8-2020

The Long-Term Persistence and Tissue Tropism of Brazilian Zika Virus in *Monodelphis domestica*

Marisol Morales
The University of Texas Rio Grande Valley

THE LONG-TERM PERSITENCE AND TISSUE TROPISM OF BRAZILIAN ZIKA VIRUS IN MONODELPHIS DOMESTICA

A Thesis

by

MARISOL MORALES

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

AUGUST 2020

Major Subject: Biology

THE LONG-TERM PERSISENCE AND TISSUE TROPISM OF BRAZILIAN ZIKA VIRUS IN MONODELPHIS DOMESTICA

A Thesis
by
MARISOL MORALES

COMMITTEE MEMBERS

Dr. John M. Thomas III
Chair of Committee

Dr. Robert Dearth
Committee Member

Dr. John VandeBerg
Committee Member

August 2020

ABSTRACT

Morales, Marisol, The Long-Term Persistence and Tissue Tropism of Brazilian Zika Virus in Monodelphis domestica. Master of Science (MS), August 2020, 45 pp., 3 tables, 5 figures, references, 63 titles.

Zika virus (ZIKV) rose as a major public health concern due to the congenital abnormalities of infants born to infected mothers during the 2015 Latin American outbreak. While animal models such as transgenic mice and nonhuman primates are used to study ZIKV pathogenesis, they display various limitations. Additionally, the persistence and tissue distribution of long-term ZIKV infection remains unknown. We describe the laboratory opossum, *Monodelphis domestica,* as a potential new model for ZIKV pathogenesis research to address these issues. We utilized immunohistochemistry, RT-PCR, and ELISA to show that: infant animals inoculated intracerebrally were susceptible to infection, ZIKV infection persisted through juvenile age in the brain, sex organs, and spleen, and that some animals developed an immune response to ZIKV infection.

DEDICATION

This thesis is dedicated to my dad, Jose Angel Morales, for sharing his own experiences as a graduate student as a way to show his support throughout this process.

ACKNOWLEDGEMENTS

I would like to acknowledge my mentor and chair of my committee, Dr. John Thomas III, for his guidance and patience as I completed my master's thesis. I am thankful for is dedication while assisting me in carrying out the research, as well as helping me develop and edit each section of this manuscript amid a global pandemic.

I would also like to acknowledge Dr. John VandeBerg and his research team led by Susan Mahaney, for providing, handling, and processing the unique animals utilized in these experiments, and for providing data on experimental protocols and tissue pictures. Thanks as well to Dr VandeBerg and Dr. Andre Pastor for providing ELISA data and methodology mentioned in this manuscript. Additionally, many thanks to Dr. Robert Dearth, for his guidance in carrying out the immunohistochemistry experiments and providing insight into the physiology of the abnormal experimental tissues. This study would not have been possible without their dedication.

Finally, I would like to thank the members of my laboratory for their help in processing samples for this study during times when I could not attend lab: DionnCarlo Silva, Oscar Quintanilla, Kush Maheshwari, Jonathan Kasofky, Kevin Gongora, and Bridger Southwell. Special thanks to Juan Garcia, the laboratory leader who took the time to teach me most of the procedures utilized in this experiment and helped with the protocols for viral RNA extraction and RT-PCR.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Zika virus (ZIKV) is an enveloped, single-stranded positive-sense RNA virus belonging to the family *Flaviviridae.* The name originates from the Ziika forest of Uganda, wherein the virus was first isolated in 1947 from a rhesus monkey, and subsequently from the *Aedes africanus* mosquito (Dick et al., 1952). The most common mode of ZIKV horizontal transmission is now attributed to *A. aegypti* and *A. albopictus* mosquitos, which pose the greatest threat to human health due to their feeding behavior and wide distribution across warm, urban environments (Powell and Tabachnick, 2013). ZIKV emerged to the forefront of research following the 2015 Brazilian outbreak, during which an increase of infants with neurologic abnormalities, particularly microcephaly, were born to infected mothers (Zanluca et al., 2015). The link between ZIKV and neurologic abnormalities in infants resulting from vertical transmission is supported by signs of ZIKV infection detected in the neurologic tissue of microcephalic fetuses, as well as the amniotic fluid of pregnant mothers (Calvet, et al., 2016). Additionally, ZIKV displays a wide range of tissue tropism, particularly in immune-privileged sites such as the brain, ocular tissue, and sex organs. Viral infection of the sex organs is of particular importance, as this could contribute to the sexual transmission of ZIKV and may have detrimental effects on fertility (Ma et al., 2016; Miner and Diamond, 2017). Due to the various consequences of ZIKV infection, animal models are an important tool to study its biology and pathogenesis, which remains poorly understood. While animal models such as transgenic mice

and non-human primates (NHPs) are used in ZIKV research, they display limitations such as long developmental times, and lack of an intact immune system. Therefore, a more suitable model is needed for ZIKV research. We describe the laboratory opossum, *Monodelphis domestica,* as a new model for the study of ZIKV pathogenesis. At birth, these marsupials are developmentally equivalent to a 5-week-old human embryo and subsequently begin to develop a robust immune system. This makes them a convenient extrauterine model for experimental manipulation from an early developmental point. Pilot studies utilizing the Puerto Rican strain of ZIKV (ZIKV-PR) in the laboratory opossum show that wild-type animals are permissive to infection and result in brain pathology analogous to that of humans and other animal models (Thomas and VandeBerg, personal communication). While initial studies support the suitability of laboratory opossum as a model for ZIV research, more investigation is needed to further analyze the consequences of ZIKV infection as it pertains to long-term infection coupled with tissue distribution.

The long-term effects of viral infection in children infected *in utero* during the 2015 Brazilian outbreak remain unknown at this time. Currently, it is unclear whether viral infection persists long-term in animal models, from an early developmental period through juvenile age. This period of time is difficult to study in NHPs and transgenic mice. At the onset of sexual maturity, healthy brain and sexual organ development is crucial. It is unknown whether these tissues retain long-term infection. The purpose of this study was to determine the permissiveness of the laboratory opossum to Brazilian Zika virus (ZIKV-BR) infection, as well as the viral tissue distribution for animals that retain long-term infection. This study mimics the timeline of children infected *in utero*, as animals were initially inoculated during what is equivalent to the first trimester of human development, and sacrificed at 22 and 26 weeks of age, during which

sexual maturity begins. The tissues we analyzed for viral infection were the brain, sex organs, and spleen. This study provides insight into the long-term persistence and tissue distribution of ZIKV-BR, and further establishes the *Monodelphis domestica* as a model for ZIKV research.

CHAPTER II

REVIEW OF THE LITERATURE

Flaviviruses

The flaviviridae is a family of viruses comprised of four genera: *Pestivirus, Hepacivirus, Pegivirus,* and *Flavivirus*. *Flavivirus* is the largest and contains over 70 viruses, particularly arboviruses of clinical relevance due to their pathogenicity in humans (Suchetana et al., 2005). Viruses belonging to this genus include yellow fever virus (YFV), West Nile virus (WNV), dengue virus (DENV), and Zika virus (ZIKV). Despite causing a range of clinical symptoms, flaviviruses share a similar genomic structure and replication strategy. ZIKV outbreaks in Asia and South America during the last decade have led to an renewed interest in the pathogenesis of this arbovirus, particularly as it pertains to neonatal health when vertical transmission occurs from pregnant mother to child.

Virus Architecture and Genome Organization

The spherical ZIKV particle is approximately 50 nm in diameter and encompasses three structural proteins: the capsid (C), pre-membrane/ membrane (prM/ M), and envelope (E) (Hamel et al., 2015; Suchetana et al., 2005). The capsid encompasses multiple monomers of α-helices of C protein that run anti-parallel to each other to form the nucleocapsid core (NC); this structure houses the viral genome (Suchetana et al., 2005; Fernandez-Garcia, 2009). Surrounding

the NC is a lipid bilayer derived from the host's endoplasmic reticulum (ER). The lipid bilayer contains the E and M glycoproteins, which form heterodimers tethered by their respective transmembrane domains, giving rise to the smooth, outer surface of the virus particle (Kuhn et al., 2002; Yun and Lee, 2017).

The NC of the virus particle houses a single stranded, positive-sense RNA genome of ~11 kb in length (Apte-Sengupta et al., 2014). Starting at the 5'- end, the genome contains a type 1 cap structure (m7GPPAmp) followed by an untranslated region (UTR), a single open reading frame (ORF), and a second UTR adjacent to the 3'-end, which lacks a poly-A tail (Yun and Lee, 2017). The ORF encodes a polyprotein precursor that is co- and post-translationally cleaved into the three structural and seven non-structural (NS) proteins, which are oriented from N-terminus to C-terminus on the polypeptide molecule: C, pr/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Iglesias et al., 2009). The NS proteins are associated with virus assembly, RNA replication, and host immune response (Fernandez-Garcia, 2009; Yun and Lee, 2017).

Life Cycle and Replication

While information regarding ZIKV-specific replication is lacking, the general flavivirus life cycle is well-characterized. Upon entering the host, the virion will attach to the surface of a target cell and gain access via receptor-mediated endocytosis (Suchetana et al., 2005; Yun and Lee, 2017). Following internalization, acidic conditions trigger a conformational change of the endosome, particularly the outer E protein (Yun and Lee, 2017), which allows fusion of virus and host membranes, followed by disassembly of the viral particle (Suchetana et al., 2005). Subsequently, the NC is released into the cytoplasm, where the viral RNA strand will dissociate and serve as mRNA to drive genome replication on intracellular membranes and virus particle assembly on the endoplasmic reticulum. Immature viral particles travel through the trans-Golgi

network, after which maturation occurs as a result of cleavage by the host protease furin; the mature virions are then released from the target cell via exocytosis (Suchetana et al., 2005; Yun and Lee, 2017; Apte-Sengupta et al., 2014).

ZIKV Pathology in the Developing Fetus

Clinical symptoms associated with ZIKV infection encompassed a mild, febrile illness (Simpson, 1964), and it is now estimated that only 19% of individuals display symptoms (Petersen et al., 2016; Minder and Diamond 2017). However, recent outbreaks in French Polynesia and South America described additional manifestations of disease, such as Guillain-Barré syndrome in adults (Cao-Lormeau, et al., 2016) and congenital abnormalities in 1 to 13% of infections (França et al.,2016). In Brazil, 850 infants born to ZIKV-infected mothers displayed signs of microcephaly characterized by calcifications leading to irregular cortical development, as well as decreased myelination and hypoplasia of the cerebellum and brainstem (de Fatima et al., 2016). The risk of microcephaly was disproportionately greater when women became infected during the first trimester of pregnancy (Brasil et al., 2016). Additional morbidities included intrauterine growth restriction (IUGR) in 9% of ZIKV-exposed infants, as well as fetal demise and spontaneous abortion (Martines et al., 2016). Zika virus-infected neonatal mice have displayed signs of microcephaly characterized by decreased cortical thickness and a "vacuolar nuclei" appearance in neurons, as well as IUGR, when compared to mock-infected animals (Cugola, et al., 2016; Miner et al., 2016). In non-human primates, fetal brain pathology resulting from ZIKV infection is less severe and lacks the cortical and cerebellar malformations seen in human and mice (Adams et al., 2016). However, animals inoculated with more clinically relevant virus strains have displayed significant fetal demise, reduced brain volumes, and brain lesions such as microcalcifications and gliosis (Magnani et al., 2018; Martinot et al., 2018).

Congenital abnormalities linked to ZIKV are supported through post-mortem examination of fetal tissue, as well as experimental infection of different animal models.

ZIKV Tissue Distribution

Zika virus displays a range of tropism, particularly in immune-privileged sites including neural, ocular (Nelson et al., 2019), and reproductive tissues. Zika virus RNA has been identified in amniotic fluid (Calvet et al., 2016) and Hofbauer cells of placental tissue, as well as in microcephalic fetal brains (Bhatnagar et al. 2017; Miner and Diamond, 2017), supporting the link between ZIKV infection and microcephaly via the vertical transmission from mother to child. Particularly, ZIKV infects cortical neural progenitor cells in humans, NHPs, and mice, attenuating their growth and attributing to the brain pathology observed in neonates infected during early pregnancy (Tang et al., 2016; Li et al., 2016; Martinet, 2018). While most data concerning fetal infection focuses on the CNS, evidence of viral dissemination to other organs has been described. Zika virus RNA has been found in the brain, spleen, kidney, and liver of neonatal mice, with the highest viral loads in the brain, followed by the spleen (Cugola et al., 2016). In NHP fetuses, antigen has been observed in the brain, lymph nodes, lungs, intestines, and in sex organs including the prostate, seminal vesicles, and uterus (Nguyen et al., 2017).

Studies with adult animal models provide more insight into the tropism of ZIKV beyond the CNS, as evidence of infection in the spleen, liver, kidneys, and sex organs has been shown (Miner and Diamond, 2017). The reproductive tract is a key topic of study due to the implications of ZIKV related to sexual transmission (Musso et al., 2015; Counotte et al., 2018) and fertility. In humans, ZIKV can persist in cervical secretions (Prisant et al., 2016) and semen, which can shed virus for up to 6 months post-infection (Atkinson et al., 2016; Barzon et al., 2016). Zika virus has been shown to infect the Leydic and Sertoli cells of the testes, and

epithelial cells of the vagina (Miner and Diamond, 2017). Studies in mice have shown a high viral load in the testes resulting in impaired sex hormone levels, which has been attributed to testicular damage and infertility (Ma et al., 2016; Govero et al., 2016; Lazear et al., 2016). Female gonads have also displayed high viral loads in NHP ovaries (Hirsh et al., 2017) and in the ovarian follicles of mice (Duggal et al., 2018), further suggesting an association between viral replication and tissue tropism. Interestingly, ovarian infection and damage in mice is less prominent than that of the testes, and no long-term implications on fertility have been observed in this model (Morelli et al., 2020).

Monodelphis domestica as a Model for Research

In this study, we utilized a novel animal model for ZIKV pathogenesis research. The gray short-tailed opossum, *Monodelphis domestica*, is a small marsupial native to South America. While its role in infectious disease research has not yet been widely established, the laboratory opossum has been utilized as a competent lab model in genetics, reproduction, and developmental biology research (Samollow, 2006). Extensive insight into human metabolism has also been provided using the laboratory opossum to study the effects of diet on lipoprotein composition (Rainwater and VandeBerg, 1992; Kushwaha et. al, 2004), as well as genetic factors that affect intestinal absorption of cholesterol (Chan et al., 2010). The laboratory opossum is particularly valuable in oncogenic research, as neonatal pups have been shown to act as natural vessels for xenografted human cancer cells of the skin, colon, and prostate, allowing for *in vivo* studies (Wang et al., 2008). This finding suggests favorable characteristics of the neonatal opossum's immune system that render it receptive to the establishment of xenogeneic tumors as a foreign antigen. This attribute can be exploited for a new area of biomedical research using the *Monodelphis domestica*, particularly in the realm of viral pathogenesis.

Advantages of the Laboratory Opossum

The laboratory opossum is the most widely used marsupial in laboratory settings (Samollow, 2006) owing to its various favorable characteristics. The opossums are docile and small, typically 80-120 grams, and are highly prolific induced-ovulators. Females can produce litters of 6-13 pups three to four times a year, as allowed by gestation and weaning periods (VandeBerg and Blangero, 2010). These characteristics make them more economically viable than NHPs and provide greater sample sizes over a shorter time period. Unique to marsupials is their birth at an embryonic stage of development. In addition, females of the genus *Monodelphis* do not have a pouch, enabling newborn pups to be easily manipulated experimentally. Newborn pups are developmentally equivalent to a 5-week-old human embryo (Cardoso-Moreira et al., 2019) and attach to the mother's nipple, where they remain attached as they continue the first 14 days of embryonic and fetal development (VandeBerg and Blangero, 2010). Neonatal pups essentially serve as an exteriorized fetus model, providing a unique opportunity for virus inoculation during what is equivalent to the first trimester of human pregnancy, a crucial time period for ZIKV infection (Caine et al., 2018). These characteristics make the laboratory opossum a convenient model to handle during experimental procedures.

Finally, through initial studies utilizing the PRVABC59 strain of ZIKV (ZIKV-PR), neonatal opossums have been shown to harbor the virus and survive post-infection, without the need for immunologic abatement (Thomas and VandeBerg, personal communication). The viral NS5 protein antagonizes human STAT2 as part of the type 1 interferon (IFN) response, but has no effect on mouse STAT2 (Grant et al., 2016). Mice therefore do not serve as natural hosts for ZIKV. Consequently, there is a need to actively adapt the mouse model to ZIKV research. This has been accomplished by utilizing embryonic, 1-day-old mice, and transgenic mice lacking the

IFN α/β receptor. These models are permissive to ZIKV infection, but often do not survive long enough for extensive long-term studies (Pawitwar et al., 2017). Studies with the laboratory opossum have shown the susceptibility of the animal to infection, while also displaying brain pathology and occasional growth restriction in infected animals (Thomas and VandeBerg, personal communication), parallel to that seen in humans, NHPs, and mice. Infected laboratory opossums at 10 weeks of age continued to show signs of infection (Thomas and VandeBerg, personal communication), indicating that long-term persistence of the virus can be analyzed in this model. Without the need to remove host immunity factors, this model allows us to thoroughly study the repercussions of infection long-term, while maintaining normal immune development.

Limitations in ZIKV Research

Although insight into ZIKV biology has been acquired through various animal models, each displays some limitations from a practical and biological aspect, as previously described. Additionally, studies with ZIKV have not combined the infection of early embryonic animals with the resulting tissue distribution as it pertains to long-term persistence into young adulthood. Studies that investigated ZIKV infection from a gestational stage did not evaluate animals beyond 10-20 days post-infection in mice (Manangeeswaran et al., 2016; van Den Pol et al.,2017). Similarly, in NHPs, animal evaluation did not occur at the juvenile stage (Mavigner et al., 2018). Juvenile age is relevant to ZIKV biology due to the maturation of the sex organs, which have been shown to serve as host tissues. Long-term studies in mice up to 4 months of age address the persistence of ZIKV in the brain, kidney, liver, spleen and testes, but animals were initially exposed between 4 to 7 weeks of age (Lazear et al., 2016; Govero et al., 2016) and not during gestation. One study evaluated ZIKV infection in immunocompetent, neonatal mice all

the way to adulthood and found significant impacts in brain pathology (Nem de Oliveira Souza et al., 2018), but failed to mention infection in other tissues.

Statement of the Problem

The long-term effects and tissue distribution of ZIKV infection in children infected *in utero* during the 2015 Brazilian outbreak are unknown at this time. It is also unclear whether viral infection persists in animal models from an early developmental period through juvenile age, a critical point in sexual maturity. This period of time is difficult to study in NHPs and transgenic mice, and therefore a more suitable animal model is needed.

Purpose of the Study

The purpose of this study addresses three key points. First, the laboratory opossum was evaluated for its susceptibility to the Brazilian strain of Zika virus (ZIKV-BR), supplementing pilot studies that utilized the Puerto Rican strain (ZIKV-PR) for the first time in this model. Second, the persistence of infection is evaluated from an early "gestational" stage through the onset of young adulthood at 22 and 26 weeks of age. Lastly, we looked at the tissue distribution resulting from long-term persistence, particularly in the brain and sex organs, which are of key importance in adolescent development. The spleen was also evaluated due to its high viral load in the initial ZIKV-PR studies (Thomas and VandeBerg, personal communication). Immunohistochemistry and RT-PCR were used to analyze the persistence and location of infection at the end of the experimental timepoint. Additionally, ELISA titers were included in the analyses to determine whether any animals produced antibodies against ZIKV infection.

Based on initial studies with this model, it is expected that the laboratory opossum will be susceptible to ZIKV-BR infection, and that viral dissemination will occur in the sex organs and

spleen following intracerebral inoculation. While persistence of infection has not been studied beyond a 10-week time period in this model (Thomas and VandeBerg, personal communication), it is also hypothesized that evidence of infection will remain detectable in the juvenile animals, as has been seen in long-term studies with adult mice (Lazear et al., 2016; Govero et al., 2016). Animals infections took place between 3-8 days of age, so a negative ELISA titer is expected for most serum samples.

CHAPTER III

MATERIALS AND METHODS

Animals

The laboratory opossums used in this study were produced in the breeding colony maintained at The University of Texas Rio Grande Valley and maintained under standard conditions (VandeBerg and Blangero, 2010).

Ethics Statement

All animal work described herein was subject to review and approval by the UTRGV Institutional Animal Care and Use Committee (IACUC), as well as oversight provided by the UTRGV Department of Laboratory Animal Resources (LAR). LAR maintains compliance with the National Institutes of Health Office of Laboratory Animal Welfare (NIH OLAW) Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals; PHS Assurance number A4730-01, and the United States Department of Agriculture (USDA); USDA Assurance number 74-R-0216. The animal protocol for this work was approved and conducted under the IACUC protocol of Dr. John Thomas (#2016-005-IACUC)

Virus Preparation and Cells

Virus preparation was done by senior research lab member, Juan Garcia at the University of Texas Rio Grande Valley. ZIKV isolate BR1911 was used for the inoculations. Vero cells (CCL-81; ATCC, USA) were used for virus titration, and C6/36 cells (CRL-1660; ATCC, USA) derived from *Aedes albopictus* were used to amplify lyophilized virus for scale-up. Virus generated from the initial reconstituted lyophilized stock was passaged once in C6/36 cells, and the resulting supernatant was clarified and purified over a sucrose cushion. Virus supernatants were quantified in duplicate by plaque assay, as described previously (Shan et al., 2016). Aliquots were stored at -80°C for further use (John Thomas and Juan Garcia, personal communication).

Animal Infections

All animal infections took place under the direction of Dr. John VandeBerg and his research associates led by Susan Mahaney at the South Texas Diabetes & Obesity Institute, University of Texas Rio Grande Valley. Animals were intracranially injected with 2µm of ZIKV-BR (BR1911) at 10^5 PFU, or mock-infected with 2µm of sterile PBS. Viral infections took place when animals were between 3-8 days of age, and animals were sacrificed at 22 or 26 weeks of age. One animal, P1967 was euthanized early at 19 weeks due to an inflamed, bleeding scrotum. Another animal, P1968 was euthanized at 21 weeks of age as an approximate age-matched control for P1967. Both of these animals are considered to be in the 22-week-age group. At the time of animal processing, blood was collected for serum and organs were harvested and placed either in 10% formalin or kept frozen at -80°C for storage until the time of tissue analysis (Susan Mahaney, personal communication).

Tissue Fixation and Sectioning

Dissected tissue was fixed in sterile PBS (Gibco, USA) + 4% formaldehyde solution and stored at room temperature. Fixative was then cleared from tissue by performing three quick washes in sterile PBS+Tween 20 (PBTB) followed by three 10-min washes in sterile PBTB. Next, the tissue was incubated for 60 min in 33% OCT mounting media: sterile PBS, followed by 3x quick washes in sterile PBTB. Tissue was then incubated for 60 min in 66% OCT: sterile PBS, followed by 3x quick washes in PBTB. Finally, tissue was incubated overnight in 100% OCT. Tissue was mounted in OCT and cooled to -20°C for sectioning by a cryostat (Leica Biosystems, USA). Sections of 5 µm were mounted onto Frost + microscope slides and stored at -20°C.

Antibody Staining

Tissues analyzed through immunohistochemistry (IHC) included the brain, sex organs, and spleen of 22- and 26-week-old animals. Mounted sections of tissue were incubated in PBTB (sterile PBS + .01% Tween20 + 0.2% BSA) for 1 hour followed by incubation in 1:500 dilution of primary antibody (Arigo Biolaboratories, Taiwan), for either 1 hour at room temperature or overnight at 4°C. Anti-ZIKV monoclonal antibody directed against NS1 protein constituted the primary antibody. Primary antibody was removed by washing 3X quickly, then 3X for 10 min each in PBTB. Tissue was then incubated in 1:200 dilution of AlexaFluor (546 – Thermo Fisher Scientific, USA) conjugated secondary antibody in PBTB for 1 hour. Secondary antibody was removed in the same manner as primary antibody, except that DAPI (Thermo Fisher Scientific, USA), and AlexaFluor 488 (Thermo Fisher Scientific, USA) conjugated phalloidin were included in the first 10-min wash at 1:1000 and 1:200 dilution, respectively. Tissue was imaged

using an Olympus FV10i confocal microscope. Protocol for Antibody staining was provided by Dr. Matthew D. Terry, associate professor at University of Texas Rio Grande Valley.

Validation of the anti-NS1 antibody was done by analyzing one tissue slide with primary antibody, and another slide of the same tissue from the same animal without primary antibody. The rest of the staining protocol was followed as stated above. Tissue was imaged using an Olympus FV10i confocal microscope, and images were compared to detect the presence/ absence of primary antibody. Once it was determined that no non-specific binding of NS1 was occurring, ovary for ZIKV-infected P2141 animal was utilized as a positive control for remaining immunohistochemistry procedures. PBS-mock infected tissues were utilized as a negative control.

Viral RNA Extraction of Tissue and RT-PCR Analysis

Tissues from 22-week-old animals frozen at -80°C were extracted for viral RNA utilizing one of two kits, depending on the tissue type. Brains were considered fatty tissue and were extracted following the steps for the QIAGEN RNeasy® Lipid Tissue Mini Kit (50), while spleen, ovary, vagina, testis, and epididymis were extracted following steps for the QIAGEN RNeasy® Fibrous Tissue Mini Kit (50). Samples were lysed using the TissueLyser II from QIAGEN. Following extraction, samples were analyzed for concentration of RNA and run on the 7500 Fast RT-PCR machine.

The SuperScript III Platinum One-Step qRT-PCR (Ref. 11732-088) was utilized to complete the reactions. A primer/probe mix working stock was made for ZIKV-NS5 (Pabbaraju et al., 2016). Reagents and tissue samples were loaded on to a 96-well reaction plate (Ref. 4346906) in sets of duplicate wells. DH20 was utilized as a negative and NTC control, and ZIKA

NS5 RNA was utilized as a positive control. The plate was sealed with and optical adhesive cover and centrifuged using the Allegra X-15R centrifuge at 1000 rpm for 3 minutes. The reaction took place and standard cycle settings (Pabbaraju et al., 2016). Results were evaluated for amplification at C_T values between 12 and 38, as recommended by CDC guidelines under the Trioplex Real-time RT-PCR Assay (Juan Garcia, personal communication). Results were displayed as either positive (+), in which both wells per tissue displayed amplification within the detectable C_T range; mixed (+/-), in which one well fell within the detectable C_T range while another fell out of the detectable C_T range; negative (-) in which both wells per tissue fell out of the detectable C_T range.

ELISA

ELISA analyses were established and conducted by Dr. Andre Pastor and Dr. John VandeBerg at the South Texas Diabetes & Obesity Institute, University of Texas Rio Grande Valley. This analysis included 22- and 26-week old animal serum. ELISA was conducted in 96-well plates using, in each well, 10^2 PFU of inactivated ZIKV [PRVABC59] as the capture antigen, 100 µL of opossum serum diluted 1:50 in PBS-T supplemented with 1% BSA (PBS-T/B), and 100µL of goat anti-opossum IgG (H+L)-HRP conjugate (Alpha Diagnostic) diluted 1:1,000 in PBS-T/B as the detection antibody. The plates were read at 450 nm using a Thermo Multiskan FC. The result for each sample was expressed as an "endpoint titer," which is defined as the mean optical density of the sample assayed in triplicate divided by the negative/positive cut-off optical density. The cut-off optical density was determined for each plate by a mathematical manipulation of the mean optical density of multiple wells containing a negative serum pool. Samples with endpoint titers greater than 1.000 were scored as positive, samples with endpoint titers of 0.900 – 1.000 inclusive were scored as indeterminate (probably negative),

and samples with endpoint titers < 0.900 were scored as negative (John VandeBerg, personal communication).

CHAPTER IV

RESULTS

Antibody Stain

To determine animal susceptibility to ZIKV-BR infection and viral tissue distribution over a long-term period, immunohistochemistry (IHC) was first conducted on 22- and 26-week-old males and females. Tissues evaluated for ZIKV-NS1 protein via immunofluorescent microscopy included the brain, spleen, testes, epididymis, vagina, and ovaries. For the viral NS1 protein validation experiments, ZIKV-infected tissue stained without anti-NS1 antibody did not display signal of infection. ZIKV-infected tissue from the same animal stained with anti-NS1 antibody showed a distinct, punctate red pattern within various nuclei of the tissue (Figure 1).

In total, 38% of the tested animals displayed viral NS1 signal in at least one tissue type, indicating persistent infection at the time of organ harvesting. The teste showed the highest infection rate (40%), followed by the epididymis (33.33%), the female sex organs (25%), brain (23.08%), and finally the spleen (7.69%). Two 22-week females, P2142 and P2141, displayed signs of ZIKV-infection in the brain, ovary, and vagina, but not the spleen (Figure 2). The remaining females did not display NS1 signal in any of the four evaluated tissues. In the male 22-week-age group, P1968 only displayed NS1 signal in the teste, and P1967 displayed signs of NS1 protein in the brain, teste, epididymis, and the spleen. All animals that displayed positive

signs of viral infection in at least one tissue were infected at day 5 and belonged in the 22-week-age group, except for P1937, which was inoculated at day 7 and displayed viral infection in the epididymis at 26 weeks (Table 3).

One 26-week male displayed infection in the epididymis, which was the only tissue analyzed in this animal (Table 1). P1967 displayed an abnormal morphological appearance in the sex organs, which appeared necrotic, dark brown, and tumor-like (Figure 3). No other infected males displayed a similar morphology. Mock-infected animals displayed normal testicular appearance, which were smooth, round, and white (not pictured). The four age- and sex-matched PBS mock-infected control animals analyzed for antibody stain did not display any signs of NSI protein in the brain, spleen, testes, epididymis, vagina, or ovaries.

RT-PCR

Next, to genetically confirm susceptibility of the animals to ZIKV-BR and viral tissue distribution over a long-term period, viral RNA was extracted from tissue and RT-PCR was conducted on 22-week-old animals. Frozen tissue, parallel to those used in the immunohistochemistry analysis (brain, spleen, and sex organs), were evaluated for the ZIKV NS5 gene via RT-PCR. Ten of the twelve 22-week animals in the study had frozen tissue available for analysis. A mixed result indicated that one well amplified for viral NS5 gene within the detectable Ct range, while the duplicate well did not amplify within the same range. A mixed result was considered positive since NS5 amplification was detected in at least once instance. Out of this group, 100% of the animals displayed evidence of viral infection to some capacity (+ or mixed +/- result) in at least one tissue type (Table 2). The tissue with the highest infection rate analyzed through RT-PCR was the spleen (77.78%), followed by the epididymis (66.67%), teste (60%), ovary (50%), brain (44.4%), and finally vagina (25%). All tissue types displayed NS5

amplification to some degree (Table 2). All of the animals that displayed a positive RT-PCR result in at least one tissue type were inoculated at either 5 or 8 days of age (Table 3). No PBS-infected frozen animal tissue was available for this analysis. The positive control ZIKV-NS5 RNA amplified in duplicate within the detectable C_T range, and the DH20 negative control did show amplification withing the detectable C_T range (data not shown).

ELISA

To determine whether any animals developed an antibody response to ZIKV infection at the time of inoculation, serum was collected, and ELISA titers were obtained for animals in the 22- through 26-week age group (Figure 4). Three of the animals of the 22-week age group, P2142, P2141, and P2241, displayed a positive ELISA titer of 1.085, 1.043, and 1.663, respectively. These animals were infected at 5, 5, and 8 days of age, respectively. In the 26-week-group, three animals P1934, P1937 and P2187 had positive endpoint titers of 1.226, 1.475, and 1.143, respectively. These animals were infected at 7, 7, and 3 days of age, respectively. Surprisingly, one mock-infected PBS animal displayed and unexpected titer of 2.90; the rest of the animals had titers too low to be considered positive.

CHAPTER V

DISCUSSION

This study evaluated the long-term persistence and tissue distribution of ZIKV-BR in the juvenile laboratory opossums that were inoculated intracerebrally as infants with the virus; this is a novel model for ZIKV research. A basic requirement for developing a new model for disease transmission is its ability to harbor pathogen infection and replication. Moreover, long-term studies of infection from an age of early embryonic and fetal development through juvenile age are not feasible in other animal models. The nature of the laboratory opossum's developmental biology can be exploited for this type of study. To determine the susceptibility of the laboratory opossum to ZIKV-BR infection, as well as viral persistence and organ distribution, we analyzed tissues with IHC and RT-PCR.

Analysis of fixed tissue supported the presence of viral NS1 protein within the nuclei of various tissues in 38% of the infected animals that were evaluated through IHC (Table 1). The NS1 protein is shared among flaviviruses and is involved in viral replication (Young et al., 2000). Its detection through antibody staining shows that the laboratory opossum supports long-term ZIKV replication in the brain, testes, epididymis, ovaries, and vagina (Table 1; Figure 5). Non-specific binding of the anti-ZIKV NS1 antibody was ruled out through validation tests (Figure 1). Although animals were infected with an injection to the brain, evidence of viral replication is further shown by its dissemination to the sex organs. Three out of four animals that displayed NS1 signal were infected at 5 days old and sacrificed at 22 weeks, whereas only one

infected at 7 days of age and harvested at 26 weeks displayed NS1 signal (Table 3). More research needs to be conducted to determine any correlation between age of inoculation and viral persistence, as developmental time point could be relevant to persistence of infection. Animals nearing 6 days of age are developmentally equivalent to about a 12-week-old human fetus (Cardoso-Moreira 2016), the end point of the first trimester where fetal tissue may be more reactive to ZIKV infection. The results obtained from IHC overall indicate that the laboratory opossum was permissive to viral infection and replication of ZIKV-BR, and that chronic infection remained present in the brain, spleen, and sex organs at the time of tissue harvesting.

Unexpected findings in the analyzed tissue include the small number of samples that were observed to have viral NS1 signal. Possible explanations for this discrepancy can be attributed to a number of factors. First, we consider the possibility that some animals did not become infected at the time of inoculation. One limitation of this study is that animals were not screened for ZIKV infection prior to the study endpoint at 22 and 26 weeks. To the author's knowledge, there is not yet an established rate of successful infection for ZIKV in the neonate laboratory opossum. However, RT-PCR results that showed most 22-week-old animals had viral RNA to a limited extent in their tissues. This supports the idea that infection rates were higher than that observed through immunohistochemistry. RT-PCR also removes the possibility that animals did not retain infection long-term. This study also used ZIKV-BR for the first time in this model, while pilot studied have utilized the ZIKV-PR strain at different concentrations. Genetic differences can play a role, although both strains share similar nucleotide sequences (Sheridan at al., 2018).

The low number of infected tissues observed through antibody staining may be due to the amount of time tissues spent in fixative prior to their embedding, sectioning, and staining. At the time of tissue processing, samples had been fixed in 10% formalin for over a year. A study evaluating the effects of prolonged formalin fixation determined that for certain antigens, a decrease in the efficacy of immunohistochemical analysis may be observed after 7 weeks and they emphasize the use of antigen retrieval strategies to overcome prolonged fixation (Webster et al., 2009). While this study did not include the NS1 viral antigen, it is important to consider how long-term storage in formalin may have affected tissue integrity for antibody staining. Another study found that cross-linking between RNA, DNA, and proteins in tissue resulting from prolonged formalin fixation lasting one year limited the detection of nucleic acid from viral pathogens when analyzed with in situ hybridization (Mostegl et al., 2011). They also mention the use of proteinase K to reverse the effects of cross linking and found that it recovered antigen detection (Mostegl et al., 2011). While our study was still able to detect viral NS1 antigen despite prolonged fixation, a future direction may implement strategies to recover antigen and give more complete results for immunohistochemistry.

In establishing the *Monodelphis domestica* for ZIKV pathogenesis, various methodologies to test for infection remain to be optimized. One such analysis includes viral RNA extraction from tissue and subsequent RT-PCR analysis. We utilized RT-PCR to further evaluate the permissiveness and long-term tissue distribution of ZIKV-BR in our model. Previous attempts for RNA extraction of infected laboratory opossum tissues were unsuccessful when analyzed through RT-PCR. This could be in part due to the use of a viral RNA extraction kit that was not specific for fibrous and fatty tissue, such as those analyzed in this study. Previous studies with NHPs and mice have utilized RT-PCR for quantification of viral load in

tissues (Lazear et al., 2016; Li et al., 2016). To a very limited extent, we attempted RNA extraction with kits specific for fatty and fibrous tissue (discussed in the methods section) in the frozen tissues available for 22-week-old infected animals. No mock-infected animals were used in this procedure because frozen tissue was unavailable at the time of this analysis.

Detection of viral RNA occurred to some degree across all tissue types: brain, ovary, testis, epididymis, and spleen (Table 2 and 3; Figure 5). Interestingly, the tissue with the highest infection rate was the spleen (Figure 5). This correlates with findings that utilized RT-PCR also reported the spleen as one of the tissues with the highest viral load (Li et al., 2016), followed by the brain and testes (Lazear et al., 2016). We observed amplification of viral RNA in the same tissue types, as well as in female sex organs, which are also prone to ZIKV. Animals where persistent infection was determined were inoculated at 5 and 8 days of age (Figure 3), not showing a distinct preference for date inoculation as it relates to long-term persistence. Like in IHC findings, more studies need to be conducted to identify any correlations between developmental age of infection and persistence into juvenile age. While we could not develop a standard curve based on our limited data, detecting ZIKV RNA to some extent in our target tissues is a positive first step in developing and optimizing RNA extraction procedures for RT-PCR analysis. This may provide an opportunity to determine viral load in individual tissues.

A large number of mixed (+/-) results were observed in this analysis (Table 3), in which one well amplified for viral RNA and the other well in the duplicate set did not amplify within a valid C_T range, even though both samples were derived from the same tissue eluate. This finding can probably be attributed to the amount of time that passed from the time of tissue collection to the time of analysis, which occurred two years apart. While DNA is stable and may be stored for long-term at freezing temperatures, RNA is less stable and more prone to degradation by various

ribozymes that can retain enhanced reactivity at -70°C (Fabre et al., 2014). The tissues in this study may have experienced RNA degradation and fragmentation due to their long storage. C_T values for tissues displaying mixed amplification of viral RNA often reached the minimal threshold of detection, meaning that there was only a small amount of detectable RNA present. Mixed and negative data may therefore not be a result of lack of viral infection, but more of a question of tissue integrity and RNA preservation. More studies need to be conducted utilizing the method of RNA extraction used in this experiment with properly stored samples in order to validate an assay for RT-PCR in the laboratory opossum.

Overall, more tissues were identified to contain ZIKV-BR infection through RT-PCR analysis than with IHC staining procedures (Figure 5). While the IHC staining was validated and NS1 was not determined to have non-specific binding (Figure 1), RT-PCR seemed to be more sensitive in detecting viral infection in tissues. Given the overall question of integrity of the analyzed tissues, inconsistent findings in infection between tissues of the same animal analyzed through different methods is not surprising. For this study, both methods provide an overall view into the different types of tissues that retained infection long-term.

To detect whether any animals developed antibodies against ZIKV at the time of infection, ELISA was conducted on the serum of 22- and 26-week-old animals. The laboratory opossum gives birth to underdeveloped pups that lack immunologic organs and an adaptive immune system (Old and Deane, 2000). During the first two weeks post-partum, pups latch on to the mother's teat and do not begin to produce their own antibodies until about day 7 (Belov et al., 2007). Subsequently, the pups detach and undergo intermittent suckling for about 20 days, during which exposure to new pathogens occurs, as well as an increase in immunocompetence (Belov at al., 2007). The animals in this study were exposed to ZIVK-BR between 3 and 8 days

of age. It was expected that newborn (embryonic) pups would be too young to recognize a foreign pathogen, would become immunologically tolerized to it, and would not develop antibodies against ZIKV even after the development of their immune system. ELISA results utilizing ZIKV-PR (PRVABC59) as the capture antigen showed that indeed, most ZIKV-infected animals had a negative endpoint titer (Figure 4). However, 6 infected animals had positive titers. These animals were infected throughout the entire range of age post-partum (3,5,6,7,8 days). This result suggests that those animals were not tolerized and they became capable of recognizing ZIKV as foreign as their immune systems developed, and that the date of inoculation did not have an effect in antibody production in these animals. More research is needed to evaluate any correlation between age of inoculation and production of antibodies against ZIKV. However, for the purposes of this study, animals with positive titers further support the notion that at least some of the infant laboratory opossums developed ZIKV infection. Additionally, the production of antibodies in this analysis was directed against ZIKV-PR, whereas animals were infected with ZIKV-BR. These results indicate the possibility of cross-reactivity between antibodies produced against different strains of ZIKV. This result is not surprising, as ZIKV-PR and ZIKV-BR strains both belong to the Asian lineage and vary by less than 4% in their nucleic acid sequence (Sheridan et al., 2018). It should be noted that one mock-infected animal produced a positive endpoint titer. It remains unclear as to why such a result occurred, but it may be attributed to the inadvertent exposure of this specific animal to ZIKV during the long experimental time period of 6 months. IHC analysis and RT-PCR remain to be conducted to further analyze evidence of infection in this animal.

The long-term nature of this study is unique to this model and mimics the infection pattern of infants who were exposed to ZIKV as a result of vertical transmission. Pups were

intracerebrally injected at 3-8 days old and were evaluated between 19-22 weeks of age. The laboratory opossum reaches sexual maturity at about 6 months of age (VandeBerg and Blangero, 2010), meaning that the animals in this study were reaching this timepoint. The onset of sexual maturity is a critical period in the health of humans, the repercussions of which remain unknown in children that were infected in utero during the 2015 ZIKV outbreaks. The sexual transmission of ZIKV can be explained by the findings of viral RNA in the sex organs and secretions of humans, mice, and NHPs (Musso et al., 2015; Atkinson et al., 2016; Lazear et al. 2016; Hirsh et al., 2017). One study noted that infected male mice displayed ZIKV in the testes, which led to orchitis and epididymitis, as well as a dark-brown appearance and reduced testosterone levels (Ma et al., 2016). Interestingly, this correlated with one of the males in our study group, P1967, which was euthanized prior to the experimental endpoint due to an inflamed scrotum. Upon closer examination during necropsy, its sexual organ appeared as a necrotic, tumor-like mass and displayed viral NS1 protein in the brain, teste, and epididymis (Figure 3). It is likely that this morphological abnormality is a result of ZIKV infection, as has been described in other models.

It is important to consider whether infected infants continue to harbor infection into puberty, as this could potentially have detrimental impacts on their sexual health. Moreover, the possibility of vertical transmission must be considered in these individuals. Anecdotal evidence in the laboratory opossums has shown incidences of animals infected at an early developmental stage reaching sexual maturity, breeding, and giving birth to pups that remain infected with ZIKV-BR. This study provides some insight into the persistence of viral infection in the tissues crucial to the sexual development of males and females: the brain, testis, epididymis, ovary, and vagina.

Due to the novel nature of the laboratory opossum in realm of arboviral research, there is room for improvement and future studies. Based on the limitations of this study, future directions in research can implement the use of antigen retrieval strategies for improved recovery of antigen in fixed tissue. This study also displayed promising RT-PCR results as a new method of tissue RNA extraction was implemented. Future studies could optimize this procedure. Moving forward, studies to determine the sexual health of infected juveniles, such as determining how sex hormone levels are affected, could provide insight into the significance of ZIKV in juvenile lives and the possibility for future vertical transmission.

Through immunohistochemistry, we were able to identify signs of ZIKV infection across the following tissues: Brain, spleen, ovary, vagina, testis, and epididymis. The ELISA data supports the susceptibility of neonatal pups to ZIKV infection, as some were shown to develop antibodies against ZIKV. Moreover, RT-PCR showed to a limited extent that genetic confirmation of ZIKV can be determined in tissues of infected animals. Each individual analysis conducted in this study, while limited to some capacity, together display support for the *Monodelphis domestica* as a competent model for ZIKV pathogenesis research. Moreover, we showed that animals infected at a neonatal stage can retain infection long-term into juvenile age, where target organs such as the brain and sex organs continue to display signs of infection. Overall, the laboratory opossum continues to show promise as a model for *in vivo* ZIKV replication studies.

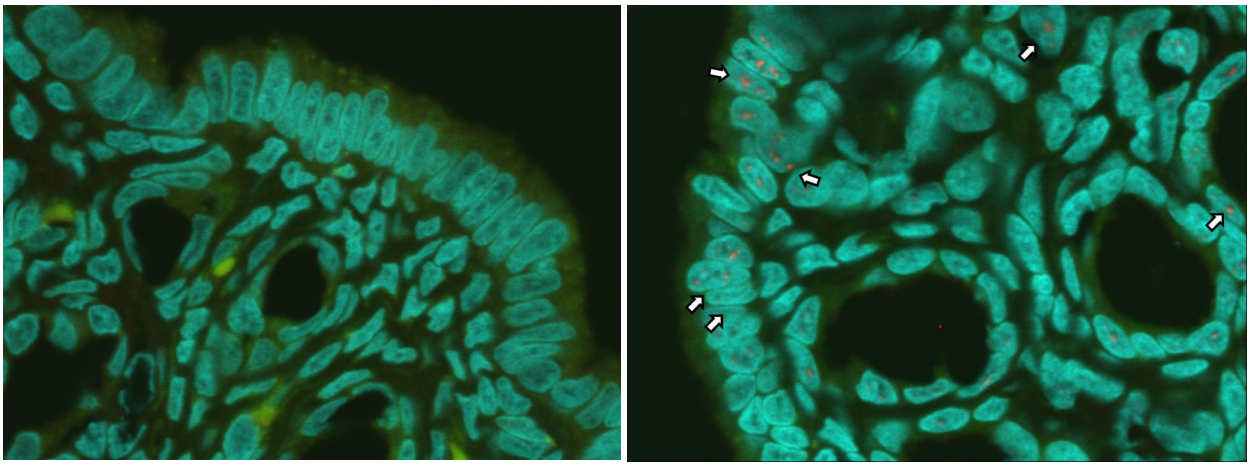


Figure 1. Confirmation of NS1 Protein in Infected Ovary. Immunofluorescence staining of brain section from ZIKV-infected 22-week-old female (P2141) incubated without primary anti-NS1primary antibody (left) and with primary anti-NS1 antibody (right) at 60x (picture zoomed in for detail). White arrows point out nuclei displaying signs of ZIKV infection (red). Sections stained with anti-ZIKV NS1 monoclonal antibody (red). Cytoskeleton is stained green; nuclei are blue.

				Immunofluorescent Signal Detection (Y/N)					
ID number	Age(wks)	Sex	Treatment	Brain	Ovary	Vagina	Spleen	Teste	Epididymis
P2133	22	F	BZV	N	N	N	N	-	-
P2142	22	F	BZV	Y	Y	Y	N	-	-
P2141	22	F	BZV	Y	Y	Y	N	-	-
P2241	22	F	BZV	N	N	N	N	-	-
P1965	22	F	BZV	N	N	N	N	-	-
P2087	22	F	BZV	N	N	N	N	-	-
P1967	19	M	BZV	Y	-	-	Y	Y	Y
P1968	21	M	BZV	N	-	-	N	Y	N
P2138	22	M	BZV	N	-	-	N	N	N
P2146	22	M	BZV	-	-	-	-	-	-
P2246	22	M	BZV	-	-	-	-	-	-
P2090	22	M	BZV	N	-	-	N	N	N
P2275	26	F	BZV	N	N	N	N	-	-
P2276	26	F	BZV	N	N	N	N	-	-
P1937	26	M	BZV	-	-	-	-	-	Y
P2279	26	M	BZV	N	-	-	N	N	N
P2300	22	F	PBS	N	N	N	N	-	-
P2306	22	M	PBS	N	-	-	N	N	N
P1945	26	F	PBS	N	N	N	N	-	-
P2296	26	M	PBS	N	-	-	N	N	N

Table 1. Detection of ZIKV-NS1 Viral Protein in Animal Tissues Intracerebrally Inoculated with Virus or PBS. Presence of NS1 protein indicated by the observation of secondary antibody AlexaFluor 546 (Thermo Fisher Scientific, USA). BZV: Brazilian Zika Virus; PBS: Phosphate buffer solution; Y: Yes- immunofluorescent signal was detected; N: No- immunofluorescent signal was not detected; Not stained: tissues were embedded and sectioned, but not stained due to insufficient time.

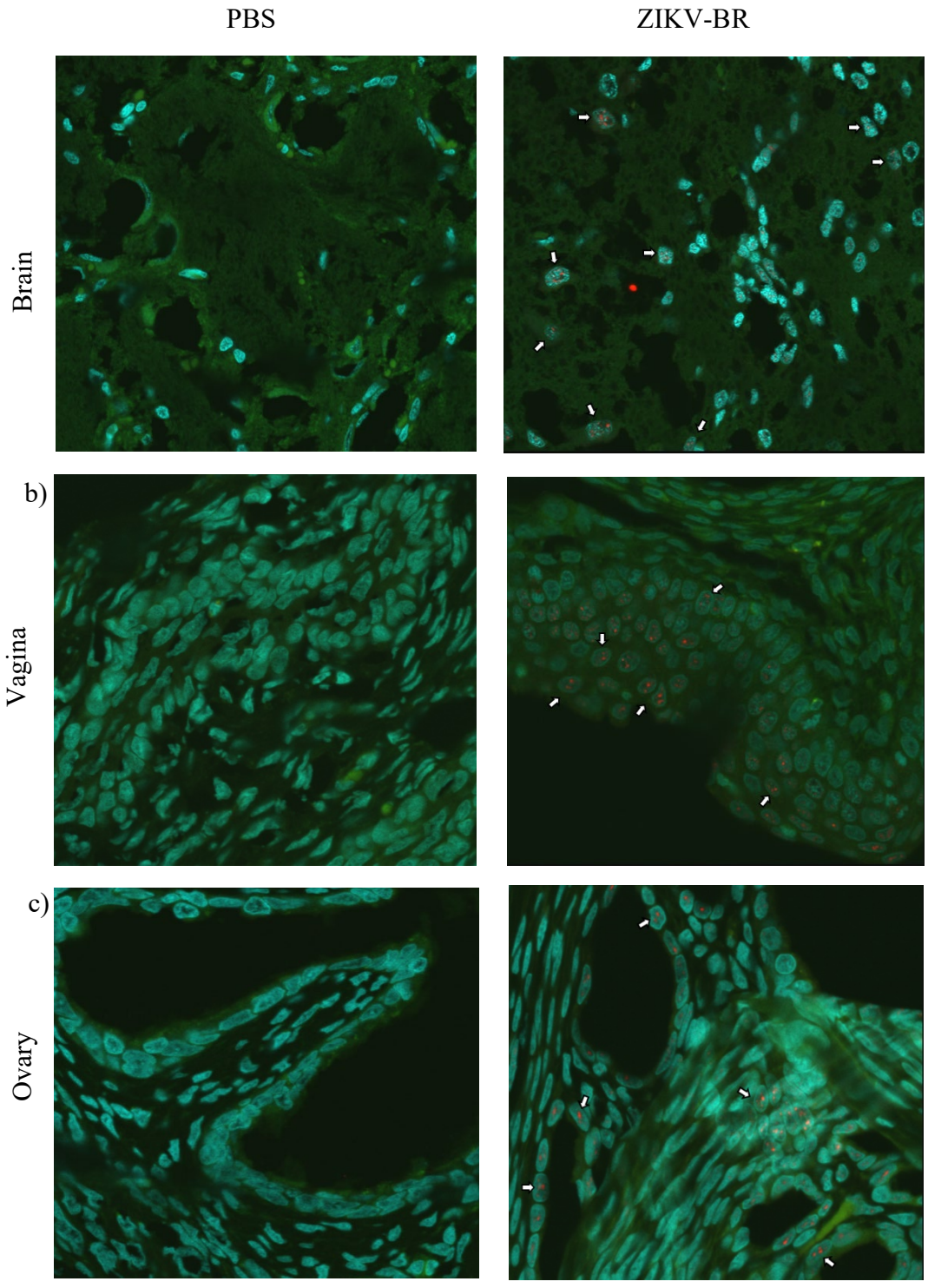


Figure 2. Immunohistochemical Detection of ZIKV in Tissue. (a) Immunofluorescence staining of brain section from mock-infected female (left) and BZV-infected female (right) at 60x. **(b)** Vagina section from mock-infected female (left) and BZV-infected female (right) at 60x. **(c)** Ovary section from mock-infected female (left) and BZV-infected female (right) at 60x.

White arrows point out nuclei displaying signs of ZIKV infection (red). Sections stained with anti-ZIKV NS1 monoclonal antibody (red). Cytoskeleton is stained green; nuclei are blue. Picture zoomed in for detail.

a) b) c)

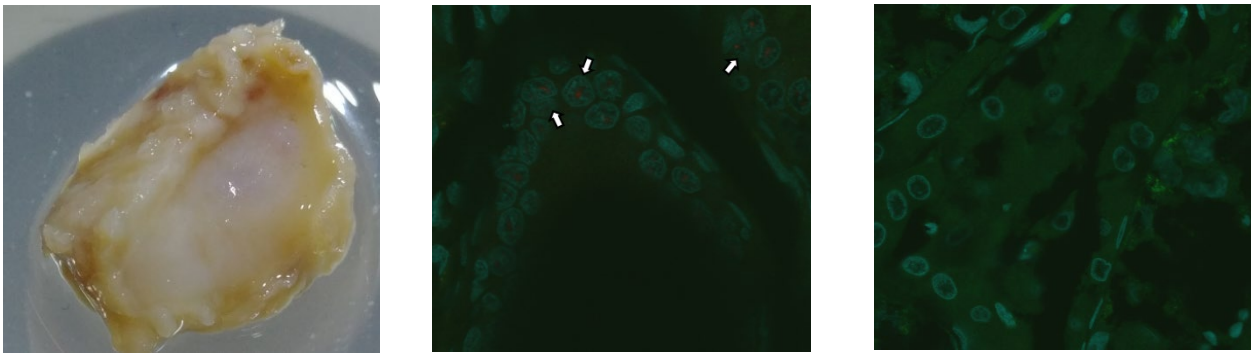


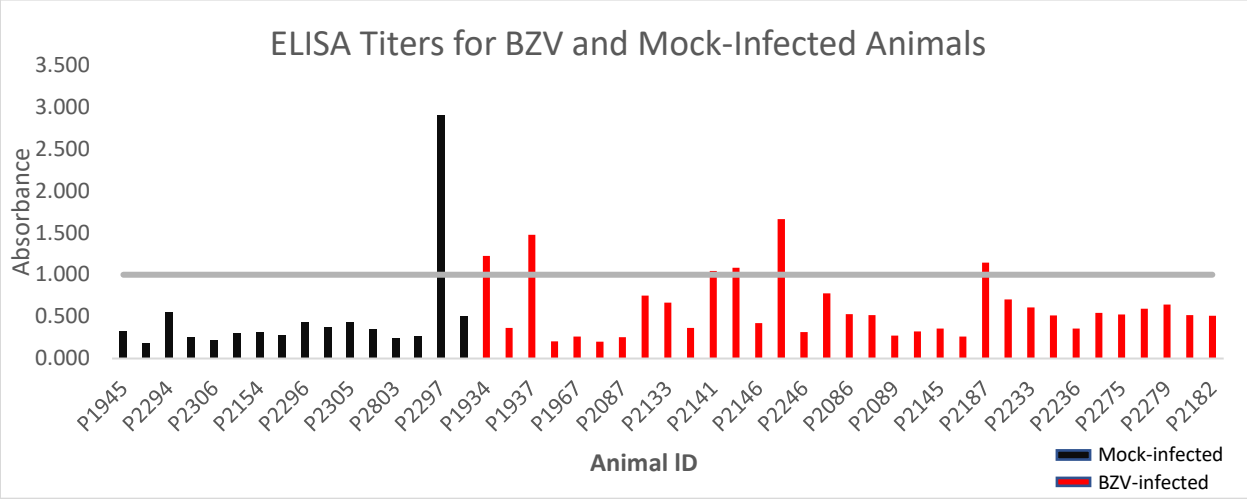
Figure 3. Immunohistochemical detection of ZIKV in abnormal teste from animal P1967. **(a)** Image shows necrotic tumor-like appearance of reproductive tissue prior to sectioning. **(b)** Immunofluorescence staining of sectioned tissue from panel (a) at 60x. White arrows point out nuclei displaying signs of ZIKV infection (red). **(c)** Immunofluorescence staining of aged-matched mock-infected control (P2306) at 60x. Sections stained with anti-ZIKV NS1 monoclonal antibody (red). Cytoskeleton is stained green; nuclei are blue.

			RT-PCR Results					
ID Number	Age(wks)	Sex	Brain	Ovary	Vagina	Spleen	Teste	Epididymis
P2133	22	F	-	+	+	/	/	/
P2241	22	F	-	-	-	mix (+/-)	/	/
P1965	22	F	/	mix (+/-)	-	mix (+/-)	/	/
P2087	22	F	mix (+/-)	-	-	mix (+/-)	/	/
P1967	19	M	-	/	/	mix (+/-)	*	*
P1968	21	M	-	/	/	-	+	mix (+/-)
P2138	22	M	mix (+/-)	/	/	-	mix (+/-)	-
P2146	22	M	+	/	/	+	-	mix (+/-)
P2246	22	M	-	/	/	mix (+/-)	-	mix (+/-)
P2090	22	M	mix (+/-)	/	/	mix (+/-)	mix (+/-)	+

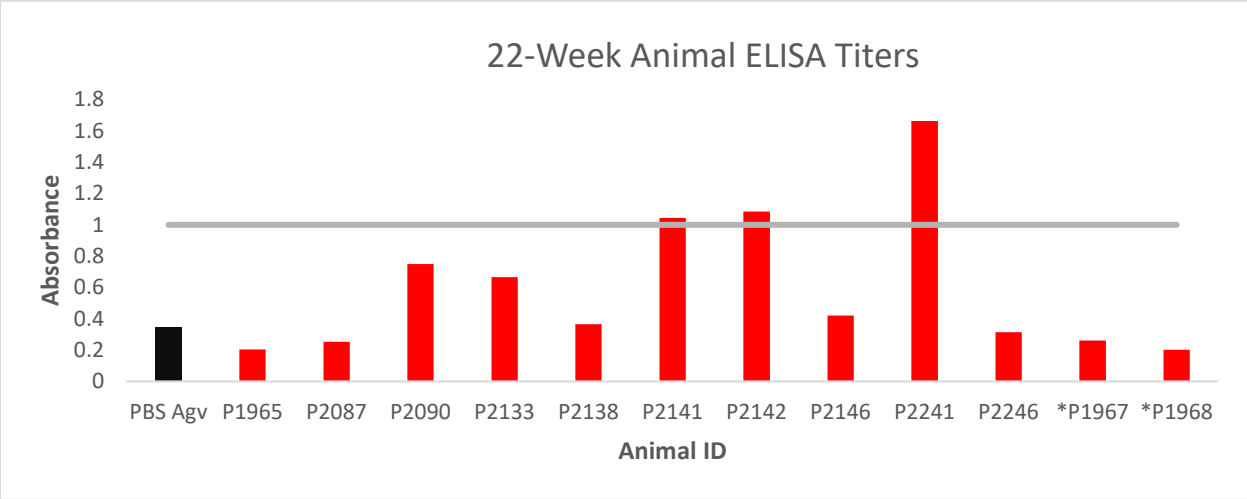
Table 2. RT-PCR Results for 19-22-Week-Old Animals. Each set of tissues was run in duplicate wells on PCR 96-well plate. BZV RNA was used as positive control and gave a valid C_T value (data not shown). DH20 was used as negative control and gave a valid C_T value (data not shown). Negative sign (-) indicates C_T values that fell out of the detectable amplification range. Positive sign (+) indicates C_T values that fell within detectable amplification range. Mix (+/-) indicates one set of values that fell within detectable range, while the second set fell outside of the detectable amplification range. Slash (/) indicates tissue was not analyzed for RT-PCR. *P1967 frozen tissue not available for analysis due to unusual reproductive morphology. Definitive positive results are highlighted.

Figure 4.

a)


ELISA Titers for BZV and Mock-Infected Animals
Absorbance
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
P1945
P2294
P2306
P2154
P2296
P2305
P2803
P2297
P1934
P1937
P1967
P2087
P2133
P2141
P2146
P2246
P2086
P2089
P2145
P2187
P2233
P2236
P2275
P2279
P2182
Animal ID
Mock-infected
BZV-infected

b)


22-Week Animal ELISA Titers
Absorbance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS Agv
P1965
P2087
P2090
P2133
P2138
P2141
P2142
P2146
P2241
P2246
*P1967
*P1968
Animal ID

c)

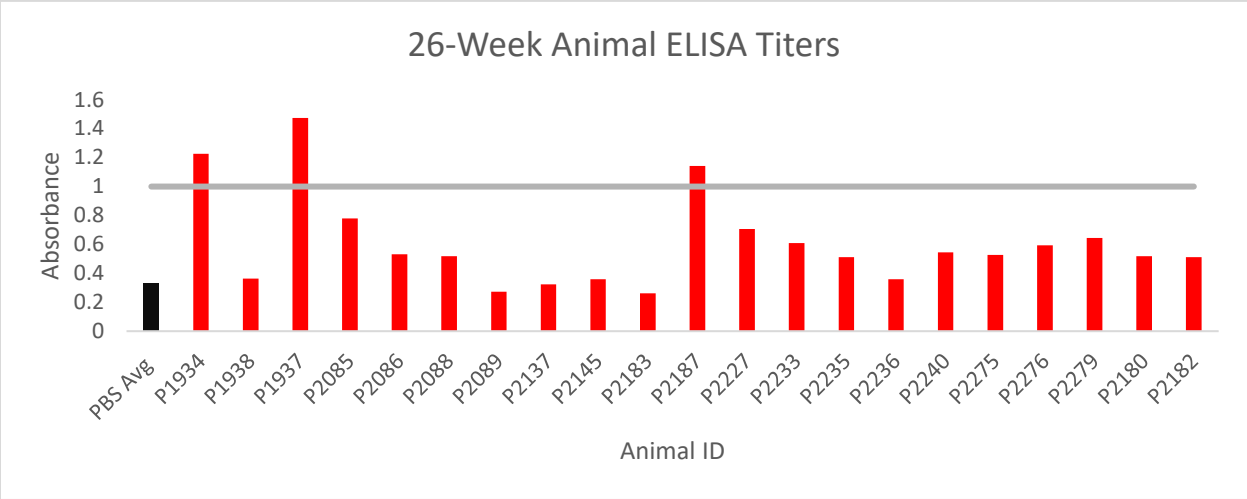

26-Week Animal ELISA Titers
Absorbance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS Avg
P1934
P1938
P1937
P2085
P2086
P2088
P2089
P2137
P2145
P2183
P2187
P2227
P2233
P2235
P2236
P2240
P2275
P2276
P2279
P2180
P2182
Animal ID

Figure 4 ELISA Results. (a) ELISA absorbance values for serum of BZV-inoculated and PBS-inoculated animals. **(b)** 22-week-age group only. **(c)** 26-week-age group only. Values above 1.00 are considered positive titers. Values between 9.00 and 1.00 are considered inconclusive. Values below 9.00 are considered negative. PBS values indicate the average titers of all PBS-inoculated 22-week-old animals, except P2297, which showed an unexpected high titer. *P1967 and P1968 are 19 and 22-week-animals, respectively.

ID	Inoculation Age (Days After Birth)	Age at Necropsy (Wks)	Treatment	Sex	Persistent Infection Detected (Yes/No)		ELISA Titer (+/-)
					IHC	RT-PCR	
P2133	5	22	ZIKV-BR	F	N	Y	(-)
P2142	5	22	ZIKV-BR	F	Y	N/A	(+)
P2141	5	22	ZIKV-BR	F	Y	N/A	(+)
P2241	8	22	ZIKV-BR	F	N	Y	(+)
P1965	5	22	ZIKV-BR	F	N	Y	(-)
P2087	8	22	ZIKV-BR	F	N	Y	(-)
P1967	5	19	ZIKV-BR	M	Y	Y	(-)
P1968	5	21	ZIKV-BR	M	Y	Y	(-)
P2138	5	22	ZIKV-BR	M	N	Y	(-)
P2146	5	22	ZIKV-BR	M	N/A	Y	(-)
P2246	8	22	ZIKV-BR	M	N/A	Y	(-)
P2090	8	22	ZIKV-BR	M	N	Y	(-)
P2275	6	26	ZIKV-BR	F	N	N/A	(-)
P2276	6	26	ZIKV-BR	F	N	N/A	(-)
P1937	7	26	ZIKV-BR	M	Y	N/A	(-)
P2279	6	26	ZIKV-BR	M	N	N/A	(-)
P2300	6	22	PBS	F	N	N/A	(-)
P2306	6	22	PBS	M	N	N/A	(-)
P1945	7	26	PBS	F	N	N/A	(-)
P2296	6	26	PBS	M	N	N/A	(-)
P1934	7	26	ZIKV-BR	F	N/A	N/A	(+)
P1937	7	26	ZIKV-BR	M	N/A	N/A	(+)
P2187	3	26	ZIKV-BR	M	N/A	N/A	(+)
P2297	6	22	PBS	M	N/A	N/A	(+)

Table 3. Summary of Results for ZIKV- and Mock-Infected Animals. Table shows results for three assays described in this study: antibody staining (IHC), RT-PCR, and ELISA. ELISA titers are only shown for animals that were analyzed by IHC and/or RT-PCR, except for three

26-week animals displaying positive titers, and one 22-week PBS mock-infected control that displayed an unexpected high titer. Date of inoculation of either ZIKV-BR or PBS is included. F-female; M-male; Y-yes, evidence of infection was detected; N-no, evidence of infection was not detected; N/A-tissues not analyzed with the indicated assay; (+)-positive ELISA titer >1.000; (-)-negative ELISA titer <0.900. RT-PCR results displaying (Y) indicate that at least one well of tissue sample amplified for ZIKV-NS5 gene within the detectable C_T range (12-38); RT-PCR results displaying (N) indicate that no wells amplified for ZIKV-NS5 gene within the detectable C_T range.

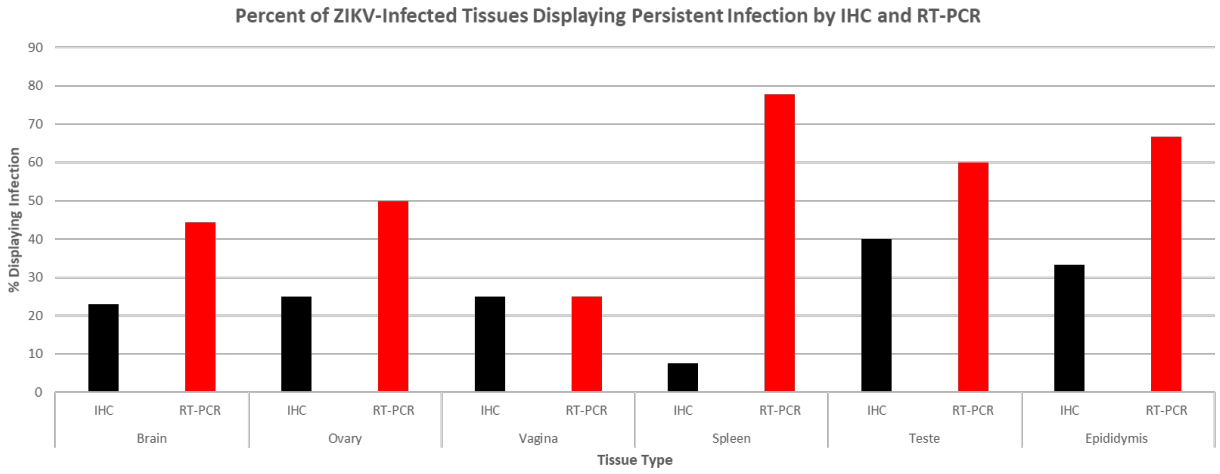


Figure 5. Comparison of Infected Tissue Analyzed through IHC and RT-PCR. Black bars represent IHC-analyzed tissue; red bars represent RT-PCR-analyzed tissue.

REFERENCES

Adams, Waldorf, et al. “Fetal Brain Lesions after Subcutaneous Inoculation of Zika Virus in a Pregnant Nonhuman Primate.” *Nature medicine* 22.11 (2016): 1256–1259.

Atkinson, Barry, et al. “Detection of Zika Virus in Semen.” *Emerging infectious diseases* 22.5 (2016): 940–940.

Apte-Sengupta, et al. “Coupling of Replication and Assembly in Flaviviruses.” *Current opinion in virology* 9 (2014): 134–142.

Barzon, Luisa, et al. “Infection Dynamics in a Traveller with Persistent Shedding of Zika Virus RNA in Semen for Six Months after Returning from Haiti to Italy, January 2016.” *Euro surveillance : bulletin européen sur les maladies transmissibles* 21.32 (2016): n. pag.

Bhatnagar, Julu, et al. “Zika Virus RNA Replication and Persistence in Brain and Placental Tissue.” *Emerging infectious diseases* 23.3 (2017): 405–414.

Brasil, Patrícia, et al. “Zika Virus Outbreak in Rio de Janeiro, Brazil: Clinical Characterization, Epidemiological and Virological Aspects.” *PLoS neglected tropical diseases* 10.4 (2016): e0004636–.

Brasil, Patrícia, et al. “Zika Virus Infection in Pregnant Women in Rio de Janeiro.” *The New England journal of medicine* 375.24 (2016): 2321–2334.

Caine, Elizabeth, et al. “Animal Models of Zika Virus Infection During Pregnancy.” *Viruses* 10.11 (2018): 598–.

Calvet, Guilherme, et al. “Detection and Sequencing of Zika Virus from Amniotic Fluid of Fetuses with Microcephaly in Brazil: a Case Study.” *Lancet Infectious Diseases, The* 16.6 (2016): 653–660.

Cao-Lormeau, Van-Mai, et al. “Guillain-Barré Syndrome Outbreak Associated with Zika Virus Infection in French Polynesia: a Case-Control Study.” *Lancet, The* 387.10027 (2016): 1531–1539.

Cardoso-Moreira, Margarida, et al. “Gene Expression Across Mammalian Organ Development.” *Nature (London)* 571.7766 (2019): 505–509.

Chan, Jeannie, et al. “Differential Expression of Hepatic Genes Involved in Cholesterol Homeostasis in High- and Low-Responding Strains of Laboratory Opossums.” *Metabolism* 57.5 (2008): 718–724

Counotte, Michel Jacques, et al. “Sexual Transmission of Zika Virus and Other Flaviviruses: A Living Systematic Review.” *PLoS medicine* 15.7 (2018): e1002611–.

Cugola, Fernanda R., et al. “The Brazilian Zika Virus Strain Causes Birth Defects in Experimental Models.” *Nature (London)* 534.7606 (2016): 267–271

de Fatima Vasco Aragao, Maria, et al. “Clinical Features and Neuroimaging (CT and MRI) Findings in Presumed Zika Virus Related Congenital Infection and Microcephaly: Retrospective Case Series Study.” *BMJ* 353 (2016): i1901–.

Dick, G.W.A, S.F Kitchen, and A.J Haddow. “Zika Virus (I). Isolations and Serological Specificity.” *Transactions of the Royal Society of Tropical Medicine and Hygiene* 46.5 (1952): 509–520.

Duggal, Nisha K., et al. “Sexual Transmission of Zika Virus Enhances in Utero Transmission in a Mouse Model.” *Scientific reports* 8.1 (2018): 4510–8

Elong Ngono, Annie, and Sujan Shresta. “Immune Response to Dengue and Zika.” *Annual review of immunology* 36.1 (2018): 279–308.

Fabre, Anne-Lise, et al. “An Efficient Method for Long-Term Room Temperature Storage of RNA.” *European journal of human genetics : EJHG* 22.3 (2014): 379–385.

Fernandez-Garcia, Maria-Dolores, et al. “Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell.” *Cell host & microbe* 5.4 (2009): 318–328.

França, Giovanny, V.A., et al. “Congenital Zika Virus Syndrome in Brazil: a Case Series of the First 1501 Livebirths with Complete Investigation.” *The Lancet (British edition)* 388.10047 (2016): 891–897.

Grant, Alesha, et al. “Zika Virus Targets Human STAT2 to Inhibit Type I Interferon Signaling.” *Cell host & microbe* 19.6 (2016): 882–890.

Govero, Jennifer, et al. “Zika Virus Infection Damages the Testes in Mice.” *Nature (London)* 540.7633 (2016): 438–442.

Hamel, Rodolphe, et al. “Biology of Zika Virus Infection in Human Skin Cells.” *Journal of virology* 89.17 (2015): 8880–8896.

Hirsch, Alec J., et al. “Zika Virus Infection of Rhesus Macaques Leads to Viral Persistence in Multiple Tissues.” *PLoS pathogens* 13.3 (2017): e1006219–.

Iglesias et. al. Ch.3 Flaviviruses. In: Viral Genome Replication. Cameron, Gotte, Ramsey (editors) 2009. Springer.

Kuhn, R. J. et al. (2002) Structure of Dengue Virus. Cell. [Online] 108 (5), 717–725

Kushwaha, Rampratap S, Jane F VandeBerg, and John L VandeBerg. “Effect of Dietary Cholesterol with or Without Saturated Fat on Plasma Lipoprotein Cholesterol Levels in the Laboratory Opossum (Monodelphis Domestica) Model for Diet-Induced Hyperlipidaemia.” British journal of nutrition 92.1 (2004): 63–70.

Li, Cui, et al. “Zika Virus Disrupts Neural Progenitor Development and Leads to Microcephaly in Mice.” *Cell stem cell* 19.5 (2016): 672–672.

Li, Xiao-Feng, et al. “Characterization of a 2016 Clinical Isolate of Zika Virus in Non-Human Primates.” *EBioMedicine* 12.C (2016): 170–177.

Lazear, Helen M., et al. “A Mouse Model of Zika Virus Pathogenesis.” *Cell host & microbe* 19.5 (2016): 720–730.

Ma, Wenqiang, et al. “Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice.” *Cell (Cambridge)* 167.6 (2016): 1511–1524.e10.

Magnani, Diogo M., et al. “Fetal Demise and Failed Antibody Therapy During Zika Virus Infection of Pregnant Macaques.” *Nature communications* 9.1 (2018): 1–8.

Manangeeswaran, Mohanraj, Derek D. C Ireland, and Daniela Verthelyi. “Zika (PRVABC59) Infection Is Associated with T Cell Infiltration and Neurodegeneration in CNS of Immunocompetent Neonatal C57Bl/6 Mice.” *PLoS pathogens* 12.11 (2016): e1006004–.

Martines, Roosecelis Brasil, et al. “Notes from the Field : Evidence of Zika Virus Infection in Brain and Placental Tissues from Two Congenitally Infected Newborns and Two Fetal Losses — Brazil, 2015.” *MMWR. Morbidity and mortality weekly report* 65.6 (2016): 159–160.

Martinot, Amanda J., et al. “Fetal Neuropathology in Zika Virus-Infected Pregnant Female Rhesus Monkeys.” *Cell (Cambridge)* 173.5 (2018): 1111–1122.e10.

Mavigner, Maud, et al. “EMERGING INFECTIONS: Postnatal Zika Virus Infection Is Associated with Persistent Abnormalities in Brain Structure, Function, and Behavior in Infant Macaques.” *Science translational medicine* 10.435 (2018): n. pag.

Miner, Jonathan J., et al. “Zika Virus Infection During Pregnancy in Mice Causes Placental Damage and Fetal Demise.” *Cell (Cambridge)* 165.5 (2016): 1081–1091.

Miner, Jonathan J, and Michael S Diamond. “Zika Virus Pathogenesis and Tissue Tropism.” *Cell host & microbe* 21.2 (2017): 134–142.

Morelli, Fabrício, et al. “Zika Virus Infection in the Genital Tract of Non-Pregnant Females: a Systematic Review.” *Revista do Instituto de Medicina Tropical de São Paulo* 62 (2020): e16–15.

Mostegl, Meike M., et al. “Influence of Prolonged Formalin Fixation of Tissue Samples on the Sensitivity of Chromogenic in Situ Hybridization.” *Journal of Veterinary Diagnostic Investigation* 23.6 (2011): 1212–1216.

Musso, Didier, et al. “Potential Sexual Transmission of Zika Virus.” *Emerging infectious diseases* 21.2 (2015): 359–361.

Nelson, B.R., et al. “Immune Evasion Strategies Used by Zika Virus to Infect the Fetal Eye and Brain.” *Viral immunology* (2019): n. pag.

Nem de Oliveira Souza, Isis, et al. “Acute and Chronic Neurological Consequences of Early-Life Zika Virus Infection in Mice.” *Science translational medicine* 10.444 (2018): eaar2749–.

Nguyen, Sydney M., et al. “Highly Efficient Maternal-Fetal Zika Virus Transmission in Pregnant Rhesus Macaques.” *PLoS pathogens* 13.5 (2017): e1006378–.

Old, J.M, and E.M Deane. “Development of the Immune System and Immunological Protection in Marsupial Pouch Young.” *Developmental and comparative immunology* 24.5 (2000): 445–454.

Pabbaraju, Kanti, et al. “SIMULTANEOUS DETECTION OF ZIKA, CHIKUNGUNYA AND DENGUE VIRUSES BY A MULTIPLEX REAL-TIME RT-PCR ASSAY.” *Journal of Clinical Virology* 83 (2016): 66–71.

Pawitwar, Shashank S, et al. “Overview on the Current Status of Zika Virus Pathogenesis and Animal Related Research.” *Journal of Neuroimmune Pharmacology* 12.3 (2017): 371–388.

Petersen, Lyle R., et al. “Zika Virus.” *The New England journal of medicine* 374.16 (2016): 1552–1563.

Prisant, Nadia et al. “Zika Virus in the Female Genital Tract.” *The Lancet infectious diseases* 16.9 (2016): 1000–1001.

Powell, Jeffrey R, and Walter J Tabachnick. “History of Domestication and Spread of Aedes Aegypti - A Review.” *Memórias do Instituto Oswaldo Cruz* 108.suppl 1 (2013): 11–17.

Rainwater, David L, and John L VandeBerg. “Dramatic Differences in Lipoprotein Composition Among Gray Short-Tailed Opossums (Monodelphis Domestica) Fed a High Cholesterol/saturated Fat Diet.” *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism* 1126.2 (1992): 159–166.

Samollow, Paul, “Status and application of genomic resources for the gray, short-tailed opossum, Monodelphis domestica, an American marsupial model for comparative biology.” *Australian Journal of Zoology* 10.1071 (2006): n. pag.

Shan, Chao, et al. “An Infectious cDNA Clone of Zika Virus to Study Viral Virulence, Mosquito Transmission, and Antiviral Inhibitors.” *Cell host & microbe* 19.6 (2016): 891–900.

Simpson, D.I., "Zika Virus Infection in Man." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 58.4 (1964): 335–38.

Suchetana Mukhopadhyay, Richard J. Kuhn, and Michael G. Rossmann, "A Structural Perspective of the Flavivirus Life Cycle." *Nat Rev Microbiol* 10.1038 (2005): 13-22.

Tang, Hengli et al. "Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth." *Cell stem cell* 18.5 (2016): 587–590.

VandeBerg, J.L. and Williams-Blangero S. "The Laboratory Opossum." The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals. (2010): Jan 19;8:246-61

van den Pol, Anthony N., et al. "Zika Virus Targeting in the Developing Brain." *The Journal of neuroscience* 37.8 (2017): 2161–2175.

Wang, Zhiqiang, et al. "The Laboratory Opossum (Monodelphis Domestica) as a Natural Mammalian Model for Human Cancer Research." *International journal of clinical and experimental pathology* 2.3 (2009): 286–299.

Young, Paul R et al. "An Antigen Capture Enzyme-Linked Immunosorbent Assay Reveals High Levels of the Dengue Virus Protein NS1 in the Sera of Infected Patients." *Journal of clinical microbiology* 38.3 (2000): 1053–1057.

Yun, Sang-Im, and Young-Min Lee. "Zika Virus: An Emerging Flavivirus." *The journal of microbiology* 55.3 (2017): 204–219.

Zanluca, Camila, et al. "First Report of Autochthonous Transmission of Zika Virus in Brazil." *Memórias do Instituto Oswaldo Cruz* 110.4 (2015): 569–572.

BIOGRAPHICAL SKETCH

Marisol Morales earned her Master of Science degree in biology at the University of Texas Rio Grande Valley in 2020, where she focused on characterizing a new animal model for Zika virus pathogenesis research. During her time at UTRGV, Marisol also participated in the processing and testing of patient samples for COVID-19 under the direction of Dr. John Thomas. In December of 2015, Marisol received her Bachelor of Science degree in animal science from Texas A&M University, where she worked as a clinical pathology student technician. After graduating, Marisol worked in the virology section of the Texas A&M Veterinary Medical Diagnostic Laboratory until 2017. Marisol's research interests include the study of viruses and the resulting host pathology. Marisol will be pursuing her PhD in Integrated Biomedical Sciences at the University of Texas Health Science Center San Antonio, where she plans to continue research in microbiology and immunology.

Marisol can be reached at: morales.marisol93@gmail.com.