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INFECTION AND PERSISTENCE OF ZIKA VIRUS IN FEMALE IMMUNE-COMPETENT GRAY SHORT-TAILED OPOSSUMS *(Monodelphis domestica).*

A Thesis

by

OSCAR QUINTANILLA

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

DECEMBER 2020

Major Subject: Biology

INFECTION AND PERSISTENCE OF ZIKA VIRUS IN FEMALE IMMUNE-COMPETENT

GRAY SHORT-TAILED OPOSSUMS *(Monodelphis domestica).*

A Thesis
by
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December 2020

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ABSTRACT

Quintanilla, Oscar, Infection and Persistence of Zika Virus in Female Immune-Competent Gray Short-Tailed Opossums Master of Science (MS), December 2020, 38pp, 1 table, 5 figures, references, 43 titles.

Zika virus (ZIKV) is a class IV RNA virus transmitted by *Aedes* species of mosquitoes. It produces flu-like symptoms, but recent studies have associated additional, more severe pathologies with ZIKV like microcephaly. Currently, no effective treatment options or vaccines exist. This is partly due to limitations of current animal models. *Monodelphis domestica* (the gray short-tail opossum) may serve as a proper model for ZIKV research. Here we test the susceptibility of *M. domestica* to ZIKV infection. Female, immune-competent subjects were injected with live ZIKV and analyzed serologically and histologically for evidence of ZIKV replication. ELISAs performed on sera confirmed that subjects injected with ZIKV generated anti-ZIKV antibodies. Additionally, ZIKV nonstructural protein 1 was detected in sex organs through immunohistochemical analysis up to 26 weeks post-infection. These data suggest that infection was established and persisted in sex organs. *M. domestica* may therefore serve as a practical animal model for ZIKV research.

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CHAPTER I

INTRODUCTION

Zika virus (ZIKV) is a pathogen most notorious for its association with microcephaly in infants (Cauchemez et al., 2016), and with Guillain-Barre syndrome (Barbi et al., 2018; Şahiner et al., 2017) and ocular pathologies (de Paula Freitas et al., 2017; Şahiner et al., 2017) in adults. It is transmitted primarily through arthropod vectors like *Aedes* species of mosquitoes but has also been shown capable of vertical transmission transplacentally and horizontal transmission between sexual partners (Sharma and Lal, 2017). It was discovered in 1947 in the Zika forest of Uganda (Dick, 1952a) and shown to infect humans in 1952 (Dick, 1952b). At the time, this raised very little concern since only mild pathologies were observed in humans. As a result, ZIKV was largely ignored by the scientific community for decades (Wikan and Smith, 2016). Recent outbreaks, however, have uncovered more severe manifestations of ZIKV infection (Weaver et al., 2016). Many cases of infants born with microcephaly emerged during the 2013/2014 French Polynesia outbreak and the 2015/2016 Brazil outbreak (Cauchemez et al., 2016; Weaver et al., 2016). The Brazil outbreak in particular attracted much attention from health professionals and the general public alike, and the World Health Organization (WHO) considered ZIKV enough of a threat to declare it a public health emergency of international concern. The emergency status was lifted the same year, but these events nevertheless inspired a wave of research focused on characterizing ZIKV pathogenesis. Much has been learned about ZIKV because of this wave of research, but there remain many gaps in our knowledge. We have

not fully characterized its pathogenesis, and there is no effective vaccine approved for human use at the time of this study. This is in part due to ZIKV being novel from a research perspective. Compared to many other pathogens for which we have treatments and vaccines, ZIKV has only been studied for a short time. Additionally, there exists a phenomenon known as antibody-dependent enhancement (ADE) whereby immunity to one viral strain predisposes an individual to an exacerbated infection by a different, but antigenically similar, strain of virus. This is a concern with ZIKV immunization since ZIKV is phylogenetically and antigenically similar to Dengue virus (DENV), which is well-known for ADE (Katzelnick et al., 2017), and some studies have shown antigenic cross-reactivity between ZIKV and DENV (Giraldo-García and Castaño-Osorio, 2019). Vaccines developed against ZIKV must therefore be tested rigorously, preferably in multiple animal models, before being approved for widespread human use. Results from certain vaccine challenge studies have shown promise but demonstrating vaccine effectiveness in multiple animal models would be more predictive of that effectiveness translating to humans (Vannice et al., 2019). As such, access to a variety of suitable animal models would hasten vaccine and treatment development. Unfortunately, only few animal models are currently available for ZIKV research, and those that do exist have various limitations.

Here, we explore the potential of *Monodelphis domestica* (the gray short-tailed opossum) as a model for ZIKV research. *M. domestica* opossums are native to South America. They sometimes also referred to as laboratory opossums given their use as model organisms for certain forms of biomedical research (VandeBerg and Robinson, 1997). Various features make these organisms well-suited for ZIKV research. Firstly, they are practical to work with, especially compared with models like non-human primates (NHP's). They are described as docile (VandeBerg and Robinson, 1997), and they are relatively easy to care for. Secondly, like mouse

models, these opossums are small and produce large litters, making it feasible to achieve high sample sizes and to reproduce experiments. Finally, they are marsupials and therefore finish embryonic development after birth. This makes them ideal for developmental studies which have utmost relevance in ZIKV research considering the association between ZIKV and microcephaly. Of course, these animals should be susceptible to ZIKV infection, ideally in an immune-competent state, if they are to serve as models for ZIKV research. The purpose of this study, therefore, was to address two questions: first, whether immune-competent *M. domestica* opossums are susceptible to ZIKV infection; and second, whether viral replication persists in pathologically relevant organs. To test this, we challenged female opossums with two separate doses of live ZIKV: one dose at 18 weeks of age and one dose at 20 weeks of age. Opossums were then sacrificed at either 22 weeks of age or 46 weeks of age and their sera and sex organs were analyzed. Immunohistochemical (IHC) analysis of sex organs like the uterus, ovaries, and vagina revealed presence of ZIKV nonstructural 1 (NS1) protein. Sera were screened for anti-ZIKV antibodies via ELISA. The ELISA results confirmed that immune-competent opossums challenged with ZIKV recognized the pathogen and mounted an adaptive immune response. Adaptive immunity generally takes two weeks to develop (Alberts et al., 2002). Traditionally, pathogens are cleared from the host once adaptive immunity is engaged, but some pathogens are known to circumvent adaptive countermeasures and persist long after immunologic memory is established (Charles A Janeway et al., 2001). Since these opossums were sacrificed well after they generated adaptive immunity, presence of viral protein in various organs strongly suggest persistence of ZIKV.

CHAPTER II

REVIEW OF THE LITERATURE

Classification of Zika Virus

Zika virus (ZIKV) can be classified in various ways, but two are especially relevant for this study. Firstly, it can be classified phylogenetically as a *Flavivirus*. Other notable members of the *Flavivirus* genus include dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV) (Kuno et al., 1998). Like ZIKV, these other members are also transmitted by arthropod vectors. In addition to mode of transmission, members of the genus also share many similarities regarding their replication strategy and genome organization (Hansen et al., 2019). Understanding the close genetic relatedness between ZIKV and other members of the genus is important when discussing ZIKV biology as there are many molecular aspects conserved within the genus.

Secondly, ZIKV can be classified by the type of genome it has and, by extension, the replication strategy is has adopted. Based on the Baltimore classification scheme, ZIKV has a class IV genome (Lodish et al., 2000). A class IV genome is defined as a single strand ribonucleic acid (RNA) genome of positive sense orientation. Positive sense RNA genomes have open reading frames (ORF) that are directly translated by host ribosomes into the polypeptide encoded in the genome; without the need for an intermediate transcript (Berman, 2012). In fact, from the perspective of the host cell, a *flavivirus* genome is essentially a messenger RNA molecule, complete with a 5’ methylguanosine cap and a 3’ untranslated region

(UTR) that folds into secondary structures that functionally mimic eukaryotic poly-A tails (Mazeaud et al., 2018).

Zika virus biology and intracellular replication

All viruses consist of a genome encapsidated by a protein shell, collectively referred to as a nucleocapsid. Additionally, some viruses have lipid membranes enveloping the core nucleocapsid, typically with proteins embedded. ZIKV is one such enveloped virus, and the envelope proteins it has determine the cells it can attach to and infiltrate. In fact, attachment to host cells is the first step in the replication cycle of all viruses and is highly dependent on the host receptors that viral surface proteins can recognize and bind to (Dimitrov, 2004). In the case of flaviviruses, viral particles (virions) are believed to accumulate on cell surfaces via electrostatic interactions between viral envelope (E) protein and host attachment factors like glycosamino glycans (GAG's) (Perera-Lecoin et al., 2013). ZIKV E proteins subsequently bind to Tyro 3 and AXL receptors (Mohd Ropidi et al., 2020) as well as C-type lectin receptors like DC-SIGN, which are commonly found on the surfaces of myeloid cells (Agrelli et al., 2019; Hamel et al., 2015; Perera-Lecoin et al., 2013). Binding to these receptors induces internalization of the surface-bound virions via clathrin-coated endocytosis (Agrelli et al., 2019; Fernandez-Garcia et al., 2009; Perera-Lecoin et al., 2013). The endosomes subsequently fuse with lysosomes in the cytoplasm which result in acidification of the resultant endolysosome. The drop in pH induces conformational changes in the viral E protein which allow for the fusion of the viral envelope with the membrane of the endolysosome, expelling naked nucleocapsids in the process (Fernandez-Garcia et al., 2009; Mohd Ropidi et al., 2020). They then preferentially travel to the rough endoplasmic reticulum (RER) where RER-bound ribosomes translate the viral genomes. The ZIKV genome encodes a polypeptide that is cleaved into ten proteins (eleven if

the prM protein is considered two proteins). These proteins vary in function, but all (either directly or indirectly) contribute to the replication and amplification of the viral genome as well as the assembly of new virions (Fernandez-Garcia et al., 2009). Newly assembled virions are trafficked through the golgi where they mature and are then released into the extracellular environment (Fernandez-Garcia et al., 2009; Sirohi and Kuhn, 2017). Released virions may then attach to other adjacent, susceptible cells and begin the cycle anew.

Zika virus genome and proteins

The ZIKV genome is approximately 11kb in length and encodes a long polypeptide that is cleaved into ten functional proteins. Although each protein has a highly specific function, they can be broadly categorized as structural and nonstructural (NS) proteins. Structural proteins are incorporated into new virions, and they include the capsid (C), membrane (M), and E proteins. NS proteins are responsible for replicating genomic strands, remodeling the host intracellular architecture to promote viral replication, subverting intracellular antiviral machinery, and facilitating the assembly of virions (Fernandez-Garcia et al., 2009; Mohd Ropidi et al., 2020; Sirohi and Kuhn, 2017). NS proteins are not incorporated into virions and therefore are not usually present in cells unless translation of viral genome strands has occurred. There are a total of seven NS proteins and are named numerically as NS1 through NS5 (NS2 and NS4 have "a" and "b" variants).

Translation of the ZIKV genome primarily occurs along the RER membrane; some regions of the polypeptide face the cytosol whereas others face the lumen of the RER (Fernandez-Garcia et al., 2009; Wang et al., 2017). Cleavage of the polypeptide is mediated by both host cell proteases and by translated viral proteases (Wang et al., 2017). Following cleavage, NS proteins induce remodeling of RER architecture to produce tubular networks and

invaginations of the RER membrane(Cortese et al., 2017). These structures act as sheltered sites where replication complexes can form. Replication complexes are highly organized congregation of NS proteins that recruit and replicate viral genomes. Essentially, they act as viral genome factories. The resultant genomes can either be translated (resulting in the production of more replication complexes) or bound to structural proteins to form nascent virions.

Current model of Zika virus pathogenesis and tissue tropism

Although not fully characterized, we have a generalized model of how ZIKV disseminates throughout the mammalian host. Traditionally, ZIKV virions bypass epidermal barriers by being deposited into dermal layers by mosquito vectors (Sharma and Lal, 2017). In the dermis, virions infect various cells such as fibroblasts, keratinocytes, and myeloid cells (Hamel et al., 2015; Miner and Diamond, 2017a). Myeloid cells in particular are believed to be involved in promoting viral dissemination by acting as carriers that transport ZIKV virions from the dermis to lymphatic circulation (Aid et al., 2017; Ayala-Nunez et al., 2019). From the lymphatics, ZIKV gains access to cardiovascular circulation and disseminates to target tissues (Miner and Diamond, 2017a). Some organs and cells known to be susceptible to ZIKV infection as confirmed by case studies, in vitro studies, and in vivo studies include, but are not limited to: neural progenitor cells and glial cells in the brain; trophoblasts, Hofbauer cells, and endothelial cells in the placenta; vaginal epithelium; Leydig cells, Sertoli cells, and spermatogonia in testes; ganglion cells, bipolar neurons, and cornea of the eye; and fibroblasts, keratinocytes, macrophages, and dendritic cells in the dermis (el Costa et al., 2016; Hamel et al., 2015; Miner and Diamond, 2017b, 2017a; Shaily and Upadhya, 2019). Taking this into account, it becomes apparent how some of these susceptible tissues may be involved in transplacental transmission

and the development of neurotropically driven pathologies. It remains unclear, however, how ZIKV gains access to some of the more restricted sites like the brain and reproductive organs, though some researchers have explored various mechanisms (Ayala-Nunez et al., 2019). Nevertheless, more research is needed to fully understand the factors affecting ZIKV dissemination, infiltration of restricted and immune-privileged sites, and long-term viral persistence in lymphatic and reproductive organs.

Current animal models for Zika virus research

Studying the effects of ZIKV infection in humans relies heavily on in vivo experiments using animals as models. Mice and non-human primates (NHP's) are the most commonly used models for ZIKV research. Other animals such as guinea pigs, hamsters, pigs, sheep, and chick embryos have also been used or proposed as models and are extensively reviewed elsewhere (Dong and Liang, 2018; Morrison and Diamond, 2017; Narasimhan et al., 2020), but here the discussion will be limited to mice and NHP's as they are the most widely accepted models for this research.

Mice are the most practical and accessible model to study ZIKV. They are small, cheap, and easy to work with. Many different varieties exist, but two broad groups are used for ZIKV research: immune-competent [wild type (WT)] mice and immune-deficient mice. WT mice are very limited in applicability as only newborn mice exhibit a pathologic response after being challenged with ZIKV (Lazear et al., 2016). Nevertheless, studies using WT mice have been conducted, but the types of studies that can be conducted using these animals is limited by the narrow age range during which they are susceptible to ZIKV infection. Researchers therefore rely on mouse strains deficient in interferon (ifn) for experiments designed to study ZIKV infection in adult mammals, transplacental vertical transmission, and horizontal sexual

transmission (Morrison and Diamond, 2017). The main advantage to using ifn-deficient mice is that they reproduce some of the pathologies observed in humans. Normally, ifn is the primary defense against viral infection, so artificially ablating its function means that the model no longer simulates standard physiologic conditions. Results from studies using ifn-deficient mice are nevertheless interesting and ought to be used to formulate hypotheses for subsequent studies, but they ought not be interpreted as being wholly representative of ZIKV infection in humans.

NHP's are genetically and physiologically more similar to humans. This makes them excellent research models for biomedical research in general. Some NHP's that have been used for ZIKV research are pigtail macaques, rhesus macaques, and cynomolgus macaques (Morrison and Diamond, 2017). The main disadvantage with NHP's is that they are relatively inaccessible models for many institutions. This is in part because of their cost. Caring for one macaque throughout the course an experiment can cost upwards of $15,000 or even $25,000 in the case of pregnancy studies (Morrison and Diamond, 2017). This, combined with the fact that they have long developmental time periods compared with mice and produce fewer offspring per pregnancy, makes it difficult to achieve high sample sizes. Reproducing experiments conducted with NHP's is also time-consuming and impractical. Additionally, working with NHP's requires highly trained and qualified staff. All of this taken together make NHP's inaccessible for many researchers.

It is clear that ZIKV research is partly limited by the shortage of appropriate animal models. Treatment and vaccine development benefit from consistent results across multiple studies, preferably utilizing different models. As such, establishing an additional model for ZIKV research would broaden the number of studies that can be conducted and thereby hasten development of appropriate countermeasures against ZIKV.

Monodelphis domestica as a potential model for Zika virus research

One animal that remains largely unexplored within the context of ZIKV research, outside of a few pilot studies and a pending manuscript (Thomas et al., 2019), is *Monodelphis domestica*, the gray short-tailed opossum. *M. domestica* opossums are marsupials native to South America. They are quite small relative to their North American and Latin American *Didelphis* counterparts, more comparable to small rats in size. They produce large litters of pups which, at birth, are developmentally similar to 5-week-old human embryos (Cardoso-Moreira et al., 2019). This makes them promising models for studying the developmental effects of ZIKV research. They are also docile, practical to work with, and can be housed using relatively standard equipment like the very same cages used with mice (Rousmaniere et al., 2010), making them quite accessible. Additionally, they have already been established as research models for other forms of biomedical research like metabolic, developmental, evolutionary, and cancer studies to name a few (Rousmaniere et al., 2010; VandeBerg and Robinson, 1997; Wagner, 2018). As a result, many aspects of *M. domestica* biology have already been characterized. Taken together, these features make *M. domestica* opossums promising as candidate models for ZIKV research.

Statement of the problem

ZIKV remains a global concern as of 2020. The mosquito vectors responsible for transmitting it are widespread, inhabiting every continent except Antarctica. The pathologies associated with ZIKV range from mild flu-like symptoms to severe neurological conditions like microcephaly in newborns and Guillain-Barre syndrome in adults. Given that ZIKV research was only seriously undertaken within the past decade, its pathogenesis remains poorly understood and effective vaccines and treatment options have not been developed. Progress in

characterizing ZIKV infections is partly limited by the shortage of suitable animal models. A suitable animal model for ZIKV research ideally should have three important qualities: firstly, it should be accessible and practical to work with; secondly, it should be susceptible to ZIKV infection in an immune-competent state; and thirdly, it should exhibit a similar tissue tropism as that observed in human patients. To date, existing animal models for ZIKV research fulfil some, but not all, of the aforementioned qualities. Mice, for example, are practical to work with, but only exhibit observable ZIKV-associated pathologies when their ifn system is knocked down; and NHP's genetically and physiologically resemble humans but are expensive and difficult to work with.

Purpose of this study

The present study tests whether *M. domestica* meets all the qualities of a suitable animal model for ZIKV research. It has already been established that *M. domestica* is practical and accessible as a biomedical research model. Here, we therefore only assess whether immune-competent *M. domestica* opossums are susceptible to ZIKV infection, and we test for evidence of viral replication in pathologically relevant sites. Sites considered pathologically relevant are any which may act as sites for viral persistence or portals of transmission. Studies in NHP's have demonstrated persistence of ZIKV in lymphoid organs (Aid et al., 2017). We therefore chose the spleen as a representative organ of lymphoid tissue as it is acts as a sort of hub or crossroad for both lymphatic and cardiovascular circulation. More importantly, however, we also chose to test for viral replication in reproductive organs including the uterus, ovaries, and vagina. These structures are significant when studying ZIKV infection as they are potential portals for vertical transmission from mother to offspring and horizontal transmission between sexual partners.

CHAPTER III

MATERIALS AND METHODS

Animals

The opossum model, *Monodelphis domestica*, used in this study were produced in a colony maintained at The University of Texas Rio Grande Valley, Brownsville, Texas. All animals were bred and maintained under standard conditions (VandeBerg and Blangero, 2010).

Virus Preparation and Cells

Virus preparation was performed by research lab manager, Juan Garcia, at the University of Texas Rio Grande Valley, Edinburg, Texas. ZIKV isolate BR1911 (ZIKV-BR) was used for this study. Virus titration was performed using Vero-76 cells (CCL-81; ATCC, USA). Lyophilized virus was passaged in C6/36 cells (CRL-1660; ATCC, USA) derived from *Aedes albopictus* to produce working stocks of live ZIKV-BR. Virus obtained from the initial reconstituted lyophilized stock was passaged once in C6/36 cells. The resultant supernatant was clarified and purified over a sucrose cushion. Virus supernatants were quantified in duplicate by standard plaque assay (Shan et al., 2016). Aliquots of live ZIKV-BR were prepared and stored at -80°C (John Thomas and Juan Garcia, personal communication).

Animal Infections

All animal infections were conducted under the direct supervision of Dr. John VandeBerg and his research associates led by Susan Mahaney of the Department of Human Genetics,

University of Texas Rio Grande Valley, Brownsville, Texas. Animals were injected intraperitoneally, intramuscularly, or subcutaneously with either 5µL of ZIKV-BR (BR1911) at 10^5 PFU, or mock-infected with 5µL of sterile phosphate-buffered saline (PBS) placebo. There were 64 animals injected with ZIKV-BR and 26 animals injected with PBS, for a total of 90 animals. Animals within each group received two identical doses two weeks apart: one at 18 weeks of age and a second dose at 20 weeks of age. Some animals were sacrificed at 22 weeks of age, but others were saved for mating and instead sacrificed at 46 weeks of age. Five of animals injected with ZIKV-BR and three of animals injected with PBS died before the intended sacrifice date and were excluded from the study. Sacrificed animals were exsanguinated for serum isolation and organs were dissected for immunohistochemical analysis. Organs were either preserved in 10% formalin or kept frozen -80°C for preservation until the time of tissue analysis (Susan Mahaney, personal communication).

ELISA

"ELISA was conducted in 96-well plates using, in each well, 102 PFU of inactivated ZIKV-BR (BR1911) (prepared in Dr. John Thomas' laboratory) as the capture antigen, 100 µL of opossum serum (Alpha Diagnostic) 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 a titer defined as the mean optical density of the sample assayed in triplicate divided by the positive/negative 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 titers greater than 1.000 were scored as positive, samples with titers of 0.900 – 1.000 inclusive were scored as indeterminate (probably negative), and samples with titers < 0.900 were scored as negative." (John VandeBerg, personal communication).

Tissue fixation and slide preparation

Dissected organs were fixed in a solution of sterile PBS (Gibco, USA) and 4% formaldehyde and stored at room temperature. When organs were selected for analysis, fixative was thoroughly washed from tissue using a mixture of sterile PBS and Tween 20 (PBTB). Three initial quick washes were performed followed by three slow washes; each slow wash involved a 10-min incubation of the organs in sterile PBTB. Next, the tissue samples were incubated for 60 min in 30% OCT mix (30 % OCT mounting media and 60% sterile PBS), followed by 3x quick washes in sterile PBTB. Organs were then incubated for 60 min in 60% OCT mix (60% OCT mounting media and 30% sterile PBS), followed by 3x quick washes in PBTB. Finally, organs were incubated overnight in 100% OCT mounting media. When ready for sectioning, organs were frozen in small, cuboidal molds at -22°C in 100% OCT mounting media. The resultant frozen OCT block-embedded organs were sectioned using a cryostat (Leica Biosystems, USA) to produce 5 μm slices which were mounted onto Frost + microscope slides. Two slices were mounted per slide and stored at -20°C until ready for immunohistochemical analysis.

Immunohistochemistry staining

Organs of interest were analyzed through immunohistochemistry (IHC). There organs included reproductive organs and spleens. Each slide contained two mounted organ sections. Organ sections were individually surrounded with a hydrophobic barrier to contain reagents at

the sites where sections were mounted and to prevent spillover of reagents between the two sections on a given slide. Sections were blocked to prevent nonspecific binding of antibodies. Blocking involved incubation of mounted sections in a blocking solution consisting of sterile PBS + .01% Tween20 + 0.2% BSA (PBTB) for 1 hour. Blocking solution was removed from sections and replaced with a solution of monoclonal anti-ZIKV NS1 primary antibody at a dilution of 1:500 (Arigo Biolaboratories, Taiwan) for 1 hour at room temperature. Primary antibody was removed from sections by washing 3X quickly, then 3X slowly (10 min incubation) in PBTB. Tissue was then incubated in a solution of secondary antibody at a dilution of 1:200 for two hours in a humidifier chamber at 4°C. Secondary antibody used was AlexaFluor 546-conjugated antibody directed against the primary antibody (Thermo Fisher Scientific, USA). Secondary antibody was removed by washing 3X quickly with PBTB. Sections were then incubated for 10 mins in a solution of DAPI (Thermo Fisher Scientific, USA) and AlexaFluor 488-conjugated phalloidin (Thermo Fisher Scientific, USA) dissolved in PBTB at 1:1000 and 1:200 dilutions, respectively. Sections were then washed slowly twice with PBTB. Sections were sealed with cover slips and stored at 4°C until imaging. Imaging of slides was performed using an Olympus FV10i confocal microscope. Protocol for Antibody staining was provided by Dr. Matthew D. Terry, an Associate Professor at University of Texas Rio Grande Valley.

Primary antibody specificity was validated by including a section on each slide which was not treated with primary antibody but was otherwise treated the same (antibody control). Each organ analyzed thus included an antibody control against which a section stained with the standard protocol described above would be compared.

Analysis of confocal images

Slides were visualized using confocal microscopy (Olympus FV10i). Organs were rated as being positive or negative for the presence of ZIKV NS1 depending on the fluorescent patterns observed. Slides were considered positive if non-artifact fluorescence signal was observed in concentrated regions around or within cells, preferably if in a distinct punctate pattern characteristic of viral replication sites. Slides were considered negative if no such fluorescence was observed or if the observed fluorescence was very clearly artifact. In addition to positive or negative, some images displayed fluorescence signal that resembled the pattern characteristic of positive signal, but only weakly so. These ambiguous cases were therefore labeled as weakly positive.

CHAPTER IV

RESULTS

ELISA

To assess susceptibility of immune-competent M. domestica opossums to ZIKV infection, it was first important to confirm that the subjects had functional and responsive immune systems. To this end, female subjects were challenged with either live ZIKV or a PBS placebo via intraperitoneal (IP), subcutaneous (SC), or intramuscular (IM) routes. Sera were isolated and screened using ELISA's for the presence of anti-ZIKV antibodies. ELISA titers with a value greater than 1.000 were considered positive, titers less than 0.900 were considered negative. Positive values are indicative of an adaptive immunity generated in response to ZIKV challenge and negative are indicative of no adaptive immunity generated against ZIKV. Of the 59 ZIKV-injected animals, 98% (58) of them were assigned a positive status. All (23 of 23) of the PBS-injected animals were assigned a negative status. The results of the ELISA's can be seen in **Figure 1**. The results are divided into three graphs, each corresponding to a route of administration: IP (**1A**), IM (**1B**), and SC (**1C**). In all three groups the animals treated with ZIKV showed significantly higher titers of anti-ZIKV antibodies than the animals treated with the PBS placebo. Only one animal (1397) that was treated with ZIKV had an anti-ZIKV antibody titer that was scored negative. Not included in the results are animals that died prematurely and excluded from the study.

Immunohistochemical analysis

We were interested in determining if immune-competent M. domestica opossums could sustain ZIKV infection in pathologically relevant organs. For this study, we specifically analyzed the uterus, vagina, ovaries, and spleen. To test if ZIKV replication had occurred in these organs, immunohistochemical (IHC) analysis was performed to screen for ZIKV NS1 protein. Organs from both the ZIKV-treated group and the PBS control group were analyzed.

Additionally, animals were sacrificed at two different ages: 22 weeks of age (two weeks after their last inoculation) and 46 weeks of age (26 weeks after their last inoculation). Organs from 12 animals from the ZIKV-treated group and from 6 animals from the PBS control group were analyzed. Specifically, the spleen, uterus, vagina, and ovaries were selected for their relevance in ZIKV pathogenesis and transmission.

Before analyzing IHC images and comparing organs from PBS control animals with organs from ZIKV-treated animals, we validated the specificity of our antibodies. With IHC there is always a risk that the secondary antibody will engage in non-specific binding and tag structures that don't actually carry the target antigen. This is largely mitigated by including a blocking step, but antibody validation is still necessary, both to ensure that the blocking step was successful and to establish a baseline fluorescence of the secondary antibody's conjugated probe in the absence of antigen. Validation involves the staining of a tissue section in duplicate where one section is treated with primary antibody and one section, the validation control, is not. The validation control serves to mimic a tissue section where target antigen is absent. **Figure 2** shows an example of a validation run performed on a uterus from a ZIKV-infected animal. These images are meant to highlight the difference in secondary antibody fluorescence pattern between the two sections of the same organ. The image in panel **2A** shows a tissue section

stained using standard IHC protocol. The image in panel **2B** shows a tissue section that was stained without the presence of primary antibody. All organs included in this analysis were run in duplicate, with one of the sections serving as a validation control.

The difference between tissues scored negative and tissues scored positive can be seen in **Figure 3** which compares images of organs derived from PBS-treated animals with images of organs derived from ZIKV-treated animals. Images of positive tissues display red focal points of signal, often colocalized with nuclei or peripherally associated with them. Because the counterstain used (phalloidin) tags cytoskeletal elements, but not cellular membranes, it is difficult to determine in some cases if the observed signal is intracellular or interstitial. This is most apparent in the positive spleen from panel **3B**. Here, the red signal is closely associated with nuclei, but not quite colocalized with them as is the case in panel **3D**. Interestingly, many of the reproductive organs seem to show NS1 signal highly colocalized with nuclei and it is only the spleens which show NS1 signal almost exclusively in cytosolic or perhaps interstitial locations. This difference in NS1 fluorescence patterns between the reproductive organs and the spleen can be further seen in **Figure 4** which shows representative images of each type of organ analyzed. The images are broken down into the individual fluorescence channels: blue for dapi-stained nuclei; green for phalloidin-stained cytoskeleton; red for NS1 viewed indirectly through alexafluor546-tagged secondary antibody; and a merge image with all channels displayed. Separating the channels in this way makes the NS1 signal in each image more discernable. Here again the reproductive organs, i.e. vagina (**4C**), uterus (**4D**), and ovary (**4E**), show NS1 signal in a punctate pattern colocalizing with nuclei. This was the case for many, but not all, of the reproductive organs observed. The spleen section from a ZIKV-infected animal (**4B**) shows NS1 signal between nuclei.

The co-localization of NS1 signal and nuclei of cells from reproductive organs can be better appreciated in **figure 5** panel **5A** which features highly magnified (180x) image of an ovary section from a ZIKV-infected animal. However, although the majority of the reproductive organs observed displayed this colocalization between NS1 signal and nuclei, there were some which instead exhibited a pattern more similar to that seen in the spleens from figures **3B** and **4B**. Panel **5B** shows a section of an ovary from a ZIKV-infected animal. In this image, high NS1 signal can be seen between nuclei, but little, if any, can be seen within the nuclei themselves. These ovaries were extracted from animals of the same age (22 weeks), but the injection routes differed: subcutaneous in **5A** and intramuscular in **5B**.

Table 1 shows the results of the tested organs for each animal of the 12 ZIKV-treated animals, six showed clear positive fluorescence signal in at least one of the analyzed organs. Of these six there were two animals (1426 and 1477) which showed clear positive signal in all three reproductive organs studied. An additional two animals showed clear positive signal in two reproductive organs: 1424 had a positive-scoring uterus and vagina; 1465 had a positive-scoring uterus and ovary. The remaining two (1350 and 1475) only had positive-scoring uteri. It is important to mention that all organs analyzed from these six animals scored positive. In other words, the organs that did not score positive were not analyzed, and therefore did not score negative either. In addition to these six, there were four which showed weak positive signal in at least one organ. 1397 and 1493, for example, had a weak positive-scoring vagina and uterus, respectively. No other organs from those two animals were analyzed. 1341 and 1349 had weak positive-scoring spleens, but also negative-scoring uteri. Finally, the remaining two ZIKV-infected animals did not display positive signal in any of the observed organs and actually had

negative-scoring spleens and uteri. Of the 6 PBS-treated animals, 5 showed no positive signal in any of the analyzed organs, but one showed positive signal in the uterus (data not shown).

CHAPTER V

DISCUSSION

This study sought to address whether M. domestica opossums were susceptible to ZIKV infection with the intent of establishing them as animal models for ZIKV research. Efforts to develop countermeasures against ZIKV would greatly benefit from an additional model, and so we explored the potential of these animals for this role. They have been used for numerous biomedical studies, but there are no publications as of yet that use M. domestica for ZIKV research.

An ideal quality for a ZIKV research model would be susceptibility to ZIKV infection within the context of an intact immune system. Researchers often use mouse models deficient in ifn for ZIKV research, which limits the conclusions that can be drawn from data generated by these experiments. We therefore used WT M. domestica opossums for this study. Moreover, the functionality and responsiveness of their immune systems were confirmed experimentally. The ELISA results demonstrated that these animals generate strong adaptive immunity when challenged with ZIKV. This is an important premise for any conclusions drawn from subsequent results demonstrating ZIKV replication in this model. Successful ZIKV infection and replication despite a functional and responsive immune system offers more meaningful insight into the mechanisms ZIKV employs to evade host defenses than does an event of infection within the context of a partially functional immune system. Viral infection and the response of the host involve a complex interplay between different but overlapping systems, so removing a central

and highly involved component (i.e ifn) may drastically change the progression and outcome of infection.

Having established that the immune system of M. domestica successfully recognizes and responds to ZIKV, we next looked for evidence of viral replication in pathologically relevant organs. These include the spleen and, more importantly, reproductive organs like the uterus, vagina, and ovaries. The spleen was chosen because it is a key lymphoid organ, and some studies suggest that the lymphatic system is involved in ZIKV dissemination and persistence. Reproductive organs are highly relevant when studying ZIKV as they may sustain viral replication long after adaptive immunity is established and may also be involved in vertical and horizontal transmission. To determine if these organs were sustaining viral replication, we performed IHC analyses to test for the presence of ZIKV NS1 protein. NS1 was chosen because it is a nonstructural protein. This means that NS1 is not incorporated into ZIKV virions. When new virions are formed within a host cell, only structural proteins are incorporated. Presence of structural proteins therefore indicate presence of virions. Presence of virions in or around a host cell, however, does not necessarily indicate that replication has occurred in that particular cell. Viral replication cycles involve multiple steps, many of which are upstream of translation of viral proteins and replication of viral genome. Virions may fail to attach to cells, or they may attach but not infiltrate. Even after infiltrating, there is a chance that virions will not proceed to genome translation or replication. Structural proteins may therefore be detected in tissues

without viral replication necessarily occurring. NS proteins, on the other hand, are typically only found in a host cell if the viral genome was translated within that cell. Additionally, Flavivirus NS1 protein has been shown to be a component of viral replication complexes. Replication complexes are assemblies of viral proteins that work together to amplify viral genome. Finding ZIKV NS1 protein in an organ is therefore highly suggestive of viral replication within that organ.

Of the animals challenged with ZIKV injection, 12 had their organs analyzed for presence of ZIKV NS1 protein. NS1 was confidently detected in organs of at least 6 of these animals. It is possible that the other 6 also had ZIKV NS1 as four of them exhibited faint signals when imaged but were not counted as positive as it is also likely that these faint signals may have been the result of incomplete blocking of tissue or simply artifact. Additionally, not all organs of interest were analyzed in all animals due to time constraints. It is therefore possible that some of the ZIKV-treated animals that were scored as negative may in fact have been harboring viral infection in tissues that were not imaged. Nevertheless, it is quite promising that evidence of viral replication was detected in pathologically relevant organs despite incomplete analysis, especially since some of the animals with NS1-positive organs were animals sacrificed at 46 weeks of age. These subjects had 26 weeks to develop an adaptive immunity and clear infection. Most pathogens are quickly cleared by the host once adaptive immunity is engaged, but some viruses are able to hide from or subvert host immunity through a variety of mechanisms. It is unclear how ZIKV may persist after adaptive immunity is generated.

Despite these promising results, it is important to address some limitations of this study. Firstly, as mentioned above, not all organs were analyzed in the animals selected for IHC. Moreover, not all animals that were included in the study were selected for IHC. This greatly

limited any inferences that could be drawn regarding differences between inoculation route or differences in ages at which the animals were sacrificed. It is clear from table 1, for example, that within the ZIKV-infected group of animals analyzed via IHC, those infected via IM route were overrepresented. Half (6) of the animals from that group were infected via IM route while only three of them were infected via IP route and three via SC route. Additionally, the group of analyzed animals which were sacrificed at 46 week those infected via SC route were underrepresented, with only one subject included. Nevertheless, some trends do emerge from these limited results, albeit not statistically supported. For example, it was more common to find puncta of ZIKV NS1 signal colocalizing with the nuclei of cells from reproductive organs than in the spleens. Of course, there were some samples analyzed which did not follow this trend, such as the ovary seen in **Figure 5B**. Because of the small sample size and multiple variables (treatment, route of administration, and age), it is difficult to determine what accounts for the difference in NS1 signal patterns. It would follow from comparing the majority of the images of reproductive organs to the images of spleens that the difference would have some histological basis. But this is complicated by the few samples that did not follow this trend. It can also be reasoned that the age at which the animals were sacrificed may be responsible for this trend, but both ovary images in **figure 5** were extracted from animals of the same age (22 weeks) and their NS1 patterns differed. It is also possible that the patterns observed in **figure 5B** and the spleens is not so uncommon, but simply did not emerge often in this study because of the small sample size. Nevertheless, this observation, while not an inference of the data, can inspire hypotheses for future experiments. It is possible that route of administration, for example, may have a significant effect on early steps of infection. Equal representation of these administration routes, in addition to a larger sample size, may shed light on NS1 distribution in organs. It would also

be interesting to compare viral tropism between the different age groups since adaptive immunity-driven clearance of virions from circulation may affect late events of infection and, by extension, viral persistence. In addition to not analyzing all subjects, another limitation of this study lies within the only viral detection method employed. IHC analysis is highly specific and therefore unlikely to produce false positives if the antibodies used are properly designed and validated. We included validation controls in every slide analyzed, so we are confident when we score organs as being positive for ZIKV NS1. It is important to mention that one of the PBS-treated animals was scored as positive for ZIKV NS1, but we believe this to be the result of experimental error involving cross-contamination or miss-labeling of slides rather than a false positive IHC reading. Unfortunately, viral infection may not be evenly distributed throughout an organ. Consequently, it is possible to slice off a clean, non-infected section from an otherwise infected organ. This introduces the possibility of reporting false negatives. As a result, organs scored as negative should be interpreted more so as inconclusive than as truly negative. Taking serial sections and analyzing multiple regions of the same organs certainly would reduce the possibility of reporting false negatives, but this approach was not employed because we prioritized achieving a higher sample size within our constrained timeframe.

Nevertheless, the results of this study strongly suggest that *M. domestica* may serve as a good animal model for ZIKV research. The data demonstrate that these animals are susceptible to ZIKV infection and that viral replication may persist even months after initial exposure. Because of the inherent weaknesses in this study, this work should be taken less as a conclusive verdict on the applicability of *M. domestica* as a model for ZIKV research and more as a stepping-stone for future studies. This experiment, despite its limitations, successfully addressed the questions it sought to answer and produced results that are more than mere proof-of-concept.

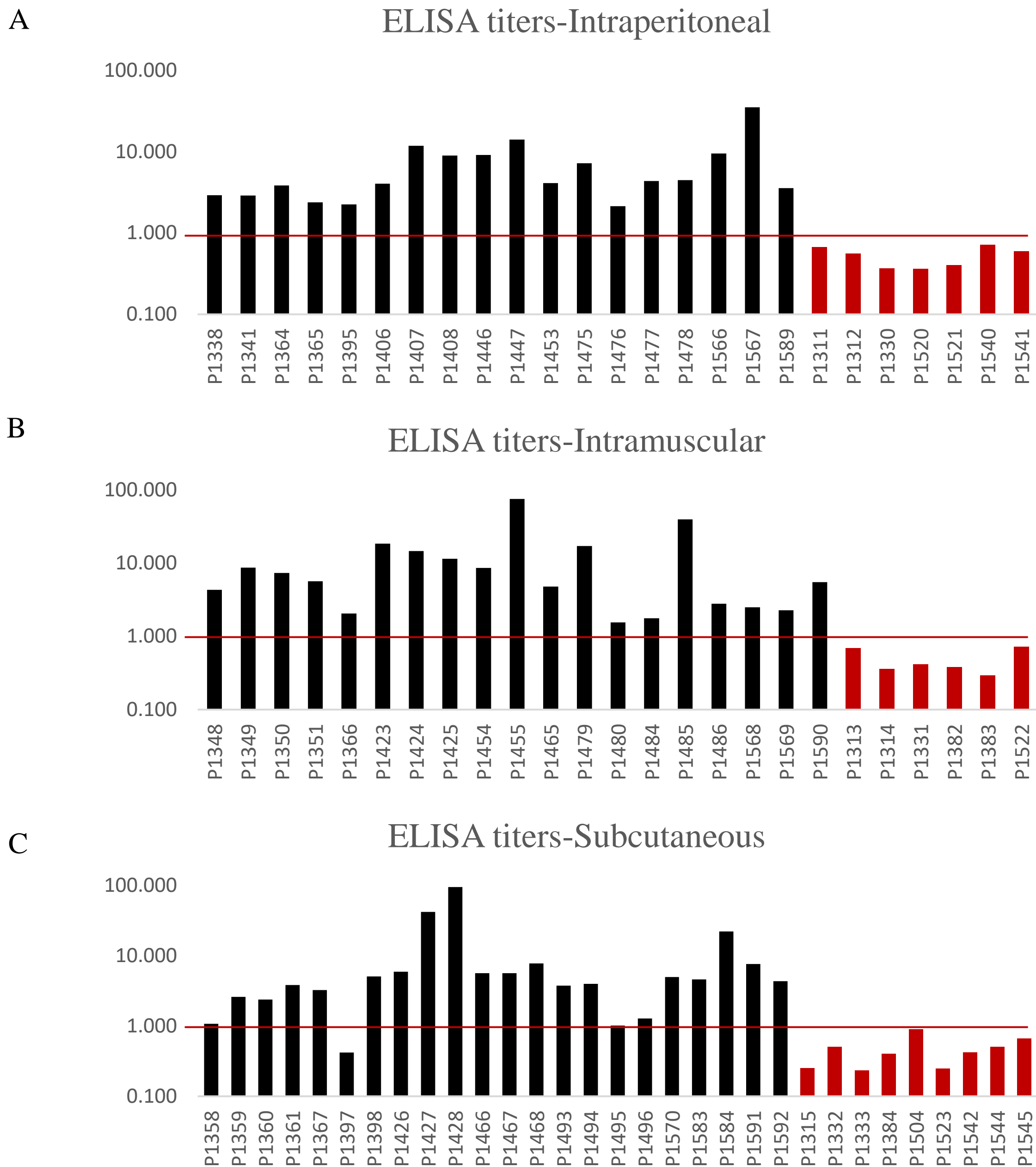

Figure 1. ELISA titers. ELISA titers for each animal are shown above, separated by routes of administration; intraperitoneal (A), intramuscular (B), and subcutaneous (C). Y-axis follows logarithmic scale to minimize skew induced by outliers. Black bars represent animals that were inoculated with ZIKV-BR. Red bars represent animals inoculated with PBS placebo. Horizontal red lines in each graph represent the cutoff titer. Titers above the line are considered positive and titers below the line are considered negative.

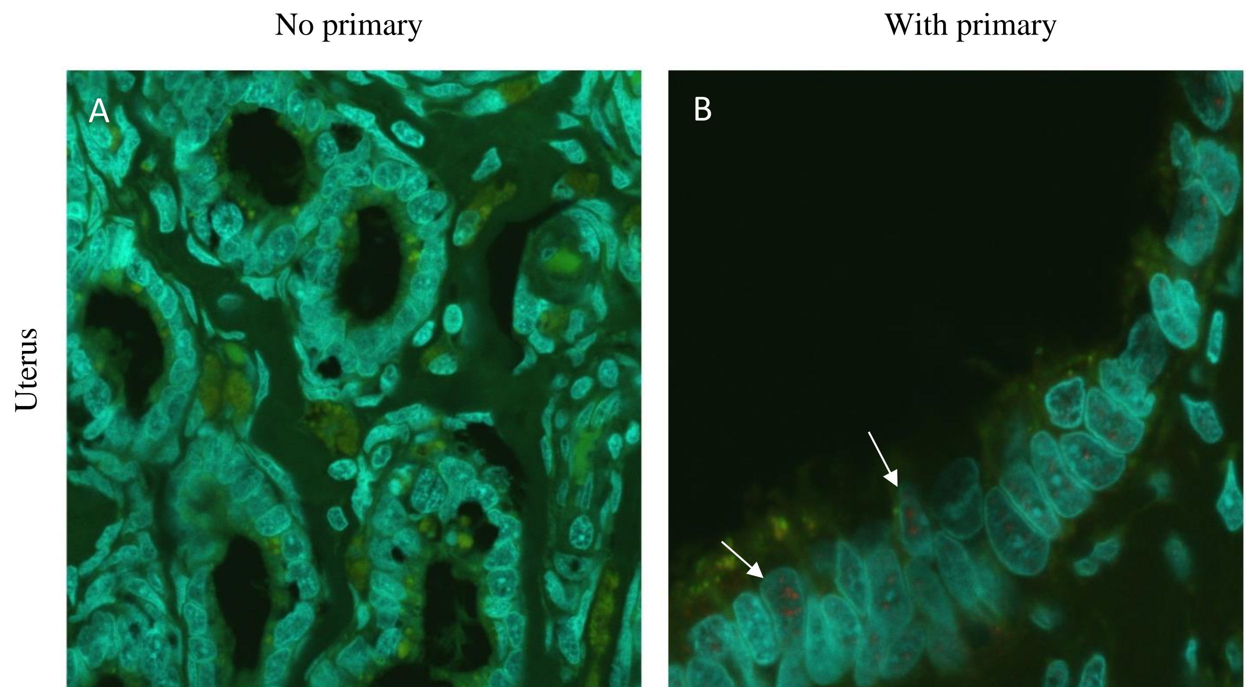

Figure 2. Validation of primary antibody. Primary antibodies were validated by including organ sections not treated with primary antibody but otherwise stained the same. Figure shows confocal images of a uterus from a 46 week old, female opossum injected with ZIKV-BR intraperitonially. Panel A shows a tissue section which was stained without including primary antibody. Panel B shows a section of the same uterus which was treated with primary antibody during the staining process. White arrows indicate focal points of NS1 signal.

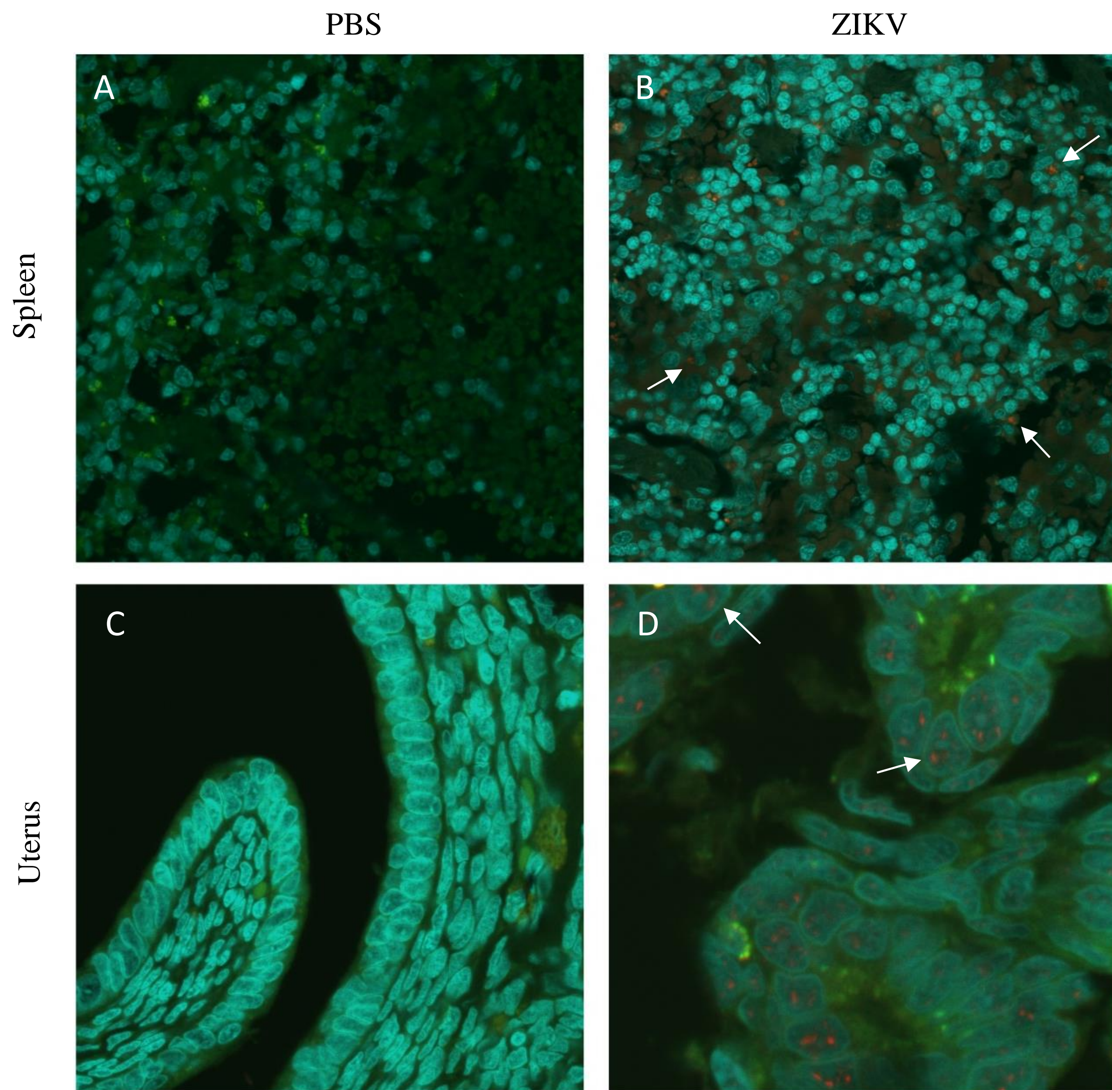

Figure 3. Comparison between organs of PBS-treated animals and organs of ZIKV-treated animals. Image shows the difference between organs from PBS-treated animals and organs from ZIKV-BR-treated animals. Panels A and B display a PBS spleen (46 weeks) and a ZIKV-BR spleen (46 weeks), respectively. Both spleen images are at 60x magnification. Panels C and D display a PBS uterus at 100x magnification (46 weeks) and a ZIKV-BR uterus (46 weeks) at 210x magnification, respectively. White arrows indicate focal points of NS1 signal.

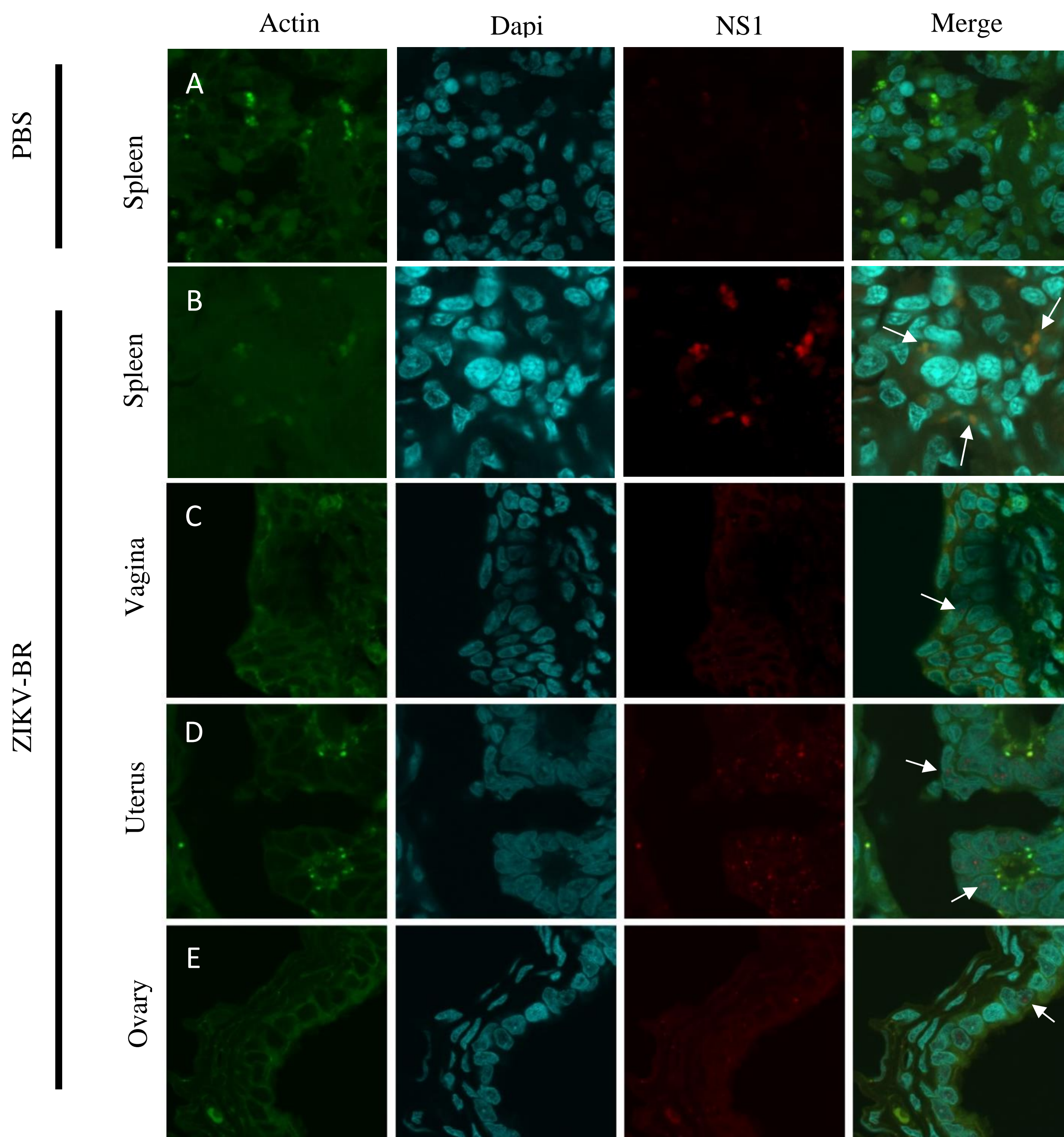

Figure 4. Representative examples of organs. Image depicts representative examples of each type of organ analyzed. Top row (A) shows a spleen from a PBS-treated animal (46 weeks) at 180x magnification. All other rows depict organs from ZIKV-BR-treated animals. (B) shows a ZIKV-BR-treated spleen (46 weeks) at 280x magnification. (C) shows a ZIKV-BR-treated vagina (46 weeks) at 180x magnification. (D) shows a ZIKV-BR-treated uterus (46 weeks) at 210x magnification. (E) shows a ZIKV-BR-treated ovary (46 weeks) at 180x magnification. White arrows indicate focal points of NS1 signal.

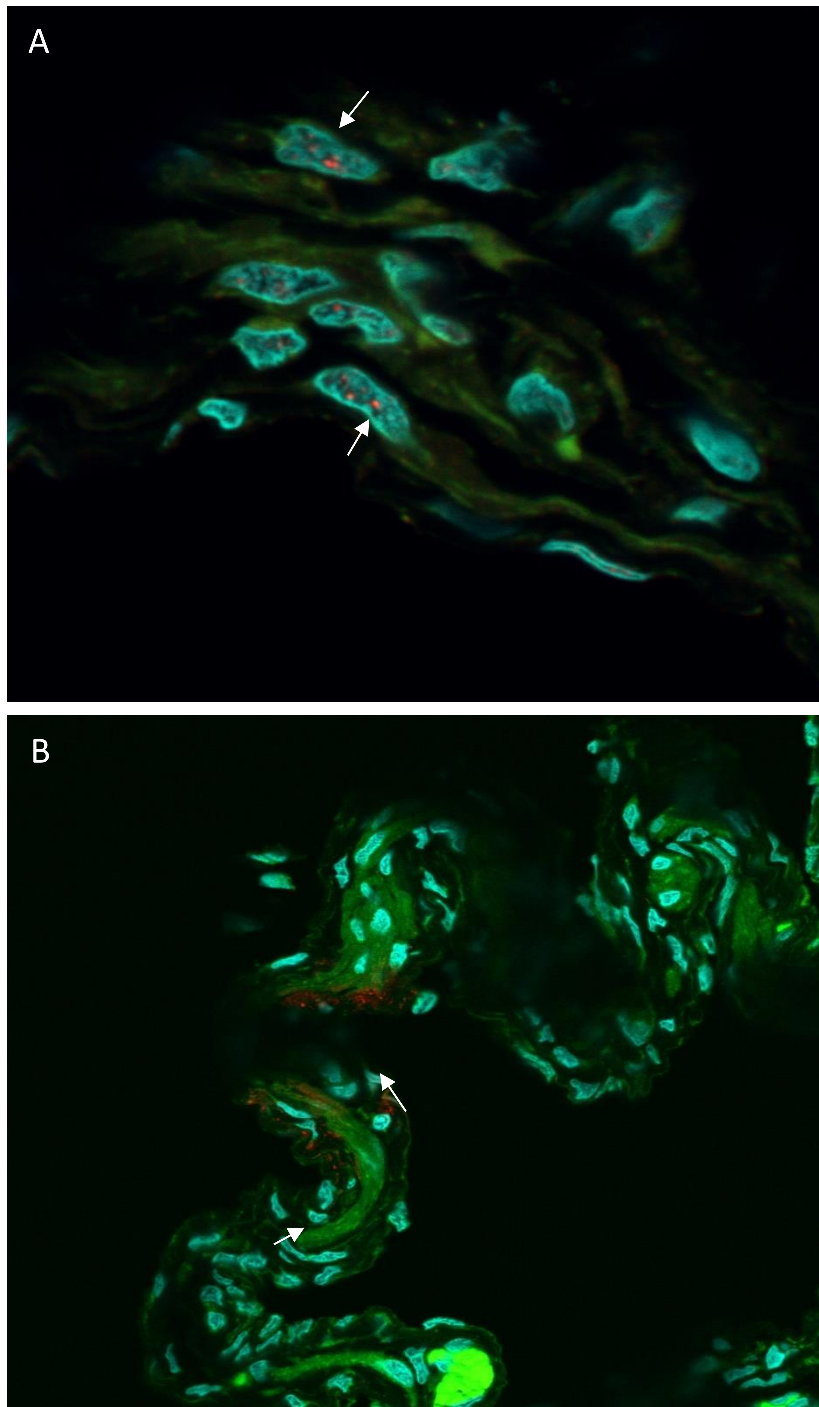

Figure 5. Different punctate NS1 signals observed in ovaries. Image depicts ovaries from age-matched ZIKV-BR-infected females. (A) ovary from ZIKV-BR-infected animal (22 weeks) which exhibits NS1 puncta colocalized with the visible nuclei. Animal was injected via subcutaneous route. (B) ovary from ZIKV-BR-infected animal (22 weeks) which exhibits NS1 puncta which are not colocalized with the visible nuclei. Animal was injected via intramuscular route.

	Animal ID	ELISA TITER	Sex	Treatment	Route	Age sacrificed	Organs analyzed			
							Uterus	Vagina	Ovary	spleen
22 weeks old	1330	0.369	F	PBS	IP	22	N	-	-	N
	1397	0.419	F	ZIKV-BR	SC	22	-	w	-	-
	1366	2.053	F	ZIKV-BR	IM	22	N	-	-	N
	1493	3.727	F	ZIKV-BR	SC	22	w	-	-	-
	1465	4.764	F	ZIKV-BR	IM	22	Y	-	Y	-
	1426	5.924	F	ZIKV-BR	SC	22	Y	Y	Y	-
	1475	7.237	F	ZIKV-BR	IP	22	Y	-	-	-
46 weeks old	1521	0.404	F	PBS	IP	46	N	-	-	N
	1542	0.418	F	PBS	IM	46	N	-	-	N
	1541	0.594	F	PBS	IP	46	-	-	-	N
	1545	0.663	F	PBS	SC	46	Y	-	-	-
	1540	0.719	F	PBS	IP	46	-	-	N	N
	1341	2.893	F	ZIKV-BR	IP	46	N	-	-	w
	1477	4.365	F	ZIKV-BR	IP	46	Y	Y	Y	-
	1351	5.65	F	ZIKV-BR	IM	46	N	-	-	N
	1350	7.304	F	ZIKV-BR	IM	46	Y	-	-	-
	1349	8.634	F	ZIKV-BR	IM	46	N	-	-	w
	1424	14.617	F	ZIKV-BR	IM	46	Y	Y	-	Y

Table 1. Summary of IHC results. Table showing how organs from each animal was scored. Animals are organized firstly by age, with 22-week old animals at the top half of the table and 46-week old animals at the bottom half. Each group was then organized by treatment, with PBS-treated animals being displayed above ZIKV-BR-treated animals. Organs scored as positive through IHC analysis were labeled as (**Y**), meaning that positive fluorescence NS1 signal was observed. Organs scored as negative through IHC analysis are labeled as (**N**), meaning that positive fluorescence NS1 signal was not observed. Organs that were scored as weak are labeled (**w**), meaning that only weak fluorescence NS1 signal was observed. Organs labeled (**-**) were not analyzed. Animal ID's that are highlighted represent subjects that had at least one organ scored as (Y) or (W). **IP**, intraperitoneal; **SC**, subcutaneous; **IM**, intramuscular

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BIOGRAPHICAL SKETCH

Oscar Quintanilla received his Bachelor of Science degree in biology from the University of Texas Rio Grande Valley (UTRGV) in December of 2018. He then went on to conduct research in Dr. John M Thomas III's virology lab. His research involved the characterization of a novel animal model for Zika virus research. In 2019, Oscar was admitted into the biology graduate program at UTRGV and earned his Master of Science degree in Biology in December of 2020. During his time in the graduate program, he also served as a teaching assistant for general biology and cellular biology instructional lab. Additionally, Oscar was hired by UT Health Rio Grande Valley to assist with the diagnostic testing of patient samples for COVID-19. Oscar aspires to earn a PhD in either pathology or immunology and attain a professorship position at a University where he can lead a research as well as educate future generations. For professional inquiries, please contact author via email: oscar.quintanilla02@utrgv.edu.