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Inoculation of Juvenile Monodelphis domestica with Zika Virus Using Various Routes of Infection

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INOCULATION OF JUVENILE MONODELPHIS DOMESTICA WITH ZIKA VIRUS USING VARIOUS ROUTES OF INFECTION

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ABSTRACT

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Monodelphis domestica is a small marsupial from South America that has been previously been used in human metabolism research. However, their suitability as models for Zika virus (ZIKV) research, is currently unknown. Preliminary studies have shown *M. domestica* susceptible to ZIKV through the intracerebral route. We utilized immunohistochemistry, and analyzation of serological responses to show that juvenile *Monodelphis domestica* can be infected with ZIKV by using various routes of infection that include subcutaneous, intraperitoneal, and intramuscular. ELISAs performed on sera confirmed that juveniles injected with ZIKV by all three routes generated anti-ZIKV antibodies. Additionally, ZIKV nonstructural protein 1 was detected through multiple organs that include brain, eye, spleen, heart, and sex organs through immunohistochemical analysis. The data suggests that infection was established, persisted in multiple organs and had general immune responses regardless of route. These results demonstrate *M. domestica* as a susceptible new model for ZIKV infection *in vivo*.

DEDICATION

This Thesis is dedicated to Dr. John M. Thomas III and Juan Garcia, the two men who taught me everything I know.

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I would like to acknowledge Dr. John M. Thomas III; his mentorship and guidance is the reason that I am here today. I am thankful for his dedication to teaching, and the opportunity that he gave me to learn under his tutelage since I was an undergraduate student. I will always appreciate his honesty and his tenacity of getting work done.

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CHAPTER I

INTRODUCTION

Zika virus (ZIKV), a member of the family Flaviviridae and genus flavivirus, was first isolated in 1947 from an *Aedes africanus* mosquito trapped in the upper canopy of the Ziika forest in Uganda (Dick et al., 1952). ZIKV is a small, enveloped, single-stranded positive-sense RNA virus that is typically transmitted in a zoonotic cycle that alternates between a vertebrate host and an invertebrate vector. ZIKV was relatively obscure until outbreaks in Yap and French Polynesia in 2007 and 2013, respectively, and the 2015-2016 pandemic in Brazil. This event led the World Health Organization to declare ZIKV as a public health emergency of international concerns (Jamil et al., 2016). The 2015 Brazilian outbreak saw an increase of infants with neurological abnormalities born to ZIKV-infected mothers (Zanluca et al., 2015), and there was also a significant increase in Guillain-Barre syndrome and microcephaly observed during the outbreak (Musso et al., 2016). Analysis of fetal tissue collected from ZIKV-infected infants supports a causal relationship between ZIKV and neurological abnormalities, as ZIKV has been detected in brain tissue of microcephalic fetuses, as well as in amniotic fluid of pregnant women (Calvet et al., 2016). Due to the dramatic increase of these abnormalities, animal models have become an important tool for the better understanding of the ZIKV infection mechanism and has brought an urgent need for the development of *in vivo* models (Pawitwar et al., 2017). To date, the principal animal models for accessing ZIKV pathology have been nonhuman primates

(NHPs) and transgenic mice; limited studies also have been conducted with chicken embryos (Pawitwar et al., 2017).

The NHP model is the most relevant in terms of reproducing the pathology in vivo compared to what is known about ZIKV-induced pathologies in humans (Dudley et al., 2016) (Waldorf et al., 2016). The use of rhesus macaques as a nonhuman primate model has shown that the animal model does succumb to ZIKV infection and undergo rapid viral replication in the blood and display signs of immune activation (Dudley et al., 2016). ZIKV has also been detected in urine and saliva of the macaques, and occasionally in the cerebral spinal fluid or vaginal secretions of the female macaques (Dudley et al., 2016). However, the cost associated with the use and maintenance of NHPs precludes large-scale experimentation. This limitation, together with the long duration of time required to investigate effects of ZIKV infection during gestation on development during infancy, adolescence, and into adulthood and old age in NHPs, suggests that additional animal models are required.

The mouse model is predominantly based on using immunocompromised animals lacking the receptor for type I interferon (IFN α/β)(A129 mice) or types I and II IFN (IFN α/β/γ)(AG129 mice)(Ifnar 1 -/-).This is because initial peripheral inoculation studies show no disease signs or infection with wild-type (WT) C57BL/6, BALB/c, or CD-1 mice infected with African and Asian ZIKV isolates (Morrison et al., 2017). Studies have shown that ZIKV antagonizes the human type I interferon (IFN) response, in part through its NS5 protein, which promotes degradation of STAT2 (Grant et al., 2016). However, NS5 does not promote the same degradation in mouse STAT2 (Grant et al., 2016). Mice with genetic deficiencies in type I IFN signaling pathway are highly susceptible to ZIKV infection and sustain infection with high viral loads in the brain and spinal cord, consistent with the severe neurological manifestation of ZIKV

in humans, and high viral loads in the testes, relevant to the sexual transmission observed with ZIKV in humans (Aliota et al., 2016, Dowall et al., 2016; Rossi et al., 2016). However, because these animals lack receptors for Type I and Type II IFN they are not able to test for vaccines that rely on intact IFN pathways (Pawitwar et al., 2017). Furthermore, the pathogenesis is more severe than that experienced in humans and mice that do survive exposure to virus are protected from re-challenge (Pawitwar et al., 2017).

Therefore, due to the limitations of existing animal models for ZIKV-induced pathogenesis, we explored the potential of a marsupial model to circumvent those limitations. *Monodelphis domestica* (*M. domestica*), also known as the laboratory opossum, is a gray short-tailed marsupial native to Brazil and surrounding countries (Vandeberg et al., 1997). Laboratory opossums are widely used as models in many fields of biomedical research, and they possess some characteristics that render this model suitable and, in some respects, unique, for studying the pathogenesis of ZIKV *in vivo.* It is docile, breeds readily in captivity, and produces large litters (typically 6-13 pups per litter) (Vandeberg et al., 1997). At birth, these marsupials are developmentally equivalent to an 8-week-old human embryo and subsequently begin to develop a robust immune system. *M. domestica* complete embryonic and most of fetal development while attached to the mother's nipples over a 2-week period, which means that the pups are easily accessible for experimental research. *M. domestica* are small, weigh about (80-120g) as adults, are easy to manipulate and can be maintained cost-effectively. These animals cannot only produce large numbers, but they also have a fully intact immune system as they develop beyond the fetal stage. These advantages enable robust statistical analysis for between-group comparisons, as well as robust assessment of within-group variations in outcome of ZIKV infection (Thomas and Vandeberg, personal communication).

The purpose of this study was to assess the utility of *M. domestica* as a model for ZIKV pathogenesis, to determine if using different routes of injection (IP, IM, SC) has any impact on viral infection and dissemination in vivo.

CHAPTER II

REVIEW OF THE LITERATURE

Flaviviruses

The family *Flaviviridae*, named from the latin "*flavus*" for the hallmark jaundice caused by infection with yellow fever virus (YFV), is comprised of the genera *Flavivirus*, *Pestivirus*, *Pegivirus* and *Hepacivirus* (Huang et al., 2014). Flaviviruses are the largest genus and is comprised of more than 70 viruses (Huang et al., 2014). Mosquito-borne flaviviruses, such as yellow fever virus, dengue virus serotypes 1-4, Japanese encephalitis virus, West Nile virus and Zika virus are responsible for significant human morbidity and mortality in affected regions (Huang et al., 2014). Flaviviruses share a similar genomic structure and replication strategy.

Virus Architecture and Genome Organization

ZIKV contains a single-stranded, positive-sense RNA of about 10,000 nucleotides, encompassing a coding region of 10 genes (Pearson and Kielian et al., 2013; Marano et al., 2016). ZIKV contains a single open reading frame (ORF) that encodes for a polypeptide which is further processed post-translationally by both host and viral proteases into 3 structural proteins. The polyproteins are organized from 5' to 3' with both ends of the ORF covered by a 100nt untranslated region (UTR) (Chambers et al., 1990). The viral structural proteins consisting of the capsid (C), pre-membrane (prM), and envelope (E), are located towards the 5' end, while the seven non-structural (NS) proteins consisting of NS1, NS2A, NS2B, NS3, NS4A, NS4B, and

NS5 are found at the 3’ portion (Kuno and Chang et al., 2007; Hulo et al., 2011; Cunha et al., 2016). The NS proteins are associated with virus assembly, RNA replication, and host immune response (Fernandez-Garcia et al., 2009; Yun and Lee et al., 2017).

Life Cycle and Replication

The life cycle of ZIKV is similar to other known flaviviruses: virions attach to the surface of the host cell by interactions between viral surface glycoproteins and cell surface receptors, enter the cell by receptor-mediated endocytosis and are then internalized into clathrin-coated pits. This causes the viral RNA to be released into the cytoplasm following fusion of the viral and host membranes (Hamel et al., 2015). The positive-sense genomic RNA is translated into a single polyprotein that is processed co-translationally and post-translationally by cellular and viral proteases (Hamel et al., 2015). This cleavage makes a total of three structural proteins and seven non-structural proteins. Genome replication occurs on transport vesicles, which helps transport ZIKV to the Golgi apparatus, thus facilitating the assembly of the viral replication complex (Gratton et al., 2019). Viral replication occurs on the surface of the endoplasmic reticulum, these new particles travel alongside the host secretory pathway through the trans-Golgi network, where virion maturation occurs and then is released by exocytosis (Lindenbach and Rice et al., 2003; Roby et al., 2015).

ZIKV Tissue Distribution

In human studies, ZIKV RNA has been detected in both maternal and fetal tissues, including cord blood, several placental cell types, amniotic fluid, and the developing fetal and neonatal human brain. ZIKV RNA has also been detected in the brain and placenta of spontaneously aborted human fetuses in the first and second trimesters (Bhatnagar et al., 2017).

After congenital infection of pigtail macaques with a Cambodian ZIKV isolate, viral RNA was isolated from the maternal brain, eye, spleen, and liver, with the highest amounts observed in the placenta (Adams and Waldorf et al., 2016), suggesting that, as in mice and humans, ZIKV infects cells in the placenta of primates. *In vitro* infection studies of human placental cells have shown that ZIKV replicates in placental macrophages (Hoffbauer cells), trophoblasts, and fetal endothelial cells and induces expression of antiviral genes (Quicke et al., 2016; Tabata et al., 2016). In one study, human trophoblasts isolated from a term placenta were relatively resistant to infection, in part due to inhibition mediated by type III IFN-l (Bayer et al., 2016). Gestational age and genetic variation in host factors within the placenta (e.g., expression of virus attachment or immune restriction factors) may affect the relative vulnerability of placental cell types to ZIKV infection (Tabata et al., 2016). Human and animal model studies have demonstrated that ZIKV infection can result in persistence of infectious virus and viral nucleic acid in several body fluids (e.g., semen, saliva, tears, and urine) and target organs, including immune-privileged sites (e.g., eyes, brain, and testes) and the female genital tract. (Miner et al., 2017).

Monodelphis domestica as a model for ZIKV Research

M. domestica is a small marsupial whose role in infectious disease research has not yet been fully established. The laboratory opossum has been previously used in developmental biology, reproduction, genetics, and human metabolism research. They have been used to study the effects of diet on lipoprotein composition, as well as genetic factors that affect intestinal absorption of cholesterol (Rainwater and Vandeberg et al., 1992). Its true value has been shown in oncogenic research where pups have been shown to act as natural vessels for xenografted human cancer cells of the skin, colon, and prostate, allowing for the opportunity of *in vivo* studies (Wang et al., 2008). The laboratory opossum is a docile creature typically between 80-

120 grams in body weight that can produce litters of 6-13 pups three to four times a year (Vandeberg and Blangero et al., 2010). These high numbers of lab animals make the laboratory opossum an economically viable animal model compared to NHPs model. Unlike other marsupials, female *M. domestica* do not have a pouch which makes the manipulation of newborn pups very easy to do. Newborn pups are developmentally equivalent to a 5-week-old human embryo (Cardoso-Moreira et al., 2019). They attach to the mother's nipple where they will remain attached for the first 14 days of the embryonic and fetal development (Vandeberg and Blangero et al., 2010). *M. domestica* are marsupials, which means that their pups are born incompletely developed and are typically carried and suckled in a pouch on the mother's belly. That type of access creates opportunity for viral inoculations on pups that are equivalent to the first trimester of human pregnancy, a crucial time for ZIKV infection (Caine et al., 2018). These unique advantages make the laboratory opossum a convenient model for experimental procedures.

Statement of the Problem

The long-term effects of ZIKV infection are still unknown currently. NHPs and mouse models have significant limitations and due to those limitations a more suitable animal model is needed. *M. domestica* could be that new model that can circumvent those limitations, but to confirm this theory, further studies need to be established.

Purpose of the Study

The purpose of this study was to assess the utility of *M. domestica* as a model for ZIKV pathology by determining if ZIKV, specifically the Puerto Rican strain Z-PRV, can replicate using the standard routes of administration. *M. domestica* were injected using five different

routes of inoculation that included, Intramuscular (I.M.), Intraperitoneal (I.P.), and Subcutaneous (S.C.). Intracardiac (I.H.) and Intratesticular (I.T.) were collected but were not processed due to time constraints. Each route received an inoculation of Z-PRV at 10^5pfu/dose spaced at 2-week intervals over a 56-day period, for a total of four injections. Afterwards, a total of 51 animals (male and female) were collected for tissues and 210 serum samples were recovered. Tissues that were examined included brain, spleen, eye, heart, and sex organs. Serum was collected at day 0 (pre-boost), and then at days 14, 28, 42, and 56 of the experiment and were used for serological studies. Previous research has shown that intracranial injection was a successful route of infection for *M. domestica*, which helps support the notion that *M. domestica* are susceptible to ZIKV infection.

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

Z-PRV was used for 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. Six well plates were seeded with Vero cells (CCL-81; ATCC, USA) the night prior for plaque assays. 90% confluent monolayer was infected with tenfold serial dilutions of Z-PRV, incubated for 96 hours, fixed with 4% PFA, and stained with crystal violet. 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.

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 infected with Z-PRV through five different routes that include S.C., I.P., I.M., I.H., and I.T. Each injection was 50µL Z-PRV, 10^5 PFU, with a 27g needle, and a 1mL syringe. Control animals were injected the same with 50µL of PBS. *M. domestica* were injected starting at 18 weeks of age, given a booster shot every two weeks afterwards, and necropsied after 26 weeks of age. There was a total of 51 animals used for this study. Serum samples were also collected at day 0, 14, 28, 42, 56, and stored at -80°C. Tissues were placed either in 10% formalin or kept frozen at -80°C until the time of tissue analysis.

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, tissues were 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 30 μm were mounted onto Frost + microscope slides and stored at -20°C.

Immunohistochemistry

Tissues analyzed through immunohistochemistry (IHC) included the brain, sex organs, and spleen of 18- 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. 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. Tissues were 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 09344 animal was utilized as a positive control for remaining immunohistochemistry procedures. Three serial sections were used for each tissue to determine positive or negative. Two slides would be stained normally, the third slide would be stained without the primary antibody. PBS-mock infected tissues were utilized as a negative control.

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). The animals that were assayed include the 30 animals used for the three main routes. The animals stained for immunohistochemistry are also included in this assay. The data that was gathered from the ELISA was used for statistical analyses and used for ANOVA with the use of JMP®, Version Pro 13. SAS Institute Inc., Cary, NC, 1989-2019.

CHAPTER IV

RESULTS

Viral Concentrations through Plaque Assays

Plaque assays were done to determine the viral concentration that was used for the inoculations. The result of this show plaque formation for Z-PRV on Figure **1**. To determine the viral titer of the stock we used the equation Pfu/mL=((Average # of plaques)/(Dilution)(Volume of diluted virus added to the plate)). The average number of plaques we calculated was 3.8 at 10^-5 with 100µL of volume used. When we add that to the equation, we get 3.8 x 10^6 pfu/mL, when divided by 1000µL we get 3.8 x 10^3 pfu/µL. When we multiply 3.8 x 10^3 pfu/µL with 50µL/dose we get a total of 1.9 x 10^5 pfu/dose.

Analysis of Serological Responses

ELISAs were done to evaluate the antibody response of ZIKV infection in *M. domestica*. Each route was able to deliver the virus and infect *M. domestica* throughout the 56 days (Figure 2). The result of each serum sample was expressed as an "endpoint titer." Samples with endpoint titers greater than 1.00 are considered positive as shown as a line in the y axis on the graphs of Figure **2A-C**. *M. domestica* serum had positive endpoint titers by day 14 in all three routes of infection. An interesting note to point out is that all *M. domestica* animals had positive endpoint titer responses through the I.M. route as seen in Figure **2B**. The averages of the serum samples collected for day 28, 42, and 56 can be seen in Figure **2D**. Each booster shot was able to increase

the titer significantly. This increase in antibody response is similar to what past studies have shown for NHP models (Liang et al., 2017). Day 14 serum samples also when through statistical analyses to determine if there was a significant difference between routes of administration. Data shows that there was no significant difference between routes (Figure 3).

Immunohistochemistry

To determine animal susceptibility to Z-PRV infection and viral tissue distribution over a long-term period, immunohistochemistry (IHC) was conducted on a subset of 26-week-old males and females. Three *M. domestica* from each route (2 females, 1 male) were evaluated for ZIKV-NS1 protein via immunofluorescent microscopy which included the brain, heart, eye, spleen, and sex organs (epididymis, vagina, uterus, and ovaries). For the viral NS1 protein validation experiments, PBS animals for each route were stained the same as the infected tissue and showed no immunofluorescence when compared to infected tissue. A total of 12 animals were antibody stained for NS1 (Table 1). Each *M. domestica* showed infection in certain tissues. NS1 protein was detectable in the brain, eye, heart, spleen and sex organs (Figure 4). *M. domestica* stains show that not only were they susceptible to Z-PRV, but also that the virus was able to disseminate throughout the body and infect certain organs dependent on the individual animal. Table 3 shows seven of the nine animals selected from the subset were able to be confirmed through antibody staining. Six of the nine animals had punctate NS1 signal show on the cells of sex organs. 09344, 09348, and 09343 had NS1 signal detected on the spleen; we also detected NS1 signal in the eye of 09375, 09346, and 09373. Three animals had NS1 signal in the brain which included 09344, 09348, and 09347. The only animals that didn't show signal were 09374 and 09342. There was no NS1 signal detected in any of the organs, however, more staining needs

to be done on these tissues. Majority of the samples tested positive for immunohistochemistry and produced positive endpoint titers (Table 4).

CHAPTER V

DISCUSSION

This study was to determine the utility of *M. domestica* as a model of ZIKV pathogenesis by determining if Z-PRV is able to replicate within the same standard routes that have been used before in other animal models such as the NHPs and mouse models. *M. domestica* have been previously used in obesity research and various other studies, but they have never been used in ZIKV research. Establishing this model would benefit the goal of understanding ZIKV at a greater scale. To determine the validity of the model, we injected 18wk old *M. domestica* with four shots of Z-PRV every 2 weeks for a total of 56 days. Serum was collected every two weeks prior to the shots and after 56 days (26wks old) tissues were extracted and stored in 10% formalin. Tissues were analyzed with IHC staining and serums went through ELISAs assays.

Plaque assays were performed to determine the titer of the stock and calculations were made to create 1.9 x 10^5/dose. Since the particle/infectivity ratio has yet to be established we decided to inoculate at a max rate to guarantee infection and strong immune response. Similar studies have been done with Adult Rhesus Macaques at 10^6 pfu/mL (Woollard, et al., 2018). Plaque formation from Z-PRV can be seen in Figure **1**.

The data that was gathered from ELISAs showed a very strong immune response to ZIKV from *M. domestica* juveniles. The data shows a strong immune response starting at day 14 after the first prime shot, majority of the animals had a positive endpoint titer for routes S.C., I.P., and I.M. (Figure **2A-C**). One thing to note is that all the animals in the I.M. route had a

positive endpoint titer at day 14 compared to the other two routes. This is similar to what is seen in NHPs and mouse models for vaccine injections. I.M. delivery has been used for mRNA vaccines in Rhesus macaques and was shown to generate strong antibody responses (Liang et. al, 2017). The S.C. route has also been used with purified inactivated virus vaccines for Z-PRV and have also demonstrated strong antibody responses (Abbink et al., 2016). The most common routes for ZIKV infection have been the S.C. and I.P. routes for both animal models.

Furthermore, the mouse model needs to have genetic deficiencies in the type I IFN signaling pathway in order to sustain ZIKV infection, this is not the case for *M. domestica*. NHPs on the other hand are easily susceptible to ZIKV but are limited by the cost of NHPs, and the lack of facilities that are equipped to handle NHP's to determine more data. *M. domestica* does not seem to have that limitation, it is a marsupial that has an immune system that is susceptible to ZIKV infection and create strong antibody responses. Despite only using 12 animals for the staining, that is still more than double than what NHP's studies can use. The study has shown that *M. domestica* not only is susceptible to Z-PRV, it can also create a strong immune response, and by the second shot all 3 routes of transmission were confirmed with positive endpoint titers by day 28 of the serum collections. Each consecutive shot afterwards as well saw a substantial increase of OD450nm antibody titers (Figure **2D**). This correlates with previous data that has been seen in NHP's and type I IFN deficient mouse models (Nazerai et al., 2019).

However, statistical analyses of day 14 serum show no significant difference between the routes of administration (Figure 3). ANOVA was performed for all samples and showed no significant difference. ANOVA data for Day 28, 42, 56 are not shown. This shows that any route of infection is viable for infection, which is good news for this brand-new animal model.

The next phase of the project was to determine if viral replication had occurred in relevant organs. NHP's when inoculated with ZIKV through the S.C. or I.P. route have shown replication in several different organs that include the uterus, heart, vagina, brain, and spleen (Woollard et al., 2018). To determine if any of the *M. domestica* organs sustained any ZIKV replication, we performed IHC stains to detect any presence of nonstructural protein 1 (NS1) with the use of a monoclonal antibody. A subset of 12 animals were used to detect ZIKV NS1 protein in tissue. For each route 3 animals (2 female and 1 male) were stained including one PBS animal for each route which were served as negative controls. *M. domestica* organs that were stained for NS1 include the brain, eye, heart, spleen, and sex organs (ovary, vagina, uterus, epididymis). A representative stain of each internal organ can be seen in Figure 4. From the nine infected animals that were stained and verified, seven of those animals had NS1 detection in at least one of the extracted organs. NS1 was consistently detected in the sex organs (male and female) (Table 2). This correlates with ZIKV pathology in NHPs and immunodeficient type I IFN mouse models (Woollard, et al., 2018). More staining needs to be done to determine the susceptibility of *M. domestica*.

When antibody stains and ELISA results were compared, we saw that the I.P. route which had the weakest immune response also had the most organs come out positive for antibody staining (Table 3 & 4). S.C. and I.M. on the other hand had stronger immune responses but similar positive antibody stain results. This could be due to the peritoneum having a higher concentration of IgG, which could be causing a delay on Z-PRV delivery.

Nevertheless, as promising as these results are, there were several limitations that need to be addressed. The first limitation was that not all the animals used in the experiment were used for IHC staining. There are still plenty of tissues that need to be antibody stained and confirmed

for ZIKV NS1 protein. We are only getting a small picture of the study and more data needs to be produced before we can suggest the best route to take for *M. domestica*.

A second limitation that occurred was the size of tissue that we transferred to the slide. When we first started the project, we were slicing 30µm sections into the slides. Normal thickness should be between 5-10µm, because our tissue was thicker than normal that made detection for NS1 more difficult. The only detection we could see was the top layer of the tissue, but despite that issue we were still able to detect NS1 in all three routes.

Despite the limitations we were still able to observe susceptibility of *M. domestica* through positive ELISA titers and IHC detection (Table 3). The data in this study suggests that these animals are a good model for ZIKV pathology, they can be susceptible through any of the three routes and have the ability to create a strong immune response as seen in the literature with other ZIKV animal models. Future studies should include RT-PCR confirmation of the tissues, more high-volume staining of animal organs, and better Z-PRV RNA recovery methods. *M. domestica* is on the right path of being recognized as a suitable animal model for ZIKV replication and future studies will one day support that notion.

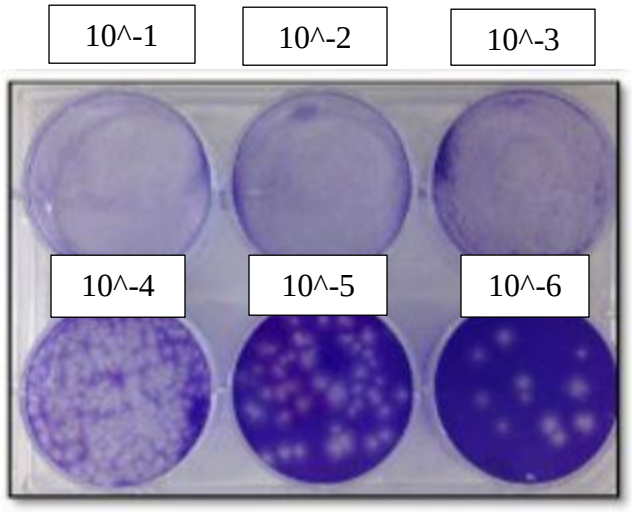


Figure 1. Plaque Assay staining for Z-PRV. The average number of plaques we calculated was 3.8 at $10^{\wedge -5}$ dilution with a volume of 100µL. This gave us a titer of 3.8 x $10^{\wedge 6}$, which then gave us a final concentration of 1.9 x $10^{\wedge 5}$ pfu/dose which was used for inoculations.

Z-PRV inoculation of *M. domestica*							
Animal ID	**Inj. Vol (µL)**	**Sex**	**Age at Time of Inj.**	**Age at Death**	**Injection Site**	**Treatment Group**	**Group**
*O9342	50 x 4	F	18 wks	26 wks	S.C.	Z-PRV	1
*O9346	50 x 4	F	18 wks	26 wks	S.C.	Z-PRV	1
O9369	50 x 4	F	18 wks	22 wks	S.C.	Z-PRV	2
*O9373	50 x 4	M	18 wks	26 wks	S.C.	Z-PRV	2
O9441	50 x 4	M	18 wks	26 wks	S.C.	Z-PRV	3
O9460	50 x 4	M	18 wks	26 wks	S.C.	Z-PRV	4
O9524	50 x 3	F	18 wks	26 wks	S.C.	Z-PRV	5
O9535	50 x 4	F	18 wks	20 wks	S.C.	Z-PRV	6
O9539	50 x 3	M	18 wks	26 wks	S.C.	Z-PRV	6
*O9575	50 x 4	F	18 wks	26 wks	S.C.	PBS	7
*O9343	50 x 4	F	18 wks	26 wks	I.M.	Z-PRV	1
*O9347	50 x 1	F	22 wks	22 wks	I.M.	Z-PRV	1
*O9374	50 x 4	M	18 wks	26 wks	I.M.	Z-PRV	2
O9435	50 x 4	F	18 wks	26 wks	I.M.	Z-PRV	3
O9442	50 x 4	M	18 wks	26 wks	I.M.	Z-PRV	3
O9455	50 x 4	F	18 wks	26 wks	I.M.	Z-PRV	4
O9461	50 x 4	M	18 wks	26 wks	I.M.	Z-PRV	4
O9525	50 x 3	F	18 wks	26 wks	I.M.	Z-PRV	5
O9540	50 x 3	M	18 wks	26 wks	I.M.	Z-PRV	6
*O9572	50 x 4	F	18 wks	26 wks	I.M.	PBS	7
*O9344	50 x 2	F	18 wks	22 wks	I.P.	Z-PRV	1
*O9348	50 x 4	F	18 wks	26 wks	I.P.	Z-PRV	1
*O9375	50 x 4	M	18 wks	26 wks	I.P.	Z-PRV	2
O9436	50 x 4	F	18 wks	26 wks	I.P.	Z-PRV	3
O9443	50 x 4	M	18 wks	26 wks	I.P.	Z-PRV	3
O9462	50 x 4	M	18 wks	26 wks	I.P.	Z-PRV	4
O9522	50 x 2	F	18 wks	20 wks	I.P.	Z-PRV	5
O9526	50 x 3	F	18 wks	26 wks	I.P.	Z-PRV	5
O9541	50 x 3	M	18 wks	26 wks	I.P.	Z-PRV	6
*O9573	50 x 1	F	18 wks	18 wks	I.P.	PBS	7

Table 1. **Z-PRV inoculation of *M. domestica***. Animals were inoculated with Z-PRV starting at 18 weeks and euthanized at 26 weeks. Animals used for IHC are marked with * symbol.

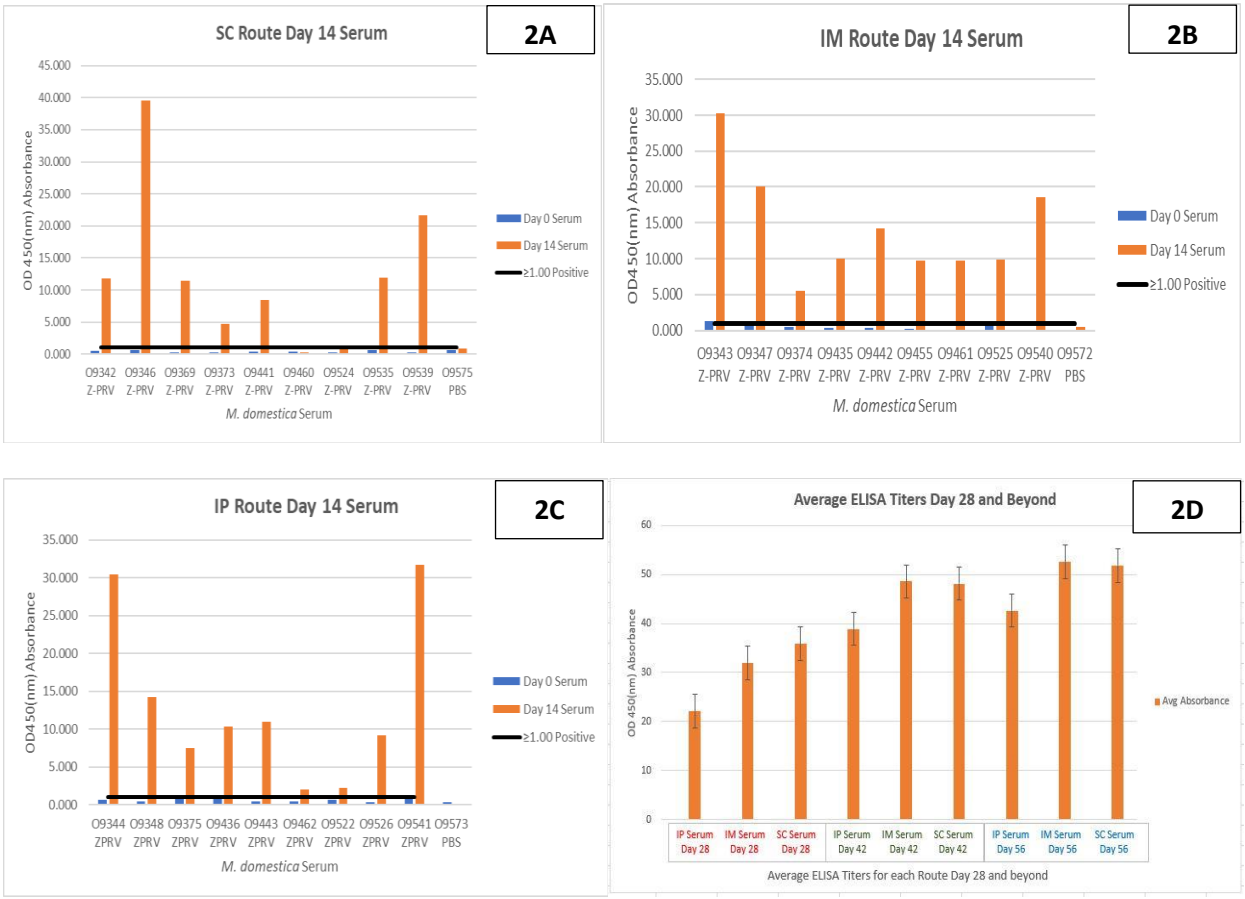
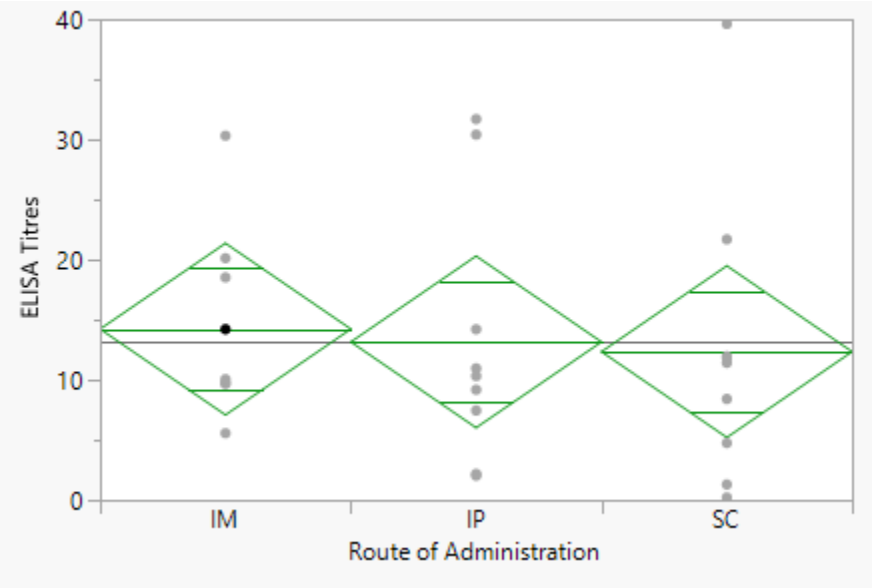


Figure 2. Serological responses of *M. domestica* to Zika virus. 2A.) Day 14 serum of animals inoculated through subcutaneous route. **2B.)** Day 14 serum of animals inoculated through intramuscular route. **2C.)** Day 14 serum of animals inoculated through the intraperitoneal route. **2D.)** Average ELISA Titers for serums that were collected for day 28, 42, and 56. Data was provided by Dr. Andre Pastor and Dr. John Vandeberg.



ANOVA Table for Day 14

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Route of Administration	2	16.0125	8.006	0.0743	0.9286
Error	24	2586.3348	107.764		
C. Total	26	2602.3473			

Summary of Statistics

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
IM	9	14.2328	3.4603	7.0910	21.375
IP	9	13.1633	3.4603	6.0216	20.305
SC	9	12.3523	3.4603	5.2106	19.494

Figure 3. Statistical Analysis of Day 14 ELISA data. Data shows that there was no significant difference between the routes of administration. JMP®, Version Pro 13. SAS Institute Inc., Cary, NC, 1989-2019.

Organs stained for NS1 through IHC								
ID	**SEX**	**Inj Site**	**Virus or PBS**	**Brain**	**Eye**	**Spleen**	**Heart**	**Sex Organ**
O9344	F	IP	V	+	-	+	+	+
O9348	F	IP	V	+	-	+	-	+
O9375	M	IP	V	-	+	-	-	+
O9343	F	IM	V	-	-	+	+	+
O9347	F	IM	V	+	-	-	-	+
O9374	M	IM	V	-	-	-	-	-
O9342	F	SC	V	-	-	-	-	-
O9346	F	SC	V	-	+	-	-	-
O9373	M	SC	V	-	+	-	-	+
O9572	F	IM	PBS	-	-	-	-	-
O9573	F	IP	PBS	-	-	-	-	-
O9575	F	SC	PBS	-	-	-	-	-

Table 2. Organs stained for NS1 through Immunohistochemistry. Seven of the nine animals show repeated confirmation of Z-PRV through antibody staining.

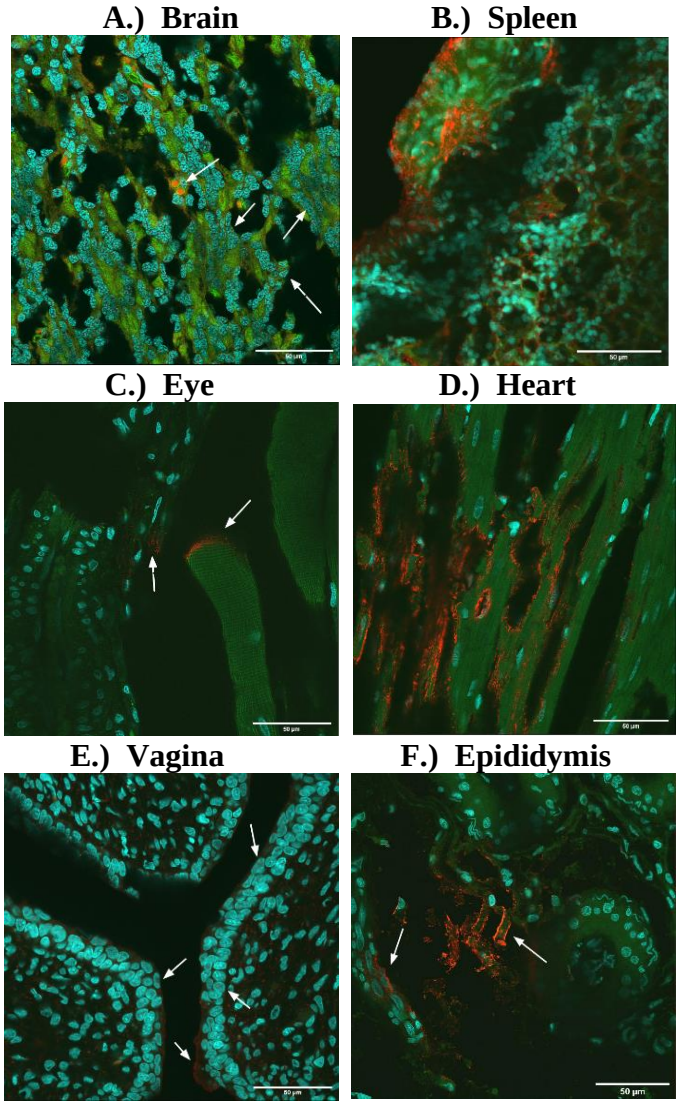


Figure 4. NS1 stain representation of each organ. 4A.) 9347 IM positive brain stain at 60x. **4B.)** 9344 IP positive spleen stain at 60x. **4C.)** 9373 SC positive eye stain at 60x. **4D.)** 9343 IM positive heart stain at 60x. **4E.)** 9348 IP positive vagina stain at 60x. **4F.)** 9373 IM positive epididymis stain at 60x. Colors represent green for cytoplasm, blue for DAPI and red for NS1 signal. Arrows point to NS1 signal.

Summary of Results						
ID	SEX	Inj Site	Virus or PBS	Ab stain	ELISA	% of Positive Tissue
O9344	F	IP	V	+	+	80 %
O9348	F	IP	V	+	+	60 %
O9375	M	IP	V	+	+	40 %
O9343	F	IM	V	+	+	60%
O9347	F	IM	V	+	+	40%
O9374	M	IM	V	-	+	0%
O9342	F	SC	V	-	+	0%
O9346	F	SC	V	+	+	20%
O9373	M	SC	V	+	+	40%
O9572	F	IM	PBS	-	-	0%
O9573	F	IP	PBS	-	-	0%
O9575	F	SC	PBS	-	-	0%

Table 3. Summary of Results for ZIKV infected animals injected in three different routes. Antibody stains compared to ELISA assay show consistent infection.

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BIOGRAPHICAL SKETCH

Juan Garcia Jr earned his Master of Science degree in Biochemistry and Molecular Biology at the University of Texas Rio Grande Valley in 2021, where he focused on characterizing a new animal model for Zika virus pathogenesis research and vaccine development. During his time in UTRGV, Juan Garcia Jr managed and participated in the processing and testing of patient samples for COVID-19 under the direction of Dr. John M. Thomas III. In May of 2017, Juan Garcia Jr received his Bachelor of Science degree in Biology from UTRGV, where he worked as a Teacher assistant and Research Associate I. After graduating, Juan Garcia Jr worked as a zika public health technician for the Hidalgo County Health Department. Juan Garcia Jr is currently the Clinical Lab Manager for the UT-Health clinical lab located in UTRGV. Juan's research interests include the study of viruses and vaccine development. Juan will be pursuing his Ph.D. in Integrated Biomedical Sciences at the University of Texas Health Science Center San Antonio, where he plans to continue research in Microbiology and Immunology,

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