DESIGN AND EVALUATION OF A NON-STRUCTURAL PROTEIN 1-BASED
DIAGNOSTIC FOR ZIKA VIRUS INFECTION

AN ABSTRACT SUBMITTED ON THE TWENTY-EIGHTH DAY OF FEBRUARY
TWO THOUSAND TWENTY TO THE DEPARTMENT OF MICROBIOLOGY AND
IMMUNOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE
GRADUATE SCHOOL OF TULANE UNIVERSITY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY BY

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ABSTRACT

Zika virus (ZIKV), a member of the *Flaviviridae* family, was the cause of a large viral outbreak reaching across the Americas during 2015 and 2016. Discovered in 1947, it has historically been a neglected disease, due to its emergence in humans on a large scale being recent. At the time of the outbreak, no FDA approved ZIKV diagnostics existed, and those that were able to detect the virus were unable to distinguish it from related viruses such as Dengue virus (DENV), and at this time, no approved therapeutics or vaccines are available. We investigated the ability of diagnostics targeted toward both anti-NS1 antibodies and NS1 antigen circulating during infection to detect current or past ZIKV disease, as well as the capability of NS1 to produce a protective response. Our studies suggest anti-NS1 diagnostics are feasible, though some populations may display an immune response reminiscent of a prior infection. Levels of circulating NS1 were lower than those produced during DENV infection, though were still detectable with our assay. Additionally, intraperitoneal immunization with NS1 produced an anti-ZIKV NS1 response that coincided with a decrease in viremia, though further work was needed to discern life-prolonging effects. Together, this work furthers the development of the tools necessary to combat future outbreaks of ZIKV in vulnerable populations.
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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Garry. Without the resources, time, effort and ideas he offered in my years at Tulane, I would not have gotten to this point. I would also like to thank Dr. Luis Branco for the laboratory skills needed to proceed with the work necessary, as well as the past and present members of the Garry lab for making it enjoyable and motivating to come into work every day. Thank you to my committee for giving me your valuable time and knowledge during this process. Learning from such experts in multiple areas is very much appreciated. The members of the Viral Hemorrhagic Fever Consortium who gave me advice along the way deserve thanks as well, with a particular thanks to Dr. Matt Boisen for his guidance. Finally, thank you to my wife Julia for always being there with support, and often with Taco Bell, during this entire process.
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Zika Virus Discovery and Epidemiology

Zika virus is named for the Zika forest in Uganda, where it was first isolated from a sentinel monkey in 1947 during an epidemiological study of Yellow Fever virus [1]. Soon after, it was isolated from mosquitoes in the same area during a parallel Yellow Fever study [2]. Follow-up surveys, predominantly serological studies, revealed Zika to be widespread throughout much of Africa, including Nigeria [3–5], Sierra Leone [6], Senegal [7, 8], Gabon [9, 10], Cote d’Ivoire [11], the Central African Republic [12], and Egypt [13]. It was also found in serological surveys in Asia, with evidence of the virus in Pakistan [14], India [15], Thailand and North Vietnam [16], Malaysia [17], Philippines [18] and Indonesia [19]. Subsequently, the virus itself was isolated from mosquitoes in Malaysia [20], lending increased support to the serological evidence and showing Zika was widespread in both Africa and Asia.

The first reported natural Zika virus infection in humans was reported in 1964, when a scientist was infected while isolating virus from mosquitoes in Uganda [21, 22]. Between then and 2007, only a handful of human infections were found via viral isolation in Nigeria [4, 5]. Other infections reported were based on serological evidence in febrile patients [23]. Infections reported before 1964, including an experimental human infection, were found to be erroneously attributed to Zika virus [24–26]. Infections reported before 2007 were confined to Africa and Asia (Figure 1-1).

The first known incidence of Zika outside Africa and Asia was in 2007 in Yap State, Micronesia [27], in what became the first large outbreak recorded [28]. Laboratory
IgM testing indicated an infection caused by Dengue virus but was a clinically separate disease. Testing for neutralizing antibody responses and viral RNA lead to identifying 49 cases of Zika during the outbreak. Cross-neutralizing antibody responses with a positive IgM result were classified as probable Zika virus disease, with 59 individuals categorized this way. Overall, it is believed 73% of residents of Yap Island 3 years are older were infected, though no infections resulted in significant morbidity [29].

In 2010, one case of Zika virus infection was found in Cambodia via viral sequencing [30], and one case in 2012 in the Philippines identified the same way [31]. A case in Indonesia was confirmed during a 2014-2015 Dengue outbreak [32], as well as two cases of Zika infection acquired during travel to Indonesia, one of which was a probable monkey bite [33, 34]. About this time, travelers from Thailand were found with Zika infection [35–38], with a subsequent study finding multiple infections across Thailand, confirming its circulation in the country [39]. This was reinforced via a more recent immunological survey of serum samples [40]. A 2014 case was also identified in a traveler, acquiring Zika from either Malaysia or Borneo [41].

In 2013, cases of Zika began being reported in French Polynesia. After a year, 294 cases were confirmed via RT-PCR [42], with 383 cases being confirmed via serology, and 11.5% of the population seeking health care advice for Zika-like symptoms [43]. In total, 8746 cases were estimated to have occurred during the outbreak, making it the largest outbreak of Zika virus to that date [44]. In a later seroprevalence survey, it was found that before the outbreak, the only seropositive samples were found in French Polynesia residents who had traveled abroad, and no positive samples were found in those who had not traveled, indicating the introduction of Zika was about the same time
as the start of the outbreak [45]. Several travelers subsequently were infected with Zika upon travel to French Polynesia [46–49].

In 2013, New Caledonia began reporting cases of Zika, with eventual autochthonous transmission being found. Cases were found from French Polynesia, but also from other Pacific islands. Eventually, almost 1400 cases were found [44, 50]. The Cook Islands had an outbreak with 50 confirmed cases and 932 suspected cases [44], with an exported case to Australia [51]. 2014 saw an outbreak on Easter Island, with 51 confirmed cases of Zika virus infection out of 89 suspected cases [52]. The outbreak is thought to originate from a festival held on the island [53]. Figure 1-2 summarizes the Pacific travel of Zika virus.

Beginning in early 2015, cases of Zika virus infection were reported in Bahia, Brazil [54]. A second introduction of Zika was likely introduced into Brazil at about the same time, in Natal, and it has been suggested the World Cup was responsible for initial introduction [55]. The World Cup was held in Natal and Salvador, with Salvador reporting Zika-like symptoms in 2015 [56]. Other speculation suggests possible introduction during a World Championship canoe race in 2014 in Rio de Janeiro [53]. Another study estimates introduction in mid to late 2013, more than a year before detection in Brazil [57]. Early estimates were of 440,000 to 1.3 million cases [58], with an eventual estimate of over 500,000 cases of Zika [59] and 73% of the population exposed in some areas [60].

Other countries in the Americas also reported Zika cases during this time. Colombia reported over 100,000 suspected cases [61], with a total of 27 countries in the Americas reporting cases, including Mexico, Guatemala, Dominican Republic and
Panama [62]. Eventually, Zika cases were reported in the continental United States, with multiple introductions of Zika into Florida [63], as well as Texas cases. These cases in Texas and Florida were locally acquired, indicating at least some level of circulation in local mosquito populations [64]. Afterward, Zika made its way to Cape Verde, Samoa, the Solomon Islands and surrounding region [22, 65] (Figure 1-3). In all, 87 countries were affected by the pandemic, with cases continuing after the large outbreak, including 30,000 cases in 2018 [59]. With outbreaks continuing, it remains a possibility that Zika virus has established itself as endemic to many more regions than it was prior to the outbreak, and may continue to infect humans routinely into the future.

Zika Virus Natural History

Zika infection results in a self-limited illness that, in up to 80% of cases, is asymptomatic [66]. The incubation period, or time between viral exposure and beginning of symptoms, is between 3 and 14 days [67]. Typical presentation consists of low fever that lasts for several days [68], though the fever may reach above 40°C [69]. Fever may not always be present [70]; likely in just under 75% of cases [71]. The most common symptom is a maculopapular rash, occurring in over 80% of cases [71], often on the face, torso and upper arms [70, 72]. Another common symptom is arthralgia in about 62% of cases, often with involvement of small joints [36, 71], but can be larger joints as well [70, 73]. Conjunctivitis is seen in roughly half of patients, with both eyes commonly being affected [71, 74]. Other symptoms may be seen, such as myalgia, headache, nausea, vomiting, sore throat and cough [75]. Symptoms resolve within a week and are generally not fatal [76], provided there is not a comorbidity, such as sickle cell anemia [69].
Symptoms may present similarly to other infections commonly found in arbovirus endemic areas (Figure 1-4), such as the skin rash, high fever and joint pain associated with Chikungunya infection, though in the case of the latter infection symptoms are generally more debilitating and long lasting [77]. Dengue fever also can mimic some of the Zika virus disease symptoms, with myalgia, arthralgia, headaches, fever, nausea, vomiting, and a rash of varying intensity. In some cases, Dengue infection can cause hemorrhagic fever symptoms of petechiae and vascular leak. Zika does not result in hemorrhaging to that level, instead resulting in less severe bleeding symptoms, such as hematospermia [70, 78, 79]. Spondweni virus infection can be easily confused with Zika infection as well, with both viruses belonging to the same serogroup within the virus family *Flaviviridae*, and co-circulating in many areas [80].

Before 2013, no significant complications were reported from infections with Zika virus [21]. Upon the outbreak in French Polynesia, increased reports of multiple complications began. During the French Polynesia outbreak, multiple cases of Guillain-Barré Syndrome (GBS) were reported [22, 81, 82]. GBS is a neurological disorder presenting as a rapid ascending paralysis that results in respiratory failure in 20-30% of cases [83]. Usually preceded by exposure to an infectious agent, such as Zika virus, it is the most common cause of acute paralysis in children [84]. Typically, symptoms will begin 1-2 weeks after infection, with the peak neurological weakness occurring between 2 and 4 weeks. The condition often requires ICU care, especially in those where the condition rapidly progresses, or involves respiratory anatomy. Recovery can last months or years between the immune response decline and repair of the nerve damage responsible for the weakness [83].
Another complication seen from Zika infections, microcephaly, occurs in a developing fetus in infected pregnant women. Defined as a neurological malformation resulting in decreased head circumference due to death of neural progenitor cells, microcephaly has been implicated as a result of Zika infections in 8 individuals during the French Polynesia outbreak [85]. The outbreak in Brazil resulted in many more reports of congenital microcephaly, prompting the World Health Organization to declare a Public Health Emergency of International Concern on February 1, 2016 [86].

Microcephaly, whether caused by Zika or another infectious disease, has lifelong implications for children of infected mothers, resulting in delays in many milestones, or disabilities [87]. Infection during the first trimester is associated with an increased risk to the fetus [85]. During the outbreak in Brazil, a 20-fold increase in reports of congenital microcephaly cases occurred, with an estimate of 100 in every 100,000 live births [88]. A more recent study used a definition of $\geq 3$ SD below mean head circumference for age and size, rather than the previously used $\leq 33$ cm circumference, and determined the rate of congenital microcephaly to be 28 per 100,000 live births, which still significantly exceeds the 6 per 100,000 in non-affected areas [89].

Other complications include conditions such as optic neuropathy, uveitis and congenital glaucoma resulting in loss of vision [90, 91]. Some of these can occur after birth, some occur in utero [92]. Other neural complications include ventriculomegaly, or enlargement of the lateral cerebral ventricles in the brain, which can hinder brain development via increased cerebrospinal fluid pressure [93, 94]. Lissencephaly, or an absence or insufficiency in the folds and grooves of normal brain anatomy, can also result from infection [95]. This can lead to developmental delays, seizures, and sometimes
death early in life [96]. Finally, those infected as adults can experience negative cardiac manifestations such as atrial fibrillation [97], potentially leading to stroke or heart failure [98].

**Transmission**

Zika virus is transmitted by the mosquito vector, much like other closely related Flaviviruses such as Yellow Fever and Dengue viruses. The predominant mosquito vector for Zika includes multiple *Aedes* spp., including *Aedes aegypti*, *Aedes albopictus*, *Aedes hensilii*, *Aedes africanus*, *Aedes polynesiensis*, *Aedes furcifer*, *Aedes apicoargenteus*, *Aedes luteocephalus*, and *Aedes vitattus* [11, 20, 99, 100]. *A. hensilii* was the predominant species identified during the Yap Island outbreak of Zika, yet was never found with any viral RNA present, so has only been identified as the probable vector [29]. A non-*Aedes* species, *Culex quinquefasciatus*, has also been found to contain Zika virus [101].

*A. albopictus* and *A. aegypti* are the likely vectors for the outbreak in Brazil [102], with both species being present in much of the Southern aspect of the United States, and Hawaii [73]. With previous work demonstrating an incubation period of 10 days in *A. aegypti*, high levels of virus present for 60 days, and transmission up to 72 days after a blood meal, there is a high potential for Zika spreading in the United States in the future [103]. Climate change occurring in a continuously escalating pattern will contribute to an enlarged potential for Zika to cause outbreaks due to the increased range of the *Aedes*
mosquito, as well as the increased breeding season resulting from increasing warmth [104].

Most infections by Zika result from a mosquito bite, but there are other routes of infection. Sexual transmission has been documented from person to person. A scientist returning to Colorado, USA from Senegal was infected with Zika virus and transmitted it to his wife, with symptoms occurring 10 days after intercourse [70]. Other infections have been noted through semen contact [79], with ZIKV RNA being detected in semen for 188 days following infection [105], and infectious Zika virus being detected 30 days after infection [106, 107]. Most samples of semen are negative for ZIKV RNA 4 months after infection [108]. This lead to the US CDC to recommend condom use for 6 months following infection by Zika virus [109]. Viral RNA has been detected in salvia [110], urine [35, 111] and breast milk [112] as well, with virus recovered from urine and breast milk, but no infections have been associated with exposure to these fluids. Zika has also been acquired via blood transfusion [113].

Zika virus has been found in non-human primates, believed to be responsible, along with mosquitos, for maintaining the virus in nature [114]. Multiple other animals have been found to contain ZIKV antibodies, including elephants, sheep and hippos [115], with small mammals also being seropositive [14]. It is likely humans are an incidental host for Zika, though with the likely ability for urban transmission cycles to form without the typical non-human primate reservoirs present [29, 116]. A summary of the ZIKV transmission cycle can be seen in Figure 1-5.
ZIKV Genome Arrangement and Structure

The Zika virus genome consists of a 10.8 kb single-stranded positive-sense RNA molecule with a single 10 kb open reading frame consisting of a single polyprotein. This polyprotein is processed by various viral and cellular proteases after translation into three structural proteins consisting of capsid (C), precursor membrane (prM), and envelope glycoprotein (E). Also processed are seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Figure 1-6) [117, 118]. NS5 is an RNA dependent RNA polymerase responsible for ultimately replicating the viral genome, as well as capping the genome once synthesized. The other nonstructural proteins are responsible for assisting in replication and processing of the viral RNA (vRNA). The virus consists of an icosahedral capsid protein arrangement surrounded by a spherical host-derived lipid envelope with M and E proteins anchored [119] (Figure 1-6).

Zika Virus Entry and Replication

Zika virus utilizes multiple attachment factors to mediate cell adhesion in order to allow for entry, including glycosaminoglycans such as heparin sulfate and chondroitin sulfate [120]. Binding to attachment factors aids in localization to the cell surface but does not initiate cell entry. This is accomplished via binding to surface receptors [121]. C-type lectin receptors such as DC-SIGN can serve as entry receptors via interaction with envelope protein, but the main receptor used for entry is the phosphatidylserine receptor Axl, part of the TAM family [122]. This receptor is bound to the viral membrane through an intermediate, or bridging molecule, known as Gas6 [123]. Because of the wide range of entry receptors, the virus exhibits the ability to infect a multitude of host cells,
including spermatogonia, Sertoli and Leydig cells of the reproductive tract [124], and neural cells from the cerebral cortex and hippocampus [125]. Human cells from skin fibroblasts[122], uterine fibroblasts [126], placental trophoblasts [127], Hofbauer cells [128], endometrial stromal cells [129], and neural progenitor cells [130, 131] are also targets.

Clathrin mediated endocytosis is the main internalization method utilized by Flaviviruses. Following the binding to the receptors, the clathrin-coated pits containing the receptor-virus complex is endocytosed [132]. Once endocytosis has occurred, the clathrin is released from the endosome, and it fuses with an early endosome. This early endosome matures into a late endosome, during which the endosomal pH is significantly lowered [133]. This lowering of the pH initializes a conformational change in envelope protein, forming a homotrimer from the normal homodimers on the surface which results in fusion of the endosomal membrane with the viral membrane, and release of the viral genome into the cytosol [132, 134].

Once released into the cytosol, the positive-sense viral RNA associates with the endoplasmic reticulum (ER) and translation of the viral genome beings. NS5 (the viral RNA dependent RNA polymerase) synthesizes negative-strand RNA from this positive strand, and high copy numbers of positive-strand RNA is made from these negative-strand RNAs [135]. This is dependent on both NS5 and NS3 capping the 5’ end of the vRNA in order for initiation of translation to occur [119, 136]. NS3 also provides helicase activity, along with its co-factor NS2B [137], as well as processing viral polyprotein [138].
Once protein production has commenced, a replication complex forms within the ER in order to aid in genome replication. This is formed from the nonstructural proteins of the virus, with NS4A and B, along with NS1, anchoring the complex to the membrane, NS5, NS3, and interactions with NS2A and B, replicating the genome [139] (Figure 1-7A. The viral genome then gets packaged into virions also containing prM, E, capsid, and envelope. At this stage, prM/E is trimeric, before passing through the Golgi network, which cleaves pr, leaving dimeric M/E on the mature virion, which is then exocyted [117, 121] (Figure 1-7B).

**ZIKV Nonstructural protein 1**

Following ZIKV polyprotein expression and processing, NS1 is glycosylated in the ER, followed rapidly by dimerization [140]. This dimer is partially hydrophilic, and associates with the ER membrane, as well as vesicle packets in order to play a role in anchoring the replication complex [141, 142]. Dimeric NS1 is also trafficked to the cell surface, as well as secreted [140, 143]. Some NS1 is trafficked to the Golgi, where a hexameric species is formed, as well as the carbohydrate moieties being modified. This hexamer picks up a lipid component in its central channel and is secreted from the cell [144, 145] (Figure 1-8).

During the disease process, NS1 can, in some Flavivirus species, accumulate to high levels in circulation. NS1 can provide a protective response in the case of vaccinations and natural infections but can also contribute to the disease process. Dengue NS1, as well as antibodies generated against NS1, contributes to vascular permeability via direct interactions with endothelial cells, platelets, extracellular matrix, integrins and
proteins responsible for blood clotting [139]. ZIKV does not elicit these symptoms, so more work must be done to elucidate how DENV NS1 differs from ZIKV NS1 [146].

**ZIKV Pathogenesis and Immune Response**

Once released by the mosquito into the host, ZIKV infects Langerhans cells, which are dendritic cells in the skin, via the receptor DC-SIGN. Other cells can be infected in the area as well, including fibroblasts and keratinocytes, using the Axl receptor [122]. The virus then travels to the lymph nodes and, after amplification there, is released into the circulation for exposure to other cell types, such as neural cells, myocardium and fetal tissues, presumably through the placenta [147–150]. Infection in the brain shows glial cell and neuronal replication, cellular infiltrates, and brain softening [1, 147, 151]. Infection of the fetus through the placenta has major implications for the future neural health of the fetus, with risks being highest in the first trimester and falling by 46% with each subsequent trimester [152, 153].

Autophagy, the normal lysosomal degradation of specific substrates that occurs continuously in cells, can play a role in ZIKV infection [154]. Autophagic structures have been found in skin fibroblasts infected by ZIKV, as well as autophagy-associated proteins accumulating in infected cells [122]. In this case, autophagy is associated with increased replication of Zika virus, with murine models deficient in autophagy-associated genes showing decreased ZIKV replication in the placenta, implicating autophagy in infection or complications within the fetus. This may implicate impaired autophagy in vertical transmission and congenital malformations associated with ZIKV infections, as inhibition of autophagy inhibits this process in mice [155, 156].
Upon infection, Zika virus elicits antiviral immune responses by stimulating interferon (IFN) Type I pathways. Toll-like Receptors (TLRs) 3 and 7 sense viral RNA along with RIG-1 and MDA5, in conjunction with cGAS that recognizes incorrectly localized dsDNA. Recognition of infection by these stimulate the IFN response and result in Interferon Stimulated Gene (ISG) production [157]. IFN Type I responses are the most predominant during ZIKV infection, with Interferon Response Factors (IRFs) 3, 5, and 7, alongside NFκB, resulting in generation of IFN Type I [158]. IFN Type I then results in signaling to generate ISG production through the action of STAT1 and STAT2 [159] (Figure 1-9).

ZIKV can evade host immune responses via the action of several viral nonstructural proteins. NS4A blocks the signaling from MDA5 and RIG-1 [160]. NS1, NS2A, NS2B, and NS4B can block kinase activity that activates IRF transcription, and along with NS4A and NS5, can block activation of IFN type I transcription via lack of IRF3 action [161–163]. NS1 and NS2B3 can block action and downstream effects of cGAS [164, 165]. NS2B3 can block activation of JAK1, and NS5 can block activation of STAT1 and STAT2, along with targeting STAT2 for proteasomal degradation [162, 166, 167]. These effects can make it more difficult to control viral replication [157]. A summary of these evasion strategies can be seen in Figure 1-9.

Activation of the adaptive immune response likely plays a role in resolving infection by ZIKV. Non-human primate studies indicate a role for CD4+ and CD8+ T cells in reducing replication during infection [168]. Murine studies that abrogate the IFN response during infection show the adaptive response is critical in keeping ZIKV in check, as well as keeping it from spreading to gonadal tissues and neural tissue. During
pregnancy, anti-ZIKV CD8+ T cell activation is weaker, potentially facilitating vertical transmission [169]. This indicates a necessity of the adaptive response in preventing the worst sequelae of infection, as well as providing secondary protection [170, 171].

Antibody responses play a significant role in Flavivirus infections, including ZIKV and DENV. Infection with either results in neutralizing antibodies largely targeting the E protein [172], with the overall antibody pool also targeting prM and NS1 [173]. During a primary infection, the antibody seen first is Immunoglobulin M (IgM), which then drops in levels along with a rise in Immunoglobulin G (IgG) later. These play a role in protection, pathogenesis, and can be used for diagnostic purposes [174, 175] (Figure 1-10). During a primary DENV infection, neutralizing IgG develops to that serotype that generally prevents recurrence. A secondary DENV infection, coming from a separate serotype, then elicits that same neutralizing response against the first. This response is subneutralizing for the second serotype, and can elicit an antibody dependent enhancement (ADE) of the infection, enhancing replication and potentially resulting in Dengue Hemorrhagic Fever, and Dengue Shock Syndrome [176, 177].

Previous exposure to Dengue can influence the antibody response an individual will mount during a Zika virus infection. A Zika infected individual who is DENV experienced will generate antibodies that resemble a secondary Dengue infection [178]. Cross-reactive B cell populations are higher early during ZIKV convalescence, with conversion to a more ZIKV specific population later [60]. Pre-existing immunity to DENV may influence congenital outcomes in pregnant women, with lower incidence of abnormal birth events for DENV IgG-positive women [179] and immunity to more than one DENV serotype is protective against Congenital Zika Syndrome (CZS) [180].
Antibodies may also play a role in ZIKV infiltration of the placenta, with Hofbauer cells being the main resident cell type infected by ZIKV. Cross-reactive antibodies from DENV result in placental transcytosis of ZIKV in an FcRn-dependent manner [181].

ZIKV exposure may also alter virulence during DENV infection. Studies in mice show increased replication and mortality to ZIKV disease when immunocompromised mice are treated with DENV-experienced human serum [182, 183], though the relevance of this has been called into question [175]. Exposure to ZIKV can enhance DENV virulence as well, shown by increased DENV mortality in immunocompromised mice, newborn mice, and nonhuman primates who were Zika experienced or were administered Zika antibodies, including those maternally acquired [184–186]. Since DENV and ZIKV co-circulate, more information is needed in order to fully establish the significance of prior exposure to either virus during infection with the co-circulating species [175].

**Evolution of Zika Virus**

ZIKV strains identified prior to 1966 were solely African strains. The first Asian strain of the virus was identified in Java, Malaysia in 1966 [20]. Since then, there have been two lineages identified: African and Asian [187], though there may be three when taking E and NS5 variations into account [188]. Asian lineages account for the epidemics associated with the Pacific Island outbreak in 2007, and all other outbreaks since [189]. Though ZIKV has a low mutation rate, which may account for its lack of human infection prevalence before 2007, there are multiple differences between the two lineages [116]. The mutation rate increased during the outbreak in the Americas, though that may be short lived due to outbreak conditions [190].
The main differences between the African and Asian lineages lies in the proteins NS1 and NS5, though strain variation within each lineage are distributed across the genome [190–192]. An NS1 mutation (A188V) thought to drive the outbreak due to an increase in infectivity was found to be present in South Asian strains before the outbreak, though this substitution has become fixed in the viral population since [191]. The NS5 mutation (M60V) is only found in outbreak strains, suggesting a possibility of it playing a role in emergence [192]. A prM mutation, S139N, may contribute to the increase in the ability of the strains from the outbreak to cause fetal microcephaly, with envelope mutations also contributing to neurovirulence [193, 194].

Prevention, Therapeutics, and Vaccines

Prevention

The best way to prevent contact with Zika Virus is to avoid mosquito bites. Vector control can consist of long sleeved clothing, insect repellent such as DEET, window screens, and draining standing bodies of water [195]. A new approach may be useful in the future, with genetically modified mosquitos being released that produce larvae that cannot reach adulthood. This may be an effective vector control tool to lower population levels, due to their ability to compete with wild population members, as shown in Brazil to control A. aegypti-vectored Dengue Fever [196]. Use of insect repellant for 14 days after going to an affected area is encouraged, as this may prevent local mosquito populations in Zika-free areas from acquiring the virus [197].

Avoiding spread of the virus through non-vector means should be attempted, as well. Any sexual contact with males should be with the use of condoms for the six month
period following virus exposure [109]. Pregnant women who have traveled to these areas should be screened for ZIKV exposure and, if positive, undergo fetal ultrasound every 3 to 4 weeks during pregnancy [198].

**Therapeutics**

There are currently no approved treatments for Zika virus disease, though work is being performed in the area. Multiple nucleoside inhibitors (e.g. Sofosbuvir) already approved by the FDA have been tested as potentially effective by causing premature termination of synthesis of vRNA [199, 200], along with entry inhibitors [118, 201]. Interferon treatment may prove effective in limiting ZIKV replication as well [202]. Small molecules are being investigated with effects such as inhibiting viral entry by preventing acidification of the endosome [203]. Drugs such as chloroquine and ivermectin have shown anti-ZIKV activity as well, with some (e.g. Daptomycin) being of potential use during pregnancy [124].

Another route of potential treatment development has been through the use of antibody therapy. Targeting of the envelope protein may have protective effects, as long as ADE is not observed to a significant degree. The fusion loop has been targeted, which prevents entry of the virus [204]. Another avenue of therapy is anti-envelope antibody directed toward the intra-dimer interface, which prevents rearrangement of envelope and blocks ZIKV entry [205]. Antibodies that target NS1 have the potential ability to provide protection without the subsequent danger of ADE [206].

**Vaccines**
Though there is no approved vaccine, there are multiple currently in development. These are comprised of an assortment of targets: Three candidates are whole virus, six are prME, two are E alone. Others are VLP-DNA, Dengue recombinant inactivated, and recombinant inactivated adenovirus [118, 207].
Figure 1-1: Estimated Range of Zika Virus Before 2007.

Before 2007, Zika Virus was only known to occur in Africa and Asia. Taken from [22].
Figure 1-2: Tracking the Pacific Epidemiology of Zika Virus.

Zika Virus travel through the Pacific region, beginning with the introduction of Zika to the Yap State in 2007. Taken from [65].
Figure 1-3: Distribution of Zika Virus Infections during the 2015-2016 Global Pandemic.

The numbers listed represent early estimates of cases. The virus circulated from the Pacific Islands into South America and eventually back to Africa, causing much of its morbidity in Brazil and other South American countries. Taken from [65].
Figure 1-4: Map showing co-circulation of Zika, Dengue and Chikungunya Viruses

Viruses with symptoms similar to, and easily confused with, Zika infection circulate in many of the same areas, which can lead to misdiagnosis. Taken from [78].
Zika is maintained in the sylvatic (enzootic) cycle between non-human primates and mosquitos. Transmission is possible from mosquito to human, as well as between humans. Transmission may be possible between non-human primates as well but has not been observed. Human epidemics can start when there is transmission to a human and there is sufficient human presence to transmit between only humans and mosquitos.

Taken from [65].
Figure 1-6: Genome Arrangement of Zika Virus

The genome consists of 5’ (107 nucleotides) and 3’ (428 nucleotides) untranslated regions (UTRs) and a polyprotein that is processed into 3 structural proteins and 7 nonstructural proteins. Taken from [118].
Figure 1-7: Replication of Zika Virus

A: Replication of Zika virus genome is located within the ER, where the replication complex is located within a vesicle packet. This complex consists of host factors and viral nonstructural proteins. Taken from [139]. B: Process by which Zika virus is replicated from entry to exocytosis of mature virions. Taken from [121].
**Figure 1-8: Cellular Production of NS1**

NS1 is initially produced in the ER, where it exists shortly as a monomer (1). It is glycosylated via high mannose carbohydrate residues (2) and rapidly dimerizes to become hydrophobic and associates with the membrane (3). This, along with a subset of NS1 that is GPI anchored (4) is trafficked to the cell surface, the Golgi (5), or to vesicle packets for replication (9). In the Golgi, the high mannose CHO is trimmed and converted to a complex CHO, and the NS1 becomes hexameric (6). This hexameric NS1 picks up a lipid cargo (7) and is secreted from the cell (8). This results in both dimeric and hexameric NS1 being present in extracellular spaces during infection. Taken from [139].
Figure 1-9: Immune Evasion by Zika Virus

Strategies by Zika Virus to evade the immune response consist of evasion of IFN Type I induction via blocking of RIG-1/MDA5/cGAS action, as well as downstream blocking of transcription of IFN genes. Evasion of IFN signaling can occur through the blocking of STAT1 and STAT2 action, as well as degradation of JAK1 and STAT2. Taken from [157].
Figure 1-10: Timeline for Zika Virus Diagnostic Methods

Diagnostic techniques for Zika Virus include cell culture (CC), Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Enzyme Linked Immunosorbent Assay (ELISA) for IgM and IgG, Immunofluorescent Assay (IFA) and Neutralization Testing (NT). Given are the tissue types and time frames relevant to the diagnostic techniques. Taken from [197].
CHAPTER 2: The effect of mutagenesis of conserved regions of NS1 on binding of antibodies from ZIKV patient serum

Introduction

Infection with Zika virus is detected using various methods in common use in clinical laboratories. Serological assays, such as IgG and IgM detection, are commonly used as a diagnostic for Flavivirus infections. IgM, the first antibody seen during an infection, becomes elevated as soon as 3 days post onset of symptoms [27, 208]. IgM may continue to be present for 3 months post infection [208–210]; therefore, a positive result beyond those 14 days cannot infer acute infection. A collection date prior to 7 days post symptom onset does not indicate a negative test if IgM titer is the sole criterion used [211]. Testing of IgM coupled with nucleic acid tests is preferable within 7 days [211], though most reports indicate lack of RNA found beyond 5 days in serum [76, 212].

IgG levels increase after IgM levels, with some patient samples being IgG positive 10 days post symptom onset in an individual with no previous Flavivirus exposure [51, 208]. These levels are elevated from months to years post infection and cannot be used to determine acute infection status, though may be useful for recent infection status (not acute) if the useful window for IgM detection has lapsed, though may also indicate past infection if taken under 45 days post onset [213, 214]. The recommended collection of samples consists of serum collected two weeks apart to measure seroconversion from IgM to IgG specific to ZIKV. This coupled serological data may help with potential cross-reactivity issues present in Flavivirus diagnostic testing [27].
Plaque Reduction Neutralization tests (PRNTs) are used in many laboratories to discern between related Flavivirus infections and are considered the “gold standard” for Flavivirus diagnostics [27, 29, 81, 215–218]. They are recommended by the CDC for use in conjunction with other serological tests to determine infection status [211] (Figure 2-1). These tests are time, and labor, intensive, often requiring a week or more to complete. They require individuals highly trained in their use and are low throughput, thus they are not used everywhere [215]. A newer method of PRNT for ZIKV has been developed using a luciferase reporter-encoded Zika virus that utilizes a 96-well plate approach, thus increasing its throughput and decreasing both interpretation time and supply usage [219, 220]. Typically, laboratories will use a ratio of ZIKV PRNT\textsubscript{90}( the amount of serum needed to reduce virus plaque number by 90%) to DENV PRNT\textsubscript{90} of at least 4 to determine specific ZIKV infection [29]. Depletion of IgG in a patient sample before assay increases the ability of the PRNT to discern between recent and past infections [221].

Cross-reactivity between the various Flaviviruses is a common problem for diagnostics, especially ones used for serological analysis such as immunoassays [222–226]. This causes false positive and false negative interpretations in detection of Flaviviruses, as well as playing a role in disease severity in cases of Dengue Fever [227]. The need for sensitive diagnostic tests is high in the case of vaccine trials, and the need for sensitive and specific tests is crucial for clinical needs. Assays need to span the breadth of antibody type and functionality; however, in order to characterize the protective responses elicited by vaccination and natural infection [222].
Diagnostic assays early during the 2015-2016 Zika epidemic were sparse, with laboratories specializing in Flaviviruses possessing in-house ELISAs to detect antibodies to the virus, often with middle-of-the-road specificities of about 85% [228]. Euroimmun IgM and IgG ELISA tests were available commercially rapidly following the outbreak, with the IgM sensitivity of 37% [229], 60% [230] and 12.5% [231] in various studies, which were improved when combined with the IgG ELISA data [229]. A specificity of 98.3% was recorded for acute phase specimens. The Euroimmun IgG assay specificity was low, at 32% [231]. Many of these diagnostics were not commercially available at the time the epidemic began, but have been made available in some cases, as well as multiple other assays now being available for purchase.

There are currently five serological assays (IgM and IgG) available commercially that have FDA Emergency Use Authorization (EUA), allowing them to be used during an ongoing public health emergency, even without formal final approval [222]. One of the kits uses inactivated virus, the CDC MAC-ELISA, captures IgM from the serum before detecting via NS1-based monoclonal antibody [228]. Another, which recently gained full FDA approval [232], the InBios ZIKV Detect 2.0, uses the same approach using the ZIKV envelope protein. The remaining tests, including the Advia Centaur, DPP and Liaison XL tests utilize detection of antibodies to NS1 protein (Table 2-1) [222]. The Advia Centaur and Liaison XL tests are also approved for marketing within the US by the FDA [232]. These are all IgM detection-based assays, as no assays for detection of IgG have been validated yet [222].

One major assay used before and during the 2015-2016 epidemic was the CDC MAC-ELISA. This assay has been the comparison used for the development of multiple
more recent assays, often with better performance. This assay performed similarly to the predecessor assay to the InBios EUA assay, with a specificity of 100% for PRNT-confirmed infections for patients with primary ZIKV exposure. In contrast, in cases of previous DENV exposure, it drops significantly, and has trouble discriminating between ZIKV and DENV infections [229]. The revised assay, which had EUA status, is now FDA approved for marketing in the US. The InBios ZIKV Detect 2.0 showed increased ability to detect non-ZIKV IgM positive samples, with 95% specificity for ZIKV/DENV seronegative samples and PRNT confirmed non-ZIKV samples. Limited testing has occurred to date; however, and more evaluation needs to be done to determine the true potential of this test [233]. The need still has not been met for adequate, approved, and available diagnostics that have been tested thoroughly.

We set out to design a diagnostic test for Zika virus using NS1 protein with reduced cross-reactivity. We utilized site-directed mutagenesis to modify Zika NS1 in select areas to reduce sequence similarity with related Flaviviruses, such as Dengue, West Nile and Yellow Fever. Areas that are exposed on the outer surface of NS1 were targeted for mutation as immunodominant regions, or regions containing epitopes preferentially targeted for antibody production during infection (Figure 2-2A, B). One area, in region 2, was found to contain an immunodominant region in both natural infection and vaccination, and mapped to the wing domain with a conserved amino acid sequence from aa 114-119 (Figure 2-2A, B) [234–236]. Monoclonal antibodies against this region are capable of inducing protection across multiple species within Flaviviridae [226].

Immunodominant region 4 in the NS1 protein corresponds to an area of NS1 that is exposed above the beta-ladder on the protein surface and is also an area of high
antibody activity. This region elicited strong serum binding responses upon vaccination of mice with NS1 of DENV2 plus adjuvant, as well as natural infection with DENV2, and cross-reacted with DENV3. This points to this region being conserved among some DENV strains, and makes it a target for removal of cross-reactivity in diagnostics. [236].

We hypothesized that mutation of NS1 to remove these amino acid sequences, conserved among Flaviviruses related to ZIKV, would decrease the potential for cross-reactivity to non-ZIKV species for diagnostic purposes. We investigate this possibility via site-directed mutagenesis of the above regions, followed by employing NS1-based ELISA assays to determine differences in binding of serum collected from ZIKV-infected patients from the 2015-2016 epidemic in Colombia and the Dominican Republic. We demonstrate the differences elicited by these mutations in this chapter, as well as characteristics of serum collected from both of these countries.

Materials and Methods

Zika Gene Synthesis and PCR amplification

The full length Zika NS1 gene (NCBI Accession Number LC002520) was synthesized in pUC-IDT (Amp) (Integrated DNA Technologies, Iowa, USA). The NS1 gene was then amplified using primers NS1 WT Forward (Table 1) with the underlined upstream BamHI site and NS1 WT Reverse (Table 1) with the underlined HindIII site added downstream, as well as an E. coli STOP codon. PCR was performed using a reaction containing 1 unit Phusion polymerase (New England BioLabs, Massachusetts, USA), 200 µM dNTPs, 0.5 µM forward and reverse primers, 1X Phusion HF Buffer, and 3% DMSO
using an ABI thermal cycler (Applied Biosystems, California, USA). Reaction conditions consisted of an initial denaturation at 98ºC for 30 seconds, followed by 35 cycles of denaturation at 98ºC for 10 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 30 seconds, with a final extension of 72ºC for 10 minutes. DENV2 NGC NS1 (NCBI Accession Number KM204118) was synthesized under the same conditions, except usage of NotI instead of HindIII restriction enzyme.

Cloning of NS1 into Expression Vector and Expression Screening

The full-length amplified Zika NS1 gene was ligated into the expression vector pET-45b(+) (Novagen, USA) using the BamHI and HindIII restriction sites. This results in an N-terminally hexahistidine-tagged Zika NS1 protein under the control of the T7 promoter with minimal vector-encoded amino acids. The plasmid was transformed into Rosetta 2 (DE3) E. coli cells and plated on LB agar containing 100 µg/mL carbenicillin and incubated overnight at 37ºC. Colonies were screened by PCR amplification of gene insert followed by confirmation via restriction digestion using BamHI and HindIII. Colonies confirmed by digestion were further confirmed by sequencing of the insert using primers for T7 promoter and T7 terminator sequences (GeneWiz, New Jersey, USA). Colonies from a confirmed plasmid were screened for expression levels by plating on LB agar containing 100 µg/mL carbenicillin and growing in LB broth. At an OD$_{600}$ of 0.7, expression was induced using different concentrations of IPTG (0.1, 0.25, 0.5 and 1 mM) and pelleted after a period of 3 hours. Colonies were diluted to 100x the final OD$_{600}$ with water and lysed with addition of Laemmli sample buffer (Bio-Rad, California, USA) to working concentration, followed by brief sonication. Lysed samples
were analyzed by SDS-PAGE followed by total protein staining via Coomassie Blue G250.

**Mutagenesis of Zika rNS1 Protein**

Site-directed mutagenesis was performed to generate mutated rNS1. Primers were designed to mutate residues in regions of NS1 with high sequence similarity to other members of *Flaviviridae*. PCR was performed using a reaction consisting of 20 ng wild-type plasmid template, 0.5 µM forward and reverse primers, 1X Pfx AccuPrime Reaction Mix, 2.5 units AccuPrime Pfx polymerase (ThermoFisher, USA). Reaction conditions consisted of 1 cycle of initial denaturation of 95°C for 5 minutes, 12 cycles of denaturation at 95°C for 30s, annealing at 56°C for 1m, and extension at 68°C for 8 minutes. This was followed by a final annealing step of 56°C for 1m, and a final extension of 68°C for 30m. The PCR products were treated with DpnI (ThermoFisher, USA) for 1 hour and purified using PureLink Quick PCR purification Kit (Invitrogen, Germany). Purified products were then transformed into JM109 (Promega, Wisconsin, USA) or DH5α (Invitrogen, USA) cells via heat shock and plated for overnight incubation on LB agar containing 100µg/mL carbenicillin at 37°C. Colonies were picked and screened by PCR amplification as per the protocol for wild-type NS1.

**Optimization of Expression Conditions and Protein Localization**

Glycerol stock from the highest expressing colony was inoculated into LB and grown to an OD$_{600}$ of 0.7. Appropriate concentrations of IPTG were added and the media was placed at differing temperatures (25, 30 and 37°C) and sampled at various times (0, 1, 2,
3, 4, 5, 6 and 16 hours). Following appropriate times, cells were pelleted and frozen at -20°C. Different conditions were analyzed via SDS-PAGE after lysis as before to determine expression efficiency. To determine location of the recombinant protein, the pellet from an expression culture was lysed with water, followed by centrifugation at 10,000xg for 40 minutes. The resulting fractions were analyzed by SDS-PAGE.

**rNS1 Production and Solubilization**

1 L cultures of recombinant NS1 were grown to an OD\textsubscript{600} of 0.7 and induced with appropriate amounts of IPTG. After induction for previously determined amounts of time, cultures were harvested at 10,000xg for 10 minutes. Pellets were suspended in cell lysis buffer (10mM Tris, 10mM EDTA, 100mM NaCl, 100µg/mL Lysozyme, 1% Triton X-100, and protease inhibitor cocktail) for 1 hour at 4°C. Pellets were sonicated for 5 minutes at 40% power (10s on/30s off). The solution was centrifuged at 10,000xg for 40 minutes and resuspended in IB wash buffer (10mM Tris, 200mM NaCl, 1M Urea, pH 6.0). Suspension was sonicated briefly and rocked for 1 hour at 4°C and centrifuged at 10,000xg for 40 minutes. The resulting inclusion body was resuspended in IB solubilization buffer (10mM Tris, 500mM NaCl, 100mM NaH\textsubscript{2}PO\textsubscript{4}, 8M Urea, and 10mM Beta-mercaptoethanol, pH 8.0) overnight at 4°C after a brief sonication. The resulting solution was centrifuged at 3,220xg for 30 minutes and used for purification of rNS1.

**Purification by Immobilized Metal Affinity Chromatography**

The addition of the N-terminal His\textsubscript{6} tag allowed for purification of rNS1 by immobilized metal affinity chromatography (IMAC) using HisPur Ni-NTA spin columns (Thermo
Scientific, USA). The column was equilibrated with 2 bed volumes of IB solubilization buffer and the solubilized rNS1 was loaded onto the column. The protein was allowed to bind for 1 hour at room temperature on a rotary shaker. Unbound protein was allowed to flow through via gravity, and the resin was washed with 10 bed volumes of wash buffer (500mM NaCl, 100mM NaH$_2$PO$_4$, 8M Urea, 10mM beta-mercaptoethanol, 20mM imidazole, pH8.0). Protein was eluted by passing 10 bed volumes of elution buffer (500mM NaCl, 100mM NaH$_2$PO$_4$, 8M Urea, 10mM beta-mercaptoethanol, 250mM imidazole, pH6.0) over the column. Fractions were collected for later SDS-PAGE analysis, and pure eluted fractions were pooled together for refolding.

**Refolding of recombinant NS1**

The concentration of the pooled protein was adjusted to 100µg/mL in IB solubilization buffer and added to a 10 MWCO dialysis flask (Thermo Scientific, USA). The protein was refolded at 4°C over the course of 3 days in refolding buffer (50mM Tris, 0.4M L-arginine, 1mM reduced glutathione, 0.1mM oxidized glutathione, pH 8.0) in a 10-fold volume excess, changing refolding buffer once a day. Dialysis was subsequently performed into PBS (pH 7.4) using 3 changes of buffer, with a final overnight exchange.

**Western Blot analysis of refolded protein**

Refolded rNS1 was analyzed by western blot by first electrophoretically separating on SDS-PAGE gels and transferring to nitrocellulose via wet transfer by XCell II Blot Module (Thermo Scientific, USA). The membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in PBS and 0.05% Tween-20 (PBST). The blots
were incubated with mouse anti-His<sub>6</sub> monoclonal antibody (GE Healthcare, USA) or anti-ZIKV NS1 polyclonal antibody (GeneTex, USA) overnight at 4°C with gentle rocking, followed by washing with PBST three times for 5 minutes each. Incubation with anti-mouse HRP antibodies followed for one hour at room temperature, and another wash with PBST three times for five minutes each, followed by development with Novex ECL chemiluminescent reagent (Invitrogen, USA).

**Evaluation of rNS1 via Antibody Capture ELISA**

Determining the viability of an NS1-based antibody capture diagnostic was done by Enzyme-Linked Immunosorbent Assay (ELISA). Purified and refolded rNS1 proteins were diluted to 10 µg/mL in CBC and coated on 96-well Nunc Maxisorp plates (100 µL/well) and incubated overnight at 4°C. Wells were blocked with 1.5% BSA in PBS for 4 hours at room temperature, and dried overnight at room temperature before being stored at 4°C until use. Serum samples were diluted 1:100 and added to antigen-coated wells for 1 hour at room temperature, followed by washing with PBS containing 0.05% Tween-20 (PBST) three times. Washed wells were incubated with anti-IgM HRP or anti-IgG HRP at appropriate dilution for 1 hour at room temperature, followed by three PBST washes. Color was developed by 100 µL/well TMB-H₂O₂ substrate (Sigma, USA) for 20 minutes, and the reaction was stopped by the addition of 100 µL 0.36N H₂SO₄. Absorbance was measured at 450nm.

**Competition ELISA**
To evaluate the differences in binding affinity of patient antibodies to Dengue and Zika rNS1 proteins, competition ELISA was performed. For each well of ELISA, 100 µL of a 30 µg/mL solution of Dengue or Zika wild type rNS1 was incubated with patient serum (1:100 dilution) overnight at 4⁰C. Afterward, the serum was evaluated for antigen binding by ELISA using plates coated with the opposite species’ antigen using the protocol noted previously. Non-competitive samples were evaluated at the same time as competitive samples for comparison.

**IgG Avidity ELISA**

Antibody avidity for ZIKV rNS1 was evaluated using the antibody capture ELISA as per mentioned protocol except as follows. Plates were arranged with samples mirrored in the top and bottom halves. After the one hour sample incubation and wash, one half of the plate was incubated with 7M Urea while the other half was incubated with PBS for ten minutes at room temperature. The plate was washed again, and the rest of the ELISA protocol was continued unaltered.

**Generation of ZIKV rNS1 antiserum**

Antiserum against ZIKV rNS1 proteins was generated via immunization of goats using all three of the rNS1 constructs (ProSci, CA, USA). Goats were immunized with 1.5 mg of each protein four times at intervals of three weeks. A pre-immunization bleed was collected plus 4 post-immunization bleeds. Two goats were used for each protein, with the last bleed for each protein being used for experiments.
Evaluation of ZIKV rNS1 antiserum binding

Antibody binding of goat serum to rNS1 proteins was evaluated via antibody capture ELISA per previous protocol except as follows. Samples were diluted in sample diluent 1:1,000 followed by 1:5 in sample buffer. Both goats from each protein immunization were combined before dilutions. Pre-immunization bleeds from the same individuals were treated identically and run side-by-side with post-immunization bleeds for background comparison.

Results

Beginning our investigation into diagnostics of Zika virus necessitated production of recombinant non-structural protein 1 (NS1) of the virus, as well as the related Dengue Virus. The sequence for NS1 was amplified and cloned into pET-45b(+) using restriction enzymes encoded via primers (Table 2-2). This vector allows for addition of an N-terminal His$_6$ tag for purification. Added restriction sites differ per protein based on internal sequences that may match certain enzymes. Digestion via SacI enzyme after ligation of NS1 sequence into the expression vector increased the number of NS1-positive colonies during screening, due to SacI removal during initial vector digestion (Figure 2-3).

Mutations of the sequence were introduced as changes of amino acids internal to the ZIKV NS1 sequence into triple alanine repeats in order to minimize impacts on folding of the protein structure. This was designed to lower reactivity of the protein to antibodies specific to non-ZIKV and increase specificity of a diagnostic toward ZIKV. Mutations were targeted toward sequences in the protein in immunodominant regions.
(Figure 2-2A, B) that also shared sequence identity with related Flaviviruses (Figure 2-2C).

Screening of these colonies after plating yielded good numbers of positives (Figure 2-4A), with gene insert being confirmed via Sanger sequencing. After induction of protein production, multiple colonies were screened for expression by SDS-PAGE, with expression appearing to be near peak at 4 hours post induction (Figure 2-4B). Upon expression, localization was confirmed by sonication of an induced pellet followed by centrifugation to pellet insoluble mass and resuspension of that mass. This revealed the vast majority of our protein to be in the insoluble fraction (inclusion body).

Solubilization of the inclusion body was performed using urea-based buffers in order to denature the protein and pull it into solution. Following the protein through multiple purification steps shows the protein is not soluble in either the initial Triton X-100 solubilization buffer, or 1M urea washes, but must be solubilized in 8M urea (Figure 2-5A).

Ni-NTA purification of protein based on the added hexahistidine tag was performed on the 8M urea soluble fraction with washes using 20mM imidazole to eliminate non-specific binding. Elution was performed with 250mM imidazole to outcompete the binding of the protein to the resin and allow the protein to flow through the column. This resulted in protein that was over 90% pure as assessed on SDS-PAGE via coomassie staining (Figure 2-5B).

Once solubilization of the protein was complete, refolding was performed in order to return the protein to its native form. This was accomplished via dialysis in refolding buffer designed to introduce the protein into mildly oxidizing conditions using a 10:1
ratio of reduced glutathione and oxidized glutathione in order for disulfide bonds to form as folding occurs slowly during the process. The buffer also contained L-arginine in order to counteract the phenomenon of aggregation commonly seen during refolding. Aggregation was also combated by diluting the protein to a low concentration of 100 µg/mL in denaturing buffer before addition to the dialysis membrane.

Once folded to its native form, we analyzed the rNS1 proteins by Western Blot in order to assess size. Probing used an antibody directed at the N-terminally introduced His$_6$ tag used for purification. This blot revealed a main fragment at just above 40kDa, as expected, but also showed a lower band at slightly under 20kDa (Figure 2-6A). Probing from the C-terminal end, with no tag addition, we find no such additional fragment (Figure 2-6B).

In order to assess the possibility of incorporation of this fragment into the structure of the refolded protein, we sought to separate the fragments via size exclusion chromatography. The A280 analysis of protein elution from the column over time, indicating separation of proteins based on size, shows no significant separation of lower and upper bands of the protein (Figure 2-7A). Attempts to separate these bands via centrifugation-based filters of 30kDa (almost two times the size of the lower fragment) indicate no flow of the fragment through these filters (Figure 2-7B).

Antigenic recognition of the protein was assessed via construction of an antibody capture ELISA assay. This assay utilizes an antigen (in this case rNS1) to bind antibodies in a sample, e.g. patient serum. These antibodies are then detected in order to evaluate the likelihood of previous exposure to the infectious disease of which the antigen under analysis is part. This process relies partly on how much native character is
present in the protein utilized for analysis. For this analysis, we utilized protein refolded via the previously mentioned method. Worth noting is our attempt to utilize the rapid refolding method using sarkosyl as a detergent for solubilization of the inclusion body. This resulted in good solubilization of the protein, though the resulting signals arising from this protein were insufficient for use in the ELISA assay.

Before patient samples were utilized on the antibody capture ELISA, we sought to optimize the conditions of the assay, in order to maximize the signal to noise ratio in the data arising from high background. Conditions important in this assay that we optimized were buffer used for coating the protein onto the plate, plate type used for coating, and protein amounts coated onto the plate. Coating protein onto Maxisorp plates using carb-bicard (CBC) buffer showed a higher ratio signal generated from a positive serum sample versus signal generated from a negative sample than either Polysorp or Medisorp plates (Figure 2-8A). Maxisorp plates are designed to bind molecules with high hydrophilicity, such as antibodies and glycoproteins, while Medisorp plates are designed with a slightly hydrophobic coating to bind a range of molecules. Polysorp plates bind very hydrophobic molecules well, such as something with high lipid content.

Optimization for coating amount consisted of coating rNS1 onto Maxisorp plates in varying amounts from 10 µg down to 0.125 µg per well. The 5 µg/well coating amount performed the best, with a 475% signal to noise ratio of positive/negative serum samples, versus a bit over 400% for 10, 2.5 and 1.25 µg/well coating amounts. The 0.125 µg/well coating performed barely above the buffer-only wells (Figure 2-8B). In order to ensure protein is maintaining stability while coating onto the plate, we coated 1µg/well of protein onto Maxisorp plates in various coating buffer overnight. The buffers chosen
were at least 1 pH unit over the isoelectric point of the protein (5.9) in order to maintain the structure. Carb-bicarb buffer coating resulted in higher S/N than HEPES, PBS or Tris-based buffers, though none were significantly detrimental to protein reactivity (Figure 2-8C).

Performing the optimized antibody capture ELISA on serum samples from patients with suspected cases of Zika virus infection was performed in order to determine circulating levels of IgG and IgM. High levels of IgM antibody indicate an infection or exposure recently, while high levels of IgG indicate an exposure months to years ago. We acquired serum samples from patients during the 2015-2016 Zika outbreak in Colombia and the Dominican Republic, as well as panel of IgM positives and negatives (SeraCare, USA). The samples from Colombia had higher IgM titers than the Dominican Republic, with 50% of samples being positive from Colombia (Figure 2-9). IgM detection in the Dominican Republic serum did not increase upon removal of the IgG via protein G (data not shown). In comparison, nearly 100% of Colombia samples were positive for ZIKV NS1 IgG antibody (Figure 2-10). This is in contrast to the Dominican Republic samples, which had a positive IgM in only 30% of samples (Figure 2-11), and a positive IgG in 68% of samples (Figure 2-12). Due to low numbers of well characterized samples, cut-off was set as the limit of detection, and calculated as mean plus 1.96* standard deviation of the negative control.

To determine if the lower western blot band in the wild type NS1 contributed to differences in serum reactivity, reactivity comparisons were made by calculating the ratios of protein binding of the mutants to that of the wild type ZIKV NS1 protein, with a ratio of over 1 indicating increased binding to the mutant, and ratio of under 1 indicating
increased binding to the wild type protein. IgG binding ratios for the Colombia samples show no differences in binding between wild type and either mutant, and IgM binding ratios indicate increased binding to the wild type protein. Samples from the Dominican Republic show a different trend between mutants, with IgG ratios showing increased binding to the ZIKV NS1 W117A, G118A, K119A mutant and decreased binding to the ZIKV NS1 H227A, T228A, L229A mutant (Figure 2-13A). Both mutants bind serum IgM more readily in the Dominican Republic samples than the wild type (Figure 2-13B).

Assessing the contribution of the lower band of protein in the wild type preparation of rNS1 to antibody development was performed by generation of antiserum from goats by immunization with ZIKV NS1 wild type and mutant proteins. Antibodies developed against each protein were assayed for binding via antibody capture ELISA using pooled serum from two goats immunized for each protein separately. Antibodies generated from wild type rNS1 binds wild type and mutant protein less readily than those generated from mutant proteins (Figure 2-14A, B, and C). All proteins bind DENV NS1 much less than ZIKV NS1, with over 4 times less signal generated from the A450. The trend of less binding occurring from wild type protein antiserum continues in the DENV plate, however (Figure 2-14D).

Serum samples from the Dominican Republic possessing significantly lower circulating IgM (Figure 2-15A) was investigated. Assessing the binding avidity (functional affinity) of circulating IgG to ZIKV NS1 wild type was performed by ELISA with and without incubation in 7M urea in order to dissociate any IgG not possessing high avidity to rNS1, similar to an assay performed to distinguish primary and secondary Dengue infections [237]. In order to compare binding avidities, the ELISA was
performed on both ZIKVNS1 WT and DENV NS1 WT, with an avidity index being calculated as the binding ratio of non-urea A450 measurements and A450 measurements from urea-incubated wells of the same protein and sample multiplied by 100. For example, an avidity index of 100 indicates equal binding of the urea and non-urea incubated antibodies, equating to the highest avidity IgG. Comparing the avidity indices of ZIKV and DENV NS1 indicates significantly higher avidity IgG for DENV rNS1 than that of ZIKV (Figure 2-15B).

Antibody binding preference was also investigated via the competition ELISA. Serum samples incubated in sample buffer in parallel, with one set of samples spiked with either ZIKV or DENV rNS1 WT was incubated overnight, followed by ELISA performed on 96-well plates from the species opposite the one spiked (e.g. the ZIKV-spiked samples were run on DENV plates). Binding that is significantly decreased upon spiking indicates antibodies with a binding preference for the other species. We see a significant decrease in binding of serum antibodies from Dominican Republic samples in those spiked with DENV protein versus those spiked with ZIKV protein (Figure 2-15C). We did not see a significant decrease in binding when we spiked with DENV NS1 and ran samples on a ZIKV plate (Figure 2-15C). Though the data is not shown, we saw no significant differences when spiking proteins of the same species as those of the plate assays (e.g. running ZIKV-spiked samples on ZIKV plates).

Discussion

The results presented in this chapter demonstrate the capability of E. coli to efficiently produce ZIKV and DENV nonstructural protein 1. The protein is not soluble
when produced using this method but can be refolded upon solubilization via 8M urea buffer, and remains stable in PBS. Mutation of rNS1 resulted in more of the protein solution containing full-length protein than wild type, as demonstrated via Western Blot of both protein ends showing a C-terminal fragment. This lower band fragment is not produced separately in solution, but is instead folded into the larger protein structure, as assessed by the inability to purify the lower band from the upper band after refolding of the protein.

This difference in structure changes the reactivity of the protein to antibodies circulating in patient serum, as well as antibodies generated in goats via direct immunization. We see increases in binding of serum antibodies to mutant proteins, especially ZIKV NS1 W117A, G118A, K119A in IgM and IgG from individuals in the Dominican Republic. We see the opposite in those samples from Colombia during the same time frame. This may be a result of differences in general antibody reactivity, with those from the Dominican Republic reacting to the N-terminus of the protein more than those from Colombia. If individuals in Colombia react predominantly to sequences from the N-terminus, it could result in lowered binding to the mutants, due to the majority of the protein they react to being present in the wild type. In fact, this area of the protein will be present in higher amounts than in the mutants, due to the protein being quantified in total, which will leave the wild type protein enriched in the N-terminal region versus the mutant being present in equal amounts across the protein sequence.

Differences in reactivity are present significantly in immunized goat serum, as well. Goats immunized with the wild type protein produce antibodies binding to all three variants of ZIKV NS1, as well as DENV NS1, less strongly than antiserum produced
from mutant ZIKV NS1 proteins. The lessened reactivity, along with the data indicating high binding of the wild type protein with antiserum from mutants reinforces the idea that there is a significant difference in the wild type protein from the mutants. The mutants bound each of the other proteins in amounts not significantly different, indicating that the mutations had little effect on binding of the NS1, at least in goats.

The lower number of IgM positive sera coming from patients in the Dominican Republic was investigated. Patient IgM concentrations were significantly lower than that coming from Colombia, indicating a potential secondary response rather than a primary, IgM centered, response seen from many infections. The data coming from our IgG avidity experiments indicates that the IgG circulating in patients consists of DENV-specific antibodies, rather than ZIKV-specific antibodies. This is confirmed when we compete out the fraction of antibodies that are binding to DENV NS1. They do not switch binding post-DENV adsorption in order to bind ZIKV NS1 on plates. On the other hand, when adsorbing with ZIKV NS1, followed by assaying against DENV NS1 on plates, the antibodies do end up binding DENV NS1, indicating a switch in binding due to preference.

Taken together, this data indicates that during the Dominican Republic outbreak, these patients generated DENV-specific IgG, rather than ZIKV-specific IgM, that resembled a response to a secondary DENV exposure, rather than a primary ZIKV exposure. This indicates that simply assaying for IgM or IgG for ZIKV will not provide a broad enough serological picture of an individual presenting with a possible ZIKV infection.
We must work to generate tools in order to be able to characterize sera with better accuracy in order to give differential diagnoses of Flavivirus infections a lower margin for error. IgG-based diagnostics have made progress in a similar fashion to IgM diagnostics since the start of this work, specifically the blockade of binding (BOB) assay that relies on antibodies competing for binding of ZIKV antigen [238]. Later in this work, we develop an analytical tool for further specific characterization of serum to complement current serological tests. This will serve to enhance diagnostic capacity in the event of future outbreaks, as well as a surveillance tool for epidemiological and vector control studies.

**Future Directions**

Although we were able to effectively detect IgM and IgG from infected ZIKV patients, we lack samples that have been well characterized, including patient history related to previous Flavivirus infections or exposure. Collection of well-characterized sera from groups in a position to contribute should be a high priority. These groups include the Sustainable Sciences Institute, responsible for developing the B.O.B. assay mentioned previously, and the Centers for Disease Control and Prevention Division of Vector Borne Diseases, which is the reference laboratory for arboviral diseases.

Characterization of our current serum collection could also be aided by the development of a neutralization assay dependent on less material than traditional plaque assays. We propose development of a pseudovirus system displaying the envelope protein of ZIKV and all four DENV serotypes, included in separate systems. This system would include a reporter system such as luciferase. As mentioned before, a ZIKV
reporter virus has been developed [219]. Our proposed system would be replication competent only for one round, thus making it safe for use by any personnel, including pregnant women. This would allow for comparative measurements between ZIKV and all four DENV serotypes using a similar amount of serum utilized in one round of traditional plaque assays. This type of system has been utilized previously, with one version being in current use in our lab [239].

Further mutagenesis of relevant sites within the NS1 protein may result in lowered cross-reactivity without the need for complicated systems such as the MAC-ELISA. A series of mutations combining our current sites with a combination of others can be screened for activity with ZIKV NS1 remaining at a significant level, while lowering reactivity to DENV NS1 and WNV NS1.
Figure 2-1. Testing algorithm recommendations from the U.S. Centers for Disease Control and Prevention.

Current recommended sequence of diagnostic testing for non-pregnant individuals with suspected Zika virus infection. NAAT: Nucleic Acid Amplification Test. IgM: Immunoglobulin M. PRNT: Plaque Reduction Neutralization Test. Figure taken from [211].
Table 2-1. Zika Serological Diagnostics currently approved or possessing Emergency Use Authorization from the U.S. Food and Drug Administration.

Adapted from [222, 232].

<table>
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<tr>
<th>Zika Diagnostic Test</th>
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<th>Target</th>
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<td>Zika MAC-ELISA</td>
<td>CDC</td>
<td>February 26, 2017*</td>
<td>Inactivated cell culture or VLP antigen</td>
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<tr>
<td>ZIKV Detect 2.0 IgM Capture ELISA</td>
<td>InBios International</td>
<td>May 23, 2019**</td>
<td>Envelope</td>
</tr>
<tr>
<td>LIASON XL Zika Capture IgM Assay</td>
<td>DiaSorin Incorporated</td>
<td>October 28, 2019**</td>
<td>NS1</td>
</tr>
<tr>
<td>ADVIA Centaur Zika</td>
<td>Siemens Healthcare Diagnostics</td>
<td>July 17, 2019**</td>
<td>NS1</td>
</tr>
<tr>
<td>DPP Zika IgM Assay System</td>
<td>Chembio Diagnostic Systems</td>
<td>September 27, 2017*</td>
<td>NS1</td>
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Figure 2-2. Mutagenesis of conserved regions of related Flaviviruses.

Regions within ZIKV NS1 were mutated in immunodominant regions 2 and 4 that are exposed on the outer surface of the protein and elicit large antibody responses (A and B). C: Sequences aligned to related Flaviviruses show similarities within the ZIKV NS1. Red boxes depict areas that were mutated to alanine residues. A and B were taken from [236].
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<td><strong>YFV</strong></td>
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Table 2-2. Primer sequences used for the generation and mutation of NS1 proteins.

Underlined areas indicate added restriction sites.

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<td>ZIKV NS1 WT F</td>
<td>5'-ATCGATGGATCCCGGTGGGTTGCTCAGTGGAC-3'</td>
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<tr>
<td>ZIKV NS1 WT R</td>
<td>5'- ATCGATAGCTTTAGACCCCGCTGTCACCA-3'</td>
</tr>
<tr>
<td>DENV2 NS1 WT F</td>
<td>5'- ATCGATGGATCCCGATAGTTGTGCCGTGTTGT-3'</td>
</tr>
<tr>
<td>DENV2 NS1 WT R</td>
<td>5'- ATCGATGGCGCCGCCTAGGTGACCAAGGAGC-3'</td>
</tr>
<tr>
<td>ZIKV NS1 117, 118, 119-AAA F</td>
<td>5'- ATGAGCTGCCCCATGGCTGGAAAGCCGCTGCAGCATC GTATTTTGTAGG3'</td>
</tr>
<tr>
<td>ZIKV NS1 117, 118, 119-AAA R</td>
<td>5'- CTGTTGTTGATTCCCGGCCCTAAACAAAAATACGATG CTGCAGCCGCTTTC CAGCCG-3'</td>
</tr>
<tr>
<td>ZIKV NS1227, 228, 229-AAA F</td>
<td>5'- GAATGGCCAAGTGCTGCGGCTGCAGTGAGATGGA GTAGAAGAAAGTGCTGTCATACCC-3'</td>
</tr>
<tr>
<td>ZIKV NS1227, 228, 229-AAA R</td>
<td>5'- CTCATCTGTCCAGGCAGGCGAGACATTGGCCCATTC ACATGTTTTCATCTCAATCAGGTTG-3'</td>
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Figure 2-3: Map of the pET-45b(+) Vector.

The Zika NS1 gene is inserted in the area depicted between the removed sequence between restriction sites, thus removing the SacI cleavage site (circled in red), which was then used to linearize self-ligated vector. Base vector map taken from Novagen (EMD Millipore).
Figure 2-4: Production of rNS1 protein using a bacterial expression system.

NS1 protein was produced using Rosetta 2 DE3 E. coli cells. A: Agarose gel screening five colonies with NS1 gene inserted after ligation reaction showing all colonies positive for insert. B: Protein expression using IPTG over time shown via SDS-PAGE stained with coomassie brilliant blue G-250. Numbers indicate hours post IPTG induction.
A

1650 bp

B

40kDa
Figure 2-5: Visualization of the purification process of rNS1.

A: SDS-PAGE gel stained with Coomassie blue shows the process of locating the protein fraction as well as washing before final solubilization in 8M Urea. Sol: Soluble fraction. W1-W3: Washes in 1M urea to remove slightly insoluble proteins. 8M: Protein dissolved in 8M urea solution. B: Coomassie stained SDS-PAGE gel shows Ni-NTA purification of rNS1. FT: Unbound protein flow-through of Ni-NTA column. W1-W4: Washes of the column using 20mM imidazole. E1-E5: Elution fractions containing purified protein using 250mM imidazole.
Figure 2-6: Western Blot of NS1 mutants.

A: Blot was probed with anti-His6 antibody targeted toward the protein N-terminus. A protein band below 20 kDa is seen in the wild type protein (Lane 1) that is not present in the mutants (Lanes 2 and 3). B: Blot was probed with anti-ZIKV NS1 mAb targeted toward the C-terminus. No bands below the monomer are seen in any protein.
Figure 2-7: ZIKV rNS1 WT lower protein band is incorporated into the larger structure of the protein

A: A280 time course of protein elution from size exclusion column of ZIKV NS1 WT protein. Protein fragments show lack of separation from each other during chromatography run. B: SDS-PAGE showing fractions collected from multiple passes of a 30kDa Amicon ultra spin filter. Protein fragments do not separate through the column. Ladder: Novex Sharp Pre-Stained. FT: Flow-through.
Figure 2-8: Optimization of Protein Coating conditions for Antibody Capture

ELISA

Optimization was performed using A: Type of plate, B: Amount of rNS1 WT protein coated in each well of a Maxisorp plate, and C: Coating buffer type using a Maxisorp plate. Data depicted in ratio of signal from a positive sample to a negative sample, each run at 1:50 dilution. PBS: Phosphate Buffered Saline. CBC: Carb-Bicarb Buffer.
A  Plate Type Optimization

Positive/ Negative Signal (%)

Maxisorp  Medisorp  Polysorp

B  Protein Coating Amount Optimization

Positive/ Negative Signal (%)

10ug  5ug  2.5ug  1.25ug  0.125ug  0ug
Coating Buffer Optimization

Positive/ Negative Signal (%)

- PBS pH 7.4
- CBC pH 9.2
- Tris pH 7.5
- HEPES pH 7.4
Figure 2-9: IgM Data from samples gathered in Colombia during the 2015-2016 Zika virus Outbreak.

Data represents serum assayed via Antibody Capture ELISA using ZIKV Wild type NS1. Blue line indicates assay cut-off for positivity. Cut-off was calculated as the Mean $A_{450}$ of the Negative control (negative serum) + 1.96*SD of the mean.
Figure 2-10: IgG Data from samples gathered in Colombia during the 2015-2016 Zika virus Outbreak.

Data represents serum assayed via Antibody Capture ELISA using ZIKV Wild type NS1. Blue line indicates assay cut-off for positivity. Cut-off was calculated as the Mean $A_{450}$ of the Negative control (negative serum) + 1.96*SD of the mean.
Figure 2-11: IgM data from the 2015-2016 ZIKV Outbreak in the Dominican Republic

Samples were from A: Dominican Republic and from B: SeraCare ZIKV IgM Panel.

Data represents serum assayed via Antibody Capture ELISA using ZIKV Wild type NS1. Blue line indicates assay cut-off for positivity. Cut-off was calculated as the Mean A$_{450}$ of the Negative control (negative serum) + 1.96*SD of the mean. Bars indicate SE of the mean.
A

ZIKV NS1 WT IgM

Dominican Republic Samples

B

ZIKV NS1 WT IgM

SeraCare Samples
Figure 2-12: IgG data from the 2015-2016 ZIKV Outbreak in the Dominican Republic.

Samples were from A: Dominican Republic and from B: SeraCare ZIKV IgM Panel. Data represents serum assayed via Antibody Capture ELISA using ZIKV Wild type NS1. Blue line indicates assay cut-off for positivity. Cut-off was calculated as the Mean A$_{450}$ of the Negative control (negative serum) + 1.96*SD of the mean. Bars indicate SE of the mean.
Figure 2-13: Ratios of mutant vs. wild type binding of rNS1 using patient samples

A: Ratios of mutant/wild type binding from IgG in samples show some preferential binding to 117-119 mutant NS1. B: IgM shows preferential binding of mutant NS1 proteins in samples from the DR. Colombia= Colombia samples from suspected ZIKV infection. Dom Rep= Dominican Republic samples from suspected ZIKV infection. 117-119= ZIKV NS1 W117A, G118A, K119A. 227-229= ZIKV NS1 H227A, T228A, L229A.
**A**

IgG Binding Ratios to Mutant ZIKV NS1

**B**

IgM Binding Ratios to Mutant ZIKV NS1
Figure 2-14: Serum from goats immunized with ZIKV mutants show differential binding to NS1 proteins.

Antibody capture ELISA was performed on serum from a mixture of two goats immunized with each ZIKV NS1 protein. Binding of WT ZIKV NS1 showed lower binding to WT (A), both mutants (B and C), as well as DENV2 WT NS1 (D). Data represents samples run in duplicate and repeated for a total of three runs. *: Significant difference seen between WT serum and 117-119 serum. **: Significant difference seen between WT serum and 227-229 serum.
**Figure 1**

(A) ZIKV NS1 WT Plate

(B) ZIKV NS1 W117A, G118A, K119A Plate
Figure 2-15: Binding Characteristics of ZIKV patient antibodies.

A: IgM binding analysis shows low levels of IgM produced in patients from the DR. B: Patient antibodies from suspected ZIKV cases in the DR show higher levels of avidity (B) and competitive binding (C) to DENV NS1 protein than ZIKV NS1 protein. Col: Colombia. DR: Dominican Republic.
**Figure A: IgG/IgM ELISA**

- **Dr IgG**, **Dr IgM**, and **Col IgM** are plotted against **Absorbance at 450 nm (A450)**.
- The data points are shown with a p-value of less than 0.05, indicating statistical significance.

**Figure B: IgG Avidity ELISA**

- **ZIKV NS1** and **DENV NS1** are plotted against **IgG Avidity Index**.
- The data points show a p-value less than 0.0001, indicating strong statistical significance.
C

Competition ELISA

- ZIKV Plate No Spike
- ZIKV Plate DENV Spike
- DENV Plate No Spike
- DENV Plate ZIKV Spike

A450

p < 0.0001

ns
CHAPTER 3: Development and Initial Evaluation of Non-structural Protein 1

Antigen Capture Assay for Zika Virus Infection

Introduction

Antigen capture diagnostic assays targeting non-structural protein 1 (NS1) have been utilized in the past for Dengue virus infection, with NS1 being established as an early biomarker for Flavivirus infection [142, 240–242]. Assays are often in Enzyme Linked Immunosorbent Assay (ELISA) format, though other technologies have been employed for detection [243–247]. This assay type is recommended by the Centers for Disease Control and Prevention for the diagnosis of Dengue infection during the first 7 days of illness [248].

One of the assays commonly employed for the early diagnosis of Dengue is the InBios DENV Detect NS1 ELISA Kit. Recently received approval by the FDA for marketing, it has a self-reported sensitivity of 86.8% and specificity of 97.8% [249]. One potential issue may be the lack of sensitivity in secondary Dengue cases, with one study indicating 100% sensitivity in primary infections, and 10% sensitivity in secondary infections [242]. This is likely due to circulating anti-NS1 antibodies present during secondary infections [250]. This demonstrated success with NS1-based detection of Dengue infection means it is an attractive option for detection of infection with other Flaviviruses.

The Zika epidemic of 2015-2016 spurred a development period resulting in multiple antigen capture assays being developed on a research level [251–253], as well as some becoming commercially available, though for research use only [254]. Some of the
diagnostics exhibit low limits of detection, but as yet are untested in the field [252], while others had sensitivities and specificities between 70% and 100% [255]. Generally, these tests are based on the platform of monoclonal antibodies capturing the NS1 protein. Along with the previously mentioned Dengue diagnostics based on NS1 capture being susceptible to interference from antibodies circulating in patient serum, other diagnostics have exhibited the same issue.

A diagnostic based on capture of West Nile Virus (WNV) NS1 was developed using a monoclonal antibody. Capture of recombinant NS1 was deemed sensitive, with a detection limit of 0.5 ng/mL soluble NS1. Detection in serum after day 7, even in mice during a primary infection, was decreased. This was presumably due to the increase in IgM generated against WNV NS1. After separation of the antigen-antibody complex, NS1 detection increased significantly [256]. This presents a potential difficulty with using strictly monoclonal antibody capture NS1 diagnostics, with the possibility of a decreased window of detection.

This work aims to generate an NS1 capture diagnostic that is sensitive, specific, and has little to no interference from antibodies generated against NS1 from the early immune response in a patient. This will be accomplished via the use of polyclonal antibodies generated against ZIKV NS1. This will further the goal of making available the tools necessary to make ZIKV infection diagnoses possible at every window of an infection, thus giving the clinician valuable information for future treatment.

Materials and Methods
Flavivirus Gene Synthesis and PCR amplification

The full length NS1 gene from Yellow Fever, West Nile, and St. Louis Encephalitis viruses (NCBI Accession Numbers KF769016.1, DQ211652.1, and KX258461.1, respectively) was synthesized in pUC-IDT (Amp) (Integrated DNA Technologies, Iowa, USA). The NS1 gene was then amplified using primers NS1 WT Forward (Table 3-1) with the underlined upstream restriction site and NS1 WT Reverse (Table 3-1) with the underlined restriction site added downstream, as well as an E. coli STOP codon. Restriction sites differed based on whether each enzyme cut site was contained within the gene of interest; if so, a different enzyme was chosen. For SLEV, BamHI and HindIII were used, with YFV using MfeI and HindIII, and WNV using BamHI and NotI. PCR was performed using a reaction containing 1 unit Phusion polymerase (New England BioLabs, Massachusetts, USA), 200 µM dNTPs, 0.5 µM forward and reverse primers, 1X Phusion HF Buffer, and 3% DMSO using an ABI thermal cycler (Applied Biosystems, California, USA). Reaction conditions consisted of an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension of 72°C for 10 minutes.

Cloning of NS1 into Expression Vector and Expression Screening

The full-length amplified NS1 gene was ligated into the expression vector pET-45b(+) (Novagen, USA) using the added restriction sites. This results in an N-terminally hexahistidine-tagged NS1 protein under the control of the T7 promoter with minimal vector-encoded amino acids. The plasmid was transformed into Rosetta 2 (DE3) E. coli
cells and plated on LB agar containing 100 µg/mL carbenicillin and incubated overnight at 37°C. Colonies were screened by PCR amplification of gene insert followed by confirmation via restriction digestion using the enzymes used for insertion. Colonies confirmed by digestion were further confirmed by sequencing of the insert using primers for T7 promoter and T7 terminator sequences (GeneWiz, New Jersey, USA). Colonies from a confirmed plasmid were screened for expression levels by plating on LB agar containing 100 µg/mL carbenicillin and growing in LB broth. At an OD$_{600}$ of 0.7, expression was induced using 1 mM IPTG and pelleted after a period of 3 hours. Colonies were diluted to 100x the final OD$_{600}$ with water and lysed with addition of Laemmli sample buffer (Bio-Rad, California, USA) to working concentration, followed by brief sonication. Lysed samples were analyzed by SDS-PAGE followed by total protein staining via Coomassie Blue G250.

rNS1 Production and Solubilization

1 L cultures of recombinant NS1 were grown to an OD$_{600}$ of 0.7 and induced with 1 mM IPTG. After induction for 4 hours, cultures were harvested at 10,000xg for 10 minutes. Pellets were suspended in cell lysis buffer (10mM Tris, 10mM EDTA, 100mM NaCl, 100µg/mL Lysozyme, 1% Triton X-100, and protease inhibitor cocktail) for 1 hour at 4°C. Pellets were sonicated for 5 minutes at 40% power (10s on/30s off). The solution was centrifuged at 10,000xg for 40 minutes and resuspended in IB wash buffer (10mM Tris, 200mM NaCl, 1M Urea, pH 6.0). Suspension was sonicated briefly and rocked for 1 hour at 4°C and centrifuged at 10,000xg for 40 minutes. The resulting inclusion body was resuspended in IB solubilization buffer (10mM Tris, 500mM NaCl, 100mM
NaH$_2$PO$_4$, 8M Urea, and 10mM Beta-mercaptoethanol, pH 8.0) overnight at 4ºC after a brief sonication. The resulting solution was centrifuged at 3,220xg for 30 minutes and used for purification of rNS1.

**Purification by Immobilized Metal Affinity Chromatography**

The addition of the N-terminal His$_6$ tag allowed for purification of rNS1 by immobilized metal affinity chromatography (IMAC) using HisPur Ni-NTA spin columns (Thermo Scientific, USA). The column was equilibrated with 2 bed volumes of IB solubilization buffer and the solubilized rNS1 was loaded onto the column. The protein was allowed to bind for 1 hour at room temperature on a rotary shaker. Unbound protein was allowed to flow through via gravity, and the resin was washed with 10 bed volumes of wash buffer (500mM NaCl, 100mM NaH$_2$PO$_4$, 8M Urea, 10mM beta-mercaptoethanol, 20mM imidazole, pH8.0). Protein was eluted by passing 10 bed volumes of elution buffer (500mM NaCl, 100mM NaH$_2$PO$_4$, 8M Urea, 10mM beta-mercaptoethanol, 250mM imidazole, pH6.0) over the column. Fractions were collected for later SDS-PAGE analysis, and pure eluted fractions were pooled together for refolding.

**Refolding of recombinant NS1**

The concentration of the pooled protein was adjusted to 100μg/mL in IB solubilization buffer and added to a 10 MWCO dialysis flask (Thermo Scientific, USA). The protein was refolded at 4ºC over the course of 3 days in refolding buffer (50mM Tris, 0.4M L-arginine, 1mM reduced glutathione, 0.1mM oxidized glutathione, pH 8.0) at a 10-fold
volume excess, changing refolding buffer once a day. Dialysis was subsequently performed into PBS (pH 7.4) using 3 changes of buffer, with a final overnight exchange.

**Generation of Polyclonal Antibodies against ZIKV NS1**

Antiserum generated against ZIKV NS1 117, 118, 119-AAA were purified using affinity chromatography. CarboxyLink Coupling Resin (Thermo Scientific, USA) was coupled to 5-10 mg ZIKV117, 118, 119-AAA in PBS using a 3-fold increase of the suggested amount of EDC to compensate for phosphate-containing buffer. Coupled resin was washed and the flow-through combined with the wash was analyzed at A280 to determine coupling efficiency by comparing this with the protein solution applied to the resin. Coupled resin was added to a column suitable for chromatography and stored at 4ºC in PBS + 0.5% sodium azide. Antiserum against rNS1 was diluted 1:1 with PBS and filtered through a 0.22 µm syringe filter before applying to the column. Once applied to the column, the solution was allowed to flow via gravity until column exit. Fractions were collected in 1 mL aliquots for analysis via A280. Columns were washed using 10 resin bed volumes of PBS, followed by elution of the bound antibodies via 0.1M glycine, pH 2.7. Elution fractions (1 mL each) were combined with 60 µL of 1M Tris-HCl, pH 9.0 for antibody affinity protection. The column was immediately washed with at least 10 resin bed volumes of PBS to remove the glycine, followed by storage of the column in PBS + 0.5% sodium azide at 4ºC.
Purification of ZIKV specific Antibodies

Anti-ZIKV NS1 polyclonal antibodies were cross-adsorbed against DENV2 NS1 using affinity chromatography against DENV2 NS1 coupled to CarboxyLink resin via the same protocol as before. Antibodies eluted from the ZIKV NS1-coupled column were mixed 1:1 with PBS and applied to the DENV2 NS1-coupled column. The column was washed with 10 resin bed volumes of PBS, followed by elution of the bound fraction by 0.1M glycine, pH 2.7. These fractions were combined for later experimental use as non-adsorbed ZIKV NS1 polyclonal antibodies. The fractions that were collected in the flow-through and wash fractions were combined and applied to the DENV2 NS1-coupled column again. Flow-through and wash fractions were collected and combined to be used experimentally as cross-adsorbed ZIKV NS1 polyclonal antibodies. Both of the cross-adsorbed and non-adsorbed solutions were concentrated via Amicon Ultra 30,000 MWCO spin filters (Millipore, Ireland). A portion of polyclonal antibodies were biotinylated via the EZ Link Sulfo-NHS Biotin Kit (Thermo Fisher, USA).

Antigen Capture ELISA

Evaluation of ability of purified polyclonal antibodies to bind NS1 proteins was done via Antigen Capture Enzyme Linked Immunosorbent Assay (ELISA). Affinity purified polyclonal antibodies were coated on Nunc Maxisorp 96-well plates overnight at 10 µg/mL in CBC at 4°C. Wells were blocked with 1.5% BSA for four hours at room temperature before plates were dried overnight at room temperature and stored at 4°C. Serum samples to be analyzed were diluted at 1:10 in sample diluent before addition to
the antibody-coated plate and incubated at 37°C for one hour. Plates were washed with PBS containing 0.05% Tween-20 (PBST) four times before addition of biotinylated-NS1 pAbs (5 µg/mL) for 30 min at room temperature. After another PBST wash, a 1:5,000 solution of high sensitivity Streptavidin-HRP (Thermo Fisher, USA) was added and the plate was incubated at room temperature for 30 min. After another PBST wash, the plate was incubated with TMB substrate (Sigma, Germany) in the dark for 15 min. The reaction was then stopped with 0.36N H$_2$SO$_4$ before reading the absorbance of each well at 450 nm. Standards were run on each plate consisting of ZIKV rNS1 diluted from 2000 ng/mL down to 0.49 ng/mL using 1:4 dilutions, plus a negative control consisting of normal human serum diluted 1:10 in sample diluent.

NS1-Antibody Complex Dissociation ELISA

Serum samples were incubated in an alkaline detergent solution in order to release antibodies from the antigen (NS1) to examine the possibility of antibody interference of antigen detection. Serum (55 µL), or soluble NS1 in the case of the standard curve, were mixed with 55 µL of dissociation solution (1M Tris base, pH 10.5, 2% Triton X-100, and 150 mM NaCl) and incubated for 1.5 hours at 37°C. After incubation, the alkalinity was neutralized via addition of 20 µL of 2M HCl and incubation for one hour at 37°C. After incubation, 100 µL of 150 mM NaCl solution was added to dilute the detergent percentage for more efficient binding to the capture antibodies. After sample addition to the plate, the plates were incubated overnight at 4°C and subjected to the rest of the above ELISA procedure the next day. Dissociated and non–dissociated samples were run on the same plate for comparison. Non-dissociated samples were diluted in pre-neutralized and
diluted dissociation buffer. Standards consisted of soluble NS1 run side-by-side on the same plate using dissociated and non-dissociated conditions.

Results

Development of the antigen capture diagnostic began by generating nonstructural protein 1 antigens from the related Flaviviruses Yellow Fever, West Nile and St. Louis Encephalitis in order to determine how ZIKV NS1 capture antibodies would cross-react. Sequences generated synthetically were cloned into the pET-45b(+) vector using primers adding appropriate restriction sites into the NS1 protein sequence (Table 3-1). As with our previous ZIKV and DENV NS1 constructs, this allowed for insertion of an N-terminal His6 sequence in order to allow for downstream blotting and purification. Removal of the SacI sequence allowed for more efficient positive sequence selection after ligation. Protein staining of colonies induced with IPTG alongside uninduced colonies via Coomassie Blue reveals NS1 bands in sizes as expected (Figure 3-1).

As found previously, the NS1 proteins were located predominantly in the inclusion body. Solubilization of the inclusion body was performed using urea-based buffers in order to denature the protein and pull it into solution. Ni-NTA purification of protein based on the added hexahistidine tag was performed on the 8M urea soluble fraction with washes using 20mM imidazole to eliminate non-specific binding. Elution was performed with 250mM imidazole to outcompete the binding of the protein to the resin which allowed the protein to flow through the column.
Once solubilization of the protein was complete, refolding was performed in order to return the protein to its native form. This was accomplished via dialysis in refolding buffer designed to introduce the protein into mildly oxidizing conditions using a 10:1 ratio of reduced glutathione and oxidized glutathione in order for disulfide bonds to form as folding occurs slowly during the process. The buffer also contained L-arginine in order to counteract the phenomenon of aggregation commonly seen during refolding. Aggregation was also combated by diluting the protein to a low concentration of 100 µg/mL in denaturing buffer before addition to the dialysis membrane.

In order to generate a polyclonal antibody solution that is specific to ZIKV NS1, without cross-reactivity to related viruses, we began with goat anti-ZIKV NS1 serum generated from inoculation with the full-length ZIKV NS1 W117A, G118A, K119A mutant. Using a CarboxyLink column coupled with the same mutant NS1, we purified anti-ZIKV pAbs via affinity chromatography. This resulted in a pool of antibodies with high levels of binding toward ZIKV NS1, but also possessed affinity for DENV2 NS1. This was removed via affinity chromatography using a similar column set-up but coupled with DENV2 NS1 wild type protein. This cross-adsorption was repeated once, resulting in a polyclonal antibody solution with high affinity to ZIKV NS1 and no reactivity toward DENV2 NS1 (Figure 3-2).

The anti-ZIKV NS1 pAbs generated were coated onto plates in order to perform antigen capture ELISA targeted toward ZIKV NS1. Purified ZIKV NS1 W117A, G118A, K119A was diluted 1:4 in buffer beginning at 2000 ng/mL and going down to 0.49 ng/mL. This assay provided a good range of values corresponding with dilutions of NS1, with a limit of detection of between 1.95 and 7.8 ng/mL, defined as the mean of the
negative control plus 1.96* standard deviation of control (Figure 3-3A). Linear regression analysis shows a good fit of data, with an $r^2$ value of 0.97 (Figure 3-3B). Comparison of runs using the same solution of pAbs in both runs results in a p value of 0.9956 (Figure 3-4A), while using two separate stocks of purified antibodies results in a p value of 0.3524 (Figure 3-4B), as determined via the Sum of Least Squares F test.

Cross-reactivity of anti-ZIKV NS1 pAbs was analyzed via antigen capture assay using cross-adsorbed antibodies. The ELISA assay was performed with ZIKV, DENV, SLEV, WNV and YFV NS1 proteins in parallel. Binding values ($A_{450}$) were significantly lower in concentrations of NS1 of ZIKV vs. DENV, SLEV and YFV from 2000 ng/mL to 7.8 ng/mL, and between ZIKV NS1 and WNV NS1 from 2000 ng/mL to 31.25 ng/mL (Figure 3-5A). West Nile Virus NS1 had cross-reactivity assessed via percentage of ZIKV NS1 binding at just above 20%, with all other NS1 proteins being at or below 20% cross-reactivity (Figure 3-5B). Assessing cross-reactivity of non-adsorbed antibodies was performed as well. In this case, the cross-reactivity of WNV NS1 was over 70%, with other proteins falling between that and 50% (Figure 3-6A). Non-cross-adsorbed antibodies react to ZIKV NS1 at approximately 55% of the level of cross-adsorbed antibodies in the same assay (Figure 3-6B).

We utilized the ZIKV NS1 antigen capture assay to determine circulating NS1 levels in patient serum from the 2015-2016 ZIKV outbreak. Levels found circulating range from not detectable to over 2.2 µg/mL (Table 3-2). This is 20 times lower than the highest levels found in Dengue Fever patients [257]. In the Colombian samples, 52.6% of samples were positive for NS1, while the Dominican Republic samples showed a
higher positivity of 67.5%. Overall, 55.7% of samples collected from symptomatic ZIKV patients during the outbreak were positive (Table 3-3).

We next asked if the detection of NS1 could be improved by dissociating the antigen from any circulating anti-Flavivirus NS1 antibodies patients may have developed from previous infections. If so, it would pose a challenge for detection of ZIKV infection in Flavivirus endemic areas. Using an alkaline detergent method, we separated the antigen-antibody complex and performed NS1 antigen capture assay as done previously. This method showed little effect on detection of NS1 in solution (Figure 3-7A). Performing the assay on samples from the Dominican Republic shows no significant change in detection of NS1 whether associated to circulating antibodies or not (Figure 3-7B).

Discussion

The work here shows that a diagnostic utilizing a polyclonal antibody approach to NS1 antigen capture may be an effective tool that will add to those currently available for detection of ZIKV infection and discrimination from related Flavivirus infections. Antibodies generated against ZIKV NS1 mutant W117A, G118A, K119A were able to be purified using a CarboxyLink column coupled with the protein. Using the same approach with DENV2 NS1 WT, the antibodies were effectively cross-adsorbed to yield a polyclonal solution specific to ZIKV NS1. Cross-adsorption also increased the reactivity of our antibodies, with a near double signal generated versus non cross-adsorbed pAbs.
An NS1 antigen capture ELISA utilizing this polyclonal antibody solution was able to effectively quantify soluble protein, with a limit of detection between 1.95 ng/mL and 7.8 ng/mL. This compares favorably to comparable DENV NS1 capture ELISAs, such as SD Bioline’s detection limit between 16 and 63 ng/mL NS1 [258]. This assay is consistent between runs, as well as between different preparations of the polyclonal solution. This is commonly thought of as a negative with regard to usage of polyclonal antibodies, but the variability is not significant in our assay [259].

Production of the NS1 proteins of the related Flaviviruses, Yellow Fever, West Nile and St. Louis Encephalitis proceeded as previously with ZIKV and DENV NS1. Utilizing these proteins to investigate the cross-reactivity of our diagnostic reveals high ZIKV specificity, with the highest cross-reactivity coming from WNV, with only 20% of the signal generated from ZIKV NS1 at the highest level of protein tested. This is a decrease of 50% signal intensity from non-cross-adsorbed antibodies, indicating that cross-adsorption pulls a large portion of cross-reactive antibodies from the solution, leaving a high signal generating, specific pool of pAbs. This assay, tested using human serum from acute cases of ZIKV infection, detected ZIKV infection on par with similar DENV antigen capture diagnostics [260].

This assay is effective at detection of ZIKV NS1 even when antigen is present with co-circulating non-ZIKV antibodies. This circumvents a problem that can be encountered with monoclonal diagnostics in use in areas with endemic Flaviviruses that cross-react with ZIKV proteins. This tends to mask detection of the antigen if patient antibodies share epitopes with diagnostic antibodies. This limitation is not present in our
assay. Our assay contributes to solving the need for more ZIKV-specific assays that must be in place to differentiate ZIKV infections from similar and related infections.

**Future Directions**

Furthering our assay’s ability to detect NS1 circulating in patient serum, we propose generating a rapid diagnostic test in order to satisfy a need for point of care diagnostics able of deploying to the sites of outbreaks and being performed by personnel without extensive training [261]. This could also be utilized in a side-by-side antigen and antibody (IgM) detection platform, as is available for Dengue infection [242, 262]

Evaluation of our diagnostic would benefit from further serum samples that are well characterized including patient history related to previous Flavivirus infections or exposure. Collection of well-characterized sera from groups in a position to contribute should be a high priority. These groups include the Sustainable Sciences Institute and the CDC Division of Vector Borne Diseases.

Evaluation of different methods of antibody generation may be suitable for the future. Rabbits are recently commonly used to generate antibodies, and possess enhanced binding capabilities [263, 264]. Utilization of rabbit antibodies in NS1 antigen capture may require less cross-adsorption, as well as result in low non-specific interactions during lateral flow rapid tests. Generation of protein via mammalian cells may be of benefit as well, as mammalian cells result in protein more relevant to that produced during infection, which may aid in antibody recognition. Recent advances in mammalian cell protein production promise amounts sufficient for our purposes [265, 266].
Table 3-1: Primers used for the Generation of Flavivirus NS1 Proteins

Underlined sequences indicate added restriction sites.

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEV NS1 WT F</td>
<td>5’- ATCGATGGATCCGCTGATTGGATGTCGCA -3’</td>
</tr>
<tr>
<td>SLEV NS1 WT R</td>
<td>5’- ATCGATAAGCTTTTAAGCTGACTGAGATTT – 3’</td>
</tr>
<tr>
<td>WNV NS1 WT F</td>
<td>5’- ATCGATGGATCCCCGACACTGGGTGGCCATA -3’</td>
</tr>
<tr>
<td>WNV NS1 WT R</td>
<td>5’- ATCGATGCGGCCGCTTAAGCATTCACTTGTGACTG – 3’</td>
</tr>
<tr>
<td>YFV NS1 WT F</td>
<td>5’- ATCGATCAATTGCAAGGATGCGCCATCAAC -3’</td>
</tr>
<tr>
<td>YFV NS1 WT R</td>
<td>5’ - ATCGATGCGGCCGCTTATATTTCCTCCAGCTGTAAC – 3’</td>
</tr>
</tbody>
</table>
Figure 3-1: Production of Flavivirus proteins related to ZIKV NS1.

Coomassie-stained SDS-PAGE images of induced (I) and uninduced (U) cultures from NS1 proteins of Yellow Fever (YF), West Nile (WN) and St. Louis Encephalitis (SLE). Bands indicate proteins similar to DENV and ZIKV NS1 WT production.
Figure 3-2: Process for purifying and cross-adsorbing antibodies toward ZIKV NS1 protein.

A gravity flow column with ZIKV NS1 protein is used to purify initial antibodies to the protein. A: The bound and eluted fraction is then put through a column with DENV NS1 two times. The unbound and eluted (non-DENV reactive) fraction is then taken as the cross-adsorbed, ZIKV-specific fraction. B: Antibodies flowing through the column initially bind ZIKV at low signal strength. Following ZIKV NS1 column elution, strength of binding of the solution goes up, but DENV NS1 reactivity is present. Upon cross-adsorption against DENV NS1, specificity of the pAb solution goes up, as well as its binding avidity to ZIKV NS1.
A

Purification against ZIKV NS1: Take the bound/eluted fraction

Purification against DENV2 NS1: Take the unbound/eluted

Repeat DENV2 NS1 Purification: Take the unbound/eluted
B

![Bar chart showing A450 values for different samples: ZIKV FT, ZIKV Elution, DENV Elution, and ZIKV-Specific pAbs. The chart compares ZIKV NS1 Binding and DENV2 NS1 Binding.](chart.png)
Figure 3-3: Antigen Capture ELISA Assay quantifies ZIKV NS1 to a low concentration that correlates well to a standard curve.

A: ZIKV rNS1 shows binding significantly higher than BSA down to 7.8 ng/mL. Limit of detection of the assay is between 7.8 and 1.95 ng/mL NS1. B: Linear regression of NS1 detection via ELISA demonstrates assay can quantify protein across a range of values.
A

ZIKV Antigen Capture

\[
\begin{align*}
A450 & \quad \text{Ag (ng/mL)} \\
2000 & \quad 500 \quad 125 \quad 31.25 \quad 7.8 \quad 1.95 \quad 0.49 \quad 0
\end{align*}
\]

* *
* *
* *
* *

B

\[
\begin{align*}
\log (A450) & \quad \log (rNS1 \text{ concentration}) \\
R^2 & = 0.97
\end{align*}
\]
Figure 3-4: Antigen capture assay shows consistency between runs and antibody preparations

. A: Two runs using the same preparation of anti-ZIKV NS1 pAbs. \( p = 0.9956 \). B: Two runs using separate preparations of pAbs. \( p = 0.3524 \). P values determined through Sum of Least Squares F Test.
ELISAs Using the Same pAb Prep

ELISAs Using Separate pAb Preps
Figure 3-5: ZIKV NS1 Antigen capture assay lacks cross-reactivity to related viruses.

Recombinant NS1 antigens were produced and assayed. A: Even at low levels of NS1 detection, the assay is specific to ZIKV NS1. B: Relative binding of each related virus to ZIKV NS1, set at 100%, as evaluating using the highest level of NS1 in the assay. **: Significant difference between ZIKV NS1 and all other assayed proteins. *: Significant difference noted between ZIKV NS1 and other assayed proteins except for WNV NS1.
**Cross-reactivity vs. Related Viruses**

**A**

![Graph showing A450 vs. Ag (ng/mL) for different viruses.](image)

**B**

![Bar chart showing % ZIKV Reactivity for different viruses.](image)
Figure 3-6: Reactivity of NS1 antibodies without cross-adsorption.

A: Related Flavivirus reactivity using NS1 antibodies non-adsorbed against DENV NS1 shows binding at a high percentage of ZIKV NS1 levels. B: Comparison of ZIKV NS1 reactivity using adsorbed and non-adsorbed NS1 antibodies shows higher signal intensities of cross-adsorbed antibodies to ZIKV NS1 protein.
A  Cross-reactivity of Related Viruses using Non-Adsorbed pAbs

% ZIKV Reactivity

ZIKV  YFV  WNV  SLEV  DENV

B  ZIKV NS1 Reactivity Before and After Cross-adsorption

% ZIKV Reactivity

ZIKV NS1pAbs

Adsorbed  Non-adsorbed
With DR originate from the Dominican Republic. Sample ID numbers beginning with Z originate from Colombia, while those beginning with DR originate from the Dominican Republic.

**Table 3-2: NS1/IgM/IgG Levels in Surveyed Patient Serum**

Sample ID numbers beginning with Z originate from Colombia, while those beginning with DR originate from the Dominican Republic.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IgM WT</th>
<th>IgM 117-119</th>
<th>IgM 227-229</th>
<th>IgG WT</th>
<th>IgG 117-119</th>
<th>IgG 227-229</th>
<th>NS1 Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-10</td>
<td>0.18675</td>
<td>0.13835</td>
<td>0.12285</td>
<td>1.20945</td>
<td>1.11725</td>
<td>1.14445</td>
<td>82.05</td>
</tr>
<tr>
<td>Z-100</td>
<td>0.18045</td>
<td>0.12485</td>
<td>0.12275</td>
<td>0.59875</td>
<td>0.7307</td>
<td>0.61055</td>
<td>83.98</td>
</tr>
<tr>
<td>Z-101</td>
<td>0.1868</td>
<td>0.1559</td>
<td>0.10545</td>
<td>1.88775</td>
<td>1.86325</td>
<td>1.82465</td>
<td>ND</td>
</tr>
<tr>
<td>Z-102</td>
<td>0.1221</td>
<td>0.1034</td>
<td>0.0889</td>
<td>1.41885</td>
<td>1.4985</td>
<td>1.4237</td>
<td>221.8</td>
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<tr>
<td>Z-103</td>
<td>0.5427</td>
<td>0.34535</td>
<td>0.1813</td>
<td>0.12735</td>
<td>0.0831</td>
<td>0.07875</td>
<td>73.8</td>
</tr>
<tr>
<td>Z-104</td>
<td>0.19075</td>
<td>0.1023</td>
<td>0.0616</td>
<td>0.4231</td>
<td>0.4824</td>
<td>0.4387</td>
<td>11.68</td>
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<tr>
<td>Z-105</td>
<td>0.3016</td>
<td>0.19225</td>
<td>0.12755</td>
<td>1.77325</td>
<td>1.7653</td>
<td>1.76545</td>
<td>10.48</td>
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<tr>
<td>Z-106</td>
<td>0.5318</td>
<td>0.31145</td>
<td>0.1454</td>
<td>1.05435</td>
<td>1.18865</td>
<td>1.1215</td>
<td>60.99</td>
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<td>Z-107</td>
<td>0.1959</td>
<td>0.10525</td>
<td>0.0644</td>
<td>0.8615</td>
<td>1.04225</td>
<td>0.93125</td>
<td>54.11</td>
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<tr>
<td>Z-110</td>
<td>0.35795</td>
<td>0.2078</td>
<td>0.09665</td>
<td>1.78745</td>
<td>1.78155</td>
<td>1.7858</td>
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<td>Z-115</td>
<td>0.18385</td>
<td>0.1088</td>
<td>0.04575</td>
<td>1.86325</td>
<td>1.82935</td>
<td>1.6451</td>
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<tr>
<td>Z-118</td>
<td>0.1003</td>
<td>0.0811</td>
<td>0.07025</td>
<td>1.9695</td>
<td>2.05525</td>
<td>1.97125</td>
<td>50.31</td>
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<tr>
<td>Z-119</td>
<td>0.11065</td>
<td>0.07645</td>
<td>0.05305</td>
<td>1.9428</td>
<td>1.92395</td>
<td>1.95725</td>
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</tr>
<tr>
<td>Z-12</td>
<td>0.1454</td>
<td>0.10885</td>
<td>0.17605</td>
<td>2.13385</td>
<td>1.99755</td>
<td>2.00915</td>
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<td>Z-120</td>
<td>0.5909</td>
<td>0.8051</td>
<td>0.55625</td>
<td>1.6434</td>
<td>1.65535</td>
<td>1.632</td>
<td>72.17</td>
</tr>
<tr>
<td>Z-121</td>
<td>0.1013</td>
<td>0.09255</td>
<td>0.05955</td>
<td>1.7243</td>
<td>1.7405</td>
<td>1.69705</td>
<td>81.26</td>
</tr>
<tr>
<td>Z-122</td>
<td>0.26625</td>
<td>0.1755</td>
<td>0.11025</td>
<td>1.46965</td>
<td>1.56365</td>
<td>1.5706</td>
<td>101.9</td>
</tr>
<tr>
<td>Z-123</td>
<td>0.32225</td>
<td>0.2368</td>
<td>0.1847</td>
<td>1.9463</td>
<td>1.983</td>
<td>1.97375</td>
<td>ND</td>
</tr>
<tr>
<td>Z-124</td>
<td>0.10315</td>
<td>0.0859</td>
<td>0.0675</td>
<td>1.85715</td>
<td>1.8266</td>
<td>2.00635</td>
<td>17.93</td>
</tr>
<tr>
<td>Z-125</td>
<td>0.10775</td>
<td>0.09165</td>
<td>0.0798</td>
<td>0.14495</td>
<td>0.13275</td>
<td>0.11665</td>
<td>ND</td>
</tr>
<tr>
<td>Z-126</td>
<td>0.59</td>
<td>1.13835</td>
<td>1.14065</td>
<td>1.8974</td>
<td>1.90265</td>
<td>1.86705</td>
<td>ND</td>
</tr>
<tr>
<td>Z-127</td>
<td>0.28025</td>
<td>0.341</td>
<td>0.3252</td>
<td>1.9503</td>
<td>1.9229</td>
<td>1.84155</td>
<td>21.25</td>
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<tr>
<td>Z-128</td>
<td>0.2137</td>
<td>0.1842</td>
<td>0.14925</td>
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Table 3-3: Ag Capture Results per Outbreak Region

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<td>27 (67.5%)</td>
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<td>Overall</td>
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<td>108 (55.7%)</td>
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Figure 3-7: ZIKV NS1 Capture ELISA does not display interference from substances in patient serum.

Antibodies against NS1 were dissociated from antigen via detergent and assayed for increased activity using the antigen capture ELISA. A: The assay was still able to detect ZIKV NS1 well in both N-ELISA (dissociation solution was neutralized before addition of NS1) and D-ELISA (dissociation solution was neutralized in the presence of NS1). B: No significant difference was seen in ZIKV NS1 detection in patient samples between N-ELISA and D-ELISA conditions.
A

N-ELISA ($R^2 = 0.9678$)
D-ELISA ($R^2 = 0.9924$)

B

N-ELISA
D-ELISA
CHAPTER 4: Evaluation of Non-structural Protein 1 as a Vaccine Antigen for Protection Against ZIKV Infections

Introduction

Immunization against DENV NS1 has been shown to protect mice from DENV infections, as well as vascular leak associated with antibody dependent enhancement [267]. Immunization with NS1 rather than a structural protein, such as Envelope, may avoid the potential for antibody dependent enhancement prominent in Flavivirus infections [226, 268], especially Dengue, as has been seen recently with the Dengvaxia vaccine [269]. Dengvaxia is recommended for those that have experienced previous Dengue infection and should not be given to those that are naïve [270]. Monoclonal antibody therapy targeting DENV NS1 protein also protects mice against vascular leak and mortality from DENV infection [267]. ZIKV vaccines based on genetic material have been successful previously for conferring protection against infection [268], with potential for NS1-based protection utilizing a DNA based approach [271]. Currently, the vaccine candidates advanced enough to undergo trials are DNA, mRNA, or inactivated virus [207]. We sought to understand whether an approach based on a protein only vaccine would confer protection as well as these non-protein approaches.

For the study of Zika pathogenesis, mouse models have been produced that allow for infection of the normally non-susceptible animal. Some have been useful in the past for the study of DENV infections [267]. Two models used for studying pathogenesis of ZIKV infection, A129 and AG129 mice, are deficient in Type I Interferon receptor and Type I/II Interferon receptor, respectively [207, 272, 273]. These models have the benefit of replicating disease progression, including neurological changes, other organ
damage, and ZIKV found in many tissues, as well as possessing functional adaptive immunity, making them ideal candidates for vaccine studies [272]. Other mouse models, such as the CD1 mouse, are used as well, and can replicate disease progression in an intrauterine model, but must often be inoculated intracerebrally for studies, thus making them more difficult and less relevant [274, 275].

More recently, a mouse model lacking a STAT2 protein was developed that mimics ZIKV pathogenesis well. Viral RNA levels in tissues are similar to the A129 mouse model, including brain levels. Relevant organ damage was observed, with disease replication present among multiple strains of virus, and increased virulence noted among African strains. This model is also likely to exhibit normal adaptive immunity [276, 277].

We chose the ifnar−/− mouse model for our study. This model is the same knockout for Type I Interferon receptor as A129, but on a C57BL/6 instead of 129S7/SvEvBr [278]. This allows for infection with Zika virus, unlike wild type mice, without using intracerebral inoculation, as well as intact adaptive immunity for vaccine response evaluation.

**Materials and Methods**

**Viruses and Cells**

VeroE6 cells were propagated in Dulbecco’s Modified Eagle Medium containing 4500 mg/L glucose, L-glutamine, 3.7 g/L sodium bicarbonate, and 10% FBS. Zika virus strain MR766 was propagated on VeroE6 cells via adsorption in serum free DMEM for 1
hour at 37°C in 5% CO₂ with rocking every 15 minutes to distribute liquid. Once adsorption was complete, growth medium with 2% FBS was added and propagation was continued until CPE was displayed across a majority of the monolayer.

Titration of Zika virus was accomplished via plaque assay on VeroE6 cells. 12-well plates were seeded with VeroE6 cells until 80-90% confluency was attained before beginning. Zika virus supernatant was diluted serially in serum free DMEM and added to wells in duplicate in amounts of 150 µL and allowed to adsorb for 1 hour at 37°C in 5% CO₂ with rocking every 15 minutes. Once complete, medium was removed and 1 mL of an overlay medium comprised of Minimum Essential Medium with 2.2 g/L sodium bicarbonate, L-glutamine, 2% FBS, and 1.2 g/L Avicel was added. Plates were incubated 4 days at 37°C in 5% CO2 before fixing with 10% formalin. Plaques were visualized by addition of 0.1% crystal violet and titers were calculated as PFU/mL.

**Ethics Statement**

All work was undertaken according to the National Institutes of Health Guidance for the Care and Use of Laboratory Animals. All procedures were approved by the Tulane University School of Medicine Institutional Animal Care and Use Committee (IACUC) under protocol # 4458: Characterization of Non-Structural Protein 1 Immunization on Pathogenesis of Zika Virus Disease.

**Mice**

Mice deficient in the IFN α/β receptor on a C57/BL6 background were obtained from a breeding colony at Jackson Laboratories. Male and female mice in equal numbers were used at 8 weeks old.
**Vaccination Studies**

Mice were immunized on days 0, 14 and 42 intraperitoneally with 100µL containing 20µg Zika NS1 protein (Native Antigen, United Kindgom) or ovalbumin (OVA) with 1µg MPLA in Addavax adjuvant (Invivogen, CA, USA). On day 56, mice were infected with $1.0 \times 10^6$ PFU of Zika virus strain MR766 in PBS in a volume of 40 µL injected subcutaneously above the ankle of each hind leg. Control mice received injections of the same volume of PBS.

On day 3 post-challenge, 2 control mice and 2 experimental mice (1 male and 1 female) were euthanized via CO$_2$ asphyxiation and chest wall opening. The remaining mice were euthanized upon meeting the humane euthanasia criteria. 4 mice died overnight in their cages, which prevented blood collection. Upon euthanasia, blood was collected for viremia and antibody titer determination, as well as spleen, testes, ovaries, and brain.

**Quantitative RT-PCR**

Tissue samples were weighed and homogenized in PBS via the TissueRuptor II (Qiagen, UK) until completely smooth. The RNeasy RNA extraction kit (Qiagen, UK) was used with the maximum allowable volume of homogenate. Blood was extracted via the same procedure. Samples were passed through a QIAshredder before extraction (Qiagen, UK). 1 µL of RNA from blood was used for determination of viral load. PCR was performed using the SuperScript III Platinum One-step qRT-PCR kit (Thermo, US) consistent with kit protocols. Primers utilized were ZIKV specific (5’-CCGCTGCCAACAACAAG-3’) and (5’-CCACTAACGTTTTTGCAGACAT-3’).
with (5’-56-FAM/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ/-3’) as probe [27] using the QuantStudio 6 Flex Real Time PCR System (Thermo, US).

Antibody Capture ELISA

Determination of the production of antibodies to ZIKV via NS1 vaccination was done by Enzyme-Linked Immunosorbent Assay (ELISA). Purified and refolded rNS1 proteins were diluted to 10 µg/mL in CBC and coated on 96-well Nunc Maxisorp plates (100 µL/well) and incubated overnight at 4⁰C. Wells were blocked with 1.5% BSA in PBS for 4 hours at room temperature, and dried overnight at room temperature before being stored at 4⁰C until being used. Serum samples were diluted 1:100 and added to antigen-coated wells for 1 hour at room temperature, followed by washing with PBS containing 0.05% Tween-20 (PBST) three times. Washed wells were incubated with anti-mouse IgG HRP at appropriate dilution for 1 hour at room temperature, followed by three PBST washes. Color was developed by 100 µL/well TMB-H₂O₂ substrate (Sigma, USA) for 20 minutes, and the reaction was stopped by the addition of 100 µL 0.36N H₂SO₄. Absorbance was measured at 450nm.

Results

Evaluation of NS1 immunization on Zika virus infection in mice proceeded as expected, with mouse weight loss and obvious signs of discomfort for several days post challenge. On post-challenge day 3, four mice were sacrificed to monitor infection progress. Four mice on day 5 were found deceased in their enclosures, with three mice
meeting euthanasia criteria on day 6, and one on day 7. Control mice died at no different rates than NS1 vaccinated individuals (Figure 4-1).

After euthanasia, blood and tissue samples were taken. RNA extraction was performed on serum separated from the blood samples. qPCR performed on serum sample extracts were used to calculate genome copy numbers per unit volume. Genome copy numbers trended lower in the individuals that were vaccinated with NS1 from ZIKV, except for the blood taken on day 3. Mice vaccinated with OVA had generally higher copy numbers (Figure 4-2A). Antibodies directed toward NS1 were detected via antibody-capture ELISA utilizing anti-mouse secondary detection antibodies. Mice vaccinated with NS1 displayed higher antibody titers than control mice toward ZIKV NS1 (Figure 4-2B).

Discussion

Our work shows vaccination with NS1 from Zika virus results in anti-ZIKV antibody titers above that of sham inoculated individuals. This coincided with lowered genome copy numbers in those individuals, indicating lowered amounts of ZIKV viremia after challenge. This did not increase the life expectancy of the mice included in this study, though the numbers were low due to the study’s pilot nature.

We decided to end the study due to the lack of extended life expectancy in vaccinated mice, as well as other studies accomplishing some of our indented goals. DNA-based vaccines producing NS1 provide protection with a single immunization that provides both IgG-based and CD8+ T-cell-based protection, and is protective of life
No vaccine, to our knowledge, has been advanced that is administered using NS1 protein alone. Perhaps this indicates the administration of NS1 alone has been found insufficient for protection of life by others, and NS1 must be produced in larger amounts or over a longer time period to provide protection, which is solved via the DNA vaccine route. Overall, we show that an NS1 only vaccine can produce an immune response that is sufficient to potentially lower viremia.

**Future Directions**

If we were to continue this avenue of investigation, a few things would be interesting to determine. Determining viral titers in multiple tissues based off of NS1 protein-only immunization would be one of the first steps. Determining if those tissues have resident infectious virus or only genome would tell us more about protection potential, particularly for uterine and brain tissues.

Experimenting with different doses and adjuvants may reveal protection that is lacking in our approach. More protein, or a different adjuvant, or a different booster schedule may provide life-prolonging protection. We would also seek to determine if a mammalian expressed protein was essential for protection, or if a non-glycosylated protein expressed from bacteria would prove similar, as in previous work with a combined Envelope and NS1 vaccine [279]. If so, does mutant NS1, with its higher percentage of full-length character, produce a response similar to wild type, or can it produce neutralizing responses in lower amounts? There are still avenues to explore within the NS1-only vaccine approach, if such investigations were to continue.
Figure 4-1: Mouse death numbers days post infection.

Mice from day 3 were euthanized for data collection. Mice the following days were euthanized once the humane euthanasia criteria were met. Four were found deceased in cage.
Figure 4-2: Viremia is lowered in mice with anti-ZIKV antibody response.

qPCR and Ab Capture ELISA show decreasing genome copy number (A) corresponds with anti-ZIKV antibodies (B) found in the blood of infected mice. Mice immunized with OVA showed both no antibody response, and higher amounts of circulating virus.
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BIOGRAPHY

Brandon Joel Beddingfield was born in Tyler, Texas on July 15th, 1986 to George “Joe” and Donna Beddingfield. Throughout his childhood, he was always interested in medical science, likely sparked by Leonard “Bones” McCoy and Spock of Star Trek fame. During the completion of his Bachelor of Science degree from the University of Texas at Tyler, he became interested in microbiology as a field. While completing his Master of Science degree at the University of Texas at Tyler, he undertook research focusing on the pathogenesis of *Pseudomonas aeruginosa* under Drs. John “Cliff” Boucher and Ali Azghani, which cemented his decision to pursue the field at the Ph.D. level. After acceptance to the Biomedical Sciences program at Tulane University, he joined the lab of Dr. Robert F. Garry. During his tenure in this lab, he was able to participate in exciting research, including travel to Sierra Leone and aiding in the Lassa Fever vaccine development initiative through the Center for Epidemic Preparedness Innovations. Following completion of the Biomedical Sciences program, he hopes to continue to work with Dr. Garry and the Viral Hemorrhagic Fever Consortium to push forward the development and deployment of tools to aid affected individuals throughout the world.