Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Teresa Smith Date

Evaluation and application of novel serologic tools for assessing ZIKV-specific immunity for surveillance and translational research

By

Teresa Smith
Master of Public Health
Global Epidemiology

_____ [Chair's signature]
Gonzalo Vasquez-Prokopec, PhD
Committee Chair

_____ [Member's signature]
Matthew Collins, MD, PhD
Committee Member

Evaluation and application of novel serologic tools for assessing ZIKV-specific immunity for surveillance and translational research

By

Teresa C. Smith
B.S. Biochemistry & Cell Biology
Rice University
2019

Thesis Committee Chair: Gonzalo Vasquez-Prokopec, PhD

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Global Epidemiology
2021

Abstract

Since the beginning of the Latin American Zika epidemic first recognized in 2015, autochthonous transmission of Zika virus (ZIKV) has been reported in 87 different countries. Although most ZIKV infections are mild or asymptomatic, there is risk of developing Guillain-Barré syndrome and adverse pregnancy outcomes including congenital malformations, stillbirth, and pre-term births. Although RT-PCR testing is available, ZIKV RNA is detectable during a narrow window in patients presenting with symptoms. Serology can detect recent or remote infections, but traditional assays are complicated by cross-reactive antibodies elicited by other co-circulating flaviviruses like dengue virus (DENV). Thus, there is a critical need for serologic tools for reliable diagnosis and surveillance of ZIKV infection as well as to gain a better understanding of protective immunity for vaccine development. We developed a blockade-of-binding (BOB) ELISA using A9E and G9E, two ZIKV envelope protein-binding monoclonal antibodies (mAbs), which are strongly neutralizing against ZIKV but do not bind to DENV. We assessed BOB performance by ROC curve analysis after running a panel of positive and negative control sera. At the optimal cutoff, the A9E BOB ELISA has a sensitivity of 93.5% (95% CI: 79.3,98.9) and specificity 97.8 (95% CI: 92.2,99.6). The G9E BOB ELISA has a sensitivity and specificity of 100% (95% CI: 89.0,100.0) and 100% (95% CI: 95.9,100). We then applied these assays to test samples from surveillance cohorts in Risaralda, Colombia. Finally, the assay was applied to samples from participants in a phase 1 randomized controlled trial for a ZIKV DNA vaccine candidate. Serum samples collected 30-day post-vaccine administration exhibited significantly less A9E and G9E blockade than those with natural ZIKV immunity. In conclusion, A9E and G9E ZE-BOB are sensitive and specific assays that may be useful tools for diagnosis of recent or remote ZIKV infections and clinical decision making. Further development of A9E and G9E BOB assays as potential serologic correlates of protective immunity against ZIKV is well justified.

Evaluation and application of novel serologic tools for assessing ZIKV-specific immunity for surveillance and translational research

By

Teresa C. Smith
B.S. Biochemistry & Cell Biology
Rice University
2019

Thesis Committee Chair: Gonzalo Vasquez-Prokopec, PhD

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Global Epidemiology
2021

Acknowledgements

I would like to acknowledge Dr. Matthew Collins and Dr. Gonzalo Vasquez-Prokopec for sharing their expertise with me and supporting my efforts over this past year.

I am also grateful for my roommates Serena Agrawal and Piper Shifflett who made virtual graduate school in a pandemic bearable.

Finally, thank you to my wonderful family who have been supporting my academic and career endeavors since the very beginning.

Table of Contents

Chapter I: Literature Review

Flaviviruses and the emergence of Zika as a public health threat

Zika virus (ZIKV) was first isolated in 1947 from a rhesus macaque in Uganda's Ziika Forest, and a few years later it was first recognized as being able to cause human illness *(1, 2)*. ZIKV is a member of the flavivirus genus, which is comprised of mosquito and tick-borne single stranded positive-sense RNA viruses. Other notable flaviviruses include dengue (DENV), yellow fever (YFV), Japanese encephalitis (JEV) and West Nile (WNV) viruses *(3)*. They have a vast geographic distribution, with over 2 billion people living in dengue-endemic areas alone *(4)*. Over time, certain environmental changes have allowed flaviviruses and some of their mosquito vectors to flourish and spread, increasing the potential for large epidemics. Some of these factors include changing global climate, poorly planned urbanization and the increase in intercontinental travel *(3, 5)*.

The first well-documented, large outbreaks of Zika were in the Micronesia and Polynesia between the years 2007-2014. ZIKV was first identified in the Americas in Bahia, Brazil in 2015. It subsequently spread to other countries in South and Central America and has now been documented in 87 countries around the world *(6, 7)*. The Brazilian and French Polynesian outbreak led to the observation of a substantial uptick in fetal abnormalities including microcephaly caused by ZIKV infection of pregnant women and transmission to the developing fetus, a condition later described as congenital Zika syndrome (CZS) *(7)*. The 2015-2016 epidemic that spanned the Americas, led to thousands of infants diagnosed with CZS. In Brazil alone there were over 200,000 probable cases and over 8,600 babies born with malformations. Though incidence of ZIKV fell dramatically in 2017, the sheer numbers of susceptible hosts

living in areas where competent *Aedes* vectors are endemic, along with identification of viable mammalian reservoirs, make future ZIKV transmission highly likely *(8)*.

Zika epidemiology and diagnosis

ZIKV transmission can occur through a sylvatic or suburban-urban transmission cycle. Nearly all cases are attributed to transmission from *A*. *aegypti* or *A*. *albopictus* mosquitos. Both have similarly low vector competence. However, *A*. *aegypti* is thought to have higher vectorial capability due to ecological and behavioral factors. Although most infections occurs from mosquito-borne transmission, ZIKV can also be transmitted from mother to fetus during pregnancy and can be sexually transmitted *(7)*.

The incubation period of ZIKV ranges from 3-14 days *(9)*. In one epidemiologic study in Micronesia, it was found that only 19% of persons who had evidence of ZIKV infection had had symptoms. When illness is present, common symptoms include, rash, fever, arthritis, conjunctivitis, myalgia, and headache *(7)*. In rare cases, those infected may experience Guillain-Barré syndrome, an autoimmune disorder that can lead to nerve damage, muscle weakness, paralysis and respiratory failure *(7, 10)*.

ZIKV diagnosis is typically done by RT-PCR and IgM detection via MAC-ELISA. Viremia in nonpregnant people is transient and generally only is detectable by RT-PCR for the first week after symptom onset. In contrast, viral mRNA is detectible in the blood of pregnant women as late as 10 weeks post-infection. Cross-reactive antibodies from recent DENV infection can lead to false positive ZIKV MAC-ELISA results as well. This makes specific ZIKV diagnosis and surveillance from dengue difficult given that DENV co-circulates essentially throughout ZIKV's entire geographical range, and similar non-specific symptoms are caused by both viruses *(3, 7)*.

Other ELISA based assays attempt to capture ZIKV IgG but cross-reactivity can lead to false positive results. People also use E protein subdomains such as EDIII that are less well conserved between viruses as the antigenic target *(11)*. A blockade-of-binding competitive ELISA has also increase specificity compared to whole virus capture ELISAs *(12)*. Despite advances in ELISA based screening methods, P/FRNT remain the gold standard for diagnostics and surveillance despite being labor-intensive and requiring BSL-2 conditions *(13)*.

ZIKV biology

The ZIKV genome encodes 3 structural (C,E prM) and 7 non-structural proteins. Viral particle assembly requires interaction of prM and E protein in the endoplasmic reticulum as well as encapsulation of genetic material by the C protein. prM is later cleaved from the immature virion surface by furin, triggering viral release. However, virions may be released immaturely with incomplete prM cleavage, resulting in the host's production of immature ZIKV conformations as well *(14)*. Mature virions consist of 90 E protein dimers. Each E protein monomer consists of three domains (EDI, EDII, EDIII). Anti-EDIII antibodies of flaviviruses tends to, virus-specific, and EDIII is a frequently a target of vaccine development. Moreover, studies in ZIKV-infected patients have found that many monoclonal antibodies (mAbs) target DI or DII and quaternary epitopes *(15)*. Because of genomic and amino acid homology, antibodies elicited by one flavivirus infection often cross-react with other viruses in the genus, leading to complications for creating specific ZIKV diagnostics and understanding the nuances of host-pathogen interactions *(16)*. One study found that memory B-cell responses from DENV infections of all four serotypes resulted in similar amounts of cross-reactivity to ZIKV, however samples from patients with a history of secondary DENV infection showed higher levels of cross-reactivity compared to serum with a monotypyic DENV infection history*(17)*. Another

study found that four ZIKV-immune serum samples had antibodies that bound DENV1-4 E protein at about the same levels in an ELISA format *(18)*. This study also found that in the same four ZIKV-immune patients, 65% of the E DI/DII protein mAbs isolated from ZIKV patients were cross-reactive to all four DENV serotypes *(18)*. However, ZIKV immune patients have neutralization titers highest for ZIKV with much lower titers to the four DENV viruses. Similarly DENV patients tend to have neutralization titers four fold lower than the lowest DENV titer *(19)*. Although the two viruses are distinct, the large amount of serologic cross-reactivity leads to complications for creating specific ZIKV diagnostics, understanding correlates of protection for vaccine development, and understanding the nuances of host-pathogen interactions *(16)*.

Complications of flavivirus cross-reactivity

Due to extensive cross-reactivity observed for antibodies elicited to DENV and ZIKV, traditional serologic diagnostics such as capture ELISAs often fail to differentiate between the two viruses. Moreover, due to low and short-lived viremia, infection is often hard to acutely capture with molecular testing. This creates diagnostic and surveillance complications since the two viruses often co-circulate *(7, 20)*. One study found an inhibitory ELISA reporting total antibody had a sensitivity of 68.3% and specificity of 58.3%. The same study found that an IgM MAC-ELISAs are quite sensitive during early convalescence infection (~94%) and high specificity however sensitivity wanes over time. Although this may be relevant for symptomatic diagnostics, this is less practical for use in surveillance settings. A more accurate alternative is an NS1 blockade-of-binding (BOB) ELISA that was found to have a high specificity (>90%) while being able to maintain a high sensitivity into late convalescence making it the most optimal for surveillance *(20)*. This assay uses NS1 as the antigenic target and a labeled anti-NS-1 mAb (ZKA 35) as a detection probe *(12)*. Despite this, a confirmatory PRNT is currently

recommended by the CDC and WHO for ZIKV diagnosis, which is much more expensive and labor intensive *(13, 21)*.

Because ZIKV and DENV have structural homology, there is great interest in understanding consequences of heterologous infection between ZIKV and DENV. Secondary DENV infections result in a more potent cross-reactive ZIKV neutralizing antibody immune profile compared to primary DENV infection *(17)*. In fact, some mAbs isolated from secondary dengue infection were potently neutralizing to both DENV and ZIKV by binding quaternary epitopes *(22)*. However, there is *in vitro* evidence of DENV antibodies could enhance ZIKV infection in mice *(23)*. Despite this, prior DENV immunity has not been associated with enhanced ZIKV infection in large observational epidemiologic human population studies *(24, 25)*. Moreover, a pediatric cohort study found that people with previous ZIKV infection were more likely to a symptomatic severe DENV 2 infection during the a large outbreak in the region *(26)*. In summary, there is potential for infection enhancement in patients with a DENV infection with a history of ZIKV infection. However, the enhancement is asymmetric, in patients with ZIKV infection, previous DENV infection does not appear to enhance ZIKV infection and in some cases appears to be protective. Understanding these nuances is critical given that nearly all ZIKV endemic places are also DENV endemic *(27)*. Thus, clearly deciphering what constitutes a protective ZIKV immune profile and how it may modify a person's risk for other future severe flavivirus infections is a critical consideration when establishing strategies for vaccine development.

Features of natural immunity

The E protein contains the predominant epitope targets for flavivirus neutralizing antibodies *(22)*. The fusion loop, which is a well-conserved region of E critical for host cell

infection, is a common target of antibodies that are cross-reactive among flaviviruses. Binding to E protein domain three (EDIII) has been found to be more virus specific *(11, 28)*. It is also believed that antibodies that bind to specific quaternary epitopes, which may include amino acid residues over multiple E protein monomers and are only present on the intact virion, are important to establishing protection *(22)*. This concept was clearly demonstrated in one study that examined neutralizing activity of late convalescent plasma from 4 people following primary ZIKV infection. Depleting all antibodies that bound to ZIKV E protein monomers had resulted in small to no reduction in neutralizing antibody titers, whereas depleting plasma with ZIKV virus like particles (VLP) nearly completely abolished neutralizing activity *(29)*. This point may be critical to understanding of correlates of protection for natural immunity that will be key for effective vaccine design and development. Non-structural protein 1 (NS1) is also an antigenic target of ZIKV protective neutralizing antibodies *(31)* Antibodies to ZIKV NS1 are generally virus-specific and have been a target for serologic diagnostics previously mentioned *(12, 31)*.

Vaccine development and challenges

Essentially every vaccine platform is being attempted to develop a safe and effective ZIKV vaccine. These include DNA, RNA, recombinant protein, live attenuated, inactivated, and live and non-live vector vaccines. Many of these vaccines specifically use ZIKV prM/E protein as immunologic targets including most advanced stage DNA vaccines, and a whole virus inactivated vaccine. *(33, 34)*.

The unsustained transmission of ZIKV globally makes it difficult to conduct prospective clinical trials *(35)*. Challenge studies have also been considered but face some ethical resistance and it is unclear how efficacy in animal models translate to efficacy in humans, thus making it difficult to acquire the necessary data for vaccine efficacy evaluation and approval. Mouse

models do not replicate the clinical disease seen in humans but have been helpful in vaccine challenge studies using viremia as a primary endpoint. Additionally, some candidates have also been evaluated in non-human primate (NHP), using FRNT50 titer higher than 1:100 as a correlate for protection as it has resulted in no detectable viremia post-challenge *(36)*. However, NHPs do not experience the same clinical features of ZIKV infection compared to humans so generalizing these results to humans. Additionally, studies of two different DNA vaccine candidates (VRC 5288 and VRC 5283) that code for the ZIKV E protein found that although the two produced similar neutralization titers in humans, plasma from recipients of one was significantly more protective against ZIKV challenge in a murine model. Further investigation revealed that this may be due to inefficient prM cleavage by the vaccine-induced VLPs, as nAbs to mature E protein virion have been shown to be most dominant in serum from patients with previous ZIKV infection *(37)*. Other sources have noted that residual prM may complicate inactivated virus vaccine immunogenicity by creating a target for non-neutralizing antibodies *(22)*.These results indicate that simple measures of neutralizing antibody levels may not be reliable measures of immunity and that better understanding the quality or specific characteristics of neutralizing antibodies is required to define robust correlates of protection for Zika

While the NS-1 BOB ELISA performs well as a diagnostic test, its sensitivity may be limited over time *(38)*. In addition to diagnosing infection, simple serologic assays that measure correlates of immunity are critical and currently lacking for epidemiologic purposes and vaccine development. Antibodies that neutralize ZIKV by binding to epitopes on E are the most likely target for such assays; the role for ZIKV-specific anti-NS1 antibodies in immunity to ZIKV is unclear. Furthermore, several vaccine candidates do not include NS1 antigen; thus, the NS1 BOB assay would not have a role in assessing vaccination status, though it could potentially be used to

differentiate ZIKV immunity derived from natural infection versus vaccination. Clear, convenient correlates of protection are needed both to understand natural immunity, as well as support vaccine design, development, and efficacy evaluation.

A9E and G9E monoclonal antibodies and their potential use in a ZE-BOB ELISA format

A9E and G9E are two potently neutralizing mAbs isolated from the same patient who had a primary ZIKV infection after travel in Salvador, Bahia Brazil in 2015 *(12)*. Both mAbs are ZIKV-specific and recognize unique quaternary epitopes on the ZIKV E protein. In competition BOB ELISA assays, six different flavivirus cross-reactive mAbs were found to not interfere with A9E and G9E blockade signal. EDE1 mAbs showed partial blockade to G9E, and ZKA 190 strongly blocked A9E signal. In an assessment of a small sample DENV and ZIKV immune sera, most ZIKV immune serum samples exhibited strong blockade to A9E and G9E. Most DENV-positive samples exhibited distinctively less blockade signal then the ZIKV- positive samples, and the few with exceptionally high signal were samples collected during early convalescence when cross-reactive antibodies are at their highest *(29)*.

Moreover, due to their specificity and potent neutralizing activity, A9E and G9E are excellent candidates for use as a tool for diagnostics, surveillance and vaccine development. There are more than 50 ZIKV candidate vaccines using ZIKV E protein as the primary antigenic target, and thus correlates of sterilizing immunity to E protein are needed to be able to evaluate vaccine immunogenicity at all stages of vaccine development *(15)*.

Chapter II: Manuscript

Introduction

The 2015-2016 epidemic that spanned the Americas led to thousands of newborns diagnosed with congenital Zika syndrome *(39)*. In Brazil alone there were over 200,000 probable cases and over 8,600 babies born with congenital malformations *(39)*. Since the 2016 epidemic, global incidence of ZIKV infection has fallen dramatically *(39, 40)*. However, it is estimated that billions of people remain at risk for ZIKV infection based on lack of immunity and geographical distribution and environmental suitability of the primary mosquito vector *Aedes aegypti (41)*. Although approximately 75% of cases are inapparent, the population risk of Guillain-Barré syndrome and the extensive risk to pregnant women and their unborn fetuses remain a pressing global public health threat *(7)*. Due to low and short-lived viremia, ZIKV is challenging to detect solely by molecular testing *(7)*. However, due to extensive cross-reactivity observed for antibodies elicited by ZIKV and other flaviviruses (most notably the four dengue virus (DENV) serotypes), traditional serologic diagnostics such as ELISAs often fail to differentiate between them, creating complications for diagnostic and surveillance testing, particularly where DENV and ZIKV co-circulate *(7, 42)*.

ZIKV encodes 3 structural (C,E, prM) and 7 non-structural proteins *(43)*. The surface of mature virions comprises 90 E protein dimers. Each E protein monomer consists of three domains (EDI, EDII, EDIII). EDIII of ZIKV is an antigenic target for ZIKV-specific antibodies *(11, 18)*. However, studies in ZIKV-infected patients have found that DI, DII and quaternary epitopes are also important antigenic targets *(15)*. Quaternary epitopes that bind to ZIKV envelope protein appear to be important targets for humoral immunity resulting from natural infection *(29)*.

There were over 50 vaccine candidates by 2018, but progress stagnated when ZIKV transmission dramatically decreased globally, precluding large vaccine efficacy trials. Alternative approaches to evaluating and approving ZIKV vaccines, such as human challenge studies, have not been met with unified support *(15)*. Neutralizing antibodies as measured by plaque (or focus) reduction neutralization testing (P(F)RNT) are likely key components of a ZIKV-protective immunity *(37, 44)*. These assays are labor and reagent intensive that require the handling of infectious virus, making it a less than ideal assay to conduct in low-resource settings *(7)*. Moreover, there is evidence for both ZIKV and DENV, that commonly used neutralizing assays may not account for important properties of neutralizing antibodies that impact protective efficacy *(37, 45)*.

A9E and G9E are two potently neutralizing monoclonal antibodies isolated from a patient following primary ZIKV infection acquired in Bahia, Brazil in 2015 *(29)*. Both mAbs are ZIKV-specific and recognize distinct quaternary epitopes on the ZIKV envelope (E) protein. G9E binds across the E dimer (Adams, *et al.*, under review) similar to other described monoclonals (Z20, Z-117 and ZIKV-195) *(46–48)*. A9E exhibits binding inhibition in the presence of ZIKA-190, indicating a binding region involving or near to the lateral ridge of EDII, or the EDI/III linker region. It should be noted that the lateral ridge has been found to be an important target of DENV E protein as well*(49)*. Both mAbs exhibited protection against lethal WT challenge in an immunocompromised mouse model. *(29)*.

A9E and G9E were used in a blockade of binding (BOB) ELISA format. A similar strategy has been employed using ZIKV NS1 antigen and ZKA 35 as the probe and is a sensitive and specific test for past ZIKV infection *(12)*. We are interested in understanding if the same

can be applied to E-protein specific monoclonals to see if they may serve as correlates of protection for both natural and vaccine induced immunity.

Here we use a well-defined sample set with known flavivirus immune profiles to assess both A9E and G9E ZE-BOB assay performance as a serodiagnostic test and surveillance tool, with the ultimate goal of detecting previous ZIKV infection with minimal cross-reactivity to primary and secondary dengue infections.

Materials and Methods

Human serum and plasma samples

Most samples analyzed in this study were archived specimens previously collected under IRB approved studies led by collaborators. These deidentified samples were made available for testing under a standard lab protocol. Sample sources are briefly described below and in Table 1:

TWS (Emory IRB# 103363) Immunocompetent adults seen in pre-travel consultation prior to international travel were recruited at Emory's TravelWell Center. Pre and post-travel sera were assessed DENV- or ZIKV-reactive IgG by antigen capture ELISA as previously described *(29)*. IgG+ samples were tested further by neutralization assays to confirm infection and determine the infecting virus(es).

AVE and ZIKA pilot (Emory IRB# 110683). Serum samples were collected from adults in the Atlanta area with a suspected past or acute emerging infection. Participants with confirmed ZIKV infection were recruited to donate longitudinal convalescent samples at the Emory Hope

Clinic. Furthermore, serum from a subset of participants who came in for unrelated infection and confirmed to be flavivirus naïve were used as negative controls in analysis.

ArboTrav (UNC IRB# 08–0895). Serum was collected from North Carolina residents with suspicion for flavivirus infection based on symptoms and recent travel history. Specimen were serologically characterized by virus-capture ELISA and FRNT*(24, 33)*.

ZIKA TS (UNAN-León Acta 37, 2016 and UNC IRB#16-0541). Serial serum specimens were collected in León, Nicaragua during the 2016 Zika epidemic from people presenting to local health centers with fever or rash illness as part of a prospective cohort study. Serum samples were collected during acute illness and 2, 3, 4, 8, 12, and 24 weeks post symptom onset. Subjects were tested for ZIKV RT-PCR performed on the presentation sample (acute) and by paired acute and convalescent serology testing for ZIKV and DENV IgM and IgG.

YFV vax study (ZIKV -) (Clinical Trial: NCT00254826). Serum was collected from participants 18-40 in a randomized, double blind clinical trial comparing the efficacy of the yellow fever vaccine with the vaccine administered with human immunoglobulin. Participants were recruited from the metro Atlanta area with no history of travel to yellow fever endemic areas. Serum was collected at days 5, 11, 30 and 91 days post vaccine administration *(51)*.

Colombia AIP. Serum was collected from mothers age 18-43 in Risaralda, Colombia between November 2017 and June 2019 on the day they gave birth. Samples were tested for DENV and ZIKV immunity by antigen capture IgG ELISA and eFRNT.

VRC320 (Clinical Trial NCT02996461). Serum samples were acquired from a phase 1, randomized clinical trial for the VRC 320 ZIKV DNA vaccine candidate. Participants were healthy adults 18-50 and were recruited at the NIH Clinical Center. They received 3 doses of VRC5283 on weeks 0,4 and 8. The participants were split into three groups, each with a different vaccine delivery scheme *(52)*.

Lab Experiments

Production and labeling of 9E monoclonal probes. A9E and G9E were isolated from PBMCs of a participant in the UNC Dengue Travelers cohort using a process previously described*(29)*. A9E and G9E mabs were labeled with alkaline phosphatase for use in the BOB ELISA assay using the LYNX Rapid Alkaline Phosphatase Antibody Conjugation Kit® (LNK012AP).

Antigen capture IgG ELISA. Levels of ZIKV-binding IgG were measured using a virus-capture ELISA. ZIKV was captured with plates coated with 4G2 monoclonal antibody. Plates were blocked with 3% nonfat milk-TBS-T. IgG were detected by measuring optical density (OD) at 405nm after incubation with a *p*-nitrophenyl phosphate. This protocol was also modified by capturing Z-EDIII instead of whole virus to detect Zika EDIII specific IgG *(11)*. For the EDIII assay, 3% nonfat milk-TBS-T was used as a blocking buffer and the plate was coated with streptavidin instead of 4G2.

ZIKV envelop protein-blockade of binding (ZE-BOB) assay. The Blockade of Binding assay performed has been previously described *(29)*. ZIKV was captured using monoclonal antibody 4G2. Plates were blocked with 3% nonfat milk in PBS-T. Different dilutions of antigen, serum

and labeled monoclonal probe and the following protocol was set from those results (Fig 1). Sera were plated in duplicate at a 1:10 dilution and incubated at 4°C overnight. 10ng/well of alkaline phosphate conjugated G9E or A9E was added to each well and incubated shaking for one hour at room temperature. *p*-Nitrophenyl phosphate was added, and OD was measured at 405nm. Percentage of blockade of binding was calculated using the following equation: (100 − [OD of sample/optical density of control]) × 100. Controls consisted of two naïve serum specimen that were consistent plate to plate. Furthermore, this assay was adapted from methods used to conduct NS-1 BOB assays *(12)*.

Focus forming neutralization testing (FRNT.) FRNT was performed as previously described and modified as detailed below for some experiments *(29)*. In brief, neutralization IC50 titers were determined using a 96-wee mico FRNT format. Titers were determined using the sigmodal dose response equation in PRISM Graphpad 8.4.3.

estimated FRNT (eFRNT), The FRNT assay was abbreviated to increase testing throughput and has been previously described *(53, 54)*. Four 4-fold serial dilution (1:20, 1:80, 1:320, 1:1280) were assessed. FRNT50 values were assigned to the most dilute sample with neutralization greater than 50% compared to the negative control.

Statistical Analyses

ROC curve analysis. The A9E and G9E ZE-BOB assays were validated by ROC curve analysis using Prism GraphPad 8.4.3. Positive sera came from ZIKV-confirmed participants from studies conducted in ZIKV endemic countries as well as from travelers in the United States. The negative sample set came from participants in studies that were designated as negative based on immune profile and/or travel history.

Linear regression. Simple linear regression was used to assess the association between ZE-BOB (% blockade compared to naïve control) and neutralization titers determined by either eFRNT or FRNT to better understand the qualities of the ZE-BOB activity and how they are correlated with these assays. GraphPad 8.4.3. was used to conduct the analysis. R^2 and F-test p-values were reported when appropriate.

T-test. An unpaired t-test was used to assess if there was a difference in ZE-BOB response in DENV-naïve and DENV immune individuals. The two-tailed p-value was reported and post-hoc power analysis was conducted.

ANOVA. Brown-Forsythe and Welch ANOVA tests were used to test the differences between ZE-BOB activity in the three vaccine delivery groups in the VRC320 trial. Dunnett's multiple comparison tests were used to do pairwise comparisons between groups

Results

ZE-BOB Assay Optimization and Validation

A9E and ZE-BOB assays were optimized using a known positive and negative ZIKV controls. After varying antigen, serum, labeled probe dilution level, the protocol was set and used consistently throughout (Fig 1). The A9E and G9E ZE-BOB assays were validated using serum from persons with confirmed recent ZIKV infection, including those with (n=14) and without (n=17) prior DENV infection. Negative controls also came from healthy travelers (n=24) or travelers with a history of monotypic (n=9) or polytypic DENV infection (n=12). Additionally, sera from a cohort of flavi-naïve participants who had received the YLFV vaccine (n=44) as part

of a randomized control trial were included in the validation set of negative controls. ZIKV positive samples consistently showed more reactivity than all other subgroups (Fig 2 A-B). The average mean BOB difference between the ZIKV positive controls and ZIKV negative controls for the A9E BOB ELISA was 51.1% and was 56.3% for the G9E BOB ELISA. Two polytypic DENV positive cases showed cross-reactivity in A9E BOB. Both reported having no symptoms associated with dengue in the last 20 years. Naïve and YFV-vaccinated specimen showed very little ZE-BOB activity. These data were used in receiver operating characteristic (ROC) curve analyses to determine the optimal positivity threshold for the two ZE-BOB assays (Fig 2 C-F) (AUC A9E ELISA = 0.992, AUC G9E ELISA =1.00). The first timepoint after 30 days post symptom onset (DPSO) was selected for ROC curve analysis if a participant donated samples at multiple timepoints. The optimal cutoffs were 25.75% and 17.58% blockade for the A9E and G9E ZE-BOB ELISA respectively. At the optimal cutoff, the A9E BOB ELISA has a sensitivity of 93.5% (95% CI: 79.3,98.9) and specificity 97.8 (95% CI: 92.2,99.6). The G9E BOB ELISA has a sensitivity and specificity of 100% (95% CI: 89.0,100.0) and 100% (95% CI: 95.9,100).

Characteristics of the A9E and G9E response

We examined the durability of the A9E and G9E blockade response using a subset of longitudinal samples from travelers with confirmed ZIKV infection from the ZIKV pilot study (Table 1). Although there is individual variability in the magnitude of ZE-BOB signal, BOB activity for A9E or G9E is detectable in the majority (7/8) of people in late convalescence (200-300 days post symptom onset, DPSO (Fig 3 A-D). Signal is increasing or stable for 4/8 samples in A9E BOB, with the other 4 exhibiting waning signals, to the point of seroreversion in 2 samples. For G9E BOB, 6/8 samples exhibit increased to stable signal and only 1 seroreversion of the two samples with waning signal. Interestingly, Zika1 seroreverted for both assays (Figure 4 A,C). Although, there is some waning in that participant's FRNT IC50 over time (data not

shown), it is not enough to explain why seroreversion is seen in both assays, and is likely do to individual heterogeneity in immune response.

Next, we were interested in knowing if A9E and G9E responses differed in ZIKV-positive persons who were DENV-naïve compared to ZIKV-positive persons who were DENV-immune (Fig 4 A-B). Samples from the ZIKV Pilot Cohort (n=4) and in the ZIKV TS Nicaragua cohort (n=14) that were collected between five- and seven-months post-symptom onset were used for this analysis. There was no significant difference detected in the A9E (p=0.25) and G9E (p=0.51) response of ZIKV positive specimen that are DENV naïve vs DENV immune using an unpaired two sample t-test.

Colombian Mother Surveillance Cohort

We were interested in understanding the performance of the ZE-BOB assays in an endemic surveillance context where previous infection status was not so precisely defined preemptively. The ZE-BOB assays were applied to a cross-sectional surveillance cohort study based in Risaralda, Colombia. There was a significant, although weak association between ZIKV eFRNT50 and both ZE-BOB ELISA responses using simple linear regression (A9E:R^2=0.12, p=0.0003, G9E: R^2=0.24, p<0.0001, Fig 5A-B).

ZIKV positivity was assigned for any samples with an eFRNT50 greater than or equal to 1:200. DENV was assessed the same way but was further differentiated to monotypic or polytypic. A sample was assigned as monotypic if no other DENV eFRNT50 was within 4-fold of the highest eFRNT 50 serotypes, otherwise it was assigned as polytypic. The ZE-BOB response was assessed between ZIKV positive and negative samples stratified by DENV status (Fig 5C-D, Table 2). The A9E and G9E ELISA false positivity rates were 14% and 34% respectively when using eFRNT results as the gold standard (Table 2). Specificity was higher when meeting both the A9E and G9E threshold was required for positivity (Specificity = 90.4%).

The specificities that were lower than estimated during validation are likely do to the high levels of polytypic DENV (49%) among those who were ZIKV- in the cohort compared to 12.4% in the validation sample set. Sensitivity of the assay in the Colombia cohort also ranged between 66% and 81% depending on positivity criteria, which is lower than what was observed in the validation sample set(Table 2). This may be explained if misclassification of ZIKV positives occurred due to DENV cross-reactive neutralizing antibodies.

VRC 320 Phase 1 Clinical Trial Recipients

Immunity induced by natural infection provides the gold standard benchmark for assessing vaccine-induced immunity. To determine whether vaccination with a PrM/E-encoding ZIKV DNA vaccine elicited A9E and G9E-competing Abs seen in most after natural infection, we tested sera from the VRC 320 Phase I vaccine trial *(52)*. The vaccine candidate used the VRC5283 ZIKV wildtype plasmid. Samples from 30 days post-vaccination had only modest ZE-BOB activity (Fig 6A-B). Only 5 samples had A9E or G9E BOB activity above the positivity threshold and only one specimen was positive for both A9E and G9E BOB (Fig 6 B-C). Furthermore, when grouped by delivery system, both A9E and G9E ZE-BOB assays had similar patterns compared to neutralization activity seen by the Collins lab and by the results published by trial team (Fig 6C-D) *(52)*. Group 1 (G1) received a full dose (4mg) in 1 deltoid by needle at each timepoint , Group 2 (G2) received split doses (2mg) at each timepoint by needle in syringe in both deltoids and group 3 (G3) received split doses (2mg) one by needle and syringe and one by a needle-free device at each timepoint. A Dunnet's D3 multiple comparisons test found there to only be a significant FRNT IC 50 titer difference between G1 and G3 ($p = 0.0001$) (Table 3). Likewise, there was only a statistically significantly A9E-BOB activity between G1 and G3 ($p=0.04$) (Table 3). The groups did not have significantly different G9E-BOB activity (Table 3). Furthermore, ZE-BOB results were compared to ZIKV FRNT50 results (Table 3). A sample's

percent blockade for A9E or G9E BOB assay showed no association with neutralization activity (A9E:R^2=0.04, p=0.17, G9E: R^2=0.03, p=0.26, Fig 6B-C). An ED3 antigen ELISA was also conducted to compare the reponse to neutralization and ZE-BOB activity. ED3 OD_{405} and the A9E response were found to not be significantly correlated (R^2=0.06, p=0.21, Fig 6E). ED3 OD_{405} was significantly but weakly correlated with the G9E BOB response (R^2=0.17, p=0.006, Fig 6F). FRNT IC50 titers were then compared to the ZIKV ED3 ELISA response. These were found to be significantly, but weakly associated (R^2=0.11, p=0.03) (Fig 6G).

Discussion

The ZIKV epidemic of 2015-2016 exposed profound public health shortcomings, particularly in the ability to protect pregnant women and children from emerging infectious diseases. Five years later, the threat of future ZIKV outbreaks looms large, and there are still no licensed antivirals, vaccines or other proven methods of preventing ZIKV infection. To advance work to meet critical public health goals for Zika, we aimed to develop and apply novel serologic tools based on two strongly neutralizing, ZIKV-specific mAbs to increase understanding of human immunity to ZIKV infection in natural infection and vaccination.

A9E and G9E were isolated from PBMCs of one ZIKV+ patient infected during travel to Bahia, Brazil. The two monoclonals were well characterized *in vitro* and found to be protective in one mouse challenge experiment *(29)*. ROC analysis in a well-defined samples indicated that both assays were sensitive and specific(Fig 2). Further, both assays had AUC values above 0.99. When applying the optimized ZE-BOB assays to a population level, we find A9E and G9E-like responses are present in the vast majority of ZIKV+ sample tested and these responses consistently persist into late convalescence (>6-12 months post infection) (Fig 2-3). This indicates that A9E and G9E reactivity are a public, immunodominant response. Furthermore, this

BOB ELISA platform framework shall be applied to other human ZIKV neutralizing mAbs to understand the landscape of important immunologic targets that provide protection to natural infection and can guide vaccine development and evaluation. Additionally, a serologic correlate of protection is greatly lacking and urgently needed. Although neutralizing antibody titers are the typical outcome measure in immunogenicity studies, there is evidence that the quality, not only the quantity of nAbs is important to establish protection *(37)*. More investigation in animal model challenge studies is needed to understand if ZE-BOB ELISAs could fill this gap.

Defining ZIKV-specific antibody responses could also facilitate the development of novel diagnostic assays. Traditional serologic diagnostic tools are complicated by other flavivirus antibody cross-reactivity, particularly DENV, which is endemic in essentially everywhere ZIKV has emerged *(3, 7)*. Some tools have been developed to fill this gap including the NS1 BOB ELISA *(12)*. The A9E and G9E ZE-BOB is distinct because it is based on detecting antibody responses that react to the ZIKV surface protein via epitopes known to be targets of neutralizing antibodies. Thus, ZE-BOB may simultaneously convey diagnostic information while also reflecting a relevant immunologic state. The A9E and G9E ZE-BOB assays has high sensitivity and specificity in the validation sample set (Fig 2). After applying the assay to a surveillance cohort of pregnant women in Colombia, we found that the sensitivities and specificities of the ZE-BOB assay when using ZIKV eFRNT as the gold standard were lower than the estimates we saw in the validation sample set (Table 2), demonstrating that the assay may not be as accurate when applied to certain sample sets. This is likely a result of high levels of polytypic DENV immune profiles in places where ZIKV is endemic, such as in Risaralda, Colombia. Sensitivity was also lower however; this may be because of specimen being misclassified as ZIKV positive because of polytypic DENV that can lead to ZIKV eFRNT IC50

> 1:200. Moreover, the assays may still be useful in clinical decision-making among pregnant women in US traveler settings when previous travel history can rule out a secondary DENV infection.

This analysis had several limitations. First, the validation sample set contained only 11 polytypic DENV cases (12.4%) among the negative controls. Because this group was the most likely to show ZE-BOB cross-reactivity, and endemic regions have high levels of DENV seroprevalence, it is important to have a larger sample of polytypic DENV cases to gain a better understanding of how the assay behaves in this group. Additionally, due to small sample sizes in some sub-analyses, some tests were underpowered to detect differences between groups. When comparing BOB activity between DENV naïve and DENV immune persons who were ZIKV positive, post-hoc power analysis yielded a power of 22% for the unpaired t-test for the A9E BOB ELISA and 8.9% for the G9E BOB. Moreover, although using eFRNT screens is a more efficient way to characterize serum samples, it may lead to some misclassification since it is not perfectly correlated with FRNT IC50s.

In summary, this work demonstrates the diverse utility of a novel serologic tool measuring ZIKV-specific antibody responses and provides further insight into the quality of humoral immunity elicited by ZIKV infection and vaccination. The findings and approaches in this study serve as a proof-of-principle of a conceptual, technical and analytic framework that paves the way for future work including the further characterization and application of other ZIKV-specific neutralizing mAbs as well as the application of ZE-BOB assays to samples from recipients of other vaccine candidates. This approach will also be useful in studying other emerging infectious diseases since the need for accurate diagnostics, efficient surveillance tools and effective vaccines holds true for all infectious diseases.

Figures

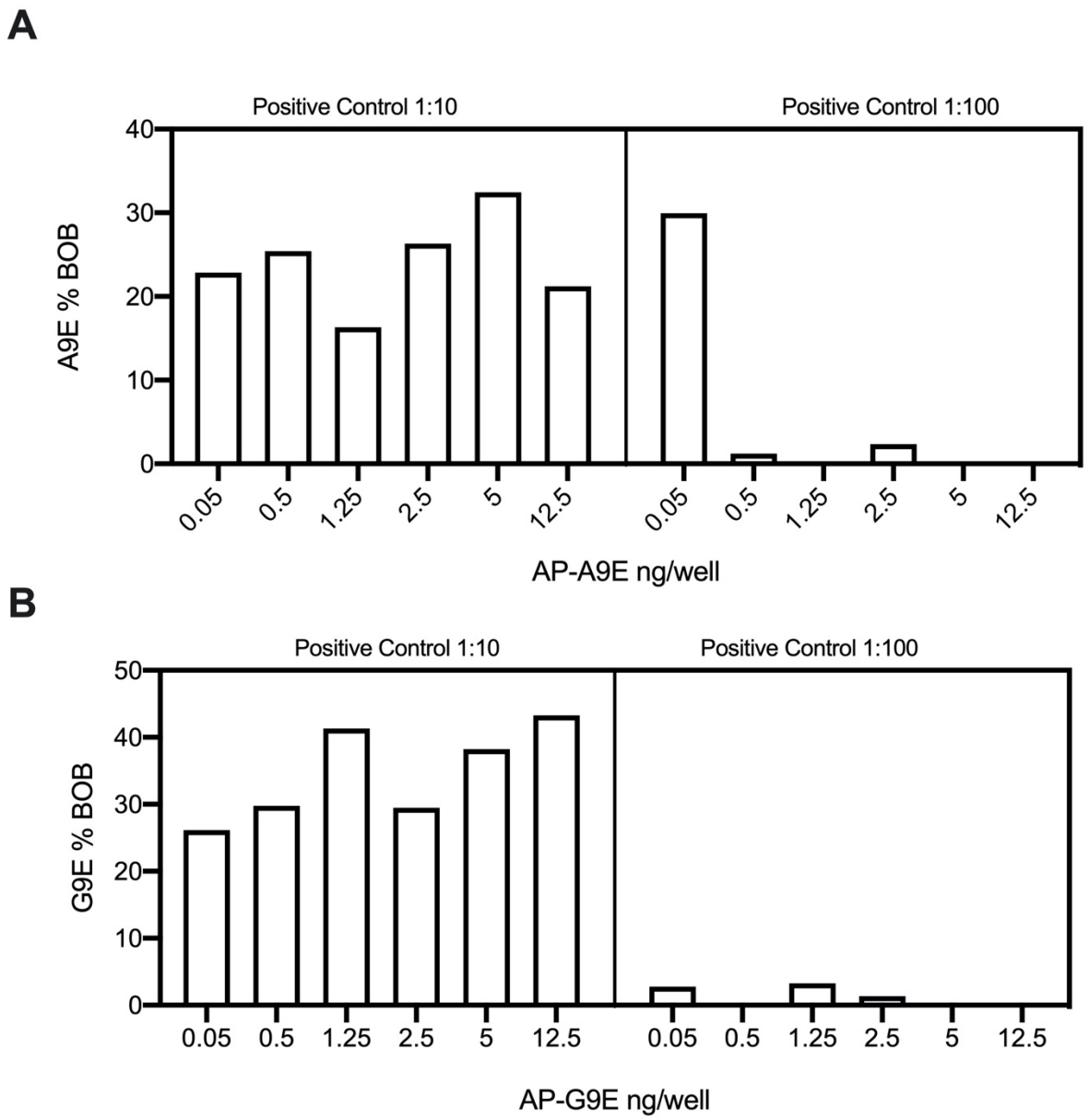

Figure 1 – A9E (A) and G9E (B) BOB ELISA results varying labeled mAb and serum dilutions. The percent blockade of binding BOB was reported for each.

Table 1. Human Serum Specimens			
Study Source	**Number of subjects**	**Total number of specimens**	**Characteristics**
Used for ROC Curve Analysis			
ArboTrav	34	34	Samples from 9 ZIKV+, 9 primary DENV, 11 polytypic DENV, and 5 naïve with and without flavivirus vaccine exposure
ZIKV TS	14	14	Samples 28 DPSO and 6 months DPSO of subjects with PCR confirmed ZIKV infection in Nicaragua
ZIKV Pilot	8	29*	Specimen from confirmed ZIKV infection in US travelers, longitudinal samples range 5-740 days post symptom onset
YFV vax	44	44	Serum specimens collected following YFV vaccination in flavivirus-naïve subjects
TWS	18	18	Serum from adults seen in pre-travel consultation at the Emory TravelWell Clinic prior to international travel that were determined to be flavi-naïve
AVE	2	2	Serum samples from adults in the Atlanta area with a suspected past or acute emerging infection. Both these participants tested negative for DENV and ZIKV and and the average OD readings for these samples were used as plate controls to calculate % BOB.
Surveillance and Vaccine Cohorts			
Colombia AIP	102	102	Cross-sectional sample set of recent mothers from a flavivirus-endemic area in Colombia
VRC320	43	43	Subjects in the VRC 320 RCT who received PrM/E DNA vaccine – 30 days post vaccination

*Table 1. Description of sample sets used for ROC analysis and cohorts to which the assays were applied. *Only 1 timepoint was used for each participant in the ZILV pilot study.*

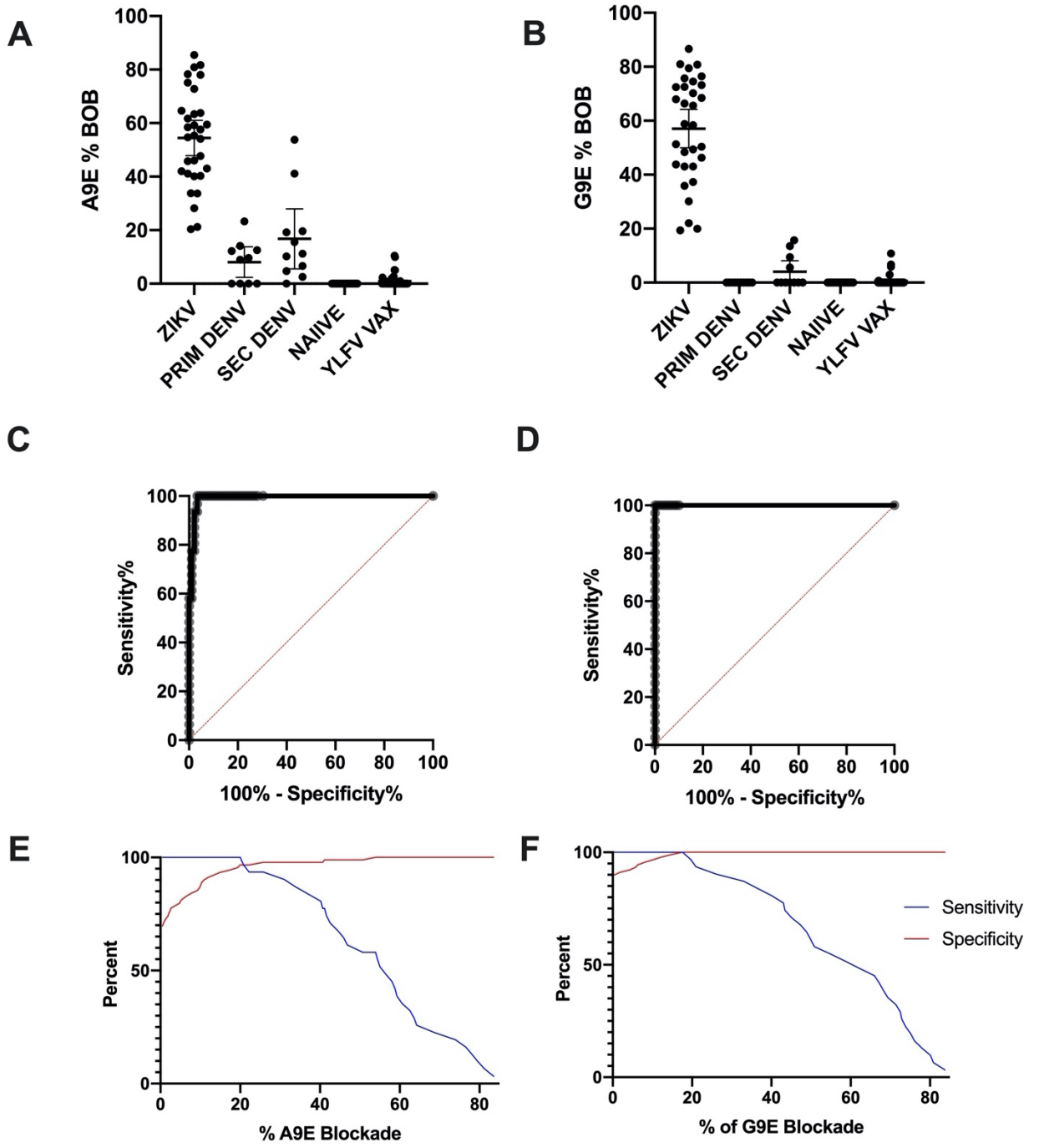

Figure 2. A9E and G9E BOB results in validation sample set. (A-B) Percent A9E and G9E blockade grouped by flavi-immune status. (C-D) ROC curves depicting results from samples in validation set (AUC = 0.992 for A9E BOB ELISA and AUC = 1.0 for G9E BOB ELISA). (E-F) Sensitivity and specificity and plotted at % blockade cutoff points based on ROC analysis results.

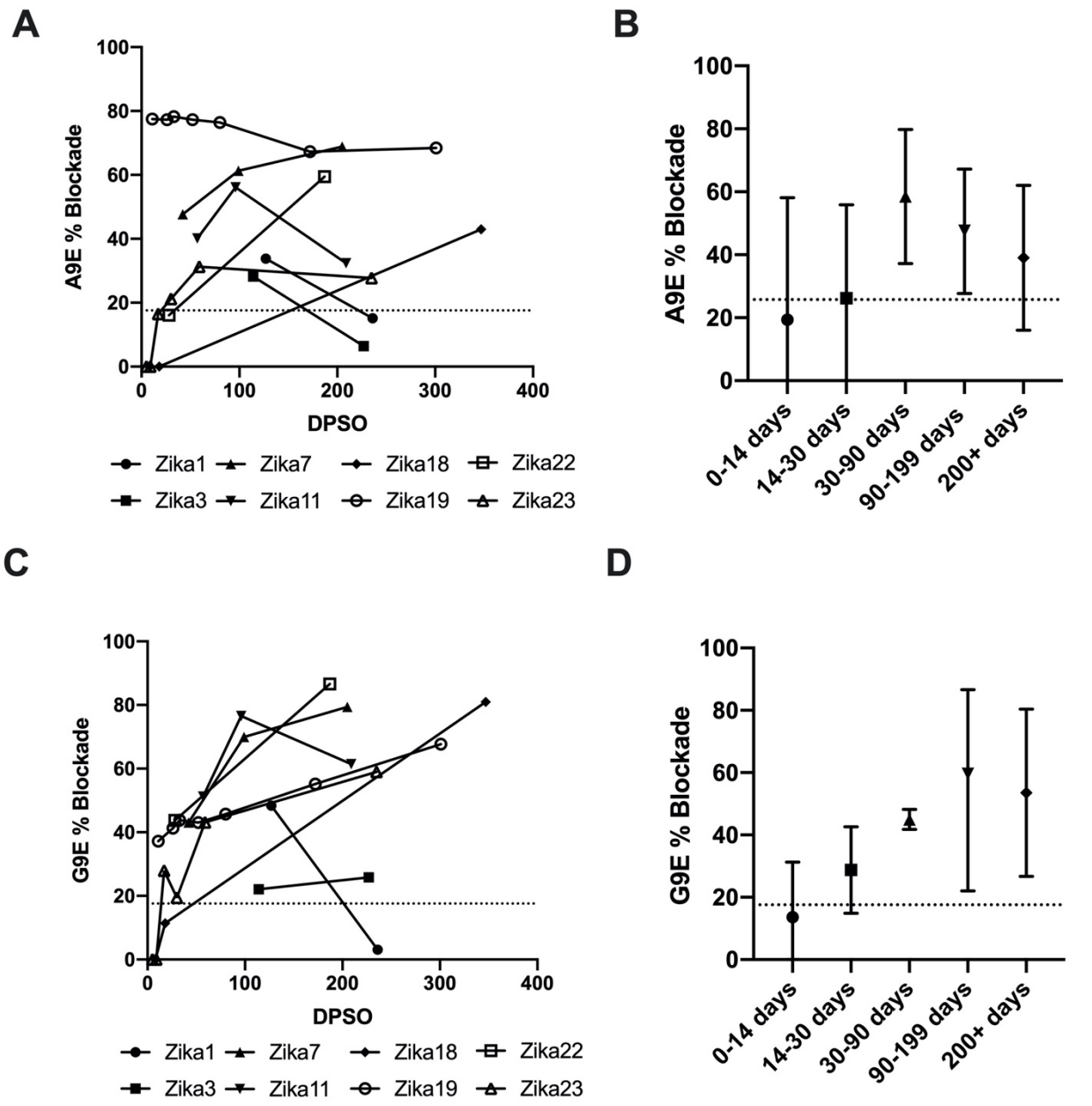

Figure 3. Longitudinal analysis of A9E BOB and G9E BOB durability. All samples in this analysis are from the ZIKV pilot study. (A) % A9E BOB and (C) % G9E BOB reported by days post symptom onset (DPSO). (B) %A9E BOB and (D) %G9E BOB for the same samples summarized by DPSO groupings. Error bars represent +/- the standard deviation of the mean.

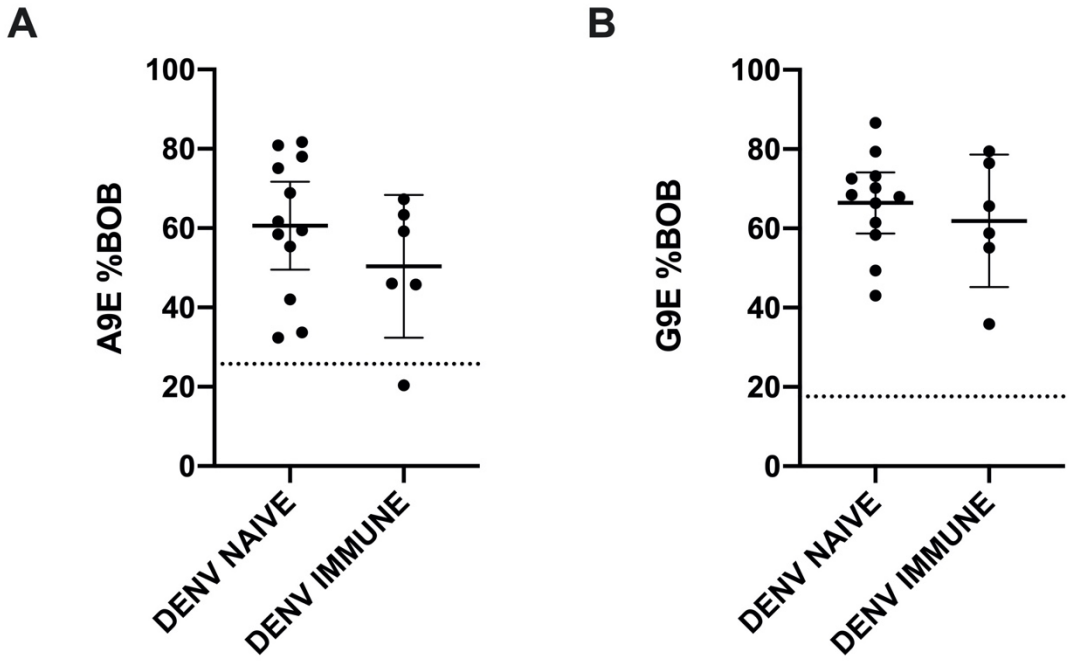

Figure 4. (A) % A9E and (B) % G9E BOB for DENV naïve and immune participants among those that were ZIKV+. Only samples from Zika Pilot and ZIKV TS samples were used and all samples had a DPSO between 7-9 months. Error bars represent the 95% CI of the mean. An unpaired t-test comparing the DENV naïve group to the DENV immune group yielded p-values of 0.25 and 0.50 for a A9E and G9E respectively.

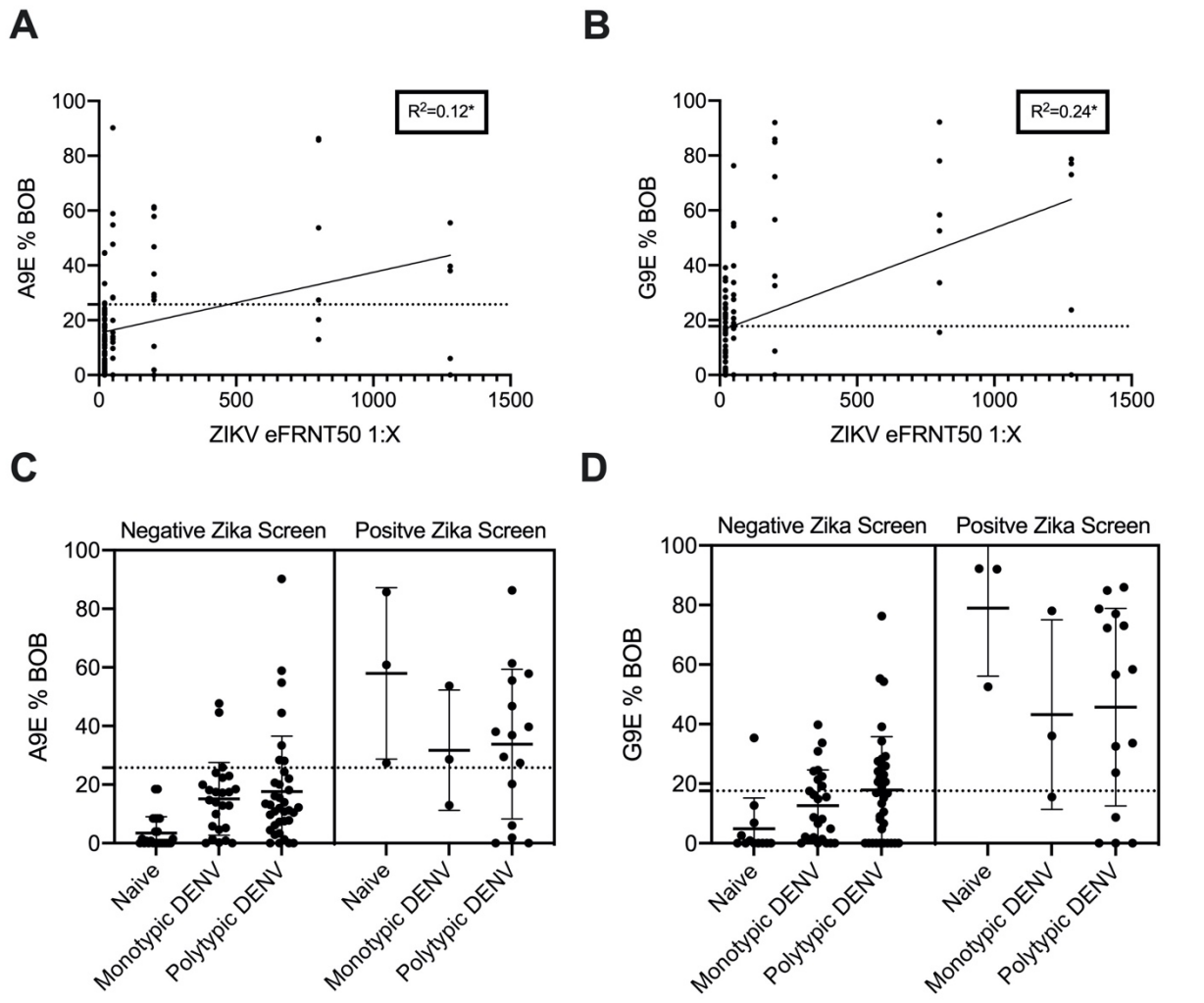

Figure 5. Application of the A9E and G9E assays to the Colombia AP sample set (n=102). (A) Correlation between ZIKV eFRNT50 and %A9E BOB and (B) % G9E BOB for the Colombia AIP samples. R-squared values are reported and significant correlation at an alpha level of 0.05 is stared. (C) %A9E BOB and (D) % G9E BOB grouped by ZIKV and DENV eFRNT classification. Dotted lines represent positivity threshold determined by ROC analysis and error bars +/- the standard deviation around the mean.

ZIKV Screen Status	DENV Screen Status	% A9E BOB Positive	% G9E BOB Positive	% A9E AND G9E BOB Positive	% A9E OR G9E BOB Positive
ZIKV Negative	Naïve	0% (0/12)	8.3% (1/12)	0% (0/12)	8.3% (1/12)
	Monotypic DENV	12% (3/25)	32% (8/25)	8% (2/25)	36% (9/25)
	Polytypic DENV	19% (7/36)	44% (16/36)	14% (5/36)	50% (18/36)
	Total	14% (10/73)	34% (25/73)	9% (7/73)	38% 28/73
ZIKV Positive	Naïve	100% (3/3)	100% (3/3)	100% (3/3)	100% (3/3)
	Monotypic DENV	66% (2/3)	66% (2/3)	66% (2/3)	66% (2/3)
	Polytypic DENV	66% (10/15)	73% (11/15)	60% (9/15)	80% (12/15)
	Total	71% (15/21)	76% (16/21)	66% (14/21)	81% (17/21)

Table 1. % A9E BOB ELISA positivity rate by ZIKV and DENV eFRNT classification based on thresholds set by previous ROC analysis.

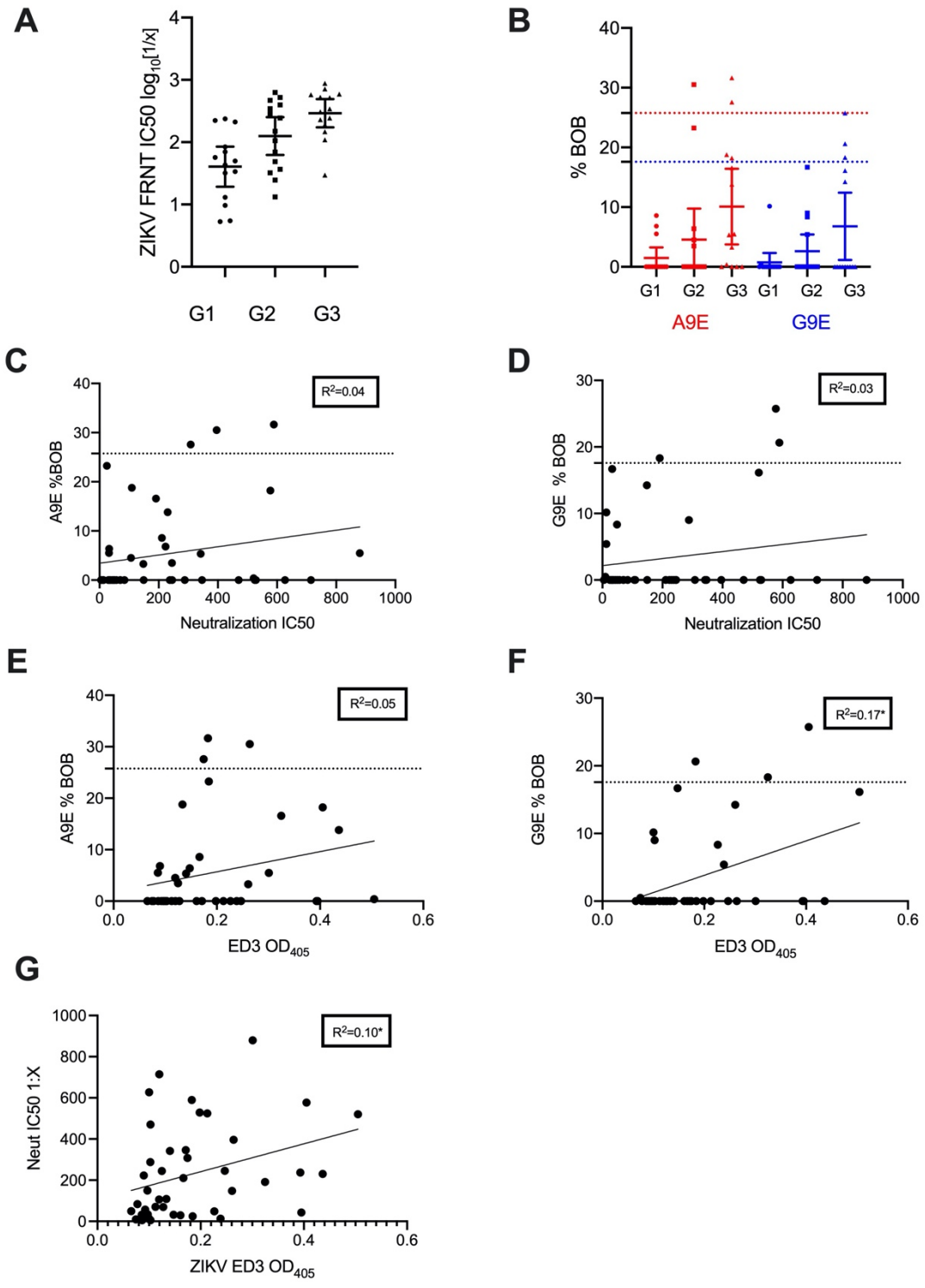

Figure 4. (A-B)FRNT IC50, A93 % BOB, and G9E %BOB for the three groups in the VRC320 ZIKV DNA vaccine trial. Groups were compared using a Dunnett's T3 multiple comparisons test (Table 3). (C) Correlation between A9E % BOB and (C) G9E % BOB with FRNT IC50. Dotted linen indicates positivity threshold as determined by ROC analysis (E-F) Correlation between ZIKV EDII ELISA OD_{405} and A9E and G9E % BOB. (G) Correlation between ZIKV EDIII ELISA OD_{405} and FRNT IC50. R-square values are reported and significance at an alpha of 0.05 are starred. Dotted lines represent positivity threshold for each assay.

Dunnet's T3 Multiple Comparisons Test: VRC320 ZIKV FRNT, mean differences (adj p-value)			
	Group 1	Group 2	Group 3
Group 1		-148.5 (0.053)	-309.9 (0.001)*
Group 2			-161.5 (0.19)

Dunnet's T3 Multiple Comparisons Test: A9E BOB ELISA , mean %BOB differences (adj p-value)			
	Group 1	Group 2	Group 3
Group 1		-3.05 (0.56)	-8.59 (0.037)*
Group 2			-5.54 (0.39)

Dunnet's T3 Multiple Comparisons Test: G9E BOB ELISA , mean %BOB differences (adj p-value)			
	Group 1	Group 2	Group 3
Group 1		-1.87 (0.51)	-6.03 (0.12)
Group 2			-5.54 (0.42)

Table 2. Dunnet's multiple comparison tests for VRC320 ZIKV candidate vaccine groups. Mean difference between groups and the adjusted p-value are reported for each comparison. Comparisons were done between groups for FRNT IC50, %A9E BOB and % G9E BOB.

Chapter III: Public Health Implications

Although current global ZIKV incidence is low, both the 2015-2016 Zika epidemic and the 2020-2021 COVID-19 pandemic have shown us how quickly a pathogen can go from unknown to an issue of global proportions. We must be prepared if and when ZIKV reemerges, particularly to prevent against the harms of congenital Zika syndrome. To achieve this, an effective vaccine, safe for pregnant women is urgently needed, and we will need clear correlates of protection to expedite vaccine development efforts. Serologic competition ELISAs using known potently neutralizing mAbs are an attractive and leading option for this purpose.

Exploring the nuances of the A9E and G9E ZE-BOB response have advanced our understanding of host-pathogen interactions. Studies such as those presented here have and will continue to give us insight on the qualities of protective antibody immunity to ZIKV, how exposure to related flaviviruses may affect ZIKV host-pathogen interactions and the most promising approaches for Zika vaccine development. It appears that A9E and G9E ZE-BOB assays may not be sensitive and specific enough to justify development as a broadly applicable serodiagnostic test for clinical purposes. However, A9E and G9E may still prove useful in the evaluation of vaccine candidates with ZIKV E protein as the primary antigenic target and be a valuable adjunctive serologic tool in translational and epidemiologic research. Importantly, the concepts and methods developed in this work focused on A9E and G9E provide a critical framework for additional research. This approach can be readily applied to other mAbs to develop diagnostics and assays measuring correlates of protection for ZIKV, or for similar work to combat other emerging viral pathogens.

References

1. G. W. A. Dick, Zika virus. II. Pathogenicity and physical properties, *Trans R Soc Trop Med Hyg* **46**, 521–534 (1952).

2. F. N. MacNamara, Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria, *Transactions of the Royal Society of Tropical Medicine and Hygiene* **48**, 139–145 (1954).

3. T. C. Pierson, M. S. Diamond, The continued threat of emerging flaviviruses, *Nature Microbiology* **5**, 796–812 (2020).

4. S. Bhatt, P. W. Gething, O. J. Brady, J. P. Messina, A. W. Farlow, C. L. Moyes, J. M. Drake, J. S. Brownstein, A. G. Hoen, O. Sankoh, M. F. Myers, D. B. George, T. Jaenisch, G. R. W. Wint, C. P. Simmons, T. W. Scott, J. J. Farrar, S. I. Hay, The global distribution and burden of dengue, *Nature* **496**, 504–507 (2013).

5. S. Ali, O. Gugliemini, S. Harber, A. Harrison, L. Houle, J. Ivory, S. Kersten, R. Khan, J. Kim, C. LeBoa, E. Nez-Whitfield, J. O'Marr, E. Rothenberg, R. M. Segnitz, S. Sila, A. Verwillow, M. Vogt, A. Yang, E. A. Mordecai, Environmental and Social Change Drive the Explosive Emergence of Zika Virus in the Americas, *PLoS Negl Trop Dis* **11**, e0005135 (2017).

6. C. Zanluca, V. C. A. de Melo, A. L. P. Mosimann, G. I. V. D. Santos, C. N. D. D. Santos, K. Luz, First report of autochthonous transmission of Zika virus in Brazil, *Mem Inst Oswaldo Cruz* **110**, 569–572 (2015).

7. L. R. Petersen, D. J. Jamieson, A. M. Powers, M. A. Honein, Zika Virus, *N Engl J Med* **374**, 1552–1563 (2016).

8. A. S. Siraj, T. A. Perkins, Assessing the population at risk of Zika virus in Asia – is the emergency really over?, *BMJ Global Health* **2**, e000309 (2017).

9. E. R. Krow-Lucal, B. J. Biggerstaff, J. E. Staples, Estimated Incubation Period for Zika Virus Disease - Volume 23, Number 5—May 2017 - Emerging Infectious Diseases journal - CDC, , doi:10.3201/eid2305.161715.

10. Guillain-Barré Syndrome | Campylobacter | CDC (2019) (available at http://www.cdc.gov/campylobacter/guillain-barre.html).

11. L. Premkumar, M. Collins, S. Graham, G.-J. A. Liou, C. A. Lopez, R. Jadi, A. Balmaseda, J. A. Brackbill, R. Dietze, E. Camacho, A. D. De Silva, C. Giuberti, H. L. dos Reis, T. Singh, H. Heimsath, D. Weiskopf, A. Sette, J. E. Osorio, S. R. Permar, M. J. Miley, H. M. Lazear, E. Harris, A. M. de Silva, Development of Envelope Protein Antigens To Serologically Differentiate Zika Virus Infection from Dengue Virus Infection, *J Clin Microbiol* **56** (2018), doi:10.1128/JCM.01504-17.

12. A. Balmaseda, K. Stettler, R. Medialdea-Carrera, D. Collado, X. Jin, J. V. Zambrana, S. Jaconi, E. Cameroni, S. Saborio, F. Rovida, E. Percivalle, S. Ijaz, S. Dicks, I. Ushiro-Lumb, L. Barzon, P. Siqueira, D. W. G. Brown, F. Baldanti, R. Tedder, M. Zambon, A. M. B. de Filippis, E. Harris, D. Corti, Antibody-based assay discriminates Zika virus infection from other flaviviruses, *Proc. Natl. Acad. Sci. U.S.A.* **114**, 8384–8389 (2017).

13. CDC, Zika Virus*CDC* (2019) (available at http://www.cdc.gov/zika/laboratories/types-of-tests.html).

14. F. X. Heinz, K. Stiasny, The Antigenic Structure of Zika Virus and Its Relation to Other Flaviviruses: Implications for Infection and Immunoprophylaxis, *Microbiol. Mol. Biol. Rev.* **81** (2017), doi:10.1128/MMBR.00055-16.

15. H.-H. Lin, B.-S. Yip, L.-M. Huang, S.-C. Wu, Zika virus structural biology and progress in vaccine development, *Biotechnology Advances* **36**, 47–53 (2018).

16. R. Peters, M. Stevenson, Zika virus diagnosis: challenges and solutions, *Clin Microbiol Infect* **25**, 142–146 (2019).

17. P. Andrade, P. Narvekar, M. Montoya, D. Michlmayr, A. Balmaseda, J. Coloma, E. Harris, Primary and Secondary Dengue Virus Infections Elicit Similar Memory B-Cell Responses, but Breadth to Other Serotypes and Cross-Reactivity to Zika Virus Is Higher in Secondary Dengue, *J Infect Dis* **222**, 590–600 (2020).

18. K. Stettler, M. Beltramello, D. A. Espinosa, V. Graham, A. Cassotta, S. Bianchi, F. Vanzetta, A. Minola, S. Jaconi, F. Mele, M. Foglierini, M. Pedotti, L. Simonelli, S. Dowall, B. Atkinson, E. Percivalle, C. P. Simmons, L. Varani, J. Blum, F. Baldanti, E. Cameroni, R. Hewson, E. Harris, A. Lanzavecchia, F. Sallusto, D. Corti, Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection, *Science* **353**, 823–826 (2016).

19. M. Montoya, M. Collins, W. Dejnirattisai, L. C. Katzelnick, H. Puerta-Guardo, R. Jadi, S. Schildhauer, P. Supasa, S. Vasanawathana, P. Malasit, J. Mongkolsapaya, A. D. de Silva, H. Tissera, A. Balmaseda, G. Screaton, A. M. de Silva, E. Harris, Longitudinal Analysis of Antibody Cross-neutralization Following Zika Virus and Dengue Virus Infection in Asia and the Americas, *The Journal of Infectious Diseases* **218**, 536–545 (2018).

20. A. Balmaseda, J. V. Zambrana, D. Collado, N. García, S. Saborío, D. Elizondo, J. C. Mercado, K. Gonzalez, C. Cerpas, A. Nuñez, D. Corti, J. J. Waggoner, G. Kuan, R. Burger-Calderon, E. Harris, Comparison of Four Serological Methods and Two Reverse Transcription-PCR Assays for Diagnosis and Surveillance of Zika Virus Infection, *J Clin Microbiol* **56** (2018), doi:10.1128/JCM.01785-17.

21. WHO, Laboratory testing for Zika virus infection (2016) (available at https://www.who.int/publications/i/item/laboratory-testing-for-zika-virus-infection).

22. V. Dussupt, K. Modjarrad, S. J. Krebs, Landscape of Monoclonal Antibodies Targeting Zika and Dengue: Therapeutic Solutions and Critical Insights for Vaccine Development, *Front Immunol* **11** (2021), doi:10.3389/fimmu.2020.621043.

23. S. V. Bardina, P. Bunduc, S. Tripathi, J. Duehr, J. J. Frere, J. A. Brown, R. Nachbagauer, G. A. Foster, D. Krysztof, D. Tortorella, S. L. Stramer, A. García-Sastre, F. Krammer, J. K. Lim, Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity, *Science* **356**, 175–180 (2017).

24. A. C. B. Terzian, A. S. Schanoski, M. T. de O. Mota, R. A. da Silva, C. F. Estofolete, T. E. Colombo, P. Rahal, K. A. Hanley, N. Vasilakis, J. Kalil, M. L. Nogueira, Viral Load and Cytokine Response Profile Does Not Support Antibody-Dependent Enhancement in Dengue-Primed Zika Virus-Infected Patients, *Clin Infect Dis* **65**, 1260–1265 (2017).

25. D. Michlmayr, E.-Y. Kim, A. H. Rahman, R. Raghunathan, S. Kim-Schulze, Y. Che, S. Kalayci, Z. H. Gümüş, G. Kuan, A. Balmaseda, A. Kasarskis, S. M. Wolinsky, M. Suaréz-Fariñas, E. Harris, Comprehensive Immunoprofiling of Pediatric Zika Reveals Key Role for Monocytes in the Acute Phase and No Effect of Prior Dengue Virus Infection, *Cell Reports* **31**, 107569 (2020).

26. A. Gordon, L. Gresh, S. Ojeda, L. C. Katzelnick, N. Sanchez, J. C. Mercado, G. Chowell, B. Lopez, D. Elizondo, J. Coloma, R. Burger-Calderon, G. Kuan, A. Balmaseda, E. Harris, Prior dengue virus infection and risk of Zika: A pediatric cohort in Nicaragua, *PLoS Med* **16** (2019), doi:10.1371/journal.pmed.1002726.

27. L. C. Katzelnick, S. Bos, E. Harris, Protective and enhancing interactions among dengue viruses 1-4 and Zika virus, *Current Opinion in Virology* **43**, 59–70 (2020).

28. H. Zhao, L. Xu, R. Bombardi, R. Nargi, Z. Deng, J. M. Errico, C. A. Nelson, K. A. Dowd, T. C. Pierson, J. E. Crowe, M. S. Diamond, D. H. Fremont, Mechanism of differential Zika and dengue virus neutralization by a public antibody lineage targeting the DIII lateral ridge, *J Exp Med* **217** (2020), doi:10.1084/jem.20191792.

29. M. H. Collins, H. A. Tu, C. Gimblet-Ochieng, G.-J. A. Liou, R. S. Jadi, S. W. Metz, A. Thomas, B. D. McElvany, E. Davidson, B. J. Doranz, Y. Reyes, N. M. Bowman, S. Becker-Dreps, F. Bucardo, H. M. Lazear, S. A. Diehl, A. M. de Silva, Human antibody response to Zika targets type-specific quaternary structure epitopes, *JCI Insight* **4** (2019), doi:10.1172/jci.insight.124588.

30. M. H. Collins, E. McGowan, R. Jadi, E. Young, C. A. Lopez, R. S. Baric, H. M. Lazear, A. M. de Silva, Lack of Durable Cross-Neutralizing Antibodies Against Zika Virus from Dengue Virus Infection, *Emerg Infect Dis* **23**, 773–781 (2017).

31. M. J. Bailey, F. Broecker, J. Duehr, F. Arumemi, F. Krammer, P. Palese, G. S. Tan, Antibodies Elicited by an NS1-Based Vaccine Protect Mice against Zika Virus, *mBio* **10** (2019), doi:10.1128/mBio.02861-18.

32. C. Shan, X. Xie, P.-Y. Shi, Zika Virus Vaccine: Progress and Challenges, *Cell Host & Microbe* **24**, 12–17 (2018).

33. S. J. Thomas, A. Barrett, Zika vaccine pre-clinical and clinical data review with perspectives on the future development, *Human Vaccines & Immunotherapeutics* **16**, 2524–2536 (2020).

34. R. A. Larocca, P. Abbink, J. P. S. Peron, P. M. de A. Zanotto, M. J. Iampietro, A. Badamchi-Zadeh, M. Boyd, D. Ng'ang'a, M. Kirilova, R. Nityanandam, N. B. Mercado, Z. Li, E. T. Moseley, C. A. Bricault, E. N. Borducchi, P. B. Giglio, D. Jetton, G. Neubauer, J. P. Nkolola, L. F. Maxfield, R. A. De La Barrera, R. G. Jarman, K. H. Eckels, N. L. Michael, S. J. Thomas, D. H. Barouch, Vaccine protection against Zika virus from Brazil, *Nature* **536**, 474–478 (2016).

35. K. S. Vannice, M. C. Cassetti, R. W. Eisinger, J. Hombach, I. Knezevic, H. D. Marston, A. Wilder-Smith, M. Cavaleri, P. R. Krause, Demonstrating vaccine effectiveness during a waning epidemic: A WHO/NIH meeting report on approaches to development and licensure of Zika vaccine candidates, *Vaccine* **37**, 863–868 (2019).

36. G. Young, K. J. Bohning, M. Zahralban-Steele, G. Hather, S. Tadepalli, K. Mickey, C. S. Godin, S. Sanisetty, S. Sonnberg, H. K. Patel, H. J. Dean, Complete Protection in Macaques Conferred by Purified Inactivated Zika Vaccine: Defining a Correlate of Protection, *Scientific Reports* **10**, 3488 (2020).

37. S. Maciejewski, T. J. Ruckwardt, K. M. Morabito, B. M. Foreman, K. E. Burgomaster, D. N. Gordon, R. S. Pelc, C. R. DeMaso, S.-Y. Ko, B. E. Fisher, E. S. Yang, D. Nair, K. E. Foulds, J. P. Todd, W.-P. Kong, V. Roy, M. Aleshnick, S. D. Speer, N. Bourne, A. D. Barrett, M. C. Nason, M. Roederer, M. R. Gaudinski, G. L. Chen, K. A. Dowd, J. E. Ledgerwood, G. Alter, J. R. Mascola, B. S. Graham, T. C. Pierson, Distinct neutralizing antibody correlates of protection among related Zika virus vaccines identify a role for antibody quality, *Science Translational Medicine* **12** (2020), doi:10.1126/scitranslmed.aaw9066.

38. E. J. M. Nascimento, M. I. Bonaparte, P. Luo, T. S. Vincent, B. Hu, J. K. George, G. Áñez, F. Noriega, L. Zheng, J. W. Huleatt, Use of a Blockade-of-Binding ELISA and Microneutralization Assay to Evaluate Zika Virus Serostatus in Dengue-Endemic Areas, *Am J Trop Med Hyg* **101**, 708–715 (2019).

39. Zika: the continuing threat, *Bull World Health Organ* **97**, 6–7 (2019).

40. M. J. Siedner, E. T. Ryan, I. I. Bogoch, Gone or forgotten? The rise and fall of Zika virus, *The Lancet Public Health* **3**, e109–e110 (2018).

41. J. P. Messina, M. U. Kraemer, O. J. Brady, D. M. Pigott, F. M. Shearer, D. J. Weiss, N. Golding, C. W. Ruktanonchai, P. W. Gething, E. Cohn, J. S. Brownstein, K. Khan, A. J. Tatem, T. Jaenisch, C. J. Murray, F. Marinho, T. W. Scott, S. I. Hay, M. Jit, Ed. Mapping global environmental suitability for Zika virus, *eLife* **5**, e15272 (2016).

42. L. Priyamvada, K. M. Quicke, W. H. Hudson, N. Onlamoon, J. Sewatanon, S. Edupuganti, K. Pattanapanyasat, K. Chokephaibulkit, M. J. Mulligan, P. C. Wilson, R. Ahmed, M. S. Suthar, J. Wrammert, Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus, *Proc Natl Acad Sci U S A* **113**, 7852–7857 (2016).

43. V. A. Kostyuchenko, E. X. Y. Lim, S. Zhang, G. Fibriansah, T.-S. Ng, J. S. G. Ooi, J. Shi, S.-M. Lok, Structure of the thermally stable Zika virus, *Nature* **533**, 425–428 (2016).

44. P. Abbink, R. A. Larocca, K. Visitsunthorn, M. Boyd, R. A. De La Barrera, G. D. Gromowski, M. Kirilova, R. Peterson, Z. Li, O. Nanayakkara, R. Nityanandam, N. B. Mercado, E. N. Borducchi, A. Chandrashekar, D. Jetton, S. Mojta, P. Gandhi, J. LeSuer, S. Khatiwada, M. G. Lewis, K. Modjarrad, R. G. Jarman, K. H. Eckels, S. J. Thomas, N. L. Michael, D. H. Barouch, Durability and Correlates of Vaccine Protection Against Zika Virus in Rhesus Monkeys, *Sci Transl Med* **9** (2017), doi:10.1126/scitranslmed.aao4163.

45. S. R. Hadinegoro, J. L. Arredondo-García, M. R. Capeding, C. Deseda, T. Chotpitayasunondh, R. Dietze, H. I. H. Muhammad Ismail, H. Reynales, K. Limkittikul, D. M. Rivera-Medina, H. N. Tran, A. Bouckenooghe, D. Chansinghakul, M. Cortés, K. Fanouillere, R. Forrat, C. Frago, S. Gailhardou, N. Jackson, F. Noriega, E. Plennevaux, T. A. Wartel, B. Zambrano, M. Saville, CYD-TDV Dengue Vaccine Working Group, Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease, *N Engl J Med* **373**, 1195–1206 (2015).

46. S. S. Hasan, A. Miller, G. Sapparapu, E. Fernandez, T. Klose, F. Long, A. Fokine, J. C. Porta, W. Jiang, M. S. Diamond, J. E. Crowe, R. J. Kuhn, M. G. Rossmann, A human antibody against Zika virus crosslinks the E protein to prevent infection, *Nat Commun* **8**, 14722 (2017).

47. F. Long, M. Doyle, E. Fernandez, A. S. Miller, T. Klose, M. Sevvana, A. Bryan, E. Davidson, B. J. Doranz, R. J. Kuhn, M. S. Diamond, J. E. Crowe, M. G. Rossmann, Structural basis of a potent human monoclonal antibody against Zika virus targeting a quaternary epitope, *PNAS* **116**, 1591–1596 (2019).

48. Q. Wang, H. Yang, X. Liu, L. Dai, T. Ma, J. Qi, G. Wong, R. Peng, S. Liu, J. Li, S. Li, J. Song, J. Liu, J. He, H. Yuan, Y. Xiong, Y. Liao, J. Li, J. Yang, Z. Tong, B. D. Griffin, Y. Bi, M. Liang, X. Xu, C. Qin, G. Cheng, X. Zhang, P. Wang, X. Qiu, G. Kobinger, Y. Shi, J. Yan, G. F. Gao, Molecular determinants of human neutralizing antibodies isolated from a patient infected with Zika virus, *Sci Transl Med* **8**, 369ra179 (2016).

49. R. de Alwis, S. A. Smith, N. P. Olivarez, W. B. Messer, J. P. Huynh, W. M. P. B. Wahala, L. J. White, M. S. Diamond, R. S. Baric, J. E. Crowe, A. M. de Silva, Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions, *PNAS* **109**, 7439–7444 (2012).

50. M. H. Collins, H. A. Tu, C. Gimblet-Ochieng, G.-J. A. Liou, R. S. Jadi, S. W. Metz, A. Thomas, B. D. McElvany, E. Davidson, B. J. Doranz, Y. Reyes, N. M. Bowman, S. Becker-Dreps, F. Bucardo, H.

M. Lazear, S. A. Diehl, A. M. de Silva, Human antibody response to Zika targets type-specific quaternary structure epitopes, *JCI Insight* **4** (2019), doi:10.1172/jci.insight.124588.

51. S. Edupuganti, R. B. Eidex, H. Keyserling, R. S. Akondy, R. Lanciotti, W. Orenstein, C. del Rio, Y. Pan, T. Querec, H. Lipman, A. Barrett, R. Ahmed, D. Teuwen, M. Cetron, M. J. Mulligan, YF-Ig Study Team, A randomized, double-blind, controlled trial of the 17D yellow fever virus vaccine given in combination with immune globulin or placebo: comparative viremia and immunogenicity, *Am J Trop Med Hyg* **88**, 172–177 (2013).

52. M. R. Gaudinski, K. V. Houser, K. M. Morabito, Z. Hu, G. Yamshchikov, R. S. Rothwell, N. Berkowitz, F. Mendoza, J. G. Saunders, L. Novik, C. S. Hendel, L. A. Holman, I. J. Gordon, J. H. Cox, S. Edupuganti, M. A. McArthur, N. G. Rouphael, K. E. Lyke, G. E. Cummings, S. Sitar, R. T. Bailer, B. M. Foreman, K. Burgomaster, R. S. Pelc, D. N. Gordon, C. R. DeMaso, K. A. Dowd, C. Laurencot, R. M. Schwartz, J. R. Mascola, B. S. Graham, T. C. Pierson, J. E. Ledgerwood, G. L. Chen, S. Plummer, P. Costner, K. Zephir, J. Casazza, A. Ola, M. Victorino, C. Levinson, W. Whalen, X. Wang, J. Cunningham, O. Vasilenko, M. B. Florez, S. Hickman, I. Pittman, L. Le, B. Larkin, C. Andrews, P. Apte, R. Hicks, C. T. Cartagena, P. Williams, C. R. Boyd, M. Conan-Cibotti, J. Stein, F. Kaltovich, H. DeCederfelt, S. McAdams, P. Renehan, W. Chen, N. Greenberg, N. Wymer, L. Wadsworth, M. Billington, T. Robinson, C. Boyce, F. P. Brown, L. Chrisley, A. Kwon, P. Patel, P. Kominou, B. Dorsey, S. Eddington, S. Telscher, M. Lee, R. Mosely, A. Ross, G. Ford, B. Domjahn, J. Xu, A. Beck, R. Fineman, S. Heeke, J. Winter, S. Nagar, C. Kelley, M. Mulligan, S. Plummer, P. Costner, K. Zephir, J. Casazza, A. Ola, M. Victorino, C. Levinson, W. Whalen, X. Wang, J. Cunningham, O. Vasilenko, M. B. Florez, S. Hickman, I. Pittman, L. Le, B. Larkin, C. Andrews, P. Apte, R. Hicks, C. T. Cartagena, P. Williams, C. R. Boyd, M. Conan-Cibotti, J. Stein, F. Kaltovich, H. DeCederfelt, S. McAdams, P. Renehan, Safety, tolerability, and immunogenicity of two Zika virus DNA vaccine candidates in healthy adults: randomised, open-label, phase 1 clinical trials, *The Lancet* **391**, 552–562 (2018).

53. A. C. Willcox, M. H. Collins, R. Jadi, C. Keeler, J. B. Parr, D. Mumba, M. Kashamuka, A. Tshefu, A. M. de Silva, S. R. Meshnick, Seroepidemiology of Dengue, Zika, and Yellow Fever Viruses among Children in the Democratic Republic of the Congo, *Am J Trop Med Hyg* **99**, 756–763 (2018).

54. M. H. Collins, O. Zepeda, B. Blette, R. Jadi, M. Morales, R. Pérez, G.-J. A. Liou, M. Montoya-Cruz, E. Harris, S. Becker-Dreps, A. M. de Silva, J. Stringer, F. Bucardo, E. Stringer, Serologic surveillance of maternal Zika infection in a prospective cohort in Leon, Nicaragua during the peak of the Zika epidemic, *PLoS One* **15**, e0230692 (2020).