DEVELOPING NOVEL SEROLOGICAL TESTS TO DISTINGUISH FLAVIVIRAL INFECTIONS

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ABSTRACT

Our study developed serological tests to distinguish dengue (DENV) and Zika (ZIKV) virus infections. We expressed the precursor peptide of DENV1, yellow fever virus, West Nile virus (WNV), and ZIKV using two expression systems. Using the pr protein of DENV1, we were able to distinguish DENV and ZIKV infections with high specificity and sensitivity. Furthermore, we improved upon our previous work that a combination of three enzyme-linked immunosorbent assays (ELISAs) based on non-structural protein 1 (NS1) can distinguish DENV and ZIKV infections by replacing the DENV1-NS1 ELISA with a pooled DENV1-4-NS1 ELISA. This increased the overall sensitivity and specificity of our assays. Lastly, we developed a WNV-NS1 ELISA that can be used in combination with DENV- and ZIKV-NS1 ELISAs to distinguish secondary flaviviral infections. With the current limitations of serodiagnosis of DENV and ZIKV due to cross-reactivity, our findings provide a promising addition to the existing CDC recommendations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
ADE	Antibody-dependent enhancement
C	Capsid
CDC	Center for Disease Control and Prevention
CHIKV	Chikungunya virus
D1-4	Dengue virus serotypes 1-4
DC	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DENV	Dengue virus
DENV1-4	Dengue virus serotypes 1-4
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FRNT	Focus reduction neutralization test
HEK 293T cells	Human embryonic kidney cells 293 transformed with large T antigen of simian virus 40
His	Histidine
I	Induction
IRB	Institutional Review Board
JEV	Japanese encephalitis virus
Kb	Kilo-base

Kd	Kilo-Dalton
M	Membrane
mAbs	Monoclonal antibodies
MAC-ELISA	IgM antibody capture ELISA
Neg	Negative
NIAID	National Institute of Allergy and Infectious Diseases
Non-I	Non-induction
NS	Nonstructural
NS1	Nonstructural protein 1
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pDENV	Primary dengue virus
pr	Precursor peptide
prM	Precursor membrane
PRNT	Plaque reduction neutralization test
PSO	Post-symptom onset
pWNV	Primary West Nile virus
pZIKV	Primary Zika virus
rER	Rough endoplasmic reticulum
RIG-I	Retinoic acid-inducible gene
RNA	Ribonucleic acid
rNS1	Recombinant nonstructural protein 1
rOD	Relative optical density
RT	Room temperature
SD	Standard deviation
sDENV	Secondary dengue

sNS1	Secreted soluble nonstructural protein 1
STST	*Strep*-tag II
TM	Transmembrane domain
TLR	Toll-like receptor
VLP	Virus-like particle
WB	Western blot
WHO	World Health Organization
WNV	West Nile virus
WNVwpFla	West Nile virus with previous flavivirus
WT	Wild type
YFV	Yellow fever virus
ZIKV	Zika virus
ZIKVwpDENV	Zika with previous dengue

CHAPTER 1

INTRODUCTION

Flavivirus

The genus *Flavivirus* belongs to the family *Flaviviridae* and is made up of over 70 members, all of which are enveloped, single-stranded, positive-sense RNA viruses roughly 50 nm in diameter containing genomes encode for ten proteins. Within the genus, there are several members causing significant human diseases and they belong to different serocomplexes. The four serotypes of dengue virus (DENV) belong to the DENV serocomplex; West Nile virus (WNV) and Japanese encephalitis virus (JEV) to the JEV serocomplex; yellow fever virus (YFV) as a single member; Zika virus (ZIKV) in its serocomplex; and tick-borne encephalitis virus (TBEV) serocomplex (Lindenbach et al. 2013). While these viruses share similar genome organization and replication strategy, they are capable of causing a variety of severe diseases in humans ranging from vascular leakage and hemorrhage to encephalitis (Fernandez-Garcia et al., 2009). Flaviviruses have received increasing public attention in recent years due to their re-emergence and increasing global spread. With environmental changes projected to further extend the reach of both known and unknown flaviviruses, research in this field is of great importance and interest (Bollati et al., 2010).

DENV

First isolated in 1943 in Japan, DENV has caused outbreaks in Asia and the South Pacific for centuries. It has since spread throughout the tropical and subtropical regions around the world following the spread of its mosquito vector, *Aedes aegypti*, and its incidence has increased 30-fold in the last 50 years (Guzman & Harris, 2015; Messina et al., 2014). The four serotypes (DENV1 to DENV4) are phylogenetically and antigenically distinct but related, sharing only 60-75% identity at the amino acid level. Different serotypes and genotypes within each serotype have been associated with varying clinical manifestations. While most DENV infections are inapparent, approximately 25% infected individuals develop symptoms exhibiting mild, self-limiting disease known as dengue fever (DF) or severe, sometimes fatal disease known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Guzman & Harris,

2015). Secondary DENV infection with a heterologous serotype, along with viral strains and other risk factors, have been associated with DHF/DSS. Based on the revised 2009 WHO case definitions, clinical manifestations are classified as dengue without warning signs, dengue with warning signs, and severe dengue (WHO 2009). Estimates of annual DENV infections and case numbers vary; previously the WHO reported 50-100 million infections each year while Bhatt et al. estimated 390 million infections each year. This discrepancy is largely due to underreporting and the difficulty in tracking inapparent cases (Bhatt et al., 2013).

Genome

DENV contains positive-sense, single-stranded RNA genome of approximate 10.7kb in length. Flanked by 5' and 3' untranslated regions, the single reading frame of the genome encodes a precursor polyprotein, which is cleaved during and after translation by both host and viral proteases into ten proteins: three structural proteins and seven non-structural proteins (Fig. 1B) (Lindenbach et al., 2013). The structural proteins are made up of the capsid (C), premembrane (prM), and envelope (E) proteins, and the non-structural proteins, which are involved in viral replication, are comprised of NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 1A) (Fernandez-Garcia et al., 2009).

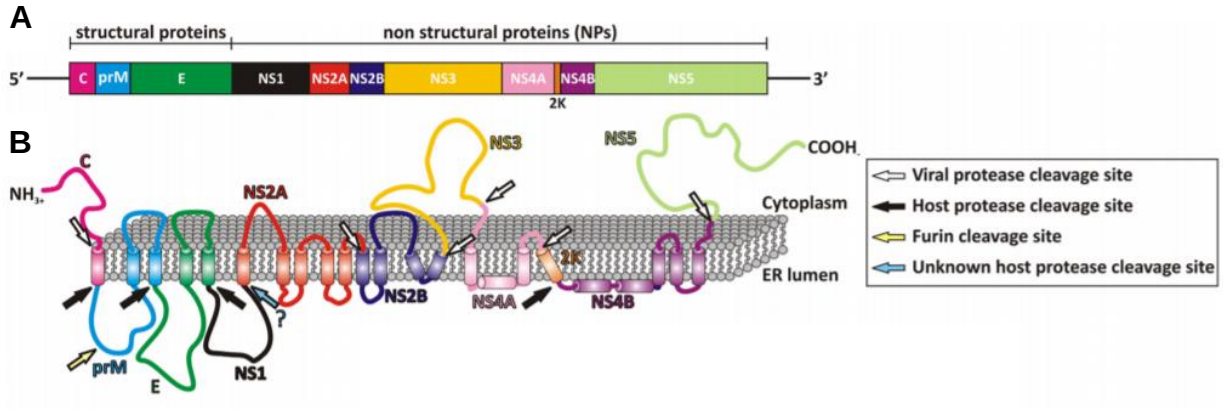

Fig. 1. Diagram of flavivirus polyprotein organization and processing. (A) Linear organization of structural and nonstructural proteins. (B) Membrane topology of polyprotein with cleavage sites (arrows) (Assenberg et al., 2009).

Replication cycle

Mosquito-borne flaviviruses such as DENV, ZIKV, WNV, and YFV are introduced to the host by an infected female vector during a blood meal. The virus is capable of entering a variety of cell types in different host species; in humans, the infection of dendritic cells (DC) by binding with a C-type lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) is thought to be particularly important. Other receptors identified as potential flavivirus receptors include $\alpha_v\beta_3$ integrin, GRP78, and CD14 (Lindenbach et al., 2013). After receptor binding, the virus is internalized by clathrin-mediated endocytosis. The acidic environment in the endosomes triggers conformational changes in the E protein that induces the fusion of the viral and endosomal membranes, releasing the viral RNA into the cytoplasm (Fernandez-Garcia et al., 2009).

Translation of the single open reading frame takes place in the ribosomes on the rough endoplasmic reticulum (rER), resulting in a polyprotein that is then co- and post-translationally cleaved into ten proteins: three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Fig. 1) (Lindenbach et al., 2013). After initial translation, a replication complex is assembled on the ER-derived membranes where viral replication takes place. The positive-sense viral genome is transcribed into negative-sense RNA by NS5, an RNA-dependent RNA polymerase, which then serves as a template for producing new positive-sense viral RNA. At this point, additional translation can occur or the viral components can be assembled into virions. Packaging of virions also takes place on the membrane of the endoplasmic reticulum (ER), where the structural proteins and the associated viral RNA bud off and are released into the lumen of the ER. The immature virions are transported through the trans-Golgi where furin cleaves prM into precursor (pr) and membrane (M) proteins, generating mature, infectious virus particles that are released by exocytosis (Fig. 2) (Fernandez-Garcia et al., 2009). However, there are variations in the efficiency of this cleavage, resulting in the release of a mixture of immature, partially immature, and mature virus particles (Guzman & Harris, 2015; Lindenbach et al., 2013).

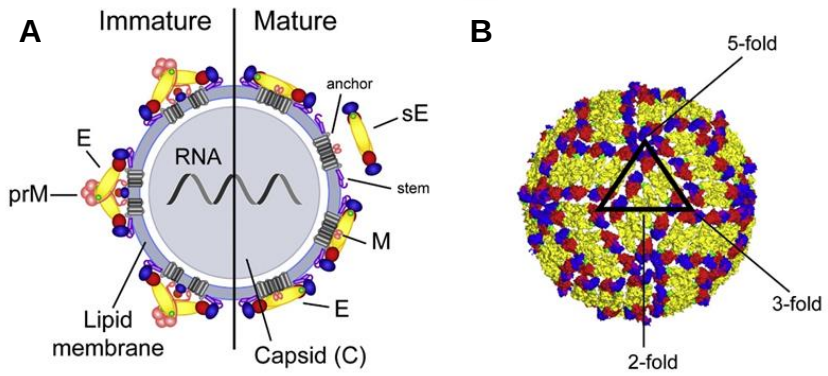

Fig. 2. Flavivirus structure. (A) Left: immature virions covered by spiky complexes of 60 trimers of prM-E heterodimers. Right: cleavage of prM results in smooth-surfaced particles. (B) Virion structure of 90 E protein dimers on the surface of mature virions based on cryo-EM. The triangle indicates 2-, 3-, and 5-fold symmetry axes (Heinz & Stiasny, 2012).

NS1 protein (DENV1-4)

The NS1 protein is a glycoprotein of roughly 48 kD and its intracellular and secreted forms play important roles in viral replication and immune evasion, respectively. Initially translated as a monomer, NS1 protein forms a dimer at which point it takes one of four paths: associates with the viral replication complex on the ER membrane; associates with the plasma membrane on infected cells; forms soluble lipophilic hexamers to be secreted by infected cells; or forms hexamers and binds to the surface of uninfected cells (Beatty et al., 2015; Lindenbach et al., 2013). Secreted soluble NS1 (sNS1) protein can bind directly to complement components and inhibit complement activation in solution and on the cell surface (Beatty et al., 2015). The sNS1 protein and the immune complex formation of sNS1 and anti-NS1 antibodies, which activates the Fcγ receptors on various immune cells, has been associated with DENV-associated vascular leakage and increased disease severity, triggering an inflammatory cytokine production that is seen in DHF and DSS. It is also used as a diagnostic marker for acute DENV infection (Guzman & Harris, 2015; Rastogi et al., 2016; Screaton et al., 2015).

Because of the important role NS1 protein plays in disease pathogenesis, it has become an

interesting target for developing inhibitors. Soluble NS1 in patient serum is also useful as a diagnostic marker in flaviviral infections; NS1 antigen ELISAs have been used in diagnosing JEV, WNV, and DENV infections. Other technologies such as biosensor-based approaches, fluorescent nanoparticles, and electrochemical detection are being developed as tools to quantify NS1 in patient samples. Furthermore, NS1-based subunit and DNA vaccines have been developed preclinically against JEV, DENV, and YFV with partial success (Rastogi et al., 2016).

pr protein

The precursor of the prM protein, pr, is a 91-amino-acid glycoprotein. Despite its small size, it plays a critical role as a chaperone by assisting in the proper folding of the E protein. Furthermore, its cleavage during virus maturation by host cell protease furin converts the immature virus particle into mature virions, although the cleavage is often inefficient, resulting in a mixture of mature, partially immature, and immature virions (Fig. 2) (Lindenbach et al., 2013; Luo et al., 2015; Yoshii et al., 2012). Previous studies have found that antibodies against prM do not neutralize DENV well and can transform non-infectious immature DENV particles into infectious ones through a mechanism of enhancement by antibody. Other studies have also shown a positive correlation between circulating prM antibody levels and disease severity (Luo et al., 2015).

Epidemiology

DENV is estimated to infect up to 390 million people each year, and of the 96 million individuals who have symptomatic, acute illness, roughly 500,000 develop life-threatening complications including hemorrhage and shock (Bhatt et al., 2013). Taking into consideration both fatal and non-fatal outcomes of DENV infection, in 2013 alone, DENV was responsible for 1.14 million disability-adjusted life-years (Stanaway et al., 2016). As a result, DENV places a heavy economic burden on local and global economies. In the Americas alone, DENV costs $2.1 billion a year on average excluding vector control, and this is expected

to worsen with population growth, increasing movements of people, urbanization, climate change, and inadequate public health and vector control programs (Guzman & Harris, 2015). Studies mapping the geographic distribution of the four serotypes of DENV have shown that until the 1980s, most areas reported having only one or two serotypes of DENV circulating; however, by the early 2000s many areas had all four, indicating an expansion of DENV endemicity (Fig. 3) (Messina et al., 2014).

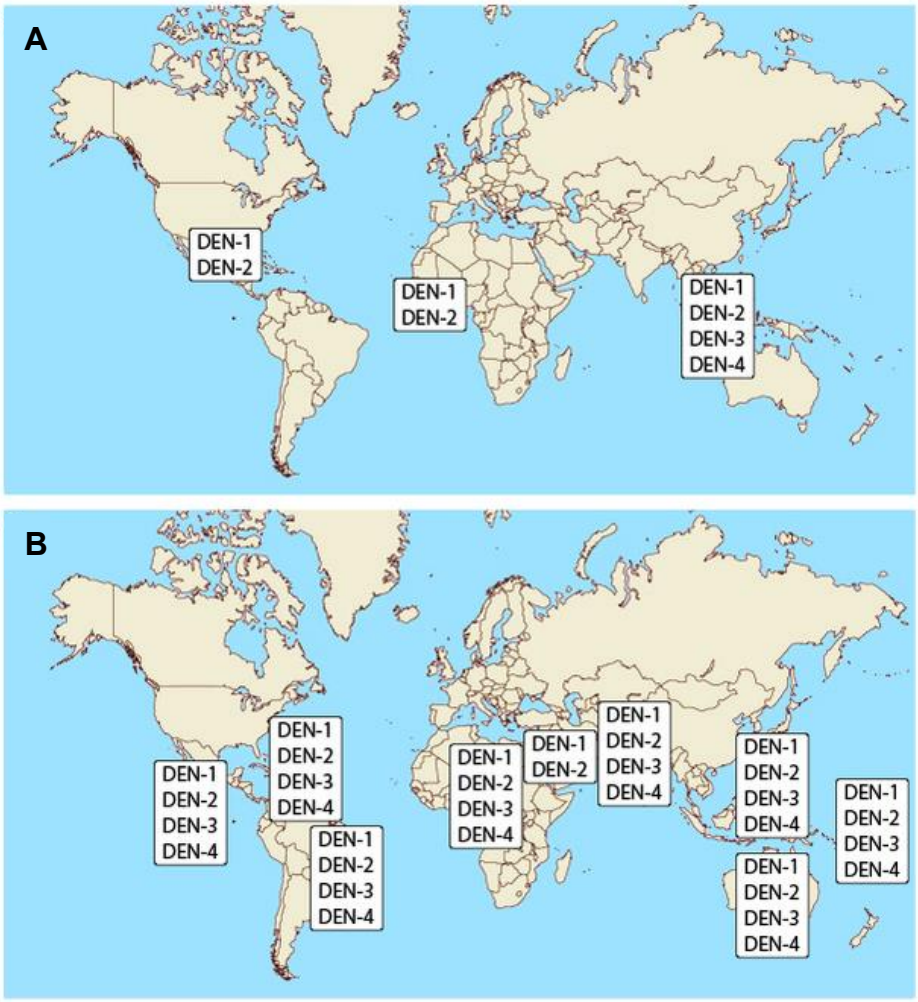

Fig. 3. The global distribution of DENV serotypes. (A) Distribution in 1970. (B) Distribution in 2004 (Guzman et al., 2010).

Vectors

Many flaviviruses are arthropod-borne. While tick-borne encephalitis virus is transmitted by ticks, others such as DENV, ZIKV, and WNV are transmitted by mosquitoes. The primary vector for DENV and ZIKV is the urban *Aedes aegypti* mosquito, although *A. albopictus* are also capable of transmission. Both mosquito species are widely distributed across tropical and subtropical regions around the world. The globalization of trade, particularly of tires from vehicles, is thought to have contributed significantly to the spread of eggs and immature vectors, as has the urbanization of endemic regions in Asia and Latin America, resulting in increased population density and vector-breeding sites in urban communities. While vector control through chemical and biologic targeting is a widely accepted method of DENV prevention, this approach has not been successful in stopping transmission in endemic regions (Simmons et al., 2012).

Clinical features

Clinical symptoms emerge after an incubation period of about 3 to 7 days and present themselves in three phases: an initial febrile phase, a critical phase, and a spontaneous recovery phase (Fig. 4). The first phase is characterized by high temperatures along with headache, retro-orbital pain, myalgia, joint pain, and macular rash. The second phase occurs in a small number of patients, usually in children and young adults, and is characterized by vascular leakage with increasing hemoconcentration, pleural effusions, and ascites. Hemorrhagic manifestations, as seen mainly in DHF and DSS patients, occur most often during this phase. The vascular leakage is often short-lived and at this stage, the patients are considered to be in the third and final recovery phase where their symptoms rapidly improve (Simmons et al., 2012). Lifelong immunity is developed after infection with one of the four serotypes of DENV; however, this immunity is type-specific and does not protect against heterologous serotypes (Bhatt et al., 2013).

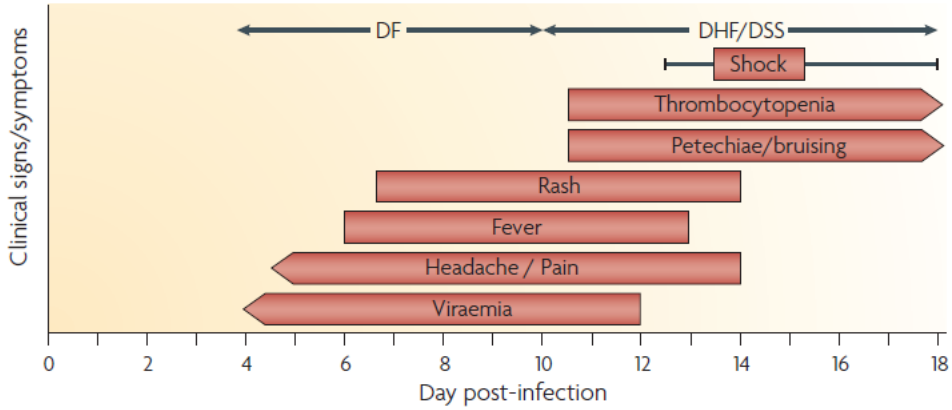

Fig. 4. Time course and clinical signs and symptoms of DENV infection (Whitehead et al., 2007).

Immune response

The first line of defense against DENV infection is the activation of the innate immune response through pattern recognition receptors such as Toll-like receptors (TLRs) and intracellular sensors such as melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I) recognizing viral RNA (Guzman & Harris, 2015). One of the downstream effects of activating these pathways is the induction of the interferon *α*/*β*-mediated antiviral response, which limits early viral replication and spread (Fernandez-Garcia et al., 2009). To counter these mechanisms, certain nonstructural proteins of flaviviruses have functions that undermine interferon induction as well as interferon-induced signaling (Lindenbach et al., 2013).

After primary DENV infection, the serotype-specific neutralizing antibody response is targeted towards certain epitopes in the hinge region or domains of the E protein. However, most of the anti-DENV antibodies that are targeted towards the E, NS1, and prM/M proteins are cross-reactive amongst the different DENV serotypes (Guzman & Harris, 2015). This is particularly the case immediately after primary infection; however, over time, the neutralizing antibody response becomes more DENV type-specific (Katzelnick et al., 2015). While primary DENV infection provides lifelong protection against the infecting serotype and transient cross-protection against other serotypes, severe manifestations of DENV infections such as DHF and DSS have been associated with secondary DENV infection with a

heterologous serotype (Guzman & Harris, 2015).

Antibody-dependent enhancement (ADE)

Individuals who have secondary DENV infections are at an increased risk of developing DHF and DSS, and this is thought to be caused by a phenomenon known as ADE. ADE occurs when virus-antibody complexes bind to Fcγ receptor-expressing cells, which results in an increase in the uptake of viral particles and thus an increase in infected cells and viremia (Guzman & Harris, 2015). Mononuclear phagocytes are considered the main target cell for DENV infection, and disease severity is thought to be correlated with the extent of mononuclear phagocyte infection. In vitro studies have shown that DENV titers in mononuclear phagocytes are higher when cultured with either heterotypic DENV antibodies or with homotypic antibodies at concentrations too low for efficient neutralization, as compared with DENV titers grown in similar conditions but without DENV-specific antibody. ADE has been described in DENV1-4, WNV, ZIKV, YFV, and other flaviviruses *in vitro*. Furthermore, *in vivo* studies have shown enhanced viremia in rhesus monkeys initially infected with DENV1, 2, 3, or 4 and challenged with a heterologous DENV serotype. Other studies have shown increased virus loads in immune animals compared to naïve animals, as well as shorter incubation periods and greater incidence of severe disease (Halstead, 1988). The role of non-neutralizing antibodies in ADE has been of great interest and recent studies have shown that a significant portion of all circulating antibodies after a first heterotypic DENV infection are directed at prM, targeting immature and partially immature DENV via the pr peptide. Antibodies against prM are poorly neutralizing and highly cross-reactive within the DENV serocomplex and have been shown to enhance the infectivity of noninfectious, immature DENV particles (Halstead, 2014).

Vaccines and antivirals

Due to the concern over ADE and its role in severe DENV clinical manifestations, effective DENV vaccines would need to be in tetravalent formulations capable of eliciting strong yet balanced immune

responses against all four serotypes. Vaccine candidates currently in development include live-attenuated and inactivated viruses, recombinant proteins, and DNA. An ideal vaccine would be safe in both children and adults regardless of their DENV immune status, would avoid ADE, require only one or two doses, and induce long-lasting protection against all four serotypes. Development of vaccines have been hampered by several factors including the lack of a suitable small animal model for DENV efficacy studies, the gaps in knowledge in the correlates for protection, and the risk of subsequent infection which may lead to severe DENV (Guzman & Harris, 2015).

Sanofi Pasteur's live-attenuated, chimeric yellow fever-dengue tetravalent dengue vaccine (CYD-TDV) known as Dengvaxia was first approved by the World Health Organization (WHO) in 2015 (Villar et al., 2015). Since then, it has been licensed for use in individuals nine years and older with varying upper age range in 20 DENV-endemic countries including Mexico, El Salvador, Philippines, and Brazil (WHO, 2018). Administered in three doses (0, 6, and 12 months), it has advanced the furthest out of all DENV vaccine candidates and has completed phase 3 clinical trials in Asia and Latin America (Villar et al., 2015). A recent report released by Sanofi Pasteur in November 2017 indicated an increased risk of severe dengue in dengue-naïve individuals who received the vaccine, resulting in the halt of the immunization campaign in the Philippines (Iacobucci, 2018). Previous studies showed that the vaccine is more efficacious in children who were seropositive at baseline than those who were seronegative (83.7% vs. 43.2%) and is more efficacious against serotypes 3 and 4. Furthermore, their previous results from the phase 2b study in Thailand showed that despite eliciting similar levels of neutralizing antibody, efficacy of the vaccine varied significantly, further emphasizing a need for better immune correlates of protection (Villar et al., 2015). As a result, the WHO has updated their recommendation in which only DENV seropositive individuals should be vaccinated and that pre-screening using serological testing should be conducted (Iacobucci, 2018).

There are currently no U.S. Food and Drug Administration (FDA)-approved antivirals against DENV but several drugs are being repurposed or developed to target different stages and players in the DENV replication cycle. Monoclonal antibodies against the E protein and fusion inhibitors are under development to prevent viral entry. Drugs targeting viral enzymes such as NS2B/3 protease, NS3

NTPase and helicase, and NS5 methyltransferase and RNA-dependent RNA polymerase are also under development and would inhibit viral replication. Drugs such as tetracycline and doxycycline have shown to reduce cytokine levels in patients with DHF/DSS, showing how targeting host factors responsible for increased cytokine levels that contribute to severe disease can improve patient outcome (Guzman & Harris, 2015).

ZIKV

While the primary mode of transmission for ZIKV is by the bite of an infected mosquito, specifically *A. aegypti* and *A. albopictus*, there have been reported cases of sexual, perinatal, and blood transfusion-transmissions. Like DENV, only 20-25% of infected individuals develop symptoms and when they do, they tend to develop around 6 to 11 days after infection and are relatively mild. ZIKV has become particularly concerning in recent years due to its association with Guillain-Barré syndrome, and infection during pregnancy has been linked to microcephaly and other congenital birth defects. There are currently no vaccines or antiviral drugs available for ZIKV (Faye et al., 2014).

Genome

The ZIKV genome is made up of a single-stranded positive-sense RNA that is 10.8 kb in length and encodes for a single open reading frame. Flanked by two non-coding regions in the 5’ and 3’ ends, the genome is translated into a polyprotein that is cleaved into three structural proteins: C, prM, and E; and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 1B) (Faye et al., 2014).

Epidemiology

Prior to the 2015 outbreak that began in Brazil, ZIKV received little attention since its discovery in 1947 in

a rhesus monkey in Uganda. Its ability to cause disease in humans was confirmed in 1953 in Nigeria and the first known outbreak occurred in 2007 on the Yap Island in the Federated States of Micronesia where roughly 73% of the population were infected and experienced only mild symptoms. No further outbreaks occurred despite occasionally reported cases until 2013 in French Polynesia, where 66% of the population was infected accompanied by an unprecedented spike in cases of Guillain-Barré syndrome, marking ZIKV's first association with neurologic sequelae (Lessler et al., 2016). After this outbreak, ZIKV spread throughout the South Pacific, ultimately reaching northeastern Brazil where the earliest confirmed case was reported in late 2014 alongside an increase in cases of Guillain-Barré syndrome and microcephaly (Fig. 5) (Musso, 2015). As of January 2018, there have been 223,477 confirmed autochthonous cases, 6,329 imported cases, and a total of 3,720 cases of congenital syndrome associated with ZIKV infection in the Americas between 2015 and 2018 (PAHO/WHO, 2018).

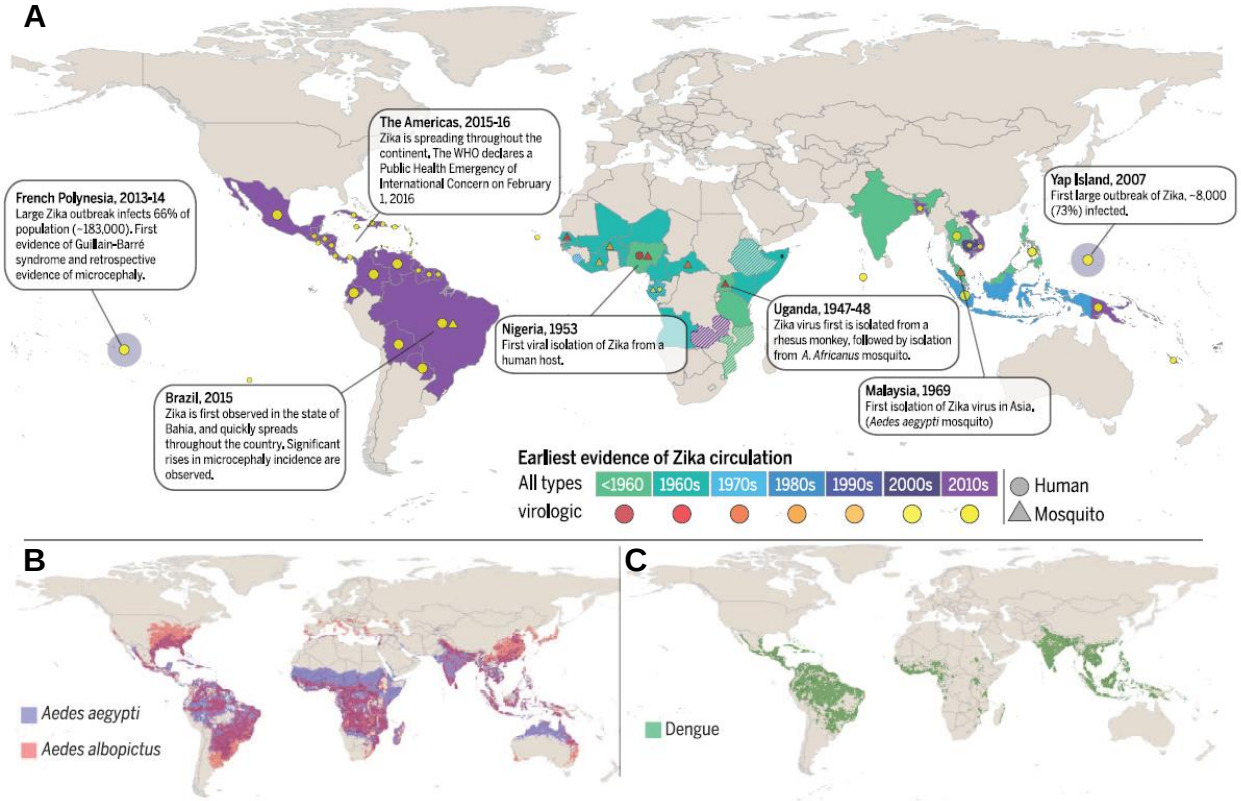

Fig. 5. Historical global distribution of flaviviruses. (A) Historical spread of ZIKV globally. (B) Global vector distribution. (C) Distribution of DENV (Lessler et al., 2016).

Clinical features

Like DENV, the majority of ZIKV infections (75-80%) do not present symptoms; however, when they do, they tend to be nonspecific, mild, and often self-limiting. Symptoms can include maculopapular rash, fever, arthritis, nonpurulent conjunctivitis, myalgia, headache, retro-orbital pain, edema, and vomiting, and these tend to resolve within one to two weeks after symptom onset. ZIKV infections have also been associated with Guillain-Barré syndrome, a neurological sequela characterized by ascending weakness, mild sensory loss, and paralysis. Infection during pregnancy has been one of the greatest concerns surrounding ZIKV, as it has been linked to fetal microcephaly and other congenital birth defects such as intracranial calcifications, ventriculomegaly, ocular impairment, brainstem hypoplasia, intrauterine growth restriction, and even fetal demise, collectively known as Congenital Zika Syndrome (Lessler et al., 2016).

Vaccines and antivirals

While there are currently no FDA-approved vaccines or antivirals available for ZIKV infection, several candidates are under development. While the vaccine approaches are varied, many of them are based on other flaviviral vaccines currently available. However, the tissue tropism and unique manifestations of ZIKV pose a unique challenge as the vaccine must protect both mother and fetus. Two vaccine candidates developed with the support of the National Institute of Allergy and Infectious Diseases (NIAID) are currently undergoing clinical trials: a DNA-based vaccine containing wild-type prM and E proteins from a 2013 French Polynesia isolate has entered phase 2 clinical trial; and ZPIV, a purified inactivated ZIKV vaccine based on a Puerto Rican isolate, is currently undergoing phase 1 clinical trial (NIAID, 2017; Pierson, 2016). Other vaccines under development include live-attenuated vaccines, including those attenuated using chimeric flaviviruses; mRNA vaccines; vesicular stomatitis virus-based vaccines; and AGS-v, an investigational vaccine containing recombinant proteins from mosquito salivary glands providing protection against a range of mosquito-transmitted diseases (NIAID, 2017; Pierson, 2016).

Abbink et al. are developing multiple ZIKV vaccine platforms including a purified inactivated virus vaccine, a DNA vaccine, and a single-shot recombinant rhesus adenovirus serotype 52 vector DNA vaccine containing ZIKV prM and E proteins, all of which were able to protect against ZIKV challenge in rhesus monkeys. This group has also found that adoptive transfer of ZIKV-specific IgG from monkeys vaccinated with a purified inactivated virus vaccine into both mice and rhesus monkeys conferred protection when challenged (Abbink et al., 2016). Recombinant subunit vaccines have also shown promise. Immunization of three mouse strains using ZIKV E protein with adjuvant resulted in high levels of specific antibody and neutralizing antibody and protection upon challenge (To et al., 2018). One of the biggest considerations in developing vaccines for both DENV and ZIKV is the potential impact of cross-reactive antibodies against other flaviviruses. Cross-reactive antibodies for ZIKV and DENV have been described and DENV-specific antibodies have been shown to increase ZIKV replication *in vitro* and *in vivo* (Abbink et al., 2016; Dejnirattisai et al., 2016; Bardina et al., 2017). There are no specific treatments for ZIKV infection and management is mainly supportive, including fluids, rest, antipyretics (no nonsteroidal anti-inflammatory drugs until DENV can be ruled out), and analgesics (Plourde & Bloch, 2016).

Diagnosis

Clinical evaluation alone is not a reliable way to diagnose DENV or ZIKV because they share clinical presentations with other viral infections such as Chikungunya (CHIKV) and they all tend to circulate in similar geographic regions (Plourde & Bloch, 2016). Therefore, the use of laboratory diagnostic tests is critical in clinical management, surveillance, and research. Determining which assay to use depends on the timing of sample collection as well as the purpose of the diagnostic test. In DENV cases, patients are viremic for four to five days after fever onset and viremia is closely associated with the duration of fever. During primary DENV infections, anti-DENV IgM is detectable five days after fever onset and lasts up to three months. Anti-DENV IgG levels increase slowly, with low titers between eight to ten days after fever onset. However, during secondary infections, anti-DENV IgG titers develop rapidly and can be detected early on after fever onset. In these cases, IgM may be undetectable (Guzman & Harris,

2015).

DENV testing is most commonly performed on serum and plasma, and ZIKV testing is commonly performed on serum/plasma, urine and/or saliva. However, ZIKV has been detected for extended periods of time in the cerebral spinal fluid, amniotic fluid, and semen of individuals infected with ZIKV. The bodily fluid tested for ZIKV diagnosis can vary depending on the timing of sampling and the diagnostic marker being sought. For serotype-specific DENV diagnosis of viral isolates, immunofluorescence assays using serotype-specific monoclonal antibodies can be performed. RT-PCR is a more common method capable of determining the infecting serotype, whereby viral RNA extracted from the aforementioned body fluids or tissue can be amplified and detected (Guzman & Harris, 2015). RealStar and Trioplex Zika Virus RT-PCR test are available for diagnosis of ZIKV, DENV and CHIKV, and has been given Emergency Use Authorization in the U.S. (Plourde & Bloch, 2016). The limitation to this method is that it is generally limited to the period during which viral RNA is present. Secreted NS1 protein can be detected up to nine days after fever onset, making them a useful marker for DENV detection. Rapid tests and ELISAs for detecting circulating NS1 are commercially available, although their sensitivity can range from 54 to 93% and sensitivity is particularly low in secondary infections (Guzman & Harris, 2015). The ELISA format is also used for detecting anti-DENV antibodies. IgM ELISAs identify recent infections, and the Center of Disease Control and Prevention (CDC) has developed and authorized the use of the E protein-based IgM antibody capture ELISA (MAC-ELISA) for ZIKV diagnosis (Plourde & Bloch, 2016). However, their sensitivity and specificity can vary greatly. IgG ELISAs are useful tools for testing previous exposure or testing convalescent-phase samples (Guzman & Harris, 2015). In both IgM and IgG E protein-based ELISAs, the greatest limiting factor is the cross-reactivity of antibodies between different flaviviruses, which is particularly problematic in secondary infections. Therefore, it is recommended that ELISA testing should be followed with neutralization assays (Plourde & Bloch, 2016).

Of all the available tests, neutralization assays are the most specific test to measure anti-DENV antibodies following primary infection. The plaque reduction neutralization test (PRNT) is most commonly used but still has limitations. Due to variations in cell lines, expression of cell receptors, complement, virus propagation cell lines, maturation state of the virus, temperature, and incubation time between

laboratories, the reproducibility is low. Furthermore, PRNTs are time consuming and require trained technicians. As a result, other neutralization tests have been developed such as focus reduction neutralization tests and flow cytometry-based neutralization assays. However, the cross-reactive antibodies after secondary infections or even those receiving vaccines against YFV or JEV can still prevent differentiation between DENV and ZIKV infections (Plourde & Bloch, 2016). Other methods being developed include microsphere-based immunoassays such as Luminex, nano-diagnostic and immunosensors, and microarray-based technology (Guzman & Harris, 2015).

The current CDC guidelines for ZIKV diagnosis recommend initial testing of serum, saliva, urine, and other bodily fluids by RT-PCR. Testing by RT-PCR should be done as soon as possible; while viremia can be detected up to several weeks after exposure, the test is most sensitive in the first two weeks. A diagnosis can be made at this point if the result is positive for DENV, CHIKV, or ZIKV. If the test is negative for all three viruses, serological tests using the E protein-based MAC-ELISA can be performed for DENV and ZIKV. Because the antibody response against the E protein is cross-reactive, it is common for individuals to be positive for both DENV and ZIKV at this stage. This test is then followed by a PRNT, at which point a final diagnosis can be made for those experiencing ZIKV or DENV infection for the first time. However, in individuals who have been exposed to multiple flaviviruses, PRNT assays can give positive results for both ZIKV and DENV and therefore cannot distinguish between DENV and ZIKV (CDC, 2018). An alternative to the E protein-based ELISA is the NS1-based ELISA, which is much less cross-reactive (Matheus et al., 2016). However, this does not address whether serum from secondary flaviviral infections can cross-react to ZIKV-NS1 and whether it can distinguish between primary DENV (pDENV), secondary DENV (sDENV), primary ZIKV (pZIKV) and ZIKV with previous DENV (ZIKVwpDENV) infections.

Hypothesis and Specific Aims

Specific Aim 1: Express the pr protein of DENV and other flaviviruses and develop pr-based serological tests for ZIKV.

Hypothesis: Antibodies generated against flaviviral prM protein are specific to each serocomplex. Therefore, the use of pr protein in serological tests will help to distinguish DENV and ZIKV infections.

Rationale: The antibody response during flavivirus infection primarily targets the E protein, followed by NS1 and then prM. While antibodies against the E protein are highly cross-reactive, antibodies against NS1 and prM are not (Whitehead et al., 2007; Lai et al., 2008). M protein is not exposed and pr protein is the major target of anti-prM antibodies.

Experimental Plan: Expression and production of DENV1- and ZIKV-pr will be tested *in vitro* by Western blot (WB) and ELISA. Antigenicity will be tested by ELISA using confirmed DENV- and ZIKV-immune sera and plasma.

Specific Aim 2: Develop NS1-based serological tests to distinguish ZIKV from DENV and other flavivirus infections.

Hypothesis: Antibodies generated against flaviviral NS1 protein are specific to each serocomplex. Therefore, the use of NS1 protein in serological tests will help to distinguish DENV, ZIKV, and WNV infections.

Rationale: Previously we reported that combination of DENV1-NS1 and ZIKV-NS1 IgG ELISAs can distinguish pZIKV, ZIKVwpDENV, pDENV1 and sDENV infections. Whether DENV2-NS1, DENV3-NS1 or pooled DENV1-4-NS1 can detect pDENV2 and pDENV3 panels and distinguish with pZIKV and ZIKVwpDENV panels remains unclear. In addition, whether combination of DENV, ZIKV and WNV NS1 IgG ELISAs can distinguish DENV, ZIKV, and WNV infections remains to be explored.

Experimental Plan: We plan to establish DENV2-, DENV3-, DENV4- and pooled DENV1-4-NS1 IgG ELISAs and test with serum or plasma samples from confirmed cases with pDENV1, pDENV2, pDENV3, sDENV, pZIKV, ZIKVwpDENV or primary WNV (pWNV) infections.

Significance and innovation. Due to the cross-reactivity of antibodies to the E protein between DENV, ZIKV, WNV, and other flaviviruses, an E protein-based test alone cannot distinguish these infections. Detection of anti-NS1 and anti-pr antibodies can be used to differentiate flaviviruses infection, especially in regions where multiple viruses are circulating.

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CHAPTER 2

MATERIALS AND METHODS

Preparation of Virus-Like Particles (VLPs)

DENV1 VLPs were previously generated in our lab by transfecting HEK293T cells, which have been seeded at 5 x 10^5 cells per 10-cm dish one day prior, with 10 ug of DNA constructs expressing the prM/E proteins of DENV1 by Lipofectamine 2000 (Life Technologies). At 48 h post-transfection, culture supernatants were collected and centrifuged at 1,250 x *g* at 4°C for 20 min, followed by filtration through a 0.22 µm membrane (Millipore), and layered above a 20% sucrose solution for ultracentrifugation at 65,000 x *g* at 4°C for 5 h. The resulting pellets containing VLPs were re-suspended in 30 µm 1 x phosphate-buffered saline (PBS) buffer (Hsieh et al., 2014).

The cell lysates were prepared by collecting the 48 h post-transfection HEK293T cells, washing with 1 x PBS, and treating with 1% NP40 lysis buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitors [Roche Diagnostics]). The resulting mixture was centrifuged at 20,000 x *g* at 4°C for 30 min, after which the pellets were discarded to obtain the final cell lysates (Hsieh et al., 2014).

Constructs of recombinant NS1 protein

Recombinant ZIKV NS1 (rNS1) protein expression plasmid was previously generated by our group using pMT/Bip-based vectors (Invitrogen) containing a Drosophila codon-optimized NS1 gene (amino acids 1-352) and a histidine (His) tag at the C-terminus (Integrated DNA Technologies) and expressed in Drosophila S2 cells (Tsai et al., 2017). The NS1 gene insertion was verified by restriction enzyme digestion and DNA sequencing patterns of the whole insert. These DNA plasmids were amplified using DH5α competent *Escherichia coli* and purified using endotoxin-free plasmid DNA Purification Kit (Omega Biotek, Qiagen). DENV1-4 rNS1 proteins were purchased from The Native Antigen Company.

Constructs of recombinant pr protein

Recombinant DENV1- and ZIKV-pr protein expression plasmids were generated using similar methods with pMT/Bip based constructs containing a truncated, codon-optimized pr gene (amino acids 1-86 for DENV1, 1-88 for ZIKV) and a histidine (His) tag for DENV1 and a *Strep*-tag II for ZIKV at the C-terminus (Integrated DNA Technologies). The pr gene insertions were verified in the same way as the rNS1 proteins by restriction enzyme digestion and sequencing of the insert, then amplified and purified using the methods described above.

Expression and purification of rNS1 and pr proteins

Serum-free adapted drosophila S2 cells (kindly provided by David Clements at Hawaii Biotech) cultured in 10-cm dishes were co-transfected with our pMT/Bip-based rNS1 or pr constructs and pCoHygro selection plasmid at 27°C using Lipofectamine LTX (Life Technologies) to express rNS1 or pr. Stable clones were established by hygromycin B selection for 1.5-2 wks in 10 ml of serum-free medium, followed by an expansion to 600 ml in two 1000 ml Erlenmeyer flasks. The supernatants were then harvested and purified through a 1 ml His-trap column for DENV1 rNS1 and pr, and a *Strep*-Tactin affinity column for ZIKV pr using the AKTA system (GE Life Sciences) (Tsai et al., 2017). The purified proteins were concentrated into 2-3 ml volumes of 1 x PBS with protease inhibitor and concentrations of the proteins were quantified using spectrophotometry. At this stage, the rNS1 proteins were stored at -80°C. The pr proteins, on the other hand, underwent anion exchange using a HiTrap Q HP anion exchange chromatography column, which contains a Q Sepharose High Performance strong quaternary ammonium anion exchange resin (GE Life Sciences). The proteins were eluted using a salt gradient buffer.

Preparation of prVLPs

DENV1-prVLP expression plasmids were generated using pCB-based constructs containing the

truncated pr protein plus the stem anchor region of the E protein (amino acids 394-495), bridged by a linker region. The pr gene insertions were verified by restriction enzyme digestion and sequencing of the inserts, then amplified and purified in the same way as rNS1 and pr proteins described above.

DENV1-prVLP plasmid was transfected into HEK293T cells, which have been seeded at 5 x 10^5 cells per 10-cm dish one day prior, with 10 ug of plasmid DNA using Lipofectamine 2000 (Life Technologies). At 72 h post-transfection, culture supernatants were collected and centrifuged at 1,110 x *g* at 4°C for 20 min, followed by ultracentrifugation at 111,000 x *g* at 4°C for 5 h. The resulting pellets containing pr-VLPs were re-suspended in 30 µl 1 x PBS buffer.

Polyacrylamide gel electrophoresis (PAGE) and WB analysis

PAGE was ran by initially preparing cell lysates and supernatants with 4 x non-reducing loading buffer (2% SDS, 0.5M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue [final concentrations]) and loading them on 12% gels using an electrophoresis chamber (Bio Rad) at 80-120 V. The gels were transferred onto a nitrocellulose membrane (Bio Rad) at 1.3 A for 7 m (Bio Rad Trans-Blot Turbo Transfer System) and blocked with blocking buffer (4% milk in wash buffer; 10 mM Tris [pH 7.4], 150 mM NaCl, 0.2% Tween-20 [final concentrations]) on a rocker for 1 h at room temperature (RT) (Lai et al., 2008; Tsai et al., 2015). The membrane was then incubated at 4°C overnight with primary antibody (anti-His mouse mAb at 1:2,000; anti-*Strep*-Tactin rabbit antibody at 1:1,000; human sera at 1:1,000 or 1:5000). The following day, after the membrane was washed three times at 10 m per wash with 1 x wash buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.2% Tween-20 [final concentrations]), the membrane was incubated at RT for 1 h with secondary antibody (IRDye 680RD or 800CW anti-mouse IgG at 1:2500; anti-rabbit IgG at 1:2,500; anti-human IgG at 1:5,000, LI-COR). After washing again three times at 10 m per wash, the signals were detected by a near infrared fluorescent imaging system (Licor Odyssey CLx) using both the 700 nm and 800 nm channels.

Three-layer rNS1 ELISA

The three-layer rNS1 ELISA was performed on human sera or plasma samples from ZIKV-, DENV1-, DENV2-, DENV3-, and WNV-infected individuals. 96-well ELISA plates were coated with 6 ng/well of ZIKV-NS1, 16 ng/well of DENV1-, DENV3-, or WNV-NS1, or 8 ng/well of DENV2- or DENV3-NS1 in a total volume of 50 µl carbonate coating buffer (pH 9.5) and incubated overnight at 4°C. The next day, 400 µl of blocking buffer (Starting Block™, ThermoFisher Scientific) was added to each well and the plate was incubated at RT for 1 h. Following removal of blocking buffer, primary antibody (mouse anti-His at 1:5000 dilution or human sera or plasma at 1:400 dilution [Johnson et al., 2000]) was added in a total volume of 50 µl and the sealed plate was incubated at 37°C for 2 h. After incubation, the plate was washed four times with 300 µl per wash of ELISA wash buffer (0.5% Tween-20 in 1 x PBS) and followed by the addition of secondary antibody (goat anti-mouse IgG at 1:5000 dilution or donkey anti-human IgG at 1:10,000 dilution, Jackson Laboratory) in a total volume of 50 µl. The plate was incubated at 37°C for 1 h, followed by six washes. 100 µl TMB was added and the plates were incubated at RT for 15 m, followed by 100 µl of stop solution (2N H_2SO_4). Absorbance (or optical density [OD]) was measured at 450 nm with a reference wavelength of 650 nm using an ELISA plate reader (ELx50, BioTek) or at 650 nm on a multilabel plate reader (VICTOR, Perkin Elmer) (Lai et al., 2013; Tsai et al., 2015). For the urea wash, 100 µl urea (6M) was added to each well at room temperature for 5 min between the second and third washings of NS1-IgG ELISA after the primary antibody, and other steps were as described above. Each ELISA plate utilized the inner 60 wells and included two positives (OD higher than 1; two confirmed samples), eight negatives (4 flavivirus-naïve sera and 4 flavivirus-naïve plasma), and samples (all in duplicates). The cut-off was defined by the mean OD value of negatives plus 12 standard deviations, which gave a confidence level of 99.9% from 4 negative controls (Frey et al., 1998; Jarmer et al., 2014). The OD values were divided by the mean OD value of one positive control (OD close to 1) in the same plate to calculate the relative OD (rOD) values for comparison between plates. The rOD readouts were analyzed using GraphPad Prism 6 and the two-tailed Mann-Whitney test was used to determine the *p*-value comparing rOD values between two groups (Tsai et al., 2015).

Three-layer pooled DENV1-4 rNS1 ELISA

The three-layer combined rNS1 ELISA was performed on human sera or plasma from ZIKV-, DENV1-, DENV2-, DENV3-, and WNV-infected individuals. 96-well ELISA plates were coated with 8 ng/well of DENV1- and DENV3-NS1, and 4 ng/well of DENV2- and DENV3-NS1 using coating buffer. The remainder of the pooled DENV1-4 rNS1 ELISA protocol is identical to what has been described above.

Three-layer pr and prVLP ELISA

The three-layer pr and prVLP ELISAs were performed on human sera or plasma from ZIKV-, DENV1-, DENV2-, DENV3-, and WNV-infected individuals. 96-well ELISA plates were coated with 1072 ng of pr peptide per well or 50 ul of 1:1000 dilution of prVLP antigen prepared in coating buffer. The remainder of the pr and prVLP ELISA protocol is identical to what has been described above.

Clinical Samples

The study of coded serum or plasma samples was approved by the Institutional Review Board (IRB) at the University of Hawaii (CHS #17568, CHS #23786). Table 1 summarizes the basic information of the serum and plasma samples in this study. Forty convalescent-phase samples from RT-PCR confirmed ZIKV cases that were DENV-naïve (n=20) or previously DENV-exposed (n=20), designated as pZIKV and ZIKVwpDENV infection panels, respectively, were kindly provided by Dr. Eva Harris at the University of California Berkeley from the Pediatric Dengue Cohort Study in Managua, Nicaragua between July and September of 2016 (Kuan et al., 2009). The study was approved by the IRBs of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Nineteen convalescent-phase samples from patients who presented symptoms compatible with ZIKV and had detectable anti-DENV IgG antibodies, designated at probable ZIKVwpDENV infection panel, were kindly provided by Dr. Carlos Brites at the Federal University of Bahia, Salvador, Brazil between November 2015 and May 2016. The

study was approved by the IRB of Federal University of Bahia.

Convalescent-phase or post-convalescent phase dengue samples were from RT-PCR confirmed dengue cases including 40 from Kaohsiung, Taiwan between 2001 and 2009 (Wang et al., 2006), 18 from Nicaragua (three cases with sequential samples) between 2006 and 2008 (Lai et al., 2013), and 12 from the Big Island, Hawaii during the 2015 DENV1 outbreak. Flavivirus-naïve samples (n=52) were from a recent serosurvey in Taiwan (Lai et al., 2013). Forty plasma samples from blood donors with RT-PCR confirmed WNV infection were kindly provided by Dr. Susan Stramer at the American Red Cross.

Table 1. Basic information of serum/plasma panels in this study.

Panels[a]	Category	Number of subjects/ samples	Sampling time PSO[b]	Source of samples and year
pDENV1	convalescent to post-convalescent	22/26	19 days−15 months	Taiwan, 2006-9 Hawaii, 2015 Nicaragua, 2006-8
pDENV2	convalescent to post-convalescent	6/12	19 days−96 months	Taiwan, 2001-2
pDENV3	convalescent to post-convalescent	4/10	3−19 months	Taiwan, 2006-9
pWNV	early convalescent	37/37	NA	U.S. ARC, 2006-15
pZIKV	convalescent	20/20	14−24 days	Nicaragua, 2016
	post-convalescent	18/18	3−4 months	
ZIKVwpDENV	convalescent	20/20	14−19 days	Nicaragua, 2016
	post-convalescent	15/15	3−4 months	
probable ZIKVwpDENV	convalescent	19/19	6−14 days	Brazil, 2015-16
sDENV	convalescent	24/24	8−35 days	Taiwan, 2001-2
sDENV	post-convalescent	6/6	3−4 months	Taiwan, 2006-9 Nicaragua, 2006-8
sDENV	post-convalescent	18/18	12−12 months	Nicaragua, 2006-8
sDENV	post-convalescent	14/14	18−24 months	Taiwan, 2006-9 Nicaragua, 2006-8
sDENV	post-convalescent	5/5	67−72 months	Taiwan, 2006-9
Flavivirus-naive	NA	52/52	NA	Taiwan, 2015 serosurvey

[a]pDENV1, primary DENV1 infection; pDENV2, primary DENV2 infection; pDENV3, primary DENV3 infection; pWNV, primary WNV infection; pZIKV, primary ZIKV infection; sDENV, secondary DENV infection; ZIKVwpDENV, ZIKV infection with previous dengue; ARC, American Red Cross.
[b]PSO, post-symptom onset

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CHAPTER 3

RESULTS

Specific Aim 1: Express the pr protein of DENV and other flaviviruses and develop pr-based serological tests for ZIKV.

Experimental plan

In order to test the antigenicity of pr, DNA constructs were generated for DENV1 (D1), YFV, WNV, and ZIKV in two different expression systems: a Drosophila S2 cell-based system (Fig. 6A) and a HEK 293T cell-based system (Fig. 6B). The in vitro expression and production of pr proteins were examined by WB analysis followed by ELISA. Sera or plasma samples were tested in the ELISA formats to evaluate the antigenicity of pr.

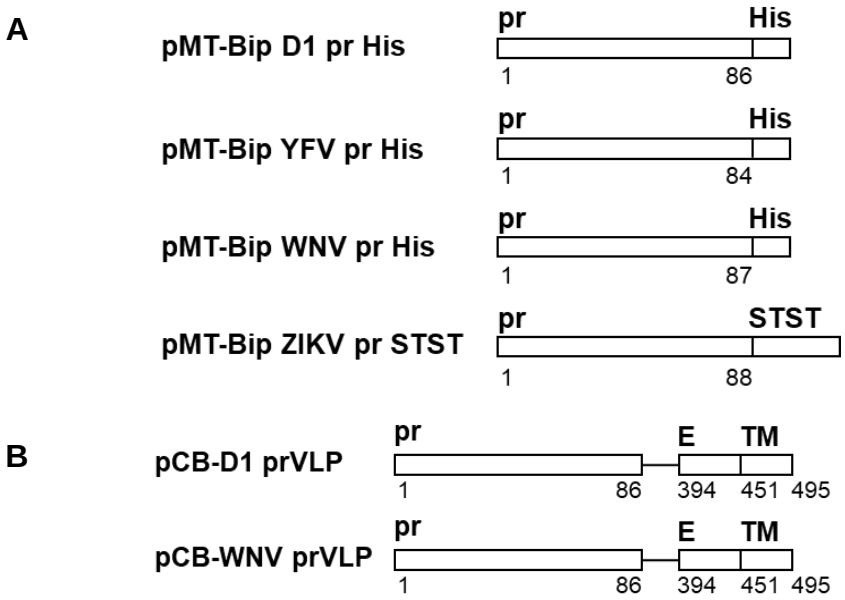


Fig. 6. Schematic drawings of the pr constructs. (A) S2 cell-optimized pMT-Bip-based constructs of pMT-D1-pr, pMT-YFV-pr, and pMT-WNV-pr with histidine (His) tags at the C-terminus, and pMT-ZIKV pr with a *Strep*-tag II (STST). (B) Schematic drawings of the pCB-D1-prVLP and pCB-WNV-prVLP constructs with truncated pr and E stem-anchor (transmembrane [TM] domains) connected by a six-amino acid glycine-serine linker sequence, GGSGSG.

Expression and purification of pr proteins generated in S2 cells

After transfection of Drosophila S2 insect cells with S2 cell-optimized pMT-Bip constructs of D1-, YFV-, WNV-, and ZIKV-pr, stable clones were established by Hygromycin B selection. Expression was confirmed by Coomassie blue staining and WB analysis of culture supernatants (Fig. 7). In the WB analysis, the expression of D1-, YFV-, and WNV-pr proteins was confirmed by anti-His mouse mAb that detected the histidine tag (Fig. 7A). The observation of bands with molecular weight higher than the monomer suggest that pr prefers to form dimers and other oligomers or binds to cellular proteins over remaining in its monomer form. This also suggests that the pr protein is unstable as a monomer, perhaps due to its small size and its intended role as a chaperone protein that forms heterodimers with the E protein (Lindenbach et al., 2013). Coomassie blue staining was performed for ZIKV-pr but its expression could not be confirmed at this stage (Fig. 7B).

The collected supernatants of D1-, YFV-, and WNV-pr His were purified using a His-trap column and ZIKV-pr STST was purified using a *Step*-Tactin affinity column to obtain pure pr proteins (Fig. 8). The resulting fractions were analyzed by Coomassie blue staining and WB. Multiple pr oligomers could be seen in in the YFV-pr His and WNV-pr His WB but the dominant population for D1-pr His appeared to be in dimer form. The ZIKV-pr STST had much higher expression overall compared to the histidine-tagged constructs, but also had multiple high molecular bands seen in the WB. The fractions with the most protein of interest while having minimal non-specific proteins were chosen to undergo buffer exchange to further concentrate the pr proteins (Fig. 9). The buffer exchange was followed by anion exchange chromatography using the AKTA purification system. The resulting fractions from the AKTA purification and ion exchange were tested by Coomassie blue staining, Western blot, and ELISA.

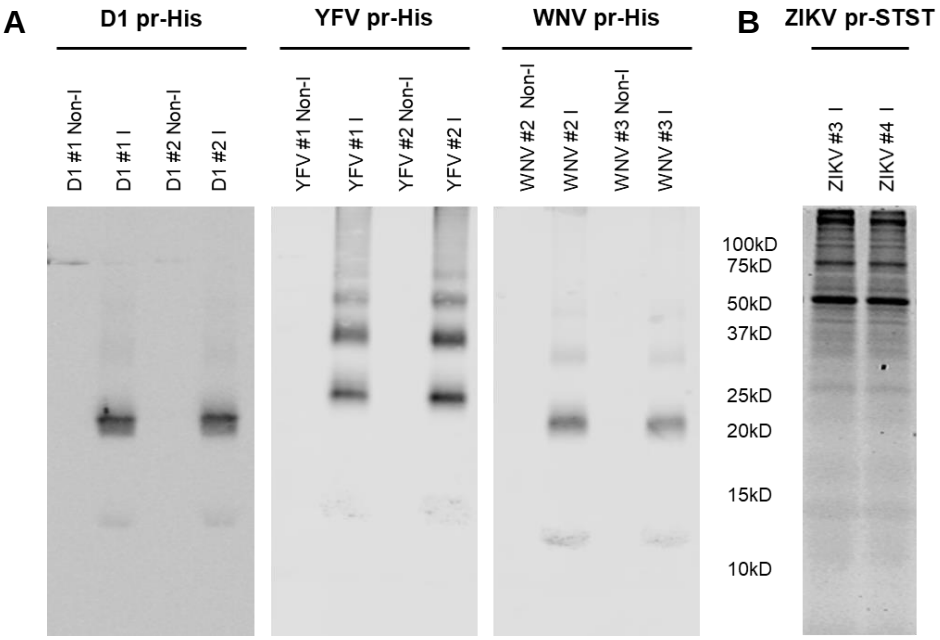


Fig. 7. PAGE analysis. (A) Western blot using anti-His mAb to detect the expression of pMT-Bip D1-, YFV-, and WNV-pr His in the culture supernatants with (I) or without (non-I) six days of induction with $CuSO_4$. (B) Scanned image of a Coomassie blue-stained gel of the pMT-Bip ZIKV-pr STST expression six days post-induction.

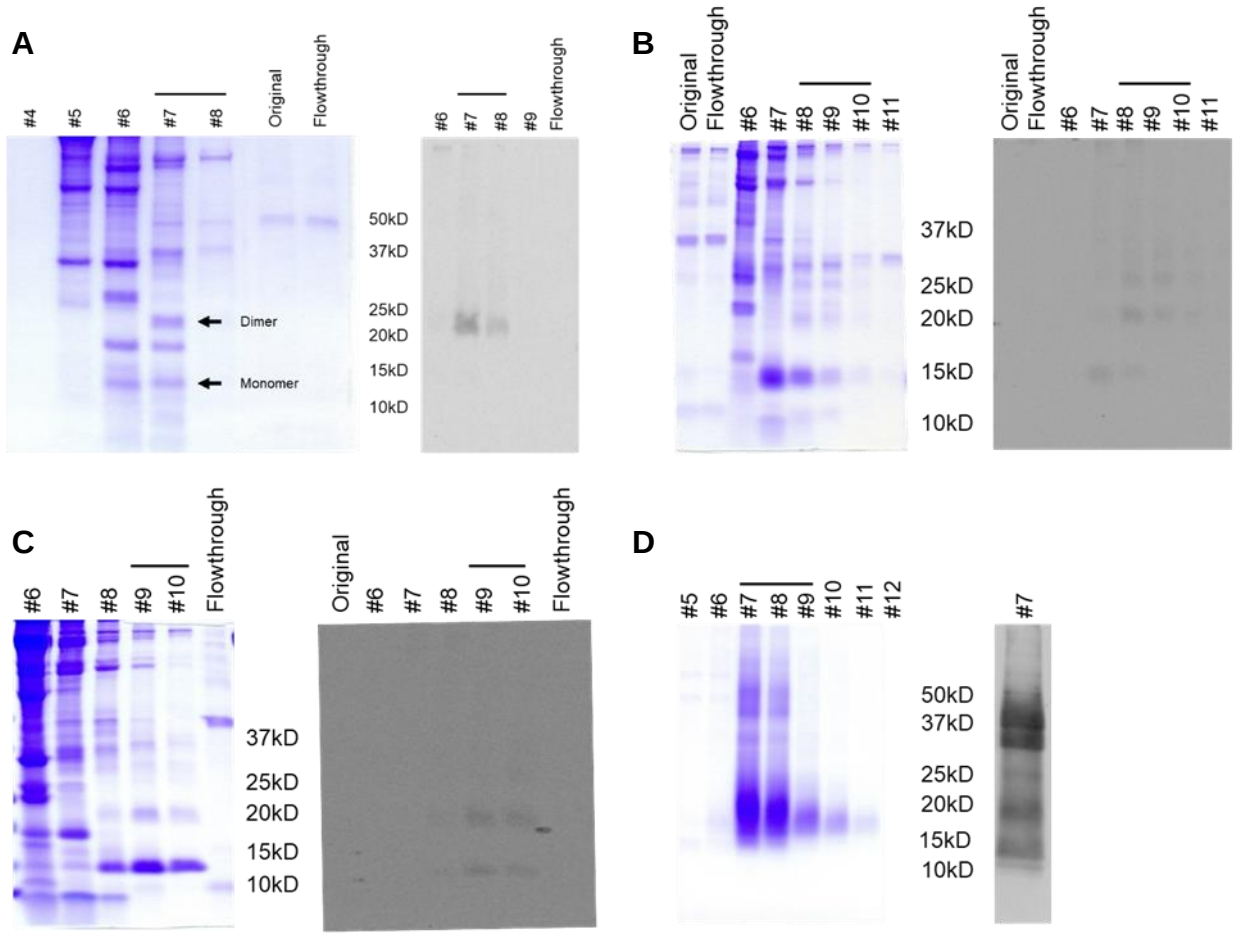


Fig. 8. Coomassie blue staining (left) and WB analysis (right). (A-C) WB analysis using anti-His mAb to detect (A) D1-, (B) YFV-, and (C) WNV-pr in the S2-cell culture supernatants after purification through a HisTrap column. (D) Coomassie blue staining (left) and WB analysis (right) using anti-*Strep*-Tactin mAb to detect ZIKV-pr STST in the S2 cell culture supernatants after purification through a *Strep*-Tactin affinity column. Numbers on the top indicate fraction number. Arrows indicate possible oligomerization of pr (A). The horizontal lines indicate fractions that were pooled together for further purification and concentration.

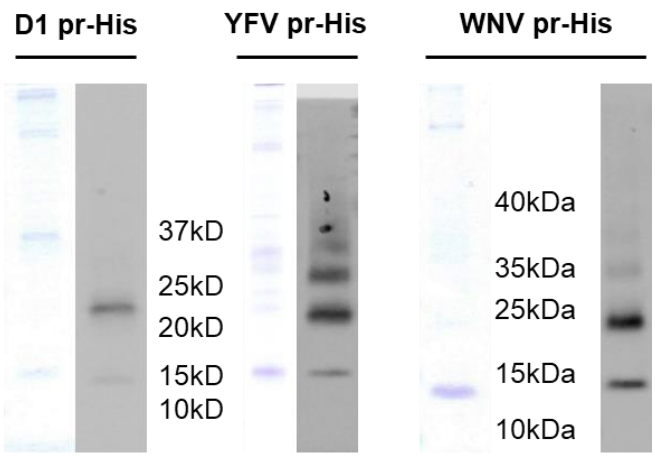


Fig. 9. Coomassie blue staining (left) and WB analysis (right) of the pooled and concentrated fractions from Fig. 8 of D1-, YFV-, and WNV-pr His using anti-His mAb.

Expression and purification of prVLP generated in HEK293T cells

To produce D1-prVLP and WNV-prVLP, pCB-based constructs containing truncated pr and an E protein stem-anchor region connected by a linker were generated (Fig. 6B). The protein was expressed by transfection in HEK 293T cells and the prVLP pellets were collected after ultracentrifugation of culture supernatants. Expression was confirmed by WB analysis and ELISA using clinical samples (Fig. 10).

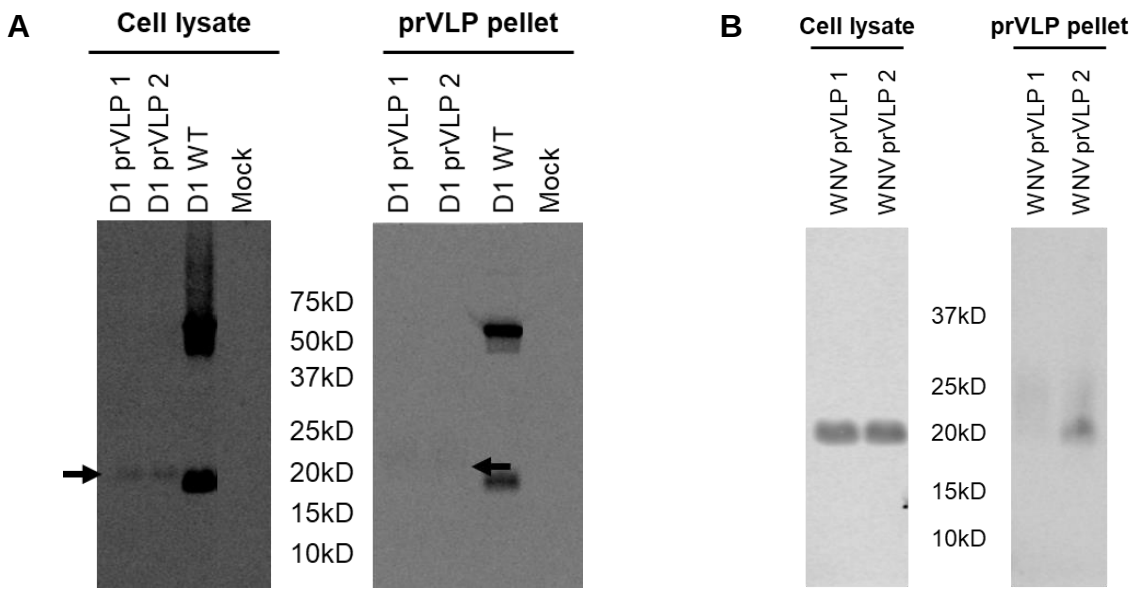


Fig. 10. WB analysis. (A) D1-prVLP and (B) WNV-prVLP expression in cell lysates (left) and prVLP pellets (right) using DENV- and WNV-immune human sera, respectively.

Testing the antigenicity of recombinant pr proteins

The antigenicity of pr protein was tested by WB analysis and ELISA using panels of sera and plasma from patients with pDENV1, pWNV, and pZIKV infections; YFV vaccinees; and flavivirus-naïve negative samples (Neg). In certain cases, WB analysis was performed using these clinical samples.

Table 2 summarizes the ELISA for the antigenicity of D1-pr His in comparison to the WB score. Due to the high non-specific reactivity of negative sera 3 (Neg S3) and negative plasma 3 (Neg P3), the cut-off for DENV-immune sera is elevated at 0.099. As a result, only two out of the nine pDENV sera tested were positive. These sera were also the highest-scoring by WB, demonstrating that they have strong antibody responses to pr protein (Table 2). However, the same sera were unable to detect D1-pr His by WB, suggesting the recognition of pr protein is affected by different experimental conditions. While WB and ELISA antigenicity tests were performed for YFV pr-His and WNV pr-His proteins, neither the YFV vaccinee sera nor the pWNV panel of sera were able to detect the recombinant YFV or WNV pr proteins. This suggests that perhaps the antigenic sites were not maintained, maybe due to the misfolding

of the pr proteins or the incorrect conformation at the experimental conditions (such as the ELISA coating and binding buffers) in which antibody binding would have taken place.

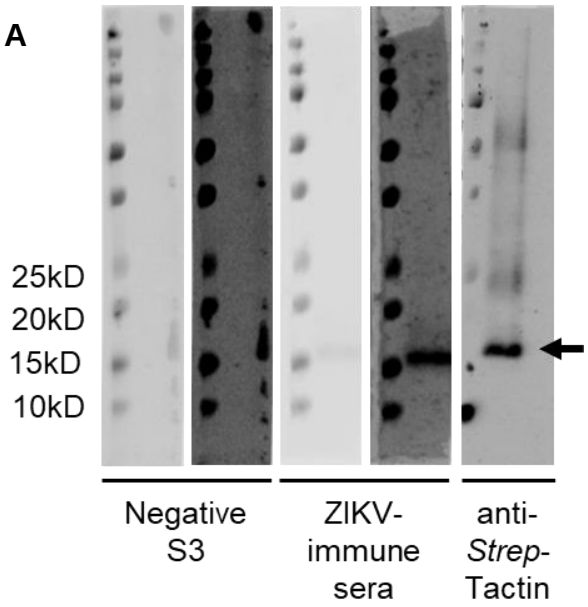
ZIKV-pr STST, on the other hand, was detected by ZIKV-immune sera in both WB and ELISA testing (Fig. 11). The clinical sample only detected the monomer conformation of ZIKV-pr STST in the WB, whereas the anti-*Strep*-Tactin mAb recognized multiple high molecular bands. These bands may represent oligomerization of pr or pr binding to cellular proteins. Taken together, this suggests that the antigenic sites on the ZIKV-pr STST may be correct or exposed only in the monomer form (Fig. 11A). The fact that pr oligomers are not naturally-occurring may provide one explanation as to the lack of recognition by human sera. These results were reflected in the ELISA, where both anti-*Strep*-Tactin mAb and the ZIKV-immune sera were able to recognize ZIKV-pr STST (Fig. 11B). However, Neg S3 had extremely high reactivity to fraction 8 where most of the pr protein was concentrated despite not observing any non-specific binding in the WB (Fig. 11A). Unlike Neg S3, Neg S2 had minimal reactivity to fraction 8 (Fig. 11B).

Table 2. The antigenicity of D1 pr-His protein tested by ELISA[a].

Primary Antibody[b] 1:400	OD	WB score[d]
Neg S1	0.017	-
Neg S2	0.009	-
Neg S3	0.062	-
Neg S4	0.015	-
Neg P1	0.015	-
Neg P2	0.009	-
Neg P3	0.051	-
Neg P4	0.014	-
K1003	0.414[c]	+++
Palau #8	0.264	+++
H1002	0.024	++
H1004	0.037	++
H1001	0.025	+
H1003	0.027	+
K1002	0.012	+
K1017	0.029	-
KS #38	0.019	-

[a]Coating concentration was 1072 ng/well of D1 pr-His protein.
[b]Human sera/plasma from negative and pDENV panels.
[c]The OD cut-off is the mean of negative sera/plasma plus 3 standard deviations (0.099). Yellow indicates positive and orange indicates high background levels.
[d]WB score indicates the intensity of prM protein band in WB detected by sera. +++: the strongest prM band is stronger than the weakest E protein band. ++: the strongest prM band is as strong as the weakest E protein band. +: the strongest prM band is weaker than the weakest E protein band.

A



B

	OD		
1°Ab	#6	#7	#8
anti-*Strep*-Tactin	0.017	0.569	1.304
ZIKV-immune sera	0.097	0.158	1.004
Negative S2	0.042	0.061	0.105
Negative S3	0.023	0.051	1.974

Fig. 11. Analysis of purified and ion-exchanged supernatants. (A) WB analysis of purified and ion-exchanged supernatant fraction #8 of ZIKV-pr STST using flavivirus-naïve (negative) sera, ZIKV-immune sera, and anti-*Strep*-Tactin mAb in short and long exposures. (B) ELISA of ZIKV-pr STST purified and ion-exchanged supernatant fractions 6, 7, and 8 using anti-*Strep*-Tactin rabbit mAb, ZIKV-immune human sera, and two negative sera. Yellow indicates positive; orange indicates high background levels.

Testing the antigenicity of prVLPs

The antigenicity of D1-prVLP was tested by ELISA using panels of sera/plasma from patients with pDENV, sDENV, pWNV, pZIKV, and ZIKVwpDENV infections, and flavivirus-naïve negative samples (Fig. 12). Three out of 49 negative samples were positive, indicating a minimal background reactivity. Four pWNV, two pZIKV, and one ZIKVwpDENV samples were positive, resulting in a specificity of 93.1%. While the sensitivity for pDENV panel was 63%, the sensitivity for sDENV panels collected in the early convalescent phase (up to 6M) was 100% and that for post-convalescent phase was 83%. The sensitivity dropped to 43% after 1.5 years. While antigenicity testing was done for WNV-prVLP, the pWNV panel was unable to recognize the WNV-prVLP. This may have been due to misfolding, conformational issues, or the experimental conditions preventing the recognition of antigenic sites on the WNV-prVLPs.

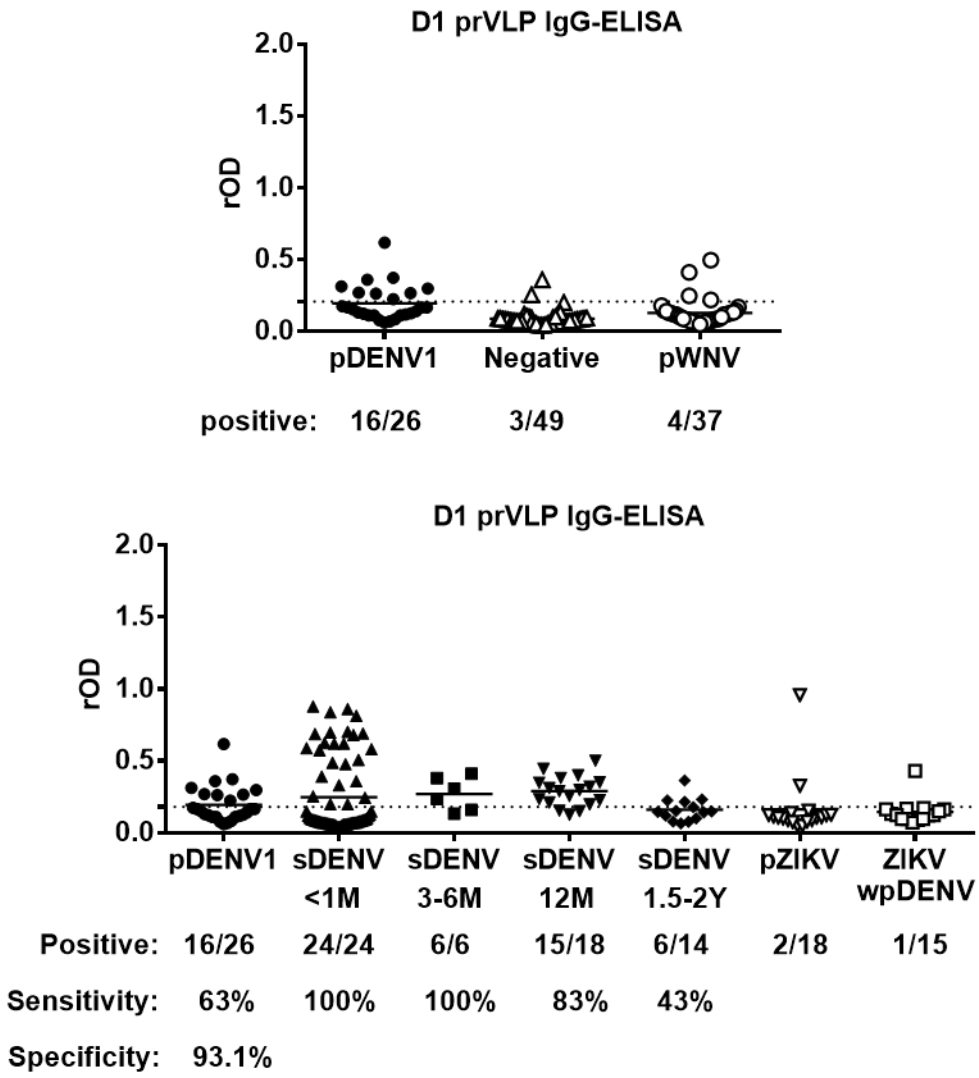


Fig. 12. IgG ELISA results of D1-prVLP. Panels of pDENV, sDENV, pWNV, pZIKV, ZIKVwpDENV, and flavivirus-naïve samples were tested, and the sensitivity and specificity were determined. Dot lines indicate cut-off values.

Specific Aim 2: Develop NS1-based serological tests to distinguish ZIKV from DENV and other flavivirus infections.

Experimental plan

In order to establish and validate the DENV2-, DENV3-, DENV4-, pooled DENV1-4-, and WNV-NS1 IgG ELISA, sera and plasma samples from confirmed pDENV, sDENV, pZIKV, ZIKVwpDENV, and pWNV infections were tested. To determine if the antibody response against DENV is serotype-specific, additional ELISAs were performed using pDENV1, pDENV2, and pDENV3 samples.

E protein-based versus NS1-based ELISA

Antibodies generated against the E protein are highly cross-reactive to different flaviviruses, making it challenging to distinguish the infecting virus relying on an E protein-based IgG ELISA alone (Fig. 13A-C). We developed ZIKV-, DENV-, and WNV-NS1-based ELISA capable of successfully distinguishing pDENV1, pWNV, and pZIKV infections as the antibodies generated against the NS1 protein are specific to the infecting virus in the situation of primary infection (Fig. 13D-F).

Validation of DENV2-, DENV3-, DENV4-, and DENV1-4-NS1 ELISAs with pDENV panels

Our previous work used DENV1-NS1 IgG ELISAs to detect all DENV samples, including those of pDENV1, pDENV2, pDENV3, and sDENV infections. We therefore wanted to determine the specificity and sensitivity of DENV1-, DENV2-, and DENV3-NS1 IgG ELISAs to recognize their respective clinical samples (Fig. 14). All of the pDENV1 and pDENV3 samples recognized their respective NS1 with minimal reactivity to pWNV, pZIKV, and negative samples, and 10/12 of pDENV2 samples recognized DENV2-NS1 (Fig. 14). Next, we tested each pDENV panel with different DENV-NS1 ELISAs. pDENV1 panel recognized DENV-NS1 with highest detection rate, followed by DENV2 and DENV3. pDENV2 panel

recognized DENV2-NS1 best, followed by DENV1 and DENV3. pDENV3 panel recognized DENV1 and DENV3 equally well, followed by DENV2 (Fig. 15). Therefore, by relying on DENV1-NS1 alone, the capacity of our DENV-NS1 ELISA to detect different DENV infection was diminished, especially in pDENV2 infection. Using a pooled DENV1-4-NS1 ELISA therefore would increase the ability for our DENV-ELISAs to detect different DENV infections, as seen in Fig. 15. For all three pDENV panels, the use of the pooled DENV1-4-NS1 ELISA led to an improvement in the detection rate as compared to using only NS1 of one DENV serotype.

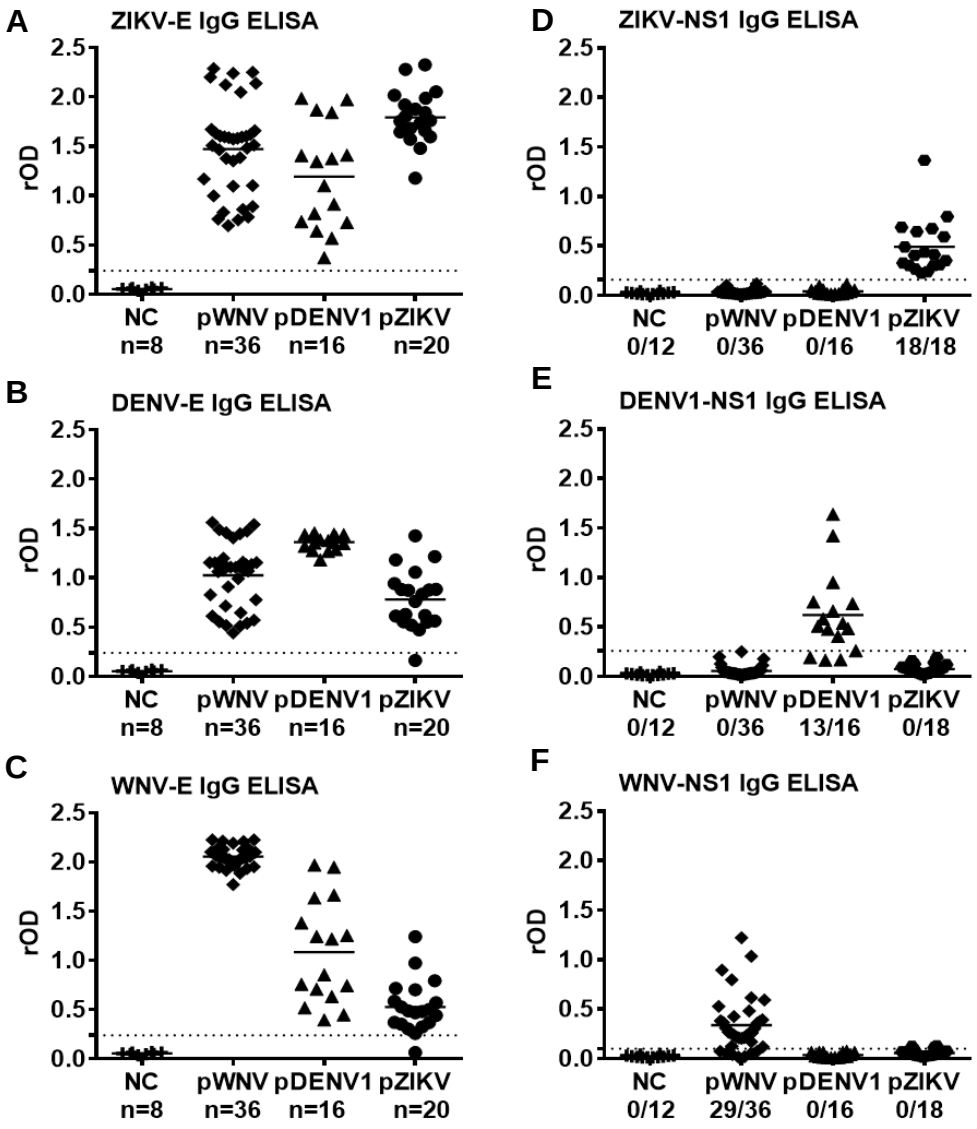


Fig. 13. E protein-based and NS1-based IgG ELISAs for pZIKV, pDENV1 and pWNV panels. (A) ZIKV-, (B) DENV-, and (C) WNV-E protein IgG ELISAs detected cross-reactive anti-E antibodies in pWNV, pDENV1, and pZIKV panels. (D) ZIKV-, (E) DENV-, and (F) WNV-NS1 IgG ELISAs can distinguish pWNV, pDENV1, and pZIKV infections.

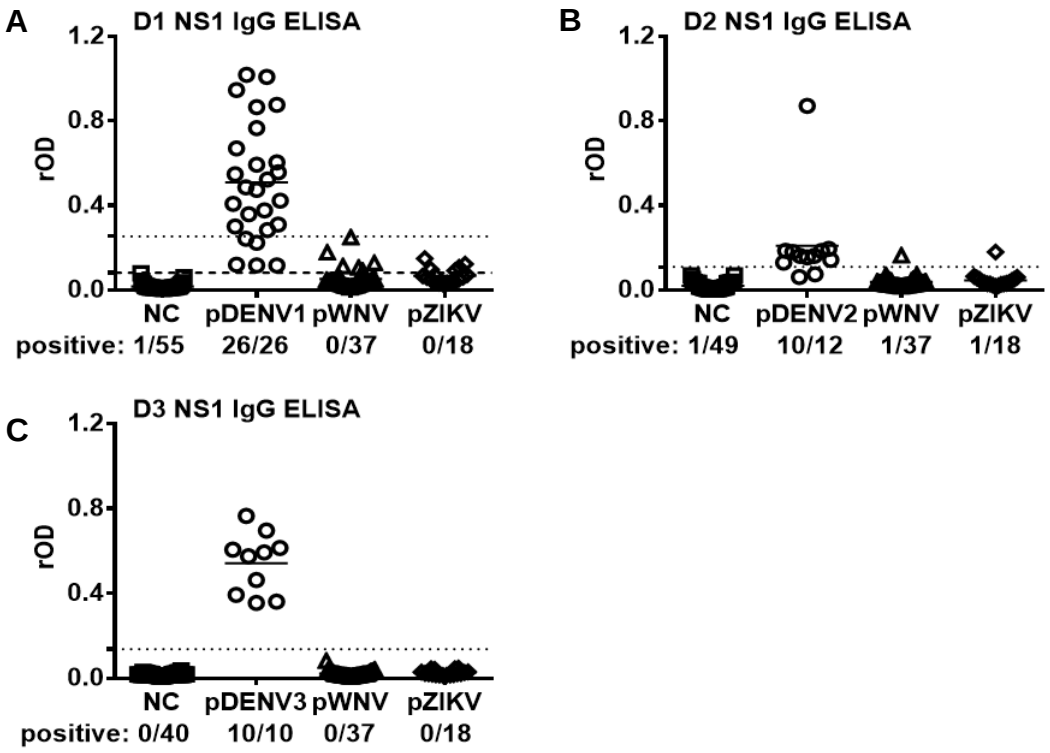


Fig. 14. Comparison of three DENV-NS1 IgG ELISAs. (A) DENV1-, (B) DENV2-, and (C) DENV3-NS1 IgG ELISA showed the specificity to the respective infected serotypes in primary infection sera. Dot lines indicate cut-off values.

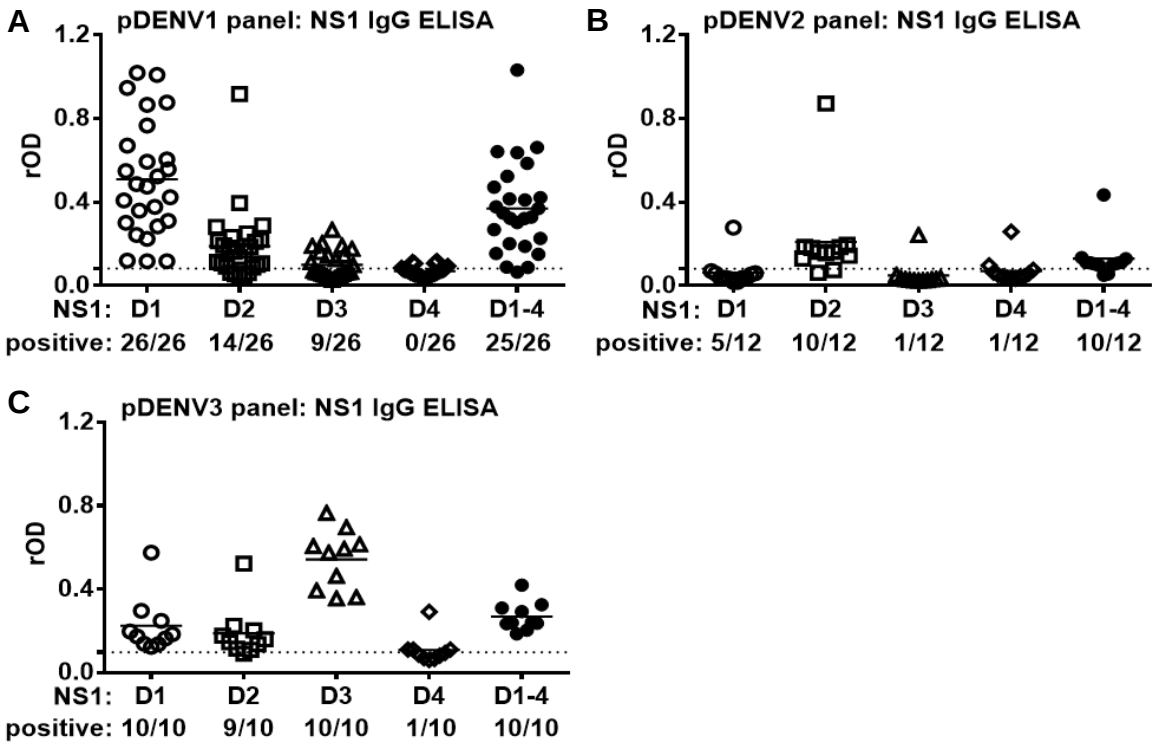


Fig. 15. Comparison of five DENV NS1 IgG ELISAs for the detection of different primary DENV infections. (A) pDENV1, (B) pDENV2, and (C) pDENV3 panels demonstrated the highest detection rate in NS1 IgG ELISAs using NS1 of the infecting serotype or pooled DENV1-4-NS1 compared with NS1 of non-infecting serotypes. Dot lines indicate cut-off values.

Validation of DENV2-, DENV3-, DENV4-, and DENV1-4-NS1 ELISA with secondary flaviviral infection panels

The antibody response following a secondary exposure to a flavivirus is known to be more robust and more cross-reactive (Screaton et al., 2015). As a result, using serological methods to distinguish the most recent flavivirus infection from a past infection can be challenging. Fig. 16A shows that the DENV1-4-NS1 ELISA is capable of detecting sDENV samples just as well as DENV1-NS1 ELISA alone regardless of the timing of sampling during the convalescent and post-convalescent phases. Fig. 16B shows an increase in the detection rate of DENV1-4-NS1 ELISA for the ZIKVwpDENV panel compared with DENV1-NS1

ELISA, confirming the cross-reactive nature of antibodies generated after secondary flaviviral infections. To distinguish sDENV infections from ZIKVwpDENV, our group previously developed an algorithm involving calculation of the relative OD ratio of the ZIKV-NS1 OD values to the DENV-NS1 OD values (Tsai et al. 2017). The cut-off was determined to be 0.24; anything equal or greater would be considered ZIKVwpDENV; anything less than 0.24 would be considered sDENV. Using this algorithm, our DENV1-4-NS1 ELISA can distinguish sDENV infections from ZIKVwpDENV infections with a sensitivity of 92% and a specificity of 89% (Fig. 17).

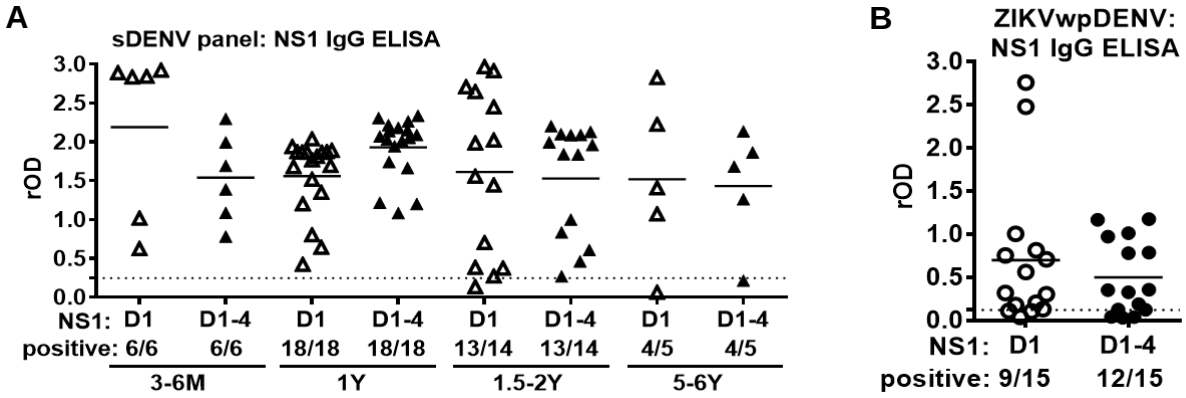


Fig. 16. Comparing detection rate of DENV1-NS1 ELISA versus DENV1-4-NS1 ELISA. (A) sDENV samples and (B) ZIKVwpDENV samples. Dot lines indicate cut-off values.

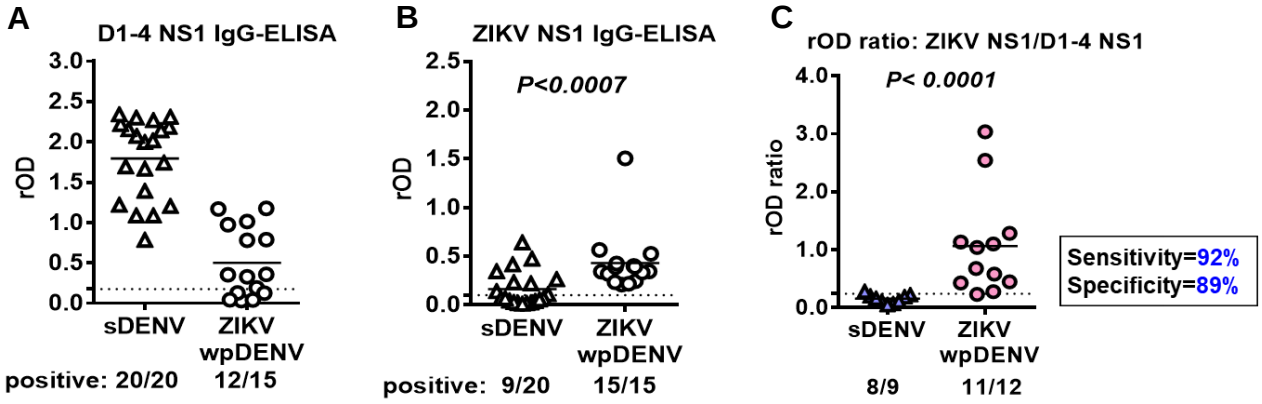


Fig. 17. Distinguishing sDENV and ZIKVwpDENV using two ELISAs. (A) Pooled DENV1-4-NS1 ELISA. (B) ZIKV-NS1 ELISA. (C) rOD ratio = ZIKV-NS1/rOD DENV-NS1. The rOD ratio algorithm applied to distinguish the two sera panels. Dot lines indicate cut-off values.

Table 3. Summary of the detection rate of each NS1 ELISA.

ELISA	naïve	pWNV	pDENV1	pDENV2	pDENV3	pZIKV	sDENV	ZIKV wpDENV
D1 NS1 IgG	1/55 (1.8%)	0/37 (0%)	26/26 (100%)	5/12 (41.7%)	10/10 (100%)	0/38 (0%)	64/67 (95.5%)	26/35 (74.3%)
D2 NS1 IgG	1/49 (2.0%)	1/37 (2.7%)	14/26 (53.8%)	10/12 (83.3%)	9/10 (90%)	1/18 (5.6%)		
D3 NS1 IgG	0/40 (0%)	0/37 (0%)	9/26 (34.6%)	1/12 (8.3%)	10/10 (100%)	0/18 (0%)		
D1-4 NS1 IgG	0/55 (0%)	1/37 (2.7%)	25/26 (96.2%)	10/12 (83.3%)	10/10 (100%)	0/38 (0%)	64/67 (95.5%)	30/35 (85.7%)
ZIKV NS1 IgG	0/12 (0%)	0/37 (0%)	0/26 (0%)	0/12 (0%)	0/10 (0%)	19/38 (50%)[a]	32/67 (47.8%)	34/35 (97.1%)

[a]For pZIKV panel <1M PSO, the detection rate is 5% (1/20), whereas for those >3M, it is 100% (18/18)

Testing of WNV-NS1 ELISA with secondary flaviviral infection panels

We showed earlier how the WNV-NS1 ELISA can distinguish pDENV, pZIKV, and pWNV infections (Fig. 13F). However, in cases where secondary flaviviral infections are involved, distinguishing sDENV, ZIKVwpDENV, and pWNV can be challenging due to the cross-reactive antibodies (Fig. 18A). The use of a 6M urea wash in ELISA, which was a successful method used in our previous work, had no effect as it dampened the signal across the board (Fig. 18B). As a result, we were not able to distinguish pWNV, sDENV, and ZIKVwpDENV panels using the WNV-NS1 IgG ELISA alone. However, the addition of the WNV-NS1 ELISA does not interfere with the previously-established algorithm and allows for differentiation of WNV with previous flavivirus infection (WNVwpFla) or sDENV from ZIKVwpDENV infection (Fig. 19).

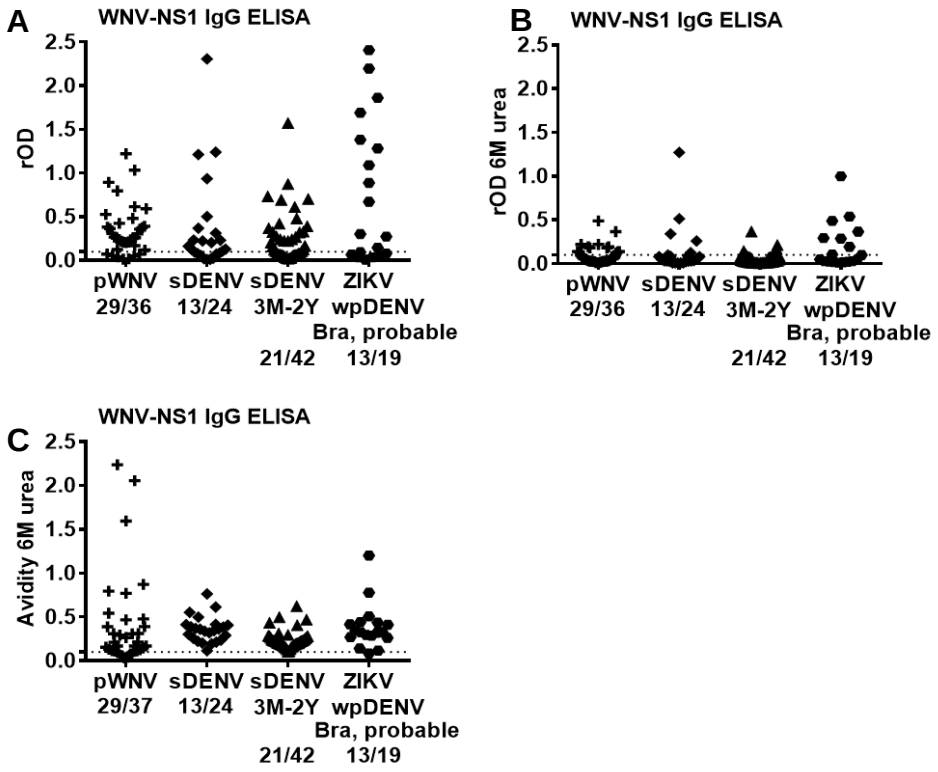
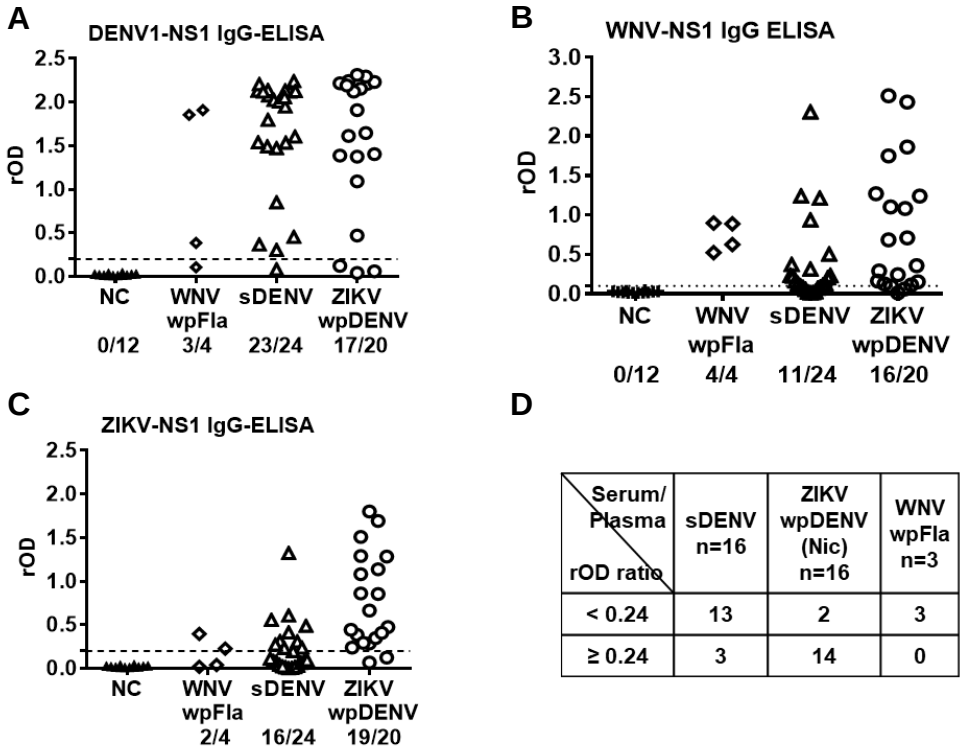


Fig. 18. WNV-NS1 IgG ELISA for pWNV, sDENV, and ZIKVwpDENV panels. (A) WNV-NS1 IgG ELISA. (B) 6M urea wash WNV-NS1 ELISA. (C) The avidity after 6M urea wash is shown. Dot lines indicate cut-off values.



Serum/ Plasma / rOD ratio	sDENV n=16	ZIKV wpDENV (Nic) n=16	WNV wpFla n=3
< 0.24	13	2	3
≥ 0.24	3	14	0

Fig. 19. Distinguishing secondary flaviviral infections using three ELISAs. (A) DENV1- (B) WNV-, and (C) ZIKV- NS1 ELISA. (D) Summary of the results using the algorithm rOD ratio = ZIKV-NS1/rOD DENV-NS1. Dot lines indicate cut-off values. WNVwpFla: WNV infection with previous flavivirus infection.

Proposed algorithm to distinguish ZIKV and DENV infections

Since the sensitivity of NS1 IgG ELISA for primary infection panel was not as good as that of E protein-based IgG ELISA (Fig. 13), our separate study has tested E protein-based IgG ELISA under higher dilution of sera and found that it can distinguish pDENV, pZIKV and pWNV panels based on the highest rOD values (data not shown). Based on these works and analysis of the results of these ELISAs (Figs. 20 and 21), we propose an algorithm to distinguish pZIKV, ZIKVwpDENV, pDENV, sDENV, and pWNV infections in endemic regions where multiple flaviviruses co-circulate (Fig. 22).

A

pDENV1	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
NDF4-1	+	–	–
NDF26-3	+	–	–
NDF31-1	+	–	–
NDF33-1	+	–	–
H1001	+	–	–
H1002	+	–	–
H1003	+	–	–
H1004	+	–	–
K1002	+	–	–
K1003	+	–	–
K1004	+	–	–
K1017	+	–	–
K1014	–	–	–
K1015	+	–	–
K1018	–	–	–
854-1	–	–	–
	13/16	0/16	0/16

B

pZIKV	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
7613	–	+	–
5839	–	+	–
5966	–	+	–
7434	–	+	–
7658	–	+	–
5248	–	+	–
4041	–	+	–
5893	–	+	–
4445	–	+	–
1304	–	+	–
5953	–	+	–
6763	–	+	–
6563	–	+	–
6669	–	+	–
7253	–	+	–
6697	–	+	–
3758			
7444	–	+	–
6248	–	+	–
7612			
	0/20	18/18	0/20

C

pWNV	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
WR 3297	–	–	+
WR 3256	–	–	+
WR 3058	–	–	+
WR 3052	–	–	+
WR 2994	–	–	+
WR 2973	–	–	+
WR 2931	–	–	-
WR 2923	–	–	-
WR 2825	–	–	+
WR 2812	–	–	+
WR 2787	–	–	+
WR 2726	–	–	-
WR 2697	–	–	+
WR 2687	–	–	+
WR 2679	–	–	+
WR 2659	–	–	+
WR 2441	–	–	-
WR 2419	–	–	-
WR 2148	–	–	+
WR 2129	–	–	+
WR 2054	–	–	+
WR 1567	–	–	+
WR 1543	–	–	+
WR 1506	–	–	+
WR 1483	–	–	+
WR 1452	–	–	+
	0/36	0/36	29/36

Fig. 20. Summary of NS1 IgG ELISA patterns. (A) pDENV, (B) pZIKV, and (C) pWNV infections.

A

sDENV	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
2077	+	+	+
2575	+	–	–
1210	+	+	+
1323	+	+	+
1322	+	+	+
1519	+	+	–
1511	+	–	–
2680	+	–	–
2552	+	+	+
2493	+	–	–
2139	+	–	–
2705	+	–	–
1320	+	+	–
1216	+	+	+
1879	+	+	–
1673	+	+	–
1297	+	+	+
2343	+	+	–
2682	+	+	+
2099	+	+	+
2459	+	+	+
2346	+	+	+
NDF38	–	–	–
2184	+	–	–
	23/24	16/24	11/24

B

ZIKVwpDENV	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
5066	+	+	+
7487	+	+	–
6775	+	+	+
4749	–	+	+
6189	+	+	+
6625	+	+	+
7933	+	+	+
5550	+	+	–
7208	+	+	+
6547	–	+	–
5575	+	+	+
6364	+	+	+
4069	+	+	+
5962	+	+	+
4203	+	+	+
5355	+	–	+
2049	+	+	+
2056	–	+	–
2076	+	+	+
2081	+	+	+
	17/20	19/20	16/20

C

WNVwpFla	NS1-IgG ELISA		
	DENV1	ZIKV	WNV
WR 3141	+	+	+
WR 2581	–	–	+
WR 1981	+	+	+
WR 1558	+	–	+

Fig. 21. Summary of NS1 IgG ELISA patterns. (A) sDENV, (B) ZIKVwpDENV, and (C) WNVwpFla infections.

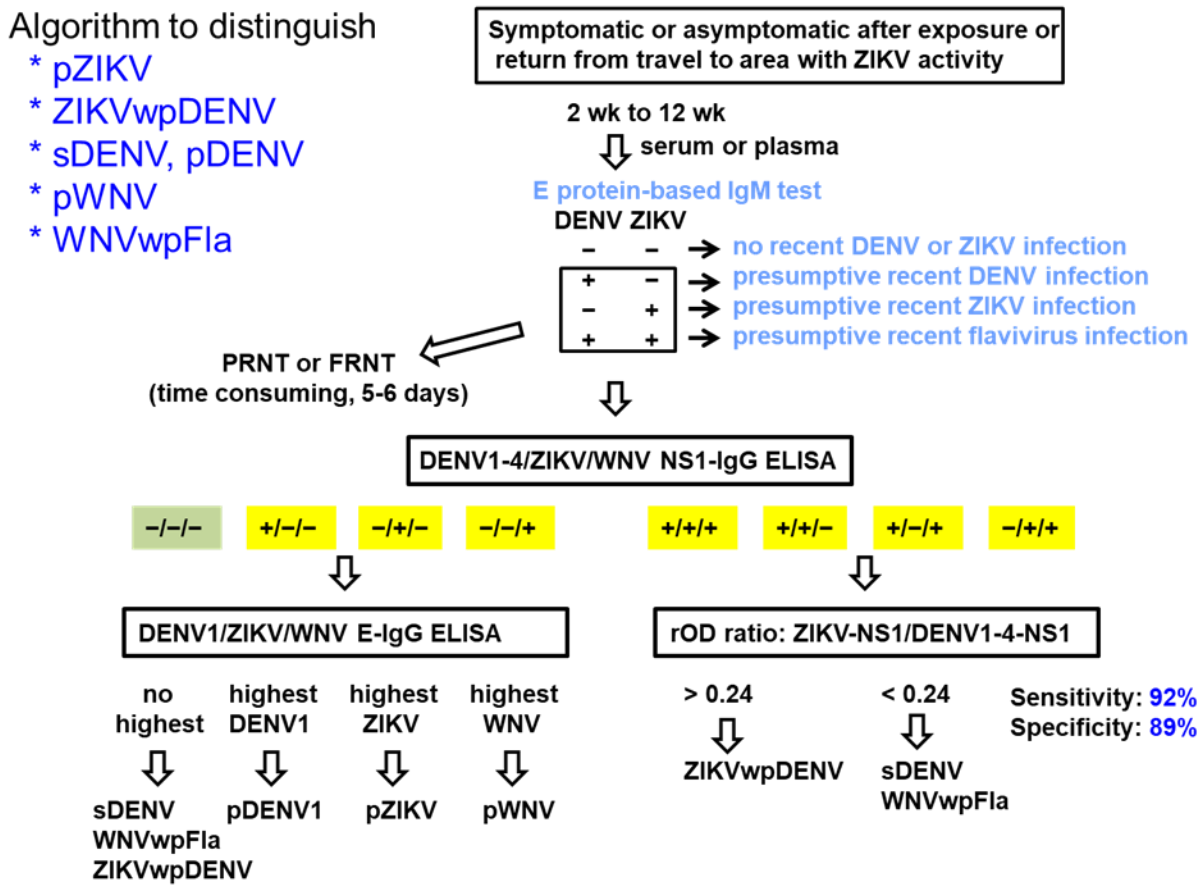


Fig. 22. Proposed algorithm of using three ELISAs to distinguish pZIKV, ZIKVwpDENV, pDENV, sDENV, and pWNV infections in endemic regions. This is based on this and previous studies (Tsai et al., 2017) in the context of the CDC guidelines for laboratory diagnosis of ZIKV infection

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CHAPTER 4

DISCUSSION

Overview

Due to the recent ZIKV outbreak in DENV-endemic areas, developing a serodiagnostic assay capable of differentiating flavivirus infections has become increasingly important. In agreement with our previous work, we showed that E protein-based ZIKV, DENV1, and WNV IgG ELISAs are unable to differentiate between pWNV, pDENV1, pZIKV, sDENV, and ZIKVwpDENV infections due to the cross-reactive antibodies generated against the E protein (Figs. 13A-C and data not shown). The antibody response against the pr and NS1 proteins tend to be more serocomplex-specific compared to the E protein, making it an attractive target for developing serological assays (Tsai et al., 2017). Here, we developed a DENV1-pr IgG ELISA and WNV-, DENV2-, DENV3-, DENV4-, and pooled DENV1-4-NS1 IgG ELISAs capable of distinguishing ZIKV from DENV and other flaviviral infections.

Two expression systems were developed to express the pr proteins: a Drosophila S2 cell-based system and a HEK 293T cell-based system. Constructs for DENV1-, YFV-, WNV-, and ZIKV-pr were designed and expressed in S2 cells successfully, as seen in the Coomassie blue staining and WB analysis (Figs. 7, 8, 9). Antigenicity testing was performed by ELISAs of all four constructs using sera and plasma samples from patients with pDENV, pZIKV, pWNV, sDENV, and ZIKVwpDENV infections, and flavivirus-naïve negative samples. Recombinant DENV1-pr protein had limited capability of being recognized by anti-pr antibodies in pDENV sera, partly due to the high background in certain negative samples which led to a high cut-off point (Table 2). Furthermore, DENV1-pr could not be recognized in ELISA by the same pDENV sera, which recognized DENV1-prM in the WB analysis, suggesting that the antigenicity of DENV1-pr is affected by different experimental conditions. The expression levels of ZIKV-pr was the most promising of the four constructs and the WB analysis showed the binding of ZIKV-immune sera to a single band as compared to the anti-*Strep*-Tactin mAb, which recognized several bands of varying sizes (Fig. 11A). However, upon performing an ELISA using the same fractions tested in the WB, we found that certain negative samples had very high non-specific binding to components of the fraction containing ZIKV-pr that were not visible in the WB (Fig. 11B). Antigenicity tests were similarly performed for YFV- and WNV-pr proteins but neither the YFV vaccinee sera nor the pWNV sera were

able to detect the recombinant YFV or WNV pr proteins. This suggests that either the conformation or folding of the pr proteins were incorrect, preventing the formation or accessibility of the antigenic sites.

Constructs for DENV1- and WNV-prVLP were designed and expressed in HEK 293T cells successfully, as seen in the WB analysis (Fig. 10). Antigenicity was tested by ELISA using the same panels of sera and plasma from patients with pDENV, sDENV, pWNV, pZIKV, and ZIKVwpDENV infections, and flavivirus-naïve negative samples. DENV1-prVLPs ELISA results show minimal background reactivity by the negative samples, positivity among different DENV panels, and minimal cross-reactivity by the pWNV, pZIKV, and ZIKVwpDENV panels (Fig. 12). The sensitivity of the assay is 100% in the <1M and 3-6M, 83% in the 12M, 43% in the 1.5-2Y samples of the sDENV panels, and 63% in the pDENV1 panel (Fig. 12B). This suggests that the antibody response against pr in pDENV1 infection may not be strong compared to that to E or NS1 protein but the anti-pr antibody can be robust in sDENV infection up to a year post-infection. While antigenicity tests were done with the WNV-prVLPs, the pWNV panel of sera was unable to detect the WNV-prVLPs, suggesting that similar misfolding or conformational issues might occur as seen in the YFV- and WNV-pr constructs.

We have shown that using a combination of ZIKV-, DENV1-, and WNV-NS1 IgG ELISAs can differentiate convalescent-phase samples of pZIKV, pDENV, and pWNV infections (Fig. 13D-F). This work relied on DENV1-NS1 to detect all pDENV and sDENV infections. We therefore wanted to determine the specificity and sensitivity of DENV1-, DENV2-, and DENV3-NS1 IgG ELISAs to detect anti-NS1 antibody in their respective panel of pDENV sera as well as panels of other DENV serotypes, and to assess whether using a pooled DENV1-4-NS1 in IgG ELISA instead would increase the detection rate of our DENV-NS1 IgG ELISA. We found that while pDENV1 and pDENV3 can be detected using DENV1-NS1 IgG ELISA, only five out of 12 pDENV2 samples could be detected using the same ELISA (Fig. 15). Therefore, by relying solely on DENV1-NS1 IgG ELISA in a serodiagnostic setting, we run the risk of missing more than half of pDENV2 infections. Table 3 summarizes the results of ELISAs for different panels. The use of a pooled DENV1-4-NS1 in IgG ELISA can greatly improve the detection rate for the pDENV2 panel with comparable results for pDENV1 and pDENV3 panels. However, sampling timing is critical and this is apparent in the pZIKV panel, where detection rate was 5% (1/20) for those collected

<1M PSO and 100% (18/18) for those >3M (Table 3). In order for our NS1 protein-based IgG ELISAs to be sensitive, the serum samples must be collected within the appropriate time frame where IgG levels can be detected. Our group previously established an algorithm capable of distinguishing sDENV and ZIKVwpDENV infections by using a combination of ZIKV- and DENV1-NS1 ELISAs, and we wanted to ensure that the use of DENV1-4-NS1 ELISA did not negatively affect the sensitivity and specificity of the test (Tsai et al., 2017). We found that the use of the pooled DENV1-4-NS1 improved the sensitivity and specificity to 92% and 89%, respectively (Fig. 17).

We have also developed a WNV-NS1 ELISA capable of distinguishing pDENV, pZIKV, and pWNV infections, and we sought to expand this ELISA to be able to distinguish secondary flaviviral infections (Fig. 13F). The addition of a 6M urea wash step in the IgG ELISA protocol removes weakly-binding antibodies, and we have had success in our previous work using this protocol in ZIKV-NS1 IgG ELISA (Tsai et al., 2018). However, we found that this did not improve our WNV-NS1 IgG ELISA as it reduced the signal across the board (of different serum panels) and as a result, we were unable to distinguish pWNV, sDENV, and ZIKVwpDENV panels using the WNV-NS1 IgG ELISA with or without 6M urea wash (Fig. 18). However, we found that the addition of the WNV-NS1 ELISA did not interfere with the previously-established algorithm, allowing for differentiation of ZIKVwpDENV from sDENV or WNVwpFla infections (Fig. 19).

A serological test capable of distinguishing different primary and secondary flaviviral infections is critically needed, especially in areas where multiple flaviviruses causing similar clinical manifestations are co-circulating. In light of the recent emergence of ZIKV in DENV-endemic regions and its association with Guillain-Barré syndrome and congenital birth defects, the ability to correctly diagnose, counsel, and treat patients has become more important than ever before. In conjunction with the recommendations made by the CDC, we have developed a serological testing algorithm to distinguish primary and secondary flaviviral infections in the context of current CDC guidelines (Fig. 22). If molecular testing by RT-PCR is inconclusive, an E protein-based IgM test will be performed. Due to the variable sensitivity and specificity of the IgM tests, any diagnosis made at this stage is only presumptive and must be followed by PRNT or other neutralization tests, which can be time consuming and requires numerous reagents and skilled

laboratory workers. Instead, we recommend performing a combination of DENV1-4-, ZIKV-, and WNV-NS1 IgG ELISA. If the clinical sample is negative for all three tests or positive for just one, a diagnosis can be made following a DENV-, ZIKV-, and WNV-E IgG ELISA substantiating the results of the NS1 ELISA. If two or more NS1 ELISA tests are positive, then a rOD ratio can be calculated and if the ratio is > 0.24, it is likely to be ZIKVwpDENV; if the ratio is < 0.24, the infection is likely to be sDENV or WNVwpFla.

Limitations and future plans

One of the biggest limitations to this study is the relatively small sample size of the tested panels of sera and plasma. We are also lacking in certain panels such as pDENV4 and YFV. Second, our panels are limited in terms of sampling time; while all of the samples are in the convalescent or post-convalescent phase, some were collected a few weeks after symptom onset, and others were collected several weeks to months after symptom onset. Third, we focused on the IgG ELISAs which is relevant and important to provide a useful tool for future studies of the epidemiology, pathogenesis and complications of ZIKV infections among individuals with different flaviviral exposure in endemic regions. However, employing these antigens in the IgM ELISA format has not been explored in this study. In addition, we hope to further expand the ability of our flavivirus ELISA to distinguish other flaviviral infections such as YFV, JEV, and TBEV as well as immunizations with currently available flaviviral vaccines, including Dengvaxia, YFV-17D, JEV and TBEV vaccines

Concluding Summary

In summary, we have successfully expressed the pr proteins of DENV1, YFV, WNV, and ZIKV in two different expression systems. We have developed a DENV1-prVLP IgG ELISA capable of distinguishing pDENV1 and sDENV infections from pWNV, pZIKV, and ZIKVwpDENV infections with an overall specificity of 93.1%. We have also successfully validated a pooled DENV1-4-NS1 IgG ELISA which

improved the sensitivity and specificity of our testing algorithm to 92% and 89%, respectively, to distinguish sDENV and ZIKVwpDENV infections. Taken together, this study provides an alternative and promising serological testing algorithm that is more cost- and time-effective and can be used in flavivirus-endemic areas.

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