Video Article

Detection of Infectious Virus from Field-collected Mosquitoes by Vero Cell Culture Assay

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URL: http://www.jove.com/details.php?id=2889
DOI: 10.3791/2889
Citation: Armstrong P.M., Andreadis T.G., Finan S.L., Shepard J.J., Thomas M.C. (2011). Detection of Infectious Virus from Field-collected Mosquitoes by Vero Cell Culture Assay. JoVE. 52. http://www.jove.com/details.php?id=2889, doi: 10.3791/2889

Abstract

Mosquitoes transmit a number of distinct viruses including important human pathogens such as West Nile virus, dengue virus, and chickungunya virus. Many of these viruses have intensified in their endemic ranges and expanded to new territories, necessitating effective surveillance and control programs to respond to these threats. One strategy to monitor virus activity involves collecting large numbers of mosquitoes from endemic sites and testing them for viral infection. In this article, we describe how to handle, process, and screen field-collected mosquitoes for infectious virus by Vero cell culture assay. Mosquitoes are sorted by trap location and species, and grouped into pools containing ≤50 individuals. Pooled specimens are homogenized in buffered saline using a mixer-mill and the aqueous phase is inoculated onto confluent Vero cell cultures (Clone E6). Cell cultures are monitored for cytopathic effect from days 3-7 post-inoculation and any viruses grown in cell culture are identified by the appropriate diagnostic assays. By utilizing this approach, we have isolated 9 different viruses from mosquitoes collected in Connecticut, USA, and among these, 5 are known to cause human disease. Three of these viruses (West Nile virus, Potosi virus, and La Crosse virus) represent new records for North America or the New England region since 1999. The ability to detect a wide diversity of viruses is critical to monitoring both established and newly emerging viruses in the mosquito population.

Protocol

1. Mosquito sorting and identification

1. The following procedures are performed on an open laboratory bench in a dedicated biosafety level-2 (BSL-2) laboratory by staff who are trained to work with live mosquitoes. A "cold-chain" is maintained throughout the procedure.
2. Anesthetize live adult mosquitoes by placing mosquito collection bag in a -20° freezer for 5 minutes. Transfer collection bag to an insulated container containing dry ice. Close lid and expose mosquitoes to carbon-dioxide for 1-3 minutes to ensure complete knock-down.
3. Tap anesthetized mosquitoes onto a pre-chilled pan placed on a bed of dry ice. Separate individual female mosquitoes using fine forceps and place in plastic portion cups. Cover with lid and place cups on wet ice.
4. Identify mosquitoes to species using descriptive taxonomic keys based on external mosquito morphology with the aid of a stereo dissecting microscope (10-60X zoom) mounted on an electronic chill table.
5. Combine identified mosquito species into pools of ≤50 individuals in 2 mL snap-cap vials containing a copper BB. Vials are labeled with a unique identifier.
6. Following identification of all mosquitoes, vials are placed in labeled bags and held in a Styrofoam cooler containing dry ice.
7. Store mosquito pools in a -80° C freezer until virus testing.

2. Biosafety considerations to isolate virus from mosquitoes

1. The following procedures are performed in a biosafety level-3 (BSL-3) laboratory by staff who are trained to work at this level of containment[1]. Laboratory facilities, equipment, procedures and practices are subject to institutional, state, and federal oversight and inspection. Seek the appropriate training and approval before attempting the following protocols.
2. Wear the appropriate personal protective equipment (PPE) for each procedure such as disposable gowns, gloves, face shields, and respirators.
3. Disinfect work surfaces with 70% alcohol before and after completing procedures.
4. Cell cultures and potentially infectious material are handled in a Class II biosafety cabinet (BSC).
5. Mosquito pools are homogenized in a mixer-mill that is placed inside a BSC.
6. Mosquito homogenates are centrifuged in an aerosol-tight microcentrifuge. If this procedure cannot be performed within a BSC, the operator should wear a respirator when operating and retrieving samples from the centrifuge.
7. Pipets and pipet tips handling material in the BSC (biosafety cabinet) are soaked in 10% bleach prior to autoclaving. All laboratory waste is decontaminated by autoclaving prior to disposal.

3. Preparation of Vero cells

1. Decant culture media from one large tissue culture flask (175 cm^2) of confluent Vero cells (Clone E6) into waste beaker containing bleach.
2. Wash Vero cells by adding a 5 mL aliquot of PBS and swirl over layer of cells and along sides of flask making sure to thoroughly cover entire monolayer.
3. Decant PBS into waste beaker.
4. Add 2.5 mL of Trypsin-EDTA and swirl over layer of cells making sure to thoroughly cover entire monolayer.
5. Incubate flask in incubator for 5 minutes at 37°C, 5% CO_2.

6. Gently shake and swirl flask to remove cells from bottom surface. The cells should slide off easily; incubate an additional 1-5 minutes if cells do not easily slide off.
7. Add 5 mL of Minimal Essential Media (MEM), 5% fetal bovine serum (FBS), using a serological pipet and pipet up-and-down 10-20 times to break up the clumps of cells.
8. Add an additional 15 mL MEM, 5% FBS for a total volume of 22.5 mL and mix well.
9. Place 40 small tissue culture flasks (25 cm^2) upright in two racks (20 flasks/rack).
10. Place the racks containing flasks inside the biosafety cabinet and remove flask caps, placing caps directly in front of flasks.
11. Dispense 4 mL of MEM, 5% FBS into each flask using a serological pipet.
12. Dispense 0.5 mL suspended cells into each flask (˜1:6 splitting ratio)Replace flask caps.
13. Add 50 mL of MEM, 5% FBS back to the original large tissue culture flask containing the remaining cell suspension (˜2.5 mLs) and return flask to the incubator. The same large tissue culture flask may be re-used up to 5 passages and then a new flask should be set-up to replace it.
14. Place small tissue culture flasks in stacks on tray. Swirl the tray to make sure that media uniformly covers the bottom of the flaks.
15. Place small flasks in the incubator at 37° C, 5% CO_2 and grow overnight until 80-90% confluent.

4. Preparation of mosquito pools

1. Keep mosquito pools cold during testing procedures. Use pre-chilled freezer racks and mixer-mill cassettes, ice-cold reagents, and a refrigerated centrifuge when processing mosquito pools.
2. Place tubes containing mosquitoes into a pre-chilled freezer rack and add 1- 1.5 mL PBS-G to each tube of mosquitoes.
3. Retrieve mixer-mill cassettes from the freezer. Place 24 tubes into each cassette and then secure in the mixer-mill.
4. Homogenize the samples for 4 minutes at 25 cycles/second. The mixer mill shakes the tubes at high velocity and a metal BB inside each tube disrupts the mosquito tissue.
5. Centrifuge tubes for 6 minutes at 7,000 rpm.
6. Place tubes back into freezer racks until inoculated into Vero cells.

5. Inoculation of Vero cells with mosquito pool homogenates

1. Check at least one tissue culture flask from each series under inverted microscope to assure adequate confluency and cell health; expect about 80-90% coverage.
2. Line up 20 small tissue culture flasks in a rack and label flasks with corresponding accession numbers from each mosquito pool.
3. Loosen caps and decant most of the media from tissue culture flasks into waste beaker. Leave enough media in the flask to completely coat cell monolayer.
4. Aliquot 100µL of supernatant from each processed mosquito pool into the corresponding tissue culture flask.
5. Replace and tighten culture flask cap.
6. Lay culture flasks flat on shaker for 5 minutes (room temperature) at 35-40 rpm.
7. Return 20 tissue culture flasks to upright position in a rack and place in the BSC.
8. Uncapping 3 tissue culture flasks at one time, dispense 4 mL growth media into each flask using a serological pipette; replace caps.
9. Be sure that the pipette tip does not contact the culture flask neck or lip. Change pipette tips as necessary if contact is made with the flask.
10. Incubate tissue culture flasks at 37°C, 5% CO_2.

6. Screening Vero cells for virus growth

1. Screen inoculated tissue culture flasks for signs of viral infection, cytopathic effect (CPE), from 3-7 days post-inoculation.
2. Visually inspect cell cultures for CPE and/or contamination by tilting the flasks and examining the media that pools at the bottom of the flask. The media will appear cloudy as the cells deteriorate during CPE or due to growth of yeast, fungus, or bacterial contamination.
3. Re-examine cell cultures that exhibit cloudy media under an inverted microscope. Viruses will cause cells to become deformed and will break loose from the culture flasks. Cell cultures with visible contaminants, e.g. yeast, other bacteria, fungus or heavy mosquito debris, are re-tested by passing the original mosquito pool through a 0.22µM syringe filter.
4. Aseptically dispense 1-2 mLs of media from virus-positive cell cultures into labeled cryotubes using a serological pipette.
5. Virus cultures are stored at -80° C until identified using the appropriate diagnostic assays.

7. Preparation of reagents

1. MEM, 5% FBS. Dissolve 37.6 g of powdered MEM in a final volume of 4 L of dd H_20. Dispense solution into 500 mL aliquots in media bottles and autoclave. When solution has cooled, aseptically add 26 mL heat-inactivated fetal bovine serum, 5.2 mL L- Glutamine, 5.2 mL anti-biotic/mycotic, and 10.4 mL 7.5% sodium bicarbonate to each bottle. Store at 4° C.
2. PBS-G. Dissolve 16 g NaCl, 0.4 g KCl, 2.3 g Na_2HPO_4, 0.4 g KH_2PO_4, and 4 mL of 0.5% Phenol Red into 1000 mL dd H_20. Adjust pH to 7.1-7.4 with 2 N NaOH (if necessary). In a separate beaker, heat 600 mL of H_20 to a boil. Remove from hot-plate, add 10 g gelatin, and dissolve. Add dissolved gelatin solution to the PBS solution and adjust final volume to 2 L with dd H_2O. Dispense 100 mL of solution into bottles and autoclave. When solution has cooled, aseptically add 42.8 mL heat-inactivated rabbit serum and 1.4 mL anti-biotic/mycotic. Store at 4° C.
3. PBS cell wash. Add 50 mL 10X Dulbecco's PBS to 400 mL dd H_2O. Adjust pH to 7.1 with 2 N NaOH. Adjust final volume to 500 mL with dd H_2 O. Filter solution with 0.2uM vacuum filter unit. Dispense into 5 mL aliquots in 8 mL screw-cap tubes. Store at 4° C.

8. Representative Results

Nine different of viruses from 4 taxonomic families were recovered from mosquitoes collected during continuous statewide surveillance in Connecticut from 1997-2010 (table 1). Five of these viruses are known to cause human disease, the most important pathogens being West Nile virus and eastern equine encephalitis virus. New discoveries include: the first isolation of WNV from mosquitoes collected in North America in 1999[2], the first isolation of Potosi virus in northeastern US in 2000[3], and the first isolation of La Crosse virus in New England in 2005[4].

Virus	Family	No. isolates	No. locations	No. years detected	Human disease	Refs.
West Nile virus	Flaviviridae	1,002	94	12	Moderate to severe, fever, encephalitis	2,5
Eastern equine encephalitis virus	Togaviridae	292	41	10	Severe, encephalitis	6,7
Highlands J virus	Togaviridae	178	39	11	Not known	6
Jamestown Canyon virus	Bunyaviridae	305	74	14	Mild to moderate, fever, meningitis	8
Potosi virus	Bunyaviridae	259	76	4	Not known	3
Cache Valley virus	Bunyaviridae	114	51	8	Mild to severe, fever, neurological illness in two documented cases	
Trivittatus virus	Bunyaviridae	83	21	11	Not known	
La Crosse virus	Bunyaviridae	1	1	1	Moderate to severe, encephalitis	4
Flanders virus	Rhabdoviridae	4	4	2	Not known	

Table 1. Viruses detected in field-collected mosquitoes by Vero cell culture assay. Mosquitoes were collected during statewide surveillance in Connecticut from 1997-2010.

Discussion

Vero cell culture assay serves as an effective method to screen field-collected mosquitoes for a diversity of viruses. This contrasts to molecular methods that specifically target one or a few viruses of interest. Moreover, we have found that Vero cell culture assay is equally if not more sensitive than RT-PCR[7] and provides us with virus isolates that are stored in our reference collection for future studies. Nevertheless, cell culture systems may not be appropriate in many instances. This approach incurs additional expense and training required to grow viruses in a BSL-3 laboratory; however, these costs may be offset by relatively inexpensive supplies for cell culture. It is also worth noting that most but not all mosquito-borne viruses produce CPE in Vero cell culture[9,10]. Dengue virus is one notable exception that should be screened by another diagnostic test. Ideally, a combination of both molecular and cell culture methods are used to test for viral infection. We are currently using conventional and quantitative RT-PCR assays[4,11,12], sometimes in conjunction with sequencing, to identify viruses isolated in cell culture, and to reconfirm infection in the mosquito pool.

Disclosures

No conflicts of interest declared.

Acknowledgements

We gratefully acknowledge the important contributions of Drs. John Anderson, Andrew Main and Shirley Tirrell to this study. This work was supported in part by grants from the Centers for Disease Control and Prevention (U50/CCU116806-01-1) and the US Department of Agriculture (58-6615-1-218, CONH00768, and CONH00773).

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