EVALUATION OF IGG ANTIBODY PRODUCTION IN RHESUS MACAQUES CHALLENGED WITH DIFFERENT GEOGRAPHICAL ISOLATES OF ZIKA VIRUS

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ABSTRACT

Zika virus was declared a public health emergency after the 2015 outbreak in South America resulted in forty-nine deaths and thousands of cases of neurological impairments, such as microcephaly and Guillain-Barré syndrome. The virus has spread from Africa through Asia and into the Americas, with different presentations of disease reported at each destination. Discussed here is the IgG host response to infection with three isolates representative of each clade. Rhesus macaques infected with $1x10^4$, $1x10^5$ or $1x10^6$ PFU of virus displayed comparable titers 10 days after challenge with slightly higher values in macaques challenged with Asian and American isolates. Titers increase significantly fifteen and thirty days after challenge with Asian and American isolates, but the African virus failed to elicit a robust response, regardless of dose. Homozygous and heterozygous re-challenge with American or Asian virus provided an immediate memory-B cell response, while re-challenge with American virus in macaques recovered from African virus infection showed no evidence of protection.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADE	Antibody Dependent Enhancement
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATCC	American Type Cell Culture
°C	Degrees Celsius
CDC	Centers for Disease Control and Prevention
DB	Dumbbell
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
F	Female
FRNT	Foci Reduction Neutralization Test
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LLOQ	Lower Limit of Quantification
M	Male
MAC	IgM Antibody Capture
nAb	Neutralizing Antibody
NS	Non Structural
OD	Optical Density
PAHO	Pan American Health Organization

PBS	Phosphate Buffered Saline
PFU	Plaque Forming Units
PRNT	Plaque Reduction Neutralization Test
RNA	Ribonucleic Acid
SL	Stem Loop
SNP	Single Nucleotide Polymorphism
ULOQ	Upper Limit of Quantification
UTR	Untranslated Region
μL	Microliter
μg	Microgram
WHO	World Health Organization

INTRODUCTION

Zika virus and its associated diseases have been a growing area of public health concern in recent years. As of March of 2018, there have been 283 confirmed cases of Zika virus related birth defects including 17 losses of pregnancy in the United States or its surrounding territories (CDC 2018). From 2016 to 2017, an estimated 220,000 confirmed cases of Zika virus infection and 3,676 related congenital deformations have been reported in South America (PAHO 2017). Local and world health agencies issued guidelines to reduce transmission, including the World Health Organization's recommendation for high risk individuals to consider delaying pregnancy (Byron and Howard 2017). Aid contributions were substantial, including a 2016 bill passed by the U.S. Government for $1.1 billion to assist in response and preparedness efforts (Epstein and Lister 2016). A coalition of South American governments and private philanthropists contributed $18 million towards the deployment of a bacterium targeting *Aedes aegypti*, the primary mosquito vector of Zika virus, designed to prevent viral transmission from insect to human (U.S. Embassy 2016). Despite these measures, transmission is ongoing and an effective vaccine is not available (Sanchez 2017).

Zika virus was first isolated in April 1947 from a rhesus macaque suspended in the canopy of the Zika forest in Uganda (Dick *et al.* 1952). A year later, transmissible virus was isolated from an *Aedes afticanus* mosquito in the same region of Entebbe (Dick 1952). The first recorded detection of Zika virus in humans occurred in 1952. Neutralizing antibodies (nAb) specific for Zika virus were found in the serum of several East African natives at a ratio sufficient to suggest infection was not a rarity (Smithburn 1952). In 1964 a researcher working with Zika virus unknowingly self-inoculated himself. He developed

a maculopapular rash and increasing neutralizing antibodies to the virus in his serum (Simpson 1964). This was the first detailed case that Zika virus causes acute infection and disease in humans.

Throughout the 1950's to 1980's cases appeared sporadically, all of which conferred mild illness in patients, (Haddow *et al.* 2012). Two distinct lineages were identified and designated as African and Asian strains. The transverse of the virus between the two continents is believed to have coincidentally occurred around the same time as its discovery in the mid 1940's (Haddow *et al.* 2012). In 2007, an outbreak occurred on the Yap Islands in the Pacific with the previously thought to be benign Asian lineage virus. Forty nine confirmed, fifty nine probable, and seventy two suspected cases of Zika virus disease were identified within a three month period. Common symptoms included rash, fever, arthralgia, and conjunctivitis; all acute effects in children and adults, with no notice of fetal abnormalities (Duffy *et al.* 2009). This was the first instance of community wide infection in a previously naïve population. The virus went on to proliferate extensively in the region, causing outbreaks in French Polynesia, The Cook Islands, New Caledonia, and neighboring islands mounting over 8,000 suspected cases (Roth *et al.* 2014).

In 2014, the virus emerged in Central America. As incidences rose, reports in Brazil of neurological impairments, such as Guillain-Barré syndrome, increased with forty nine cases as of July 2015. By October, doctors began to see a significant increase in numbers of children born with microcephaly. The following month, an infant born in Brazil with microcephaly and significant abnormalities died minutes after birth. Zika virus was detected in the child's blood and tissues. This was the first link between Zika virus and birth abnormalities. Soon after, additional mortalities began to be reported and a third clade

began to emerge in the Americas (Faria *et al.* 2016). By February 2016, Brazil reported 3,893 cases of microcephaly, 1,708 cases Guillain-Barré syndrome, and 49 deaths (WHO 2017).

As aid began to funnel into the region, Zika virus related diseases begin to decrease and health agencies rescinded emergency declarations. In November 2016, the WHO released a statement confirming their commitment to anti-Zika virus efforts, but cancelled their state of emergency (WHO 2016). Cases began to appear elsewhere in the world, such as Florida, but didn't take hold and establish widespread disease, mostly in part to fewer urban locations for the mosquito larvae to hatch (Khawar *et al.*). As the epidemic in the Americas slowed, the threat of re-emergence began to materialize. While it isn't known what sequence variations contributed to the increased pathogenicity in select American lineage strains, the fact that the strains were capable of gaining such harmful mutations was sufficient enough to alarm responsible authorities of the potential of this virus.

Zika virus is a member of the family *Flavivirridae* and has a single stranded (+) sense non-segmented RNA genome. It encodes for a single 10.7 kb polypeptide comprised of seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, and three structural proteins called envelope, capsid, and pre/membrane. Both terminal untranslated regions (UTR) have substantial secondary structure. The 5' UTR consists of ~107 nucleotides that produce two stem loop structures, denoted SL-A and SL-B. SL-A is required for correct viral polymerase placement at the AUG start codon. SL-B is required for 3'end recognition, which contributes to efficient genome replication. The 3' UTR contains 428 nucleotides which form three stem loops (SL-1, SL-2, and terminal 3'-SL), one dumbbell (DB-1) and one pseudo dumbbell (psi-DB), all of which contribute to viral

infectivity. Viral NS5 transfers a 7-methyl guanosine cap to the viral genome's 5' end, enhancing recognition by host ribosomes and protecting it from antiviral exonuclease activity (Wang *et al*. 2017). The 3' terminal end lacks a poly-A tail, however the secondary structures inhibit 3' nuclease digestion, increasing genome stability (Göertz *et al.* 2017). The virion is composed of an icosahedral shell of envelope and pre/membrane proteins surrounding the capsid protein complexed genome (Sirohi *et al.* 2016).

The virus is transmitted by the *Aedes* species mosquito, predominantly *Ae. Aegypti* and *Ae. Albopictus,* as well as i*n utero,* and through sexual intercourse (Figure 1). The incubation period typically lasts from two to ten days, which is followed by a five to seven day course of disease. Humans are incidental hosts to the virus and typically cannot sustain levels of transmission without the vector (Boyer *et al.* 2018).

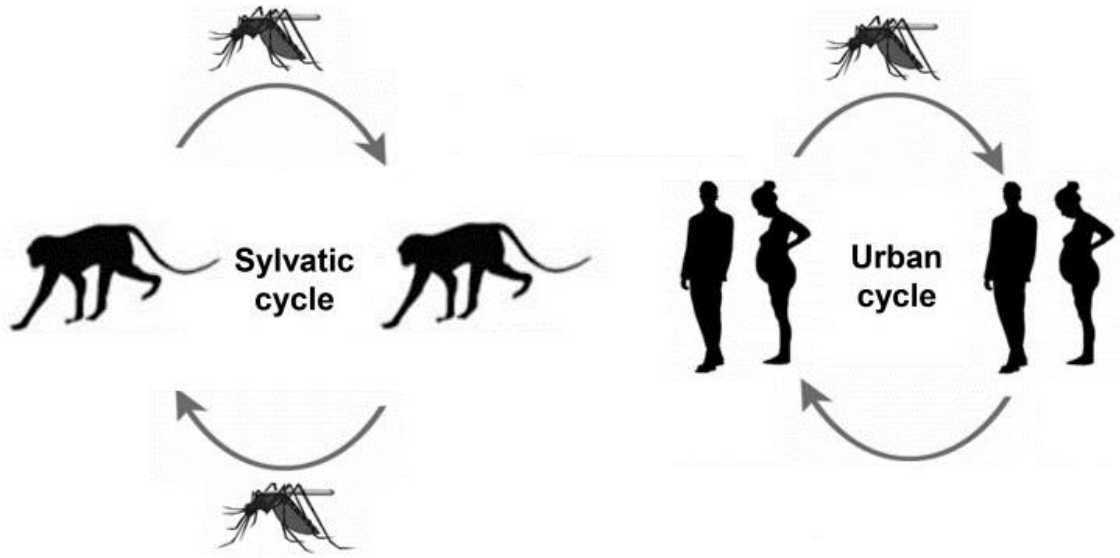

Figure 1: The Transmission cycle of Zika virus. The *Aedes* species mosquito is the primary natural vector and transmits the virus between reservoirs. Humans typically act as incidental hosts, although the virus can be spread to the fetus during pregnancy and through sexual intercourse. Image edited from graphic in Boyer *et al.* 2018.

Zika virus entry is mediated through interaction between viral envelope protein and host receptors, resulting in endocytosis of the virion into the cytoplasm. The low pH of the late endosome triggers a fusion event between the endosomal and viral membranes, releasing the RNA genome into the cytoplasm (Sharma and Lal 2017). Translation of viral RNA begins, promoting genome replication on the surface of the endoplasmic reticulum (ER). This results in an invagination on the ER surface forming regions of efficient genome production called viral factories (Cortese *et al*. 2017). Complete genomes are packaged into the ER to form immature virions, encapsulated by membrane impregnated with viral structural proteins. These particles travel through the golgi network, where furin proteases cleave pre/membrane protein causing conformational changes on the viral surface. The mature virion is then released from the cell through exocytosis (Sharma and Lal 2017).

As the exploration of host immunogenicity to Zika virus continues and vaccine candidates are evaluated, the need for a quantitative immunogenic assay becomes apparent. To evaluate such candidates, the host response to viral infection must be well-characterized in a model system. Rhesus macaques have been shown to recapitulate Zika virus disease in a semi-permissive manner and continue to be the best option to measure vaccine efficacy (Dudley *et al.* 2016). Herein is described an enzyme-linked immunosorbent assay (ELISA) for quantifying anti-Zika virus IgG antibodies in serum samples collected from rhesus macaques infected with Zika virus. Also described is a natural history study measuring anti-Zika virus IgG production in rhesus macaques challenged with three distinct geographical isolates of Zika virus, using the developed ELISA. After resolution of primary disease, animals are re-infected with the same, or different, virus, to see if antibodies capable of cross recognizing viruses of different lineage are produced.

MATERIALS AND METHODS

ELISA Optimization

This ELISA was optimized to detect anti-Zika virus IgG antibodies in non-human primate serum. The following variables were evaluated: plate binding affinity, coating conditions, coating antigen, coating antigen diluent, blocking buffer, sample dilution, secondary antibody dilution, and a method for quantification. Plate binding affinity was determined by comparing standard polystyrene plates to high binding affinity plates. Purified Zika virus lysate, recombinant envelope protein, and live virus were compared for use as coating antigens and diluted in either carbonate buffer or PBS. Blocking buffers evaluated included 5% dry milk in 0.05% Tween 20 and SuperBlock. Samples and secondary antibodies were titrated to obtain a sigmoidal range of values. Finally, data analysis softwares were reviewed for suitability.

Indirect ELISA

Purified Zika virus lysate (The Native Antigen Company, Oxfordshire, U.K.) was diluted to 0.5µg/mL in carbonate-bicarbonate buffer (Thermo Fisher Scientific, Waltham, MA). Lysate was prepared by culturing virus in Vero cells, purifying the supernatant via a sucrose gradient, lysing with detergent, and followed by heat inactivation. High binding MAXISORP™ 96-well plates (Thermo Fisher Scientific, Waltham, MA) were coated with 100µL of diluted antigen and incubated at 2-8°C overnight. Plates were washed five times with 0.05% Tween 20, then blocked for 30 minutes at 37°C using 5% dry milk (Quality Biological, Gaithersburg, MD). Plates were washed again, then 100µL of serially diluted sera samples were added to each well. Sera samples were diluted 1:100, then two-fold down the plate. Plates were incubated for 1 hour at 37°C, then washed. Secondary goat anti-human horse radish peroxidase (HRP)-conjugated IgG antibody (SeraCare, Milford,

MA) was diluted 1:2000 in 5% milk then added to all wells. Plates were again incubated at 37°C for 1 hour, then washed. Next, ABTS 1-component peroxidase substrate (SeraCare, Milford, MA) was added to each well in low light. Plates were covered and incubated at room temperature for 15 to 20 minutes, then 1% sodium dodecyl sulfate (Fisher Scientific, Hampton, NH) was added to each well. Plate absorbance optical density (OD) was read at 405nm on a SpectraMax i3 using Softmax Pro software (Sunnyvale, CA) within 15 minutes.

OD values were graphed against the log of the reciprocal of their dilution (ex. $Log_{10}100$, $Log_{10}12800$), and a line of best fit using 4 parameter non-linear regression was applied to the data using GraphPad Prism software (La Jolla, CA). Using the generated formula, data points between each dilution step were interpolated, expanding the quantifiable range from eight to one thousand values. Titers were calculated by locating an endpoint cut-off OD value on the line of best fit for each sample. The corresponding reciprocal dilution step of the identified OD value is the sample titer. If the cut off value is not located on the line of best fit, the sample titer falls outside of the quantifiable range. The quantifiable range is defined as any dilution reciprocal between or equal to 100 through 12,800. Values below or above will be denoted as less than the lower limit of quantification (<LLOQ) or greater than the upper limit of quantification (>ULOQ), respectively.

The endpoint cut-off value was defined as follows: more than twenty rhesus macaque serum samples were tested using commercially available kits for Zika Virus (XpressBio, Frederick, MD) and Dengue Virus (Calbiotech, El Cajon, CA and Abcam, Cambridge, MA). Sera testing negative for both viruses on all kits was tested using the newly developed ELISA, with the exception that samples were initially diluted, but not

titrated down the plate. OD values of the naïve sera were averaged and the cut-off point was calculated by adding two times the standard deviation of the OD value to the average OD value.

Natural History Study Phase I

Thirty-six male and female macaques were divided into nine groups each with 2 males (M) and 2 females (F). Each group was challenged with either $1x10^4$ plaque forming units (PFU), $1x10^5$ PFU, or $1x10^6$ PFU of one of three strains (Table 1).

Table 1. Group designations of rhesus macaques challenged with various doses of Zika viruses from distinct clades. Thirty six rhesus macaques distributed with two females and two males per group. Each group was challenged with $1x10^4$ PFU, $1x10^5$ PFU, or $1x10^6$ PFU of one of three Zika virus isolates.

Group Number	Animal Number	Isolate	Dose (PFU/animal)
1	4 (2M/2F)	PRVABC59	$1x10^4$
2	4 (2M/2F)	PRVABC59	$1x10^5$
3	4 (2M/2F)	PRVABC59	$1x10^6$
4	4 (2M/2F)	PLCal_ZV	$1x10^4$
5	4 (2M/2F)	PLCal_ZV	$1x10^5$
6	4 (2M/2F)	PLCal_ZV	$1x10^6$
7	4 (2M/2F)	IBH 30656	$1x10^4$
8	4 (2M/2F)	IBH 30656	$1x10^5$
9	4 (2M/2F)	IBH 30656	$1x10^6$

IBH_30656 (GenBank: HQ234500.1) is an African strain isolated in Nigeria in 1968 and shares significant homology to the first strain isolated from a human patient in 1954 (Haddow *et al.* 2012). PLCal_ZV (GenBank: KF993678.1) is an Asian strain similar to that which circulated during the French Polynesian outbreak (Pardy *et al.* 2017).

PRVABC59 (GenBank: KU501215.1) is an American strain isolated in Puerto Rico during the outbreak in the Americas and shares significant homology to the virus which affected Brazil in 2015-2016 (Figure 2). These viruses were grown and titrated in Vero cells (ATCC, Manassas VA), diluted to the appropriate concentration in virus production media (Thermo fisher Scientific, Waltham, MA) supplemented with 10% heat inactivated FBS (Sigma Aldrich, St. Louis, MO), then injected subcutaneously into the left volar forearm. Blood was collected and processed to serum for antibody titration on days 5, 15, and 30.

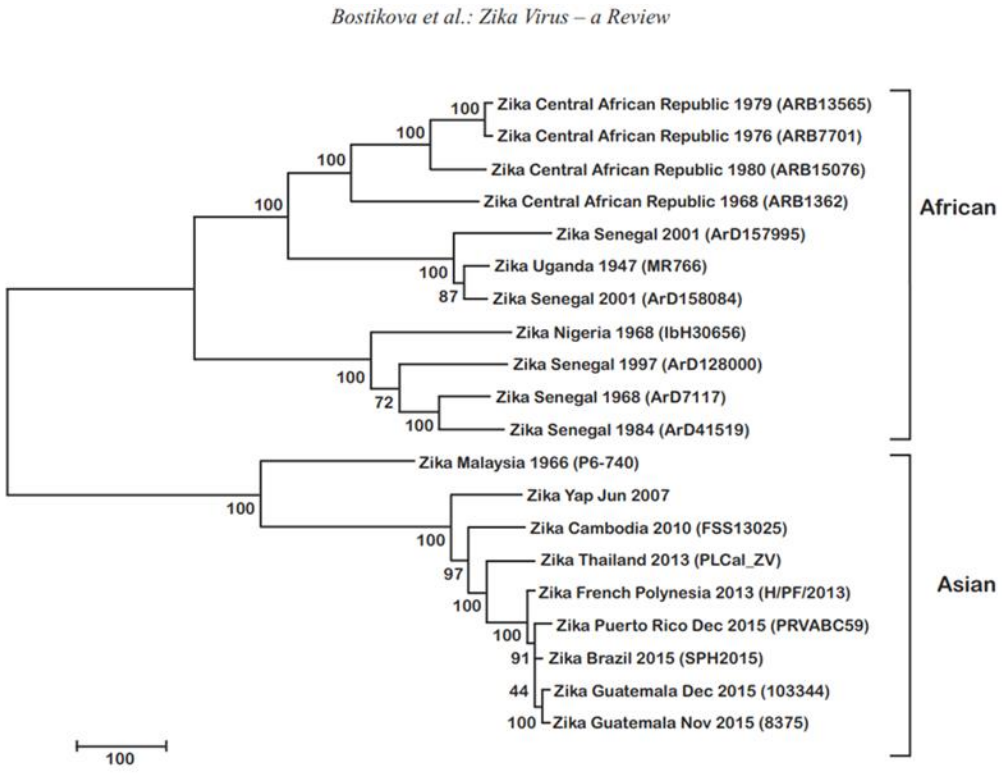

Figure 2: Phylogenetic tree of global Zika virus isolates obtained from Genbank. Analysis prepared by the University of Defense Hradec Kralove, Czech Republic.

Natural History Study Phase II

After the initial infection subsided, macaques were re-assigned into six groups of six. Each group consisted of animals challenged initially with the same isolate. Animals

were divided so that one female and one male from each of the three initial doses were included per group. On day 45, macaques were re-challenged with $1x10^6$ PFU of virus according to Table 2. Viruses were again diluted in virus production media supplemented with 10% FBS and injected subcutaneously into the left volar forearm. Each group previously challenged with either Asian or American lineage virus was again challenged with Asian or American virus. Groups previously challenged with African lineage virus were re-challenged with either African lineage or American lineage virus. Blood was collected and processed to serum for antibody titration on days 5 and 30 post re-challenge. No moribund euthanasia occurred, as macaques typically do not present clinical illness to Zika virus infection.

Table 2. Group designations of rhesus macaques for homologous or heterologous secondary challenge with various Zika viruses. Thirty six rhesus macaques were re-distributed based on prior challenge virus and dose. One male and one female from each of the initial doses are included in each group.

Group Number	Animal Number	Initial Challenge	Secondary Challenge	Dose (PFU/animal)
1	6 (3M/3F)	PRVABC59	PRVABC59	$1x10^6$
2	6 (3M/3F)	PRVABC59	PLCal_ZV	$1x10^6$
3	6 (3M/3F)	PLCal_ZV	PLCal_ZV	$1x10^6$
4	6 (3M/3F)	PLCal_ZV	PRVABC59	$1x10^6$
5	6 (3M/3F)	IBH 30656	IBH 30656	$1x10^6$
6	6 (3M/3F)	IBH 30656	PRVABC59	$1x10^6$

Statistical Analysis

Data from the study phase I was analyzed using a two-way ANOVA (analysis of variance) method paired with a Tukey's multiple comparison test using GraphPad Prism software. Datasets from each day were analyzed individually, for a total of three separate

analyses. Variables taken into consideration in each analysis included challenge dose and virus isolate identity. All variables were compared against one another within a single time point. Titers < LLOQ were included as a value of 50, while titers > ULOQ were included as a value of 15,000.

Data from the study phase II was analyzed using a one-way ANOVA method paired with a Tukey's multiple comparison test. Datasets from both time points were analyzed individually. Each analysis took all six groups into consideration. Titers outside the quantifiable range were amended in the same manner as described above.

RESULTS

Primary Anti-Zika Virus IgG Kinetics

IgG titers in serum collected from rhesus macaques following challenge with geographically diverse Zika virus isolates were determined by ELISA. Anti-Zika virus antibody titers following challenge with different Zika virus isolates are illustrated in Figure 3 and provided in Table A1 in the appendix. IgG titers were measured using the herein described ELISA method.

All animals challenged with PRVABC59 produced measurable antibody titers by day 10. The 10^6 PFU challenge group had an average titer of 1,890, the 10^5 PFU challenge group had an average titer of 1,026, and the 10^4 PFU challenge group had an average titer of 606. By day 15, all PRVABC59 challenged groups saw a significant increase in IgG titers, with the 10^6 PFU challenge group increasing by > 4-fold. By day 30, titers in all PRVABC59 challenge groups remained high. The 10^6 PFU challenged group increased to 10,486, the 10^5 PFU challenge group decreased from 7,122 to 5,127, and the 10^4 PFU challenge group increased from 3,558 to 5,573.

Challenge with PLCal_ZV resulted in similar IgG levels as challenge with PRVABC59. By day 10, all animals had detectable antibody titers, with the exception of one animal from the 10^4 PFU challenge group. The 10^6, 10^5, and 10^4 PFU challenge groups had averaged titers on day 10 of 3,494, 732, and 295, respectively. By day 15, titers of all groups increased significantly with the 10^6 PFU group increasing to 10,039. By day 30, the 10^6, 10^5, and 10^4 PFU challenge groups averaged 7,685, 7,151, and 9,341, respectively.

Animals challenged with IBH_30656 had lower detectable antibody titers compared to those challenged with PRVABC59 or PLCal_ZV. Only 25% of animals

challenged with 10^4 PFU of African virus had measurable IgG titers on day 10. Groups challenged with 10^6, 10^5, and 10^4 PFU averaged IgG titers of 717, 226, and 106, respectively. By day 15, all groups had an approximately 5-fold increase in titers with the 10^6, 10^5, and 10^4 PFU challenge group measuring at 3,331, 1,040, and 562, respectively. By day 30, all titers declined, with the 10^6 and 10^5 PFU challenge groups decreasing by nearly half.

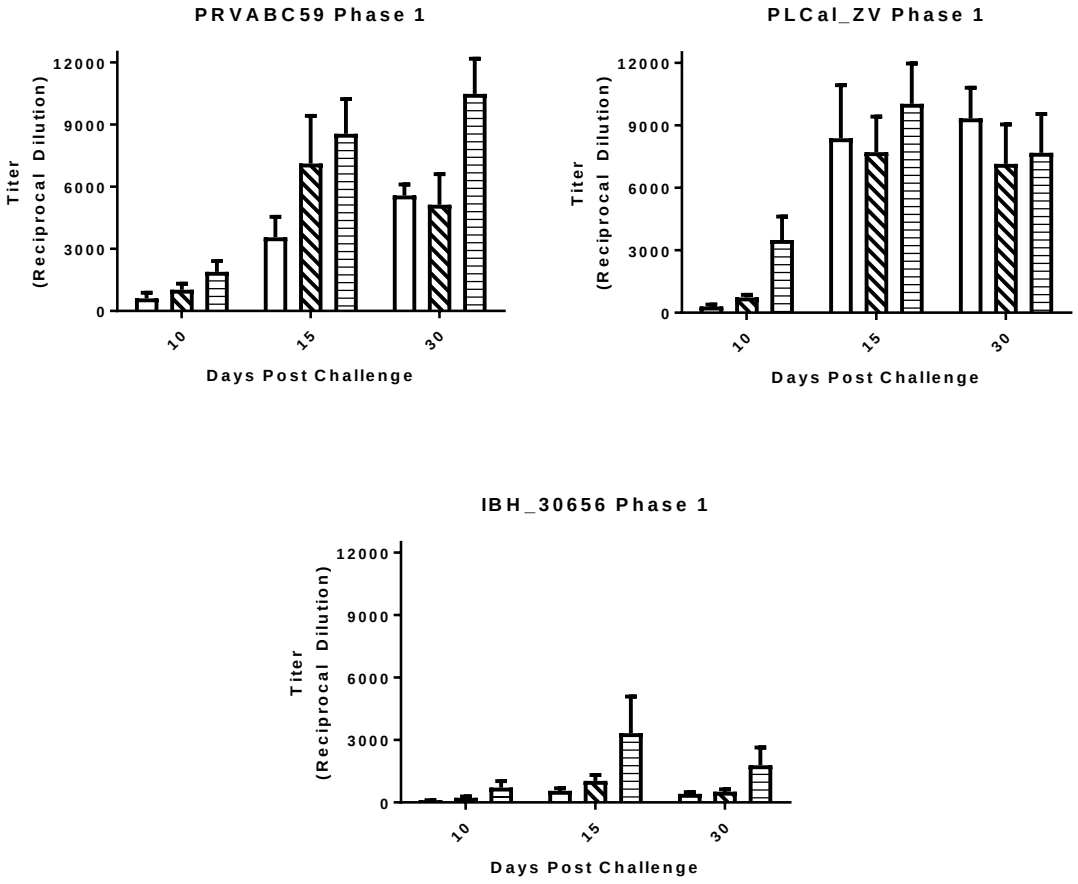

Figure 3: Anti-Zika virus IgG production following challenge with different geographical isolates of Zika virus. (A) PRVABC59; (B) PLCal_ZV; (C) IBH_30656. Each bar is the average of four data points. Bars with no fill: 1 x 10^4 PFU; Diagonal lines 1 x 10^5 PFU; Horizontal lines 1 x 10^6 PFU. Error bars represent the standard error of the means.

IgG Titers After Re-challenge

Anti-Zika virus IgG titers following re-challenge are illustrated in Figure 4 and provided in Table A2 in the appendix. After re-challenge, antibodies were detected in all animals five days post re-infection.

Animals previously challenged with PRVABC59, then re-challenged with PLCal_ZV, produced notably high titers five days post re-infection, averaging a titer of 12,099. Eighty three percent of these animals had titers above the ULOQ (12,800). By thirty days post re-infection, the average titer decreased only slightly to 10,423. Animals initially challenged with PRVABC59 then re-challenged with PRVABC59 showed a similar response five days post re-infection with an average titer of 10,141 and 50% of animals reporting titers above the ULOQ. However, by thirty days post re-infection, the average titer dropped nearly half to 6,111.

Animals previously challenged with PLCal_ZV, then re-challenged with PLCal_ZV had an average titer of 11,985 five days post re-infection, again with 83% of animals testing above the ULOQ. By thirty days post re-infection the average titer remained high at 11,248. Animals previously challenged with PLCal_ZV, then re-challenged with PRVABC59, produced an average titer of 10,672 five days post infection, which dropped to 7,093 thirty days post re-infection.

Animals initially infected with African IBH_30656, then re-challenged with IBH_30656 produced average titers of 1,203 five days post re-infection and 1,246 thirty days post re-infection. Animals initially infected with IBH_30656, then re-challenged with American PRVABC59 produced an average titer of 1,431 five days post re-infection, which grew to 10,456 by thirty days post re-infection.

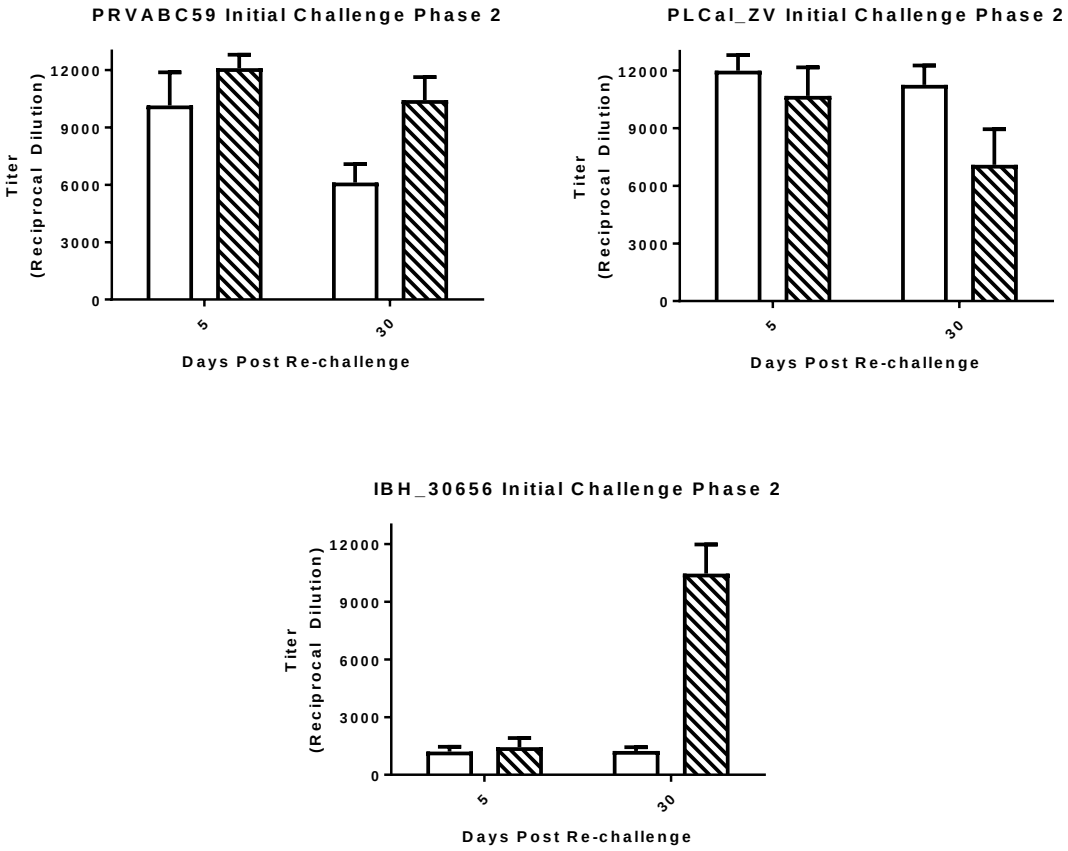

Figure 4. Anti-Zika virus IgG kinetics following re-challenge with the same or different isolate of Zika virus. (A) Initial challenge with PRVABC59 followed by re-challenge with PRVABC59 (no fill) or PLCal_ZV (diagonal lines); (B) Initial challenge with PLCal_ZV followed by re-challenge with PLCal_ZV (no fill) or PRVABC59 (diagonal lines); (C) Initial challenge with IBH_30656 followed by re-challenge with IBH_30656 (No fill) or PRVABC59 (diagonal lines). Each bar is comprised of six data points. Error bars display the standard error of the means.

Statistics

A summary of comparisons is shown in Tables A3 and A4 in the appendix. On day 10, no significant differences were observed between Asian and American viruses, within any of the challenge dose groups. A significant difference was observed in animals challenged with African IBH_30656 and Asian PLCal_ZV at the 10^6 PFU dose, but not between IBH_30656 and the American PRVABC59 virus at the same dose. Analysis of day 15 titers shows no significant differences between any viruses within each challenge

dose. No differences were detected on day 30 between the Asian and American viruses within each dose group. However, a difference is observed between PLCal_ZV and IBH_30656 at the 10^4 PFU dose, and between PRVABC59 and IBH_30656 at the 10^6 PFU dose.

Analysis of titers from day 5 post re-challenge showed a significant difference between groups who received Asian or American virus at both primary and secondary challenges and groups who were challenged with African virus during phase I. This is true whether the group was re-challenged with Asian or American virus. Interestingly, a difference is seen on day 30 post re-challenge between any group re-challenged with Asian PLCal_ZV and groups challenged twice with African IBH_30656, but not between groups re-challenged with American PRVABC59 and groups challenged twice with IBH_30656.

DISCUSSION

Zika virus has undergone a major shift in pathogenicity since its discovery in 1947, with each lineage presenting a unique disease profile. Here we describe the differences in antibody production in rhesus macaques after challenge with representative geographical isolates of Zika virus. We also provide evidence towards a degree of protection afforded to macaques recovered from pathogenic Zika virus infection and subsequently re-exposed to Asian or American virus.

Challenge with PRVABC59 or PLCal_ZV prompted IgG production as soon as ten days post infection, which grew substantially by day 15, regardless of challenge dose. Viral kinetics as shown by quantitative RT-qPCR to detect Zika virus genome in serum collected after infection indicate that replication of PRVABC59 and PLCal_ZV peaks two to three days after challenge, then drops below the LLOQ by day 8 (Rayner *et al.* 2018). Transient IgM antibodies likely provided initial neutralization to replicating virus two to four days after infection, only to be replaced by resident IgG antibodies within ten days after infection. By day 30, IgG titers dropped in both challenge groups, notably more on average in PRVABC59 infected macaques. However, IgG titers remained substantially higher than what was observed on day 10, which were sufficient to adequately suppress viral replication. The same IgG production trend was observed in the IBH_30656 infected group, although titers were considerably lower at all time points. Viral RNA was generally below the LLOQ during the entirety of the detection phase (Rayner et al. 2018). This suggests that IBH_30656 infects, but does not replicate well in the rhesus model, The resulting limited virus population stimulates maturation of a smaller B cell population compared to macaques infected with Asian and American viruses. This is consistent with

a study done by Koide *et al.* in 2016 which shows similar IgG and viral RNA kinetics in cynomologous macaques infected with similar viruses. Their team showed a substantial nAb presence in all macaques challenged with Asian or American virus fourteen days after infection, while animals infected with African virus showed no nAbs throughout the study. Peak Viral RNA observed in cynomologous macaques also mirrored what was observed in the rhesus monkeys. It's noted that the Koide team used an American strain of Zika virus in the neutralizing assay, regardless of the sample's challenge virus lineage. Therefore, nAbs must target epitopes conserved between Asian and American viruses.

Macaques challenged initially with PRVABC59 or PLCal_ZV, then re-challenged with heterologous Asian/American virus displayed elevated levels of antibodies five days after re-infection. A response of such magnitude is only possible if naïve B cells were exposed to an antigen population shared between both viruses during primary infection. As expected, a homologous primary and secondary challenge yielded a strong antibody rebound within a week. The absence of quantifiable viral RNA during the entire re-challenge phase is seen in both homologous and Asian/American heterologous re-challenged groups (Rayner *et al.* 2018). The elevated antibody titers and lack of quantifiable RNA suggests recovery from primary infection with a pathogenic strain of Zika virus affords near-complete protection to the patient. It is interesting that re-challenge with PLCal_ZV produced a substantially higher response than that observed after re-infection with PRVABC59, regardless if primary challenge was with PLCal_ZV or PRVABC59. This could be caused by single nucleotide polymorphisms (SNP) within Asian viruses that result in better viral peptide presentation in MHC-II complexes. The resulting increased presentation enhances B cell activation and secretion of antibodies.

Macaques who underwent primary challenge with African IBH_30656 and secondary challenge with PRVABC59 virus showed low IgG titers by day five post re-challenge, which grew ten-fold by day thirty post re-challenge. Quantifiable RNA was seen as early as one day after re-infection which persisted for at least three days afterwards (Rayner *et al.* 2018). Therefore, previous exposure to African virus does not confer protection against subsequent exposure to an American strain. The IgG response after primary exposure was unsubstantial, likely in part due to the virus' low replication rate inside the host. As a result, a smaller population of antigens were present, resulting in fewer circulating antibodies. Additionally, antigen homology between African and American lineages is unknown and presumably limited, further decreasing chances of cross-reactive antibodies. One explanation to the range of pathogenicity seen between African, Asian, and American viruses is that SNP variants found in Asian and American lineage viruses increased viral fitness compared to African viruses. However this gain of fitness also enhanced host immune detection and activation of antiviral responses, resulting in greater antibody production. American viruses might have further developed SNP variants enabling for recognition and entry into neural cells contributing to neurological morbidity, and enhance the virus' ability to antagonize host antiviral pathways.

This study shows the antibody production kinetics in rhesus macaques infected with either African, Asian, or American lineage Zika virus. Additionally, this study shows heterologous infection with Asian and American lineage virus elicits a robust memory B-cell response, suggesting a degree of protection is provided by cross-reactive antibodies. Information here can be used to guide decisions on vector selection for vaccine candidates, as well as provide a comparison for future efficacy studies.

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APPENDIX

Table A1: Individual and group means of anti-Zika virus IgG titers following initial challenge with PRVABC59, PLCal_ZV, or IBH_30656.

Phase I						Average		
Group	Challenge Dose (PFU/Animal)	Animal ID	Day			Day		
			10	15	30	10	15	30
1	$1x10^4$ PRVABC59	**5366**	218.6	1,755.4	4,126.5	606.4	3,558.1	5,573.2
		5362	1,431.5	6,357.5	5,769.1			
		5388	399.1	2,866.8	6,739.0			
		5376	376.5	3,252.6	5,658.1			
2	$1x10^5$ PRVABC59	**5360**	938.2	2,866.8	1,925.1	1,025.9	7,121.5	5,127.4
		5368	1,789.8	8,888.3	9,018.7			
		5389	395.3	>12,800	5,337.8			
		5378	980.1	3,930.8	4,227.9			
3	$1x10^6$ PRVABC59	**5374**	2,237.8	9,421.6	>12,800	1,890.3	8,559.7	10,486.3
		5363	3,174.6	>12,800	>12,800			
		5375	1,383.7	5,011.2	10,741.7			
		5381	765.1	7,006.0	5,603.4			
4	$1x10^4$ PLCal_ZV	**5370**	542.0	>12,800	>12,800	294.9	8,379.9	9,341.2
		5359	<100	4,008.0	8,104.8			
		5392	345.0	>12,800	6,026.8			
		5383	192.6	3,911.8	10,433.2			
5	$1x10^5$ PLCal_ZV	**5369**	1,101.3	>12,800	>12,800	732.3	7,714.7	7,151.4
		5358	633.1	6,513.8	5,769.1			
		5380	714.8	5,576.2	5,286.2			
		5384	480.0	5,968.6	4,750.5			
6	$1x10^6$ PLCal_ZV	**5367**	6,837.9	>12,800	>12,800	3,494.1	10,039.1	7,685.4
		5372	2,360.6	9,987.0	3,950.0			
		5379	1,925.1	4,569.5	7,572.1			
		5377	2,852.9	>12,800	6,419.6			
7	$1x10^4$ IBH_30656	**5361**	123.8	732.4	371.1	106.0	562.3	414.2
		5365	<100	787.7	623.9			
		5385	<100	242.0	444.1			
		5390	<100	487.0	217.5			
8	$1x10^5$ IBH_30656	**5371**	173.1	1,746.9	843.1	226.2	1,039.7	520.8
		5357	395.3	1,019.0	395.3			
		5387	227.2	980.1	511.3			
		5386	109.1	412.9	333.5			
9	$1x10^6$ IBH_30656	**5364**	765.1	3,447.8	1,664.1	717.2	3,330.9	1,790.4
		5373	1,562.3	8,263.8	4,207.4			
		5382	313.1	768.8	885.1			
		5391	228.3	843.1	405.0			

Table A2: Individual and group means of anti-Zika virus IgG titers following re-challenge with the same or different isolate of Zika virus.

Phase II						Average	
Group	Initial Challenge Virus	Re-Challenge Dose (PFU/Animal)	Animal ID	Day Post Re-challenge		Day Post Re-challenge	
				5	30	5	30
1	PRVABC59	$1x10^6$ PRVABC59	**5366**	>12,800	8,104.8	10,141.3	6,110.5
			5360	3,009.4	1,878.9		
			5374	>12,800	7,833.9		
			5388	12,731.8	7,248.3		
			5389	6,706.4	4,891.0		
			5375	>12,800	6,706.4		
2	PRVABC59	$1x10^6$ PLCal_ZV	**5362**	>12,800	12,426.4	12,098.5	10,423.0
			5368	8,591.2	7,390.4		
			5363	>12,800	>12,800		
			5376	>12,800	>12,800		
			5378	>12,800	11,005.7		
			5381	>12,800	6,115.3		
3	PLCal_ZV	$1x10^6$ PLCal_ZV	**5370**	>12,800	>12,800	11,985.1	11,247.5
			5369	>12,800	>12,800		
			5367	>12,800	>12,800		
			5392	>12,800	>12,800		
			5380	>12,800	9,106.7		
			5379	7,910.4	7,178.2		
4	PLCal_ZV	$1x10^6$ PRVABC59	**5359**	>12,800	>12,800	10,672.3	7,093.0
			5358	8,845.2	4,867.3		
			5372	3,988.5	3,009.4		
			5383	>12,800	3,284.3		
			5384	>12,800	5,797.2		
			5377	>12,800	>12,800		
5	IBH_30656	$1x10^6$ IBH_30656	**5361**	331.9	516.3	1,203.5	1,245.6
			5371	1,184.5	1,156.1		
			5364	2,205.5	1,860.7		
			5385	924.6	1,128.4		
			5387	1,330.9	1,219.5		
			5382	1,243.4	1,592.9		
6	IBH_30656	$1x10^6$ PRVABC59	**5365**	1,249.5	>12,800	1,431.7	10,455.7
			5357	1,184.5	>12,800		
			5373	3,672.5	>12,800		
			5390	302.6	4,459.9		
			5386	1,370.3	>12,800		
			5391	811.0	7,074.4		

Table A3: Summary of two-way ANOVA paired with a Tukey's multiple comparison test.

Day Post Challenge	Challenge dose	Comparison	P-Value	Significant Difference?
10	$1x10^4$ PFU	PRVABC59 vs. PLCal_ZV	0.9998	No
		PRVABC59 vs. IBH_30656	0.9943	No
		PLCal_ZV vs. IBH_30656	>0.9999	No
	$1x10^5$ PFU	PRVABC59 vs. PLCal_ZV	>0.9999	No
		PRVABC59 vs. IBH_30656	0.9368	No
		PLCal_ZV vs. IBH_30656	0.9962	No
	$1x10^6$ PFU	PRVABC59 vs. PLCal_ZV	0.2714	No
		PRVABC59 vs. IBH_30656	0.6599	No
		PLCal_ZV vs. IBH_30656	0.0047	Yes
15	$1x10^4$ PFU	PRVABC59 vs. PLCal_ZV	0.5313	No
		PRVABC59 vs. IBH_30656	0.979	No
		PLCal_ZV vs. IBH_30656	0.0942	No
	$1x10^5$ PFU	PRVABC59 vs. PLCal_ZV	>0.9999	No
		PRVABC59 vs. IBH_30656	0.3852	No
		PLCal_ZV vs. IBH_30656	0.1314	No
	$1x10^6$ PFU	PRVABC59 vs. PLCal_ZV	0.9984	No
		PRVABC59 vs. IBH_30656	0.5623	No
		PLCal_ZV vs. IBH_30656	0.4827	No
30	$1x10^4$ PFU	PRVABC59 vs. PLCal_ZV	0.6267	No
		PRVABC59 vs. IBH_30656	0.4008	No
		PLCal_ZV vs. IBH_30656	0.0078	Yes
	$1x10^5$ PFU	PRVABC59 vs. PLCal_ZV	0.9648	No
		PRVABC59 vs. IBH_30656	0.5469	No
		PLCal_ZV vs. IBH_30656	0.0815	No
	$1x10^6$ PFU	PRVABC59 vs. PLCal_ZV	0.8613	No
		PRVABC59 vs. IBH_30656	0.0055	Yes
		PLCal_ZV vs. IBH_30656	0.1559	No

Table A4: Summary of one-way ANOVA paired with a Tukey's multiple comparison test.

Day Post Re-challenge	Comparison	P-Value	Significant Difference?
5	PRVABC59/PRVABC59 vs. PRVABC59/PLCal_ZV	0.7208	No
	PRVABC59/PRVABC59 vs. PLCal_ZV/PLCal_ZV	0.7549	No
	PRVABC59/PRVABC59 vs. PLCal_ZV/PRVABC59	0.9968	No
	PRVABC59/PRVABC59 vs. IBH_30656/IBH_30656	0.0002	Yes
	PRVABC59/PRVABC59 vs. IBH_30656/PRVABC59	0.0002	Yes
	PRVABC59/PLCal_ZV vs. PLCal_ZV/PLCal_ZV	>0.9999	No
	PRVABC59/PLCal_ZV vs. PLCal_ZV/PRVABC59	0.9328	No
	PRVABC59/PLCal_ZV vs. IBH_30656/IBH_30656	<0.0001	Yes
	PRVABC59/PLCal_ZV vs. IBH_30656/PRVABC59	<0.0001	Yes
	PLCal_ZV/PLCal_ZV vs. PLCal_ZV/PRVABC59	0.9484	No
	PLCal_ZV/PLCal_ZV vs. IBH_30656/IBH_30656	<0.0001	Yes
	PLCal_ZV/PLCal_ZV vs. IBH_30656/PRVABC59	<0.0001	Yes
	PLCal_ZV/PRVABC59 vs. IBH_30656/IBH_30656	<0.0001	Yes
	PLCal_ZV/PRVABC59 vs. IBH_30656/PRVABC59	<0.0001	Yes
	IBH_30656/IBH_30656 vs. IBH_30656/PRVABC59	>0.9999	No
30	PRVABC59/PRVABC59 vs. PRVABC59/PLCal_ZV	0.2338	No
	PRVABC59/PRVABC59 vs. PLCal_ZV/PLCal_ZV	0.0571	No
	PRVABC59/PRVABC59 vs. PLCal_ZV/PRVABC59	0.9697	No
	PRVABC59/PRVABC59 vs. IBH_30656/IBH_30656	0.2685	No
	PRVABC59/PRVABC59 vs. IBH_30656/PRVABC59	0.1221	No
	PRVABC59/PLCal_ZV vs. PLCal_ZV/PLCal_ZV	0.9801	No
	PRVABC59/PLCal_ZV vs. PLCal_ZV/PRVABC59	0.6643	No
	PRVABC59/PLCal_ZV vs. IBH_30656/IBH_30656	0.0013	Yes
	PRVABC59/PLCal_ZV vs. IBH_30656/PRVABC59	0.9993	No
	PLCal_ZV/PLCal_ZV vs. PLCal_ZV/PRVABC59	0.2639	No
	PLCal_ZV/PLCal_ZV vs. IBH_30656/IBH_30656	0.0002	Yes
	PLCal_ZV/PLCal_ZV vs. IBH_30656/PRVABC59	0.9991	No
	PLCal_ZV/PRVABC59 vs. IBH_30656/IBH_30656	0.0584	No
	PLCal_ZV/PRVABC59 vs. IBH_30656/PRVABC59	0.4508	No
	IBH_30656/IBH_30656 vs. IBH_30656/PRVABC59	0.0005	Yes