interactions with the target. Lack of diversity within the aptamer, and the resulting difficulty in discriminating between targets, has been perhaps the most prevalent argument against aptamers (280, 283, 284). However, improvements to oligonucleotide synthesis techniques have made production of longer aptamers possible, and the substitution of synthetic bases for some of the standard ones in the sequence can help to increase diversity (280, 285, 286). As an added benefit, modifying aptamer bases has the added benefit of making them less sensitive to nucleases.

An advantage to aptamers is that their 3D structure is due solely to their sequences, therefore, as long as factors such as heat or salinity are not actively preventing native formation, folding is spontaneous (280, 287). Traditional antibodies, however, have to be folded inside a cell (279). Additionally, purified antibodies have a tendency to degrade or clump over time, even if stored carefully (288, 289). Once native formation has been lost, it is impossible to recover, resulting in loss of the antibody. Aptamers have no such problem, and their protein binding forms are easily recovered simply by removing them from the denaturing environment into their native folding environment (287). Additionally, aptamers can be raised against a wide range of targets, including small molecules: traditionally difficult antibody targets (290, 291).
Aptamers also have an advantage over antibodies in use in biological systems. As small pieces of nucleic acid, aptamers tend not to be very immunogenic (280, 285, 286). This gives them a clear advantage when being considered for biological therapies, as anti-antibody reactions are a concern in antibody-based therapies (292, 293). Additionally, as aptamers do not require immunization, and subsequent sacrifice, of an animal, they have an ethical advantage over antibodies. In this same consideration, aptamers do not require animals to be kept and housed, nor do they require cell culture facilities, so tend to be cheaper to generate than antibodies (290, 294).

Aptamer Utilization

1. Use of Aptamers in Laboratory Tests

There is a great variety of laboratory tests for which aptamers have been developed. There are the standard tests, such as the Western blot (295), in which aptamers have been substituted for antibodies, ELASA’s, which are standard ELISA tests with aptamers replacing at least one of the antibodies (287, 296, 297), and rapid diagnostic tests (RDT) (298, 299). However, the unique nature of aptamers has made way for some exciting new techniques.

One way in which aptamers have been uniquely applied is in affinity chromatography. In aptamer-based affinity chromatography, aptamers are immobilized on a rigid surface, which can range from magnetic beads to a gold sheet to a silicon nanotube (287, 300). Solution containing the target of interest in passed over the aptamer, in the same way it would be in antibody-based purification. To elute the target protein, aptamer purification methods do not
need to rely on introducing other chemicals to competitively bind the antibody, releasing the protein. One may simply raise the NaCl concentration, disrupting enough of the aptamer’s secondary structure for the target to dissociate. Once the target is collected, salinity can be reduced and aptamers can regain their native formation (Figure 5A). However, this methodology may be unfit to use on targets sensitive to NaCl concentrations. Thus, several labs have demonstrated aptamer-based chromatography where the target can be released by exposing the aptamers to near-UV light (301, 302). This causes a slight loss in aptamer structure, releasing the target for collection. Additionally, once the aptamers are removed from the light, their native structure returns, allowing them to be reused (Figure 5B).

**Figure 5: Aptamer Based Affinity Chromatography.** Figure adapted from Lonne, et al. (300) (A) and Smuc, et al. (287) (B). Aptamers can be covalently linked to magnetic beads (A) or a rigid surface, such as a gold nanoparticle matrix (B), and used to purify protein (A). Once protein is bound, it may be eluted by increasing NaCl concentration until the secondary structure of the aptamer is disrupted and target released. Aptamers may then be reconstituted by washing and placing them into a buffer with a lower salt concentration (A). Alternatively, some aptamers alter their shape slightly when exposed to near-UV light. In this context, one only need remove the light to reconstitute the aptamers (B).

Methods involving fluorescent tagging make further use of the nature of aptamers as oligonucleotides. Aptamers, being made of nucleic acid, are not naturally fluorescent. The only way to use them in fluorescent imaging is to attach a natural fluorophore. In this way, they are
the same as antibodies. However, aptamers with a lot of tertiary structure will sometimes undergo conformational change when they bind to their target (303, 304). Placing a fluorophore in such a location is the basis of structure-switching aptamer-based signaling. In this technique, two oligonucleotides are generated: the aptamer itself, which is tagged with a fluorophore that is placed in a location that will move when the aptamer binds its target and takes on its alternate structure. The second oligonucleotide, known as the quencher DNA (QDNA) tagged with a fluorescence quencher and designed to anneal to a region of the aptamer such that the quencher is placed in close proximity to the fluorophore only when the aptamer is in the unbound position. Thus, when the aptamer binds its target, the quencher is removed from the fluorophore and fluorescence occurs. This method of fluorescent labeling is a one-step process and has been shown to be more sensitive and faster than most antibody-based methods (291, 303, 305, 306).

A further method for exploiting aptamer characteristics to enhance sensitivity is the combination of DNA rolling circle amplification (RCA) with DNAzyme. In this technique, a DNA aptamer will be immobilized on a rigid surface such as a magnetic bead and exposed to its target. The aptamer will bind the target, and irrelevant molecules are washed away. Then a second DNA aptamer, connected to the RCA primer and circular DNA template, containing the DNAzyme sequence, will bind to the target in a sandwich. This complex will then be allowed to undergo isothermic replication, enhancing the signal (307). The DNAzyme, created by the rolling replication, has peroxidase-like activities, so it is able to mediate reduce TMB, creating a colorimetric change. This method was developed for detection of low-abundance proteins.
(308) and has been used by Pinto, et al. to increase detection capabilities of thrombin 20,000-fold (309).

2. Aptamers in Screening and Drug Delivery

As mentioned above, aptamers can be raised against cell surface markers (260, 262, 266). An application of this is raising aptamers against different types of cancer cells, using non-cancerous cells of the same type for counter-SELEX. Aptamers can be used to for screening (294, 298, 310) or to mediate drug delivery to these cells (298, 311, 312). A study performed in 2015 by Xiang, et al. demonstrated that aptamers penetrated tumors more effectively than antibodies and were better at both imaging and drug delivery than antibodies (311). Aptamers have been raised against cancers of many different types, including breast, colorectal, prostate, lung, and liver (298, 312). Generally, the aptamers bind to a cancer-specific cell surface marker on their target cell and are internalized via clatherin-coated endocytosis (313). Therapeutics or toxins attached to these aptamers are then endocytosed into the target cell with the aptamer (314–316).

Aptamers can similarly be raised against virally infected cells, purified viral particles, or purified viral proteins (260, 262, 266, 317, 318). I will focus primarily on those pertaining to flaviviral detection and treatment in this review. Perhaps the most comprehensive screen of anti-flaviviral aptamers was published by Bruno, et al. in 2012. In this paper, they generate a series of ssDNA aptamers against envelope protein from WNV and all four Dengue serotypes (319). Functionality of binding was tested by ELASA.
In 2015, Chen, et al. demonstrated that an anti-envelope aptamer, generated by their lab, was able to inhibit growth of all four Dengue serotypes (276). E was chosen as their target because it has been shown to be the primary target for neutralizing antibodies and anti-E antibodies have been shown to be protective (243). It has been suggested that aptamers specific for other structural proteins such as PrM or C might also have therapeutic uses (320), however, thus far E has been the only structural target.

There are two labs that have targeted flavivirus non-structural proteins however. Falk, et al. proposed a novel displacement-based aptamer screening method that would target the methyltransferase activity of non-structural protein 5 (NS5) (321). This methodology should apply to any flavivirus, as it relies on aptamer-based displacement of NS5 before it can methylate the guanosine cap on the RNA genome. Flaviviral non-structural protein 1 (NS1) has been linked not only to viral replication but also to multiple immune-modulatory functions (29, 54, 95, 110, 121). In 2017, Lee, et al. published an aptamer specific for Zika NS1, that can be used in an ELASA (277).

However, aptamers can also be generated to bind to viral genomes. Fletcher, et al. generated a novel system of aptamer-based fluorescence screening for Dengue. A DNA “linker” would anneal to a conserved sequence in the Dengue viral genome. The region was conserved across all four serotypes, so this technique can be used for pan-Dengue screening. The linker oligonucleotide is added first in the screen. A DNA aptamer which inhibits activity of the restriction enzyme EcoRI in its unbound conformation (Apt_EcoRI) is added next. If the linker has detected and annealed to viral RNA, Apt_EcoRI will anneal to a complementary sequence in the linker. This causes a conformational change such that the aptamer no longer inhibits EcoRI
activity. Finally, a “signaling” oligonucleotide is added. This oligonucleotide is a single stem-loop with a fluorophore on one end and a quencher on the other. The native conformation brings the fluorophore and quencher together so there is no fluorescence. The loop region of this oligonucleotide contains an EcoRI restriction site. So, if Apt_EcoRI is bound to the linker, and so unable to inhibit EcoRI activity, EcoRI cuts the loop region of the signaling oligonucleotide. This cleavage releases both the fluorophore and the quencher, causing fluorescence in the cell (322).

3. Aptamer-Based Therapies

While some labs are focusing on trying to improve aptamer resistance to nucleases (261, 280), one has incorporated that sensitivity into their treatment and turned it into an advantage. For example, Vavelle, et al. have developed an aptamer-based anticoagulant (323). The therapy, called the REG1 anticoagulation system, consists of a 31 nucleotide RNA aptamer (Pegnivacogin) that binds to Factor IXa of the blood clotting cascade, blocking its binding to Factor VIIIa. There is, therefore no Factor IXa-VIIIa dimer to activate Factor X, so the blood clotting cascade is inhibited (Figure 6A). The aptamer is modified to reduce nuclease activity and is coupled to a 40 kDa PEG carrier.
Figure 6: REG1 Aptamer Based Anticoagulation System. Figure adapted from Vavelle, et al. (323). Aptamer Pegnivacogin sterically blocks binding of clotting Factor IXa to Factor VIIIa (A), preventing blood clotting. Small RNA oligonucleotide Anivamersen is complementary to a portion of Pegnivacogin. Once annealed, it will cause a conformational change in Pegnivacogin, permanently disrupting binding to Factor IXa (A bottom and B).

One of the benefits of this therapy is its short half-life. Even with the modifications, it is vulnerable to RNAsse activity. Additionally, a 15 nucleotide RNA “antidote” (Anivamersen) was also developed which will disrupt binding of the aptamer to Factor IXa (Figure 6A bottom and B). So, if the aptamer has not been completely degraded before a medical procedure, such as open heart surgery, its effects can be neutralized within 5 minutes of application. Anivamersen was also tested and found to have no side effects in patients. The REG1 anticoagulation system has been tested in both Phase I and Phase II trials (324, 325) in patients about to receive open heart surgery. Thus far, no significant side effects have been observed.

Overview of NS1 and Aptamer Selection

Non-structural protein 1 (NS1) is a glycoprotein produced during the course of flaviviral infection. NS1 has been shown to accumulate in the cell endoplasmic reticulum (ER), where it
associates with NS4B. It helps both to anchor the flavivirus replication complex to the cytosolic side of the ER membrane and to assist NS4B in manipulating the membrane to form vesicle packets, or small invaginations within the ER membrane, where the viral replication complex generates new viral genomes (23, 31). It has also been shown to be secreted at high levels by infected cells (102) and is able to be endocytosed by and have an effect on other, uninfected cells (95, 128).

NS1 is essential for flavivirus replication (29, 31, 96), but that is not its only function. NS1 is able to inhibit immune activity directly, such as by directly inhibiting the formation of the complement membrane attack complex (119, 122, 123), and indirectly, such as by inhibiting TLR3-mediated cytokine production (95, 125). Other research in our lab implies that Dengue NS1 might also have a role in direct spread of infection, as pre-treatment with NS1 enhanced infection in monocyte-derived dendritic cells (128).

Our lab has primarily focused on the capabilities of NS1 to inhibit innate immune signaling, with particular emphasis on TLR3 cytokine signaling. To this end, a previous researcher generated a mutant West Nile virus whose NS1 was unable to inhibit TLR3 signaling but was still able to support viral replication (125). The mutation was a single nucleotide substitution in the NS1 region of the viral genome, which resulted in a change from native proline to serine at the 320 position. The 320 position of NS1 is highly conserved (326), so there was great selective pressure to revert to wild type. In Chapter 2 of this dissertation, we investigated the effects that the P320S mutation on viral fitness, when it is forcibly maintained by a full codon change in the genome.
Screening of blood for NS1 is one of the most widely used methods of screening for Dengue infection (162, 169, 175). In light of our lab’s recent findings that NS1 enhances dendritic cell infection and cytokine production, it is interesting to note that higher levels of NS1 in human Dengue infections have been directly correlated with more serious disease (177–179). Indeed, should a reliable and sufficiently quantitative method of screening for NS1 in Dengue infection be developed, it may assist physicians in early prediction of disease progression.

Current accepted methods for NS1-based Dengue screening include rapid diagnostic tests (RDT’s), which are highly portable and easy to use but are prone to false negatives and false positives (169, 175), and ELISA tests. ELISA-based NS1 screening is more reliable than RDT’s and does not require much highly specialized equipment or training, making it the best current method for Dengue screening in low resource settings (175). However, ELISA tests are antibody based.

Aptamers are single-stranded RNA or DNA oligonucleotides that form a secondary structure that allows them to bind to a target. As they are generated and synthesized entirely in vitro, their production does not require animal or cell culture facilities typical of antibody production. Thus, they can be made more cheaply than antibodies and without the added ethical concerns of animal use (290, 294). Additionally, aptamers can survive denaturing conditions, like high heat, and still be used, as they will spontaneously re-fold (287). Antibodies, on the other hand need refrigeration and are still prone to clumping, even under ideal conditions (288, 289). Additionally, denaturation will permanently destroy antibody function (279).
In Chapter 3 of this dissertation, we generate a single-stranded DNA aptamer that could be used in an ELASA to detect NS1 from Dengue serotypes 2-4. More testing as to the aptamer’s capabilities and limitations is still needed. However, as we know of no other published aptamer capable of detecting Dengue NS1, we have contributed to the repertoire of published aptamers in the literature. Additionally, if our aptamer is determined to have sufficient binding capabilities, we may have contributed to medical science as well.
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Chapter 2

Introduction of a Stable P320S Point Mutation into West Nile Virus Non-structural Protein 1 and Its Effect on Fitness

Abstract:

Our lab has previously conducted studies aimed at identifying NS1 mutations that would eliminate its ability to interfere with TLR3 signaling, while still maintaining its ability to support the West Nile Virus (WNV) replication complex. A single nucleotide mutation change was identified that changed proline at the 320 position of WNV non-structural protein 1 (NS1). However, the mutation was not stable, and the virus quickly reverted to wild-type. In this study, we introduce a full codon mutation from proline to serine at NS1 320 into a West Nile infectious clone in order to generate a more stable mutated genome and investigate that mutation’s effect on WNV biology. The codon substitution mutation is stable through several passages but has a significant fitness cost to viral growth. We also observed that, during the passaging of the 320 codon mutant, a single nucleotide compensatory mutation resulting in a change from histidine to proline at NS1 293.

Introduction:

West Nile virus is a positive sense, single stranded RNA virus in the genus Flavivirus and the family Flaviviridae. In nature, it cycles between its natural reservoir, birds, and its mosquito vector. While, upwards of 60 species of mosquito in North America have screened positive for WNV, the primary vector of transmission is the Culex mosquito, with Culex pipiens, Cx. restuans,
and *Ct. salinarius* being the most prevalent (1, 2). Humans are incidental hosts for WNV, able to become infected, but unable to produce high enough titers in the blood to infect a naïve mosquito (3–5). When infecting humans, WNV can elicit high fever and other flu-like symptoms for up to two weeks (5, 6) with a small portion of the population exhibiting encephalitis symptoms, including severe headaches, flaccid paralysis, and death (3–5).

Like all RNA viruses WNV has a high mutation rate (7, 8), allowing it to adapt quickly to new environments or hosts. Indeed, flaviviruses such as WNV will produce a variety of quasispecies within an infected cell (7), each with a slightly altered genome. The slight alterations in the quasispecies may then allow for a selective advantage, with the most fit becoming the primary one in the host (8).

During the course of infection, WNV infected cells produce ten structural and non-structural proteins. While the structural proteins form the new viral particles, the non-structural proteins primarily replicate the viral genome. Some non-structural proteins are responsible for replication of the viral genome. An example of this is NS5, which is the viral RNA-dependent RNA polymerase (9, 10). However, many of these non-structural proteins also have secondary immunomodulatory properties. WNV non-structural protein 1 (NS1) is not only essential for viral replication (10), but also able to inhibit complement mediated killing of infected cells (11, 12) and the innate immune signaling receptor Toll-like receptor 3 (TLR3) (13, 14).

TLR3 is a pattern recognition receptor that detects the pathogen associated molecular pattern (PAMP) double stranded RNA (15–17), which must be formed during replication of the
WNV RNA genome. TLR3 activation results in a signaling cascade that activates the transcription factors nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF3), which will stimulate the production of Type I interferon (15, 17–19). NFκB can initiate transcription of other pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) (20), interleukin 1β (IL-1β) (21), and IL-6 (22, 23). Additionally, TNFα can bind to its receptor and initiate further NFκB-mediated IL-6 production (23). Type I interferon has been shown to protect cells from WNV infection (24). Our laboratory has previously shown that NS1 is able to inhibit TLR3-mediated initiation of NFκB-dependent signal transduction, focusing primarily on IL-6 production (13, 14). It follows then that abrogation of NS1’s ability to inhibit TLR3 signaling might have a detrimental effect on overall growth of the virus in systems capable of mounting an antiviral response. In previous studies, our laboratory attempted to separate this inhibitory role from NS1’s role in viral replication using a trans-complementation system. NS1 was cloned into an expression vector and co-expressed with a mutant WNV genome, which had GFP substituted for NS1. In this system, if the NS1 introduced was able to support replication of the viral genome through transcomplementation, the cells would produce green fluorescence. These cells also contained a reported construct containing a red fluorescent protein (dsRed) under the transcriptional control of five copies of an NFκB response element. If the transfected NS1 was able to inhibit TLR3, these cells would produce decreased red fluorescence when stimulated with the TLR3 agonist Poly I:C (25).

In this system, our laboratory screened a series of point mutations in NS1, to test which mutations would permit replication of the viral genome without being able to inhibit TLR3 signaling. Many of the NS1 point mutations generated that were unable to suppress TLR3
mediated cytokine production were also unable to support viral genome replication. Of those that could support replication in the above-mentioned system, many were, when introduced into virus, unable to support growth to wild-type levels in the interferon competent HeLa cell line (25). The one NS1 mutant virus that was found to grow to wild-type levels, P320S, was also exceedingly unstable, reverting back to wild type sequence after only one or two passages (unpublished data). In this study, we generated a mutant WNV with a full codon change from proline to serine at the 320 position of NS1. We believed this would stabilize the mutation, as it is much less likely for the virus to undergo three simultaneous point mutations that would result in a wild-type reversion than for it to undergo a single spontaneous point mutation.

**Materials and Methods:**

**Site Directed Mutagenesis:**

PCR mediated site-directed mutagenesis was performed on WNV infectious cDNA clone #7 (IC#7), previously isolated from a human infection in Texas in 2002 (30). Mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and error prone polymerase Pfu Turbo (Agilent Technologies). Manufacturer’s directions were followed, using the primers MutSense5’-

TGTAGATCTTGCACGTACCAGCTACGTTCATCAAGGAGAAGAC-3’ and MutAntisense5’-

GTCTTCTCTTGGAAACGTAGGCTGGGTAACGTGCAAGATCTACA-3’. Quick Change primer design software was used to design primers. Primers were designed to facilitate a full codon change from CCC (Proline) to AGT (Serine) at the 320 position of NS1.
Reconstitution of Wild-Type and Mutant Infectious WNV clone:

Wild type (IC#7) or mutant (P320Sc) cDNA containing plasmid was linearized by XbaI
single restriction enzyme digestion (New England Biolabs), followed by phenol:chloroform
extraction. Digestion was confirmed by agarose gel analysis. 5’ capped RNA was generated
using MEGAscript T7 High Yield Transcription Kit (Ambion). Manufacturer’s Instructions were
followed with the following modifications to the volumes of dNTP’s used per 20 ul reaction: 2 ul
ATP, 2 ul CTP, 2 ul UTP, 2 ul G(5’)ppp(5’)G RNA Cap Structure Analog, 1 ul GTP. RNA production
was confirmed by RNA agarose gel. Viral RNA was electroporated into BHK cells, grown at 37C
in MEM base media supplemented with 1% HEPES buffer (Cellgro), 1% Fetal Bovine Serum
(Cellgro), and 1% Cellgro 10,000 IU/ml Penicillin/Streptomycin (MEM 1+1+1). Supernatants
were collected every 24 hours for 120 hours. Clarified supernatants constituted passage 0 and
were frozen at -80C until use.

Confirmation of Viral Sequence:

RNA was isolated by phenol:chloroform extraction from 72 hour viral supernatants from
passages 0 through 6. cDNA was made from RNA. NS1 cDNA from all passages was amplified
using the high fidelity AccuTaq LA DNA Polymerase (Sigma), and sequenced (Eton Biosciences).
Whole viral cDNA was sequenced from passage 0 and 4.

Virus Titration:

Viral supernatants were titered in 48 well cell culture plates (Corning), on a Vero cell
monolayer. 100 ul viral dilutions were allowed to attach to cells for 1 hour at 37C. 400 ul
1XMEM 1+1+1/1X Tragacanth Gum (MP Biomedicals) overlay were then added to cells. Cells
were incubated for 48 hours at 37C with 5% CO2. Cell monolayers were then washed 1X with Dibco’s Phosphate Buffered Saline, allowed to air dry, then fixed with 1 ml 1:1 acetone:methanol and kept at -20C until staining. For staining, fixative was removed and cells allowed to air dry. Cells were then blocked for 1 hour at room temperature with 200 ul 1% Normal Horse Serum (Gemini Bioproducts) in PBS (1% NHS in PBS). Infected cells were detected with 100 ul mouse anti-E antibody (D14G2) at 1:1000 dilution in 1% NHS in PBS, followed by 100 ul horseradish peroxidase-conjugated anti-mouse (Thermo Fisher) at 1:2000 dilution. Each antibody was incubated on cells for 1 hour at room temperature with rocking followed by 5-7 washed with PBS. Vector VIP HRP colormetric kit was used to visualize infected cells. Titers were expressed as foci forming units per milliliter (ffu/ml).

**Cell Lines**

Viruses were propagated in Baby Hamster Kidney (BHK) cells and were titered in Vero cells. Growth curves were performed in wild type HeLa, HeLa cells expressing decreased levels of TLR3 (ΔTLR3) due to small-hairpin RNA (shRNA) mediated TLR3 knock down (to be published elsewhere), and HeLa cells expressing the empty interfering RNA vector (FG12).

**WNV Growth Kinetics**

Multi-step growth curves were performed in 48 well tissue culture plates on a cell monolayer. Monolayers were infected with a multiplicity of infection (MOI) of 0.01 for 1 hour at 37C, followed by a PBS wash to remove unbound virus, and a 48 hour incubation in 500 ul 1XMEM 1+1+1 media. Viral supernatants were collected every 24 hours and stored at -80C until titration.
Results:

Full Codon Proline to Serine Mutation Introduced into WNV Genome Containing Plasmid

Previously, our lab developed a mutant WNV with a point mutation in the NS1 gene that altered the wild-type proline at the 320 position, changing it to a serine. This mutant was referred to as P320S. P320S mutant viruses generated directly from electroporated BHK cells, referred to as Passage 0, were shown to be unable to reduce TLR3 dependent cytokine production (25). However, P320S was subsequently shown to be highly unstable, reverting to the wild-type serine as early as one passage after electroporation (unpublished data). To inhibit this reversion to wild-type, a full codon mutation from proline to serine was introduced. Primers were designed to introduce the full codon serine to proline mutation into a WNV infectious clone in the pACNR plasmid backbone. We confirmed introduction of the full codon mutation by sequencing the full NS1 region of the plasmid containing the WNV viral genome (Figure 1). We then generated 5’ capped mutant and wild-type viral RNA from their respective linearized plasmids in vitro. RNA transcription was confirmed by agarose gel and electroporated mutant or wild-type RNA into BHK cells, collecting and clarifying cell supernatants for experimental use. Viral containing supernatants collected from these electroporated cells were referred to as passage 0 viruses.

Full Codon Proline to Serine Mutation Is Stable Up To Six Passages

Viruses were serially passaged by adding virus containing supernatants onto naive BHK cells, grown to 90% confluency. Viral RNA was collected from passages 0, 2, 4, and 6 supernatants via phenol:chloroform extraction. Viral cDNA was made and the region coding for
NS1 amplified by high fidelity PCR. NS1 alone was amplified from passage 0, 2, 4, and 6 viruses, and the proline to serine mutation was confirmed to be present in all passages tested (Figure 2A). To ensure that no compensatory mutations were arising in other regions of the viral polyprotein, the entire viral genome was amplified in overlapping 700 nucleotide sections for passage 0 and 4 viruses. Up to passage 4, we did not observe any additional mutations in any other regions of the infectious clone genome, apart from a single point mutation we observed, beginning at passage 2, in the NS1 region of the viral genome. The spontaneous point mutation appeared to disappear briefly at passage 4 and reappear by passage 6 (Figure 2A), however, viewing of the sequencing chromatogram confirmed that the population was mixed, with the dominate sequence varying between the native sequence and the sequence containing the point mutation (Figure 2B). The point mutation resulted in a change from the wild-type histidine at position 293 of the NS1 protein to a proline at that position (H293P). The H293P mutation was stable up to Passage 6 of the P320Sc mutant.

**P320Sc Mutation in NS1 Results in Loss of Viral Fitness**

To investigate the effect the stable 320 proline to serine mutation had on overall viral fitness in the presence or absence of TLR3 signaling, growth kinetics were determined for both the wild-type and mutant West Nile Viruses on HeLa cells competent for TLR3 (FG12) (Figure 3A) or with shRNA mediated TLR3 knockdown (ΔTLR3) (Figure 3B). To ensure uniformity of virus genomes in our mutant sample, we first generated a double mutant virus, such that the NS1 gene contained both the H293P point mutation and the P320S full codon mutation (H293P/P320S). The H293P and P320S mutations in this double mutant virus were stable for up to 4 passages (data not shown). Growth curves were determined after infection with wild-type
or mutant viruses at an MOI of 0.01. The H293P/P320S mutant showed a marked decrease in virus production at all timepoints when compared to IC#7. The decreased growth kinetics of the H293P/P320S were overall not alleviated by lack of TLR3. However, there may be partial rescue during late viral growth of the mutant in cells with decreased TLR3 functionality. Although, this may be simply due to the presence of more healthy host cells in the wells infected with mutant virus versus wild-type virus at the late timepoints.

Discussion

It was apparent to us that the 320 proline to serine mutation had great selective pressure against it. Proline is the major amino acid responsible for turns in the overall 3D structure of proteins (26, 27). Soluble NS1 has a barrel shaped structure made up of three homodimers. The pairing of each homodimer is dependent on the large β-ladder domains, which make up a significant portion of each NS1 monomer (28, 29). These β-ladders face each other in a “cross-shaped” formation, allowing the two monomers to dimerize. The 319 and 320 positions of NS1 are both highly conserved and finish a “turn” into the 9th β-strand in the β-ladder (β9A) (28). The change from the “turning” and non-polar proline to the highly polar serine was likely enough to distort the final overall structure of the NS1 protein, allowing for the observed lack of TLR3 inhibition, characteristic of properly folded NS1. However, the 320 position is not part of the dimer interface between the two β-ladders, nor is it at the end of it (28), and therefore unlikely to contribute as much to the dimerization or association between the three dimers into the hexamer. Additionally, 319 is also a proline, so it is likely that enough
of the native structure was retained that NS1 was able to fulfill its function in viral replication, albeit with some cost in efficiency. It is likely that, if a point mutation that substituted the 319 proline for another amino acid were simultaneously introduced with the P320S mutation, NS1 functions that had been previously preserved, such as replication, would be completely abrogated. Such a combination of mutations would likely prove lethal to the virus. It is also likely that the decreased fitness of the P320Sc virus is a result of this slight warping of the final geometric structure of the NS1 protein inhibiting but not preventing its function in viral replication. This would place great selective pressure on this mutation, resulting in the rapid reversion or the 293 single nucleotide substitution observed by our lab.

This rapid reversion is likely the reason that the single nucleotide mutation P320S virus was able to grow to near wild type levels. The cells were infected with viruses containing the P320S single nucleotide mutation and were able to generate wild type revertants as a quasispecies during the course of infection. These wild-type quasispecies would easily outcompete their 320 mutant counterparts, resulting in growth kinetics comparable to wild-type virus, due to the majority of viral particles being wild type. However, the NS1 generated during the early stages of infection would be the mutant 320 NS1, unable to inhibit TLR3. It is therefore conceivable that, early in infection, functional TLR3 was able to detect the dsRNA produced during viral replication, leading to the cytokine production we observed. However, fitness costs not relating to TLR3 signaling applied selective pressure to force a rapid reversion to wild-type NS1. Thus, the revertants were able to replicate to the levels we observed. However, once the 320 mutation was introduced as a full codon change, viruses were unable to revert to wild-type. This resulted in TLR3 continuing to be functional. However, shRNA
mediated inhibition of TLR3 did not result in rescue, so we conclude that whatever damage the 320 mutation has on NS1 functions, it’s effect on viral replication is not TLR3 dependant.

We believe the appearance of the histidine to proline point mutation at the NS1 293 position is a compensatory mutation acquired during generation of viral quasispecies. This mutation may have restored some of the barrel-like structure of the NS1 protein and consequently restored part of its function. However, any rescue of function appears to be slight, as H293P/P320S double mutants did not grow significantly better than P320Sc single mutants (data not shown). Additionally, there was no significant difference in the ability of H293P/P320S double mutant viruses to inhibit TLR3 mediated IL-6 production, when compared to P320Sc (unpublished data). In fact, the trend of the data was that pre-treatment with the double mutant H293P/P320S virus resulted with more TLR3 mediated IL-6 production than pre-treatment with P320Sc. It is therefore likely that whatever selective advantage the compensatory H293P mutation affords the P320Sc mutant viruses, it is not TLR3 related.

Proline is well known as a disruptor of α-helix and β-sheet internal structure (31). NS1 319 and 320 prolines are located immediately N-terminal of the 9th β-strand (28) and may be instrumental in orienting that strand within the rest of the β-ladder. We believe that the P320S mutation was not lethal to the virus because the 319 proline was able to contribute to the overall protein geometry enough to allow the β-ladder to be merely bent, rather than entirely disrupted. The H293P point mutation is located in the spaghetti loop region between the 6th and 7th β-strands (28). This mutation may have therefore restored some of the orientation strands of the β-sheet, relative to each other. This slight restoration caused the secondary mutation to be retained but did not entirely restore the native structure of the β-ladder (Figure
Thus, the H293P mutation did not rescue and did not confer enough selective advantage for quasispecies containing both mutations to outcompete those containing only the P320S mutation, resulting in a consistently mixed population.

**Figure 1: Introduction of Full Codon P320S Mutation into WNV Infectious Clone.** Proline to serine full codon mutation was introduced into our West Nile Virus infectious clone using the Agilent Technologies QuikChange Mutagenesis kit. Sense and Antisense mutation primers (black) annealed to the region to be mutated and contained the new nucleotide sequence (red). The new sequence would be incorporated upon PCR amplification with error-prone polymerase Pfu Turbo.
Figure 2: Stability of P320Sc Mutation Over 6 Passages. (A) The mutation from wild type IC#7 (WNV) proline (CCA) to serine (AGC) at NS1 320 was retained over the course of 6 passages (blue arrow). Beginning at the second passage (P320Sc_Passage_2) a point mutation (A to C) was observed that resulted in a change from histidine to proline at NS1 293 (green arrow). It disappeared briefly during passages 4 and 5 but recurred at passage 6. (B) The sequencing chromatogram from the reverse sequence of NS1 from passage 4 P320Sc. The point mutation to CCC (Proline) seemed to have reverted to native CAC (Histidine) at NS1 293, however, the population is mixed with some viral quasispecies having the native NS1 293 sequence and some having the point mutation.
Figure 3: P320Sc Mutation Incurs a Fitness Cost. To ensure that uniformity of sample, the H293P mutation was introduced into the P320Sc infectious clone. The H293P/P320S double mutant's growth kinetics were then compared to that of IC#7 in multi-step growth curves on either TLR3 competent FG12 HeLa cells (A) or the siRNA-TLR3 knockdown cell line ΔTLR3 (B). In both cell lines, H293P/P320S growth kinetics were significantly inhibited when compared to IC#7 (*P<0.05, **P<0.001).
Figure 4: Predicted Disruption of NS1 β-Ladder and Partial Restoration. We hypothesize that the P320Sc mutation introduces a disruption to the wild-type structure of the NS1 β-ladder (A) between β-strands 8 and 9 (B). We hypothesize that the compensatory H293P mutation caused a second disruption between β-strands 6 and 7, which restored some of the structure of the β-ladder (C). However, the NS1 of H293P/P320S double mutants would still retain the displacement of β-strands 7 and 8, which we believe to be the reason why the H293P mutation was maintained in only a portion of the population and why the H293P mutation did not rescue when introduced into the entire population via mutagenesis.
References


Chapter 3

Generation of a Single-stranded DNA Aptamer for Use in ELASA Based Screening of Dengue Serotypes 2-4 in Human Plasma

Abstract

Dengue is a serious human illness, with only a single vaccine, with potentially severe side effects in naïve individuals, approved for human use. Early detection is the best way of improving patient outcome, as most care is palliative. The two tests most effective for detecting Dengue early in infection are reverse-transcriptase polymerase chain reaction (RT-PCR) of the viral genome or detection of soluble non-structural protein 1 (sNS1) in patient blood. ELISA-based detection of sNS1 is considered the optimum balance between test sensitivity and ease of use, especially in low resource environments. However, ELISA’s use antibodies that require refrigeration and are expensive to make. Aptamers, single-stranded short oligonucleotides that form a three-dimensional structure that binds to a protein, do not require refrigeration and are cheaper to generate and produce. In this study, we generate a single-stranded DNA aptamer that is capable of detecting NS1 in viral supernatants of Dengue serotypes 2-4. The aptamer was raised against Dengue 1 NS1, and while it does bind purified Dengue 1 NS1, it does not bind it well enough to detect it in Dengue 1 supernatants. The aptamer is also able to detect NS1 in the West Nile Virus Australian regional variant, Kunjin virus. The aptamer underwent blocking negative selection in the presence of human plasma, so it is also able to detect NS1 in artificially infected human plasma without binding the plasma itself.
Introduction

Dengue virus is a human pathogen reported in 100 countries worldwide, putting about 40% of the world’s population at risk of infection (1). Transmitted primarily by the *Aedes aegypti* mosquito, the number of cases have steadily increased as global temperatures have risen and urbanization has expanded (2–4). Primary Dengue infection results in a prolonged high fever, malaise, and muscle aches severe enough to earn the title “break bone fever” (5, 6). Antibodies generated during primary infection confer long-term protection against re-infection with the same serotype, but not against infection with any of the other three. Indeed, secondary infection with a different serotype can result in far more serious disease or even death due to the antibodies that had previously been raised against the first dengue serotype binding the different dengue serotype without being able to inactivate it. This can result in antibody dependent enhancement (ADE) increasing the rate at which dendritic cells become infected (7, 8). The combination of enhanced infection generating enhanced proinflammatory cytokine production by dendritic cells and the proinflammatory cytokines released by activated B and T cells causes a cytokine storm. Some of the more severe symptoms associated with this cytokine storm include severe system wide inflammation, resulting in hemorrhage, dangerous drop in blood pressure, blood clots in capillaries that can easily detach and move to the lungs or brain, and shock. These conditions are called Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) respectively (7, 9–11).

There is no Dengue vaccine that has been approved for human use in the US. The tetravalent CYD-TDV vaccine was the most promising of the recent candidates (12–14). However, in its 2017 clinical trial, it was shown to stimulate a response heavily skewed toward
DV3 and DV4 (15) and has recently been shown to increase likelihood of severe dengue and hospitalization in naïve individuals after a recent attempt at implementing it in Paraguay (16). Therefore, early detection and prompt palliative care are still the most effective means of increasing the odds of patient survival (17, 18). It is therefore imperative that Dengue infection be detected as rapidly as possible. There are several widely used tests that can detect Dengue infection. While there is overlap between them, each has a window of optimum use during the course of infection. The detection of viral genome in the blood by reverse-transcriptase PCR (RT-PCR) and detection of soluble non-structural protein 1 (sNS1) in patient blood are most effective during early infection: during the viremic phase (18–20). Both can be used immediately upon onset of symptoms, allowing the cause of patient symptoms to be rapidly identified and appropriate measures taken (18, 20). While both methods are considered both effective and sensitive (19, 20), RT-PCR screening requires highly trained personnel and more specialized facilities than ELISA-based NS1 screening. Thus, ELISA-based screening of sNS1 is considered a more versatile test, and better suited for use under field conditions (20).

Dengue non-structural protein 1 (NS1) is produced and secreted in high amounts by infected cells, and it can be detected by ELISA in the blood of infected patients two days before onset of symptoms (19), with an 80% positive correlation between detectable NS1 levels and disease (21). In addition, increased levels of sNS1 have been associated with increased risk of developing more severe illness such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (22, 23). For this reason, NS1 based ELISA tests can be used to screen patients for Dengue infection and give some indication about how severe patient symptoms might be.
Aptamers are short pieces of nucleic acid, that, when folded, form a 3D structure that binds to a macromolecule. That macromolecule is often a protein, either secreted or expressed on the surface of a cell (24). Aptamers can be made from DNA or RNA and are generally single-stranded. Aptamers are largely generated using the four classical bases. However, if degradation by RNAses or DNAses is a consideration, modified nucleotides can be used (25–28). The substitution of a modified base for one of the classical ones can also be used to increase variability of the binding site, if cross-reactivity is a problem (25).

Aptamers have several advantages over antibodies. While classical antibodies need to be generated, folded, and secreted by a hybridoma or be produced in immunized animals, aptamer folding is due to the base pairing of its nucleic acid sequence, and is therefore spontaneous (29). Thus, aptamers can be easily and cheaply produced entirely in vitro (30), requiring one only to know the sequence of the aptamer to be produced. Indeed, this ease of synthesis also negates the need to have cell storage and culture facilities or the consumables required to culture hybridomas and purify the resulting antibodies from the supernatants (30, 31). Thus, aptamers themselves are much cheaper and easier to produce than antibodies. Aptamers can be used in place of antibodies in most laboratory tests, including the ELISA. When aptamers are used instead of antibodies, the ELISA become the ELASA test (enzyme-linked aptamer-sorbant assay).

In this study, we generated two new aptamers, selecting for binding to Dengue 1 NS1 protein. We characterized their limits of detection of purified Dengue 1 NS1. However, we noted that our aptamers bind best to NS1 protein from Dengue serotypes 2, 3, and 4. We chose to focus on a single aptamer, as it was more efficiently synthesized, and compared its
ability to detect NS1 from viral supernatant “infected” human plasma using an ELASA. We also found that our aptamers were also able to recognize NS1 in supernatants of the Australian WNV regional variant, Kunjin virus.

Materials and Methods

Aptamer Selection:

A random library of single-stranded DNA (ssDNA) pieces was ordered from Integrated DNA Technologies (IDT). Each ssDNA piece was 80 nucleotides long, with the terminal 20 nucleotides at both the 5’ and 3’ ends known. Thus, the ssDNA oligonucleotide pool had the structure: 5’- AGTATACGTATTACCTGCAGC-N(40)- CGATATCTCGGAGATCTTGC-3’, as previously described (54).

All positive selections were performed against His-tagged DV1 NS1, produced by stably transfected 293T cells, as previously described (32), immobilized on Ni-agarose beads (Invitrogen). 10^24 ssDNA molecules of the random library were then added to 250 ul of nuclease free water, denatured at 95 C for 30 minutes, then allowed to re-nature at room temperature for 30 minutes. ssDNA molecules were allowed to bind to immobilized NS1 for 1 hour at room temperature. Beads were then washed 3x with 1 ml PBS to remove unbound aptamers. Beads were then incubated for 30 minutes at 55 C with 50 ug/ml Proteinase K with gentle vortexing every 10 minutes to resuspend beads and ensure Proteinase K exposure on all beads, to digest NS1 and release bound aptamers. Proteinase K digestion was followed by a 30 minute 95 C heat to inactivate Proteinase K. Beads were then gently vortexed and pelleted,
with aptamer containing supernatants recovered. Beads were also washed once with 250 ul PBS with wash being recovered to ensure no aptamers remained on beads. Aptamers were then ethanol precipitated and resuspended in 30 ul nuclease free water. Bound aptamers were then PCR amplified using an error prone Taq polymerase (Invitrogen). Primers were complementary to the known constant regions of the aptamers, with the reverse primer having a 5’Biotin tag. The primer sequences are as follows:

SELEXFwd: 5’-AGTATACGTATTACCTGCAGC-3’

SELEXRev-Biotin: 5’-Biotin-GCAAGATCTCCGAGATATCG-3’

PCR amplification products were made single stranded by binding complementary strand to Streptavidin beads (Invitrogen) and then raising pH to release untagged strand as previously described (54). Streptavidin bead supernatants were recovered along with one 250 ul PBS wash, and single stranded amplified aptamers contained therein were ethanol precipitated and resuspended in 50 ul nuclease free water.

Negative selections were performed before all positive selections except the first. For negative controls, the aptamer pool was allowed to bind to irrelevant protein or virus with equal molecules of aptamer to excess irrelevant protein or equal irrelevant viruses. Negative selective binding was performed for 1 hour at room temperature, followed immediately by a positive selection. This “blocking” negative selection ensured not only that aptamers would be able to bind irrelevant targets during the one hour binding, but also that aptamers that did not bind the irrelevant target would be forced to selectively bind DV1 NS1 in the presence of an irrelevant competitor. The only exception to this technique was the negative selection against
unbound Ni beads. In this round, aptamer pool was added to unbound Ni beads in 1X PBS, incubated for 1 hour at room temperature, with gentle vortexing every 15 minutes to ensure proper access to beads. The beads, and all bound aptamers were then centrifuged, and their supernatants alone were used for the next positive selection. Negative selections performed were as follows: 50% human whole blood final concentration before second positive selection, 50% fetal bovine serum (FBS) (Cellgro) final concentration before third positive selection, 50% full cell culture media (10% FBS in Dulbecco’s Modified Essential Medium (MEM)) (Cellgro) before fourth positive selection, $10^8$ foci-forming units Yellow Fever Virus (17D strain) + $10^8$ foci forming units West Nile Virus (IC#7 strain) before fifth positive selection, $10^8$ foci forming units Sindbis Virus + $10^8$ foci forming units Adenovirus (Strain 5) before sixth positive selection, and 50 ul of unbound Ni beads (Invitrogen) before seventh positive selection.

After the seventh negative selection, the aptamer PCR amplification product was ligated into TOPO TA vector (Thermo-Fisher), following manufacturer’s instructions, and cloned into Invitrogen’s Top 10 *Escherichia coli* for cloning, as previously described (Invitrogen). Colonies were screened by whole colony PCR, as previously described (55), and agarose gel of PCR product (Sigma). Plasmids were extracted from positive colonies by Qiagen MiniPrep kit, following manufacturer’s instructions. Whole plasmids were then send to Eton Biosciences for sequencing.

**Aptamer Synthesis**

Aptamers were ordered from Integrated DNA Technologies (IDT). Aptamers were tagged at the 5’ end with Biotin.
Enzyme linked aptamer-sorbant assay (ELASA)

Targets were immobilized on standard ELISA plates overnight at 4 C with rocking. The following day, wells were shaken out and washed 5x with 200 ul ELISA wash buffer (0.5% Tween-20 in PBS), then tapped dry. Well were then blocked with 200 ul ELISA block (10% FBS in PBS) for 1 hour at room temperature with rocking. All FBS was heat inactivated.

During this time, aptamer stocks were diluted to a 2x stock (5 uM) in nuclease free water. Aptamers were then denatured at 95 C for 30 minutes in a heat block and renatured at room temperature for 30 minutes. They were then placed on ice until use.

Following 1 hour blocking of the plate, block is removed by inverting plate and tapping out remaining liquid, and re-natured aptamers were diluted to 1x stock (2.5 uM) using 20% FBS in PBS, so that aptamers are used in a final concentration of 10% FBS in PBS. 100 ul of the 1x aptamer stock is then added to each ELASA well and allowed to bind for 1 hour at room temperature with rocking. Wells are then emptied by tapping, and washed 5x with 200 ul ELISA wash. 100 ul of Streptavidin-HRP (Invitrogen) were then added to each well and incubated for 1 hour at room temperature with rocking. Wells were emptied by tapping and washed 7x with 200 ul ELISA wash buffer. 100 ul of TMB (eBioscience) were then added to each well. Once colormetric change reached detectable levels or 15 minutes after addition of TMB, reaction was stopped by adding 50 ul per well 2 M sulfuric acid (Invitrogen).

NS1

6xHis tagged Dengue 1 NS1 was previously cloned into an expression vector and stably transfected into 293T cells (32). NS1 was purified from cell supernatants by Ni column
purification (Invitrogen), as previously described (32). Harvested NS1 was then dialyzed overnight at 4 C in 1x PBS (Cellgro). NS1 was then concentrated by using polyethylene glycol (PEG) 10000 to attract water away from NS1 through the semipermeable dialysis tubing in which it was previously dialyzed. Concentrated NS1 was then dialyzed once more overnight at 4 C to 1x PBS. Purified NS1 was quantified by Pierce BCA Protein Assay (Thermo Fisher).

**Viral supernatants**

Viral supernatants were produced by infecting a monolayer of Vero cells during growth phase with low passage viral supernatants at an MOI of 0.01 for 1 hour with a low amount of media to promote viral attachment. Media was then added such that there was a standard amount of collection media in the flask. Cell supernatants were then harvested 48 hours later and clarified by centrifugation at 10,000 rpm for 5 minutes to remove cell debris. Viral collection media consisted of Dulbecco’s MEM base (Cellgro) with 1% FBS, 1% antibiotics (Cellgro Pen/Strep 10000 IU/ml), and 1% HEPES buffer (Cellgro).

**Stimulated Cell Supernatants**

293T, HeLa, and Vero cells were grown to 90% confluency in 24 well cell culture plates (Corning), then stimulated overnight with Poly I:C or R848 at a final concentration of 20 ug/ml. Supernatants were then collected and frozen at -20 C until use. Supernatants were diluted 1:1 with PBS before binding to ELISA plate overnight at 4 C. Aptamer binding to stimulated cell supernatants was then measured by direct ELASA.
**NS1 Heat Inactivation**

NS1 was heat inactivated by heating at 95 C in a heat block for 3 hours. NS1 was allowed to cool to room temperature before being bound to the plate.

**Aptamer Binding Time Course**

To test optimal binding time of aptamers to plates, ELASA plates were coated and blocked as usual. Block was then removed from wells immediately before aptamers were to be added. This was to ensure no wells became dry. Thus, the block was removed from the wells that were to have aptamer bind for 1 hour first, followed 15 minutes later by the wells to have aptamer bind for 45 minutes, and so on. This continued until the wells to be bound by aptamer for 0 minutes, in which block was removed and aptamer added, followed immediately by washing of the whole plate with ELISA buffer.

**Aptamer Binding to Decreasing Concentrations of NS1**

Each concentration of NS1 used in the ELASA was constructed using purified 6xHis-tagged DV1 NS1. NS1 was diluted from stock concentration into 10% FBS in PBS, such that the concentration of NS1 was at the concentration listed in the experiment. 100 ul of the diluted NS1 was then bound to an ELISA plate overnight at 4 C, then aptamer binding to the NS1 was tested as described above. So, if aptamer binding to 50 ug/ml NS1 was being tested, purified NS1 was added to 10% FBS in PBS to a final concentration of 50 ug/ml, with 100 ul of this mixture being added to the ELISA plate for testing.
For experiments testing “infected” human plasma, viral supernatants were mixed with human plasma in a 1:1 dilution. 20 ul of the mixture was added to each ELASA well, along with 80 ul PBS, giving a final concentration of 20% “infected plasma” in PBS per well. “Infected plasma” was bound to plate overnight at 4C with rocking. The rest of the ELASA was run as described above.

**Human Blood and Plasma**

Human blood and plasma samples were purchased from ZenBio. Both blood and plasma were collected using the anticoagulant EDTA. Whole blood was shipped fresh on dry ice and was used within 48 hours of receipt. Blood was stored at 4 C until use. Plasma was shipped frozen and was kept at -20 C until use. Both blood and plasma were screened and confirmed to be free of Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus, and Syphilis. Plasma was from a pool of two patients with O- blood types. Whole blood from two different patients was received and pooled before use.

**Aptamer Binding to Artificially Infected Plasma**

To simulate patient serum taken during early viremia, pooled human plasma (EDTA anticoagulant) (ZenBio) was mixed with equal volume of viral supernatants. 100 ul of this mixed solution was bound to an ELISA plate overnight at 4 C, then probed as described above.
Results

Single-stranded DNA Aptamer Generation

Two single stranded DNA aptamers were generated using positive SELEX (Systematic Evolution of Ligands by Exponential Enrichment) against 6xHis-Dengue 1 NS1, immobilized on Ni beads and blocking negative selection against heat-inactivated FBS, human whole blood, human plasma, West Nile, Sindbis, and Adenovirus supernatants (Figure 1A). Figure 1B shows the sequences of the selected aptamers. Aptamer 7.4.1-2 has a predicted folding structure containing two stem-loop regions; aptamer DV1NS1 5-4 has a predicted folding structure containing three stem loop regions (Figure 1C).

Aptamer Binding Specificity

We first tested the abilities of aptamers 7.4.1-2 and DV1NS1 5-4 to bind to NS1 in viral supernatants. Aptamers were able to detect NS1 in Dengue serotypes 2-4 and Kunjin supernatants (Figure 2A). To confirm that the aptamers were not binding to irrelevant secreted molecules being produced by infected cells such as proinflammatory cytokines, aptamer binding was measured against the supernatants of cells that had been stimulated with Poly I:C and R848. We observed in Figure 1A and 1B that our aptamers do not bind significantly to either FBS or cell culture media. From this we concluded that our method of “blocking” counter SELEX was effective in selecting for aptamers which would preferentially bind our target in the presence of competing proteins. There was not significant binding to these stimulated supernatants (Figure 2B). Aptamers also did not bind significantly to the flaviviruses Zika and Yellow Fever (Figure 2A). We next tested whether aptamer binding was lost when native
structure of NS1 was lost. Aptamers binding was lost when NS1 was heat inactivated at 95 C for 3 hours (Figure 2C). This was the case for purified NS1 as well as NS1 in viral supernatants.

**Aptamer Binding Characteristics**

We next investigated the effect of incubation time on NS1 detection. Aptamers were allowed to bind 200 ng purified NS1 for 1 hour, 45 minutes, 30 minutes, 20 minutes, 10 minutes, 5 minutes, and 0 minutes at room temperature with rocking. We found that signal intensity was reduced at 45 minutes and continued to drop as binding time was reduced. We therefore determined optimal binding time for aptamers 7.4.1-2 and DV1NS1 5-4 to be 1 hour (Figure 3A).

The range of sensitivity of aptamers 7.4.1-2 and DV1NS1 5-4 was determined by ELASA using decreasing NS1 concentrations ranging from 10000 ng/ml to 10 ng/ml. We found that both aptamers were effective at detecting Dengue 1 NS1 in 250 ng/ml. However, detection was not significant above background below that concentration (Figure 3B).

**Aptamer Binding to NS1 in Human Plasma**

In order to investigate the usefulness of aptamer 7.4.1-2 and DV1NS1 5-4 for use in diagnostic techniques, it was important to determine whether our aptamers could detect NS1 in the presence of human plasma. Our negative selections and all of our previous experiments had been done using heat inactivated FBS. We therefore first investigated whether human plasma needed to first have the complement factors inactivated before aptamers could be used to detect NS1. We also needed to confirm that, should heat inactivation be required, the heat would not render NS1 unable to be bound by the aptamers. We therefore added 200 ng of NS1
to 10% heat inactivated FBS in PBS and 10% native human plasma. Complement was then inactivated in some of the NS1 containing FBS or plasma by heating at 56 C for 25 min, followed by cooling to room temperature.

The capacity of our aptamers to detect Dengue 1 NS1 decreased dramatically when added to serum (Figure 3C). Aptamer binding to 200 ng NS1 in human plasma was not significant above background. Additionally, error within the experiment was high, rendering any conclusions difficult to draw. We were unable to repeat this experiment due to lack of material. Our objective in this experiment was to determine if our aptamers crossreacted with human complement proteins, which would necessitate heat inactivation of plasma before it could be screened, decreasing aptamer usefulness. However, we tested the capacity of aptamer DV1NS1 5-4 to detect NS1 in artificially infected human plasma, which contained native plasma. DV1NS1 5-4 did not bind significantly over background (Figure 4A), thus we were able to conclude that aptamer DV1NS1 5-4 did not crossreact with human compliment. Therefore, human plasma need not be heat inactivated before testing.

Aptamer Binding to NS1 in Artificially Infected Human Plasma

Due to the high G C content of aptamer 7.4.1-2, it was very difficult to synthesize, and actual purified product received post purification was routinely less than half of what was received of DV1NS1 5-4, from the same sized synthesis reaction. Since the two aptamers had performed equivalently up to this point in our experiments, we decided to focus on DV1NS1 5-4 for testing in human plasma. The reasoning for this was that aptamers are advantageous partly because they can be easily and cheaply synthesized. Aptamer 7.4.1-2 sometimes had to be put
through multiple rounds of synthesis because it did not pass quality control standards. Re-
synthesis significantly increases cost. Thus, it would be more cost effective for a company to
produce a test that incorporated aptamer DV1NS1 5-4 than aptamer 7.4.1-2.

To this end, we tested the capability of aptamer DV1NS1 5-4 to recognize NS1 in
artificially infected human plasma. Plasma and viral supernatants were mixed at equal
concentrations. The titer of each of our Dengue stocks was around $10^4$ ffu/ml. Typical viremia
levels in blood of infected patients range between $10^3$ and $10^8$ viral particles per milliliter of
blood (33). So, a 1:1 dilution would put our Dengue titers within the lower range of those
titers. We diluted all other viruses 1:1 as well, to maintain consistency, in the event protein
concentration from the media in the clarified viral supernatants had any effect on binding, such
as competing for binding to the ELASA plate, etc. This 1:1 mixture constituted our artificially
infected human plasma. For our “uninfected” control our viral collection media MEM 1+1+1
was added to human plasma at a 1:1 dilution.

We compared the binding capabilities of aptamer DV1NS1 5-4 to that of an antibody
generated in our lab against our 293T produced 6xHis-DV1 NS1 (34). Aptamer DV1NS1 5-4,
one again, did not bind well to DV1 supernatants. However, it was able to detect NS1 in
Dengue 2-4 supernatants, as well as in Kunjin supernatants (Figure 4A). The antibody, in
contrast was highly specific for DV1 alone and did not detect NS1 from any of the other
serotypes (Figure 4B). We found no significant binding to the plasma/media mixture, again
confirming the effectiveness of our “blocking” negative selections. Thus, we concluded that
DV1NS1 5-4 can be used to detect NS1 produced by Dengue serotypes 2-4 or Kunjin virus in
native human plasma.
Discussion

In this study, we investigated generating DNA aptamers to Dengue NS1, that could be used in early screening for Dengue infection. We were able to generate two single stranded DNA aptamers that will detect NS1 from Dengue serotypes 2-4 and from the West Nile variant, Kunjin virus. Our aptamers were also able to detect purified Dengue 1 NS1. However, they were not sensitive enough to Dengue 1 NS1 to detect it in clarified viral supernatants. We were unable to determine the exact concentration of NS1 in our viral supernatants, but we found that our aptamers were able to detect purified Dengue 1 NS1 down to 250 ng/ml. Aptamer DV1NS1 5-4 can also detect Dengue 2-4 NS1 in both FBS and native human plasma and could therefore be used in screening of patient plasma without any heat inactivation. NS1 screening by ELISA is one of the major tests currently used to detect early Dengue infection (20, 21), being both easy to use and relatively sensitive (35, 36). We were able to demonstrate binding in an ELASA format, so it is likely that our aptamer could be easily incorporated into current testing formats. To our knowledge, there are no other published aptamers to Dengue NS1.

Reported levels of soluble NS1 in patient blood varies widely across different studies (19, 22, 23, 37, 38). However, our detection limit of 250 ng/ml is within the upper ranges of Dengue 1 NS1 that has been reported in infected patients (19). Indeed, levels of NS1 at or greater than 600 ng/ml within the first 72 hours of infection has been associated with Dengue Hemorrhagic Fever (22). Additionally, the lower limit of detection was calculated based on binding to Dengue 1 NS1, which our aptamers were least sensitive to. Our aptamers are therefore well-suited for detecting both mild and severe cases of Dengue. Indeed, as our aptamers are much cheaper to produce than antibodies, and as they have been shown to bind
to both purified NS1 and NS1 found in viral supernatants, it may be possible to adapt them into a semi-quantitative ELASA screen by incorporating a standard curve using purified NS1 into the test. In this way, hospitals may be able to roughly predict severity of disease, as increased levels of NS1 have been correlated with increased likelihood for progression from Dengue fever to more serious complications in all four serotypes (23, 37, 39).

Additionally, our method of negative selection was modified from typical negative selection procedures. Published protocols describing negative selection describe immobilizing irrelevant proteins onto beads using antibodies or a tag conjugated to the protein (40, 41). However, as we were selecting against human blood and plasma: mediums rich in a milieu of proteins, many of them unknown. This rendered the typical negative selection protocol unfit for our purposes. We therefore took inspiration from ELISA “blocking” steps, which block extraneous binding sites on the well of the ELISA plate. We exposed our aptamer pool to our negative selection target, allowing any aptamers that bind to blood cells or any of the milieu of proteins that exist in plasma to bind before being exposed to our positive selection target. Bound aptamers were not removed before exposure to NS1. However, any aptamers that had previously bound irrelevant targets and then released that target to bind to NS1 would, by nature of them being weak binders, be selected against during positive SELEX, which amplifies the strongest binders. Thus, this new method of negative selection may be useful to other labs who are performing negative selection against a complex target.

The ELASA assay employed in this study was a direct ELASA and subject to its limitations. The most relevant limitation of a direct ELASA is binding capacity of the plate well. This is especially relevant when screening protein-rich substances such as viral supernatants or human
plasma, as a majority of the well may be occupied by irrelevant protein. Therefore, sensitivity of our aptamer may be improved by utilizing it in a capture ELASA rather than a direct ELASA. One of the issues with this type of ELASA is immobilizing the capture aptamer onto the plate, an issue that will be addressed in subsequent studies. Possible methods include tagging the aptamers and first capturing them with an anti-tag antibody that must be bound to the plate first. Alternatively, a crosslinking immunoassay plate, such as the Thermo Fisher Nunc NucleoLink plates could be used.

Current laboratory techniques for screening for Dengue infection can be divided into three general categories: PCR to detect viral genomes; tests to detect viral protein, which can include ELISA’s detecting viral structural protein or the soluble NS1 protein; and tests to detect antibody mediated immune response, including ELISA’s, plaque reduction neutralization tests, and hemagglutination tests (20, 42, 43). While the period of peak usefulness of each category of tests overlaps, there are some tests which are more useful at different stages of Dengue infection. NS1 screening and PCR detecting copies of the viral genome are most effective in early screening (18, 20, 42, 44), with higher levels of soluble NS1 present in blood being correlated with increased likelihood of severe complications such as Dengue Hemorrhagic Fever (22, 23).

Current tests for NS1 screening are antibody based (45) and therefore subject to both the benefits and drawbacks of antibody-based techniques. The first recognizable ELISA’s were performed as early as the 1960’s (46), so the technique has gone through decades of testing and fine-tuning. However, the use of antibodies, usually generated in small animals such as mice or rabbits, to target specific macromolecules has remained the cornerstone of the
technique. However, in the early 2000’s (24), labs began experimenting with substituting aptamers for antibodies in basic laboratory techniques. Since that time, aptamers have been adapted for ELISA’s, Western blots, affinity chromatography, fluorescence labeling, among others (29, 47, 48). Antibodies are generally considered to have superior binding capabilities due to the increased capacity for variability they can have in their binding regions, due to each position being filled with one of 20 potential amino acids. On the other hand, it is argued that each position in an aptamer can only be filled by one of 4 nucleotide bases. While this criticism has historically been valid, new developments in nucleotide synthesis, such as the ability to synthesize aptamers up to 200 nucleotides long or to synthesize them with modified bases, are making such criticism less compelling (49).

Additionally, the great binding specificity of antibodies becomes a disadvantage in Dengue screening, as it makes it more difficult to generate antibodies that will crossreact equally across all four serotypes. Indeed, a mouse pan-Dengue NS1 monoclonal antibody, sold by MyBioSource (Clone 8E5) for use in dot blots and ELISA’s, is not approved for diagnostic use because it is unable to recognize NS1 equally across all serotypes. The commercially available BioRad Dengue NS1 Ag Strip Kit was less adept at detecting DV3 than it was at detecting DV1 and 2 (50). One commercially available ELISA kit, The SD Dengue NS1 Ag ELISA Kit, distributed by Standard Diagnostics, Inc, South Korea, had better binding to DV3 and 4 than to DV 1 and 2, with the added complication of being outcompeted for binding by patient generated IgM (51). In this light, we believe aptamers DV1NS1 5-4 and 7.4.1-2 should be tested in competitive ELISA with anti-NS1 antibodies. In this study, we showed that aptamers DV1NS1 5-4 and 7.4.1-2 were able to recognize soluble NS1 in viral supernatants from three of the four serotypes with no
significant difference between them. Thus, it could easily be paired with only one other
aptamer to provide equivalent detection for all four serotypes.

Some additional benefits that aptamers have when compared to antibodies include
overall cost to generate, stability and resilience, and storage conditions. Classical antibodies
require immunization of an animal and generation of hybridomas from each B cell that reacts to
the target antigen. Following selection of an appropriate hybridoma, antibody production
requires cell culture facilities designed to accommodate suspension cells. So, generation of a
new antibody, or even production of an existing one is quite expensive. Some of these
expenses may be mitigated by switching to single chain antibodies (scFv antibodies). However,
both classical and scFv antibodies need to be folded by a cell, either by a hybridoma or a
bacterial cell (52, 53). Once the 3D structure of the antibody is lost, it is impossible to recover.
This usually necessitates that antibodies be refrigerated during storage. Not only are aptamers
comparatively cheap to synthesize, only needing the sequence, they fold spontaneously (29).
So, no cell culture or animal facilities are required, and the aptamers do not need to be purified
from cell supernatants. Additionally, as folding is spontaneous, as long as aptamers are stored
free from RNAses or DNAses, they do not need to be refrigerated.
Figure 1: Selection of Single-Stranded DNA Aptamers. (A) General selection strategy was to immobilize 6xHis-DV1 NS1 on Ni beads with 1 hour blocking of irrelevant binding with FBS. During the first SELEX, aptamers were added immediately to NS1-bound beads. During subsequent SELEX, counter SELEX was first performed by exposing aptamer pool to negative selection target for 1 hour prior to adding to NS1-bound beads. Following incubation, beads were washed, then NS1 digested to release bound aptamers, which were collected and amplified using error-prone PCR. (B) Using this method, we selected two aptamers able to bind purified DV1 NS1. (C) The predicted folding structure of the aptamer 7.4.1-2 contains one large, GC-rich stem loop and a second, smaller one. Aptamer DV1NS1 is predicted to have four stem-loops and is less GC-rich than 7.4.1-2.

**B**

Biotin- 7.4.1-2
5Biosg-5’-
AGTATACGTATTACCTGCAGCTGGGAGGAGGAGTGGAGCGGAGTGCTGTTAATCCTGTTGGCGATATCTCGAGATCTTGc-3’

Biotin- DV1NS1 5-4
5Biosg-5’-
AGTATACGTATTACGTGAGATATGTTAGCGAAATTTCTGCGATTATGCGTGCTGATGCACTGATTGTGCTGAAAAATTCTGCAA-3’

**C**

DV1NS1 5-4

7.4.1-2
Figure 2: Aptamers DV1NS1 5-4 and 7.4.1-2 Detect NS1 in Dengue Serotypes 2-4. (A) Aptamers DV1NS1 5-4 and 7.4.1-2 detect NS1 in virus supernatants of DV serotypes 2-4 and of the Australian WNV variant Kunjin. (B) Aptamer binding was not to some irrelevant cytokine or other molecule produced by stimulated cells. (C) Aptamer was lost with heat denaturation of NS1, either in supernatants or purified. Thus, aptamer binding was specific to NS1, and there was no non-specific binding to the plastic of the ELISA plate wells. (*P<0.05, **P<0.001).
Figure 3: Binding Capabilities of Aptamers DV1NS1 5-4 and 7.4.1-2. (A) Aptamers DV1NS1 5-4 and 7.4.1-2 bind optimally when given a full hour. (B) Aptamers DV1NS1 5-4 and 7.4.1-2 were capable of detecting purified DV1 NS1 down to 250 ng/ml. (C) While data is not significant, trends indicate that aptamers DV1NS1 5-4 and 7.4.1-2 do not bind to complement factor and are therefore able to be used to screen human plasma without complement being inactivated. (*P<0.05, **P<0.001).
Figure 4: Aptamer DV1NS1 5-4 and Anti-Dengue 1 NS1 Antibody Detection of NS1 in Artificially Infected Human Plasma. (A) Aptamer DV1NS1 5-4 detects NS1 in human plasma artificially infected with DV serotypes 2-4 and Kunjin. (B) Anti-DV1 NS1 antibody was able to detect NS1 from human plasma artificially infected with DV1 alone. (*P<0.05, ***P<0.005).
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Chapter 4
Summary and Future Directions

In this dissertation, we demonstrated that forcing the P320S mutation to remain in the West Nile Virus (WNV) genome by changing all three nucleotide positions in the codon conferred a fitness cost to the virus. We also generated two single-stranded DNA aptamers that can detect NS1 in clarified supernatants of Dengue serotypes 2-4. One aptamer was found to also be able to detect NS1 in human plasma that had been artificially infected with Dengue 2-4 and by the Australian West Nile Virus regional variant, Kunjin virus.

WNV non-structural protein 1 (NS1) is essential for viral replication (1), and is believed to interact with NS4b to help anchor the replication complex to the vesicle packets formed in the ER membrane (2, 3). However, secreted NS1 (sNS1) also has a variety of immunomodulatory functions, including inhibiting the formation of the complement membrane attack complex (MAC) via the classical, lectin, and alternate pathways (4–6). Our lab has also previously shown that sNS1 inhibits Toll-like receptor 3 (TLR3)-mediated cytokine signaling (7, 8). Our lab then generated a mutant that was unable to inhibit TLR3-mediated NFκB transcription but was still able to support viral replication (9). This mutant was generated by a single nucleotide mutation that changed the wild-type proline at NS1 320 to a serine. While this mutant was able to demonstrate a phenotype, we found that the mutation was wildly unstable, with some progeny particles having reverted to wild-type after only one passage. In this dissertation, we generate a P320S mutant in which all three nucleotides in the 320 codon have been changed to code for a serine (P320Sc). Thus, the virus would have to undergo three simultaneous mutations in order to revert to wild-type. We then showed that this mutation
was stable for 6 passages. However, we observed that a fitness cost accompanied this mutation, and we observed what we believe to be a compensatory mutation from histidine to proline at NS1 293.

NS1 320 is located immediately N-terminal to the 9th β-strand in the β-ladder, that is the primary structural domain of the protein (10). Proline is primarily known for its ability to break up secondary structures such as α-helixes or β-sheets and “turn” the protein so a new domain can begin (11, 12). Because of their placement and high conservation, we believe that the 319 and 320 prolines are important for the overall 3D structure of NS1, perhaps to orient the C-terminals of the NS1 dimer so they are in the correct position to trimerize to produce the barrel shaped hexamer that is secreted NS1 (13). We believe that the mutation of the 320 from a proline to a serine warped the geometry of the dimer. However, we believe that this warping was partially compensated for by the 319 proline, so only partial function was lost. We believe it is possible that the subsequent mutation from histidine to proline at 293 was compensatory and restored some of the 3D structure. However, the effect was not enough to restore TLR3 inhibition or to rescue replication rates.

Because of the decreased fitness of the P320Sc mutant, it is unfit for studies investigating the role of TLR3 signaling on WNV replication. However, it may form the basis of a live-attenuate WNV vaccine candidate. Even with the H293P compensatory mutation, P320Sc grew less efficiently than our wild type clone IC#7. We have also shown that the P320Sc mutation is stable up to 6 passages. However, more stability testing would be needed before it could be proposed as a vaccine. Additionally, measures would need to be taken to ensure that
it could not revert to wild-type by recombination, in the event a recipient was concurrently
infected with some strain of WNV.

If this could be successfully done, however, P320Sc would have two major advantages
as a vaccine: it would create a weak but spreading infection that would result in damage
associated molecular pattern (DAMP)-mediated immune stimulation (14), and it would be
unable to prevent TLR3-mediated cytokine signaling. Both of these pathways would adjuvant
the vaccine, likely resulting in a vaccine that required very few boosters.

According to the Center for Disease Control (CDC), Dengue virus (DV) is the most serious
mosquito-borne illness in the world (15), with around 20 million cases in 100 countries per year.
Unfortunately, the current best methods of prevention include targeted killing of the mosquito
vector (16), or a live-attenuated vaccine that is only recommended for those who have already
had a primary infection (17–19). Thus, as most care is palliative (20), early detection of Dengue
is an important factor in patient survival (21, 22).

The two most effective tests for detecting early Dengue are reverse-transcriptase PCR
(RT-PCR)-based detection of viral genome in patient blood and ELISA-based detection of sNS1
in blood (23, 24). While RT-PCR has the advantages of being quick (less than 3 hours) and very
sensitive, it is not considered ideal for a low-resource setting, as it requires highly trained
personnel and specialized facilities and equipment (25). The ELISA-based method of detecting
sNS1 is considered a good balance between ease of use and sensitivity (25). However, classical
ELISA techniques still require the use of antibodies, which are expensive to produce and need
refrigeration (26). Aptamers have been called “chemical antibodies” (27), and have been able
to replace classical antibodies in several laboratory techniques, including the ELISA (enzyme-linked aptamer-sorbant assay), which replaces at least one of the antibodies in the technique with an antibody (28, 29). Aptamers are single-stranded short RNA or DNA oligonucleotides that form a 3D structure that binds to a target and can be made relatively cheaply with no need for refrigeration (30).

In this dissertation, we generated two single-stranded DNA aptamers that can detect NS1 in Dengue serotypes 2-4 and in Kunjin virus. As one of the aptamers was very difficult to synthesize, we focused primarily on aptamer DV1NS1 5-4, which was also able to detect NS1 from artificially infected human plasma. Both of our aptamers were selected against Dengue 1 NS1 and were shown to bind as low as 250 ng/ml purified NS1, but they were not able to detect NS1 in Dengue 1 viral supernatants. We were not able to determine whether this was due to decreased NS1 production by our DV1 virus, due to our antibody only binding to purified Dengue 1 NS1. We were, however, inclined to believe it was due to our aptamer simply not recognizing Dengue 1 NS1, as our antibody is not able to detect NS1 in low levels in Dengue supernatants, but we were able to detect NS1 in the supernatants we tested, indicating at least a moderate level of NS1.

It is possible that changing the ELASA setup from a direct ELASA to a capture ELASA might increase our binding efficiency and allow our aptamers to detect Dengue 1 NS1 in viral supernatants as well. This may be done either by combining an antibody-capture with an aptamer probe or an aptamer-capture with a different aptamer probe. The problem with the latter is two-fold: to our knowledge, no other published sequences of Dengue NS1 specific aptamers exist, so we would likely have to use both aptamer DV1NS1 5-4 and aptamer 7.4.1-2.
This would raise issues of cost in a production setting, as 7.4.1-2 regularly required two or more synthesis reactions to generate a product that passed quality control. Additionally, the aptamers alone do not bind well to the plastic of the ELISA plate. To circumvent this, we propose using a crosslinking immunoassay plate, such as the Thermo Fisher Nunc NucleoLink plates. These plates would avoid the need for large protein tags that might degrade over time. This aptamer-capture/aptamer-probe sandwich-type ELISA would also avoid the use of antibodies, as the detection antibody can be directly biotinylated, as it was in our experiments, or conjugated to a DNAzyme, taking advantage of rolling circle DNA amplification to augment the signal, as proposed by Tang, et. al (31, 32).

Additionally, when testing aptamer DV1NS1 5-4 against “infected” human plasma, we were unable to acquire plasma from infected patients. We instead artificially infected human plasma by adding clarified viral supernatants. While we ensured that the number of viral foci forming units added fell within the range of reported titers during peak viremia (33), we necessarily had to add viral media and other viral or cellular factors produced during the course of infection, which will not exist in infected patient media. Therefore, the detection capabilities of aptamer DV1NS1 5-4 must be tested using plasma taken from patients infected with each of the four Dengue serotypes.

Finally, we would like to further define the binding capabilities of aptamer DV1NS1 5-4, both by testing it against purified NS1 from Dengue serotypes 2-4 and by directly comparing DV1NS1 5-4 with some of the leading commercial Dengue ELISA kits. We believe that DV1NS1 5-4 binds more readily to NS1 from Dengue serotypes 2-4, but we do not know its full
capabilities. We would also like to confirm that DV1NS1 5-4 is at least comparable to the commercially available Dengue ELISA kits in terms of binding range and signal strength.

In this dissertation, we have demonstrated that the substitution of a serine for proline at NS1 320 of WNV, while not lethal to the virus, has a fitness cost. This is an important contribution to those who study the interactions between TLR3 and WNV, as the single nucleotide mutant previously developed by our lab, P320S, grew to levels similar to our wild-type infectious clone (9). However, we now believe this is due to the high reversion rate of the single nucleotide mutant. Our findings may also contribute to understanding of sNS1 structure and how NS1 dimers trimerize to form the final barrel-like shape of the sNS1 hexamer.

We also generated two single-stranded DNA aptamers that were capable of detecting NS1 in Dengue serotypes 2-4 and Kunjin viral supernatants. Aptamer DV1NS1 5-4 was also capable of detecting NS1 in artificially infected plasma. To our knowledge, there is no published aptamer that binds Dengue NS1 protein. After further testing, an ELASA incorporating DV1NS1 5-4 may be able to replace the current commercial ELISA tests as a less expensive screen for Dengue infection. Thus, this dissertation has contributed to both the field of aptamer research and, potentially, to patient health as well.
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P320Sc Mutation is Lethal When Introduced into RepliVax Genome

Abstract

RepliVax is a single cycle replicon, generated by removing the majority of the capsid (C) coding region from the West Nile Virus (WNV) genome. Thus, RepliVax is able to infect and replicate, but not exit, so any infection cannot spread. Our lab has previously shown that a single substitution of serine for the native proline at the 320 position is able to restore Toll-like receptor 3 (TLR3)-mediated cytokine signaling. TLR3 is able to detect double stranded RNA (dsRNA), which is generated by WNV during replication. We introduced the P320Sc mutation onto the RepliVax backbone successfully, in the hope that the TLR3-mediated cytokine production by RepliVax infected cells might make RepliVax a more effective vaccine candidate. However, the P320Sc mutation was lethal in RepliVax.

Introduction

RepliVax is a single cycle replicon, generated by deletion of the majority of the C coding region of the West Nile Virus (WNV) genome. Thus, the replicon still expresses native non-structural proteins, enabling RNA to self-replicate. Native PrM and E are also produced, allowing for the production of subviral particles. If a functional capsid protein is provided in trans to the RepliVax genome, such as by the packaging cell line BHK366, new single cycle infectious particles can be produced. However, point mutations in the 3’ end of the C coding
sequence of RepliVax prevents recombination with the functional C being provided by the packaging cell line, which eliminates the risk of producing functional WNV with an intact C coding region. Thus, RepliVax particles are able to infect their target cells and replicate their genome, without allowing new viral particles to be formed. So, any infection with RepliVax is unable to spread.

RepliVax was originally designed as a vaccine candidate (1). It would be able to infect the individual being vaccinated, resulting in stimulation of the immune system and persistence of antigen: characteristic of vaccination with an attenuated virus. At the same time, RepliVax infected cells would still able to produce PrM and E containing subviral particles (2, 3), allowing for protective anti-M and anti-E antibodies to be generated (4, 5). However, unlike an attenuated virus, RepliVax infection cannot spread, making it safe to use in individuals with a weaker immune system. While RepliVax-stimulated antibodies have been shown to be protective in both the mouse and non-human primate models, animals must be immunized with a large inoculum to illicit protection. Two applications of $10^6$ focus forming units are required per animal for protection (2, 6), whereas a single challenge with $10^3$ focus forming units is the lethal dose for mice and $10^5$ being the lethal dose for non-human primates.

Attenuated viral vaccines generally stimulate longer lasting immunity and higher antibody titers than killed or subunit vaccines (7) because they generate a more robust pro-inflammatory immune response and because the immune system is exposed to the viral antigens over a longer period of time. This is the reason killed and subunit vaccines employ adjuvants. Adjuvants such as Alum will complex with the antigens, making them larger and more stimulatory (8). Oil-based adjuvants form a bolus with the antigens inside it, promoting
phagocytosis and presentation by antigen presenting cells (9, 10). Perhaps the most famous adjuvant, Complete Freund’s Adjuvant (CFA), consists of heat-killed mycobacteria in oil, and stimulates a severe, TLR-mediated, generalized pro-inflammatory response, which promotes a simultaneous response to the vaccine antigen. However, adjuvants have their own drawbacks. The very effective FCA is not approved for use in humans because it causes severe reactions at the injection site (11). The more mild adjuvants, such as alum, are not as effective at stimulating a response, necessitating booster vaccines be administered at later dates (DiPasquale). While adjuvants have enhanced effectiveness of killed and subunit vaccines to usable levels, their main advantage over attenuated vaccines is their safety. They can be given to someone with a weak immune system without worry of that person becoming ill from the vaccine itself— a risk that is always present with an attenuated vaccine.

In terms of safety, RepliVax is very similar to a killed or subunit vaccine. However, like those vaccines it does not cause the levels of tissue damage that an attenuated virus, still capable of spreading, is able to do. RepliVax itself does not cause tissue damage to the level of a spreading infection, but danger associated molecular patterns (DAMP’s) from death of host cells infected by the RepliVax particles— such as intracellular uric acid and extracellular ATP— stimulate the production of pro-inflammatory cytokines through pattern recognition receptors (PRR) (12). This signaling, coupled with signaling from virus specific PRR’s and the persistence of the viral antigens, combine to make live attenuated vaccines the most effective in types currently available. RepliVax combines many of the benefits of a live-attenuated vaccine without having the inherent risk.
WNV can be detected through several PRR’s, including Toll-like receptor 3 (TLR3), retinoic acid inducible gene-1 (RIG-I) and Melanoma differentiation-associated protein-5 (MDA-5) (13). Our lab has previously shown that WNV non-structural protein 1 (NS1) is able to inhibit TLR3 mediated cytokine production (14, 15). We have also found a single point mutation that, when introduced into the NS1 region of the WNV genome, is able to restore TLR3 mediated signaling (15). We hypothesized that RepliVax particles, containing this point mutation, might stimulate a more robust cytokine response than RepliVax that produced wild-type NS1, thereby increasing efficacy of RepliVax as a potential vaccine candidate.

Materials and Methods

Measuring NS1 Secreted from RepliVax Infected Cells

HeLa or Vero cells were infected with RepliVax at an MOI of 1 for 24 or 48 hours. Cell supernatants collected and stored at -20 C until testing. Relative NS1 was measured by ELISA. Cell supernatants were diluted 1:10 in PBS and bound to standard ELISA plates (Corning) overnight at 4 C with rocking. Plates were then washed 5x with ELISA wash buffer (0.5% Tween 20 in PBS), and blocked for one hour with 1x eBioscience Ready to Go © ELISA kit assay diluent for 1 hour at room temperature with rocking. Block was then removed. Anti-NS1 antibody (16) was diluted 1:1000 in 1x assay diluent and allowed to bind for 1 hour at room temperature with rocking. Plate was then washed 5x with wash buffer. Horseradish peroxidase conjugated antimouse antibody was diluted 1:2000 in assay diluent and allowed to bind to wells for 1 hour at
room temperature with rocking. Plates were then washed 7x with wash buffer and TMB (eBioscience) added to wells. Colormetric change was measured by spectrophotometer.

**Introduction of P320Sc Mutation into RepliVax**

We did not have the RepliVax genome in a plasmid, so we introduced the C mutation from RepliVax into a plasmid containing a full WNV genome with the P320Sc mutation.

RepliVax RNA genome was first isolated from RepliVax subviral particles using phenol:chloroform extraction. RepliVax RNA was then reverse transcribed to cDNA. RepliVax cDNA was then amplified using high fidelity Accutaq polymerase and the following primers: Fwd 5’-TGAGTGGATCTATAAGTAGTTCGCCTGTGTGAGCTGACAAACTT-3’ and Rev 5’-GGCAGAGGTCCGCAGTTATTG-3’. The forward primer incorporates a BamHI restriction enzyme site and a T7 promoter upstream of the 5’ UTR, and the reverse primer anneals downstream of a natural EcoNI restriction enzyme site present in the E region of the viral genome. The pACNR expression plasmid, which contained our P320Sc WNV genome, has a BamHI restriction site 5’ of the WNV 5’ UTR. Thus, we were then able to double digest both the P320Sc plasmid and the PCR amplified RepliVax 5’ UTR-C-PrM-E with BamHI and EcoNI (New England Biolabs). We ran both digestion products on an agarose gel and purified the bands that were the appropriate size for our desired product. We then ligated the backbone of the P320Sc-pACNR plasmid to the RepliVax 5’UTR-C-PrM-E. We cloned the product into *E. coli* to amplify. Colonies were screened by PCR for both E and NS1. Single colonies, positive for both NS1 and E, were grown with antibiotics (Kanamycin- Sigma) to amplify plasmid. Plasmids were then purified from lysed cells and analyzed by sequencing.
**Viral RNA Synthesis and Electroporation**

P320Sc RepiVax plasmid was linearized by XbaI single restriction enzyme digestion (New England Biolabs), followed by phenol:chloroform extraction. Digestion was confirmed by agarose gel analysis. 5’ capped RNA was generated using MEGAscript T7 High Yield Transcription Kit (Ambion). Manufacturer’s Instructions were followed with the following modifications to the volumes of dNTP’s used per 20 ul reaction: 2 ul ATP, 2 ul CTP, 2 ul UTP, 2 ul G(5’)ppp(5’)G RNA Cap Structure Analog, 1 ul GTP. RNA production was confirmed by RNA agarose gel. Viral RNA was electroporated into BHK366 cells, grown at 37C in MEM base media supplemented with 1% HEPES buffer (Cellgro), 1% Fetal Bovine Serum (Cellgro), and 1% Cellgro 10,000 IU/ml Penicillin/Streptomycin (MEM 1+1+1). BHK366’s were then permitted to grow for 8 days, with cell supernatants being harvested, clarified by centrifugation, and frozen every 48 hours. Cells were also visualized every 24 hours for signs of cytopathic effect consistent with WNV infection.

**Testing of Electroporated Cell Supernatants**

Supernatants harvested from electroporated BHK366 cells were tested for subviral particles by both rtPCR of the E region of the RepiVax genome and by staining infected cell monolayers with anti-E antibody. rtPCR was carried out on RNA extracted from clarified cell supernatants by phenol:chloroform. Following reverse transcription, any purified cDNA was amplified by high fidelity PCR using the primers WNVseq3fwd: 5’-GCTCACAATGACAAACGTGCT-3’ and WNVseq3rev: 5’-AGCCTTTGAACAGACGCCA-3’, that amplified a 687 nt portion of the E region of the RepiVax genome. Clarified supernatants were also serially diluted and added to
Vero monolayers to confirm the presence of subviral particles and to titer them if present.

Diluted supernatants were allowed to “infect” cells for 1 hour before a semisolid overlay of 1:1 Tragacanth Gum and 2X MEM 1+1+1 (2X MEM media+ 2% FBS+ 2% HEPES buffer+ 2% Penicillin/Streptomycin) was added. Cell monolayers were then incubated for 48 hours at 37°C with CO2. Supernatants were then removed and monolayers washed once with PBS. Monolayers were allowed to air dry and were then fixed with 1:1 Acetone:Methanol and stored at -20°C until staining.

The acetone:methanol fixative was removed and cell monolayers allowed to air dry. Monolayers were then rehydrated with 1% normal horse serum (NHS) in PBS at room temperature for 1 hour with rocking. Monolayers were then stained for 1 hour at room temperature with anti-E antibody D14G2 at a 1:1000 dilution in 1% NHS in PBS with rocking. Monolayers were then washed 5x with PBS and stained with an anti-mouse antibody that had been conjugated to horse radish peroxidase (HRP) (Thermo Fisher), at 1:2000 dilution in 1% NHS in PBS, for 1 hour at room temperature with rocking. Monolayers were then washed 7x with PBS and stained with Vector Labs Vector VIP staining kit. Foci were then observed.

Results

RepliVax Infected Cells Able to Produce NS1

Our argument for creating a better vaccine candidate by introducing the P320Sc mutation into RepliVax presupposes that RepliVax infected cells produce NS1, which would then go on to inhibit TLR3 mediated cytokine production, as we have previously shown WNV
NS1 to be able to do (14, 15). To confirm that NS1 was actually being made and secreted by cells infected with RepliVax, as WNV infected cells have been shown to do (15), we infected Vero and Hela cells with RepliVax, harvesting cell supernatants 24 and 48 hours after infection. We were able to detect NS1 by ELISA in cell supernatants 24 and 48 hours post infection (Figure 1).

**P320Sc Mutation Introduced into RepliVax Genome in pACNR Expression Plasmid**

Our lab has previously generated a proline to serine point mutation that compromises WNV NS1’s ability to inhibit TLR3 mediated cytokine signaling (15). This point mutation translates to a proline to serine substitution at the 320 position of the viral NS1 protein. In that study, a single nucleotide was changed. While the single nucleotide mutant NS1 permitted full TLR3 functionality, viral particles carrying the single mutation rapidly reverted to the wild-type proline at the 320 position. Indeed, viral particles began to revert within the first passage (unpublished data). For this study, we therefore introduced a full codon mutation translating in the change from wild type proline to serine at NS1 320. At the time of this study, our lab had just shown the full codon mutation, dubbed P320Sc, was stable for up to 4 passages (unpublished data).

We therefore used the plasmid containing the WNV infectious clone with the full codon mutation as the backbone for our P320Sc RepliVax (P320ScRV). We did not have an infectious clone of RepliVax, but we did have stocks of subviral particles, so we introduced the C mutations into our P320Sc containing WNV clone, rather than introducing the P320Sc mutation into a RepliVax genome. To do this, we first isolated and reverse transcribed the RNA genomes
from viral supernatants. We then performed a double digest with BamHI and EcoNI on both the RepliVax cDNA and the P320Sc WNV infectious clone allowed us to cut the wild type 5’ UTR, C, PrM and 5’ half of E out of the P320Sc plasmid and ligate the RepliVax 5’ UTR, C, PrM, and 5’ half of E in in its place. We confirmed proper ligation by sequencing C and NS1 regions of the ligated plasmids. We found the C sequence to be consistent with the published RepliVax C sequence (1), with the 5’ half of C deleted and having the expected single nucleotide substitutions in the 3’ half of C (Figure 2).

**Electroporation of P320Sc RepliVax RNA into BHK366 Cells Did Not Result in Subviral Particles**

We generated RNA from the P320Sc RepliVax plasmid using T7 in vitro RNA transcription and electroporated it into the RepliVax packaging cell line, BHK366 using the same procedures we have used previously to generate infectious WNV from infectious clone RNA, transcribed in vitro using the same T7 kit. However, cytopathic effect we had previously associated with wild-type RepliVax infection, such as increased intracellular membranes, did not appear in electroporated cells at any point during the 8 days of culture post electroporation. Despite media changes every 48, when supernatants were collected for testing, the cells began to show signs of cell culture crisis and overcrowding at 8 days post electroporation and were discarded. We ran reverse transcriptase PCR (RT-PCR) targeting E and NS1 regions of the Replivax genome on electroporated cell supernatants. Viral RNA was occasionally detected using this method. However, detection was not consistent across timepoints or preparations. To confirm whether subviral particles had been released into the supernatants, clarified supernatants from 48, 96, and 120 hours post electroporation were serially diluted and applied to Vero cell monolayers. Vero cells were allowed to incubate for 48 hours, at which point they were fixed and
permiabilized with acetone:methanol. We then stained the cells for E protein, which would have been inserted into the ER membrane (20). However, no cells stained positive for E protein.

**Discussion**

At the time of this study, we had not yet learned of the severe fitness cost of the P320Sc mutation. We believe that the P320Sc mutation, when coupled with the modifications to C present in RepliVax, was a lethal combination. It is possible that this data could imply some cooperation between NS1 and the C region of the viral genome, which the combination of the modifications to the C region of the genome and the mutation in the NS1 protein disrupted. Functional NS1 is essential for viral replication (17, 18), and while it is known to associate with the replication complex, it has not been shown associating with the viral genome itself. To investigate this question, we propose using CLIP in Dengue infected cells to crosslink any RNA-protein interactions (19). Using immunoprecipitation to collect all pieces of RNA associated with Dengue NS1, we could then digest with RNAse, to remove all RNA but that which is directly bound to NS1, which will be protected from RNAse activity by steric hindrance from NS1. The RNA that was protected from degradation would then be reverse transcribed and sequenced. Since NS1 seems to be most prevalent early in the course of infection, as stated above, and has seemed to be most concentrated in harvests taken when cells have been infected for less than 72 hours (unpublished data), we would recommend performing the CLIP assay at 24 and 48 hours post infection.
We believe the RT-PCR amplification we sometimes got was an artifact. We believe we were amplifying RNA leftover from the electroporation, rather than RNA from new subviral particles. This would explain why there was no discernable pattern to which samples were positive and which were negative.
Figure 1: RepliVax Infected Cells Secrete NS1. Vero and HeLa cell monolayers were infected with RepliVax clarified supernatants at an MOI of 1. Cells were incubated for 24 or 48 hours. Cell supernatants were tested for NS1 by direct ELISA.

Figure 2: RepliVax C Introduced Onto P320Sc Backbone. RepliVax C, generated from cDNA reverse transcribed in vitro from viral stocks, was ligated onto the mutant WNV infectious clone, P320Sc. The resulting infectious clone was called P320ScRV. Here we compare the sequences of wild-type WNV clone IC#7 to that of P320ScRV. We confirmed a significant portion of C to be missing, consistent with the modified C region of the RepliVax genome, and the proline to serine mutation at NS1 320 (blue arrow).
References


Appendix II

Other Single-stranded DNA Aptamers Selected Against Purified Dengue 1 NS1

Abstract

Aptamers DV1NS1 5-4 and 7.4.1-2 were the product of the last of several selection procedures. Other single-stranded DNA aptamers were generated during the previous procedures. While they were not considered optimal for our purposes, they still warrant a mention.

Introduction

Each SELEX selection procedure resulted in two or more aptamer sequences. Each of these sequences was synthesized and tested first for the ability to recognize purified NS1 from Dengue serotype 1 (DV1) in the context of a direct ELASA. Aptamers which were able to detect NS1 were then tested for the ability to recognize NS1 in clarified viral supernatants and for the ability to discriminate between NS1 produced by Dengue and other irrelevant viruses. Relative sensitivity was compared between some of the aptamers as well. The aptamers presented in this appendix were judged to be inferior to DV1NS1 5-4 and 7.4.1-2 in one or more of these categories.
Materials and Methods

Positive selection was identical to that conducted on aptamers DV1NS1 5-4 and 7.4.1-2, which is described in Chapter 3 of this dissertation, except where otherwise indicated. Negative selections vary somewhat.

Negative selection for aptamers 4.1, 4.5, 4.6, and 4.19 did not begin until the fourth round of positive selection. Before selection round 4, the aptamer pool was exposed to 10% human plasma in PBS for 1 hour at room temperature. A portion of the aptamer pool was removed, cloned, and sequenced, yielding aptamers 4.1, 4.5, 4.6, and 4.19. The rest of the pool was enriched further.

The pool was subjected to negative selection against 20% whole human blood in PBS, 50% 293T complete media containing Dulbecco’s DMEM base (Cellgro) with 10% FBS and 10% antibiotics (Cellgro Pen/Strep 10000 IU/ml), and against Ni-coated agarose beads (Invitrogen) prior to positive selection rounds 5, 6, and 10, respectively. Positive selections differed from those previously described beginning at round 7, when NS1 immobilized on the Ni beads was decreased by one fifth. The amount of NS1 on the beads was decreased by a further half for round 8. During round 9, the amount of NS1 bound to the beads was maintained from the previous round, but selection was conducted in 2x PBS buffer, instead of the 1x of all other selection procedures, to disrupt weak ionic bonds between the aptamer pool and the immobilized NS1. A sample of the aptamer pool was then cloned and sequenced after round 10, yielding aptamers 10.1, 10.3, and 10.22.
Results

Negative Selections Required for Selective Binding

Aptamers 4.1, 4.5, 4.6, and 4.19 were from early stages of selection, so did not bind NS1 as strongly as aptamers 10.1 and 10.22, which were from later in the selection process (Figure 1B). Aptamers 4.1, 4.5, and 4.6 also demonstrated an unacceptable crossreactivity to elements in cell culture media (Figure 1B). Aptamers 10.1 and 10.22 were able to detect purified DV1 NS1 with a stronger relative signal and did not crossreact with cell culture media. Aptamer 10.3 was only able to generate a weak signal, like aptamer 4.1, but it too did not crossreact to cell culture media.

Aptamer 10.1 Crossreacts with Other Viruses

We tested the ability of aptamer 10.1 to detect NS1 in viral supernatants, in a direct ELASA. We found that aptamer 10.1 did not detect all stains of Dengue NS1 equally, having difficulty detecting NS1 from serotype 3 (Figure 2). We also found 10.1 to be broadly crossreactive with both flaviviruses and non-flaviviruses.

Discussion

While the aptamers generated during our early selections were unfit for our purposes, they are nevertheless worthy of note. We are able to observe the unique selective effects of the SELEX procedure when comparing the binding capabilities of aptamer 4.1 and 10.1. The multiple rounds of selection, which are the cornerstone of SELEX, are theorized to enrich
aptamers with the best binding capacities, with more rounds of selection yielding better binding aptamers. The aptamers cloned and sequenced from selection round 4 had inferior binding capabilities to those cloned from round 10.

Additionally, the crossreactivity of aptamer 10.1 indicated to us the need for negative selections against a wide range of targets. This prompted us to include negative selection rounds against non-flaviviruses when generating aptamers DV1NS1 5-4 and 7.4.1-2. Therefore, although, these aptamers were not useful for our purposes, they did contribute to our understanding of the aptamer selection process.
**Figure 1: Negative Selections Essential to Aptamer Specificity.** (A) Sequences of all mentioned aptamers. Aptamers from round 4 positive selection had inferior binding capabilities when compared to those from round 10. Additionally, without the negative selection round, early aptamers crossreacted with cell culture media.

**Figure 2: Aptamer 10.1 Crossreacts with Other Viral Supernatants.** While aptamer 10.1 does appear to detect purified NS1 with a signal equivalent to that generated by an anti-Dengue 1 NS1 antibody previously generated by our lab, 10.1 does not detect NS1 equally across all Dengue serotypes. Additionally, 10.1 crossreacts to the mutant West Nile Virus RepliVax and the non-flavivirus Sindbis.