2015

Sindbis Virus Entry of Mosquito Midgut Epithelia...Is NRAMP Involved?

Florence Yi Ting Chim
University of North Florida, n00452021@ospreys.unf.edu

SINDBIS VIRUS ENTRY OF MOSQUITO MIDGUT EPITHELIA… IS NRAMP INVOLVED?

By

Florence Chim

A thesis submitted to the Department of Biology

in partial fulfillment of the requirements for the degree of

Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

Date 11/30/2015

The thesis of Florence Chim is approved: Date

_____ _____
Dr. Doria F. Bowers

_____ _____
Dr. David S. Waddell

_____ _____
Dr. Paul J. Linser

Accepted for the Biology Department:

_____ _____
Dr. Cliff Ross
Chair

Accepted for the College of Arts and Sciences:

_____ _____
Dr. Barbara Hetrick
Dean

Accepted for the University:

_____ _____
Dr. John Kantner
Dean of the Graduate School

Acknowledgements

I would like to thank Dr. Doria Bowers for this wonderful opportunity to work on my thesis in your lab. Without you, none of this would be possible. Thank you for your guidance and having faith in me. You have constantly demonstrate that having passion in your work will take you far in life. You are like an aunt to us in the lab, always watching out for us. You always ensured we had all the resources possible for our investigations. I am very proud to say that I earned my Master's in the Bowers' lab!

Thank you Dr. David Waddell and Dr. Paul Linser for your guidance in this investigation. When I first started the M.S. program, I had absolutely no idea what to expect. Except that I knew I wanted to surround myself with mentors that would provide the best advice. That is why I am honored that you agreed to be on my committee.

To my friends in the lab, Zoe Lyski and Michael Stephens, I really appreciate your support and advice! Thanks for allowing me to discuss ideas, shadow you, and figuring out assays with me! Jason Saredy, thanks for running assays with me, showing me how to use the fancy confocal microscope with a bagillion buttons/knobs, and ImageJ! My scale bars will be all over the place if it were not for you! Most of all, thank you everyone for catching my tears when assays just will not cooperate!

Thank you Rachel for supporting me and showing me how to BLAST!!! Your niche in genetics really helped! Pretty soon, we will be wearing matching lab coats!

To my parents, thank you for being supportive in my journey as a perpetual student and for letting me be who I want to be and do what I want to do. You have never once told me I am not to do something because you disapproved. You allowed for me to make my own decisions while providing guidance and that is the best parenting a child could hope for.

To my grandma, uncles, aunt Elsa, Ervis, and Brandi, also thank you for supporting me in my journey as a perpetual student. Not one of you ever asked, "why is it taking you so long to graduate?!". To which I would have replied, "because I am trying to identify a protein that may or may not be present!". I am almost done! Thanks for never rushing me!

To my parents-in-law, I appreciate your support and allowing me to focus solely on my research! Ti je shuma mer! Pese lart!!! Tani ha book, biskote, torte me akullore!!!

Last but never least, my husband. Thank you Arbi for your support. Without you, this journey would have been really difficult. You have filled my life with so much joy and laughter!!! Thanks for putting up with me during times of stress and taking such good care of me and our fur babies, Milo and Marsi! More dogs? Yay? Nay? JK? Oh, and thanks for tuition, food, shelter…all that good stuff!

Table of Contents

List of Figures

Abstract

Sindbis virus (SINV) is an arthropod-borne *Alphavirus* in the family Togaviridae. Sindbis virus has a broad host range that includes avian, mammalian, and human hosts; therefore, its receptor(s) is/are highly conserved. When the mosquito imbibes a viremic blood meal, the virus infects the midgut cells, disseminates into the hemolymph, and eventually infects the salivary glands. The midgut is an organ of transmission and the virus must overcome the midgut epithelia infection- and escape-barriers. Sindbis virus infection is determined by the chance collision of the glycoproteins with a compatible receptor. Research has supported the involvement of high-affinity laminin receptor and heparan sulfate in SINV binding to host cells. However, it has been suggested that not all strains of SINV are dependent on heparan sulfate for attachment/entry and that SINV could be utilizing multiple receptors. A study using *Drosophila* demonstrated that, of the nine genes that encode for proteins that enhance SINV infection, only natural resistance-associated macrophage protein (NRAMP) was conserved. A symporter of divalent metals and hydrogen ions, NRAMP is ubiquitously expressed. Overexpression of NRAMP led to an increase in SINV infection of human cells while deletion of NRAMP in mouse and *Drosophila* decreased SINV infection. Sindbis virus could be utilizing this protein to overcome the infection barriers of mosquito midgut epithelia. In this study, NRAMP was localized to *Aedes aegypti* and *Anopheles quadrimaculatus* tissues via immunofluorescence assay and TR339-TaV-eGFP was detected in the midgut epithelia and visceral muscles. We suspect that NRAMP was detected on midguts and/or Malpighian tubules of *Aedes aegypti* and *Anopheles quadrimaculatus*. The similarities between the pattern of NRAMP labeling and TR339-TaV-eGFP infection of the midgut suggest that SINV infection is influenced by NRAMP in the midgut epithelia. Because NRAMP is ubiquitously expressed, this research provides insight into the attachment and entry phase of the arbovirus lifecycle.

Chapter 1: Introduction

Arthropod-borne viruses (arboviruses) are maintained in the environment by the continuous cycle between invertebrates and vertebrates (Fields 2007). Due to the lack of protective vaccines, invertebrate resistance to insecticides, and the difficulty of containing transmission of pathogens, arboviruses remain a global health concern to humans and animals (Villarreal et al., 2000). The key to preventing outbreaks due to arboviruses is the understanding of the biology of arthropods and the virus interactions with the host.

First isolated in 1952 from mosquitoes in Sindbis, Egypt, Sindbis virus (SINV) is an *Alphavirus* that is a member of the Togaviridae family (Fields 2007). Sindbis virus is a hybrid structure composed of products originating from viral genetic information and a lipid membrane acquired during egress from its host. Its plus sense, single stranded RNA genome consists of 11,703 nucleotides, a methylated cap on its 5' end, and a 70 nucleotide long polyadenylated tail on its 3'end (Fuller 1987). The genome is housed in an icosahedral nucleocapsid, which is enveloped by a host-derived lipid bilayer studded with 240 heterodimers of E1 and E2 glycoproteins that aid in attachment to host cells. The virion contains four non-structural proteins primarily involved in enzymatic actions that are translated from genomic RNA. The three structural proteins, capsid, E1, and E2, are translated from subgenomic RNA. During translation, the capsid protein auto-cleaves from the polypeptide chain and binds to the packaging signal region of genome RNA. The growing polypeptide chain translocates to the endoplasmic reticulum where it is glycosylated and proteolytically cleaved by host's cellular signal peptidase to produce PE2, 6K, and E1. During transport to cell surface in Trans-Golgi vesicles, cleavage of PE2 by furin protease produces E2 and E3; the latter is discarded prior to encapsidation (Fields, 2007).

Entry and infection of host cells could occur by either receptor-mediated endocytosis (Helenius et al., 1980) or by fusion at the plasma membrane (Paredes et al., 2004). Sindbis virus attachment to host cells is determined by the amino acid sequence of the E2 glycoprotein (Pierro et al., 2007). Primarily, transmission of SINV occurs horizontally between the mosquito and the vertebrate host. Sindbis virus has a broad host range that includes avian, insects, mammalian, and human hosts; therefore, its receptor(s) is/are highly conserved (Bowers et al., 1995; Byrnes and Griffin 1998). Although SINV does not cause life-threatening infections in humans, several other *Alphaviruses* such as Ross River virus, Chikungunya virus, as well as eastern, western, and Venezuelan equine encephalitis viruses are fatal to humans and other lower vertebrates (Byrnes and Griffin 1998, Gubler, 1998; Weaver et al., 2012). Due to SINV's lack of pathology in humans and its persistent infection in mosquitoes, it is the prototypic *Alphavirus* (Fields 2007). The SINV consensus sequence TR339, which is derived from ancestral strain AR339, was used for this investigation.

Mosquito models used were *Aedes aegypti* (Orlando) and *Anopheles quadrimaculatus* Say. *Ae. aegypti*, the yellow fever mosquito, is a container-breeding mosquito that is endemic to the United States and dominates in urban areas of south Florida (O'Meara et al., 1995). Viruses that have been isolated from Aedine mosquitoes include dengue, yellow fever, West Nile, eastern equine encephalitis, to name a few (Benedict et al., 2007; CDC 2011).

Transmission of malaria parasite from mosquitoes to humans is only vectored by female Anopheline mosquitoes. *Anopheles quadrimaculatus* is widely distributed throughout the eastern United States and southeastern Canada. Malaria occurrences are rare in the United States, however, the distribution of *An. quadrimaculatus* and the 1850 distribution of malaria transmission suggest that it is the primary malaria vector in the United States (Levine et al.,

2004). While *Ae. aegypti* is a primary vector of SINV, *An. quadrimaculatus* is not; however, their susceptibility and widespread distribution in Florida make both species applicable for this investigation.

When mosquitoes imbibe blood, the insect directly acquires the energy and proteins needed for reproduction while incidentally transmits arboviruses. Ingested pathogens from a viremic blood meal initiate infection in the midgut, disseminate into the open hemocoel plasma (hemolymph), and then infect secondary organs. Sindbis virions must overcome the midgut infection barrier (MGIB) and midgut escape barrier (MGEB) for replication, amplification, development, and transmission (Paulson et al., 1989). Aside from the peritrophic membrane that forms during blood digestion, most of the cells that compose the simple columnar epithelium in the gut are covered in microvilli, contributing to the MGIB (Weaver and Scott 1990). The complex infoldings of the basement membrane, visceral muscles, and surrounding tracheoles contribute to the MGEB (Zieler et al., 2000; Vo et al., 2010). In addition to the morphological hindrances, the host would also have cellular responses to the virus, such as RNAi (Oviedo et al., 2011). Stresses caused by non-viremic or viremic blood meal include distention of the midgut, apoptosis, ejection of infected epithelial cells into the lumen, and/or nucleoli enlargement (Weaver and Scott 1990; Okuda et al., 2005, 2007). When Sindbis virions disseminate from the gut, they are free in the hemolymph or maintained by hemocytes while traveling and bathing other organs (Parikh et al., 2009).

Similar to the gut, SINV must overcome the salivary gland infection barrier (SGIB) and salivary gland escape barrier (SGEB) prior to transmission by bite. Once SINV emerges in the saliva, the mosquito is able to transmit the virus to permissive vertebrate hosts (Almeras et al., 2010). Salivary glands are located bilaterally in the mosquito thorax; in females, each salivary

gland is comprised of two lateral lobes and one median lobe (Janzen and Wright 1971). Enzymes produced in the proximal lateral lobes are essential to sugar digestion while those produced in the distal portion of the lateral lobes and median lobe are essential to blood feeding (Moreira-Ferro et al., 1999; Siriyasatien et al., 2005; Smartt et al., 1995). Mosquito saliva also contains proteins that impair host immune responses to arboviruses (Sim et al., 2012). Although a portion of the lateral lobes and the median lobe function similarly, unknown differences between the lobes permit the median lobe to be refractory to SINV infection (Bowers et al., 1995, 2003; James 2003; Janzen and Wright 1971; Juhn et al., 2011).

Mechanisms by which SINV infect mosquito tissues are unclear. In the 1990's, research supported high-affinity laminin receptor as a SINV receptor (Ubol and Griffin 1991; Wang et al., 1992) and heparan sulfate's involvement in SINV binding to permissive host cells (Byrnes and Griffin 1998; Klimstra et al., 1998). However, Byrnes and Griffin as well as Klimstra and colleagues both published in 1998 that not all strains of SINV are dependent on heparan sulfate for attachment/entry and suggested that SINV could be utilizing multiple receptors and multiple receptors or attachment factors could even be on the same cell type.

In 2011, Rose et al.'s work with *Drosophila* supported natural-resistance associated macrophage protein (NRAMP) as a receptor for SINV and suggested that laminin receptor and heparan sulfate promote infection ("pre-receptors") but are not necessary. Of the nine genes that encode for proteins that enhance SINV infection, NRAMP was highly conserved. A symporter of divalent metals and hydrogen ions, NRAMP is ubiquitously expressed at the organismal level. NRAMP has 12 transmembranal domains and 6 extracellular and 5 intracellular loops with its N- and C-termini in the cytoplasm (Nevo and Nelson 2006). In mammals, there are two members of the NRAMP gene family, NRAMP1 and NRAMP2; NRAMP1 is localized to phagosomal

membrane of macrophages and NRAMP2 colocalizes with transferrin at the plasma membrane, transporting iron into the cytoplasm (Gruenheid et al., 1999). Human cells that were treated with a high concentration of iron attenuated SINV infection while overexpression of NRAMP led to a 2-fold increase in SINV infection. *Drosophila* NRAMP (dNRAMP, malvolio, and MVL) transports manganese and iron, which are metals involved in signal transduction of taste perception. Deletion of NRAMP in *Drosophila* decreased SINV infection (Rose et al., 2011). Similar to *Drosophila*, NRAMP was also detected in the heads of *An. albimanus* suggesting that this protein may also play a role in taste perception in mosquitoes (Martinez-Barnetche et al., 2007). Sindbis virus may be utilizing this transmembrane protein as a receptor to overcome the infection barriers of mosquito midgut and salivary gland epithelia. In this investigation, immunofluorescence labeling of NRAMP and infection of mosquito midguts with TR339-TaV-eGFP (Sun et al., 2014) were observed to determine the correlation between SINV and its putative receptor.

Chapter 2: Methods and Materials

Hatching and Maintaining Mosquitoes

Colonized *Aedes aegypti* and *Anopheles quadrimaculatus* eggs (USDA, Gainesville, FL) were hatched and reared in the University of North Florida insectary under standard environmental conditions (25.5±.5°C, 70-80% humidity, lighting with 30 minutes of "sunrise" and 30 minutes of "sunset" bracketing a 16:8 light/dark photoperiod). *Aedes aegypti* eggs were hatched in a 1% nutrient broth (Becton Dickson Microbiology Systems, Spanks, MD) and approximately 300 first instar larvae were transferred to each plastic pan of 1.5L tap water (Bowers et al., 1995). *Anopheles quadrimaculatus* eggs were hatched directly in plastic pans of 1.5L tap water. Larvae were fed thrice weekly with a 2% aqueous beef liver powder suspension (ICN Biochemicals, Cleveland, OH). Pupae were transferred to plastic cups housed in white plastic cage topped with mosquito netting. For the carbohydrate source, honey-soaked cellucotton was placed on top of each cage; cups containing water saturated cotton balls were supplied as the hydration source. Female mosquitoes 5-15 days post-emergence were used for experiments.

Adult female mosquitoes were isolated and deprived of a carbohydrate source for 24 hours prior to blood feeding. At 5 days post-emergence, mosquitoes were offered 10mL of warmed defibrinated bovine blood (Colorado Serum Company, Denver, CO) through a bovine collagen sausage casing (22mm; The Sausage Maker Inc., Buffalo, NY) hung vertically for 1 hour (Lyski et al., 2012). When proffering a viremic blood meal, 1mL of TR339-TaV-eGFP (1.67×10^8) was diluted in 9mL of blood. Fully engorged female mosquitoes were gently isolated. Tissues from mosquitoes that imbibed on the viremic blood meal were resected at days 3-9 post-infection.

Cell culture, virus amplification, and plaque assays

Cultured C7-10 (*Ae. albopictus*) cells were grown in 25 cm^2 flasks at 28˚C, 5% CO_2 in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 5% tryptose phosphate broth (TPB; Becton Dickinson Microbiology Systems), and 20µL/mL gentamycin (Gibco). Cells were supplemented with EMEM once a week and, when preconfluent, lifted by shearing agitation and passaged.

Cultured BHK-21 (baby Syrian hamster kidney) cells were grown in 25 cm^2 flasks at 37˚C, 5% CO_2 in EMEM supplemented with 5% FBS, 5% TPB, and 20µL/mL gentamycin. Cells were lifted with 0.25% trypsin and passaged every 72 hours; cells were supplemented with fresh EMEM between passages.

When cultured BHK-21 cells were approximately 95% confluent, TR339-TaV-eGFP/virus growth media (VGM; 3% fetal bovine serum in PBS) was applied and allowed to adsorbed for 1 hour on rocker at room temperature. Virus solution was replaced with 8mL of EMEM and cells were returned to 37˚C for 24 hours. Cell media was centrifuged and supernatant was collected and transferred to -80˚F for 24 hours prior to storing at -20˚F until needed.

To quantify stock virus titer, plaque assays were performed on BHK-21 cells. Tenfold serial dilutions of virus were prepared using warmed (37˚C) VGM. Preconfluent monolayers were inoculated with virus and placed for 1 hour on a rocker at room temperature. An agarose/EMEM overlay was applied to each flask, allowing budded virions from infected cells to infect only neighboring cells. Cells were checked for cytopathic effects (CPE) every 12 hours.

Virus plaques, which are areas of lysed cells, were visualized by staining with a 0.015% neutral red solution (Gibco). Because neutral red is a vital dye, dead cells could not take up this

dye, thus plaques are areas of clearing. At low dilutions, plaques were accurately counted and calculated for plaque forming units (PFU) to determine the virus titer (Figure 1).

Figure 1 Plaques were traced and counted for calculating virus titers.

Immunofluorescent microscopy

Tissues from naïve, bloodfed, and TR339-TaV-eGFP-bloodfed mosquitoes were resected in PBS and either fixed onto microscope slides or transferred to wells of a 96-well plate for fixation. Tissues were fixed with 4% paraformaldehyde in 0.1M cacodylate buffer for 1 hour at room temperature. To permeabilize, tissues were incubated in 0.2% Tween 20 for 15 minutes following paraformaldehyde fixation or alone in 70% acetone for 2 minutes. After three washes with PBS (5 minutes each), fixed tissues were blocked with SuperBlock™ (Life Technologies, Grand Island, New York) for 1 hour at room temperature. Tissues were incubated with primary antibody overnight on rocker at room temperature and washed three times in PBS; primary antibodies included 1:100 dilution of rabbit polyclonal anti-actin (H-196) and 1:50 dilution of rabbit polyclonal anti-NRAMP2 (H-108). The secondary antibody, FITC-conjugated goat anti-rabbit IgG (sc-2012) or TX-Red-conjugated goat anti-rabbit IgG (sc-2780), was applied to tissues at a 1:100 dilution for 2 hours on rocker at room temperature. All antibodies used were from Santa Cruz Biotechnology, Inc (Dallas, TX). After 3 washes with PBS, slides were mounted

with VECTASHIELD antifade medium (Vector Laboratories, Burlingame, CA); tissues that were fixed and labeled in wells of a 96-well plate were transferred to microscope slides containing wells hand-made with glass coverslips and valap (1:1:1 of vaseline, lanolin and paraffin) and mounted in 60% glycerol in PBS (figure 2). The areas of fluorescence were analyzed using an epifluorescent and/or confocal microscope.

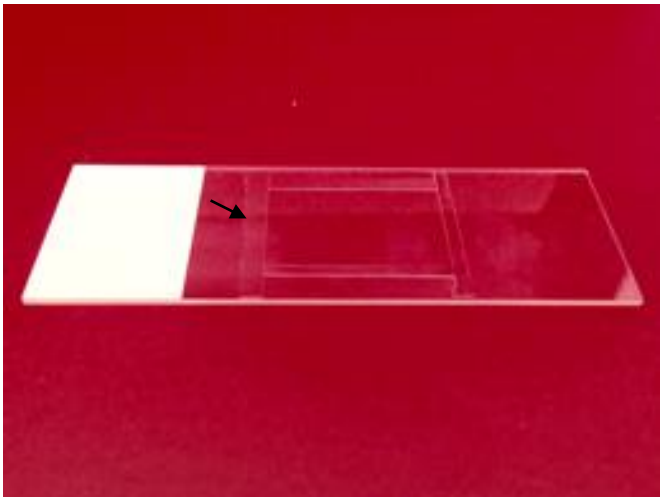


Figure 2 Glass microscope slide with well, composed of hand-cut glass coverslip sidings (→) attached with valap.

Protein analysis

Salivary glands, midguts, and Malpighian tubules (number of organs ranged from 1-8) were resected from *Ae. aegypti* and transferred to glass trituration vials each containing 10 glass beads, 300µL of calcium-deficient PBS (PBS-D), and 100µL each of *N*-α-tosyl-L-lysinyl chloromethylketone (TLCK) and *N*-tosyl-L-phenylalaninyl-chloromethylketone (TPCK). Samples were vortexed for 2 minutes at high speed in a biosafety laminar flow hood. The trituration solution (100µL) was denatured with 5µL of 5% sodium dodecyl sulfate (SDS); the supernatant was collected and stored at -80˚C until needed.

Nitrocellulose membranes (Protran® BA85, Sigma-Aldrich, St Louis, MO) were immersed in Milli-Q® (EMD Millipore, Billerica, MA) purified water for 2 minutes and

equilibrated in 1X transfer buffer (Boston BioProducts Inc., Ashland, MA) with 10% methanol for 2 minutes. Each sample (100µL) was blotted individually onto membrane with the Minifold II slot-blot system (Schleicher & Schuell, Keene, NH).

Blotted samples were blocked with SuperBlock™ blocking buffer for 30 minutes at room temperature and washed 3 times with PBS (5 minutes each) before incubating in a 1:200 dilution of primary antibody, rabbit polyclonal anti-NRAMP2, overnight at room temperature. After three rinses in PBS, the secondary antibody, HRP-conjugated goat anti-rabbit IgG (sc-2301; Santa Cruz Biotechnology), was applied to the membrane at a 1:5000 dilution for 1 hour at room temperature. The membrane was rinsed three times with PBS and the presence of HRP was detected using 3,3',5,5' tetramethylbenzadine (TMB) stabilized chromogen (Life Technologies). The reaction was terminated using a sulfuric stop solution (Life Technologies) when the desired color intensity was observed.

To identify the presence of NRAMP in mosquito midguts, the Catch and Release® v2.0 reversible immunoprecipitation system (EMD Millipore) was used. Actin antibody was used as the positive control. Midguts were resected in PBS and transferred to 300µL of homogenizing buffer (1:1000 protease inhibitor cocktail, 10mM tris-base, 1%SDS, and 10% glycerol; Sigma-Aldrich, St. Louis, MO) for at least 30 minutes. Organ samples (total number: 4, 8, 16, 32, and 64 midguts) were triturated with 5 glass beads (3mm); samples were vortexed after each midgut resection. Cell lysate and antibody, 1:100 dilution of rabbit polyclonal anti-actin or 1:50 dilution of rabbit polyclonal anti-NRAMP2, were incubated overnight on a rocker at 4˚C. Flow-throughs were collected and proteins were eluted in its denatured form for SDS-PAGE.

Resections of mosquito tissues for SDS-PAGE were conducted as previously described. After incubating in homogenizing buffer for 30 minutes, sample solutions were centrifuged

(3500g) for 15 minutes at 4˚C. The sample loading buffer (3µL) was added to sample solutions (12µL each) and warmed in a dry bath maintained at 95˚F for 7 minutes. Using a 7.5% resolving gel, proteins were separated for 4 hours at a constant current of 25mA. The gel was stained or blotted onto polyvinyl difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA).

To increase the sensitivity of silver stain, the gel was first stained with 0.003% Coomassie Brilliant Blue R-250 (Life Technologies) for 45 minutes. The stained gel was washed with destaining solution (45% methanol/ 45% deionized water/10% glacial acetic acid) at 30 minute intervals until the background of the gel was clear. Bio-Rad silver stain (Bio-Rad Laboratories, Inc.) was used to detect the presence of actin and NRAMP2 in mosquito tissues.

The proteins were transferred in Towbin buffer (Towbin et al., 1979) onto PVDF membranes at 50mA for 1 hour. Blotted proteins were blocked with SuperBlock™ for 1 hour and probed with primary antibodies, 1:100 dilution of rabbit polyclonal anti-actin or 1:50 dilution of rabbit polyclonal anti-NRAMP2, overnight on rocker at room temperature. After three rinses in PBS (5 minutes each), the secondary antibody, HRP-conjugated goat anti-rabbit IgG, was applied to the membrane at a 1:5000 dilution for 2 hours at room temperature. The proteins of interest were detected using 1-Step™ TMB-Blotting Substrate Solution (Life Technologies).

Ch. 3 Results

NRAMP localization in mosquito tissues

An antibody against NRAMP2 of human origin was used for the detection of NRAMP. Mosquito tissues were fixed with 4% paraformaldehyde in 0.1M cacodylate buffer and permeabilized with 0.2% Tween 20 following paraformaldehyde fixation or alone with 70% acetone. Areas of fluorescence were observed using an epifluorescent and/or confocal microscope. NRAMP was not detected in the salivary glands of *Ae. aegypti* (figure 3). NRAMP2 labeling of midguts were punctate and speckled; patterns of labeling were clustered, resembling SINV infection foci (figure 4). Aside from the clustered labeling of NRAMP, the punctate labeling occasionally resembled a grid; this suggests that NRAMP was detected on the peristaltic muscles (figure 5).

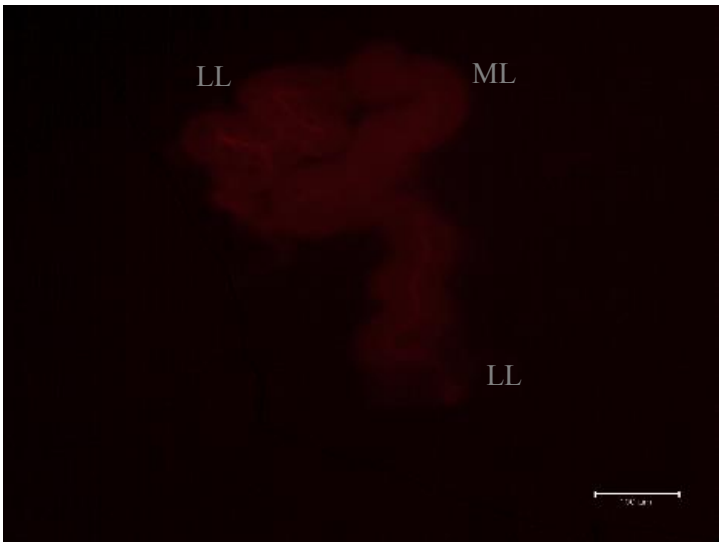


Figure 3 NRAMP was not detected in the median lobe (ML) or the lateral lobes (LL) of the salivary glands of *Ae. aegypti* when labeled with anti-NRAMP2 and TX-Red-conjugated secondary antibody; fixed with acetone, epifluorescence microscopy, 100µm scale bar.

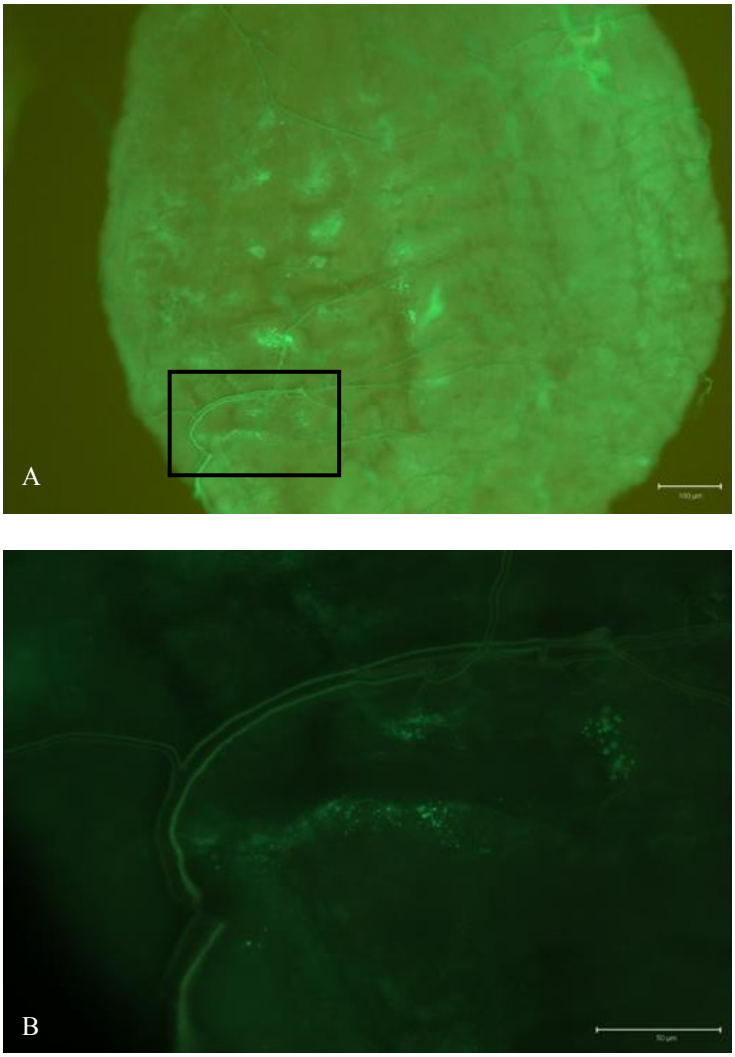


Figure 4 (A) Multiple foci-like immunofluorescence labeling of NRAMP2 in midgut of *Ae. aegypti* fixed with acetone; epifluorescence microscopy 10X, 100µm scale bar. (B) Magnified (40X, 50µm scale bar) foci-like labeling of NRAMP2 by box indicated in (A).

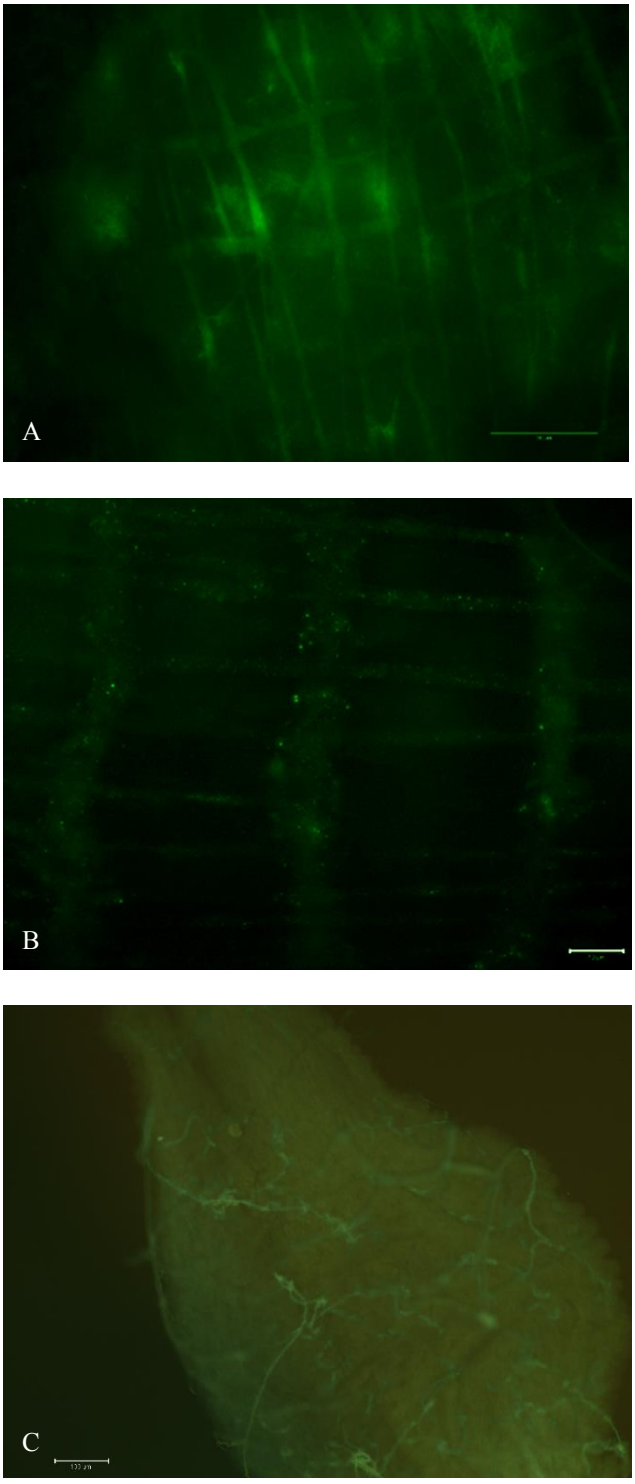


Figure 5 Immunofluorescence labeling of NRAMP2 in midgut of *Ae. aegypti* fixed with acetone; epifluorescence microscopy. (A) Note punctate and clustered fluorescent signals, 50µm scale bar. (B) Labeling confined to muscle bands, 10µm scale bar. (C) Negative control, 100µm scale bar.

NRAMP is ubiquitously expressed at the organismal level and highly conserved. Sindbis virus has evolved to utilize NRAMP as a receptor for internalization into host cells (Rose et al., 2011). NRAMP has been identified by Western blot in the head, midgut, and Malpighian tubules of *Anopheles albimanus*; AnaNRAMP was suggested to play an important role in iron metabolism (Martinez-Barnetche et al., 2007). With the midgut being the organ that directly receives and processes the blood meal, it was expected that NRAMP would be localized to the apical aspect of the midgut. Nevo and Nelson (2006) suggested that NRAMP is the main transporter of iron into the duodenum. However, NRAMP was detected on the basal side of the midgut epithelia of *Ae. aegypti* (figure 6). According to Martinez-Barnetche et al.'s study (2007), a 42% reduction in NRAMP concentration was observed in the midguts of *An. albimanus* 24 hours after a blood meal. This decrease in concentration was observed when labeling for NRAMP in midguts of *Ae. aegypti* females that imbibed on a blood meal; fluorescence was faint or could not be observed.

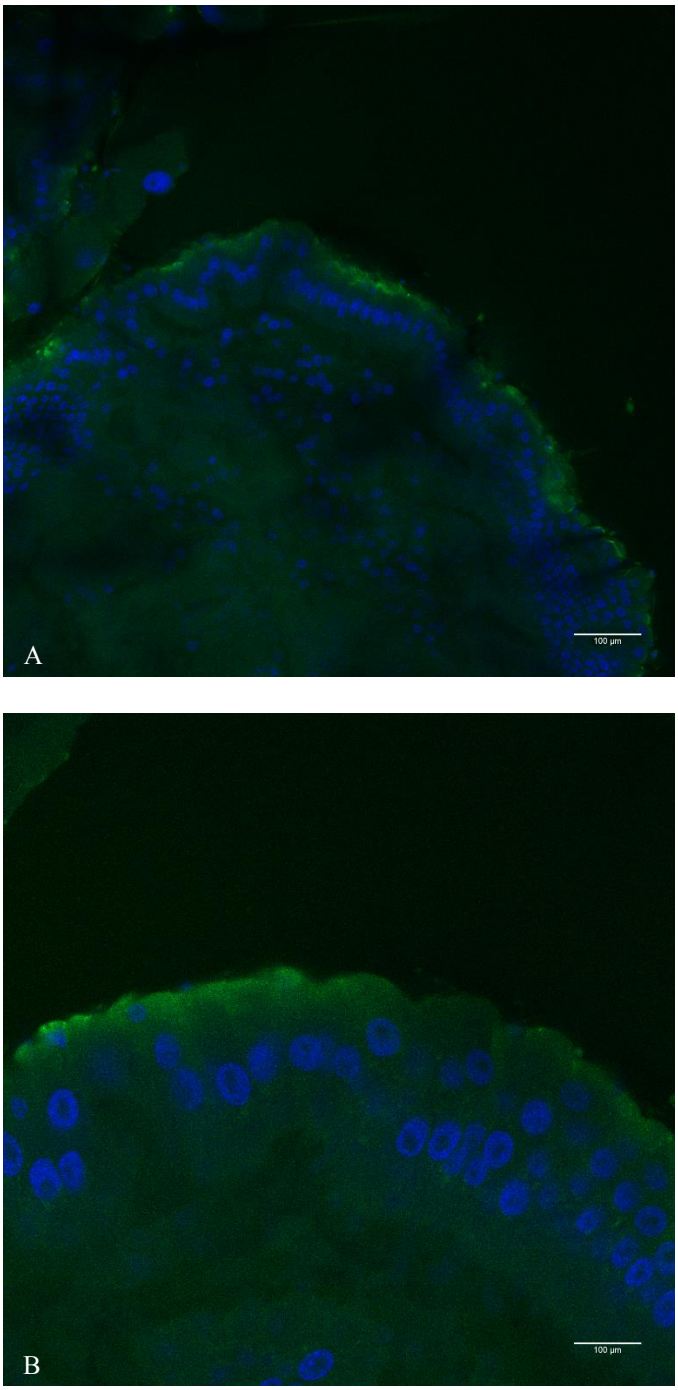


Figure 6 Optical cross-section of midgut of *Ae. aegypti* fixed with 4% paraformaldehyde/0.2% Tween 20 and immunofluorescence labeled for NRAMP2 (green) and counterstained with DRAQ5 (blue; nuclear stain); confocal microscopy, 100µm scale bar. (B) Magnified image of (A) with interest on the midgut epithelia. NRAMP labeling was localized to the basal aspect of epithelial cells.

NRAMP was detected in Malpighian tubules of unfed *An. quadrimaculatus* by immunofluorescence labeling with a polyclonal antibody against NRAMP2; NRAMP was not localized in Malphighian tubules of *Ae. aegypti*. Malpighian tubules were resected directly onto glass microscope slides and fixed in acetone for 2 minutes. Similar to NRAMP labeling in midguts, fluorescence was punctate and localized (figure 7). However, labeling in the Malpighian tubules did not occur as often as in the midguts.

Figure 7 Immunofluorescence labeling of NRAMP2 (→) in Malpighian tubules of *An. quadrimaculatus* fixed with acetone; epifluorescent microscopy, 50µm scale bar.

Protein analysis

The slotblot assay was used to assess the presence of NRAMP in mosquito tissues. Salivary glands, midguts, and Malpighian tubules from female *Ae. aegypti* were triturated, blotted onto nitrocellulose membranes, and probed with primary antibody. When TMB stabilized chromagen was applied, the reaction did not yield a color change.

Midguts of female *Ae. aegypti* and *An. quadrimaculatus* were used for SDS-PAGE. Midguts were triturated in homogenizing buffer; samples were either denatured and applied directly to the gel or immunoprecipitated for NRAMP, with actin as the positive control.

Immunoprecipitants and the flow-throughs were collected and denatured prior to SDS-PAGE. Based on the molecular weight of AnaNRAMP (63.7kDa) and actin (41.7kDa), a 7.5% polyacrylamide gel was used for this assay.

Straight denatured samples, denatured immunoprecipitants, and denatured flow-throughs were assessed with SDS-PAGE, enhanced with 0.003% Coomassie Brilliant Blue, and stained with Bio-Rad silver stain. There were 2 protein bands located in the region of interest with molecular weights between 55-70kDa (Figure 8 & 9). Lanes containing immunoprecipitants and flow-throughs did not yield a band with a molecular weight similar to AnaNRAMP or actin.

The presence of NRAMP was not detected by Western blot using polyclonal anti-NRAMP2. Aside from the protein ladder, the proteins from midgut samples failed to transfer onto PVDF membranes. When TMB stabilized chromogen was applied, the membrane became splotchy after the reaction.

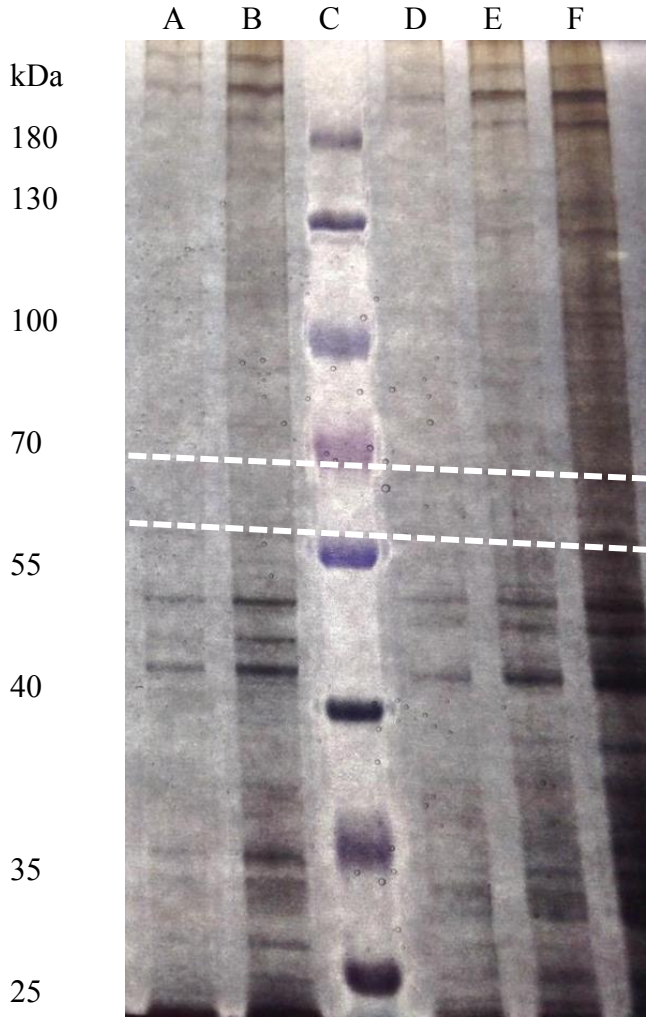


Figure 8 Proteins from triturated mosquito midguts were separated via PAGE. The gel was stained with Coomassie Brilliant Blue and counterstained with silver. Lane A (4 MGs) and lane B (8 MGs) contain *Ae. aegypti* tissue. Lane D (4 MGs), lane E (8 MGs), and lane F (16 MGs) contain *An. quadrimaculatus* tissue. Lane C corresponds to molecular ladder. NRAMP2 is 63.7kDa (area indicated by white lines).

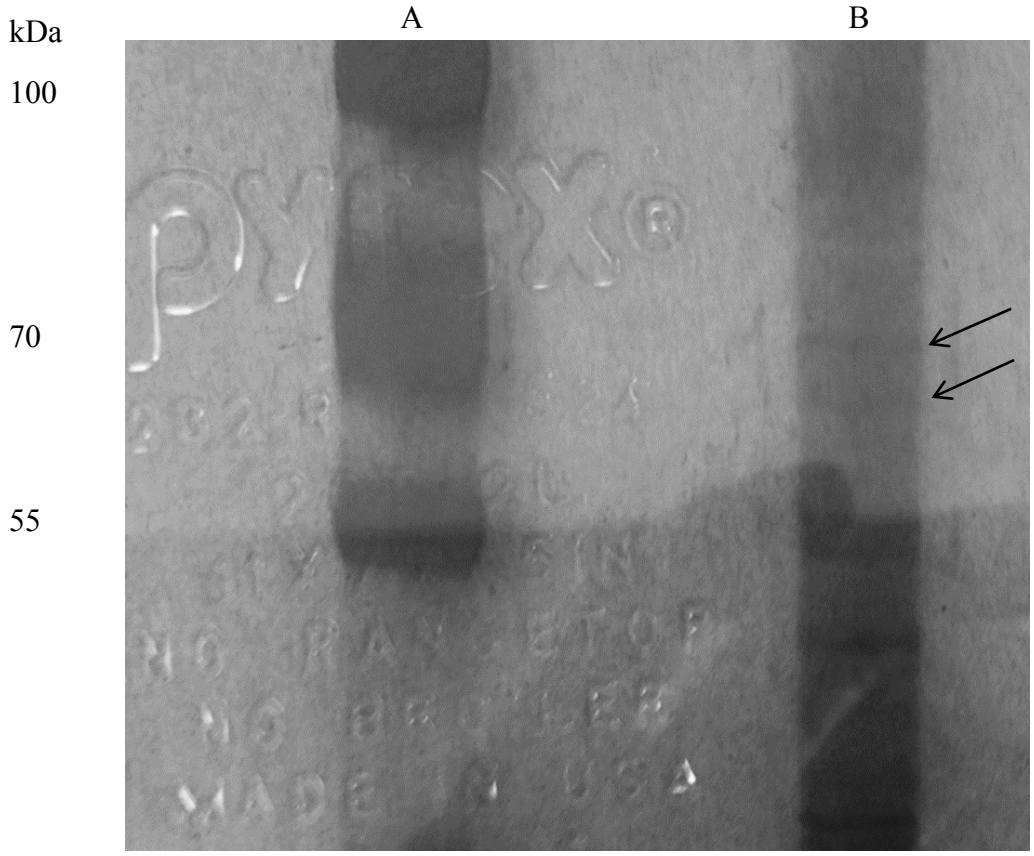


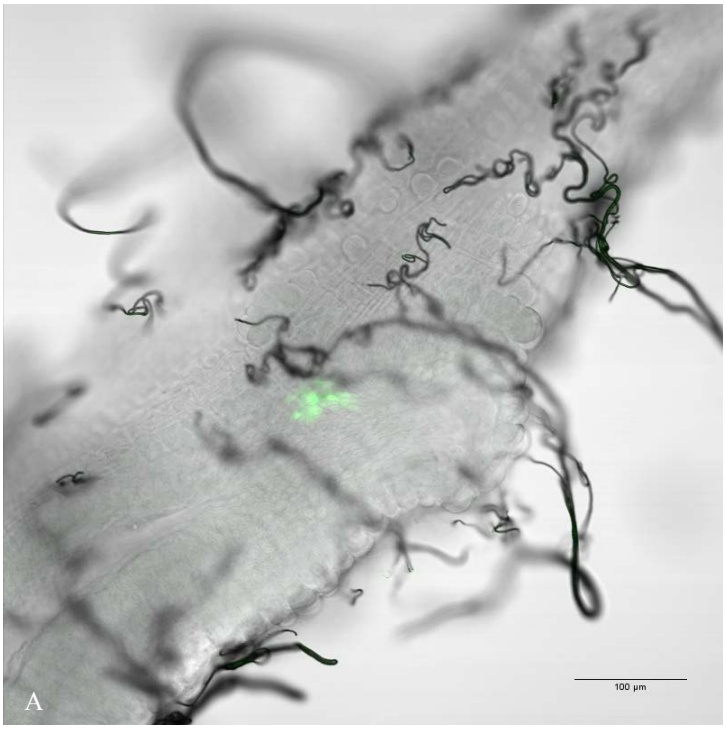
Figure 9 Proteins from triturated mosquito midguts were separated via PAGE. Gel was stained with Coomassie Brilliant Blue and counterstained with silver. Lane A corresponds to protein ladder and lane B (8 MGs) contain *Ae. aegypti* tissue. Arrows pertain to protein bands of interests with molecular weights ranging between 55-70kDa.

Sindbis virus foci in mosquito midgut

Female *Ae. aegypti* mosquitoes were exposed to TR339-TaV-eGFP through a blood meal 5 days post-emergence. Individuals that successfully imbibed on the viremic blood meal were isolated and dissected on days 3-9 post blood meal. The midguts were placed in wells of a 96-well plate and fixed with 4% paraformaldehyde in 0.1M cacodylate buffer; midguts that were immunofluorescence labeled for NRAMP were permeabilized with 0.2% Tween 20 after fixation with paraformaldehyde. The midguts were analyzed using confocal microscopy. Sites of infection were indicated by the GFP produced by TR339-TaV-eGFP (figure 10). The TR339-

TaV-eGFP foci in midguts of day 3 p.i. were small while TR339-TaV-eGFP foci were large in midguts of day 9 p.i.; foci could be made up of multiple focus that have grown in size and overlapped (figure 11). Fluorescence was detected in the midgut epithelia and visceral muscles at days 5, 6, and 9 post-infection, suggesting that SINV has disseminated from the midgut (figure 12, 13, & 14). TR339-TaV-eGFP foci were observed on every experimental date (Table 1). Virus antigens were not detected in the midguts of female *Ae. aegypti* that were proffered a non-viremic blood meal.

Infected midguts were labeled with an antibody against NRAMP2 and a TX-Red-conjugated antibody to investigate the correlation between SINV and NRAMP2 with regards to localization. NRAMP2 labeling on *Ae. aegypti* midguts was faint but was in the same area of the midgut infection focus.



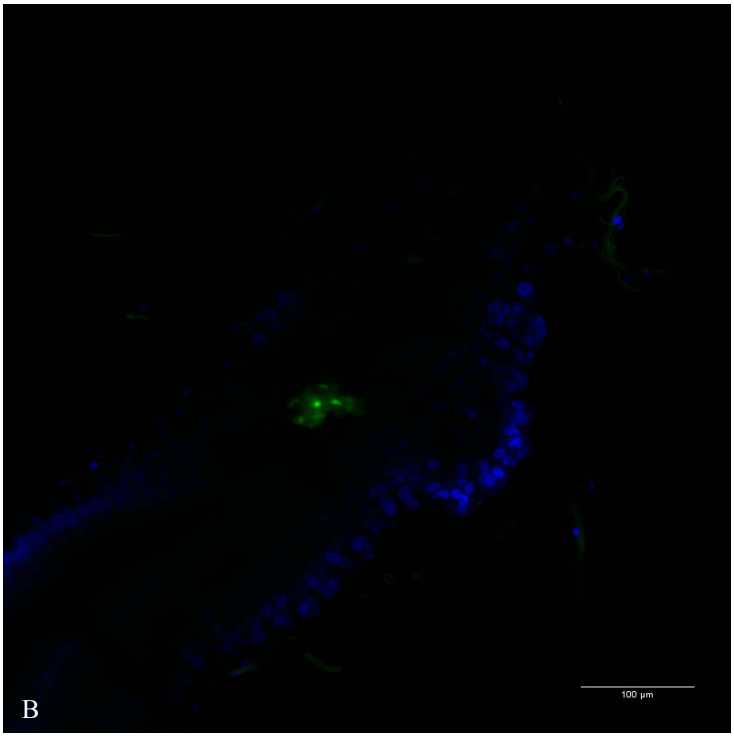


Figure 10 Confocal analysis (100µm scale bar) of midgut from *Ae. aegypti* fixed with 4% paraformaldehyde at day 3 p.i. with TR339-TaV-eGFP. (A) Localized virus focus in the midgut of *Ae. aegypti* using light and laser microscopy. (B) Confocal analysis of (A) stained with DRAQ5 (blue; nuclear stain).

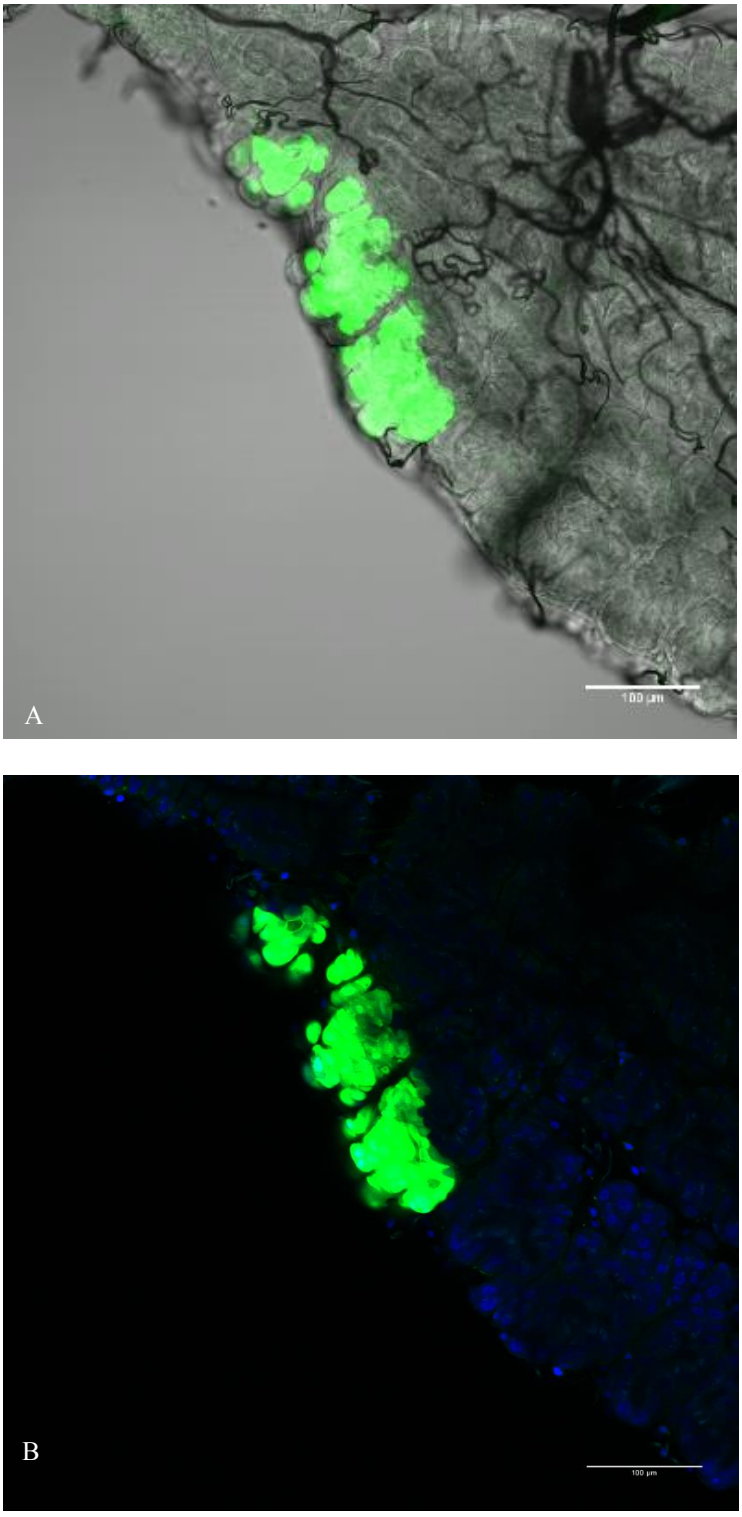


Figure 11 Confocal analysis (100µm scale bar) of midgut from *Ae. aegypti* fixed with 4% paraformaldehyde at day 9 p.i. with TR339-TaV-eGFP. (A) Localized virus focus in the midgut of *Ae. aegypti* using light and laser microscopy. (B) Confocal analysis of (A) stained with DRAQ5 (blue; nuclear stain).

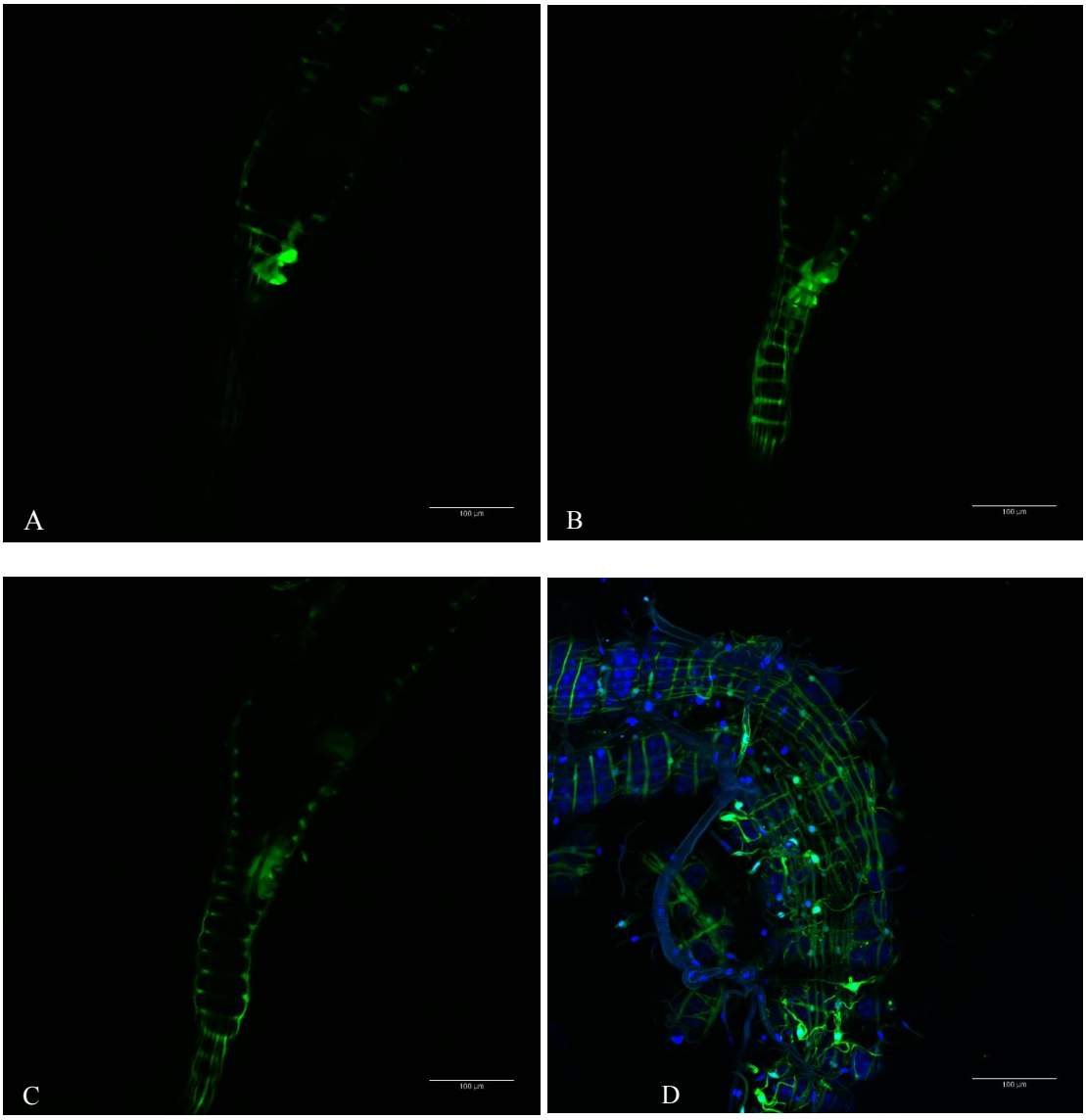


Figure 12 Optical cross-section of midgut of *Ae. aegypti* fixed with 4% paraformaldehyde/0.2% Tween 20 at day 9 p.i. with TR339-TaV-eGFP; confocal microscopy, 100µm scale bar. (A-C) Localized virus focus at anterior midgut and infected peristaltic muscles. (D) Midgut of (A-C) stained with DRAQ5 (blue; nuclear stain). Fluorescence of peristaltic muscles indicates that TR339-TaV-eGFP has disseminated from the midgut epithelia.

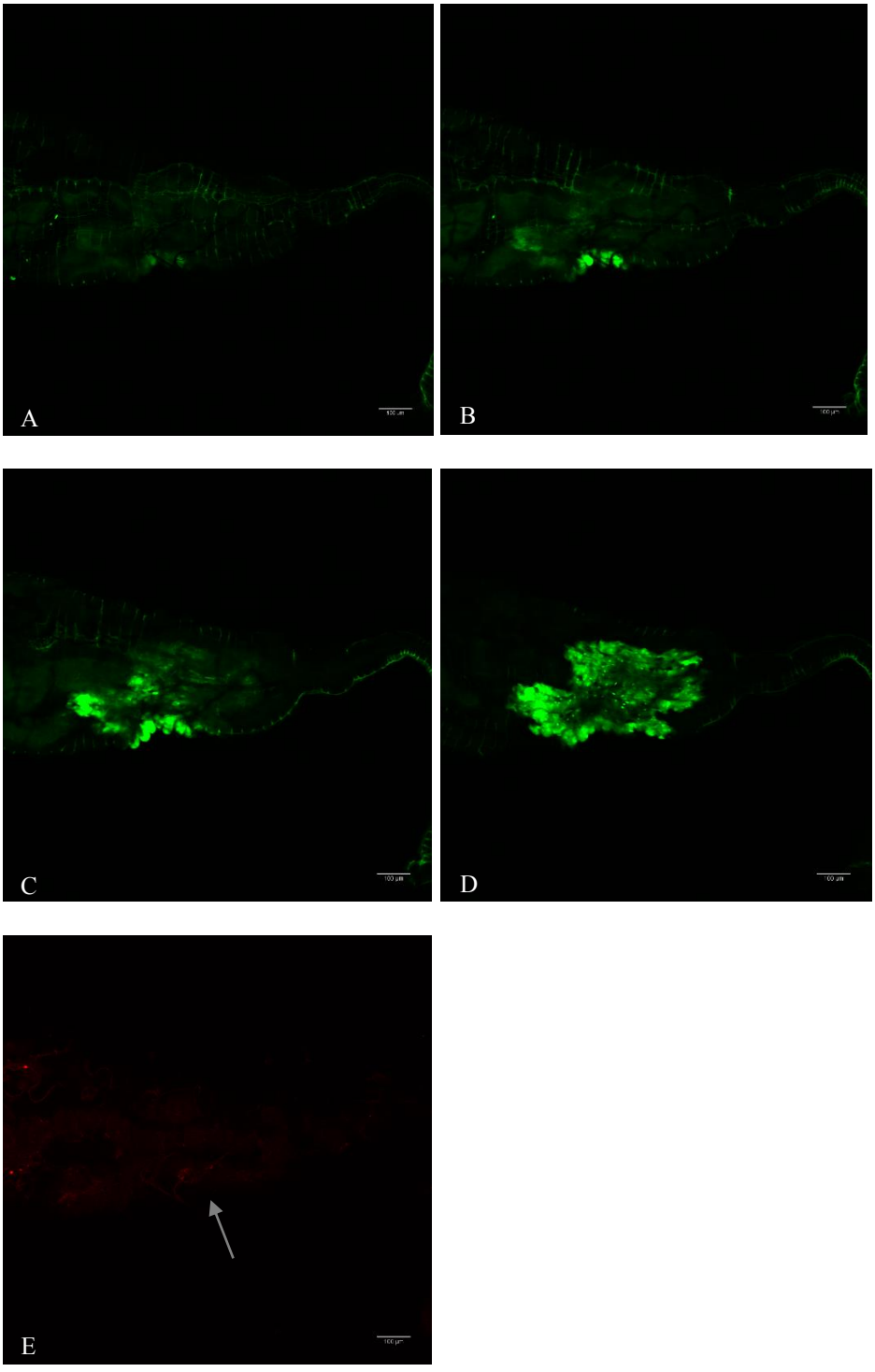


Figure 13 Optical cross-section of midgut of *Ae. aegypti* fixed with 4% paraformaldehyde/0.2% Tween 20 at day 9 p.i. with TR339-TaV-eGFP; confocal microscopy, 100µm scale bar. (A-D) Localized virus focus at midgut and infected peristaltic muscles. (E) Midgut of (A-D) labeled with an antibody against NRAMP2 and TX-Red-conjugated antibody; (→) indicate proposed NRAMP localization.

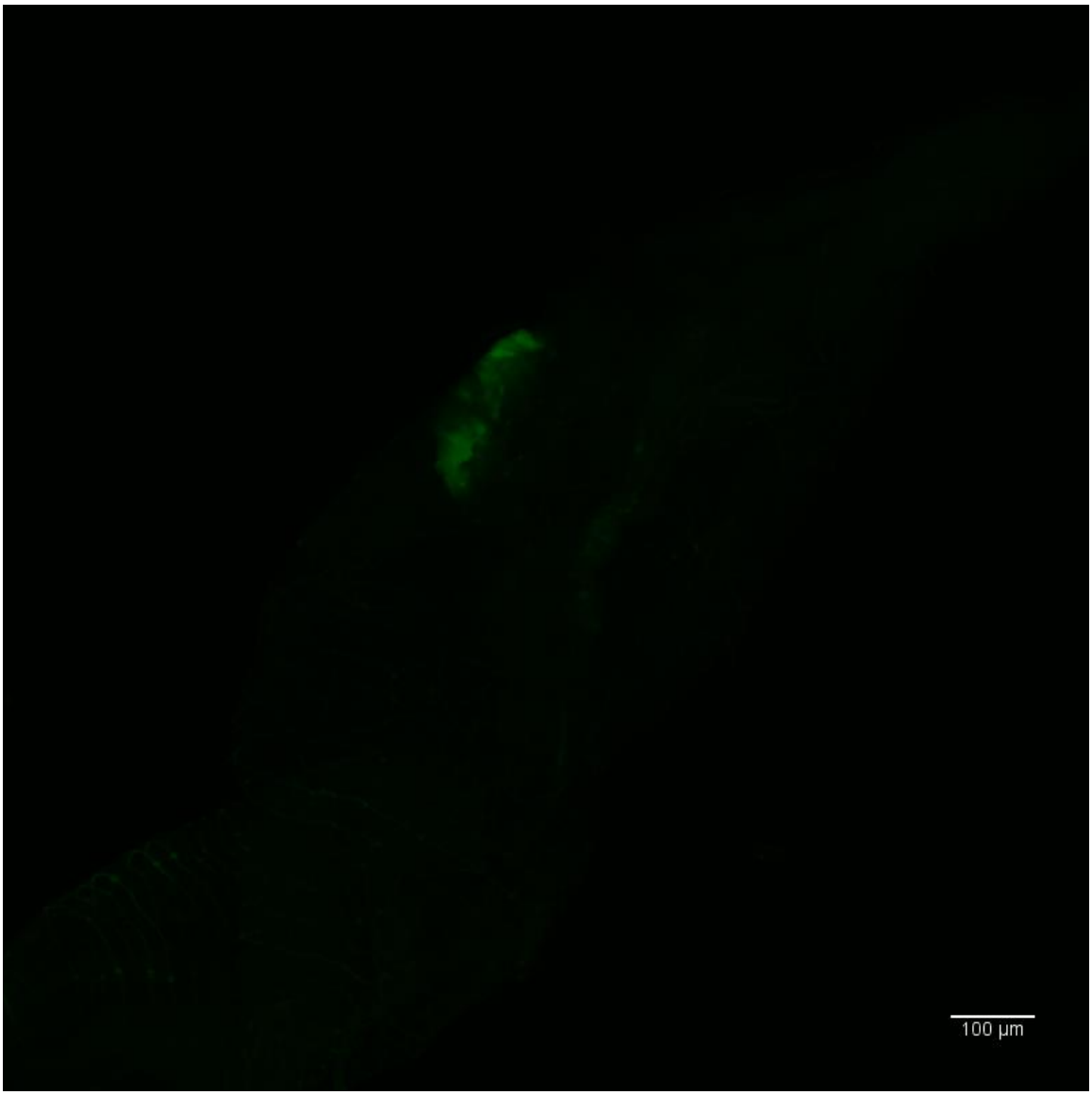


Figure 14 Midgut of *Ae. aegypti* fixed with 4% paraformaldehyde/0.2% Tween 20 at day 5 p.i. with TR339-TaV-eGFP; confocal microscopy, 100µm scale bar, Z projection. Localized virus focus at front portion of midgut and infected peristaltic muscles at the rear portion of midgut.

Table 1 TR339-TaV-eGFP infection and dissemination in midguts of *Ae. aegypti* females.

Day of Dissection	# bloodfed females	Mortality post-blood meal	# infected MG/ # resected MG	# MG with infected muscles/ # infected MG
Negative control	77% 50/65	0% 0/50	0% 0/50	0% 0/50
3 days p.i.	80% 68/85	0.01% 1/68	13% 8/61	0% 0/61
4 days p.i.	79% 81/102	0% 0/81	13% 9/70	0% 0/70
5 days p.i.	78% 59/76	0% 0/59	19% 10/52	0.02% 1/52
6 days p.i.	89% 63/71	0% 0/63	17% 10/58	0.03% 2/58
7 days p.i.	79% 68/86	0% 0/68	4% 3/68	0% 0/68
8 days p.i.	88% 95/108	0% 0/95	5% 3/65	0% 0/65
9 days p.i.	78% 61/78	0% 0/61	4% 2/53	0% 0/53
Total			9% 45/477	7% 3/45

Chapter 4: Discussion

In this investigation, NRAMP was localized in midguts of *Ae. aegypti* and virus antigens were detected in the peristaltic muscles. Punctate and speckled labeling of NRAMP was observed in this research. This pattern of labeling was similar to that seen in Gruenheid and colleagues' (1999) labeling of CHO (Chinese hamster ovarian) cells using a monoclonal antibody against NRAMP2 and Roig et al.'s (2002) labeling of HL-60 (human leukemia) cells with a polyclonal antibody against NRAMP1.

There are several different mechanisms by which the mosquito could possibly avoid infection: apoptosis of stressed epithelial cells (Weaver and Scott 1990), dense microvilli-associated network on the brush-border (Zieler et al., 2000), and binding of cytotoxic heme by the peritrophic membrane (Pascoa et al., 2002), subsequently preventing pathogens from coming into contact with epithelial cells. NRAMP labeling of mosquito midguts resembled SINV infection foci, suggesting that the reason SINV does not infect all epithelial cells of the midgut prior to dissemination was due to the lack or low density of suitable receptor(s).

When compared with unfed *An. albimanus* females, NRAMP concentration was reduced 24 hours after a blood meal. Subsequently, a 117% increase in NRAMP expression in Malpighian tubules of *An. albimanus* females was observed with Western blot, suggesting that iron from the blood in the midguts was further processed in the Malpighian tubules while NRAMP in the midguts has been utilized and recycled (Martinez-Barnetche et al., 2007). Since the immunofluorescence labeling of Malpighian tubules in this investigation was conducted with unfed *An. quadrimaculatus*, NRAMP detection was minimal.

Despite considerable efforts, NRAMP was not immunoprecipitated when using the polyclonal antibody against NRAMP2 of human origin. AnaNRAMP shares 80% identity to *An.*

gambiae but this antibody was not available commercially. *Aedes aegypti* NRAMP and *An. quadrimaculatus* NRAMP amino acid sequences were not available from National Center for Biotechnology Information, thus AnaNRAMP and *An. gambiae* NRAMP sequences were used for reference. Although *An. gambiae* NRAMP share only a 57% identity to human NRAMP2 (Figure 15), the extracellular loops of NRAMP are highly conserved and may share a greater sequence identity among species since this portion of NRAMP is responsible for iron transport (Gruenheid et al., 1999). Members of the NRAMP family have been identified in a wide range of organisms that include plants, fungus, insects, fish, mammals, and humans, to name a few (Cellier et al., 1995). NRAMP is ubiquitously expressed among a group of phylogenetically diverse species, allowing SINV to infect a wide range of hosts.

Score	Expect	Method	Identities	Positives	Gaps
375 bits(964)	9e-129	Compositional matrix adjust.	183/320(57%)	239/320(74%)	3/320(0%)

```
Query  54   IPLYIGVLITVLDTLSFLFLDKYKLRRLELLFGFLITTMAVSFGYQYIVTDIPQINVIEG  113
            IPL+ GVLIT+ DT  FLFLDKY LR+LE  FGFLIT MA++FGY+Y+     Q  V++G
Sbjct  180  IPLWGGVLITIADTFVFLFLDKYGLRKLEAFFGFLITIMALTFGYEYVTVKPSQSQVLKG  239

Query  114  MFLPWSSDYRPGTLLQAVGIIGAVIMPHNLYLHSALVKSRTINRNNVKEVKKANRYYFIE  173
            MF+P  S  R   + QAVGI+GAVIMPHN+YLHSALVKSR +NRNN +EV++AN+Y+FIE
Sbjct  240  MFVPSCSGCRTPQIEQAVGIVGAVIMPHNMYLHSALVKSRQVNRNNKQEVREANKYFFIE  299

Query  174  ASIALAVSFVINVFVVSVFAHDVYGKTNQDVIDACSNSSFADDIISAFIANNYTADINIY  233
            + IAL VSF+INVFVVSVFA   +GKTN+ V++ C+N+S        F  +N T  ++IY
Sbjct  300  SCIALFVSFIINVFVVSVFAEAFFGKTNEQVVEVCTNTSSPH--AGLFPKDNSTLAVDIY  357

Query  234  KGGLVLGCFYGGLSMYVWAIGILAAGQSSTMTGTYAGQFAMEGFLNLQWARWKRVLFTRT  293
            KGG+VLGC++G  ++Y+WA+GILAAGQSSTMTGTY+GQF MEGFLNL+W+R+ RV+ TR+
Sbjct  358  KGGVVLGCYFGPAALYIWAVGILAAGQSSTMTGTYSGQFVMEGFLNLKWSRFARVVLTRS  417

Query  294  VAIMPAFYVAFFSRLEDLTKMNDILNAVMALQLPFAAIPTVAFSSSLALMKADFVNGRLE  353
            +AI+P   VA F  +E LT MND LN + +LQLPFA IP + F+S   +M +DF NG
Sbjct  418  IAIIPTLLVAVFQDVEHLTGMNDFLNVLQSLQLPFALIPILTFTSLRPVM-SDFANGLGW  476

Query  354  KIISITLSFTVIGINLYFIV  373
            +I    L   +  IN+YF+V
Sbjct  477  RIAGGILVLIICSINMYFVV  496
```

Figure 15 Protein sequence alignment of *An. gambiae* NRAMP (query) and natural resistance-associated macrophage protein 2 isoform 3 of *Homo sapiens* (subject). NRAMP of *An. gambiae* share a 57% identity and 74% similarity with NRAMP2 of *H. sapiens*.

Localized TR339-TaV-eGFP infection foci along with infected visceral muscles indicate that only a portion of the midgut epithelia needs to be infected prior to dissemination. The reason(s) for why the entire midgut is not infected is currently unknown. TR339-TaV-eGFP foci were observed in midguts of *Ae. aegypti* 3-9 days p.i. but infected visceral muscles were only observed on days 5, 6, and 9 p.i. Visceral muscles of the entire gut were infected; however, some muscles had a greater expression of GFP while some displayed a faint presence of GFP. The area with muscle bundles that fluoresce brighter indicate that a portion of the midgut peristaltic muscles was the site of initial SINV muscle infection since SINV had a longer time period to produce GFP. Muscle bundles that fluoresce but were faint suggest that those muscles were infected with SINV at a later time. This pattern suggests that virions disseminated from the

midgut epithelia into the hemolymph then infected the peristatic muscles as it does other secondary organs. Initial infection sites of midgut muscle bundles were from virions traveling in the hemolymph; infection of remaining peristatic muscles could be from virions that were in the hemolymph or virions from neighboring infected muscle cells. If the steps to dissemination were from midgut epithelia to visceral muscles to hemolymph, then only the visceral muscles near the TR339-TaV-eGFP focus should be infected, which was not the case in this investigation.

Prerequisite to vector-borne transmission is the successful transgression of both the infection and escape barriers. In this present investigation, immunofluorescence labeling of NRAMP in mosquito midguts suggests that SINV infection of the midgut remains localized because NRAMP is not expressed in all the cells of the midgut epithelia. NRAMP detection and TR339-TaV-eGFP infection of gut peristaltic muscles further support that SINV is utilizing this transmembrane protein to overcome the MGIB and MGEB. Future research utilizing a polyclonal serum against *Ae. aegypti* and *An. quadrimaculatus* NRAMP would allow for further investigation into the presence and function of this protein. Sindbis infection focus was observed in the anterior midgut of *Ae. aegypti*. Since SINV infection is uncommon in this area of the midgut, another goal is to determine if SINV infection occurs more often in a 6specific portion of the midgut (anterior midgut or the front, center, or rear portion of the posterior midgut) and what mechanisms influence this infection pattern. This future research will continue to provide insight into tissue tropism and host permissiveness.

References

Almeras L, Fontaine A, Belghazi M, Bourdon S, Boucomont-Chapeaublanc E, Orlandi-Pradines E, Baragatti M, Corre-Catelin N, Reiter P, Pradines B, Fusai T, Rogier C. 2010. Salivary gland protein repertoire from *Aedes aegypti* mosquitoes. Vector-borne and Zoonotic Diseases. 10(4): 391-402.

Benedict M, Levine R, Hawley W, Lounibos P. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector-borne and Zoonotic Diseases. 7(1): 76-85.

Bowers D, Abell B, Brown D. 1995. Replication and tissue tropism of the *Alphavirus* Sindbis in the mosquito *Aedes albopictus*. Virology. 212: 1-12.

Bowers D, Coleman C, Brown D. 2003. Sindbis virus-associated pathology in *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology. 40(5): 698-705.

Byrnes A, Griffin D. 1998. Binding of Sindbis virus to cell surface heparan sulfate. Journal of Virology. 72(9): 7349-7356.

Cellier M, Prive G, Belouchi A, Kwan T, Rodrigues V, Chia W, Gros P. 1995. NRAMP defines a family of membrane proteins. Proceedings of the National Academy of Sciences of the United States. 92: 10089-10093.

Fields D, Knipe D, Howley P. 2007. Field's Virology 5th edition. Philadelphia: Lippincott Williams and Wilkins. 3177p.

Fuller S. 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell. 48(6): 923-934.

Gruenheid S, Canonne-Hergaux F, Gauthier S, Hackam DJ, Grinstein S, Gros P. 1999. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. Journal of Experimental Medicine. 189(5): 831-841.

Gubler DJ. 1998. Dengue and dengue hemorrhagic fever. Clinical Microbiology Reviews. 11(3): 480-496.

Helenius A, Kartenbeck J, Simons K, Fries E. 1980. On the entry of Semliki Forest virus into BHK-21 cells. The Journal of Cell Biology. 84(2): 404-420.

James A. 2003. Blocking malaria parasite invasion of mosquito salivary glands. The Journal of Experimental Biology. 206: 3817-3821.

Janzen H, Wright K. 1971. The salivary gland of *Aedes aegypti* (L.): an electron microscopy study. Canadian Journal of Zoology. 49: 1343-1345.

Juhn J, Naeem-Ullah U, Guedes B, Majid A, Coleman J, Pimenta P, Akram W, James A, Marinotti O. 2011. Spatial mapping of gene expression in salivary glands of the dengue vector mosquito, *Aedes aegypti*. Parasites & Vectors. 4(1): 1-13.

Klimstra W, Ryman K, Johnston R. 1998. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. Journal of Virology. 72(9): 7357-7366.

Levine RS, Peterson AT, Benedict MQ. 2004. Distribution of members of *Anopheles quadrimaculatus* Say s.l. (Diptera: Culicidae) and implications for their roles in malaria transmission in the United States. Journal of Medial Entomolgy. 41(4): 607-613.

Lyski Z, Saredy J, Ciano K, Stem J, Bowers D. 2011. Blood feeding position increases success of recalcitrant mosquitoes. Vector-borne and Zoonotic Diseases. 11(8): 1165-1171.

Martinez-Barnetche J, Solache MG, Lecona AN, Lopez ATT, Rodriquez MdC, Gamba G, Vazquez N, Rodriquez MH, Lanz-Mendoza H. 2007. Cloning and functional characterization of *Anopheles albimanus* DMT1/NRAMP homolog: implications in iron metabolism in mosquitoes. Insect Biochemistry and Molecular Biology. 37: 532-539.

Moreira-Ferro C, Marinotti O, Bijovsky A. 1999. Morphological and biochemical analyses of the salivary glands of the malaria vector, *Anopheles darling*. Tissue and Cell. 31(3): 264-273.

National Center for Emerging and Zoonotic Infectious Diseases [Internet]. c 2011. Center for Disease Control and Prevention [cited 2013 Feb 11]. Available from: http://www.cdc.gov/ncidod/dvbid/arbor/albopic_new.htm

Nevo Y, Nelson N. 2006. The NRAMP family of metal-ion transporters. Biochimica et Biophysics Acta. 1763: 609-620.

Okuda K, de Almeida F, Mortara RA, Krieger H, Marinotti O, Bijovsky AT. 2007. Cell death and regeneration in the midgut of the mosquito, *Culex quinquefasciatus*. Journal of Insect Physiology. 53: 1307-1315.

O'Meara G, Evans L Jr, Gettman A, Cuda J. 1995. Spread of *Aedes albopictus* and decline of *Ae. aegypti* (Diptera: Culicidae) in Florida. Journal of Medical Entomology. 32(4): 554-562.

Oviedo MVN, Romoser WS, James CBL, Mahmood F, Reisen WK. 2011. Infection dynamics of western equine encephalomyelitis virus (Togaviridae: Alphavirus) in four strains of Culex tarsalis (Diptera: Culicidae): an immunocytochemical study. Journal of Research and Reports in Tropical Medicine. 2011(2): 65-77.

Paredes A, Ferreira D, Horton M, Saad A, Tsuruta H, Johnston R, Klimstra W, Ryman K, Hernandez R, Chiu W, Brown D. 2004. Conformational changes in Sindbis virus resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. Virology. 324(2): 373-386.

Parikh GR, Oliver JD, Bartholomay LC. 2009. A haemocyte tropism for an arbovirus. Journal of General Virology. 90: 292-296.

Pascoa V, Oliveira PL, Dansa-Ptretski M, Silva JR, Alvarenga PH, Jacobs-Lorena M, Lemos FJA. 2002. *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. Insect Biochemistry and Molecular Biology. 32: 517-523.

Paulson SL, Grimstad PR, Craig GB. 1989. Midgut ad salivary gland barriers to La Crosse virus dissemination in mosquitoes of the *Aedes triseriatus* group. Medical and Veterinary Entomology. 3(2): 113-123.

Pierro DJ, Powers EL, Olsen KE. 2007. Genetic determinants of Sindbis virus strain TR339 affecting midgut infection in the mosquito *Aedes aegypti*. Journal of General Virology. 88(5): 1545-1554.

Roig EA, Richer E, Canonne-Hergaux F, Gros P, Cellier MFM. 2002. Regulation of NRAMP1 gene expression by 1α, 25-dihydroxy-vitamin D_3 in HL-60 phagocytes. Journal of Leukocyte Biology. 71: 890-904.

Rose PP, Hanna SL, Spiridigliozzi A, Wannissorn N, Beiting DP, Ross SR, Hardy RW, Bambina SA, Heise MT, Cherry S. 2011. Natural resistance-associated macrophage protein (NRAMP) is a cellular receptor for Sindbis virus in both insect and mammalian hosts. Cell Host & Microbe. 10(2): 97-104.

Sim S, Ramirez J, Dimopoulous G. 2012. Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior. PLOS Pathogens. 8(3): 1-15.

Siriyasatien P, Tangthongchaiwiriya K, Jariyapan N, Kaewsaitiam S, Poovorawan Y, Thavara U. 2005. Analysis of salivary gland proteins of mosquito *Armigeres subalbatus*. The Southeast Asian Journal of Tropical Medicine and Public Health. 36(1): 64-70.

Smartt C, Kim A, Grossman G, James A. 1995. The apyrase gene of the vector mosquito, *Aedes aegypti*, is expressed specifically in the adult female salivary glands. Experimental Parasitology. 81(3): 239-248.

Sun C, Gardner CL, Watson AM, Ryman KD, Klimstra WB. 2014. Stable, high-level expression of reporter proteins from improved *Alphavirus* expression vectors to track replication and dissemination during encephalitis and arthitogenic disease. Journal of Virology. 88(23): 2035-2046.

Ubol S, Griffin D. 1991. Identification of a putative *Alphavirus* receptor on mouse neural cells. Journal of Virology. 65(12):6913–6921.

Villarreal LP, Defillippis VR, Gottlieb KA. 2000. Acute and persistent viral life strategies and their relationship to emerging diseases. Virology Journal. 272: 1–6.

Vo M, Linser PJ, Bowers DF. 2010. Organ-associated muscles in *Aedes albopictus* (Diptera: Culicidae) respond differentially to Sindbis virus. Journal of Medical Entomology. 47(4): 215-225.

Wang K, Kuhn R, Strauss E, Ou S, Strauss J. 1992. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. Journal of Virology. 66(8): 4992-5001.

Weaver SC, Osorio JE, Livengood JA, Chen R, Stinchcomb DT. 2012. Chikungunya virus and prospects for a vaccine. 11(9): 1087-1101.

Weaver SC, Scott TW. 1990. Peritrophic membrane formation and cellular turnover in the midgut of *Culiseta melanura* (Diptera: Culicidae). Journal of Medical Entomology. 27(5): 864-873.

Zieler H, Garon CF, Fischer ER, Shahabuddin M. 2000. A tubular network associated with the brush-border surface of the *Aedes aegypti* midgut: implications for pathogen transmission by mosquitoes. The Journal of Experimental Biology. 203: 1599-1611.

Vita

EDUCATION

Spring 2009—Graduated from University of North Florida with a Bachelors of Science in Biology.

Fall 2015—Completed degree requirements for Masters of Science in Biology from the University of North Florida.

EMPLOYMENT HISTORY

Spring 2014—Graduate Teaching Assistant**,** Department of Biology, University of North Florida

CONFERENCE PRESENTATION

Florence Y. Chim, Jason J. Saredy, Dave S. Waddell, Paul J. Linser, and Doria F. Bowers. "Localization of NRAMP 2, a putative Sindbis virus receptor, in mosquitoes". Poster presentation. American Society for Virology annual conference. London, Ontario, Canada 2015.