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Editor,
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Dear Editor,

Please find attached our re-submission of the paper titled “General protocol for conducting vector competence analyses on *Aedes aegypti* mosquitoes using Zika virus”.

We thank the reviewers for their critiques and suggestions. We have responded to each comment accordingly as detailed in the accompanying point by point response.

All listed authors have contributed to and agree with the methods, executions, results, and conclusions of this manuscript, which has not been submitted for publication elsewhere. The authors report no conflicts of interest.

We thank you for your consideration of our manuscript.

Best regards,

Sasha Rafi Azar

Sasha R. Azar, Ph.D.

TITLE:

Vector Competence Analyses on *Aedes aegypti* Mosquitoes Using Zika Virus

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KEYWORDS:

mosquito, vector competence, *Aedes*, *Aedes aegypti*, arbovirus, Zika

SUMMARY:

The presented protocol can determine the vector competence of *Aedes aegypti* mosquito populations for a given virus, such as Zika, in a containment setting.

ABSTRACT:

The procedures presented describe a generalized methodology to infect *Aedes aegypti* mosquitoes with Zika virus under laboratory conditions to determine the rate of infection, disseminated infection, and transmission of the virus in the mosquito population in question. These procedures are widely utilized with various modifications in vector competence evaluations globally. They are important in determining the potential role that a given mosquito (i.e., species, population, individual) may play in the transmission of a given agent.

INTRODUCTION:

Vector competence is defined as the ability of a given arthropod, such as a mosquito, tick, or phlebotomine sand fly, to acquire and transmit an agent biologically with replication or development in the arthropod at the levels of species, population, or even an individual[1,2]. With respect to mosquitoes and arthropod-borne viruses (i.e., arboviruses), the agent is imbibed from a viremic host by a female mosquito. Following ingestion, the virus must productively infect one of a small population of midgut epithelial cells[3], overcoming various physiological obstacles such as proteolytic degradation by digestive enzymes, the presence of the microbiota (midgut infection barrier, or MIB), and the secreted peritrophic matrix. Infection of the midgut epithelium must be followed by replication of the virus and eventual escape from the midgut into the mosquito's open circulatory system, or hemolymph, which represents the onset of a

disseminated infection overcoming the midgut escape barrier (MEB). At this point the virus can establish infections of secondary tissues (e.g., nerves, muscles, and fat bodies) and continue to replicate, although such secondary replication may not be strictly necessary for the virus to infect the acinar cells of the salivary glands (overcoming the salivary gland infection barrier). Egress from the salivary gland acinar cells into their apical cavities and then movement into the salivary duct enables inoculation of the virus into subsequent hosts on biting, and completes the transmission cycle[1,2,4-7].

Given this well-characterized and generally conserved mechanism of spread within a mosquito vector, laboratory vector competence assessments are often methodologically similar, although differences in protocols do exist[1,2]. Generally, after oral virus exposure, mosquitoes are dissected so that individual tissues such as the midgut, legs, ovaries, or salivary glands can be assayed for viral infection, disseminated infection, disseminated infection/potential transovarial transmission, and disseminated infection/potential transmission competence, respectively[8]. Mere presence of a virus in the salivary glands, however, is not definitive evidence of transmission capability, given evidence of a salivary gland escape/egress barrier (SGEB) in some vector/virus combinations[1,2,4,5,7,9]. The standard method to prove transmission competence remains mosquito transmission to a susceptible animal[10-12]. However, given that for many arboviruses this necessitates the use of immunocompromised murine models[13-16], this method is often cost-prohibitive. A commonly used alternative is the collection of the mosquito saliva, which can be analyzed by reverse transcription-polymerase chain reaction (RT-PCR) or an infectious assay to demonstrate the presence of the viral genome or infectious particles, respectively. It is worth noting that such in vitro saliva collection methods may overestimate[12] or underestimate[17] the amount of virus deposited during in vivo feeding, indicating that such data must be interpreted with caution. Nonetheless, the in vitro method is highly valuable when analyzed from the perspective of mere presence of virus in the saliva, indicating transmission potential.

Two major approaches exist for determining the role of mosquito vectors in arboviral disease outbreaks. The first method involves field surveillance, in which mosquitoes are collected in the context of active transmission[18-24]. However, given that infection rates are typically quite low (e.g., the estimated 0.061% infection rate of mosquitoes in areas of active Zika virus (ZIKV) circulation in the United States[21]), incrimination of potential vector species can be heavily biased by trapping methodology[25,26] and random chance (e.g., sampling one infected individual out of 1,600 uninfected)[21]. Taking this into account, a given study may not acquire sufficient mosquitoes in both raw numbers or species diversity to accurately sample mosquitoes involved in transmission. In contrast, vector competence analyses are undertaken in a laboratory setting, allowing for strict control of parameters such as oral dose. Although not fully capable of representing the true complexity of mosquito infection and transmission capability in a field setting, these laboratory assessments remain powerful tools in the field of arbovirology.

Based on various vector competence analyses with ZIKV in several mosquito species, populations, and methods[27-32], as well as a recent review of vector competence assessments[1], we describe here several of the protocols associated with a typical vector competence workflow. In these

experiments, three *Ae. aegypti* populations from the Americas (the city of Salvador, Brazil; the Dominican Republic; and the Rio Grande Valley, TX, USA) were exposed to a single strain of ZIKV (Mex 1-7, GenBank Accession: KX247632.1) at 4, 5, or 6 $\log_{10}$ focus-forming units (FFU)/mL doses by way of artificial bloodmeals. Subsequently, they were analyzed for evidence of infection, disseminated infection, and transmission competence after various times of extrinsic incubation (2, 4, 7, 10, and 14 days) by means of dissection and a cell culture-based infectious assay. Although the present workflow/protocols are optimized for ZIKV, many elements are directly translatable to other mosquito-borne arboviruses in arthropod containment and biosafety levels 2 and 3 (ACL/BSL2 or ACL/BSL3).

PROTOCOL:

All procedures performed in these protocols were performed in full compliance with protocols approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

1. Amplify ZIKV in Vero cells

1.1. Grow Vero cells (CCL-81 or VeroE6) in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) in a humidified 37 °C incubator with 5% CO_2 to between 80−90% confluency in a 150 cm^2 tissue culture flask.

1.2. In a biosafety cabinet (BSC), remove the medium and dispose of in either 10% bleach or a working dilution of dual quaternary ammonium (**Table of Materials**). Immediately inoculate the monolayer with 1 mL of viral stock, aiming for 0.1−1 infectious viral particle per cell. Agitate the flask immediately such that the inoculum contacts the monolayer in its entirety.

1.3. Top up the medium to a volume of 5 mL using DMEM supplemented with 2% v/v heat-inactivated FBS, and 1% (v/v) penicillin-streptomycin. Then move the flask into the humidified 37 °C incubator with 5% CO_2 for 60 min.

1.4. Remove the flask from the incubator and bring it into a BSC. Add additional medium to a total volume of 15 mL using DMEM supplemented with 2% v/v heat-inactivated FBS, and 1% (v/v) penicillin-streptomycin. Move the flask into a humidified 37 °C incubator with 5% CO_2.

1.5. Examine the flask daily under phase contrast microscopy for evidence of cytopathic effects (CPE). Proceed to the viral harvest (step 1.7) when only approximately 40−50% of the cells remain in the monolayer, generally 3−5 days postinfection depending on the strain of ZIKV being utilized.

1.6. Aspirate the supernatant and place in a 50 mL conical vial. Clarify the supernatant of cellular debris by centrifugation (3,500 x *g* for 20 min).

NOTE: If multiple flasks were infected identically, supernatants from multiple flasks can be

combined into 50 mL conical tubes.

1.7. Remove the supernatant from the 50 mL conical tube to a fresh one, taking care not to disrupt the pellet. Supplement the supernatant with heat-inactivated FBS to a final 30% (v/v). Aliquot this mixture into individual screw cap tubes and freeze at -80 °C until use.

2. Preparation of artificial bloodmeals

2.1. On the day mosquitoes are to be exposed to infectious bloodmeals, turn on the power source (**Table of Materials**) in an arthropod containment facility such that the feeding units (**Table of Materials**) are preheated by the time the mosquitoes are prepared for exposure.

2.2. Prepare artificial bloodmeals using one of the methods described below.

2.2.1. Method 1: Combine freshly harvested (within a week) citrated or heparinized human blood purchased commercially 1:1 v/v with viral stock (prepared as described in section 1).

NOTE: This method is contingent on the absence of any antibodies to the virus/virus family being present in the blood.

2.2.2. If absence of antibodies cannot be confirmed or the blood source is known to have prior flavivirus exposure, wash and manually pack erythrocytes.

2.2.2.1. In a BSC, add 30 mL of whole human blood to a 50 mL conical tube and top up to 50 mL with phosphate buffered saline (PBS).

2.2.2.2. Centrifuge at 3,500 x *g* for 20 min.

2.2.2.3. Aspirate the supernatant either by gently pouring into a tray pan containing either 10% bleach or working dilution of dual quaternary ammonium, taking care to not discard the erythrocyte pellet.

2.2.2.4. Add 10 mL of PBS and gently tap the bottom of the conical tube against the bottom of the BSC such that the erythrocyte pellet has been reconstituted. Bring the volume of the suspension up to 50 mL with PBS. Mix by gentle inversion.

2.2.2.5. Repeat steps 2.2.2.1−2.2.2.4 a total of 4−6x. Confirm that the supernatant is clear or only slightly pink and no longer opaque. Remove all the supernatant using a serological pipette. Resuspend the erythrocyte pellet in 1−2 mL of PBS.

2.2.2.6. Assemble the bloodmeal: 350 µL of packed erythrocytes, 100 µL of 10% sucrose, 200 µL of heat-inactivated FBS, 900 µM recombinant ATP, and 2 mL of appropriately diluted virus stock using DMEM supplemented with 2% v/v heat-inactivated FBS, and 1% (v/v) penicillin-streptomycin.

2.3. Overlay a standard 3 mL reservoir unit (**Table of Materials**) with the skin of an uninfected mouse (other options include paraffin film, collagenous membranes, or sausage casing).

2.4. Place the covered reservoir on white paper towels. Add ~2 mL of infectious bloodmeal to the reservoir 1 mL at a time. Inspect the towel underneath the feeder for any evidence of leakage. If leaks are present, recover the bloodmeal from the feeders and discard the covers. Seal the feeders with plugs. Once again confirm that no leaks are present.

3. Backtitration of bloodmeals/plaque assay

3.1. Using the remaining volume of the prepared bloodmeal, perform a 10x serial dilution series (i.e., 6 dilutions, ranging from diluted 10x to 1,000,000x) using DMEM supplemented with 2% v/v heat-inactivated FBS, and 1% (v/v) penicillin-streptomycin.

3.2. Aliquot 100 µL of the dilutions into the wells of 24 or 12 well plates from the most dilute to most concentrated.

3.3. Incubate for 1 h in a 37 °C, 5% CO_2 incubator.

3.4. At the end of the 1 h incubation period, bring the plates back into the BSC and add 1 mL or 2 mL of methylcellulose overlay to the 24 well or 12 well plates, correspondingly.

3.5. Place overlayed plates back in a 37 °C, 5% CO_2 incubator and incubate for 3−7 days (virus strain-dependent).

3.6. Following incubation, remove the plates from the incubator and bring into the BSC. Discard the methylcellulose overlay into a tray pan containing 10% bleach or dual quaternary ammonium.

3.7. Wash each well 2x with PBS, discarding the wash into a tray pan containing 10% bleach or dual quaternary ammonium.

3.8. Add ~1 mL of methanol:acetone (1:1 v:v) and allow the cells to fix onto plate for at least 30 min in the BSC at room temperature (RT). Discard methanol:acetone according to institutional policy on organic waste.

3.9. Visualize ZIKV using one of two methods described below.

3.9.1. Following the removal of methanol:acetone, stain immediately with a crystal violet solution (0.25% w/v in 30% methanol) for 5 min. Rinse 2x in tap water and leave to dry, then directly visualize by eye for evidence of plaques or destruction of monolayer.

3.9.2. Alternatively, perform focus forming assay.

3.9.2.1. Allow the plates to air dry until no organic fixative remains.

NOTE: This should take ~2−3 h outside of the BSC but can be accelerated by air-drying in a BSC or chemical fume hood.

3.9.2.2. Wash each well 3x for 15 min each in nonsterile PBS (Mg^{2+} and Ca^{2+} free) on an orbital plate rocker. Remove PBS and add 1 mL of blocking solution (PBS + 3% FBS) to each well and rock for 15 min at RT.

3.9.2.3. Add 100 µL per well of α-ZIKV or α-flavivirus primary antibody (e.g., flavivirus group hybridoma D1-4G2-4-15 [4G2]) at a 1:2,000 dilution in blocking solution. Incubate with rocking for a minimum of 4 h (preferably overnight, not exceeding 18 h) at RT.

3.9.2.4. Remove the primary antibody and wash 3x for 15 min each with PBS (Mg^{2+} and Ca^{2+} free) on an orbital plate rocker.

3.9.2.5. Add 100 µL per well of the secondary antibody (goat α mouse HRP-labeled) diluted 1:2,000 in blocking buffer. Incubate with rocking for 1 h at RT.

3.9.2.6. Wash 3x for 15 min each with PBS (Mg^{2+} and Ca^{2+} free) on an orbital plate rocker.

3.9.2.7. Aliquot 100 µL of substrate development reagent (**Table of Materials**) per well. Rock plates at RT for 15 min.

3.9.2.8. Halt the reaction upon development of foci/plaques by removing the substrate and rinsing the plates 2x with tap water. Pour off tap water and allow the plates to air dry before quantifying.

3.9.2.9. Count viral foci to determine the number of FFU present in the given sample.

4. Administration of bloodmeals

4.1. Use *Aedes aegypti* mosquitoes 2−4 days post eclosion. Sort female mosquitoes into 0.5 L cardboard cartons with screened lids and deprive them of sugar (generally 36−48 h prior to the infectious bloodmeal). Provide ad libitum water via water-saturated cotton balls.

4.2. Remove the water-saturated cotton balls on the morning that the mosquitoes will be exposed to the infectious bloodmeal.

4.3. Attach reservoirs containing artificial bloodmeals to feeding unit leads within a clear plastic glove box.

4.4. Within the glovebox, place a 0.5 L cardboard carton with a screened lid, containing 50–100 starved *Ae. aegypti* mosquitoes, underneath the feeding unit attached to the reservoir.

NOTE: Appropriately starved mosquitoes will generally feed within 20 min. Feeding can be prolonged as needed to increase sample size in slower populations, although this should not extend beyond 60 min, because (ZIKV) viral titer can decrease within the feeder after ~60 min.

4.5. Upon completion of feeding, remove the reservoir and immerse in freshly made 10% bleach.

4.6. Cold-anesthetize mosquitoes by incubation for 30 s at -20 °C or for 5 min in a refrigerator.

4.7. Within the glovebox, pour the mosquitoes into a Petri dish on ice. Count and sort the engorged females from unengorged mosquitoes. Dispose of the unengorged mosquitoes by immersion in a 50 mL tube conical tube filled with 70% ethanol. While the mosquitoes are still anesthetized, pour them back into the 0.5 L cardboard carton and quickly cover with the screen and lid. Trim excess screen mesh off of the carton and secure the mesh with tape.

4.8. Add a cotton ball saturated with sterile filtered 10% sucrose to the screen of each carton. Place all mosquito cartons in a large plastic secondary container with a damp sponge to maintain humidity.

4.9. Place secondary container containing cartons of mosquitoes into an incubator with a temperature of 27 ± 1 °C (or as appropriate to simulate conditions in region of interest) with 80% ± 10% relative humidity and a 16:8 light:dark cycle). Maintain the mosquitoes with ad libitum access to 10% sucrose until completion of the experiments.

5. Sample acquisition and processing

5.1. On specified days postfeeding, aspirate a predetermined number of mosquitoes from the appropriate cartons using a mechanical aspirator within a glovebox. Cap the collection tube with a cotton round after the requisite number of mosquitoes is acquired.

5.2. Cold-anesthetize mosquitoes by incubation for 30 s at -20 °C or for 5 min.

5.3. Within the glovebox, pour the mosquitoes into a Petri dish on ice. Using two pairs of forceps, remove all six of each mosquito's legs and place in a prelabeled 2 mL round bottom microcentrifuge tube containing a sterilized stainless steel ball bearing (7/32") and 500 µL of mosquito collection media (MCM) composed of DMEM, 2% FBS, 1% penicillin-streptomycin, and 2.5 µg/mL amphotericin.

5.4. Gently place the mosquito onto a drop of mineral oil to restrain it, taking care to not allow any contact between the oil and the mosquito's head and proboscis.

5.5. Insert the mosquito proboscis into a 10 µL pipette tip filled with 10 µL of heat-inactivated

FBS.

NOTE: Alternatively, the pipette can be filled with sucrose, blood, or mineral oil. The oil allows for direct visualization of saliva bubbles via light microscopy.

5.6. Allow the mosquito to salivate for 30 min. Eject the micropipette tip containing FBS + saliva into a microcentrifuge tube containing 100 µL of MCM, then place the carcass into a separate 2 mL round bottom microcentrifuge tube containing a sterilized steel ball bearing and 500 µL of MCM. Ensure that the tubes used for the bodies, legs, and saliva are labeled so that it is clear all three samples originated from the same mosquito.

5.7. While the mosquito(s) are salivating, perform steps 5.3−5.5 on the remaining mosquitoes.

5.8. Transport the tubes containing the bodies and legs to a bead milling tissue homogenization device contained within a BSC. Triturate all body and leg samples at 26 Hz for 5 min to liberate viral particles into the supernatant. Clarify all samples by centrifugation at 200 x *g* for 5 min to pellet cellular debris.

NOTE: At this point, samples can be frozen at -80 °C, or assayed immediately.

6. Detection of ZIKV by infectious assay

6.1. In a BSC, prepare 24 well tissue culture plates with Vero cells (10^5 cells per well) 24 h prior to the onset of the infectious assay. Label each well with the identity of a single mosquito/sample.

6.2. If samples were frozen at -80 °C, allow to thaw.

6.3. Remove the media from the Vero cell plates prior to inoculation with samples one plate at a time.

6.4. For samples containing bodies or legs, carefully aliquot 100 µL of clarified supernatant into each well, taking care not to disturb the mosquito cell debris from the pellet.

NOTE: Saliva samples can be diluted 1:1 (v/v) with MCM prior to inoculation onto cells to conserve samples for later titration, if necessary.

6.5. Move the plates into a 37 °C, 5% CO_2 incubator and incubate for 1 h.

6.6. Return the plates to the BSC and add ~1 mL of methylcellulose overlay to each well. Place the overlayed plates back in a 37 °C, 5% CO_2 incubator and incubate for 3−7 days (virus/strain-dependent).

6.7. Perform fixation and visualization as described in steps 3.6−3.9.

6.8. Regarding scoring via a focus forming assay, quantify the positive wells by examination under a light microscope. Detection of intracytoplasmic staining of cells in a well indicates the presence of virus. Samples are scored solely as positive or negative.

REPRESENTATIVE RESULTS:

Three populations of *Ae. aegypti* from the Americas (Salvador, Brazil; the Dominican Republic; and the Rio Grande Valley, TX, USA) were exposed to an outbreak strain of ZIKV from the Americas (ZIKV Mex 1-7, Chiapas State, Mexico, 2015) over a range of bloodmeal titers (4, 5, and 6 $\log_{10}$ FFU/mL) presented in a washed human erythrocyte-based artificial bloodmeal. At days 2, 4, 7, 10, and 14 postinfection, subsets of mosquitoes were processed to determine infection, dissemination, and potential transmission rates.

At a bloodmeal titer of 4 $\log_{10}$ FFU/mL of ZIKV Mex 1−7, *Ae. aegypti* from Salvador, Brazil were infected at rates of 12.5% and 11.1% after 4 and 14 days of extrinsic incubation, respectively, with no evidence of disseminated infection observed in the assayed legs, and no virus detected in the saliva (**Figure 1a**). Increasing the titer to 5 $\log_{10}$ FFU/mL resulted in a marginal increase in infectivity with rates of 22.2%, 33.3%, and 22.2% on days 4, 10, and 14 postinfection, respectively. Similar to what was observed in the cohort exposed to 4 $\log_{10}$ FFU/mL, no disseminated infections or transmission competence was observed at any time point (**Figure 1b**). At the highest examined titer (6 $\log_{10}$ FFU/mL) no infections were identified after 2 days of incubation, but infections were observed at all other time points, peaking at 88.9% by 10 days of extrinsic incubation. ZIKV was detected in the legs of mosquitoes examined at 10 and 14 days postinfection (22.2% and 66.7%, respectively), indicating that ZIKV had disseminated into the hemocoel, although infectious ZIKV was observed in saliva at these time points (**Figure 1c**).

The *Ae. aegypti* population from the Dominican Republic proved the most susceptible to ZIKV infection and transmission competent at all tested bloodmeal titers. Some level of infection was observed at all time points at all three tested doses, with the lowest rate observed 2 days postinfection at 4 $\log_{10}$ FFU/mL (25%) (**Figure 1d**). With bloodmeal titers of 5 $\log_{10}$ and 6 $\log_{10}$ FFU/mL conditions infection rates peaked at 100%, with 100% infection observed as early as 4 days postinfection in the population of mosquitoes exposed to 6 $\log_{10}$ FFU/mL ZIKV (**Figure 1e**,**f**). Mosquitoes fed all three doses demonstrated dissemination by 7 days postinfection, peaking at 44.4% (4 $\log_{10}$ FFU/mL, 14 days postinfection), 88.9% (5 $\log_{10}$ FFU/mL, 1 and 14 days postinfection), and 100% (6 $\log_{10}$ FFU/mL, 10 days postinfection). Transmission-competence was observed after all three doses (11.1%, 22.2%, and 22.2% at 4, 5, and 6 $\log_{10}$ FFU/mL respectively), but only following a 14 day extrinsic incubation period (EIP) (**Figure 1d**−**f**).

The *Ae. aegypti* population from the Rio Grande Valley, TX, proved to be relatively refractory to infection with ZIKV. Mosquitoes exposed to bloodmeal titers of 4 $\log_{10}$ FFU/mL, were infected as early as 4 days postinfection, with infection rates between 22.2% and 44.4%. With these exposure conditions, disseminated infections were observed at 14 days postinfection at a rate of 11.1%, and no transmission competence was observed (**Figure 1g**). A ten-fold increase in bloodmeal titer produced a largely similar result, with infections observed starting 4 days postexposure (33.3% and 44.4%), while disseminated infections were found after 14 days of

extrinsic incubation at a rate of 22.2% (**Figure 1h**). Finally, in the cohort exposed to a 6 $\log_{10}$ FFU/mL bloodmeal, infection was observed beginning from the 2 day postinfection time point (22.2%) and reached peaks at 4, 10, and 14 days postinfection (66.7%). Disseminated infections in this condition began to be observed at 7 days postinfection (11.1%) and peaked at 44.4% at 14 days postinfection. Only a single mosquito (11.1%) was observed to be transmission capable at 10 days postinfection (**Figure 1i**).

FIGURE LEGENDS:

Figure 1: Representative vector competence data of various *Ae. aegypti* populations for ZIKV Mex 1–7. (**a**–**c**) Vector competence of *Ae. aegypti* from Salvador, Brazil (F2). (**d**–**f**) Vector competence of *Ae. aegypti* from the Dominican Republic (F6). (**g**–**i**) Vector competence of *Ae. aegypti* from the Rio Grande Valley, TX (F4). (**a**,**d**,**g**) *Ae. aegypti* exposed to 4 $\log_{10}$ FFU/mL of ZIKV Mex 1-7. (**b**,**e**,**h**) *Ae. aegypti* exposed to 5 $\log_{10}$ FFU/mL of ZIKV Mex 1-7. (**c**,**f**,**i**) *Ae. aegypti* exposed to 6 $\log_{10}$ FFU/mL of ZIKV Mex 1-7. At each time point (2, 4, 7, 10, and 14 days postinfection) a subset of mosquitos was collected and sampled. Infection, dissemination, and transmission rates are presented as the number of positive carcass/leg/saliva samples over the number of mosquitos assayed at that time point. Infection represented in blue, disseminated infections represented in green, and transmission rate represented in red. Data in this figure are modified from Roundy and Azar et al.[32].

DISCUSSION:

The methods described here provide a generalized workflow to conduct vector competence analyses. As a general framework, many of these methodologies are conserved throughout the literature. However, there is substantial room for modifications (reviewed in Azar and Weaver[1]). Virus (e.g., viral lineage, storage of challenge virus, viral passage history), entomology (e.g., laboratory colonization of mosquito populations, innate immunity, the mosquito microbiome/virome), and experimental variables (e.g., bloodmeal composition, sequential blood feeding, and incubation temperature) are all known to affect vector competence. Methodological variability in competence studies has proven problematic in the context of the ZIKV outbreak because it has precluded formal meta-analyses[1,33].

Within this general methodology, the importance of the appropriate starvation of mosquitoes and bloodmeal assembly cannot be overstated. Dehydration is known to drive blood feeding behavior of mosquitos in laboratory paradigms[34], underscoring the value of sugar and water starvation prior to offering an infectious bloodmeal. While deprivation of sugar for 36–48 h and water for 2–4 h prior to exposure to the bloodmeal is well tolerated by *Ae. aegypti*, it is worth noting that these mosquitoes are notoriously easy to work with in laboratory conditions[2]. Such aggressive regimens of starvation may not be nearly as well tolerated by other mosquito species, necessitating some degree of in-house optimization. Likewise, bloodmeal contents may be informed by host preference. For example, using human blood to prepare a bloodmeal for anthropophilic mosquitos like *Ae. aegypti* is entirely appropriate, but human bloodmeals made for ornithophilic species such as *Culex quinquefasciatus* may prove less effective[35]. Additionally, with respect to bloodmeal assembly, the use of relatively fresh blood products is highly advisable to minimize hemolysis of erythrocytes.

One of the greatest limitations of vector competence assessment as a whole and the procedures described herein is that these studies are largely limited to investigating viruses using mosquitoes that can be maintained in laboratory conditions. *Ae. aegypti*, while a highly relevant vector for a multitude of pathogens of clinical importance, also happens to be one of the easiest mosquitoes to rear and maintain in laboratory colonies[1,31,36,37]. Unsurprisingly, the competence of *Ae. aegypti* populations is often therefore the best characterized among common vector mosquitoes[2]. This is particularly problematic in the context of arboviruses that maintain both enzootic and urban transmission cycles[38-40], as vector competence is generally only conducted in the context of the more tractable urban mosquitoes.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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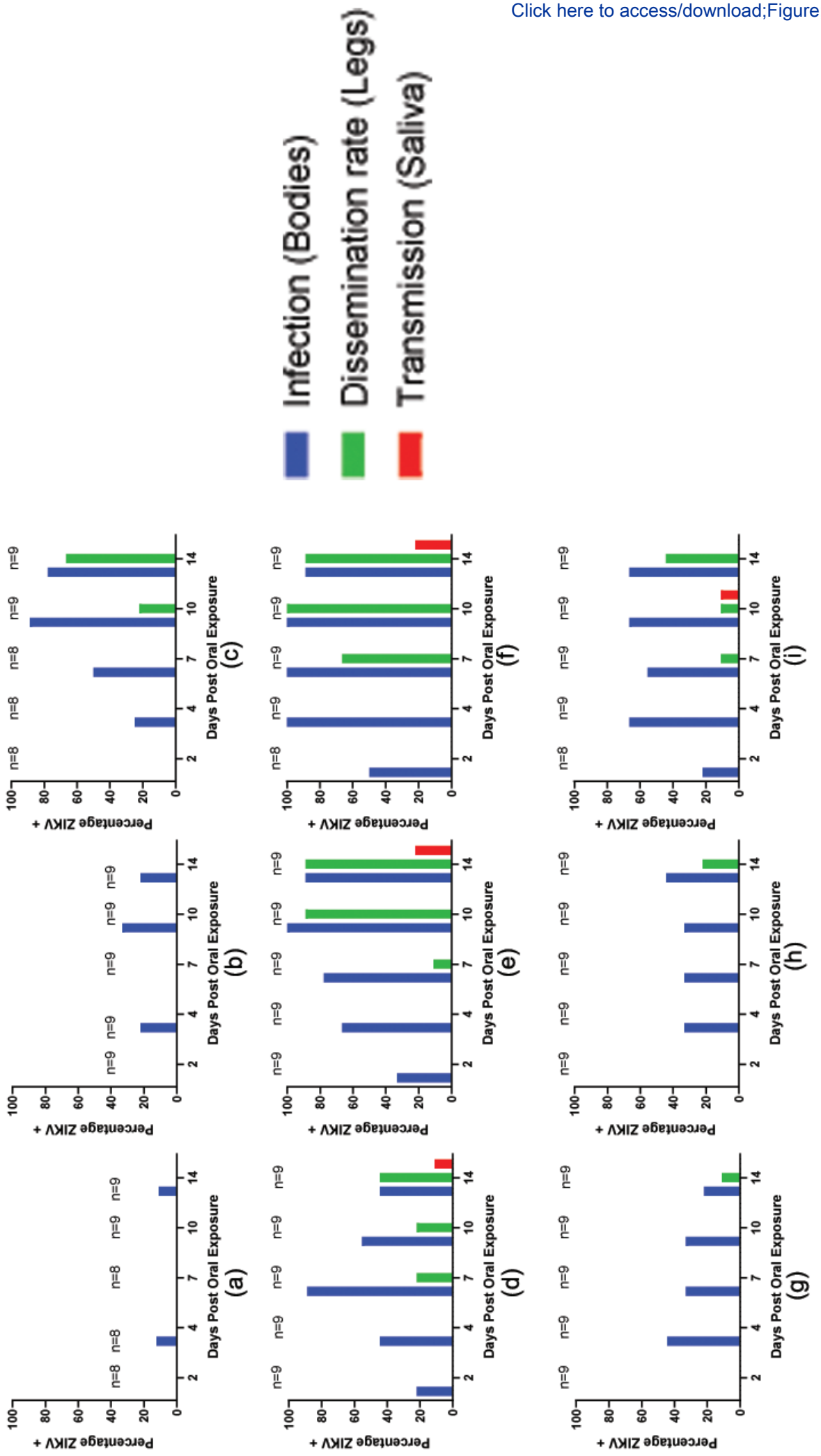

Table of Materials

Click here to access/download;Table of Materials;61112_materials_SRA_CompleteV2.xls

Name of Material/Equipment	Catalog Number	Company	Comments/Description
3mL Standard Reservoir	R37P30	Hemotek Ltd	Insectary Equipment
7/32" Stainless Steel 440 Grade C Balls	4RJH9	Grainger	Grinding Media
Acetone, Histological Grade, Fisher Chemicals, Poly Bottle, 4L, 4/Case	A16-P4	FisherScientific	Fixative
Adenosine 5'-triphospate disodium salt hydrat, microbial, BioReagent, suitable for cell culture	A6419-1G	MilliporeSigma	Reagent
Anti-Flavivirus Group Antigen Antibody, clone D1-4G2-4-15	MAB10216	MilliporeSigma	Primary Antibody for focus forming assay
Anti-Mouse IgG (H+L) Antibody, Human Serum Adsorbed and Peroxidase-Labeled, 1.0mL/Bottle	5450-0011	KPL/Seracare	Secondary Antibody for focus forming assay
Bleach	NC0427256	FisherScientific	Decontamination
Corning, Cell Culture Treated Flasks, 150cm2, Vented Cap, Case of 50	10-126-34	FisherScientific	Cell culture consumable
Costar Cell Culture Plates, 24-well, 5/bag, 100/case, Corning	07-200-740	FisherScientific	Cell culture consumable
Costar Cell Culture Plates, 96-well, 5/bag, 100/case, Corning	07-200-91	FisherScientific	Cell culture consumable
Crystal Violet	C0775-100G	MilliporeSigma	Stain
Eppendorf Snap Cap Microcentrifuge Safe-Lock 2mL Tubes, 500/Case	05-402-7	FisherScientific	Plastic consumable
Falcon 15mL Conical Centrigue Tubes	14-959-70C	FisherScientific	Plastic consumable
Falcon 50mL Conical Centrigue Tubes	14-959-49A	FisherScientific	Plastic consumable
Falcon Disposable Polystyrene Serological 10mL Pipets, 200/Case	13-675-20	FisherScientific	Plastic consumable

Falcon Disposable Polystyrene Serological 1mL Pipets, 1000/Case	13-675-15B	FisherScientific	Plastic consumable
Falcon Disposable Polystyrene Serological 25mL Pipets, 200/Case	13-675-30	FisherScientific	Plastic consumable
Falcon Disposable Polystyrene Serological 5mL Pipets, 200/Case	13-675-22	FisherScientific	Plastic consumable
Falcon Standard Tissue Culture Dishes	08-772B	FisherScientific	Plastic consumable
Fetal Bovine Serum-Premium, 500mL	S11150	Atlanta Biologicals	Cell culture reagent
Fisherbrand Economy Plain Glass Microscope Slides	12-550-A3	FisherScientific	Immobilization of Mosquitos
FU1 Feeder	FU1-0	Hemotek Ltd	Insectary Equipment; feeding units
Gibco DPBS with Calcium and Magnesium, 10 x 500mL Bottles	140-040-182	FisherScientific	Cell culture reagent
Gibco Fungizone, Amphotericin B, 250µg/mL, 50mL/Bottle	15-290-026	Fisher Scientific	Cell culture reagent
Gibco Penicillin-Streptomycin (10,000 U/mL), 100mL/Bottle, 20 Bottles/Case	15-140-163	FisherScientific	Cell culture reagent
Gibco, Tryptsin-EDTA (.25%), Phenol red, 20 x 100mL Bottles	25-200-114	FisherScientific	Cell culture reagent
Gibcom DMEM, High Glucose, 10 x 500mL Bottles	11-965-118	FisherScientific	Cell culture reagent
Human Blood, Unspecified Gender, Na-Citrate, 1 Unit	7203706	Lampire	Bloodmeal preparation
InsectaVac Aspirator	2809B	Bioquip	Insectary Equipment
Methanol, Certified ACS, Fisher Chemicals, Amber Glass Bottle, 4L, 4/Case	A412-4	FisherScientific	Fixative
Methyl cellulose, viscosity: 3,500-5,600 cP, 2 % in water(20 °C), 250g/Bottle	M0512-250G	MilliporeSigma	Cell culture reagent

Micro-chem Plus Disinfectant Detergent	C849T34	Thomas Scientific	Decontamination; working dilution of dual quaternary ammonium
Mineral Oil, BioReagent, for molecular biology	M5904-5X5ML	MilliporeSigma	Immobilization of Mosquitos
O-rings	OR37-25	Hemotek Ltd	Insectary Equipment
Plastic Plugs	PP5-250	Hemotek Ltd	Insectary Equipment
PS6 Power Unit (110-120V)	PS6120	Hemotek Ltd	Insectary Equipment; power source
Rubis Forceps, Offset blades, superfine points	4525	Bioquip	Insectary Equipment
Sarstedt Inc, 2mL Screw Cap Microtube, Conical Bottom, O-ring Cap, Sterile, 1000/Case	50-809-242	FisherScientific	Plastic consumable
Sucrose, BioUltra, for molecular biology	84097-250G	MilliporeSigma	Reagent
ThermoScientific, ART Barrier Low Retention 1000µL Pipette Tips, 100 tips/Rack, 8 Racks/Pack, 4 Packs/Case	21-402-487	FisherScientific	Plastic consumable
ThermoScientific, ART Barrier Low Retention 200µL Pipette Tips, 96 tips/Rack, 10 Racks/Pack, 5 Packs/Case	21-402-486	FisherScientific	Plastic consumable
ThermoScientific, ART Barrier Low Retention 20µL Pipette Tips, 96 tips/Rack, 10 Racks/Pack, 5 Packs/Case	21-402-484	FisherScientific	Plastic consumable
ThermoScientific, ART Barrier Low Retention, Extended Reach 10µL Pipette Tips, 96 tips/Rack, 10 Racks/Pack, 5 Packs/Case	21-402-482	FisherScientific	Plastic consumable
TissueLyser II	85300	QIAGEN	Homogenization
TrueBlue Peroxidase Substrate Kit, 200mL	5510-0030	Seracare	Developing solution for focus forming assay

Vero	CCL-81	American Type Culture Collection	Mammalian cell line to amplify virus and conduct infectious assay
Vero C1008 [Vero 76, clone E6, Vero E6]	CRL-1586	American Type Culture Collection	Mammalian cell line to amplify virus and conduct infectious assay

Dear Editor Cao,

We thank the reviewer for taking the time to consider our manuscript and provide feedback. A detailed point-by-point response is presented below with responses highlighted in red. The text of our manuscript has been revised to address these comments. Please note that any line numbers will refer to document submitted with changes tracked as opposed to the completed document.

Best wishes,

Sasha R. Azar and Scott C. Weaver

Editorial comments:
Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: The manuscript has been checked for spelling, grammar and clarity.

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

3. Protocol: Please revise it to be a numbered list following the JoVE Instructions for Authors: step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.
Response: Revised as suggested

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Microchem+, Hemotek Ltd, Tupperware, Qiagen TissueLyser II, etc.
Response: The text has been edited to remove references to commercial products.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: The text has been edited to remove asides and remain consistently in the imperative tense. Notes have been demarcated as such.

6. Please list all centrifugation speeds in terms of centrifugal g-force (x g) instead of rpm.
Response: Revised as suggested

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
Response: Please refer to responses below

8. Line 98: Please specify growth conditions (temperature, CO_2 concentration).
Response: Growth conditions added

9. Line 118: Please describe how to harvest virus.
Response: Viral harvest is detailed in the following step. The text has been modified to remove ambiguity.

10. Line 146: Please specify the blood source.
Response: The text has been revised to include the blood source

11. Line 173: Please specify the reservoir unit and covering used here.
Response: The text has been revised to provide details on both the reservoir and the covering

12. Line 248: Is the supernatant or pellet saved? Please specify.
Response: The virus is released into the supernatant and the cellular debris is pelleted. Both are saved. The language in these steps has been modified for clarity

13. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: The protocols have been edited such that some steps are now combined

14. Please include single line spacing between each numbered step or note in the protocol.
Response: Revised as suggested

15. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
Response: The text for the video has been highlighted as requested.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Response: The text for the video has been highlighted as requested.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response: The text for the video has been highlighted as requested.

18. Please reference each panel of Figure 1 in the results section.
Response: Revised as suggested

19. Discussion: As we are a methods-based journal, please discuss critical steps in the protocol, modifications and troubleshooting of the method.
Response: A paragraph noting the criticality of appropriate starvation of mosquitoes and bloodmeal assembly has been added.

20. Table of Materials: Please sort the materials alphabetically by material name.
Response: Revised as suggested

Dear Editor Cao,

We thank the reviewer for taking the time to consider our manuscript and provide feedback. A detailed point-by-point response is presented below with responses highlighted in red. The text of our manuscript has been revised to address these comments. Please note that any line numbers will refer to document submitted with changes tracked as opposed to the completed document.

Best wishes,

Sasha R. Azar and Scott C. Weaver

Reviewers' comments:
Reviewer #1:

Major Concerns:
Several thoughts to consider:
1. aegypti and ZIKV. I wonder if this shouldn't be presented as general mosquito-arbovirus methods using aegypti - ZIKV as an example?
Response: We agree with the reviewer that our methods are general and widely applicable to other agents. In the “Summary”, “Abstract” and the “Discussion” we note this.

2. Methods mentioned but not described. The authors allude to dissections to measure organ infections and to methods to imply vertical transmission, but do not go into the description of these methods in the protocols.
Response: JOVE instructions guided us to mention alternative methods. Dissection of organs is mentioned solely as an alternative approach. As the reviewer noted dissections are often not used due to being labor intensive and prone to contamination with infected hemocoel.

3. RT-PCR. I strongly recommend the inclusion of RT-PCR methods to track ZIKV RNA as a surrogate for infectious virus. I feel these method provide useful data, are safer and faster, and may be more sensitive. They certainly are more specific.
Response: We respectfully disagree. The reviewer themselves notes that RT-PCR is simply a surrogate for infectious virus. Given that transmission competence is defined as the ability for a mosquito to transmit the agent in question, the presence of viable infectious virus is far more informative than the presence of viral RNA. Specificity is not an issue as long as the mosquitoes have been tested for other viruses that could produce foci in the infectious assay. As long as infectious assay is performed on mammalian cells to preclude insect-specific viruses, it is entirely appropriate to utilize this method.

4. Data. I think a section describing how to assemble the data would be useful. The authors present results but do not clearly indicate how they calculated the percentages presented or what was the best statistical methods for comparisons. For example, the Figure shown uses the number of blood fed females as the denominator to calculate infection, dissemination and transmission rates. Because the infection rates were so low, the authors are really using uninfected mosquitoes to estimate dissemination of virus from the gut? Although this may be useful in epidemiological models, it doesn't present useful biological information. For example, some vectors with a robust gut infection barrier readily disseminate virus if infected. Similarly, should transmission be

calculated using mosquitoes with non-disseminated infections? As presented the data show the cumulative impact of all barriers.
Response: We have added the following text to the figure legend in order to clarify how the results have been derived: "At each time point (2, 4, 7, 10 and 14-days post-infection) a subset of mosquitos were collected and sampled independently. Infection, dissemination and transmission rates are presented as the number of positive carcass/leg/saliva samples over the number of mosquitos assayed at that time point". With respect to the denominator, we respectfully disagree; vector competence is often used in epidemiological modeling therefore we have chosen to present our data conservatively. The reader can derive rates of dissemination in infected mosquitoes from the data presented.

5. Controls. Back-titrating the blood meals or testing freshly fed females for virus should be done to ensure the virus titer in the challenge blood meal. This would catch errors in titration or possible unintentional virus inactivation. Sequencing the virus is useful to determine which exact variant was represented in the experiments. Similar data on the vectors is similarly useful. These sequences can be deposited for later reference. The vector competence experiment then becomes the phenotype.
Response: We thank the reviewer for catching this. We have added an additional section "Backtitration of Bloodmeals". With respect to sequencing of the virus and vectors, our experiments are conducted on strains that have previously been sequenced. We have added the GenBank Accession numbers for clarity. Our output in these paradigms is solely vector competence. The impact of variants in competence is a topic beyond the scope of this paper.

Minor Concerns:
The manuscript will need some editing prior to final acceptance. Despite the request not to, I made some suggestions on the file sent to the JoVE. Likewise specific comments were inserted using the Adobe comment tool.
Response: We thank the reviewer for their suggestions. We have implemented their grammatical suggestions as seen on the PDF. In revising some of the text to address the above major comments, other points raised in the PDF have been addressed as well.

Dear Editor Cao,

We thank the reviewer for taking the time to consider our manuscript and provide feedback. A detailed point-by-point response is presented below with responses highlighted in red. The text of our manuscript has been revised to address these comments. Please note that any line numbers will refer to document submitted with changes tracked as opposed to the completed document.

Best wishes,

Sasha R. Azar and Scott C. Weaver

Reviewer #2:
Manuscript Summary:
Mosquito-borne viruses can cause debilitating disease to humans and other mammals worldwide. Laboratory research understanding transmission of different types/strains of mosquito-borne viruses is a critical component of understanding mosquito-virus interactions.

This manuscript describes an experimental protocol to identify potential transmission ability of a mosquito-borne virus (Zika virus) with three different populations of mosquitoes from the Americas. This protocol can be easily paralleled with other mosquito species and other virus types/strains/isolates.

Major Concerns:
*No major concerns.

Minor Concerns:
*Use "mosquitoes" and not "mosquitos". Needs to be adjusted throughout the text.
Response: We have revised the text as suggested

*Line 71- Use "Two major approaches exist by which the role of mosquito..."
Response: We have revised the text as suggested

*Line 77- Add ")" after "...1,600 infected"
Response: We have revised the text as suggested

*Line 383- Italicize Ae. aegypti
Response: We have revised the text as suggested

Permission (EID)

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Reply all Delete Junk Block ···

RE: Data Reuse Permission -- Author's Deadline Past

You replied on Mon 12/9/2019 6:58 PM

EE

Richardson, Susan (CDC/DDID/NCEZID/OD) (CTR) <shr4@cdc.gov>
on behalf of
EID Editor (CDC) <eideditor@cdc.gov>
Mon 12/9/2019 2:05 PM
Azar, Sasha R.; EID Editor (CDC) <eideditor@cdc.gov>

Dear Sasha R. Azar,
Thank you for your inquiry regarding permission to reuse material originally published in *EID*.
We routinely grant such permission as we do in this instance. The reproduced material should contain a full formal citation to *EID* and include a link if possible.
D. Peter Drotman, MD, MPH
Editor-in-Chief
Emerging Infectious Diseases
http://wwwnc.cdc.gov/eid/

From: Azar, Sasha R. <srazar@UTMB.EDU>
Sent: Monday, December 9, 2019 10:51 AM
To: EID Editor (CDC) <eideditor@cdc.gov>
Cc: Weaver, Scott <sweaver@UTMB.EDU>
Subject: Re: Data Reuse Permission

Dear Susan,

I am emailing again to follow up on this [my request to reuse data from a paper that has previously been published in EID "Variation in Aedes aegypti Mosquito Competence for Zika Virus Transmission" (DOI:10.3201/eid2304.161484). To be clear, both myself and Dr. Weaver (cc'd) were authors on the manuscript].

I have contacted the editor from JOVE and asked for an extension as I am now over my deadline. Can this please be expedited?

Best wishes,
Sasha R. Azar

From: Richardson, Susan (CDC/DDID/NCEZID/OD) (CTR) <shr4@cdc.gov> on behalf of EID Editor (CDC) <eideditor@cdc.gov>
Sent: Tuesday, December 3, 2019 2:31 PM

Scott Weaver is a virologist and vector biologist who studies arthropod-borne viruses (arboviruses), their transmission by mosquitoes, and develops vaccines to control the diseases that they cause. His research encompasses the ecology and epidemiology of enzootic arbovirus transmission cycles, virus-mosquito interactions, pathogenesis, and emergence mechanisms of epidemic strains. Recently he has focused on chikungunya and Zika viruses, which in 2013 arrived in the Americas to cause major epidemics.

Sasha Azar is a virologist who studies arthropod-borne viruses (arboviruses), emphasizing investigation of the interface between the virus, vector mosquitos, and mammalian hosts. In particular, he is interested in how hematological factors of mammalian/human hosts may affect mosquito infectivity of emerging arboviruses.