Veterinary Research Scholars Program
University of Missouri

Aedes aegypti Mosquito transmission and the development of STAT2 KO hamster animal model for Zika Virus

Cassandra Tang-Wing[1], Alexis Dadelahi[2], Velmurugan Balaraman[2], Asher Kantor[2], Alexander Franz[2], Jeffrey Adamovicz[2]
[1]School of Veterinary Medicine, St. George's University, [2]Department of Veterinary Pathobiology, University of Missouri

Background

- Mosquito-borne Zika virus (ZIKV) is responsible for the recent outbreak of febrile illness that appears to be asymptomatic in many cases. According to WHO, 65 countries and territories have reported evidence of mosquito-borne Zika virus transmission since 2007. Latin America and the Caribbean are currently experiencing an overwhelming number of ZIKV diseases and congenital infections. Additionally, the CDC reports approximately 5200 cases throughout the United States and Territories.
- While many cases fail to present with clinical manifestations, the neurological implications that result in microcephaly and other aberrant neurological developments in neonates as well as associated Guillan- Barré syndrome in select individuals have raised concern regarding the spread of the virus.
- Information concerning disease transmission, immune response to infection and efficacy of antiviral components in early gestation remain unclear.
- To address these unknowns, an appropriate and effective animal model is necessary to study the pathogenesis and pathology of the virus.
- Previous studies have used immunocompetent mice, deficient in interferon α/β and γ receptors (A129 and AG129 mice) however, they do not directly mimic the pathogenicity in humans nor demonstrate the natural route of infection through mosquito bites [1,2,3,4].

Aim

To develop an animal model that recapitulates ZIKV infection cycle using both natural route by *Aedes aegypti* vector and/or an intra-dermal route that mimics human infection. By identifying the viral-induced immune response and pathology in the STAT2 KO hamsters, we expect that this will be a suitable *in vivo* model to further study ZIKV transmission and pathogenesis.

Growth characteristics of Zika virus strain MR-766

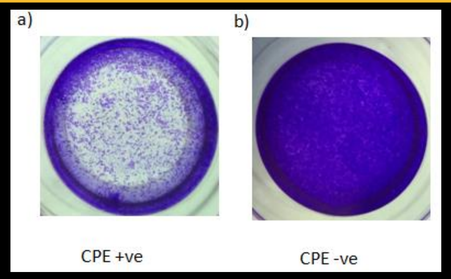

Figure 4: 6 days after Vero cells were infected with ZIKV MR-766 in a 96 well, cells were stained with 0.2 % crystal violet in 10 % Formaldehyde and 20% ethanol. a) Vero cells with marked CPE, as indicated by less intense staining (positive) b) Vero cells showing no CPE indicated by intense staining. Images were taken from dissecting microscope.

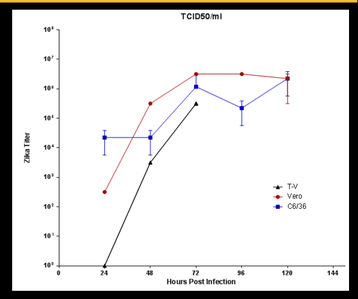

Figure 5: Three cell cultures infected with ZIKV MR- 766 (TV: from laryngeal tumor, which harbors human papillomavirus (HPV) gene sequences; Vero: from kidney of an African green monkey; C6/36: from larvae of *Ae. albopictus*). Analysis by TCID50 shows that Vero cells have highest ZIKV viral titers post-infection.

Experimental Procedures

Part 1: Prior to infecting hamsters

- **Propagating Zika MR- 766 virus on Vero cells**
  Vero cells were seeded in T25 flasks with 0.5 x 10^6 cells/ml. When confluency was determined to be ~3x10^6 cells/mL after 3 days, cells were infected with ZIKV MR-766 (African lineage), at a multiplicity of infection of 0.01. Cell monolayers were inoculated with 30 µl of stock virus mixed with 0.5 ml of growth medium (DMEM) and 10%FBS, incubated for 1h at 37°C and gently swirled every 15 min. 5mL of growth media was added to the cells after 1h. After 72 hrs, when ~40-50% cells exhibit CPE, infected cells were collected from the supernatant to give the ZIKV viral stocks and maintained at -80°C until later use.
- **Feeding and infecting mosquitoes**
  Aedes aegypti, HWE strain, are maintained on raisins and water until adulthood, before being fed defibrinated sheep's blood, supplemented with ZIKV MR-766 infected cell culture (1:1) and ATP as a phage- stimulant (figure 2). After one hour of feeding, blood is removed and fully engorged females (figure 1) are separated from un-fed mosquitoes, until later use during the hamster exposure conditions.
- **Determining viability of viral strain and competence of mosquito**
  At 14 days post blood feeding, mid-guts and carcasses are collected from infected mosquitoes. To analyze ZIKV titers, tissue culture infectious dose50 assay (TCID50) was performed. Vero cells in 24-well plates were inoculated with 150 µl of sterile-filtered saliva sample (diluted in DMEM+7%FBS). After 7 days of incubation, media was removed and 100µL of 0.2 % crystal violet in 10 % Formaldehyde and 20% ethanol was added to stain for 10-15 minutes. Wells were washed and scored for CPE effects. This was repeated daily for 3-4 days. The virus titer was calculated based on 50% endpoints using the Reed and Muench algorithm and expressed as a Log 10 TCID50/ml[5,6].

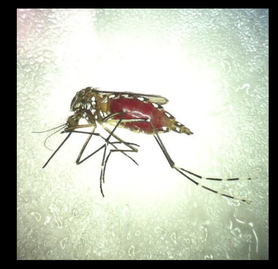

Figure 1: Fully engorged female after bloodmeal indicative of successful feeding.

Figure 2: Experimental setup of mosquitoes feeding on virus-infected cell culture supernatant with defibrinated sheep blood (1:1). Mosquitoes are allowed to feed for 1 hour, till majority are fully engorged.

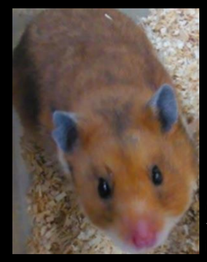

Figure 3: WT and STAT2KO Syrian Golden Hamsters to be infected by ZIKV MR-766 by mosquito bites or intra-dermal injections.

Part 2: Infecting the hamsters

To compare WT and STAT2 KO hamsters with ZIKV, Syrian Golden hamsters (*Mesocricetus auratus)* (figure 3) will each be anesthetized and infected by mosquito bites or by intra-dermal injections, to best recapitulate the natural route of infection. A 2cm x 2cm patch will be shaved on the ventrum of the hamsters. There will be three groups, with pairs of WT and STAT2 KO hamsters.

Group 1, natural route (n=6): infected mosquitoes from part I of the experiment will be exposed to anesthetized hamsters for 30 mins. 3 pairs of WT and KO, with 40, 20, 10 mosquitoes to allow different amounts of exposure and levels of infectivity.
Group 2, intra-dermal route (n=6): 30uL, 10^5-10^6pfu/mL, to be injected into the dermis at multiple sites of anesthetized hamsters.
Group 3 (n=2): WBC counts and antibody to be assessed following determination of infection results of Group 1 and 2.
Once infected, hamsters will be monitored daily for changes in behaviour, temperature and other clinical signs and disease progression for at least 14 days post infection. Blood will be collected to assess viral titers and peak viremia. Whole blood will also be analyzed by RT- PCR for ZIKV RNA, which would be used to inoculate cell cultures to isolate infectious virus. At a later time, this will allow the study of uninfected mosquitoes to feed on infected hamster and assess whether or not the mosquitoes can be infected from an infected host. Liver, spleen, neurological tissues and testes/ovaries would be collected for virus isolation and histopathology.

Competency of mosquito strain, *Aedes aegypti* HWE strain, for ZIKV MR-766

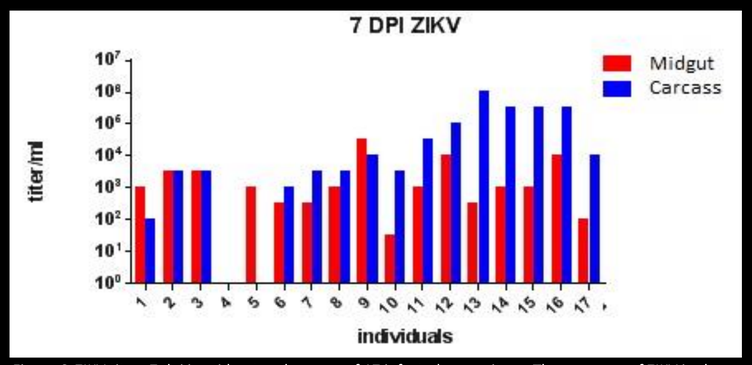

Figure 6: ZIKV titers 7 dpi in mid-gut and carcass of 17 infected mosquitoes. The presence of ZIKV in the carcass demonstrates that there is a not a midgut escape barrier for the virus, suggesting the possible competency of *Aedes aegypti* as a ZIKV MR-766 vector.

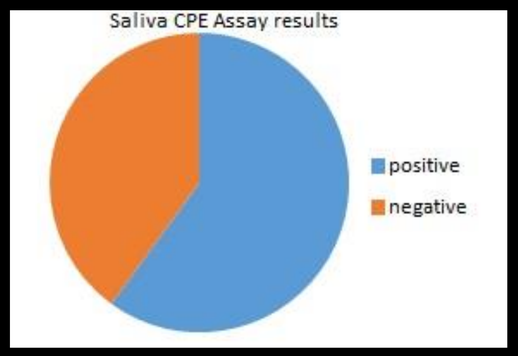

Figure 7: Vero cells assessed for presence of CPE at 3 days post-inoculation with saliva samples demonstrates 60% (18/ 30) infectivity. Suggests the possible competency of *Aedes aegypti* as a ZIKV vector.

Additional information and pitfalls

On STAT2 KO Syrian Golden Hamsters

- Advantage over other rodents; greater metabolism and physiological similarities to humans.
- STAT2 is a crucial element of Type I and III IFN signal transduction pathway.
- Type I IFN pathway is disrupted in STAT2KO hamsters; cannot up-regulate the expression of ISGs, which makes them more susceptible to viral infections [7, 8, 9].

Pitfalls: Polyomavirus in Hamsters

- Hamster 1: found dead and autolyzed
- Hamster 2: moribund and euthanized: positive HaPV - gastric mucosal necrosis, plasmacytic lyphoma, hemangiosarcoma (figure8,9)
- Remaining hamsters: PCR for parasitology (pooled fecal samples) and serology: negative HaPV

On Hamster Polyomavirus [10, 11]

DNA oncogenic virus that induces necrotizing or proliferating lesions mainly in lungs and liver. The virus uncommonly induces two neoplastic syndromes, depending on age of animal when infected. In young, virus is shed and transmitted in urine and multicentric lymphoma involving mesenteric lymph nodes and abdominal viscera is common. In adults, virus is shed and transmitted in infected epithelial cells and skin neoplasms (trichoepitheliomas) is common. Endothelial cells and other components of the vascular wall are the main target cells for oncogenic activity of the polyoma virus.

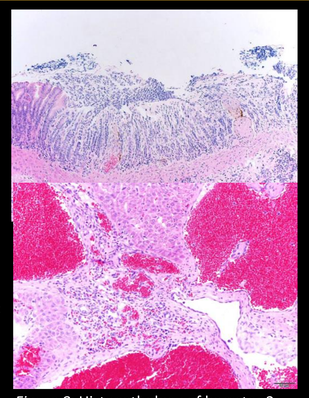

Figure 8: Histopathology of hamster 2. Gastric mucosal necrosis (top) and hemangiosarcoma (bottom) is noted. *PC: Dr. Cynthia Besch-Williford,*

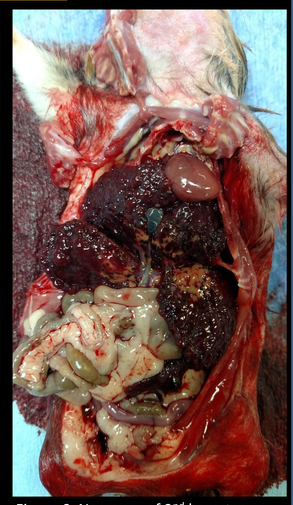

Figure 9: Necropsy of 2nd hamster shows hemangiosarcoma of liver and spleen, plasmacytoid lymphoma of liver and large areas of stomach necrosis.
PC : Dr. Daniel Montonye

Conclusions at this time

Aedes aegypti mosquitoes were infected with ZIKV MR-766 propagated on Vero cell culture. Viable ZIKV MR-766 virus was detected in mid-gut, carcass and saliva, suggestive of their ability to be competent vectors. Once STAT2 KO hamsters are infected by the mosquitoes or intradermally injected from the viral stocks, immune response and pathogenicity can be assessed.

References
1. Alliota MT, Caine EA, Walker EC, Larkin KE, Camacho E, Osorio JE (2016) Characterization of Lethal Zika Virus Infection in AG129 Mice. PLoS Negl Trop Dis 10(4): e0004682. doi:10.1371/journal. pntd.0004682
2. Dowall SD, Graham VA, Rayner E, Atkinson B, Hall G, Watson RJ, et al. (2016) A Susceptible Mouse Model for Zika Virus Infection. PLoS Negl Trop Dis 10(5): e0004658. doi:10.1371/ journal.pntd.0004658
3. Lazear et al. (2016) A Mouse Model of Zika Virus Pathogenesis. Cell Host & Microbe 19, 1–11
4. Rossi, S. et al (2016) Characterization of a Novel Murine Model to Study Zika Virus. *Am. J. Trop. Med. Hyg.*, 94(6), 2016, pp. 1362–1369
5. Higgs S, Olson KE, Kamrud KI, Powers AM, Beaty B (1997) Viral expression systems and viral infections in insects. Beard CB, Louis C, editors: Chapman and Hall, London. 459–483 p.
6. Li MI, Wong PSJ, Ng LC, Tan CH (2012) Oral Susceptibility of Singapore Aedes (Stegomyia) aegypti (Linnaeus) to Zika Virus. PLOS Neglected tropical diseases 6(8):1792.
7. Fan Z, Li W, Lee SR, Meng Q, Shi B, Bunch TD, et al. Efficient gene targeting in golden Syrian hamsters by the CRISPR/Cas9 system. PloS one. 2014; 9(10):e109755. Epub 2014/10/10. doi: 10.1371/journal.pone.0109755 PMID: 25299451
8. Fink K, Grandvaux N. STAT2 and IRF9: Beyond ISGF3. Jak-Stat. 2013; 2(4):e27521. Epub 2014/02/06. doi: 10.4161/jkst.27521 PMID: 24498542
9. Generation of transgenic golden Syrian hamsters Cell Research (2014) 24:380-382. doi:10.1038/cr.2014.
10. DORA <http://dora.missouri.edu/hamsters/hamster-polyomavirus/>
11. Defendi, V., Lehman, J (1964) The nature of the hemorrhagic lesions induced by polyoma virus i hamsters . The Wister Institue of Anatomy and Biology