ANALYSIS OF DENGUE AND ZIKA ANTIBODIES AMONG A COHORT OF PREGNANT WOMEN IN SALVADOR, BRAZIL

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES

(TROPICAL MEDICINE)

JULY 2023

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ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Sandra Chang, Dr. Mariana Gerschenson, Dr. Kevin Palmer, Dr. William Gosnell, and Dr. DeWolfe F. Miller for their guidance and direction in completing my dissertation. In addition to their scientific guidance, I'd also like to thank Dr. Mariana Gerschenson and Dr. Kevin Palmer for their personal support throughout the challenges faced during my time as a student.

Thank you to my family – Dad, Joe, Madison, Hailey, and Gia, and to my mom and stepmom whom I lost during my time as a student. Your never-ending support and unconditional love made me the person I am today.

Thank you to my husband, Jeff. I could not have done this without you by my side every step of the way.

And most of all – I dedicate this work to my daughters, Marina and Sophia.

ABSTRACT

Dengue virus has been circulating in a hyperendemic pattern in Brazil for decades and the introduction of Zika virus, a closely related member of the flavivirus genus, into the Americas has complicated this situation. Zika virus has only recently begun to be viewed as a pathogen of significant concern. A dramatic increase in the incidence of microcephaly in Northeastern Brazil was reported in late 2015, coinciding with a large increase in ZIKV infection. This unique pattern of microcephaly and other disabilities linked to infection with the Zika virus during pregnancy is known as Congenital Zika Syndrome (CZS). The pathogenesis driving this phenomenon is unknown, however, due to the similarities between Zika and dengue viruses, it is theorized that dengue virus-mediated immune enhancement in mothers may be a risk factor for the development of CZS in infants. As such, it is important to further study the immune profiles of pregnant women infected with dengue and Zika viruses. Our research analyzes the antibody response to dengue and Zika viruses among pregnant women during the 2015-16 Zika outbreak in Salvador, Brazil, and characterizes cross-reactive antibodies between dengue (DENV) and Zika (ZIKV). This study provides the unique ability to study the background levels of dengue virus immunity in pregnant women in Northeast Brazil at the time of the ZIKV epidemic. Much is unknown about the effect of prior dengue infection and its ability to confer protection or risk of enhancement of Zika virus infection, especially during pregnancy. We aim to better understand this relationship and how it correlates to protection, with the long-term goal of facilitating the development of safe and effective DENV and ZIKV vaccines.

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LIST OF ABBREVIATIONS

Abs	Antibodies
ADE	Antibody-Dependent Enhancement
CR	Cross-reactive
DENV1, DENV2, DENV3, DENV4	Dengue virus serotype 1, 2, 3, 4
ELISA	Enzyme Linked Immunosorbent Assay
FRNT	Focus Reduction Neutralization Assay
mAbs	Monoclonal antibodies
NT	Neutralizing
pDENV	Primary dengue
pZIKV	Primary Zika
sDENV	Secondary dengue
TS	Type-specific
ZIKVwprDENV	Zika with previous dengue

Chapter 1: Introduction

Genome and virion structure

Dengue and Zika viruses are members of the *Flavivirus* genus, in the family *Flaviviridae.* Dengue virus refers to a group of four serotypes (DENV-1 to DENV-4) that are genetically and antigenically related. There are over 70 viruses in the *Flavivirus* genus, grouped by their mode of transmission: mosquito-borne flaviviruses (MBFVs), tick-borne flaviviruses (TBFVs), and flaviviruses with no known vector (NKV). Mosquito-borne flaviviruses are further divided into two clades: those associated with *Culex* mosquitoes, and those spread by *Aedes* mosquitoes. Dengue and Zika belong to the latter.[1–3]

Flaviviruses share a common structure, as shown by the cryo-EM of purified virions.[4–7] Dengue and Zika are relatively small (~50 nm), spherical, positive-sense, single-stranded RNA viruses with a genome that is approximately 11,000 bases in length. The viral genome encodes a single open reading frame that is translated as a long polyprotein and then cleaved by host and viral proteases to generate three structural and seven nonstructural proteins **(Fig.1)**. The structural proteins (capsid, membrane, and envelope) make up the structure of the virion, while the nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are involved in viral replication and assembly, and immune evasion.[3,8–14]

In the core of the virion, underneath the viral envelope, lies the nucleocapsid, a structure made up of capsid (C) proteins and the viral genome.[10] The outermost layer of the virion is made up of a viral envelope studded with two proteins, envelope (E) and membrane (M), which form a protective layer that controls viral entry into human cells.

The envelope (E) glycoprotein is the major surface component of both the dengue and Zika virion. It is involved in receptor binding and membrane fusion during virus entry and is the main target of neutralizing antibodies.[2,8] The E protein is highly conserved among flaviviruses, the four DENV serotypes share a sequence homology of up to 70%, and the DENV serocomplex shares a nucleotide sequence homology of 54-58% with the Zika virus.[14–17] It contains three domains (DI, DII, and DIII), with multiple epitopes in each domain. Antibodies to these epitopes display varying degrees of cross-reactivity across to other flaviviruses. The fusion loop (FL), at the tip of DII, is the most conserved structural element among E proteins for all flaviviruses and is involved in the broad antibody cross-reactivity observed between viruses and is an antigenic site that contributes to the antibody-mediated enhancement of infection.[10,18]

Membrane protein (M), another flavivirus surface protein, is formed by cleavage of the precursor prM protein during viral maturation. PrM is present in immature particles **(Fig.2)** but is cleaved to form “pr” peptide and M protein by host cell furin during transit through the Golgi.[2,8,10,16,19] The prM protein assists with the folding and assembly of the E protein.[14,19,20] Cleavage of this protein is inefficient and the virus can be present as mature, partially mature, or immature virions.[8,10,21] The final structural protein is the capsid protein (C), which is essential for nucleocapsid formation and acts as an RNA chaperone to assist with the nucleic acid arrangement. The primary role of the capsid is to package the viral genome and release the genome during infection.[22] Capsid is one of the least conserved proteins among flaviviruses.[23]

Nonstructural protein 1 (NS1) is another highly conserved protein among flaviviruses and plays an important role in both protection and pathogenesis.[24,25] NS1 is

found as a cell membrane-bound form, on the cell surface, and as a soluble secreted hexamer. NS1, along with the precursor membrane (PrM) and envelope (E) proteins, are the principal targets of antibody response to dengue virus infections.[2] Studies have found that a higher concentration of NS1 directly correlates with increased viremia and disease severity.[26] NS1 is an important diagnostic marker and potential vaccine candidate. The other nonstructural proteins (NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are involved in various enzymatic activities, immune evasion, and assist with viral replication.[23]

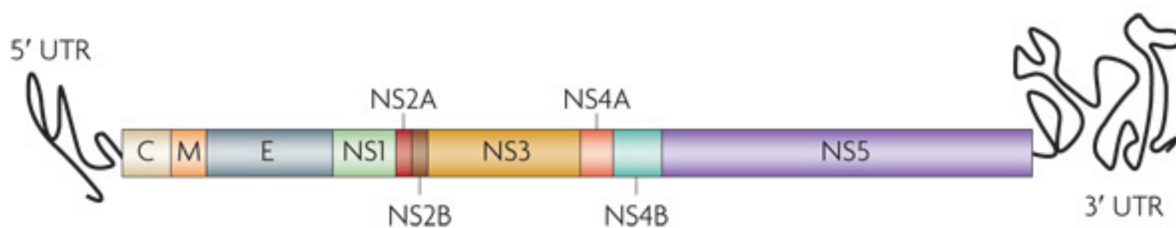


Figure 1: Flavivirus genome showing structural and nonstructural proteins.[27]

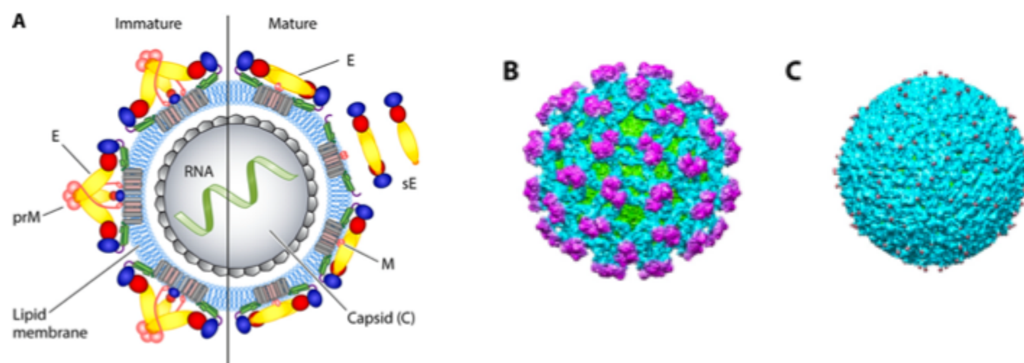


Figure 2: Structure of mature and immature flavivirus particles.[10] (A) Representation of immature (left) and mature (right) flavivirus virion. (B) Cryo-EM structure of immature dengue virus. (C) Cryo-EM structure of mature dengue virus

Replication

The flavivirus replication cycle begins with virus attachment to the host cell via interactions with various receptors on the cell surface. Several cell-surface receptors have been indicated for DENV entry, including heparan sulfate[28], DC-SIGN[29,30], CD14[31],Mannose receptor[32], TIM-1[33] , AXL[33], and others[34]. For ZIKV, the receptors DC-SIGN and AXL[35,36] appear to be the major attachment factors, however a recent publication showed that NCAM1 may be an important receptor for entry into nerve cells[37]. DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin) is an important factor for many flaviviruses as it is expressed on macrophages and dendritic cells, which are primary targets during infection.[12,29,30,38] Following attachment, virions enter the cell primarily via clathrin-mediated endocytosis.[10,34,39,40] In the endosome, acidification triggers a conformational change in the viral E protein that results in fusion between the viral envelope and the endosomal membrane and ultimately the release of the viral genome into the host-cell cytoplasm.[8,34] The positive-sense RNA genome is translated into a single polyprotein that is then cleaved by viral and host proteases into the three structural and seven nonstructural proteins. Genome replication occurs and virus assembly takes place in the lumen of the rough endoplasmic reticulum (ER). The immature virions are then transported through the trans-Golgi network (TGN) where the host protease furin cleaves prM into M, resulting in mature, infectious particles. The resulting virions are released from the cell via exocytosis.[8,10,14,34,39] This process is summarized in **Fig.3**.

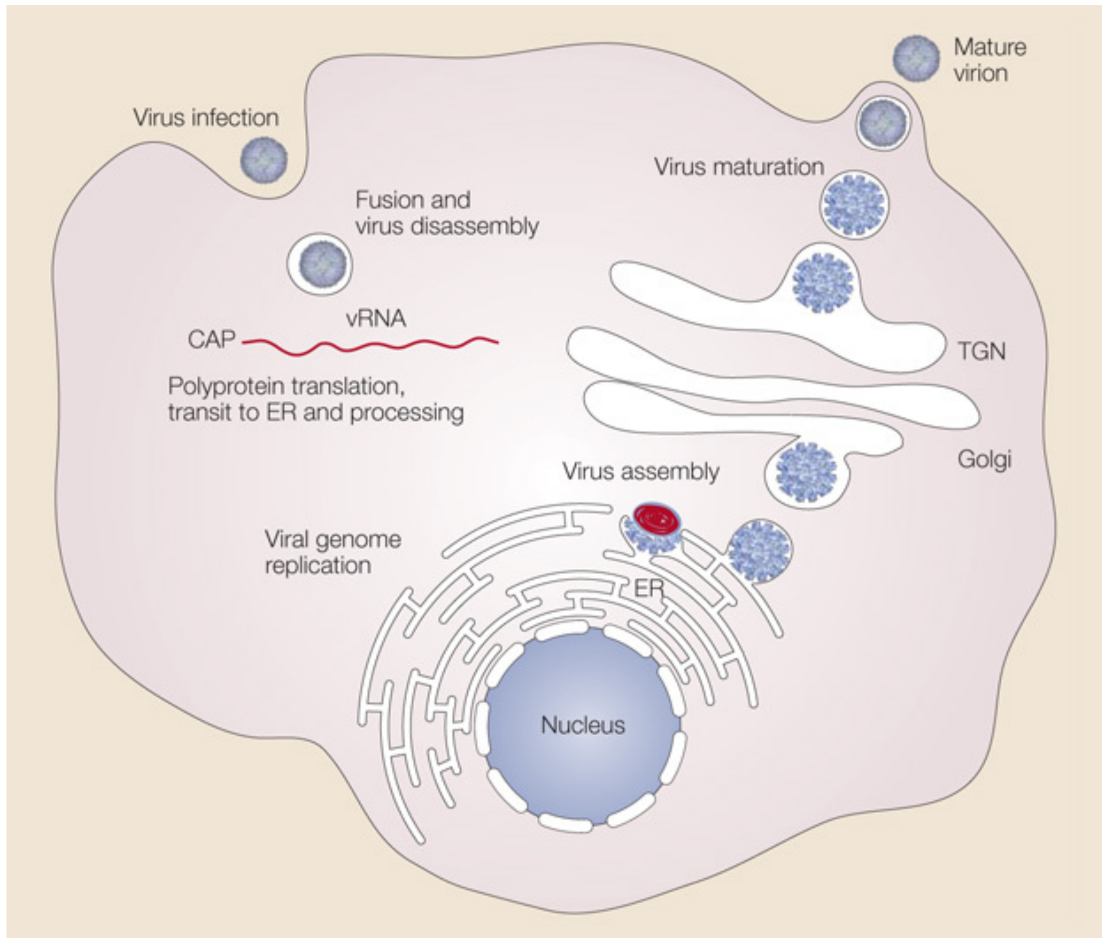


Figure 3: Dengue Virus Replication Cycle[14] Image showing steps of viral replication from initial infection to production of mature virions.

Phylogeny

The *Flavivirus* genus contains approximately 70 viruses, many of which are associated with emerging and re-emerging human diseases. This genus can be divided into three groups based on the mode transmission: mosquito-borne flaviviruses (MBFVs), tick-borne flaviviruses (TBFVs), and those with no known vector (NKV). We will focus on mosquito-borne flaviviruses. MBFVs can further be broken down into two clades: those spread by *Aedes* mosquitoes and those spread by *Culex* mosquitoes.[3]

Flaviviruses are then further divided into serocomplexes based on their antigenic properties and ability to cross-neutralize by polyclonal sera. Cross-neutralization largely correlated with amino acid sequence of the E protein, which can vary by up to 60% between viruses within the genus.[1,3,41,42] Cross-neutralization with polyclonal sera usually is lost when there is greater than 50% divergence in the amino acid sequence homology of the E protein between different flaviviruses.[10] The DENV serocomplex and Zika virus share approximately 55% sequence homology of the E protein, allowing for extensive cross-reactivity.[43]

The dengue viruses are in their own serocomplex, encompassing all four serotypes - DENV1, DENV2, DENV3, and DENV4. There are several different genotypes and lineages within each DENV serotype.[11] Zika virus is most closely related to the rarely discussed Spondweni virus, together they form the Spondweni serocomplex, which is more closely related to DENV than to the other mosquito-borne flaviviruses, as shown in **Fig.4**.[10] Zika virus exists as one serotype with two distinct lineages, the African lineage and the Asian linage that appear to have diverged in the 1940s.[44–46] Phylogenetic studies have shown that the virus involved in the ZIKV epidemic in Yap Island was from the Asian lineage.[47] The French Polynesia ZIKV outbreak was also caused by the Asian lineage Zika virus. Ultimately, a virus descended from this French Polynesian ZIKV was the virus that caused the largest Zika virus epidemic to date, in the Americas. Phylogenetic analyses indicated that this virus arrived from French Polynesia to Brazil between May and December 2013, more than a full year before the circulation of the virus was discovered.[48]

While human infections with the African or Asian Zika virus lineage typically cause similar clinical presentations, severe manifestations such as GBS or CZS have only been reported following infection with the Asian lineage.[49,50] It is still unknown whether this is a result of mutations resulting in greater virulence in this strain of ZIKV, a function of incidence – with the outbreak in the Americas being large enough to capture rare events or a result of a unique immunological profile and cross-reactivity to DENV.[49–51]

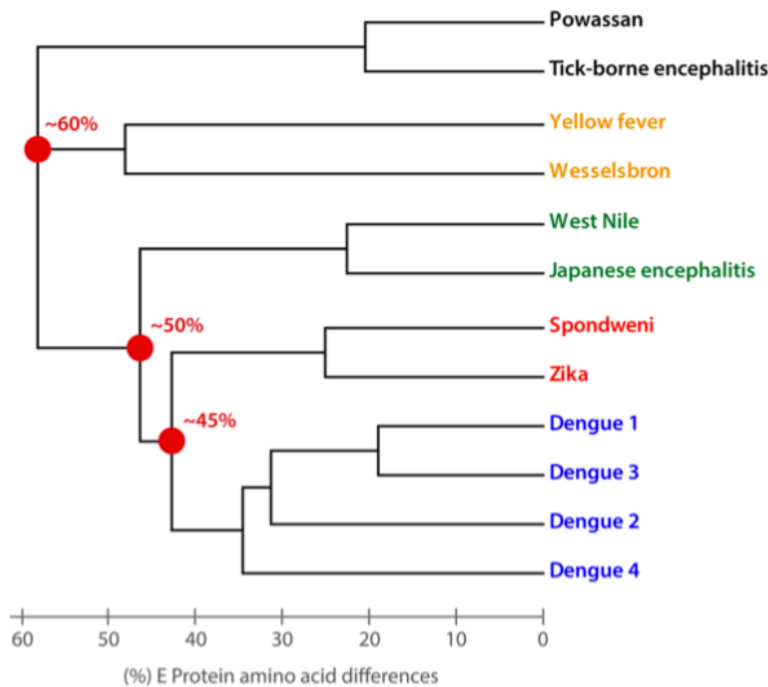


Figure 4: Phylogeny of select flaviviruses. Based on the percent amino acid sequence divergence of E proteins.[10]

Dengue Virus Epidemiology

Already known as the most prevalent mosquito-borne viral infection worldwide, the geographical range of the dengue virus is expanding even further in tropical and subtropical regions of the globe as a result of climate change, urbanization, and the movement of people and goods. The number of human infections has increased 30-fold since the 1960s, with current estimates of 390 million infections annually.[52]

Major dengue epidemics have been reported as far back as the 1700s, with reports of clinically compatible illness outbreaks dating back over one thousand years.[9,53] Between the 1700s and early 1900s, dengue virus was the cause of large but infrequent epidemics throughout the tropics and eventually became endemic in many cities across the globe (**Fig. 5C**).[9,54] Prior to 1970, only 9 countries had experienced severe dengue epidemics, but in the past 50 years dengue has expanded rapidly. Dengue is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-East Asia, and the Western Pacific and 128 countries are considered at risk for a dengue outbreak. Globally, 3.9 billion people - nearly half of the world's population, are at risk of dengue infection.[55–59]

While dengue incidence rates are increasing in many tropical and subtropical regions of the world, the Americas have seen a dramatic increase of cases during the last decades.[56,60,61] In the 1980s, 1 million dengue cases were reported in the Americas; between the years 2000 and 2007, the case count was 4.7 million from the same region.[54] Historically, dengue in the Americas can be classified as four distinct phases: (1) Introduction of dengue in the Americas (1600–1946); (2) Plan for the eradication of

the *Aedes aegypti* (*1947–1970);* (3) *Aedes aegypti* reinfestation (1971–2000); (4) *Increased dispersion of Aedes aegypti* and dengue virus circulation (2001–2010).[60]

In dengue endemic areas, it is typical for all four serotypes to co-circulate or to cyclically replace each other, meaning that multiple, sequential infections are common. Following a primary DENV infection, an individual develops lifelong immunity to the infecting serotype, but only transient immunity to the other dengue serotypes.[62,63] Dengue in the Americas has an endemoepidemic pattern with outbreaks occurring every three to five years, with increasing frequency and size.[55,60] Dengue hyperendemicity, characterized by the circulation of more than one serotype, has been reported in nearly every country in South America.[55]

The first report of a dengue-like illness epidemic in Brazil dates back to 1845, and today Brazil accounts for more than half of the dengue cases in the Americas.[55] Brazil has been experiencing hyperendemic patterns of dengue infection, characterized by the circulation of all four serotypes (DENV1-4) since 2010.[54,64–66] Hyperendemicity leads to increased numbers of severe and fatal cases, for reasons that will be discussed later. In Brazil, the highest dengue incidence and fatality are reported in the Southeast, followed by the Northeast region, including the state of Bahia.[64] Infections circulate all year round, with regional differences in serotypes reported. Owing to the large size of the country, when there is an outbreak of one serotype, it often remains regional, leading to multiple serotypes circulating simultaneously in different areas of Brazil.[55]

Zika Virus Epidemiology

Unlike dengue's centuries-long history, Zika virus is a much newer public health threat. Zika virus was first isolated in 1947 in samples from a rhesus monkey in the Zika Forest in Uganda. The second isolation of the virus was from a pool of *Aedes africanus* mosquitoes collected from the same forest.[67] In 1952, the first evidence of human infection was discovered in a serological study in Uganda but the virus was not isolated from humans until 1954, during an epidemic of jaundice in Nigeria.[68,69] For the next 50 years, Zika virus circulated quietly in Africa and Asia, without much evidence of disease. Although the virus was repeatedly isolated from mosquitoes, only 14 human cases were reported prior to 2007.[46,50]

In 2007, physicians on the island of Yap in the Federated States of Micronesia reported an outbreak of an illness involving symptoms of fever, rash, conjunctivitis, and arthralgia. Serum samples were sent to the CDC and the causative agent was identified as Zika virus. By the end of the outbreak, 73% of the island's residents over the age of 3 years had serological evidence of infection. This outbreak was the first evidence of transmission of Zika virus outside of Africa or Asia, as well as the first Zika epidemic reported.[70] This groundbreaking epidemic was followed by an even larger outbreak in French Polynesia in 2013-2014 that included 30,000 cases – an estimate which is likely far lower than the true number due to the highly asymptomatic nature of infections.[71] A retrospective serosurvey suggested an infection rate of 66%.[50] The French Polynesia epidemic was the first time that Zika virus was posited to cause neurological sequelae. There were 42 cases of Guillain-Barré syndrome (GBS) reported from November 2013 to February 2014, compared with 3 cases reported in the entirety of the year 2012.[72]

Although not noted at the time of the outbreak, retrospective analysis suggested an increase in microcephaly cases.[73]

Following the epidemics in Yap and French Polynesia, Zika virus spread throughout the South Pacific causing smaller outbreaks in New Caledonia, the Cook Islands, Easter Island, Vanuatu, the Solomon Islands, Samoa, and Fiji (**Fig. 5A**).[44] Clusters of acute exanthematous illness began being reported in late 2014 in the Americas and in March 2015, Zika was identified as the cause of this illness in Bahia, Brazil.[74] Epidemiologic evidence indicated that the outbreak began in Salvador, Bahia, and Brazil and rapidly spread throughout the country. By December 2015, the Brazil Ministry of Health estimated that 1.3 million suspected cases had occurred since the start of the epidemic.[75] In 2015, the United States began to see Zika virus cases in travelers returning from Central and South America along with sexual transmission of Zika virus.[76,77] In 2016, local transmission of Zika virus was reported in Florida and later in Texas.[49,78,79]

The introduction of the virus into the Americas was soon followed by the detection of fetal neurological disorders, termed congenital Zika syndrome (CZS) associated with maternal ZIKV infection, with a peak in microcephaly cases occurring in October 2015.[80] By 2017, ZIKV was detected in 47 American countries and territories.[81] Following this epidemic, Brazil reported over 350,000 suspected or confirmed ZIKV cases, as well as 2775 cases of CZS, the highest number reported globally.[82] Northeast Brazil is described as the epicenter of the ZIKV outbreak, with high numbers of cases in this region and evidence to support that ZIKV was first established here and spread outward throughout Brazil and the rest of the Americas.[83] The Northeastern Brazilian

city of Salvador experience rapid spread and was among the most affected regions during the 2015-2016 Zika epidemic in the Americas, with infection rates exceeding 60%.[84] As for the current picture, 2021 PAHO data shows ongoing low-level transmission of the Zika virus in the Americas, with 6,012 cases, 85% of which are reported in Brazil.

Increasing risk of arboviral infections

Dengue is considered the most widely distributed and rapidly spreading arboviral disease in the world, following its re-emergence in the 1980s. Meanwhile, the global threat of Zika remains uncertain, as information pertaining to its spread and pathogenesis continues to evolve.[44] Zika has only begun to be viewed as a public health threat in the past 15 or so years, after causing epidemics linked to neurological disorders and birth defects. Both viruses, spread by the same vector – *Aedes sp.* mosquitoes (**Fig. 5B)**, have a major impact on global public health that is predicted to increase over time. Potential exists for the re-emergence of ZIKV in all places with prior reports of transmission of this virus, indicating that Zika is likely to have significant public health consequences across Latin America and the Caribbean in years to come.[85,86] Reported dengue cases have increased 8-fold globally in the past two decades, from 505,430 cases in 2000 to 5.2 million in 2019.[58] These increases are due to a number of factors, including the change in reporting practices and the increasing ability to detect cases in developing countries. While the increased case detection is a positive reason for the increased case counts, we must also acknowledge the increasing global risk due to a number of vector and human factors.

Arboviral diseases, such as dengue and Zika, involve a complex relationship between human, mosquito, and virus factors – any of these can contribute to an increase in the spread of disease. As such, there are a number of factors that have been proposed for the increasing incidence of dengue and Zika cases globally. The world's population is expanding rapidly leading to increased urbanization, often leading to substandard housing and crowded conditions, which may lead to increased risk of mosquito exposure. Rapid urbanization often leads to the inability of water and waste systems to keep up with demand, leading to an increase in mosquito breeding sites. Dengue and Zika are often described as diseases of poverty because of their association with low GDP, poor household construction, and increased population density.[87–89] Additionally, civilizations are spreading into areas that disturb the natural ecosystem, leaving populations at risk for spillover of pathogens from the sylvatic cycle of transmission. Not only is the population expanding, but it is also getting more mobile. It is cheaper and easier than ever to travel internationally, leading to an increased movement of people and goods. This can lead to the direct introduction of infected mosquitoes into new environments, as well as the introduction of infected individuals who may be bitted by suitable mosquito vectors, leading to an outbreak. There has also been a lack of effective mosquito control and the use of ineffective spraying. *Aedes* mosquitoes are day-biters, so bed nets are not as effective for dengue and Zika virus as they are against pathogens spread by night-biting mosquitoes. There is also a lack of public health infrastructure in many places, making it difficult to detect and stop the spread of diseases early on.[9,59,88,90] Climate change is another factor that can contribute to the increased spread of a number of arboviruses as a result of expansion of mosquito

vector geographical range, increased vector competency, lengthened breeding season, and increased survivability.[55,59,91] Currently, climate change is regarded as one of the major factors enhancing the transmission intensity of dengue fever, with estimates citing that nearly 1 billion additional people over the next century will become at risk of viruses transmitted by *Aedes aegypti* and *Aedes albopictus*.[90,91]

DENV/ZIKV/CHIKV Cocirculation

Many arboviruses are endemic to Brazil, including the four DENV serotypes, yellow fever virus, Mayaro virus, and Oropouche virus, among others.[50] The 2015 Zika virus epidemic in Brazil was complicated by the re-emergence of dengue in Brazil and the recent introduction of Chikungunya into the country (**Fig. 6**). This led to an unprecedented situation in which three arboviruses that are spread by the same vector (*Aedes aegypti*) were circulating at a high incidence at the same time. There was a high burden on healthcare services and a surge in morbidity due to these three viruses, as well as challenges in clinically differentiating these three infections.[66,74,92,93]

Chikungunya is not a *flavivirus* like dengue and Zika, but rather a member of the genus *Alphavirus* and family *Togaviridae*; it has a capsid, a phospholipid envelope, and a single-stranded RNA genome. The *Alphavirus* group contains 28 known viruses including o'nyongnyong, Ross River and Mayaro.[94] These viruses overlap in the epidemiological risk factors associated with infection, due to the fact that they are spread by the same vector.[95] There is also overlap in their signs and symptoms, as further described in the clinical section of this paper.

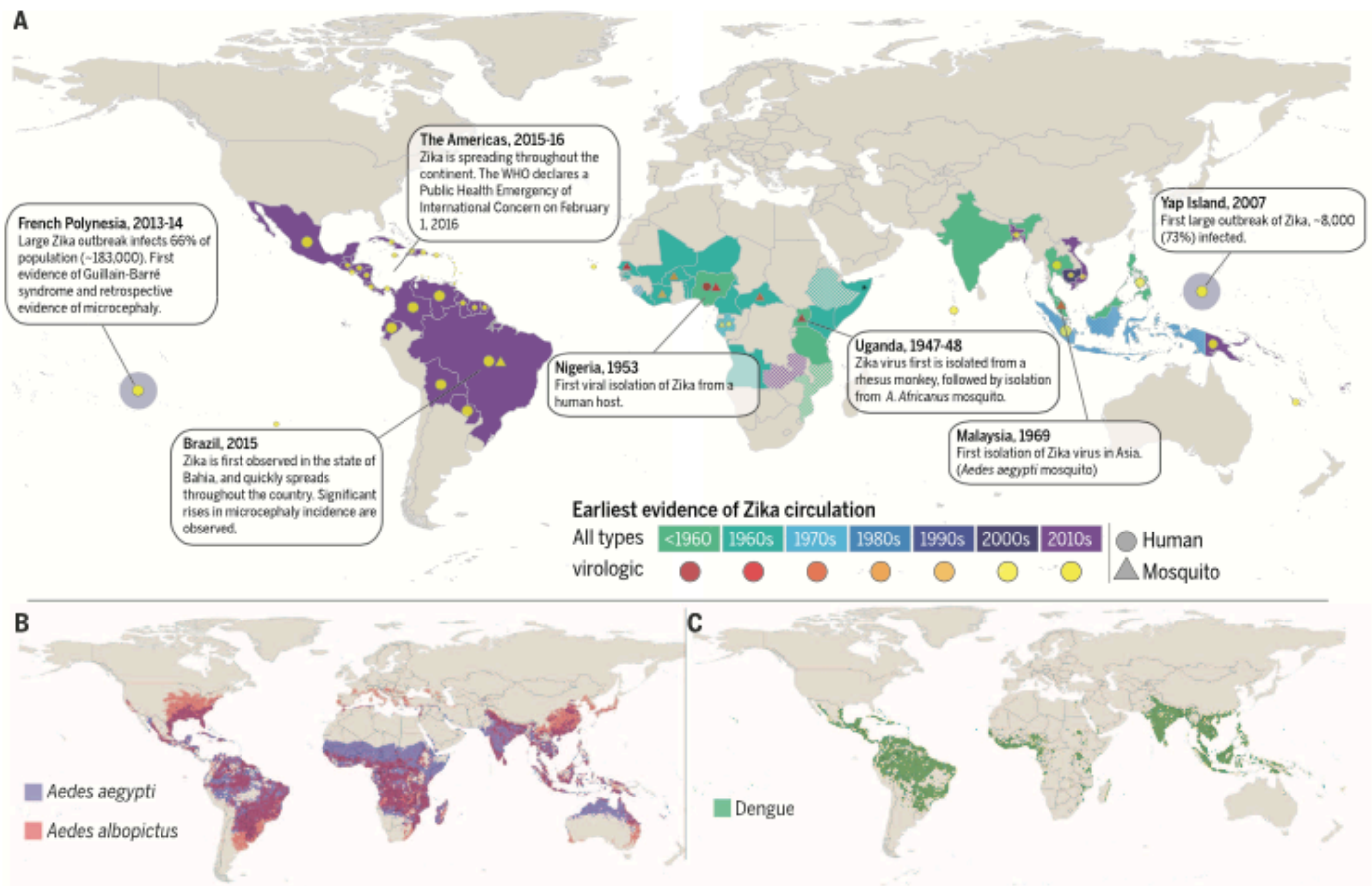


Figure 5: Global spread of Zika virus. (A) Countries with evidence of Zika circulation, colored according to decades of viral detection. (B) Distribution of vectors of DENV and ZIKV. (C) Map of areas with endemic dengue.[44]

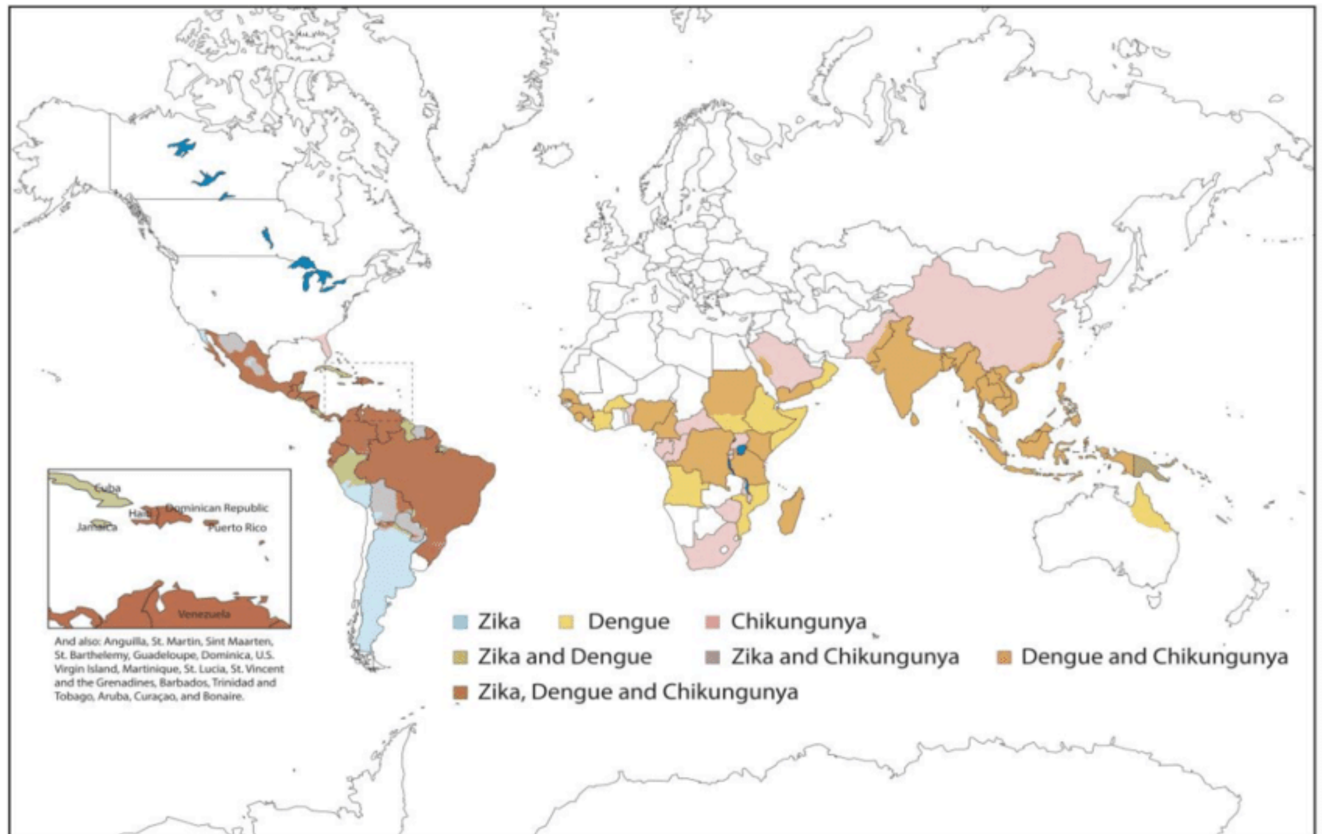


Figure 6: Global distribution of dengue, Zika, and chikungunya.[95]These three arboviruses are spread throughout the tropics and into the subtropical regions of the globe and overlap in their distribution.

Dengue Clinical Overview

Dengue infection in humans is asymptomatic in roughly 80% of cases and is globally established in both endemic and epidemic transmission cycles.[52,96] The four serotypes of dengue virus (DENV1-4) are capable of causing the same spectrum of disease, ranging from asymptomatic to classical dengue fever to more severe presentations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[9,97] The average incubation period is 4-7 days, with a range of 3 to 14 days.

For those that do show symptoms of dengue, classical dengue fever is the most common clinical syndrome. Three phases of dengue fever (DF) have been described: febrile, critical, and recovery.[16] The febrile phase of DF is characterized by fever, headache, retro-orbital pain, myalgia, rash, and lymphadenopathy.[97,98] Minor hemorrhagic manifestations, such as petechiae and nosebleeds may occur during this stage.[95] Typically, symptoms of the febrile phase resolve in less than a week without complication.[2] Following the febrile phase, approximately 5% of patients will progress to the critical phase.

The critical phase occurs after the virus is cleared from the bloodstream and the fever resolves. Lasting 1-2 days, this stage is characterized by increased vascular permeability, thrombocytopenia, loss of plasma volume, and risk of hemorrhage. Possible complications include pleural effusion, disseminated intravascular coagulation, shock, and organ damage. If untreated, mortality can be as high as 20-30%, however, symptom management and fluid replacement can reduce mortality to 1%.[16,54,95,99] For those who survive the critical phase, the recovery phase follows. Persistent symptoms such as arthralgia, fatigue, and headache have been reported in dengue patients up to

two years after illness.[100,101] Severe dengue was first recognized in the 1950s during dengue epidemics in the Philippines and Thailand. Today, severe dengue is most commonly seen in Asian and Latin American countries and is a leading cause of hospitalization and death among residents in these regions.[58]

Dengue fever, typically seen in its mild febrile form, is a nonspecific illness that may be confused with a number of other illnesses that cause fever, aches, and rash. Symptoms overlap with other mosquito-borne viruses, such as Zika and chikungunya, making clinical diagnosis difficult. Because mortality can be greatly reduced with early diagnosis and appropriate clinical management, accurate diagnosis using laboratory methods is critical.[66,99]

Dengue Pathogenesis

Dengue is spread via the bite of infected *Aedes* species mosquitoes. All four dengue virus serotypes cause the same clinical manifestations and show similar patterns of systemic dissemination.[102,103] The virus is injected into the bloodstream and infects the surrounding epidermis and dermis, where it is infects epidermal dendritic cells, called Langerhans cells, and keratinocytes.[104,105] Infected cells then migrate to the lymph nodes where macrophages and monocytes are recruited, ultimately becoming the target of infection. Dengue virus amplifies in these cells and the virus is disseminated through the lymphatic system.[106] While the mononuclear phagocytic lineage of cells (monocytes, macrophages, and dendritic cells) are the primary targets for DENV, a number of other cells have been found to be infected during in vitro experiments and human autopsy.[107] These include endothelial cells, hepatocytes, and

mononuclear phagocytic cells in the spleen, lymph nodes, lungs, liver, kidney, and stomach; indicating that three organ systems play an important role in the pathogenesis of the dengue virus: the immune system, the liver, and endothelial cell linings of blood vessels.[106,108]

The pathogenesis of dengue and the development of DHF/DSS is a complex topic that remains to be fully elucidated. There are a number of risk factors that have been implicated in the development of severe disease including age, viral serotype and genotype, genetic background of infected individual, and presence of pre-existing conditions, although secondary heterologous infection is the largest risk factor by far.[9,107,109,110] Epidemiological observations have found the risk of DHF to be up to 80 times higher in secondary dengue infection and that up to 99% of DHF cases have heterotypic antibodies to the dengue serotype causing the DHF.[109,111]

Initial infection with a serotype of dengue (primary infection) is often asymptomatic or causes a mild case of dengue fever. This infection confers lifetime protection to the homologous serotype and transient immunity to heterologous serotypes.[9,97,112,113] Although primary infections can cause severe disease in a small percentage of individuals, most cases of DHF/DSS occur when infected with a secondary heterologous serotype. Of note, many of the severe cases of primary infection are seen in infants born to DENV-immune mothers. It is believed that this is a result of non-neutralizing dengue-specific antibodies that are transferred transplacentally to infants.[114,115] The severe presentation associated with secondary dengue infection and the heightened risk of DHF/DSS in infants born to dengue-immune mothers are often cited as evidence for antibody-dependent enhancement

Antibody-Dependent Enhancement (ADE)

With dengue viruses, the phenomenon of antibody-dependent enhancement (ADE) has been discussed for decades, owing to the fact that heterotypic secondary DENV infection has been shown to be the greatest risk factor for severe disease outcomes, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[115] It is theorized that antibodies generated to the primary infecting serotype will not be of sufficient concentration or avidity to neutralize a secondary infecting serotype, but rather, at a certain concentration, they will enhance disease. This disease enhancement occurs as a result of Fc receptor-mediated endocytosis of the virus into monocytes and macrophages, which are the principal site of DENV replication, thus driving higher viral loads. ADE has been demonstrated in vitro and in mouse models, but the evidence in nonhuman primates and in humans is mixed.[116–119] Recent evidence in a human cohort study has shown that the risk of enhancement is highest within a narrow range of preexisting anti-DENV antibody titers.[120] Given the relatedness between DENV and ZIKV, and the high cross-reactivity demonstrated in serological assays, ADE between DENV and ZIKV and potential for altered disease pathogenesis require further investigation.

Zika Clinical Overview

Zika virus was thought to be an asymptomatic or mildly symptomatic infection in the decades following its discovery.[121] It's been reported that up to 80% of Zika virus

infections are asymptomatic.[122] For the remaining ~20%, ZIKV infection causes a mild clinical illness similar to dengue and chikungunya, with rash, fever, arthralgia, and conjunctivitis as the major symptoms. The self-limiting illness typically lasts 4-7 days.[50,70,75,123]

Compared with dengue, patients with Zika presented more frequently with conjunctivitis and rash. Additionally, dengue symptoms tend to be more severe and longer lasting than with Zika infection. However, there is substantial overlap and symptoms alone cannot distinguish these diseases.[124]

In the past couple of years, following large Zika epidemics in the Pacific and the Americas, more severe neurological complications have been linked to ZIKV-infections. During the 2013-2014 ZIKV outbreak in French Polynesia, there was an increase in reported cases of Guillain-Barré syndrome (GBS), an autoimmune disease causing weakness and paralysis. [125] Between October 2013 and April 2014, French Polynesia reported 42 cases of GBS – a 1400% increase from the entire year before, occurring in only 6 months.[44] Further research has estimated that the risk of GBS following Zika virus infection is around 1%.[126] Guillain-Barré may be more common in symptomatic ZIKV cases; during the French Polynesia outbreak, 88% of GBS cases reported traditional Zika virus symptoms a median of 6 days before Guillain-Barré onset.[125]

The most concerning clinical aspect of ZIKV infection is the sharp increase in infants born with microcephaly during the 2015-2016 Zika epidemic, concentrated around northeastern Brazil. The term Congenital Zika Syndrome was coined to describe the constellation of birth defects seen in infants born to mothers infected with Zika virus during pregnancy. Microcephaly is the most apparent and well-known form of

Congenital Zika Syndrome, defined as head circumferences <2 standard deviations below the mean occipito-frontal circumference for age- and sex-appropriate size.[127] CZS associated with maternal ZIKV infection, with a peak in microcephaly cases occurring in October 2015 and by the time the epidemic was over Brazil reported over 350,000 suspected or confirmed ZIKV cases, as well as 2775 cases of CZS, the highest number reported globally.[82]

While microcephaly represents the most severe end of the spectrum, it has been discovered that there are a number of long-term sequelae of congenital Zika infection.[128] Infants born to Zika-infected mothers may present as normal at birth but later be found to have developmental delays, cerebellar calcifications, ventriculomegaly, arthrogryposis, abnormal visual function, or seizures.[129] A study in Salvador, Brazil found that nearly 30% of children with suspected ZIKV infection *in utero* had evidence of significant retinal and optic nerve abnormalities.[51,130] The identification of birth defects in the years following the Zika epidemic in the Americas indicates that the true number of CZS cases is likely far higher than those counted by microcephaly alone. While adverse outcomes have been noted as a result of infection during all three trimesters of pregnancy, it appears the risk of microcephaly is greatest if infection occurs during the first trimester.[51,131–133] Zika infection is not associated with mortality in its typical course, fatality has been described in patients with congenital ZIKV infection and post-ZIKV Guillain-Barré syndrome. [51] ZIKV infection during pregnancy has been associated with increased rates of spontaneous abortion and stillbirth, although the true extent remains unknown.[134]

Zika Pathogenesis

Similar to dengue, Zika is typically transmitted by the bite of an infected *Aedes* mosquito. The virus is injected into the surrounding epidermis and dermis, where it infects epidermal dendritic cells. Infected cells then migrate to the lymph nodes where the virus is amplified, resulting in hematogenous dissemination.[51] Sexual transmission of the Zika virus was first reported in 2011 and contributed to the virus spread during the epidemic in the Americas, although the extent of which is unknown.[135,136] The contribution of sexual transmission in an area with active mosquito-borne transmission is nearly impossible to estimate.[49] Zika virus RNA can be detected in blood, urine, and semen, and infrequently detected in saliva and vaginal secretions.[137] Zika virus appears to have a broad tissue tropism, infecting monocytes and macrophages, endothelial cells, various epithelial cells, and a variety of neuronal and placental cells.[108]

Congenital Zika Syndrome

Maternal ZIKV infection during pregnancy can lead to infection of the fetus, resulting in CZS or pregnancy loss.[138] Much remains to be discovered about the measures of risk and mechanisms by which CZS is caused. The simplest mechanism for ZIKV to cause birth defects would be for the virus to cross the placenta and directly affect the nervous tissue in the fetus. There are numerous studies that have found Zika virus RNA in various placental and fetal tissues, amniotic fluid and amniotic epithelial cells, and fetal brain tissue, demonstrating the virus's ability to cross the placenta.[128,139–142] ZIKV has also been shown to infect and replicate within placental macrophages,

indicating that these cells may aid in viral dissemination throughout the placenta.[143] It is also possible that the association of maternal Zika infection and fetal birth defects may be a result of a phenomenon similar to the antibody-dependent enhancement described for the dengue virus. It is possible that the sudden occurrence of high rates of microcephaly in Northeast Brazil was due to the introduction of the Zika virus into a population with unique patterns of flavivirus immunity, thus modulating the pathogenesis of ZIKV.[51,139,144]

Cross-reactivity between DENV and ZIKV

As discussed in the genome and virion structure section of this review, DENV and ZIKV are closely related flaviviruses with ~55% sequence homology of the E protein, which gives a high degree of structural and antigenic similarity.[43] The fusion loop (FL) is the most conserved structural element among E proteins for all flaviviruses and is involved in the broad antibody cross-reactivity observed between viruses and has been shown to contribute to antibody-mediated enhancement (ADE) of infection.[10,18,41,118,144–146] While the cross-reactivity between DENV and ZIKV is known to pose a diagnostic challenge, its role in disease pathogenesis remains to be elucidated.[18,41,51,147] It is theorized that these similarities are sufficient to drive ADE in a similar to manner to what was described for heterologous secondary dengue infections.[10,44,144] Understanding the cross-reactivity of the antibody response to DENV and ZIKV will give insight into the level of protection, or risk, associated with different flavivirus serostatuses.

Historical Zika outbreaks have been largely localized within dengue-endemic areas, making the potential for preexisting dengue-induced antibodies to enhance ZIKV infection a concern. In areas that have been affected by Zika outbreaks, there is also a risk that Zika immunity could predispose individuals to an enhanced DENV disease pathology.[145]

Various studies have shown that several potent monoclonal antibodies (mAbs) derived from DENV patients can neutralize the Zika virus *in vitro.*[116–118] It has also been shown that DENV-immune sera can cross-neutralize ZIKV *in vitro*. This was mainly shown to occur in secondary DENV infections, where there is a more robust and cross-reactive antibody profile, and this ability to cross-neutralize appears to wane over time.[117,118,148] ADE has been demonstrated *in vitro* and in mice, with several studies showing that DENV-immune sera enhanced ZIKV replication.[118,119,144,145,149] However, this same enhancement was not seen in several non-human primate models.[150–152]

In terms of human studies, two cohort studies showed that prior DENV infection (as measured by IgG titer) was associated with a decreased risk of symptomatic infection.[153,154] Gordon et al. found that prior DENV infection reduced the risk of symptomatic ZIKV infection in a pediatric cohort in Nicaragua. Rodriguez-Barraquer et al. found that in their cohort in Salvador, Brazil, DENV IgG titers were correlated with protection against ZIKV infection, with a reduction in odds of disease in those who had the highest DENV titers. Of note, Katzelnick et al. found that prior Zika virus infection enhanced the risk of severe dengue disease in children in Nicaragua 2-3 years after the ZIKV epidemic in that region.[155]

In summary, DENV-immune sera can enhance ZIKV replication *in vitro* and in mice, but not in non-human primate studies. DENV-immune sera cross-neutralize ZIKV, but this effect wanes over time. Results in human cohorts are mixed. Two pediatric cohort studies showed that DENV infection may reduce the risk of symptomatic ZIKV infection, but a study of the same pediatric cohort in Nicaragua showed that ZIKV infection may enhance the risk of severe DENV in future infections. Overall, an in-depth analysis of DENV-ZIKV cross-reactive antibodies in humans and their relationship with disease outcomes, in particular for pregnant women, is lacking.

Hypothesis and specific aims

Dengue virus has been circulating in a hyperendemic pattern in Brazil for decades and the introduction of Zika virus, a closely related member of the flavivirus genus, into the Americas has complicated this situation. Zika virus has only recently begun to be viewed as a pathogen of significant concern. The dramatic increase in the incidence of microcephaly in Northeastern Brazil was reported in late 2015, coinciding with a large increase in ZIKV infection. This unique pattern of microcephaly and other disabilities linked to infection with the Zika virus during pregnancy is known as Congenital Zika Syndrome (CZS). The pathogenesis driving this phenomenon is unknown, however, due to the similarities between Zika and dengue viruses, it is theorized that dengue virus-mediated immune enhancement in mothers may be a risk factor for the development of CZS in infants. As such, it is important to further study the immune profiles of pregnant women infected with dengue and Zika viruses.

In order to close this gap in research, this study will analyze the antibody response to dengue and Zika viruses among pregnant women during the 2015-16 Zika outbreak in Salvador, Brazil, and characterize cross-reactive antibodies between dengue (DENV) and Zika (ZIKV). Much remains to be explored about this topic, including the role of background flaviviral immunity in pregnant women and how this affects the risk of congenital Zika syndrome (CZS) in infants. This study provides the unique ability to study the background levels of dengue virus immunity in pregnant women in Northeast Brazil at the time of the epidemic.

The effect of prior dengue infection and its ability to confer protection or risk of enhancement of Zika virus infection, especially during pregnancy remains to be elucidated. We aim to better understand this relationship and how it correlates to protection, with the long-term goal of facilitating the development of safe and effective DENV and ZIKV vaccines.

Specific Aim 1: To identify DENV immune status among pregnant women during the 2015-16 Zika outbreak in Salvador, Brazil.

Specific Aim 2: To characterize cross-reactive antibodies between DENV and ZIKV among pregnant women with different immune statuses.

Chapter 2: Characterizing the dengue immune status among pregnant women during the 2015-16 Zika epidemic in Salvador, Brazil.

Introduction

Dengue virus history in Brazil

The past decades have seen rising dengue incidence rates throughout the tropical and subtropical regions of the world. [56,60,61] In particular, the Americas have seen a dramatic increase in dengue cases, with an increase from 1 million cases during the 1980s to 4.7 million cases between the years 2000 and 2007.[54]

While this increase in cases may partially be attributable to increased surveillance and improved case reporting systems, it is also certainly due to the expanding geographical distribution of mosquito vectors, change in serotypes circulating, and increased population density resulting from urbanization. [156] There is a demonstrated relationship between the increased dispersion of Ae. aegypti and DENV circulation (2000–2010) characterized by a marked increase in the number of outbreaks [54,56,60] Additional factors that may play a role in the increasing disease burden include human population expansion, poor household infrastructure, barriers to accessing health services, and weather and climate changes.[157–160]

The movement of people is a factor known to influence patterns of disease.[161–163] In the Americas, a new dengue outbreak during the period 2011–2017 was observed after a great influx of people to observe four global sporting events: the 2011 Pan-American Games in Guadalajara, Mexico, and the 2013 Confederations Cup, the 2014 World Cup and the 2016 Olympics, which were held in Brazil. This outbreak was associated with an increased dengue mortality rate.[54,164,165]

Dengue cases have historically been concentrated in Southeast Asia, however, in modern times Brazil has become the country that reports the largest number of cases in the world.[166] When we look at the past 4 decades, the Americas have reported 23 million dengue cases, with 13.6 million cases occurring in Brazil.[54,167]

In dengue-endemic areas, it is typical for all four serotypes to co-circulate or to cyclically replace each other, meaning that multiple, sequential infections are common. Following a primary DENV infection, an individual develops lifelong immunity to the infecting serotype, but only transient immunity to the other dengue serotypes.[62,63] Dengue in the Americas follows an endemoepidemic pattern with outbreaks occurring every three to five years. These outbreaks are reported to be increasing in both frequency and size.[55,60] Dengue hyperendemicity, characterized by the continuous circulation of multiple serotypes, has been reported in nearly every country in South America.[55,168] Brazil has been experiencing hyperendemic patterns of dengue infection since 2010 and the number of cities reporting multiple circulating dengue serotypes is increasing.[54,64–66,169,170]

Brazil has a long and complicated history with dengue, with the first report of a dengue-like illness epidemic in Brazil dating back to 1845.[55] In Brazil, the highest dengue incidence and fatality are reported in the Southeast, followed by the Northeast region, including the state of Bahia.[64] Infections circulate all year round, with regional differences in serotypes reported. Owing to the large size of the country, an outbreak of a specific dengue serotype often remains contained within one region. This leads to multiple serotypes circulating simultaneously in different areas of Brazil.[55]

Introduction of Zika virus to the Americas

Clusters of acute exanthematous illness were reported in late 2014 in the Americas and in March 2015, Zika was identified as the cause of this illness.[74,171] Epidemiologic evidence indicated that the outbreak began in Salvador, Bahia, Brazil and rapidly spread throughout the country. By December 2015, the Brazil Ministry of Health estimated that 1.3 million suspected cases had occurred since the start of the epidemic.[74,75] In 2015, the United States began to see travel-associated cases of Zika virus in those returning from Central and South America as well as cases attributed to sexual transmission.[76,77]

The introduction of the virus into the Americas was soon followed by the detection of fetal neurological disorders, termed congenital Zika syndrome (CZS) associated with maternal ZIKV infection, with a peak in microcephaly cases occurring in October 2015.[80] By 2017, ZIKV was detected in 47 American countries and territories.[81] Following this epidemic, Brazil reported over 350,000 suspected or confirmed ZIKV cases, as well as 2775 cases of CZS, the highest number reported globally.[82] Northeast Brazil is described as the epicenter of the ZIKV outbreak, with high numbers of cases in this region and evidence to support that ZIKV was first established here and spread outward throughout Brazil and the rest of the Americas.[83] The Northeastern Brazilian city of Salvador experienced rapid spread and was among the most affected regions during the 2015-2016 Zika epidemic in the Americas, with infection rates exceeding 60%.[84] As for the current picture, 2021 PAHO data shows ongoing low-level transmission of the Zika virus in the Americas, with 6,012 cases, 85% of which are reported in Brazil.

Co-circulation of flaviviruses in Brazil

Dengue and Zika viruses have a major impact on global public health that is predicted to increase over time.[172–175] Although we do not see the sustained transmission of the Zika virus at the rates seen in the 2015 outbreak, the potential exists for the re-emergence of ZIKV in all places with prior reports of transmission of this virus.[85,176] This indicates that Zika is likely to have significant public health consequences across Latin America and the Caribbean in years to come.[85,86] As for dengue, cases reported to the World Health Organization have increased 8-fold globally in the past two decades, from 505,430 cases in 2000 to 5.2 million in 2019.[58] It is believed that this figure represents a gross underreporting of the true number of global infections due to the fact that up to 75% of cases are asymptomatic, the clinical course of the disease is similar to a number of other tropical diseases, diagnostic tests lack sensitivity, and reporting requirements (and adherence to them) vary by country.[58,177–180] One estimation puts the global disease burden as high as 390 million infections annually.[52]

Many arboviruses are endemic to Brazil, including the four DENV serotypes, yellow fever virus, Mayaro virus, Oropouche virus, among others.[50,181,182] The 2015 Zika virus epidemic in Brazil was complicated by the re-emergence of dengue in Brazil and the recent introduction of Chikungunya into the country. This led to an unprecedented situation in which three arboviruses that are spread by the same vector (*Aedes aegypti*) were circulating at a high incidence at the same time. There was a high burden on healthcare services and a surge in morbidity due to these three viruses, as well as challenges in clinically differentiating these three infections.[66,74,92,93]

The impact of previous dengue infections on the Zika disease process is unknown

The purpose of this study is to understand the dengue immune profile in non-Zika-infected individuals during the 2015 Zika virus outbreak in Salvador, Bahia, Brazil. Dengue viruses have been circulating in a hyperendemic pattern in Brazil for decades and the introduction of Zika virus, a closely related member of the flavivirus genus, into the Americas has complicated this situation.[183–185] Historical Zika outbreaks have been largely localized within dengue-endemic areas, making the potential for preexisting dengue-induced antibodies to enhance ZIKV infection a concern.[172,186]

Zika virus has only recently begun to be viewed as a pathogen of significant concern.[70,187,188] The dramatic increase in the incidence of microcephaly in Northeastern Brazil was reported in late 2015, coinciding with a large increase in ZIKV infection.[80,189] This unique pattern of microcephaly and other disabilities linked to infection with the Zika virus during pregnancy is known as Congenital Zika Syndrome (CZS).[129,190] The pathogenesis driving this phenomenon is unknown, however, due to the similarities between Zika and dengue viruses, it has been theorized that dengue virus-mediated immune enhancement in mothers may be a risk factor for the development of CZS in infants.[155,191–193]

An ideal study would involve a cohort with longitudinal samples from before, during, and after the Zika epidemic in order to assess the potential cross-reactivity and conversely, cross-protection between DENV and ZIKV.[194] Since these types of samples are not readily available, we aim to assess this question by understanding the background immunity to dengue viruses among women living in a heavily impacted region of Brazil. Much remains to be explored about this topic, including the role of

background flaviviral immunity in pregnant women and how this affects the risk of congenital Zika syndrome (CZS) in infants. This study provides the unique ability to study the background levels of dengue virus immunity in pregnant women in Northeast Brazil at the time of the epidemic.

Additionally, in areas that have been affected by Zika outbreaks, there is a risk that Zika immunity could predispose individuals to an enhanced DENV disease pathology.[145] The city of Salvador, in Northeastern Brazil, was heavily impacted by Zika virus in 2015-2016. It is important to understand the background dengue immunity in this population, in an effort to understand the impact of prior DENV infection on Zika virus outcomes. It is of particular importance to study pregnant women, due to the risk of delivering infants affected by Congenital Zika Syndrome (CZS). We aim to study the background dengue immunity, as well as the nuances of cross-reactive immunity to dengue and Zika viruses, in pregnant women living in an area that is often described as the epicenter of the Zika epidemic.

Materials and methods

Sample Collection

The study of coded serum or plasma samples was approved by the institutional review board (IRB) of the University of Hawaii at Manoa, as well as the IRB of the Federal University of Bahia. Serum samples were collected from asymptomatic parturient women receiving routine care at the Federal University of Bahia Hospital in Salvador, Brazil. Samples were collected between November 2015 and December 2016 and tested using Euroimmun ZIKV non-structural protein 1 (NS1) IgG kit. 120 samples that tested negative using this test were included in further analysis.

NS1 ELISA

Dengue virus and Zika virus NS1 IgG ELISAs were performed to determine DENV and ZIKV serostatus. Using the methodology previously described by Tsai et al., 96-well flat-bottom ELISA plates were coated with purified NS1 proteins from either DENV or ZIKV and incubated overnight at 4° Celsius.[195] All NS1 proteins were purchased from the Native Antigen Company. Plates were blocked and incubated with patient serum diluted to 1:400 for 2 hours at 37° Celsius. Plates were then washed 4 times and incubated with secondary antibodies (anti-human IgG conjugated with horseradish peroxidase, Jackson ImmunoResearch) for 1 hour at 37° Celsius before being washed 6 additional times. ELISA substrate was added for 15 minutes per well before stop-solution (2N H_2SO_4) was added. The optical density at 450 nm (OD450) was read with a reference wavelength of 630 nm. Positive (OD >1) and negative

controls were included on each ELISA plate. Serum samples were tested in duplicates on each plate and two plates were run per sample. Two positive controls and 4 negative controls were included on each plate. The mean optical density of the negative controls plus 12 standard deviations of the mean was used to calculate positivity of serum samples being tested, this methodology has previously been shown to give a confidence level of 99.9% for assessing positivity.[195,196] To standardize and compare plates, OD values were divided by the mean OD of a selected positive control and used to calculate a relative OD (rOD). Two-tailed Mann-Whitney test was used for comparisons between the 2 groups. For combination ELISA, ELISAs were completed for DENV and ZIKV NS1, and the rOD ratio of DENV:ZIKV with a cutoff of 0.24, was used to distinguish ZIKVwpDENV from sDENV infection with a sensitivity of 87.5% and specificity of 81.3% as previously described by Tsai et al.[195]

Whole Virion ELISA

These same 120 serum samples were also tested by 3-layer virion IgG ELISA to assess endpoint titers to both DENV and ZIKV. 3-layer ELISA is a type of indirect ELISA that involves the primary antibody, secondary antibody, and substrate as described below. 96-well flat-bottom ELISA plates were coated with either dengue DENV3 (H87 strain) or Zika virus (PRVABC-59 strain) and incubated overnight at 4° Celsius. Plates were then blocked with 400ul of blocking buffer and incubated at room temperature for 1 hour. Serum samples were then added in a dilution of 1:400 before plates were sealed and incubated at 37° Celsius for 2 hours. Plates were then washed 4 times and

incubated with secondary antibodies (anti-human IgG conjugated with horseradish peroxidase, Jackson ImmunoResearch) for 1 hour at 37° Celsius before being washed 6 additional times. ELISA substrate was added for 15 minutes per well before the stop-solution (2N H_2SO_4) was added. The optical density at 450 nm (OD450) was read with a reference wavelength of 630 nm. Positive (OD >1) and negative controls were included on each ELISA plate. Serum samples were tested in duplicates on each plate and two plates were run per sample.

Endpoint Titer ELISAs

Whole virion 3-layer ELISAs were used to assess endpoint titers to ZIKV and DENV3. 96-well ELISA plates were coated with either DENV3 (H87 strain) or Zika virus (PRVABC-59 strain) and incubated overnight at 4° Celsius. Plates were then blocked and samples were tested in duplicates, using serial dilutions ranging from 1:400 to 1:102400 using the methodology described above. Two positive controls and 4 negative controls were included on each plate. The mean optical density of the negative controls plus 12 standard deviations of the mean was used to calculate the positivity of the serum samples being tested. This methodology has previously been shown to give a confidence level of 99.9% for assessing positivity.[196] Each plate of duplicates was tested twice to obtain 4 data points for each dilution. Titration curves were generated based on the mean for each dilution of each sample and plotted in GraphPad Prism 5.

Virus Propagation

Dengue 1-4 and Zika viruses were grown in a monolayer of Vero cells in T75 flasks, lysed with tryptase, and purified using sucrose cushion ultracentrifugation.[197] After UV inactivation, viruses were subjected to serial two-fold dilutions (1:200 to 1:6400), coated on 96-well plates, and tested with positive control serum to determine the titer, which was the highest dilution to reach optical density (OD) of 1. The viruses were aliquoted and stored at -80° Celsius.

Microneutralization (FRNT)

Flat-bottom 96-well plates were seeded with Vero cells 24 h prior to infection to create Vero cell monolayers. Twofold serial dilutions of serum were mixed with 50 focus-forming units of DENV1, DENV2, DENV3, DENV4, or ZIKV (grown as described above) at 37°C for 1 h. After adding this mixture to the wells, the plates were incubated 48 to 70 hours depending on the virus serotype. The medium was removed and plates were fixed as described by Tsai et al.[198] Murine Mab 4G2 and a secondary antibody mixture (IRDye 800CW-conjugated goat anti-mouse IgG at 1:10,000 and the DRAQ5 fluorescent probe at 1:10,000) were then added and plates were read using the LiCor Odyssey classic imaging system (LiCor Biosciences) at a wavelength of 800 nm/700 nm fluorescence. Murine Mab 4G2 is an anti-Flavivirus Envelope protein antibody that binds to a conserved epitope on the E protein of the flavivirus family. It has been shown to recognize Dengue virus, West Nile virus, Japanese Encephalitis virus, and Zika Virus.[199] These signals were analyzed using Image Studio software to determine

percent neutralization at different concentrations and NT50 and NT90. The titers of FRNT50 and FRNT90 were determined by nonlinear regression analysis (GraphPad Prism 5.0).

Results

Serostatus to dengue and Zika viruses were determined by combined NS1 ELISA and verified by FRNT. NS1 was selected as the target for this assay due to the known issues of cross-reactivity between flaviviruses. Specifically, E protein is highly conserved among flaviviruses.[15–17,200] NS1 ELISA has previously been shown to have high specificity and a low level of cross-reactivity between ZIKV and DENV viruses.[201,202]

Among 120 women presenting for routine prenatal care at the Federal University of Bahia in Salvador, Brazil, 106 out of 120 samples (88%) were positive for dengue virus. Of note, these samples were selected based on negative Zika virus results using Euroimmun ZIKV non-structural protein 1 (NS1) IgG kit. Our methodology was more sensitive and was able to determine that Zika virus immunity existed in 6 of these 120 samples (5%). These samples were classified into serostatus groups that included primary dengue (pDENV), secondary dengue (sDENV), dengue not otherwise specified (DENV), primary Zika (pZIKV), Zika with previous dengue (ZIKVwprDENV), or naïve **(Figure 1A)** as defined by Tsai et al.[195] Overall, the dengue seroprevalence in this sample set was 88.3%. Of those, 61% were indistinguishable between one previous dengue infection (primary dengue/pDENV) and multiple previous dengue infections

(secondary dengue/sDENV) using current techniques. This is a known complication with dengue virus serology due to the cross-reactivity of closely related serotypes.[203–205] A key strength of our methodology is the ability to differentiate between sDENV and ZIKVwprDENV **(S1)**.

Samples were collected between November 2015 and December 2016, which encompassed the peak of exanthematous disease in Bahia, Brazil related to the Zika virus epidemic in the Americas **(S2).** To assess the impact of the peak of Zika virus transmission in the region, we further split the samples into an early collection group (November 2015 to March 2016) and a late collection group (April 2016 to December 2016) and assessed the percentage of samples in each of the serostatus groups designated: pDENV, sDENV, DENV, pZIKV, ZIKVwprDENV, and naïve **(Figure 1B)**. Using a two-way ANOVA table, we found no statistical significance in the serostatus patterns between the two groups. A possible limitation is the small sample size of our cohort, with only 120 total participants. Additionally, our cohort was selected based on negative Zika serology performed in Brazil and does not represent the true incidence of Zika virus infection in this region.

Using an ELISA assay including serial dilutions of serum samples, we were able to asses endpoint titers to dengue (DENV3) and Zika virus. DENV3 became the most prevalent serotype in Brazil after its introduction in the early 2000s.[206] From 2006 on, Brazil experienced multiple DENV serotypes circulating in unison.[207] In 2015, both DENV3 and DENV4 were reportedly circulating in Northeast Brazil.[206]

Each sample was run 4 times and the mean was calculated for each titration point of the samples. **Figure 2A** shows a representative sample of the pattern that was

frequently seen with our primary dengue samples. These samples showed a pattern of binding to dengue (dotted line) as well as cross-reactive binding to Zika virus (solid line) that was weaker overall. **Figure 2B** shows a representative sample of secondary dengue binding patterns seen in our titration ELISA. These samples overall had higher dengue titers than the pDENV samples, as well as higher Zika virus titers than the pDENV samples. There was significant cross-reactivity, with high titers to the Zika virus in many of these samples. The endpoints of the dengue and Zika viruses were significantly different. When confirmed by FRNT, these samples were unable to neutralize the Zika virus. Additionally, the ELISA assay used measured one specific protein, NS1, whereas the antibodies demonstrated by FRNT are likely dominated by E protein. **Figure 2C** shows the endpoint titers of our primary Zika-infected sample. This sample essentially shows the inverse pattern of the primary dengue samples, with higher endpoint titer to Zika but some cross-reactivity to dengue virus. Of note, this was the only sample in this category, but we believe that this pattern would be observed in additional samples based on the known cross-reactivity between dengue and Zika virus. **Figure 2D** is a representative sample of an individual who has had past dengue and Zika virus infections. The individuals in this group showed titers for both dengue and Zika virus. It would be helpful to repeat this study with additional Zika-exposed individuals to see if this pattern can be flushed out further with a larger sample size. It would not be possible to determine the sequence of infection using neutralization, for example, ZIKVwprDENV could not be differentiated from DENVwprZIKV. Samples were able to be classified as ZIKVwprDENV in this cohort due to the known timing of Zika virus introduction.

In **Figure** 2E, see the titration curves of our representative samples for pDENV, sDENV, pZIKV, and ZIKVwprDENV binding to dengue virus (DENV3). The primary Zika sample shows a significantly lower titer than the other samples. This pattern is expected as a result of cross-reactivity, rather than past dengue virus infection. In the samples with previous dengue virus exposure (pDENV, sDENV, and ZIKVwprDENV) the titers are higher and there is an overlap in the confidence interval of the endpoint titers of sDENV and ZIKVwprDENV. This highlights the importance of our combined NS1 ELISA methodology that allows us to differentiate between these serostatus groups. By using the previously described ratio of the optical densities of our NS1 binding ELISAs to ZIKV and DENV, we are able to determine if these samples are sDENV or ZIKVwprDENV. This methodology is much quicker and easier to perform than neutralization assays, while still providing accurate results as verified by FRNT in this study.

Figure 2F shows the titration curves of the representative samples, this time to Zika virus. We see the pDENV sample has a much lower endpoint titer to Zika virus, again owing to the cross-reactivity of these viruses. The Zika virus titers of the ZIKVwprDENV sample are significantly higher than the other groups, but there is overlap between pDENV and sDENV samples. We were unable to differentiate a number of these samples using our combined NS1 methodology and neutralization assays, creating a number of samples that were classified as dengue not otherwise specified in our serostatus groups. We aim to develop a future methodology to address this issue.

Figure 3A shows a comparison of dengue and Zika virus endpoint titers between the pZIKV sample and the ZIKVwprDENV samples, there was no statistical difference in

endpoint titers between these two groups, although the number of samples tested was quite small. We did find a statistically significant (p-value 0.005) difference in the endpoint titers between the two groups: pDENV and sDENV, as seen in **Figure 3B**. Secondary dengue infections caused a significantly higher titer to both dengue and Zika virus when compared to those with one prior dengue infection. **Figure 3C** shows the positive correlation between dengue and Zika endpoint titers. This is a pattern we expected to find, owing to the known cross-reactivity between these viruses. In **Figure 3D**, there is a comparison of endpoint titers to Zika virus, comparing the mean endpoint titer of the early collection group and the mean endpoint titer of the late collection group. There is no significant difference between these groups. This is a result of these samples being selected based on Zika seronegativity. Completing this analysis with samples not selected based on a negative Zika test would likely give different results, I would expect to see increased Zika titers in those collected during and after the peak of the epidemic. **Figure 3E** shows the grouped endpoint titers to dengue virus, and surprisingly there was a significant 4-fold increase (P-value < 0.0001) in dengue titers in the late collection group compared to those collected early on in the outbreak. This may be an indication that ongoing dengue virus transmission was occurring during this time and not reported. It is possible that the attention to the Zika outbreak led to a decrease in clinical suspicion and testing for dengue virus.

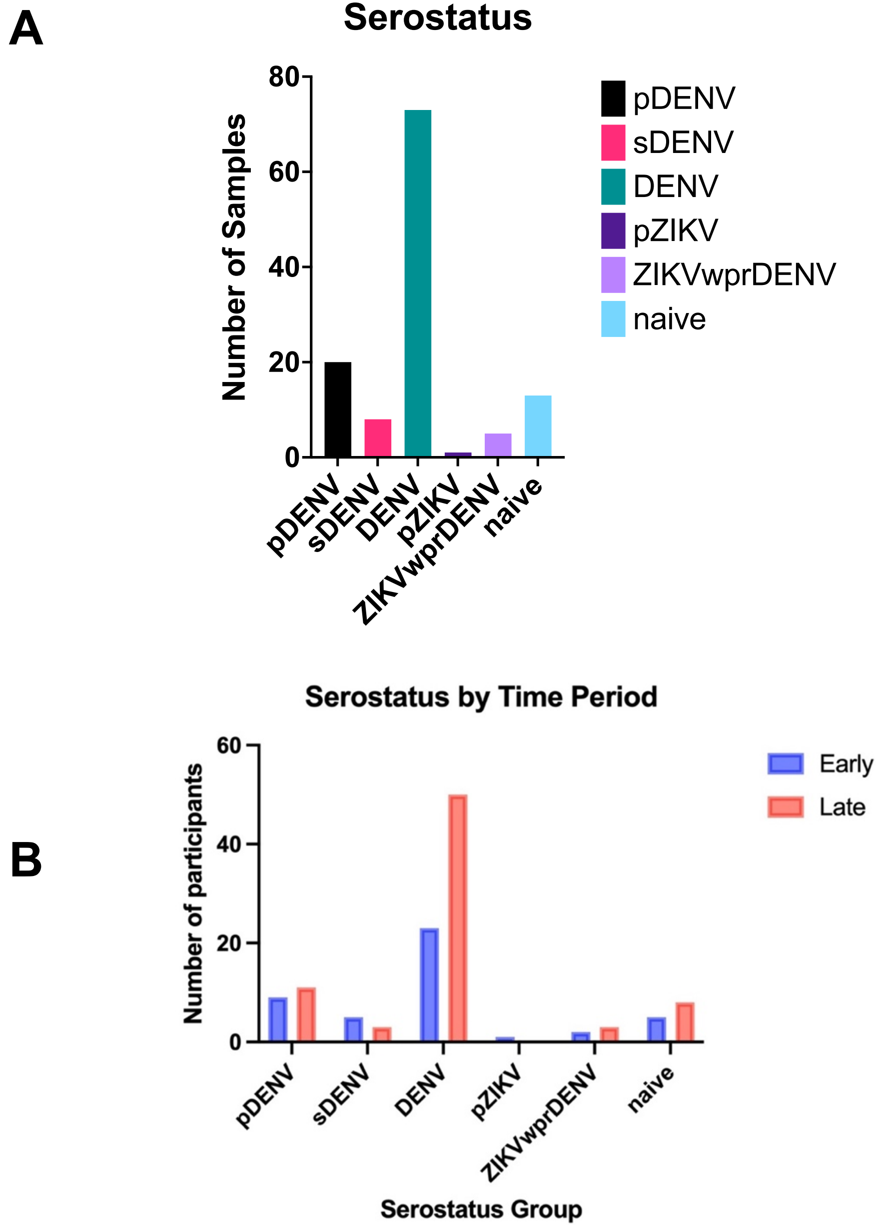
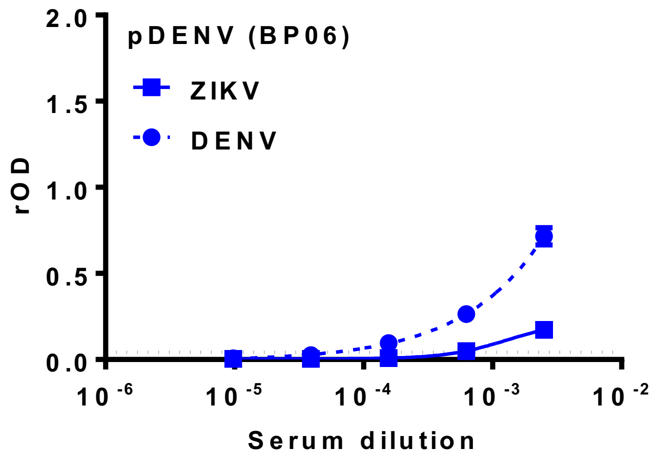
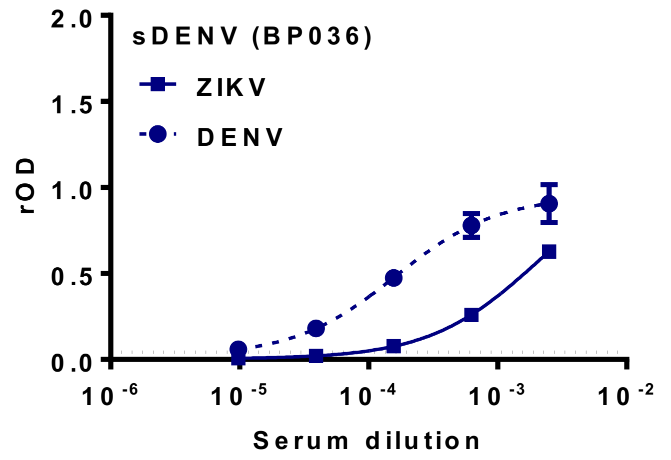
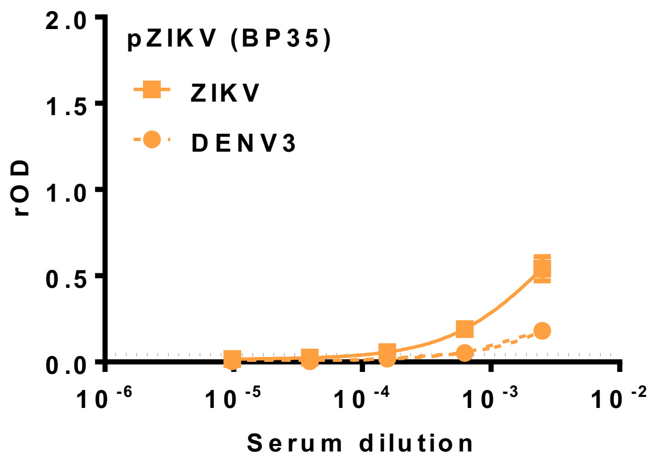
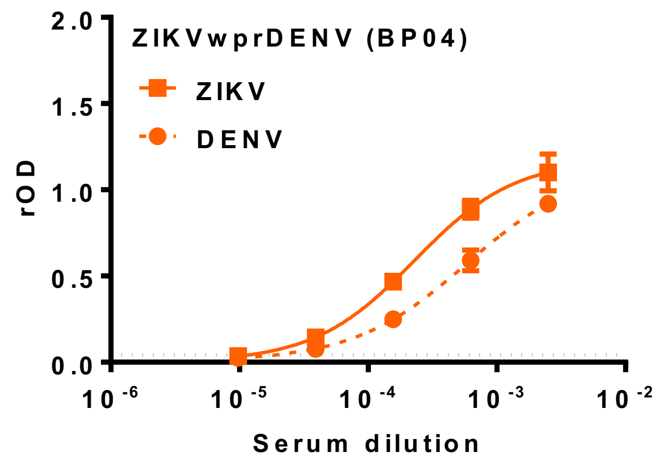


Figure 1: Serostatus of samples as determined by NS1 ELISA. **A)** Serostatus classifications (pDENV, sDENV, DENV, pZIKV, ZIKVwprDENV, naïve) of all samples. **B)** Serostatus classification by time period ("early" period refers to March 2016 and earlier, "late" period refers to after March 2016).

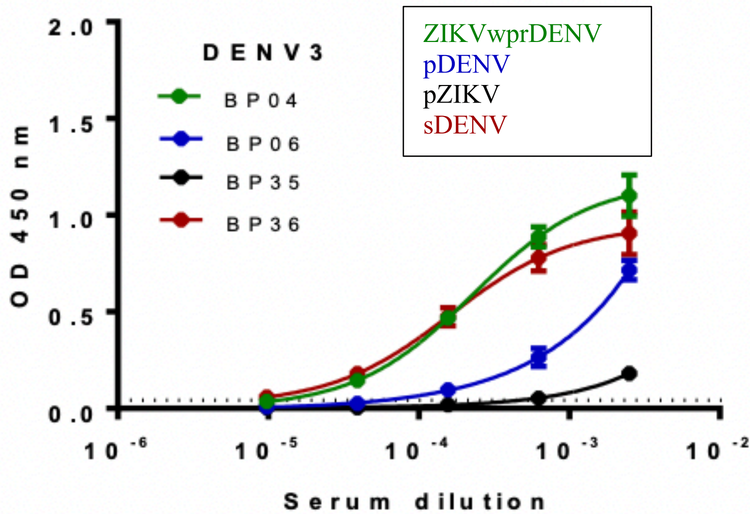

A
pDENV (BP06)
ZIKV
DENV
rOD
2.0
1.5
1.0
0.5
0.0
10 -6
10 -5
10 -4
10 -3
10 -2
Serum dilution


B
sDENV (BP036)
ZIKV
DENV
rOD
2.0
1.5
1.0
0.5
0.0
10 -6
10 -5
10 -4
10 -3
10 -2
Serum dilution


C
pZIKV (BP35)
ZIKV
DENV3
rOD
2.0
1.5
1.0
0.5
0.0
10 -6
10 -5
10 -4
10 -3
10 -2
Serum dilution


D
ZIKVwprDENV (BP04)
ZIKV
DENV
rOD
2.0
1.5
1.0
0.5
0.0
10 -6
10 -5
10 -4
10 -3
10 -2
Serum dilution

E



F

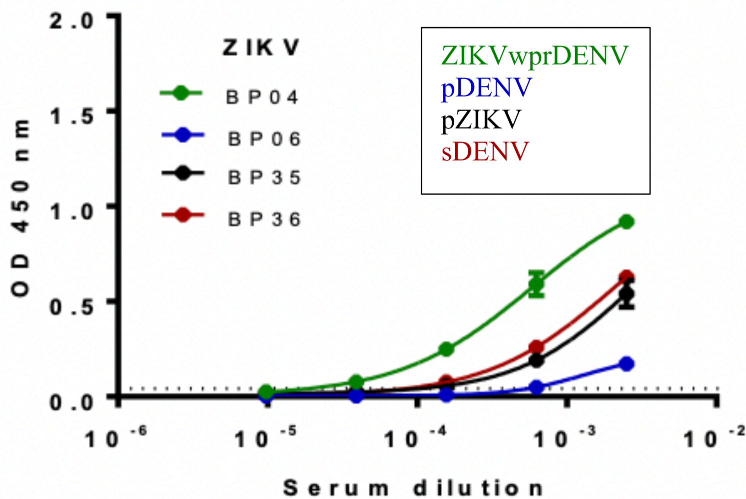


Figure 2: Titration curves of select samples to DENV3 and ZIKV. Each panel shows a representative sample of a serostatus group. **A)** Titration curves calculated for BP06, a pDENV sample. **B)** Titration curves calculated for BP036, a sDENV sample. **C)** Titration curves calculated for BP35, a pZIKV sample. **D)** Titration curves calculated for BP04, a ZIKVwprDENV sample. **E)** Titration curves of BP06, BP036, BP35, and BP04 to DENV3. **F)** Titration curves of BP06, BP036, BP35, and BP04 to ZIKV.

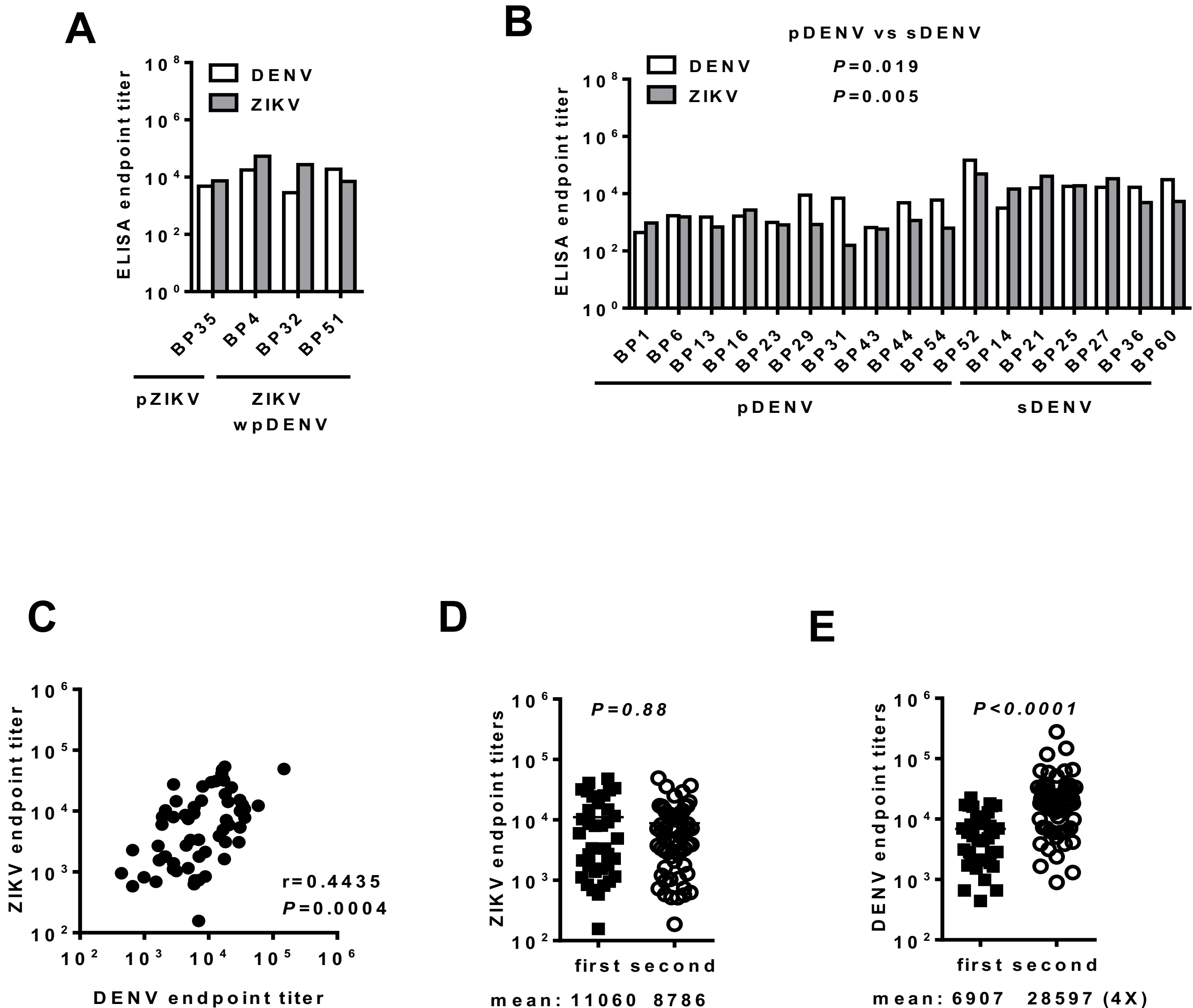
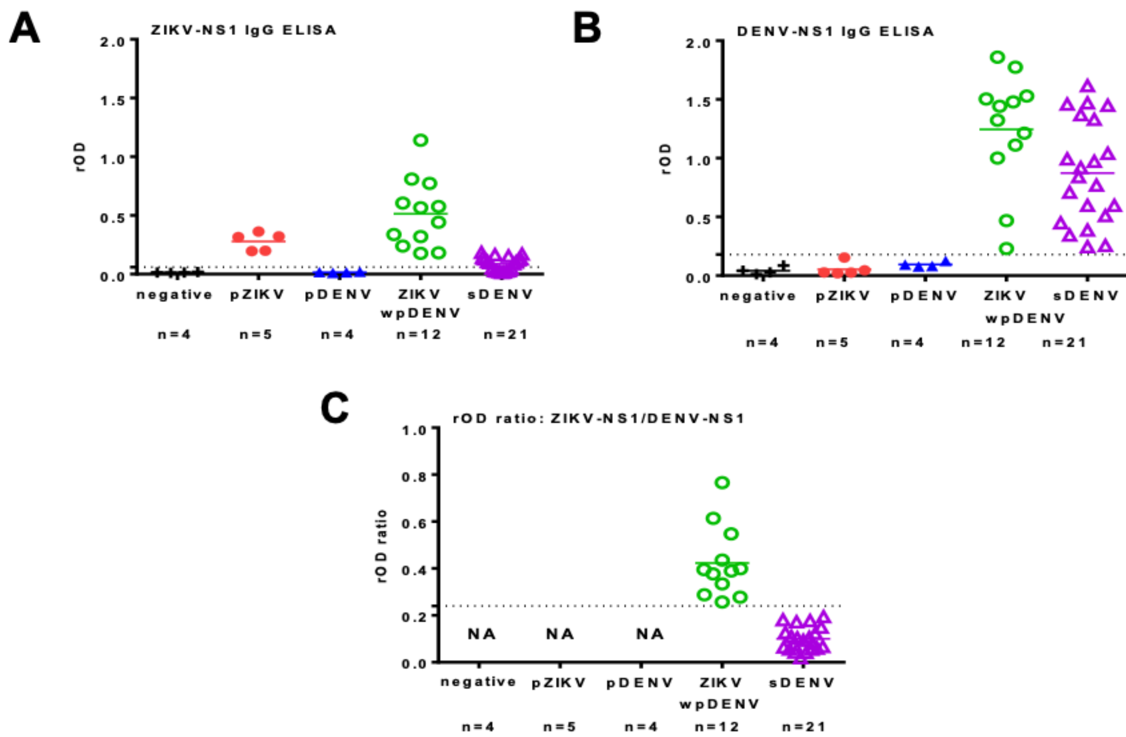
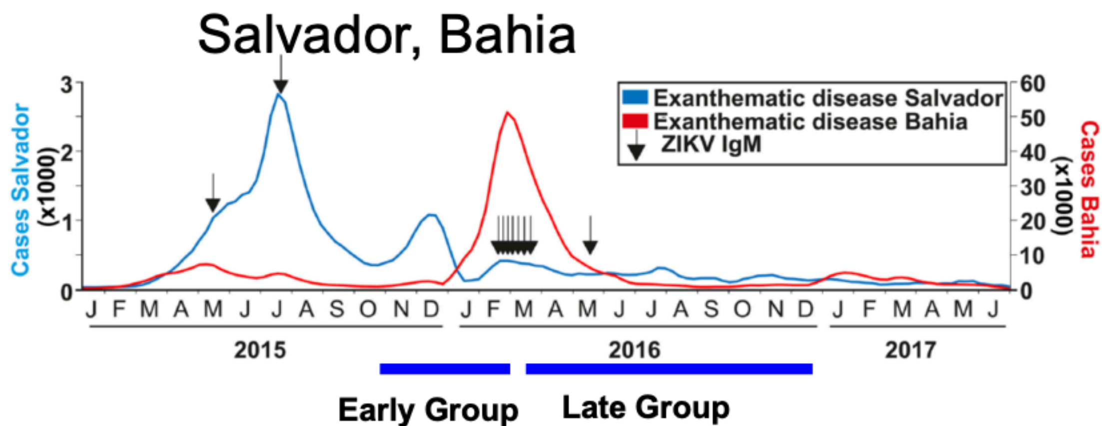


Figure 3: Endpoint titers to ZIKV and DENV as measured by ELISA. **A)** Endpoint titers to DENV and ZIKV of pDENV sample (BP35) and ZIKVwprDENV samples (BP4, BP32, BP51). **B) Endpoint titers to** DENV and ZIKV of pDENV samples (BP1, BP6, BP13, BP16, BP23, BP29, BP31, BP43, BP44, BP54) and sDENV samples (BP52, BP14, BP21, BP25, BP27, BP36, BP60). **C)** Correlation of ZIKV and DENV endpoint titers. **D)** ZIKV endpoint titers of "first" (early time period samples collected March 2016 and earlier) and "second" (late time period samples collected after March 2016). **E)** DENV endpoint titers of "first" and "second" time periods.



Supplemental Figure 1: NS1 ELISA ratios to distinguish ZIKVwprDENV from sDENV – modified from Herrera et al 2018.[208]



Supplemental Figure 2: Timeline of exanthematic disease in Salvador and Bahia, Brazil 2015-2017 – modified from Netto et al 2017.[84]

Discussion

This study aimed to assess background dengue immunity among pregnant women in Northeast Brazil. We believe that understanding the patterns of previous dengue infections in this cohort is a key component of understanding the factors that led to the emergence of Congenital Zika Syndrome.

We used a series of ELISA assays, as previously validated by Tsai et al. to be an effective measure for differentiating between those with multiple previous dengue infections versus those with previous dengue and Zika virus infections. Confirming these results with neutralization, the gold standard methodology for distinguishing dengue and Zika infections, allowed us to determine background immunity to both viruses in a cohort of asymptomatic parturient women from Salvador, Brazil – an area often called the epicenter of the Zika epidemic in the Americas. Consistent with previous reports, we found overall DENV-seroprevalence among this cohort to be 88.3%[170,209–212]

Based on the high seroprevalence of DENV in this population, there is a significant risk of severe dengue infection or dengue hemorrhagic fever as a result of ADE. This is most likely to occur in women who have had one previous dengue infection. A limitation of this study is that the methods used were unable to distinguish pDENV from sDENV in 61% of samples. As a result, it is not possible to determine the percentage of women that are at increased risk for developing severe dengue. Additionally, research has identified that ADE is more likely to occur when the subsequent dengue infection occurs 3-5 years after the initial infection.[213] Information about when the women in this study were infected with dengue is not accessible, causing further difficulty in assessing risk of ADE in this cohort.

Using titration ELISAs, we assessed endpoint titers for dengue and Zika viruses. Endpoint titers were used as a measure of the strength of antibody response. Our results showed a positive correlation between the endpoint titers to DENV and ZIKV as expected based on the known cross-reactivity of these viruses. Surprisingly, our analysis found that the DENV endpoint titers of samples collected during the later time period (after the peak of the outbreak) were 4-fold higher than those during the early time period. We believe this indicates that ongoing dengue transmission was occurring in the background of the Zika epidemic.

The increased dengue titers we found corresponded with the decline of the Zika epidemic as shown in **Supplemental Figure 2.** Our findings suggest that higher DENV antibody titers among non-ZIKV-infected individuals may have contributed to the decline of the ZIKV outbreak, though the underlying causes and mechanisms remain to be investigated. Future studies of DENV and ZIKV immunity involving longitudinal samples during and after the ZIKV outbreak are warranted. Of particular interest would be to see if Zika-infection rates were influenced by DENV-immunity.

One limitation of our study included the small sample size of 120 participants. Future studies involving a larger cohort will be valuable in assessing immunity to dengue and Zika viruses on a larger scale in Salvador, Brazil. Additionally, we were unable to differentiate between pDENV and sDENV in a number of samples using our combined NS1 methodology and neutralization assays. We aim to develop a future methodology to address this issue. Having a larger number of samples from this cohort would be beneficial for further assessing the NS1 binding patterns and endpoint titers to dengue and Zika virus represented by the various serostatus groups. Having a cohort

with known flavivirus infection history would be ideal as a comparison group for our unknown samples.

Additionally, these samples were selected based on negative Zika virus serology conducted in Brazil as we were interested in assessing background dengue immunity. Future studies to further analyze flaviviral immunity in this population and to assess the impact that pre-existing dengue immunity has on Zika virus outcomes are merited.

Chapter 3: Characterizing cross-reactive antibodies to DENV and ZIKV among pregnant women in Salvador, Brazil

Introduction

Virology

Dengue and Zika viruses are members of the *Flavivirus* genus, in the family *Flaviviridae.* “Dengue virus” refers to a group of four serotypes (DENV-1 to DENV-4) that are genetically and antigenically related.[1–3,97,214] The *Flavivirus* genus contains approximately 70 viruses, many which are associated with emerging and re-emerging human diseases.[3] Flaviviruses share a common structure, as shown by cryo-EM of purified virions.[4–7] Dengue and Zika are relatively small (~50 nm), spherical, positive-sense, single-stranded RNA viruses with a genome that is approximately 11,000 bases in length.[3,8–14]

Flaviviruses are then further divided into serocomplexes on the basis of their antigenic properties and ability to cross-neutralize by polyclonal sera.[42] Dengue viruses are grouped into one serocomplex, encompassing all four serotypes - DENV1, DENV2, DENV3, and DENV4. There are a number of different genotypes and lineages within each DENV serotype.[11] Zika virus is most closely related to the rarely discussed Spondweni virus, together they form the Spondweni serocomplex, which is more closely related to DENV than to the other mosquito-borne flaviviruses.[10] Zika virus exists as one serotype with two distinct lineages, the African lineage and the Asian lineage that appear to have diverged in the 1940s.[44–46] Phylogenetic studies have shown that the virus involved in the ZIKV epidemic in Yap Island was from the Asian lineage.[47] The French Polynesia ZIKV outbreak was also caused by the Asian lineage Zika virus. And ultimately, a virus descended from this French Polynesian ZIKV was the virus that

caused the largest Zika virus epidemic to date, in the Americas. Phylogenetic analyses indicated that this virus arrived from French Polynesia to Brazil between May and December 2013, more than a full year before the circulation of the virus was discovered.[48]

While human infections with the African or Asian Zika virus lineage typically cause similar clinical presentations, severe manifestations such as GBS or CZS have only been reported following infection with the Asian lineage.[49,50] It is still unknown whether this is a result of mutations resulting in greater virulence in this strain of ZIKV, a function of incidence – with the outbreak in the Americas being large enough to capture rare events or a result of a unique immunological profile and cross-reactivity to DENV.[49–51]

Cross-neutralization is largely correlated with the amino acid sequence of the E protein, which can vary by up to 60% between viruses within the genus.[1,3,41,42] Cross-neutralization with polyclonal sera usually is lost when there is greater than 50% divergence in amino acid sequence homology of the E protein between different flaviviruses.[10] The DENV serocomplex and Zika virus share approximately 55% sequence homology of the E protein, allowing for extensive cross-reactivity.[43] This cross-reactivity presents a challenge for serological assays, but is overcome by using more specific assays such as NS1 ELISA and neutralization assays.

Several human mAbs derived from DENV patients can neutralize ZIKV.[116–118] Additionally, DENV immune sera has been shown to cross-neutralize ZIKV in vitro. However, this was mainly seen with sera from secondary DENV infections and the cross-neutralization ability wanes over time.[117,118,148] An in-depth analysis of DENV-

ZIKV cross-reactive antibodies in humans following ZIKV infection and their relationship in disease outcomes, in particular for pregnant women, is lacking.

Similarities between Zika and Dengue Viruses

DENV and ZIKV are closely related flaviviruses with ~55% sequence homology of the E protein, which gives a high degree of structural and antigenic similarity.[43] Not only are these viruses similar in structure, they also share a disease vector and overlap in their clinical presentation.[172,214,215]

Dengue and Zika are transmitted by *Aedes mosquitoes*, mainly *A. aegypti* and *A. albopictus*.[2,3,27] These vectors are no longer confined to just tropical and subtropical regions and instead are expanding globally, increasing the risk for flavivirus emergence and reemergence.[50,216–218] Dengue is already known as the most prevalent mosquito-borne viral infection worldwide, the number of human infections has increased 30-fold since the 1960s, with current estimates of 390 million infections annually.[52]

Unlike dengue's centuries-long history, the Zika virus is a much newer public health threat. Zika virus was first isolated in 1947 in samples from a rhesus monkey in the Zika Forest in Uganda.[219] The second isolation of the virus was from a pool of *Aedes africanus* mosquitoes collected from the same forest.[67] In 1952, the first evidence of human infection was discovered in a serological study in Uganda but the virus was not isolated from humans until 1954, during an epidemic of jaundice in Nigeria.[68,69] For the next 50 years, Zika virus circulated quietly in Africa and Asia, without much evidence of disease. Although the virus was repeatedly isolated from mosquitoes, only 14 human cases were reported prior to 2007.[46,50] Zika virus continued to spread through

the Pacific, causing outbreaks in Yap, French Polynesia, New Caledonia, the Cook Islands, Easter Island, Vanuatu, the Solomon Islands, Samoa, and Fiji.[44] Clusters of acute exanthematous illness began being reported in late 2014 in the Americas and in March 2015, Zika was identified as the cause of this illness in Bahia, Brazil.[74] Epidemiologic evidence indicated that the outbreak began in Salvador, Bahia, Brazil, and rapidly spread throughout the country. By December 2015, the Brazil Ministry of Health estimated that 1.3 million suspected cases had occurred since the start of the epidemic.[75] In 2015, the United States began to see Zika virus cases in travelers returning from Central and South America along with sexual transmission of Zika virus.[76,77] In 2016, local transmission of Zika virus was reported in Florida and later in Texas.[49,78,79]

The introduction of the virus into the Americas was soon followed by the detection of fetal neurological disorders, termed congenital Zika syndrome (CZS) associated with maternal ZIKV infection, with a peak in microcephaly cases occurring in October 2015.[80] By 2017, ZIKV was detected in 47 American countries and territories.[81] Following this epidemic, Brazil reported over 350,000 suspected or confirmed ZIKV cases, as well as 2775 cases of CZS, the highest number reported globally.[82] Northeast Brazil is described as the epicenter of the ZIKV outbreak, with high numbers of cases in this region and evidence to support that ZIKV was first established here and spread outward throughout Brazil and the rest of the Americas.[83] The Northeastern Brazilian city of Salvador experience rapid spread and was among the most affected regions during the 2015-2016 Zika epidemic in the Americas, with infection rates exceeding 60%.[84] As for the current picture, 2021 PAHO data shows ongoing low-level

transmission of Zika virus in the Americas, with 6,012 cases, 85% of which are reported in Brazil.

Many arboviruses are endemic to Brazil, including the four DENV serotypes, yellow fever virus, Mayaro virus, Oropouche virus, among others.[50] The 2015 Zika virus epidemic in Brazil was complicated by the re-emergence of dengue in Brazil and the recent introduction of Chikungunya into the country. This led to an unprecedented situation in which three arboviruses that are spread by the same vector (*Aedes aegypti*) were circulating at a high incidence at the same time. There was a high burden on healthcare services and a surge in morbidity due to these three viruses, as well as challenges in clinically differentiating these three infections.[66,74,92,93] As a result of this perfect storm of overwhelmed surveillance systems, ecological factors favoring disease emergence, and the known difficulties in testing and clinically differentiating flaviviruses, much remains to be investigated about the true burden of these viruses and interactions between them during this time.

Antibody-Dependent Enhancement

Neutralization of the dengue virus occurs when antibodies prevent binding between the virion (primarily of the envelope (E) glycoprotein) and receptors on the surface of target cells.[2,8] In antibody-dependent enhancement (ADE), DENV-specific antibodies bind to the virus, but not at sufficient con concentration or avidity to neutralize the virus and prevent entry into the cell. The virus-antibody complexes that are formed by this failed attempt to neutralize attach to Fc receptors on cells such as macrophages, neutrophils, and NK cells and facilitate viral entry.[2] This results in an increased number of infected cells and increased viral output.[220]

With dengue viruses, the phenomenon of antibody-dependent enhancement (ADE) has been discussed for decades and numerous reports have identified that heterotypic secondary DENV infection is the greatest risk factor for severe disease outcomes, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[16,115,120,220]

It is theorized that antibodies generated to the primary infecting serotype will not be of sufficient concentration or avidity to neutralize a secondary infecting serotype, but rather, at a certain concentration, they will enhance disease. This disease enhancement occurs as a result of Fc receptor-mediated endocytosis of the virus into monocytes and macrophages, which are the principal site of DENV replication, thus driving higher viral loads. ADE has been demonstrated in vitro and in mouse models, but the evidence in nonhuman primates and in humans is mixed.[116–119] Recent evidence in a human cohort study has shown that the risk of enhancement is highest within a narrow range of preexisting anti-DENV antibody titers.[120]

Historical Zika outbreaks have been largely localized within dengue-endemic areas and the high level of virus homology makes the potential for preexisting dengue-induced antibodies to enhance ZIKV infection a concern.[155,193,221] In areas that have been affected by Zika outbreaks, there is also a risk that Zika immunity could predispose individuals to an enhanced DENV disease pathology.[145]

While the cross-reactivity between DENV and ZIKV is known to pose a diagnostic challenge, its role in disease pathogenesis remains to be elucidated.[18,41,51,147] It is theorized that these similarities are sufficient to drive ADE in a similar manner to what was described for heterologous secondary dengue infections.[10,44,144]

Understanding the cross-reactivity of the antibody response to DENV and ZIKV will give insight into the level of protection, or risk, associated with different flavivirus serostatuses.

Study Rationale

It is theorized that there is a risk of antibody-dependent enhancement from sequential infections with Zika and dengue viruses. Multiple studies have shown that DENV-immune sera can cross-neutralize ZIKV, but this effect wanes over time.[117,118,148] As this immunity wanes, it is possible that antibodies reach a level where they increase the risk of ADE.[155] In-vitro experiments and studies in mice have shown that DENV-immune sera can enhance ZIKV replication, but this same phenomenon was not seen in non-human primates.[118,119,144,145,149–152,222]

Results in human cohorts are mixed. Two pediatric cohort studies showed that DENV infection may reduce the risk of symptomatic ZIKV infection, but a study of the same pediatric cohort in Nicaragua showed that ZIKV infection may enhance the risk of severe DENV in future infections.[153,154]

The dramatic increase in the incidence of microcephaly in Northeastern Brazil was reported in late 2015, coinciding with a large increase in ZIKV infection.[80,80,193,223] The pathogenesis that caused this phenomenon is unknown, however, one possible theory is that dengue virus-mediated immune enhancement in mothers may be a risk factor for the development of CZS in infants.[224–227] For this reason, it is important to further study the immune profiles of pregnant women infected with dengue and Zika

viruses. An in-depth analysis of DENV-ZIKV cross-reactive antibodies in humans and their relationship with disease outcomes, in particular for pregnant women, is lacking.

In order to close this gap in research, this study analyzes the antibody response to dengue and Zika viruses among pregnant women during the 2015-16 Zika outbreak in Salvador, Brazil, and characterizes cross-reactive antibodies between dengue (DENV) and Zika (ZIKV). Much remains to be explored about this topic, including the role of background flaviviral immunity in pregnant women and how this affects the risk of congenital Zika syndrome (CZS) in infants.

This study provides the unique ability to study the background levels of dengue virus immunity in pregnant women in Northeast Brazil at the time of the epidemic. The effect of prior dengue infection and its ability to confer protection or risk of enhancement of Zika virus infection, especially during pregnancy remains to be elucidated. We aim to better understand this relationship and how it correlates to protection, with the long-term goal of facilitating the development of safe and effective DENV and ZIKV vaccines.

Methods

Sample Collection

The study of coded serum or plasma samples was approved by the institutional review board (IRB) of the University of Hawaii at Manoa, as well as the IRB of the Federal University of Bahia. Serum samples were collected from pregnant women receiving care at the Hospital Universitário Professor Edgard Santos in Salvador, Brazil between 2015 and 2017.

Virus Propagation

Dengue 1-4 and Zika viruses were grown in a monolayer of Vero cells in T75 flasks, lysed with tryptase, and purified using sucrose cushion ultracentrifugation.[197] Virus strains used were DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87), DENV4 (H241 strain), or ZIKV (PRVABC59 strain). After UV inactivation, viruses were subjected to serial two-fold dilutions (1:200 to 1:6400), coated on 96-well plates, and tested with positive control serum to determine the titer, which was the highest dilution to reach optical density (OD) of 1. The viruses were aliquoted and stored at -80° Celsius. Cell lysate was collected using tryptase and a cell scraper and stored at -80° Celsius.

Microneutralization (FRNT)

Flat-bottom 96-well plates were seeded with Vero cells 24 h prior to infection to create Vero cell monolayers. Twofold serial dilutions of serum were mixed with 50

focus-forming units of DENV1, DENV2, DENV3, DENV4, or ZIKV (grown as described above) at 37°C for 1 h. After adding this mixture to the wells, the plates were incubated 48 to 70 hours depending on the virus serotype. The medium was removed and plates were fixed as described by Tsai et al.[198] Murine Mab 4G2 and a secondary antibody mixture (IRDye 800CW-conjugated goat anti-mouse IgG at 1:10,000 and the DRAQ5 fluorescent probe at 1:10,000) were then added and plates were read using the LiCor Odyssey classic imaging system (LiCor Biosciences) at a wavelength of 800 nm/700 nm fluorescence. These signals were analyzed using Image Studio software to determine percent neutralization at different concentrations and NT50 and NT90. The titers of FRNT50 and FRNT90 were determined by nonlinear regression analysis (GraphPad Prism 5.0).

Depletion of Cross-Reactive Antibodies

To deplete cross-reactive antibodies, sera (1:20 dilution in 1× PBS) were incubated with UV-inactivated DENV1-4 or ZIKV (from Vero cells) or pellets derived from the culture supernatants of mock-infected Vero cells in 1× PBS at 37°C for 1 h and ultracentrifuged at 150,000 × *g* and 4°C for 1 h to remove bound cross-reactive antibodies. Sera were then tested by ELISA to confirm depletion was complete. The proportion (in percent) of type-specific (TS) antibodies was equal to the endpoint titer of DENV or ZIKV-depleted serum/endpoint titer of mock-depleted serum × 100. The percentage of cross-reactive (CR) antibodies was equal to 100 minus the percentage of TS antibodies.[228]

Binding Pattern ELISAs

96-well ELISA plates were coated with columns of cell lysate from virus propagation in the pattern: DENV1, DENV2, DENV3, DENV4, ZIKV, Mock. Viruses used are described in the virus propagation step above and mock refers to mock-infected cell lysate prepared in the same manner as the virus-infected cell lysates. This setup was duplicated on each plate. Samples were tested for IgG activity to each virus and controls were included in each run. Mock-infected lysates were also included as internal controls for non-specific binding.

Plates were blocked and incubated with patient serum diluted to 1:400 for 2 hours at 37° Celsius. Plates were then washed 4 times and incubated with secondary antibodies (anti-human IgG conjugated with horseradish peroxidase, Jackson ImmunoResearch) for 1 hour at 37° Celsius before being washed 6 additional times. ELISA substrate was added for 15 minutes per well before stop-solution (2N H_2SO_4) was added. The optical density at 450 nm (OD450) was read with a reference wavelength of 630 nm.

Urea ELISA

To measure serum avidity, we used a modified ELISA protocol. Plates were coated with whole virus (D1, D2, D3, D4, or ZIKV) as opposed to NS1. Before the secondary Ab (anti-human IgG conjugated with horseradish peroxidase, Jackson Immune Research Laboratory), was used, we conducted a urea wash. 100 µL urea 8 mol/L was added to each well at room temperature for 5 min between the second and third washings (total 4 washes).

Results

Serum samples from pregnant women in Salvador, Brazil were analyzed by NS1 ELISA methodology to determine serostatus groupings: primary dengue (pDENV), secondary dengue (sDENV), primary Zika (pZIKV), Zika with previous dengue (ZIKVwprDENV). Results were confirmed using the gold standard neutralization assays. Additional samples were included in this chapter to increase the number of Zika samples analyzed. Samples that were previously used begin with the prefix “BP”, additional samples that were included in these assays begin with the prefix “ZK”. All samples were collected from asymptomatic pregnant women seeking routine care at the Federal University of Bahia Hospital. Samples that were flavivirus naïve or that could not be delineated into one of these 4 serogroups were excluded from this analysis. **Figure 1** shows the serostatus groupings of this sample set: 3 women (14%) were classified as pZIKV, 3 women (14%) were classified as pDENV, 6 women (28%) were classified as sDENV, and the remaining 10 women (43%) were classified as ZIKVwprDENV. Of note, the pDENV samples included 2 women who had been infected by DENV4 and 1 woman who had been infected with DENV3 as confirmed by focus reduction neutralization (FRNT).

All samples were tested by binding pattern ELISA and results showed characteristic patterns that differed between serostatus groups, a subset of these results can be seen in **Figure 2A**. Cross-reactivity between these closely related flaviviruses was seen in this assay, as expected based on known difficulties in serological differentiation between ZIKV and DENV. pZIKV samples showed binding of various levels to all 4 DENV serotypes, although with a significantly lower strength of

binding than seen to ZIKV. The inverse is true for pDENV samples, minimal levels of binding to ZIKV were seen in pDENV samples. Of note, pDENV samples showed a high optical density (OD) reading to the presumptive infecting serotype and cross-reactivity to all other DENV serotypes. This pattern of higher reactivity to infecting serotype was repeated at much stronger OD readings in sDENV, these samples showed stronger cross reactivity to ZIKV as well as to all DENV serotypes. ZIKVwprDENV samples showed strong binding to ZIKV as well as strong reactivity to all DENV serotypes.

When compared to FRNT results (**Figure 2B**), we can see a reduction in "background noise" caused by flavivirus cross-reactivity. Although binding to all 4 dengue serotypes was seen in ELISA results of the pZIKV samples, these samples were only able to neutralize Zika virus. For our pDENV samples, the binding that was seen by ELISA was also delineated by assessing neutralization capability. These samples showed strong neutralization of the infecting dengue serotype and neutralization of other DENV serotypes and ZIKV were below the threshold for positivity determined by our methods. SDENV samples showed strong neutralization of one serotype as well as moderate neutralization of all other DENV serotypes, but no neutralization capacity for ZIKV. This pattern is likely due to "original antigenic sin", a commonly described pattern in dengue immunity where the highest neutralizing titers to in both acute and convalescent sera are to the infecting serotype, with lower cross-reactive titers seen to the other dengue serotypes [2,229–231] This is thought to play a role in ADE. ZIKVwprDENV samples showed the widest variability in neutralization results, with some samples showing very strong neutralization of ZIKV and low to moderate

neutralization capacity for the 4 DENV serotypes, while others showed strong neutralization of ZIKV as well as the DENV serotypes.

The identification of two seemingly separate neutralization patterns among the ZIKVwprDENV serogroup suggested that these women may truly fall into two separate groups: those who have been infected with Zika virus as well as multiple dengue serotypes (further referred to as sDENV-ZIKV and those who have been infected with Zika virus and one serotype of dengue (further referred to as pDENV-ZIKV).

This hypothesis was explored by utilizing a urea ELISA to assess IgG avidity, as shown in **Figure 3.** Zika virus antibodies were depleted from our samples and then the sera were tested for avidity to DENV (D1). By depleting ZIKV-reactive antibodies and comparing to the non-depleted sera, I was able to determine the percentage of type-specific versus cross-reactive antibodies. **Figure 3A** shows the urea ELISA results of representative samples of pZIKV, pDENV, sDENV, and ZIKVwprDENV. As expected, depletion of ZIKV-specific antibodies from the pZIKV sample (BP35) eliminated titers against DENV. The ZIKVwprDENV samples showed two patterns. One subset showed a large drop in IgG OD and avidity after depletion of ZIKV antibodies (BP04), we hypothesize that these samples represent pDENV-ZIKV immunity. The other group showed a negligible drop in avidity following ZIKV antibody depletion (BP112), suggesting that these samples have more durability immunity that I theorize may come from having multiple prior dengue infections (sDENV-ZIKV). Although there were different patterns in avidity seen, a decrease in IgG avidity after urea wash was not able to separate ZIKVwprDENV panel into sDENV-ZIKV and pDENV-ZIKV subgroups as shown in **Figure 3B,** as two clusterings of avidity were not seen**.** Using this

methodology on a larger sample size may help flush out these differences further. A linear relationship between the percentage of cross-reactive antibodies and a decrease in IgG avidity after urea wash was found, as shown in **Figure 3C.** This indicates that cross-reactive antibodies have a lower avidity than type-specific antibodies.

During infection with Zika virus, antibodies specific to ZIKV are generated (type-specific antibodies) as well as cross-reactive antibodies to DENV.[191,232] The inverse holds true as well, DENV infection yields DENV-specific antibodies as well as cross-reactive antibodies to ZIKV. Because the role of background flaviviral immunity is thought to play a role in the development of congenital Zika syndrome (CZS) in infants, it is important to understand the underlying antibody profiles in pregnant women from Salvador, Brazil – the epicenter of the ZIKV epidemic in the Americas. We depleted dengue virus serotypes 1-4 (D1-4) antibodies from serum samples from 22 pregnant women in order to assess the percentage of type-specific and cross-reactive antibodies. By comparing the ELISA binding of D1-4 depleted and non-depleted samples (**Figure 4**) we were able to determine that our pZIKV panel had a higher proportion of ZIKV type-specific antibody (70-82%) than ZIKVwprDENV panel (2-16%). Our ZIKVwprDENV panel had a higher proportion of DENV-ZIKV cross-reactive antibodies (84-98%) than had been previously reported.[154,155,232] This may contribute to the protective effects of previous DENV on ZIKV as well as ZIKV on DENV, as demonstrated by the low number of cases and severe infections seen during 2017-18 and enhancing effect , as demonstrated by a high number of severe dengue (DHF/DSS) and fatalities seen during 2019-20 in human cohort studies. Flavivirus infection causes temporary cross-protection to other flaviviruses, which may explain the low case numbers of DENV

and ZIKV in 2017-18. A recent publication found a dengue antibody half-life of 4 years, correlating to a peak risk of enhancement, which may explain the severe dengue phenotype seen in 2019-20.[120] **Figure 5** shows repeating this methodology with the change of depleting ZIKV antibodies. Our pZIKV samples showed 0% DENV type-specific antibodies, however, the results were highly variable among the sDENV and ZIKVwprDENV sample sets. The ZIKVwprDENV samples showed a range of 42-90% DENV type-specific antibodies remaining after ZIKV depletion, and our sDENV samples showed 27-92% DENV type-specific antibodies. In agreement with a previous report, a high proportion of ZIKV type-specific antibodies was found in pZIKV infection, as shown in **Figure 6A.**[232] In contrast to the previous literature showing predominant ZIKV type-specific antibodies following ZIKV infection, our results showed a high proportion of DENV-ZIKV cross-reactive antibodies in ZIKVwprDENV infection (**Figure 6B**).

BP35	pZIKV
ZK714	pZIKV
ZK256	pZIKV
ZK66	ZIKVwprDENV
ZK219	ZIKVwprDENV
ZK452	ZIKVwprDENV
ZK343	ZIKVwprDENV
ZK204	ZIKVwprDENV
ZK479	ZIKVwprDENV
BP4	ZIKVwprDENV
BP32	ZIKVwprDENV
BP51	ZIKVwprDENV
BP112	ZIKVwprDENV
BP36	sDENV
BP60	sDENV
BP71	sDENV
BP73	sDENV
ZK85	sDENV
ZK225	sDENV
BP6	pD4
BP67	pD4
BP31	pD3

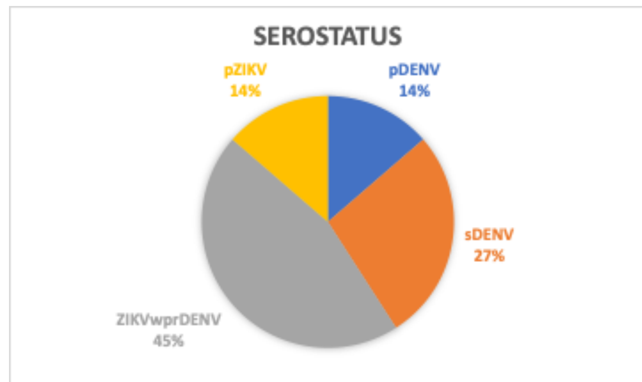
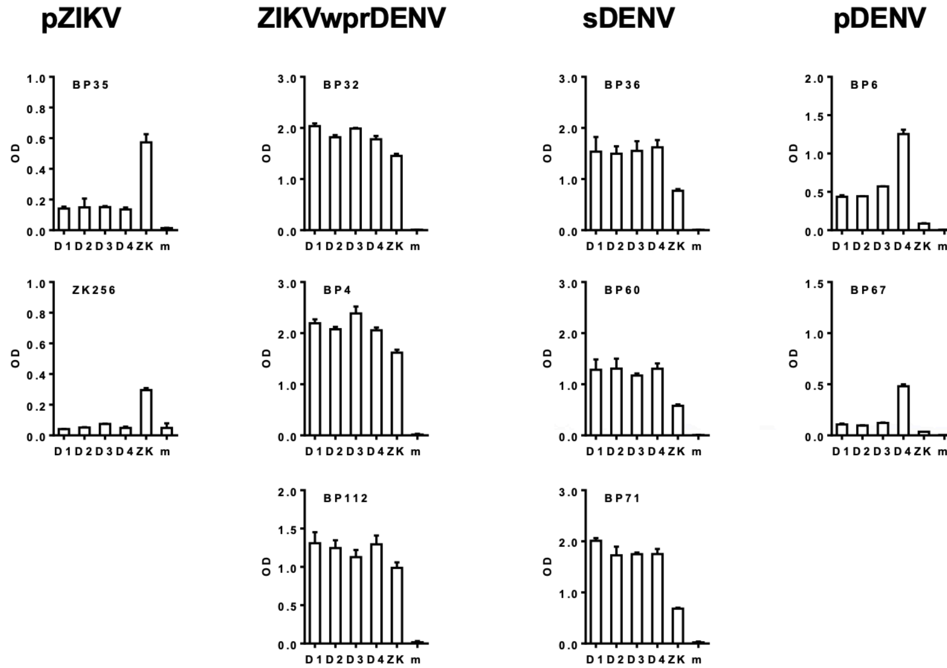


Figure 1: Serostatus of 22 samples. **A)** Table of serostatus classification for each selected sample. **B)** Serostatus group (pDENV, sDENV, pZIKV, ZIKVwprDENV) classification by percent.



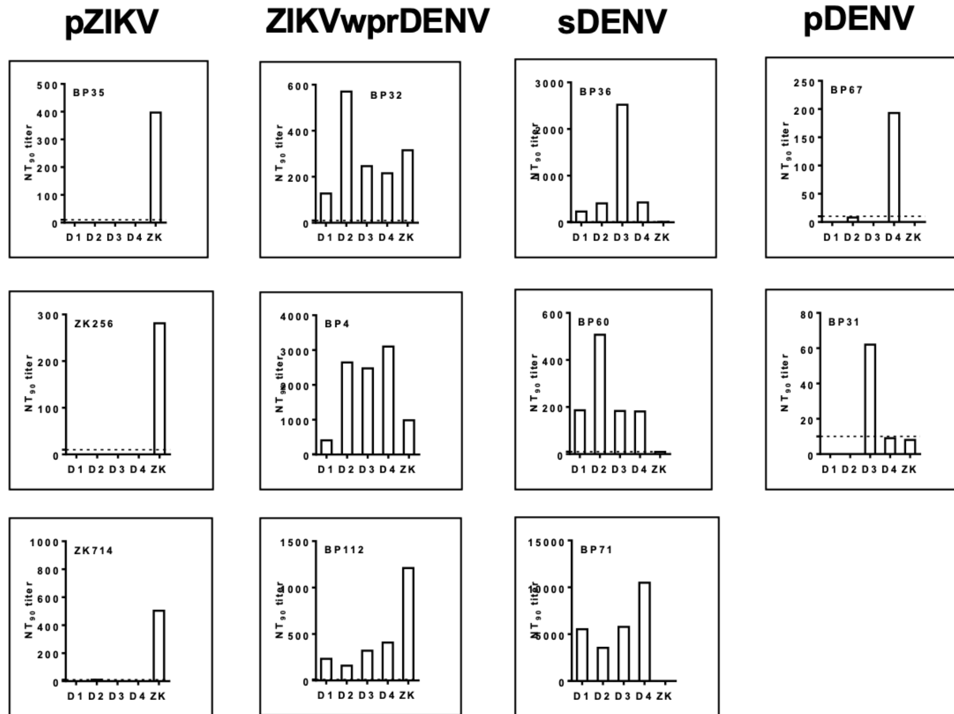


Figure 2*:* Binding Pattern ELISA and FRNT results. **A)** Binding ELISA results showing individual samples from each of the 4 serogroups included. **B)** FRNT results showing individual samples from each of the 4 serogroups included. Each sample was testing by ELISA to assess for antibody binding to Dengue virus 1-4 (D1, D2, D3, D4) and Zika virus (ZK). Mock-infected cell lysate (m) was used as an internal control.

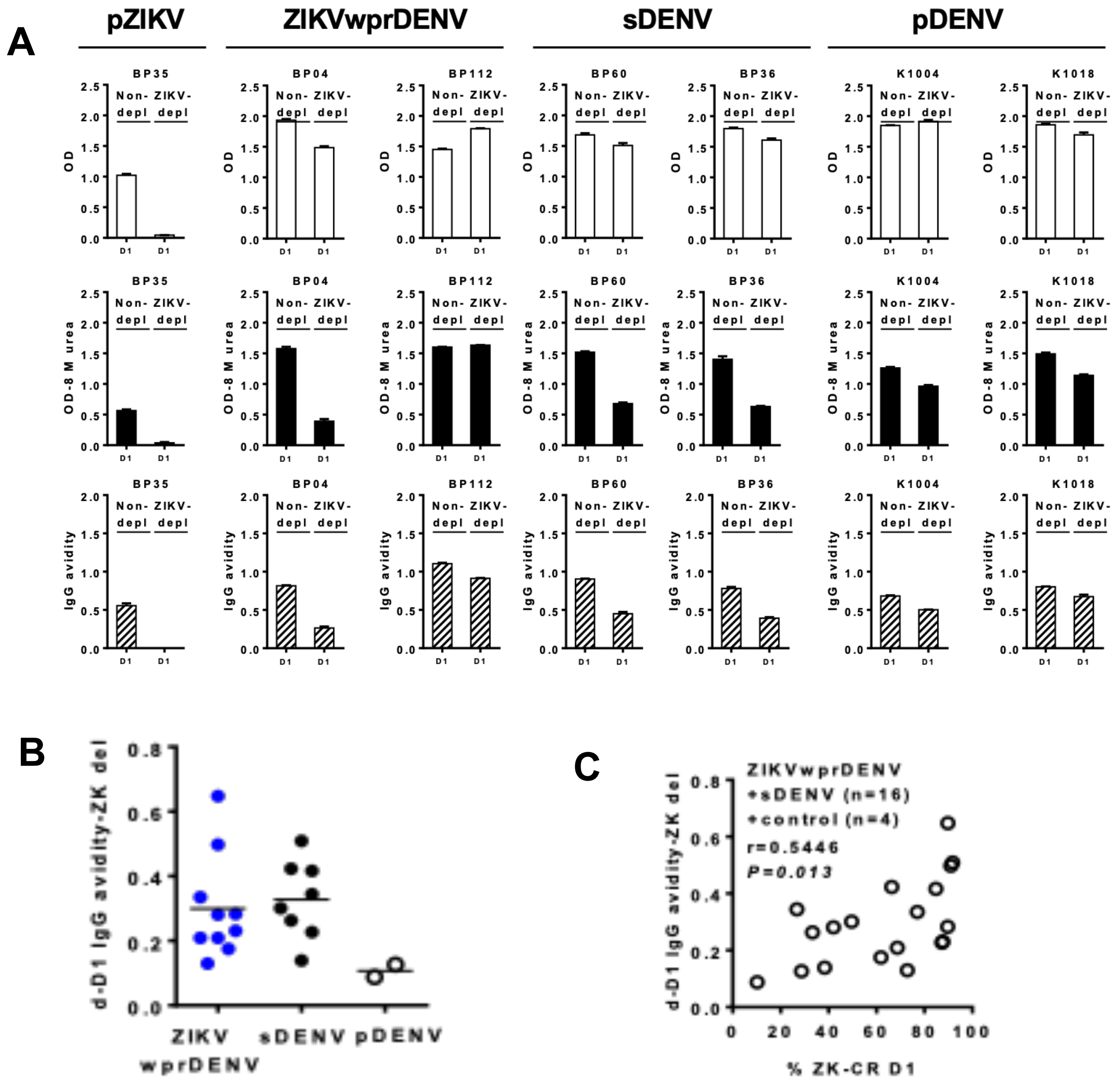


Figure 3: Avidity analysis as measured by urea ELISA. **A)** ELISA OD of original and ZIKV-depleted samples before and after urea wash. The ratio of the OD of urea washed sample and OD of the non-urea washed sample is shown as the IgG avidity. **B)** IgG avidity of samples plotted by serostatus group (ZIKVwprDENV, sDENV, pDENV). **C) Decrease in** IgG avidity of ZIKV-depleted samples plotted against the percentage of cross-reactive antibodies as determined by ELISA. Includes ZIKVwprDENV and sDENV samples.

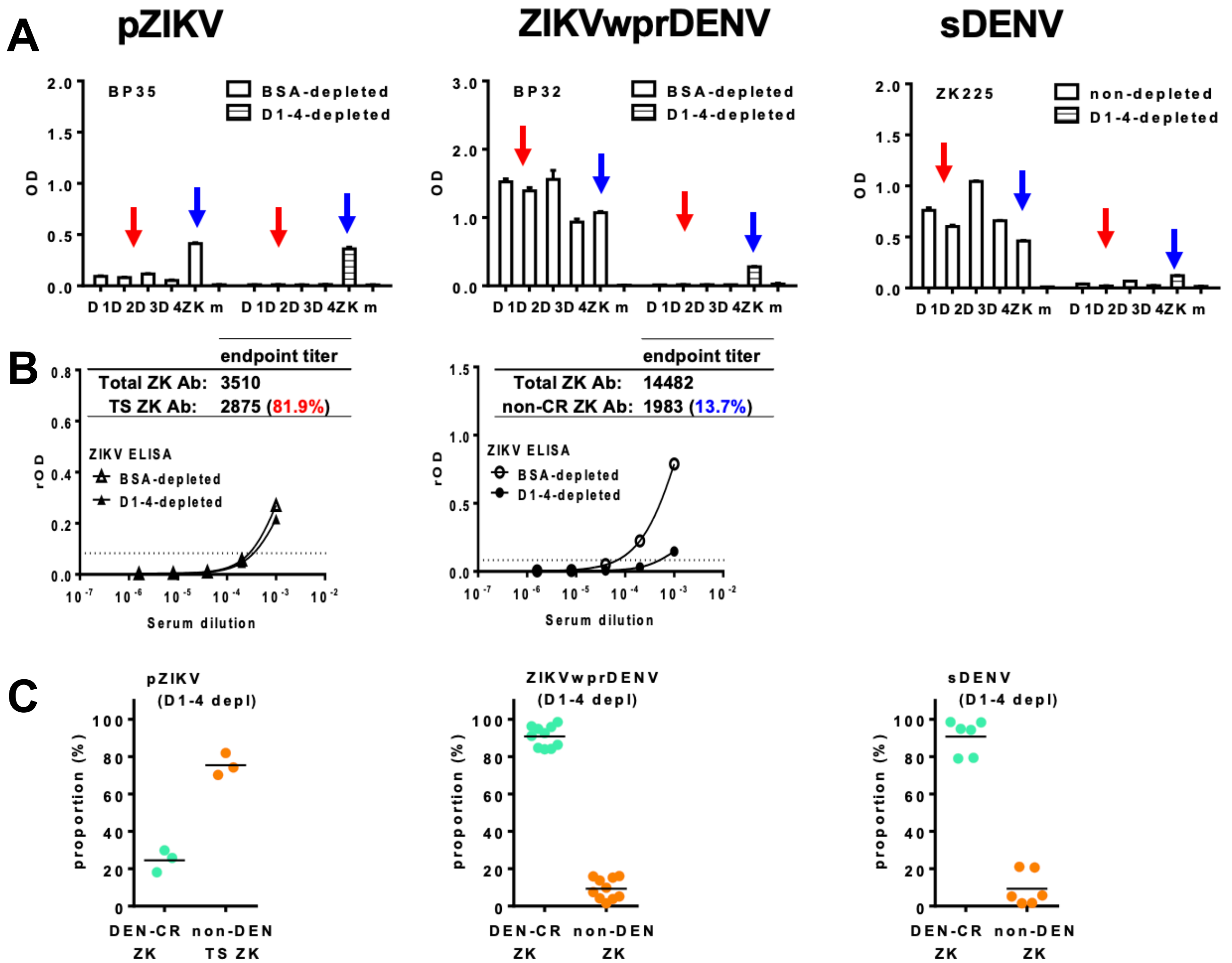


Figure 4: : Comparison of D1-4 depleted and non-depleted samples. **A)** Binding ELISA results for sera depleted with D1-4 and non-depleted samples - showing representative sample of pZIKV (BP35), ZIKVwprDENV (BP32), and sDENV (ZK225). **B)** Percentage of type-specific vs cross-reactive antibodies for pZIKV sample and ZIKVwprDENV sample. **C)** Proportion of type-specific and cross-reactive antibodies for all pZIKV, ZIKVwprDENV, and sDENV samples.

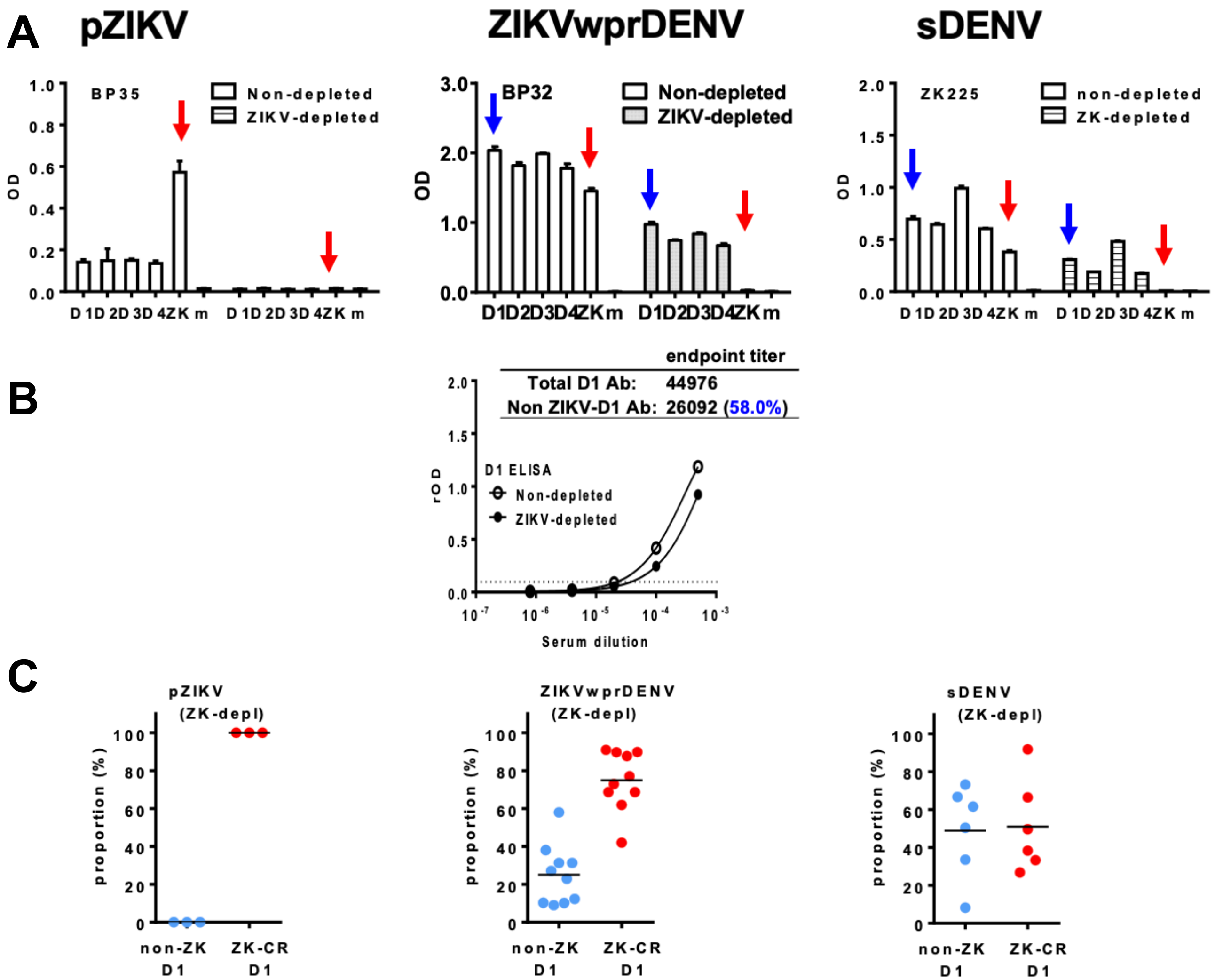


Figure 5: Comparison of ZIKV depleted and non-depleted samples. **A)** Binding ELISA results for sera depleted with D1-4 and non-depleted samples - showing representative sample of pZIKV (BP35), ZIKVwprDENV (BP32), and sDENV (ZK225). **B)** Percentage of type-specific vs cross-reactive antibodies for ZIKVwprDENV sample. **C)** Proportion of type-specific and cross-reactive antibodies for all pZIKV, ZIKVwprDENV, and sDENV samples.

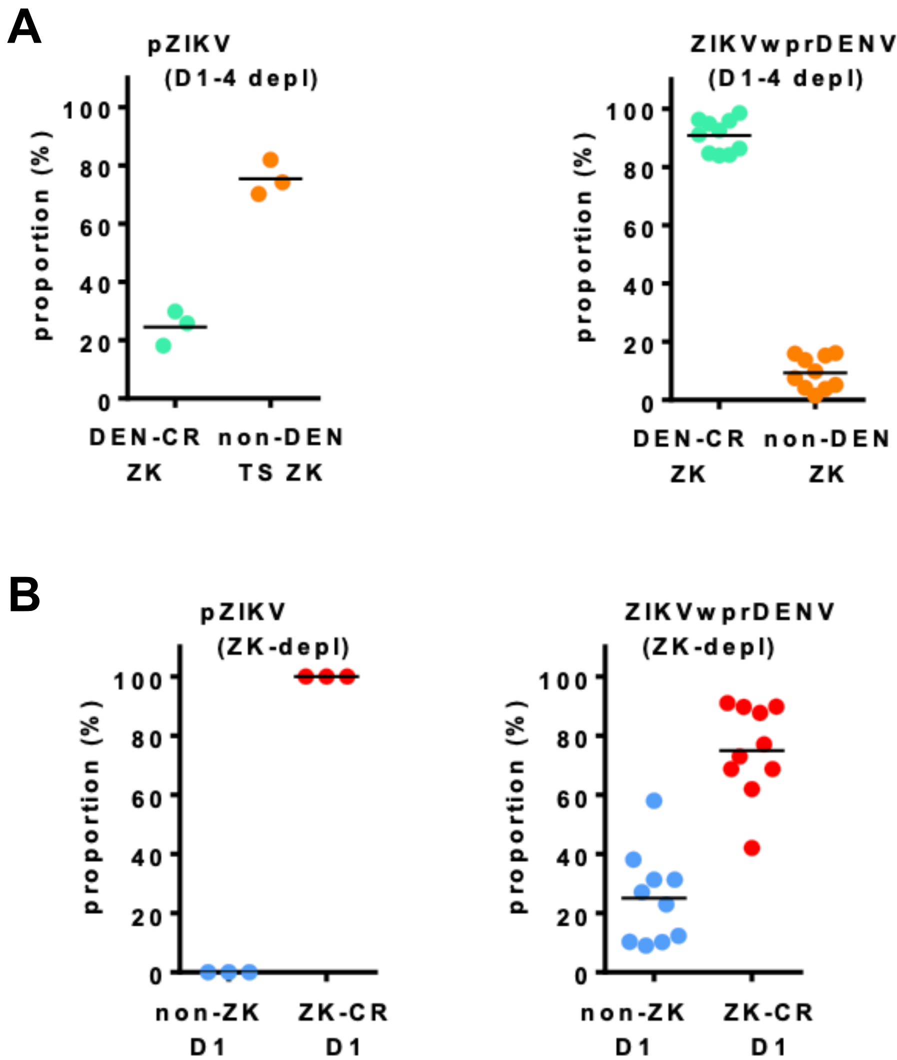


Figure 6: Comparison of antibody specificity between pZIKV and ZIKVwprDENV samples. **A)** D1-4 depleted and non-depleted samples, type-specific vs cross-reactive antibodies among pZIKV and ZIKVwprDENV samples. **B)** ZIKV depleted and non-depleted samples, type-specific vs cross-reactive antibodies among pZIKV and ZIKVwprDENV samples.

		NT90				
		D1	D2	D3	D4	ZIKV
BP35	pZIKV	<10	<10	<10	<10	397
ZK714	pZIKV	<10	10	<10	<10	504
ZK256	pZIKV	<10	<10	<10	<10	281
ZK66	ZIKVwprDENV	839	2769	369	3065	571
ZK219	ZIKVwprDENV	250	507	61	331	577
ZK452	ZIKVwprDENV	154	1035	<10	1723	368
ZK343	ZIKVwprDENV	487	291	313	155	639
ZK204	ZIKVwprDENV	232	207	161	277	611
ZK479	ZIKVwprDENV	171	760	2482	123	613
BP4	ZIKVwprDENV	408	2646	2476	3102	986
BP32	ZIKVwprDENV	127	570	246	215	315
BP51	ZIKVwprDENV	472	1826	2731	570	<10
BP112	ZIKVwprDENV	233	159	320	408	1211
BP36	sDENV	231	407	2521	426	<10
BP60	sDENV	186	507	183	181	<10
BP71	sDENV	5530	3547	5786	10488	11
BP73	sDENV	37	160	105	46	<10
ZK85	sDENV	162	259	604	323	132
ZK225	sDENV	1574	338	1996	484	244
BP6	pD4	<10	<10	ND	ND	ND
BP67	pD4	<10	<10	<10	193	<10
BP31	pD3	<10	<10	62	<10	<10

Supplemental Figure 1: FRNT results for D1, D2, D3, D4, and ZIKV for all samples, used for serostatus group classification.

Discussion

A combination of DENV and ZIKV NS1 IgG ELISAs was used to differentiate DENV and ZIKV serostatus among pregnant women during the ZIKV outbreak in Salvador, Brazil. Following this, antibodies were depleted with inactivated DENV1-4 or ZIKV, and endpoint ELISA titers were analyzed to determine the ZIKV type-specific and cross-reactive NS1 antibodies. The results showed that the pZIKV panel had a higher proportion of ZIKV type-specific antibodies (70-82%) than the ZIKVwprDENV panel (2-16%). I also found that the ZIKVwprDENV panel had a higher proportion of DENV-ZIKV cross-reactive antibodies (84-98%) than previously reported by Andrade et al. It is possible that this is a function of time since previous dengue infection or a result of having numerous prior dengue infections that have shaped a broadly cross-reactive population of antibodies.

As described by Katzelnick et al., ADE appears to be a dose-dependent phenomenon, with a high titer of cross-reactive antibody titer offering protection whereas a low titer of cross-reactive antibodies drives enhancement.[120] The high proportion of cross-reactive antibodies may have contributed a protective effect that explains the low numbers of DENV and ZIKV infections seen in 2017-18. The 2019-20 season was characterized by a severe dengue outbreak and large proportion of severe dengue cases in Northeast Brazil. This may be a result of waning antibody populations leading to ADE.[154,155,232,233]

ELISA results were confirmed with FRNT. Although cross-reactivity was seen on ELISA, pZIKV samples were only able to neutralize Zika virus. While pDENV samples showed extensive cross-reactivity to all DENV serotypes on ELISA, they were only able

to neutralize one dengue serotype – presumably the infecting serotype. ELISA results for sDENV samples showed cross-reactivity to Zika virus, but no neutralization capability was seen. Of note, cross-reactive antibodies to dengue and Zika virus are often targeted against the highly conserved E protein. NS1 ELISA is a useful tool for minimizing cross-reactivity seen on ELISA, although it does not completely remove this effect. NS1 titers and E titers may not be correlated and future studies should compare titers to these two distinct proteins in this population.

Avidity was assessed using whole DENV1 virus and identified two avidity patterns in ZIKVwprDENV samples. One group showed a large decrease in IgG avidity following urea wash, whereas the other group remained relatively unchanged. This may be due to differences in number of prior dengue infections in these individuals, but the sample size was not large enough to prove this hypothesis. Additionally, a linear relationship between the percentage of cross-reactive antibodies and a decrease in IgG avidity after urea wash was seen, indicating that cross-reactive antibodies have a lower avidity than type-specific antibodies. One limitation of this study is that the use of DENV1 virus for all assays may underestimate avidity of individuals infected with other DENV serotypes.

These samples may not be representative of a larger population, as they were collected from one hospital. It would be helpful to repeat this methodology using more samples from various healthcare centers in Northeast Brazil to increase the power of our findings. The ability to test sequential samples would be ideal, in order to understand how the type-specific (TS) versus cross-reactive (CR) antibody profile changes over time.

This study serves as a reference point for the background immunity to Zika and dengue viruses among pregnant women in an area of Brazil that was heavily impacted by Congenital Zika Syndrome. The effects of DENV-ZIKV cross-reactive antibodies on virus neutralization and antibody-dependent enhancement and disease outcomes in pregnant women remain to be studied in the future.

Chapter 4: Summary and Future Directions

Summary

The 2015-2016 Zika epidemic took the world by storm with a sudden increase in adverse fetal outcomes in Latin America. Although the vast majority of prenatal Zika virus infections may not cause harm to the fetus, a wide spectrum of congenital malformations is now recognized as associated with Zika virus infections during pregnancy - ranging from mild to life-limiting. [234] The severity and incidence of birth defects vary by trimester of infection, with the most severe outcomes reported from first-trimester infections. One study found the risk of birth defects decreases from 8% for first-trimester infections to 6% for second-trimester infections to 3.8% for third-trimester infections.[235]

While microcephaly was the finding that first drew attention to the Zika epidemic, further research identified that this was merely one part of a variable syndrome that became known as Congenital Zika Syndrome (CZS).[236,237] Outcomes are now known to include a wide spectrum of structural anomalies, ocular manifestations, intracranial calcification, ventriculomegaly, hearing loss, and functional impairments in addition to microcephaly. [129,234–237]

The long-term outcomes of CZS are not fully described. As this first generation of affected children is developing, researchers are finding that the severity of microcephaly is an important prognostic factor and case fatality rate may be as high as 10% in the first years of life.[234,236,238]

The epicenter of the Zika epidemic and subsequent cases of Congenital Zika Syndrome occurred in Brazil, where a 20-fold increase in the incidence of microcephaly

was seen in newborns in 2015.[239] The number of cases of microcephaly as a result of this epidemic is hard to definitively pinpoint due to limitations of detection and surveillance, lack of clear diagnostic criteria in the early days, and increased abortion rates of fetuses with suspected microcephaly.[219,240,241] Estimates range from 1,950 to 8,600 cases of microcephaly due to Congenital Zika Syndrome (CZS) in Brazil.[234,242,243] When you expand this to include congenital malformations of the central nervous system besides microcephaly, this number jumps to nearly 15,000 suspected cases in Brazil between 2015 and 2017.[48]

Even more elusive than the true case count of this outbreak is the pathology driving this newly reported syndrome. While Zika virus (ZIKV) was first isolated and described in 1947, the neurological effects of this virus were not widely described until this past decade when Zika virus was implicated in an outbreak of Guillain-Barré Syndrome in French Polynesia in 2013 and CZS in 2015.[219,244] Was this change the result of a more virulent strain of the virus? Some theorize that mutations of the Asian lineage Zika virus that caused these outbreaks are the cause of the more severe phenotype and some studies have shown more tropism for neural tissue in vitro and in mouse studies. [51,245–247] However, these findings do not explain why maternal ZIKV infection leads to severe microcephaly in some fetuses, but not others. [248] This also does not explain why the majority of Zika-associated microcephaly occurred in Brazil, specifically in the Northeast region of the country.[84]

The other prevailing theory is that an antibody-dependent enhancement (ADE) of Zika virus is occurring. The antigenic similarities between ZIKV and dengue virus (DENV), combined with high levels of DENV background immunity among affected

populations in recent outbreaks, suggest that anti-DENV maternal antibodies are driving ZIKV-induced neurological damage.[248]

Several human cohort studies suggest that background immunity and DENV antibody levels might affect the severity of ZIKV infections or complications.[153,154] However, it appears that the risk level is not linear. A previous study in Brazil showed that having neutralizing antibodies against a greater number of dengue serotypes was protective against having an infant with CZS.[249]

Much is still unknown about this topic as it pertains to the antibody profiles associated with the risk of enhancement versus protection. DENV-ZIKV ADE has been demonstrated in vitro and in mouse models, but the evidence in nonhuman primates and in humans is mixed.[116–119] Two recent human cohort studies suggest that background immunity and DENV antibody levels might affect the severity of ZIKV infections.[153,154] Another cohort study showed that previous ZIKV infection may impact the severity of future dengue infections, suggesting that antibody-dependent enhancement can occur from DENV to ZIKV and vice versa.[155]

An in-depth analysis of DENV-ZIKV cross-reactive antibodies in humans following ZIKV infections, and their relationship with disease outcomes, in particular for pregnant women, is lacking. In this study, we sought to determine the level of background dengue immunity, as well as the nuances of cross-reactive immunity to dengue and Zika viruses, in pregnant women living in an area that is often described as the epicenter of the Zika epidemic.

Seroprevalence

Serostatus to dengue and Zika viruses were determined by combined NS1 ELISA and verified by neutralization assays. This NS1 methodology allows us to distinguish dengue and Zika antibodies, a known challenge when analyzing flaviviral immunity, with high sensitivity. [195] Among 120 women presenting for routine prenatal care at the Federal University of Bahia in Salvador, Brazil, we found an overall DENV seroprevalence of 88.3%, which is consistent with other DENV seroprevalence reports from Northeast Brazil. [170,209–212]

Neutralization Patterns

One main goal of this study is to gain further understanding of the nuances of the antibody response to DENV and ZIKV, in terms of cross-reactive versus type-specific immunity, as well as avidity. This study identified specific patterns of neutralization antibodies across various serostatus groups (pDENV, pZIKV, sDENV, ZIKVwprDENV).

The primary Zika virus-infected samples (pZIKV) showed a clear ability to neutralize ZIKV without the ability to neutralize any of the 4 dengue serotypes. The primary dengue virus-infected samples (pDENV) showed a majority of their neutralizing activity to their infecting dengue serotype, with minimal neutralizing capacity seen in the other viruses tested. SDENV samples showed strong neutralization of one DENV serotype as well as moderate neutralization of all other DENV serotypes, but no neutralization capacity for ZIKV. This pattern is likely due to “original antigenic sin”, a commonly described pattern in dengue immunity where the highest neutralizing titers in

both acute and convalescent sera are to the initial infecting serotype, with lower cross-reactive titers seen to the other dengue serotypes. [2,229–231] This is thought to play a role in ADE.

ZIKVwprDENV, samples with prior dengue and Zika virus infections, fell into two categories of patterns. The first group showed high neutralizing capacity across the board, with high NT titers to ZIKV and all 4 DENV serotypes. The other group showed high neutralizing titers to ZIKV and lower NT titers to dengue viruses. We hypothesized that the first group, with robust neutralizing antibodies to all viruses tested, were those who had experienced Zika virus infection along with 2 or more dengue virus infections while those with the lower NT titers to dengue viruses were those with one prior Zika virus infection and one prior dengue virus infection. The lower NT titers to DENV in the ZIKwprDENV group may be due to time after last DENV infection.

Avidity Analysis

In order to better understand the two distinct neutralization patterns seen in the ZIKVwprDENV panel of samples, I assessed IgG avidity using a series of Urea ELISA tests using whole virus as antigen. One subset showed a large drop in IgG avidity after depletion of ZIKV antibodies, we hypothesize that these samples represent pDENV-ZIKV immunity. The other group showed a negligible drop in avidity following ZIKV antibody depletion, suggesting that these samples have more durability immunity that we theorize may come from having multiple prior dengue infections (sDENV-ZIKV). Although there were different patterns in avidity seen, a decrease in IgG avidity after

urea wash was not able to separate the ZIKVwprDENV panel into two distinct groups. However, this experiment showed an inverse relationship between the % of cross-reactive antibodies and IgG antibody avidity as measured by the Urea ELISA.

Cross-Reactive and Type-Specific Antibodies

I used combined DENV and ZIKV NS1 IgG ELISAs to differentiate DENV and ZIKV serostatus among pregnant women during the ZIKV outbreak in Salvador, Brazil. We found that the pZIKV panel had a higher proportion of ZIKV type-specific antibodies (70-82%) than the ZIKVwprDENV panel (2-16%). I also found that the ZIKVwprDENV panel had a higher proportion of DENV-ZIKV cross-reactive antibodies (84-98%) than previously reported by Andrade et al. It is possible that this contributed to the protective effects of previous DENV on ZIKV as well as ZIKV on DENV during 2017-18 and enhancing effect during 2019-20 in human cohort studies from Northeast Brazil.[154,155,232]

Using titration ELISAs, I assessed endpoint titers to dengue and Zika viruses. Endpoint titers were used as a measure of the strength of antibody response. The results showed a positive correlation between the endpoint titers to DENV and ZIKV as expected based on the known cross-reactivity of these viruses. Surprisingly, my analysis found that the DENV endpoint titers of samples collected during the later time period (after the peak of the outbreak) were 4-fold higher than those during the early time period. We believe this indicates that ongoing dengue transmission was occurring in the background of the Zika epidemic.

The increased dengue titers found corresponded with the decline of the Zika epidemic. These findings suggest that higher DENV antibody titers among non-ZIKV-infected individuals may have contributed to the decline of the ZIKV outbreak, though the underlying causes and mechanisms remain to be investigated. Future studies of DENV and ZIKV immunity involving longitudinal samples during and after the ZIKV outbreak are warranted.

Limitations and Alternative Strategies

This study aimed to assess background dengue immunity among pregnant women in Northeast Brazil. We believe that understanding the patterns of previous dengue infections in this cohort is a key component of understanding the factors that led to the emergence of Congenital Zika Syndrome. While this research serves as a reference point for the background immunity to Zika and dengue viruses among pregnant women in an area of Brazil that was heavily impacted by Congenital Zika Syndrome, we were unable to correlate the antibody profiles of these women with the clinical outcomes of their infants. The effects of DENV-ZIKV cross-reactive antibodies on virus neutralization and antibody-dependent enhancement and disease outcomes in pregnant women remain to be studied in the future.

Additionally, the samples used for this research may not be representative of the population as a whole, as they were collected from one hospital and were a limited number of samples. It would be beneficial to repeat this methodology using more samples from various healthcare centers in Northeast Brazil to increase the power of

our findings. Testing the DENV and ZIKV seroprevalence of this population could also be done using a Luminex assay. It would be beneficial to test these samples for a panel of related flaviviruses, as well as test for Chikungunya, which also was known to be circulating in Brazil during this time period.

We were unable to differentiate between pDENV and sDENV in a number of samples using our combined NS1 methodology and neutralization assays. We aim to develop a future methodology to address this issue. Having a larger number of samples from this cohort would be beneficial for further assessing the NS1 binding patterns, endpoint titers to dengue and Zika virus, and IgG avidity represented by the various serostatus groups. Having a cohort with known flavivirus infection history would be ideal as a comparison group for our unknown samples.

The ability to test sequential samples would be ideal, in order to understand how the type-specific (TS) versus cross-reactive (CR) antibody profile changes over time. The assessment of type-specific vs cross-reactive antibodies could be done with other methods, for example, a quantitative antibody assay. Testing memory B-cells (MBCs) would be beneficial if it is possible to obtain cryopreserved blood samples from these subjects. This could be assessed in comparison to the antibody response.

Additionally, these samples were selected based on negative Zika virus serology conducted in Brazil as we were interested in assessing background dengue immunity. Future studies to further analyze flaviviral immunity in this population and to assess the impact that pre-existing dengue immunity has on Zika virus outcomes are merited.

Future Directions

There are wide-ranging and severe societal impacts of CZS, from fear and anxiety in the general population to increased healthcare expenditures of affected countries to the physical, mental, social, economic, and educational impacts on families affected by this lifelong syndrome. Unfortunately, many basic questions regarding this disease and associated long-term consequences still remain unanswered.

The factors leading to the development of Congenital Zika Syndrome (CZS) are unknown. Previous research has found that a broader profile of neutralizing antibodies to multiple DENV serotypes was found in pregnant women who delivered healthy children compared with mothers who delivered babies affected by Congenital Zika Syndrome.[249] It is theorized that cross-reactive antibodies are driving DENV-ZIKV ADE. In this study, we found a high proportion of DENV-ZIKV cross-reactive antibodies (84-98%) among pregnant women in Salvador, Brazil who had been sequentially infected with DENV and ZIKV. A future research goal is to assess how these cross-reactive antibodies to DENV and ZIKV in pregnant women correlate to clinical outcomes in their infants.

Understanding the antibody profile of mothers at risk for delivering infants with Congenital Zika Syndrome has numerous scientific and public health impacts. This information can be used to aid in vaccine development – ensuring that vaccines do not drive ADE and more severe phenotypes of Zika-infection. It can also be used to identify a smaller population at risk for CZS, which may quell fears and population-level anxiety if another epidemic occurs. Additionally, this information can be applied to other regions

of the globe at risk for Zika virus introduction and can be used to predict which regions are at highest risk of CZS based on local patterns of dengue transmission.

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