Chapter 2

Isolation and Titration of Dengue Viruses by the Mosquito Inoculation Technique

Milly M. Choy and Duane J. Gubler

Abstract

Mosquito inoculation is a highly sensitive technique for isolation and titration of dengue virus (DENV) from sera, human tissues, wild animals, or mosquitoes. It has been underutilized since it was described 40 years ago because most dengue laboratories do not have access to an insectary to rear mosquitoes. This technique requires good eye-hand coordination while doing manipulation under a stereoscopic microscope, and extensive practice is needed to become proficient at inoculating mosquitoes. Following inoculation, mosquitoes are held for 10 days to allow dengue virus to replicate and disseminate to tissues throughout the mosquitoes. They are then harvested and examined for the presence of viral antigens in head tissue by either immunofluorescence assay (IFA) or PCR (polymerase chain reaction). The mosquito infectious dose 50 (MID₅₀) is calculated using the method of Reed and Muench to quantitate the virus. This method can be used for other arboviruses as well as for dengue.

Key words Dengue, Mosquito inoculation, 50 % end-point titration, Virus isolation, Virus titer, Arboviruses

1 Introduction

Dengue is a reemerging disease of global significance. The environmental changes associated with rapid population growth, unplanned urbanization, increased international air travel, and lack of effective mosquito control are considered to be the principal drivers for the geographic spread of both the viruses and their *Aedes* mosquito vectors [1, 2]. The public health impact of dengue is enormous, given that dengue virus (DENV) infection causes significantly more human disease than any other arboviruses. It is estimated that over 400 million infections, 100 million cases of symptomatic dengue infection, and over two million cases of dengue hemorrhagic fever/dengue shock syndrome occur worldwide annually [2, 3]. Other arboviruses such as Japanese encephalitis virus, West Nile virus, Zika virus, Ross River virus, and chikungunya virus have also emerged to cause major epidemics in recent years.

Table 1
Comparative dengue virus isolation rates using mosquito inoculation and C6/36 *Aedes albopictus* cell cultures, showing the influence of virus strains on isolation rates

Method of isolation	Aruba, 1985 DENV-1	%	Mozambique, 1985 DENV-3	%
Mosquito cells	13/29	45	2/28	7
Mosquito inoculation	14/29	48	5/28	18

DJ Gubler, 1985, unpublished data. *Note:* mosquito head squashes and cell cultures were screened for DENV by IFA [21]
The chi square values of the Aruba and Mozambique data are 0.5 and 2.67 respectively, both non-significant at 1 degree of freedom

DENV has been among the most difficult viruses to isolate and propagate because the viruses do not infect and replicate well in most laboratory animals. Studies on dengue in the 1940s to 1960s used suckling mice and various tissue culture systems for isolation and assay of DENV [4–8]. Although the use of mammalian and insect cell culture systems improved sensitivity [4, 6, 8], which is influenced by both the cell culture system and the strain of DENV, many unpassaged DENV do not produce cytopathic effects (CPE) when grown in these cells. The lack of a sensitive isolation and assay system that could be used for unpassaged wild-type viruses prevented rapid advancement in dengue research.

The development of the mosquito inoculation technique in the early 1970s provided a highly sensitive method for the isolation, propagation, and quantitation of DENV [9]. This method proved to be 10–1,000 times more sensitive in detecting dengue viruses than the commonly used plaque assay, depending on the serotype or strain of virus [9]. The influence of the virus strain is illustrated by the isolation rates of DENV from human sera in two 1985 epidemics in Aruba (DENV-1) ($\chi^2 = 0.5$, *ns*) and Mozambique (DENV-3) ($\chi^2 = 2.67$, *ns*) (Table 1). Both epidemics were explosive with severe disease and fatalities [10, 11], but isolation rates were very different. The isolation rate of DENV-1 was nearly identical by mosquito inoculation (48 %) and C6/36 *Aedes albopictus* cells (45 %), but for DENV-3 ($\chi^2 = 2.67$, *ns*), the isolation rate was much higher by mosquito inoculation (18 %) than by C6/36 cells (7 %). The higher sensitivity of mosquito inoculation is consistent regardless of the virus serotypes ($\chi^2 = 11.1$, $p < 0.001$) (Table 2). Of 193 patient sera screened by the two methods during the 1986 epidemic in Puerto Rico (DENV-1, DENV-2, and DENV-4), 106 (61.3 %) dengue cases were detected by mosquito inoculation compared to 93 (53.8 %) by C6/36 cell cultures. As DENV can be frequently recovered from serum in mosquitoes when the same serum tested negative in both mammalian and insect cell culture, the use of mosquitoes is especially important when attempting to propagate viruses with low replication efficiency from mildly symptomatic illness and to isolate viruses from sera, tissues of naturally infected humans, wild animals, or field-caught mosquitoes.

Table 2

Comparison of mosquito inoculation and C6/36 *Aedes albopictus* cell cultures for isolation of DENV-1, DENV-2, and DENV-4 from human sera during an epidemic in Puerto Rico, 1986^a

		Mosquito inoculation		
		Positive	Negative	Totals
Mosquito cell culture	Positive	93	0	93 (53.8 %)
	Negative	13	67	80 (46.2 %)
	Totals	106 (61.7 %)	67 (38.7 %)	173 (100 %)

^aDJ Gubler, 1986, unpublished data. *Note:* mosquito head squashes and cell cultures were screened for DENV by IFA [21]

The quantitation of virus in sera, human tissues, animals, mosquitoes, and cell culture is calculated using the method of Reed and Muench [12] and expressed as the dose that infects 50 % of the mosquitoes inoculated (MID₅₀). Using this method, many studies in the South Pacific islands, Indonesia, Thailand, Sri Lanka, and Puerto Rico showed a high rate of isolation of DENV from primary clinical samples. Equally important was the demonstration of considerable variation in viremia levels of different DENV strains and serotypes in patients showing a correlation with disease severity [13–19]. This technique has also been used as an assay for other arboviruses.

An accurate measure of infectious virus is critical to fully understand virus-host relationships, disease severity, viral fitness, and pathogenesis, as well as for development of effective diagnostic tests, vaccines, and therapeutics. The mosquito inoculation technique is arguably the most sensitive isolation and quantitative assay for infectious DENV in sera, autopsy tissues, mosquitoes, and in vitro systems. In this chapter, we describe the mosquito inoculation technique used to determine the MID₅₀ titer of DENV. Although the primary vector for dengue is *Aedes aegypti*, other species from the subgenus *Stegomyia* such as *Aedes albopictus* and *Aedes polynesiensis* as well as non-blood-feeding *Toxorhynchites* species can be used for this assay. Mosquitoes are reared in the laboratory under BSL-2 conditions as described previously [20].

The mosquito inoculation technique itself is technically easy, but requires good eye-hand coordination while doing the manipulation under a stereoscopic microscope. Extensive practice is required to ensure good survival of the inoculated mosquitoes. Once a person has become proficient at inoculating mosquitoes, the method becomes less labor intensive. A critical step of this proficiency is preparation of glass capillary needles, an often-overlooked step for ensuring good survival of mosquitoes after inoculation. Following inoculation, mosquitoes are placed in cartons inside security cages, provided a 10 % sucrose solution in a

locked incubator at 28 °C and held for 10 days to allow dengue virus to replicate, disseminate, and infect tissues throughout the mosquito. Surviving mosquitoes are then harvested and examined for the presence of viral antigens in head tissue by either immunofluorescence assay (IFA) [21] or polymerase chain reaction. IFA is usually used for DENV detection, as it is the most rapid and economical way to detect positive mosquitoes. Virus is quantitated by calculating the MID₅₀ using the method of Reed and Muench [12].

2 Materials

2.1 Preparation of Glass Capillary Needles for Mosquito Inoculation

1. 6-inch glass capillaries (1/0.75 OD/ID mm) (World Precision Instruments).
2. PC-10 puller (Narishge Co. Ltd)*.
3. Alcohol lamp.
4. Jeweler's forceps.
5. Stereoscopic microscope.

2.2 Mosquito Inoculation

1. Viral suspension.
2. Leibovitz's L-15 medium.
3. 3–5-day-old mosquitoes.
4. Glove box.
5. Inoculation apparatus.
 - (a) 30-ml glass syringe with Luer lock tip (Sigma-Aldrich)*.
 - (b) Plastic tubing.
 - (c) Three-way stopcock (Becton Dickinson)*.
 - (d) Brass steel needle holder with compression fittings (custom made, Sky Engineering Pte Ltd)*.
6. Glass capillary needles with markings.
7. Stereoscopic microscope.
8. Jeweler's forceps.
9. Pint holding cartons with lid (Science Supplies)*.
10. BugDorm Rearing Cage (Bioquip Product, Inc.)*.
11. Insect growth chamber (Darwin Chambers Co.)*.

2.3 Dengue Virus Detection in Mosquitoes by Indirect Immunofluorescence on Mosquito Head Squashes

1. 12-well (6 mm) teflon-coated microscope slide.
2. Razor blade.
3. Pellet pestle.
4. Flavivirus cross-reactive monoclonal antibody 4G2, HB112 (ATCC)*.

5. Sheep anti-mouse IgG FITC (Meridian Life Science)*.
6. 1× PBS.
7. Slide holder.
8. Glycerol mounting fluid.
9. Coverslips.
10. Fluorescence microscope.

*Mention of commercial products in this chapter is for information only; it does not imply recommendation or endorsement by the authors.

3 Methods

All procedures can be carried out in an air-conditioned BSL-2 laboratory unless otherwise specified.

3.1 Preparation of Glass Capillary Needles

1. Use the single-pull mode of a PC-10 puller to pull a glass capillary vertically. Set the heater level to approximately 55 °C to obtain a cone-shape tip (*see Note 1*).
2. Bend the glass capillary needle slightly over an alcohol lamp. Mark off the untapered portion of the needle under a stereoscopic microscope at 1.0 mm intervals with a thin marker and a ruler (*Fig. 1*).
3. Break off the tip of the needle at the appropriate diameter (approximately 0.5 µm), with a jeweler's forceps under a stereoscopic microscope (*see Note 2*).

3.2 Mosquito Inoculation of Dengue Viruses

3.2.1 Dengue Virus Isolation

1. Aspirate 50 3–5-day-old mosquitoes and hold them in tubes on ice for at least 30 min to anesthetize them. Separate the mosquitoes into males or females to be used for experiments into a 50-ml falcon tube (*see Note 3*).
2. Ensure all mosquitoes have been properly immobilized by holding them on ice throughout experimentation.

Fig. 1 Tip of glass needle used for inoculation. Each division is marked 1.0 mm apart

Fig. 2 Inoculation apparatus in use (a, b). Inoculation setup within a glove box (c)

Fig. 3 Needle holder holding "O" ring to obtain airtight seal

3. Set up the inoculation apparatus within a glove box (Fig. 2, *see Note 4*) and place a glass capillary needle in the needle holder (Fig. 3, *see Note 5*).
4. Load the needle with inoculum (from **Step 1**) by immersing the tip in the viral sample and withdrawing the plunger of the syringe (*see Note 6*).
5. Place 3–5 mosquitoes on a petri dish held on a tall stand under a stereoscopic microscope and position the mosquito on the dorsal aspect of its thorax.
6. Impale the mosquito on the underside of the neck (Fig. 4, *see Note 7*).

Fig. 4 Ventral aspect of head and neck of male mosquito with “X” showing preferred site of inoculation (Adapted from [9])

7. Bring the marked portion of the glass capillary needle into view under the microscope and observe the fluid meniscus when the plunger of the syringe is depressed until the desired amount has been inoculated (*see Note 8*).
8. Place mosquito within a holding carton after inoculation by using the wall of the carton or a jeweler’s forceps to dislodge the mosquito. Place the carton on ice to ensure that the mosquitoes are immobilized and contained. Block the exit of mosquitoes in the carton using cotton.
9. Inoculate 10–20 mosquitoes to form a virus seed pool.
10. Secure the holding carton with tape and store the cartons within a larger BugDorm Rearing Cage at the end of the experiment.
11. Dispose any unwanted or unused mosquito into 70 % ethanol in a 50-ml falcon tube. Place all experimental waste that came into contact with virus in a solution of Virkon for decontamination.
12. Incubate the mosquitoes at 28 °C and 80 % humidity for 10 days in an insect growth chamber. Allow mosquitoes access to 10 % sucrose and water.

3.2.2 Dengue Virus Titration

1. Perform tenfold serial dilutions (10^{-1} to 10^{-6}) of viral sample with Leibovitz’s L-15 medium. Place all viral samples on ice.
2. Perform mosquito inoculation as described (Subheading 3.2.1).
3. Inoculate enough mosquitoes so that at least 5 mosquitoes are available for testing each dilution of the viral sample.
4. Incubate the mosquitoes at 28 °C and 80 % humidity for 10 days in an insect growth chamber. Allow mosquitoes access to 10 % sucrose and water.

**3.3 Dengue Virus
Detection in
Mosquitoes by Indirect
Immunofluorescence
on Mosquito Head
Squashes**

1. After incubation for 10 days, harvest surviving mosquitoes and store at -80°C .
2. Label and number the wells of a 12-well (6 mm) teflon-coated microscope slide. Include a positive and a negative control on the slide.
3. Remove mosquitoes from the freezer and place them in a petri dish on an ice tray.
4. Remove the head of the mosquitoes using a razor blade or scalpel. Place one mosquito head per well (*see Note 9*).
5. Squash the mosquito head using a pellet pestle (*see Note 10*). Allow to dry before picking off excess chitin with jeweler's forceps.
6. Using a slide holder, submerge the slides in cold 80 % acetone. Allow cells to fix for 10 min at room temperature. Rinse once in PBS.
7. Let the slides air-dry completely. Place inside biosafety cabinet for faster drying.
8. Pipette 25 μl of flavivirus cross-reactive monoclonal antibody 4G2 (HB112) (diluted 1:10) onto each well (*see Note 11*).
9. Dampen paper towels to line a plastic incubation container.
10. Place the slides in the incubation container. Gently place the lid on the container and incubate at 37°C for 30 min.
11. Remove and rinse slides in staining dish with $1\times$ PBS. Incubate for 5 min using a magnetic stirrer.
12. Let slides air-dry.
13. Pipette 25 μl of anti-mouse IgG FITC onto each well.
14. Place the slides in the incubation container. Gently place the lid on the container and incubate at 37°C for 30 min.
15. Remove and rinse slides twice in staining dish with $1\times$ PBS. Incubate for 5 min using a magnetic stirrer.
16. Let slides air-dry completely.
17. Place a drop of mounting fluid onto the slide and gently lay down the coverslip.
18. View slides using a fluorescent microscope (*see Note 12*).
19. Record the number of infected mosquitoes for each dilution.
20. Calculate virus titers using the method of Reed and Muench and express the titers in terms of the dose that infects 50 % of the mosquitoes inoculated (MID_{50} per ml).

4 Notes

1. The needles can be pulled using a simple alcohol lamp if a needle puller is not available.
2. The tip of the needle is to be made as small as possible and yet large enough to minimize plugging with debris contained in the inocula. A small tip is advantageous both for ease in piercing the exoskeleton of the mosquito and in controlling the amount of inoculum. It is also advantageous to make the tapered portion of the needle as short as possible for better control during inoculation.
3. Male mosquitoes are used for titration experiments as they offer a significant advantage in safety since they cannot transmit the infection should they escape in the laboratory. Although male mosquitoes are as sensitive as females to dengue infection, they are less able to tolerate larger inocula and are less resistant to toxic effects of certain undiluted human sera. Female mosquitoes are only used when dealing with large inocula of up to 1 μ l or inoculation of undiluted human sera.
4. Escape of infected mosquito within the insectary is prevented by handling them in a glove box with glove ports to allow access to the containment area. The needle holder is attached by a plastic tubing to a 30-ml syringe held by the base of a tall stand. The tubing is attached to the syringe by a three-way stopcock, which allows the plunger of the syringe to be manipulated when necessary, without changing the pressure in the plastic tubing.
5. Airtight seal of the needle to the brass needle holder is made via the use of a compression fitting. The holders are not available commercially but can be made by most machine shops.
6. Start from the highest to the lowest dilution so that the same needle can be used. Change a needle after each viral sample.
7. It is also possible to inoculate female mosquitoes through the membranous area anterior to the mesepisternum and below the spiracle without compromising the survival rate of the mosquitoes after inoculation (Fig. 5).
8. A distance of 1 mm on the markings of the glass needle is normally recommended for inoculation (0.4 μ l). After inoculation, it is necessary to check that all inoculum has entered the insect and transfixation has not occurred.
9. Test at least 5 or more mosquitoes for dengue infection at each dilution of a viral sample. It is always a good practice to store the remainder of the labeled mosquito in a 1.5-ml Eppendorf tube at -80°C for future testing and possible virus isolation.

Fig. 5 Lateral aspect of thorax of female mosquito with showing the preferred site of inoculation (Adapted from [9])

Fig. 6 Example of a positive head squash (a) and a negative head squash (b)

10. Alternatively, one can coat a coverslip with Rain-X. Allow the coverslip to dry. Place the coverslip over the 12-well microscope slide with mosquito heads on them. Use the end of a pencil to squash the heads.
11. Primary antibodies specific for each dengue serotypes can also be used depending on the experiment.
12. Care should be taken in interpreting whether or not a head squash is positive. The preparation typically has a lot of tissue/chitin that autofluoresces. A distinctive positive squash should contain some doughnut-shaped cells (Fig. 6).

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