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Title: Vector Competence Analyses on Aedes aegypti Mosquitoes Using Zika Virus

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If **No**, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Olympus SZX10 StereoMicroscope

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **All potential facilities are on the Galveston campus and are walking distance from each other (~5-minute walk at most)**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sasha Azar:** In outbreaks mediated by arboviruses, countermeasures are largely limited to mosquito abatement. Vector competence analyses are critical to prevention strategies because they determine what species can potentially serve as vectors for a given arbovirus and thus should be targeted.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Sasha Azar:** Experimental vector competence evaluations offer the scientist a unique degree of control in which population or species of mosquito is exposed to a specific strain and genotype of virus at various bloodmeal titers.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Sasha Azar:** Arboviral pathogenesis can be directly affected by arthropod saliva. The basic vector competence methods can be modified to conduct mosquito transmission studies to vertebrate hosts and develop accurate animal models.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

NOTE: Step 1.3 was dropped and hence not filmed.

1.4. **Sasha Azar:** Working with infected mosquitoes requires training, experience and careful planning to minimize the risk of an escaped, infectious vector. It would be beneficial for beginners to practice some of these techniques in mock experiments with uninfected blood and mosquitos to familiarize themselves with the workflow.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

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

Protocol

2. Artificial Bloodmeal Preparation

2.1. On the day of exposure, turn on the power source in an arthropod containment facility to preheat the feeding units **[1]**. Combine freshly harvested citrated or heparinized human blood with a viral stock at a 1 to 1 volume ratio **[2]** and overlay a standard 3-milliliter reservoir unit with the skin of a naïve, uninfected mouse **[3]**. *Videographer: This step is difficult and important!*

2.1.1. WIDE: Establishing shot of talent turning on the power source of the containment facility.

2.1.2. Talent combining blood with viral stock.

2.1.2a. Added shot: Talent adding blood.

2.1.3. Talent overlaying a reservoir unit with the skin of a mouse.

NOTE: Do not use the takes with any "skin tearing".

2.2. Place the covered reservoir on white paper towels **[1]** and add about 2 milliliters of infectious blood to the reservoir 1 milliliter at a time **[2]**. Then, inspect the paper towels to confirm that there is no leakage **[3]**. *Videographer: This step is important!*

2.2.1. Talent placing the reservoir on paper towels.

2.2.2. Talent adding blood to the reservoir.

2.2.3. Talent inspecting the paper towel.

NOTE: Shot number 2.2.2. and 2.2.3. were filmed together as one shot.

2.3. Twenty-four hours prior to performing the infectious assay, prepare 24-well cell culture plates with Vero cells **[1-TXT]** and label each well with the identity of the mosquito and sample **[2]**.

2.3.1. Talent seeding Vero cells into wells. **TEXT: 10^5 cells per well**

2.3.2. Talent labeling wells.

3. Virus Assays

3.1. Remove overlayed plates from the incubator and bring them to a biosafety cabinet **[1]**. Discard the methylcellulose overlay into a tray pan containing 10% bleach **[2]** and wash each well twice with PBS, discarding each wash into the tray pan **[3]**.

3.1.1. Talent taking plates out of the incubator.

3.1.2. Added shot: Talent aspirating the methylcellulose.

3.1.3. Talent washing wells with PBS.

3.2. Remove the PBS and add 1 milliliter of methanol-acetone per well and allow the cells to fix onto the plate for 30 minutes **[1-TXT]**, then discard the fixative and leave the plates to dry **[2]**.

3.2.1. Added shot: Talent removing the PBS and adding the methanol:acetone to a few wells and leaving the plate to sit in the BSC. **TEXT: methanol-acetone (1:1 vol/vol)** *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.8.3.*

3.2.2. Talent discarding the methanol:acetone.

3.3. Wash each well 3 times with nonsterile PBS on an orbital plate rocker for 15 minutes per wash **[1]**, then add 1 milliliter of blocking solution to each well **[2]** and rock the plate for another 15 minutes **[3]**.

3.3.1. Plate on an orbital rocker.

3.3.2. Talent adding blocking solution to a few wells, with the solution container in the shot.

3.3.3. Talent putting the plate on the rocker.

3.4. Add 100 microliters of anti-Zika virus or anti-flavivirus primary antibody to each well **[1-TXT]** and incubate the plate with rocking overnight **[2]**. On the next day, remove the primary antibody and repeat the washes with PBS **[3]**.

3.4.1. Talent adding primary antibodies to a few wells. **TEXT: Dilute antibodies 1:2,000 in blocking solution**

3.4.2. Talent putting the plate on the rocker and starting it.

3.4.3. Talent adding PBS to a few wells, with the PBS container in the shot.

3.4.4. Added shot: Talent putting the plate on the rocker.

3.5. After the last wash, add 100 microliters of secondary antibodies and incubate the plate while rocking for 1 hour **[1-TXT]**. Repeat the washes with PBS, then add 100 microliters of substrate development reagent per well **[2]** and rock the plate for 15 minutes **[3]**.

3.5.1. Talent adding secondary antibodies to a few wells. **TEXT: Dilute antibodies 1:2,000 in blocking solution**

3.5.1.a. Added shot: Use the rocker shot.

3.5.1.b. Added shot: Use 3.4.3. (adding PBS)

3.5.1.c. Added shot: A shot of the rocker.

3.5.2. Talent adding substrate development reagent to a few wells.

3.5.3. Talent starting the rocker with the plate on it.

3.6. When the foci or plaques develop, halt the reaction by rinsing the plate with tap water **[1]**. Pour off the tap water and allow the plates to air dry **[2]**, then quantify the viral foci to determine the number of focus-forming units present in the given sample **[3]**. *Videographer: This step is important!*

3.6.1. Talent rinsing plate with tap water.

3.6.2. Talent pouring off water and leaving the plate to dry.

NOTE: Shots 3.6.1. and 3.6.2. were combined and filmed together as one shot.

3.6.3. Talent counting the foci.

4. Administration of Bloodmeals

4.1. Sort female *Aedes aegypti (pronounce like this)* mosquitoes into 0.5-liter cardboard cartons with screened lids **[1-TXT]**. Deprive them of sugar 36 to 48 hours prior to the infectious bloodmeal but provide ad libitum water via water-saturated cotton balls **[2]**. *Videographer: This step is difficult!*

4.1.1. Talent sorting mosquitos. **TEXT: 2 – 4 days post eclosion**

4.1.2. Talent putting water-saturated cotton balls in the carton.

NOTE: Shot number 4.1.1. and 4.1.2. were dropped and hence not filmed.

4.2. On the morning that the mosquitoes will be exposed to the infectious bloodmeal, remove the water-saturated cotton balls **[1]** and attach reservoirs with the artificial bloodmeals to the feeding unit leads with a clear plastic containment glove box **[2]**. *Videographer: This step is difficult!*

4.2.1. Talent removing the cotton balls.

4.2.2. Talent attaching the reservoirs to the feeding unit leads.

4.3. Place the cardboard carton with the starved mosquitoes into the glovebox underneath the feeding unit **[1]**. Upon completion of feeding, remove the reservoir **[2]** and immerse it in freshly prepared 10% bleach **[3]**. *Videographer: This step is important!*

4.3.1. Talent placing the carton in the glovebox.

4.3.2. Talent removing the reservoir.

4.3.3. Talent putting the reservoir in the bleach solution.

4.4. Within the glovebox, pour cold-anesthetized mosquitoes into a Petri dish on ice **[1]** and sort the engorged from the unengorged mosquitoes **[2]**. Pour the engorged mosquitoes back into the cardboard carton, quickly covering it with the screen and lid **[3]**. *Videographer: This step is important!*

4.4.1. Talent pouring the mosquitoes into the Petri dish.

4.4.2. Talent sorting the mosquitoes.

4.4.3. Talent pouring mosquitoes back in the carton and closing the lid.

NOTE: Shots 4.4.1. , 4.4.2. and 4.4.3. were combined and filmed together in one shot.

4.5. Trim the excess screen mesh off of the carton and secure the lid with tape **[1]**. Add a cotton ball saturated with sterile filtered 10% sucrose to the screen **[2]** and place the carton in a large plastic secondary container with a damp sponge to maintain humidity **[3]**.

4.5.1. Talent trimming the mesh and securing it with tape.

4.5.2. Talent adding the cotton ball with sucrose to the screen.

4.5.3. Talent placing the carton into the secondary container, with the damp sponge visible in the shot.

4.6. Incubate the secondary container at 27 degrees Celsius, allowing mosquitoes ad libitum access to the sucrose until completion of experiments **[1-TXT]**.

4.6.1. Talent placing the secondary container in an incubator and closing the door. **TEXT: 80% relative humidity ; 16:8-hour light:dark cycle**

5. Sample Acquisition and Processing

5.1. Use a mechanical aspirator to acquire a predetermined number of incubated mosquitoes from the carton **[1]**, capping the collection tube with a cotton round when finished **[2]**.

5.1.1. Talent aspirating mosquitoes.

5.1.2. Talent capping the collection tube.

NOTE: Shots 5.1.1. and 5.1.2. were combined and filmed together as one shot.

5.2. Working within the glove box, pour cold-anesthetized mosquitoes into a Petri dish on ice **[1-TXT].** Remove all 6 of each mosquito's legs **[2]** and place them in a prelabeled 2-milliliter microcentrifuge tube with a sterilized stainless-steel ball bearing and 500-microliters of mosquito collection media, or MCM **[3]**.

5.2.1. Talent pouring mosquitoes onto a Petri dish on ice.

5.2.2. SCOPE or ECU: Talent removing mosquito legs. *Videographer: Please use your judgement on whether this should be a SCOPE shot or an extreme close up. Talent doesn't need the microscope, but it might help to visualize this process.*

5.2.3. ECU: Talent putting mosquito legs in the microcentrifuge tube.

5.3. Gently place the mosquito onto a drop of mineral oil, taking care that the oil does not contact the mosquito's head or proboscis **[1]**. Insert the proboscis into a micro-pipette

tip filled with 10 microliters of heat-inactivated FBS and allow the mosquito to salivate for 30 minutes **[2]**.

5.3.1. SCOPE or ECU: Talent placing the mosquito on mineral oil.

5.3.2. SCOPE or ECU: Talent inserting the proboscis into the pipette tip and the mosquito salivating.

5.4. Eject the pipette tip with the saliva into a microcentrifuge tube with 100 microliters of MCM **[1]** and place the carcass into a separate 2-milliliter tube with a sterilized steel ball bearing and 500 microliters of MCM **[2-TXT]**.

5.4.1. Talent ejecting the pipette tip into a tube with MCM.

5.4.2. Talent placing the carcass into a different tube. **TEXT: Ensure all tubes are clearly labeled!**

NOTE: Shots 5.4.1. and 5.4.2. were combined and filmed together as one shot.

5.5. Transport the tubes to a bead milling tissue homogenization device within a biosafety cabinet **[1]** and triturate all body and leg samples at 26 Hertz for 5 minutes to liberate viral particles into the supernatant **[2]**. Clarify all samples by centrifugation at 200 x *g* for 5 minutes to pellet cellular debris **[3]**. *Videographer: This step is important!*

5.5.1. Talent bringing the tubes to the biosafety cabinet.

5.5.2. Talent using the tissue homogenizer.

5.5.3. Talent putting the tubes in the centrifuge and closing the lid.

5.6. Immediately before inoculation with samples, remove the media from the previously prepared Vero cells **[1]**. Carefully aliquot 100 microliters of clarified supernatant from the bodies or legs into each well, taking care not to disturb the mosquito cell debris from the pellet **[2]**. Then, incubate the plates for 1 hour **[3-TXT]**. *Videographer: This step is important!*

5.6.1. Talent removing the media from a plate.

5.6.2. Talent aliquoting sample into a well.

5.6.3. Talent putting the plate in the incubator and closing the door. **TEXT: 37°C, 5% CO_2** *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.8.2.*

NOTE: For shot number 5.6.3. two takes were shot with different color.

5.7. After the incubation add 1 milliliter of methylcellulose overlay to each well **[1]** and put the plates back in the incubator for 3 to 7 days **[2]**. When ready, perform fixation and visualization of the samples as previously described **[3]**.

5.7.1. Talent adding methylcellulose to a few wells.

5.7.2. *Use 4.7.3.*

5.7.3. *Use 2.4.1.*

Results

6. Results: Vector Competence Data of Various *Ae. aegypti* Populations for ZIKV Mex 1–7

6.1. Three mosquito populations were exposed to an outbreak strain of zika virus over a range of bloodmeal titers **[1]**. Over the following 2 weeks, subsets of mosquitoes were processed to determine infection, dissemination, and potential transmission rates **[2]**.

6.1.1. LAB MEDIA: Figure 1.

6.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the all blue bars when VO says "infection", all green bars when VO says "dissemination", and all red bars when VO says "potential transmission".*

6.2. At the lower bloodmeal titers, the mosquitoes from Salvador, Brazil were infected after 4, 10, and 14 days of extrinsic incubation, with no evidence of disseminated infection observed in the assayed legs **[1]**. At the highest titer, the virus was detected in the legs of mosquitoes but not in the saliva **[2]**.

6.2.1. LAB MEDIA: Figure 1 A, B, and C. *Video Editor: Label A "***4 $\log_{10}$ FFU/mL***", B "***5 $\log_{10}$ FFU/mL***", and C "***6 $\log_{10}$ FFU/mL***". Emphasize A and B.*

6.2.2. LAB MEDIA: Figure 1 A, B, and C. *Video Editor: Emphasize C.*

6.3. The mosquitoes from the Dominican Republic proved to be the most susceptible to infection and the most transmission competent at all tested bloodmeal titers **[1]**. Mosquitoes fed all three doses demonstrated dissemination by 7 days **[2]** and transmission-competence at 14 days post infection **[3]**.

6.3.1. LAB MEDIA: Figure 1. D, E, and F. *Video Editor: Label D "***4 $\log_{10}$ FFU/mL***", E "***5 $\log_{10}$ FFU/mL***", and F "***6 $\log_{10}$ FFU/mL***".*

6.3.2. LAB MEDIA: Figure 1. D, E, and F. *Video Editor: Emphasize the green bars in all 3 graphs.*

6.3.3. LAB MEDIA: Figure 1. D, E, and F. *Video Editor: Emphasize the red bars in all 3 graphs.*

6.4. Mosquitoes from the Rio Grande Valley in Texas exposed to lower bloodmeal titers were infected as early as 4 days post infection **[1]**, while disseminated infections were found after 14 days **[2]**.

6.4.1. LAB MEDIA: Figure 1 G, H, and I. *Video Editor: Label G "***4 $\log_{10}$ FFU/mL***", H "***5 $\log_{10}$ FFU/mL***", and I "*6 $\log_{10}$ FFU/mL*". Emphasize G and H.*

6.4.2. LAB MEDIA: Figure 1 G, H, and I. *Video Editor: Emphasize G and H and the green bars in G and H.*

6.5. In the cohort exposed to the highest titer, infection was observed beginning from the second day post infection and reached peaks at 4, 10, and 14 days **[1]**. Disseminated

infections were observed at 7 days **[2]** and only a single mosquito was transmission capable at 10 days **[3]**.

6.5.1. LAB MEDIA: Figure 1 G, H, and I. *Video Editor: Emphasize I.*

6.5.2. LAB MEDIA: Figure 1 G, H, and I. *Video Editor: Emphasize I and the green bars in I.*

6.5.3. LAB MEDIA: Figure 1 G, H, and I. *Video Editor: Emphasize I and the red bar in I.*

Conclusion

7. Conclusion Interview Statements

7.1. **Sasha Azar:** When attempting this protocol, it is important to work safely, not quickly. Always handle infected or potentially infected mosquitos within the confines of the glovebox UNTIL they have had legs or wings removed.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.1.1, 5.1.2, 5.2.1.*

7.2. **Sasha Azar:** The basic workflow can be adapted to produce infected mosquitos for other experiments such as transmission studies and examinations of mosquito-virus interactions.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

7.3. **Sasha Azar:** Laboratory infection of mosquitos and vector competence is a paradigm that has been in use for decades. The basic paradigm is incredibly accommodating to modifications that allow other questions to be asked such as intrahost viral diversity via NGS, viral competition experiments, and so on.

7.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.