

Propagation of Dengue Virus in Inoculated Mosquitoes

Principal Investigators : William H. Bancroft, LTC, MC
Suwana Vaithanomsat, B. Sc.
Douglas J. Gould, Ph. D.
Robert McNair Scott, MAJ, MC

Associate Investigators : Douglas R. Stutz, MAJ, MSC

OBJECTIVE: The purpose of this study was to establish a method for infecting mosquitoes with dengue virus to use in future studies of viremia in hemorrhagic fever patients.

BACKGROUND: Tissue culture methods of isolating and identifying dengue viruses are exacting, time consuming and expensive. Rosen⁽¹⁾ has developed a mosquito assay technique which does not require tissue culture and which may be even more sensitive than currently used tissue culture methods. An effort is being made to establish this assay method at SMRL to permit a prospective controlled study of both dengue isolation systems during the next hemorrhagic fever season.

DESCRIPTION: Colony-raised mosquitoes were obtained from the Department of Entomology, SMRL. All manipulation of live mosquitoes was done within a screened cubicle. Experiments used *Aedes aegypti* and *Armigares subalbatus* species. Whenever possible only male mosquitoes were used.

Seed viruses stored at -70°C were thawed and diluted in Medium 199 with 20% heat-inactivated fetal bovine serum, pH 8.2. Each virus dilution was prepared just before use and held in an ice bath.

The inoculating needle was made from a glass capillary, 0.7—1.0 mm outside diameter, graduated into 1 mm segments, drawn to a sharp tip over an alcohol flame and sterilized in an autoclave. For inoculation, a capillary was held in a stainless steel holder connected to plastic tubing leading to a 3 way stop-cock and a 30 ml glass syringe. The capillary needle was filled by submerging the point in a virus solution and carefully drawing on the syringe. The volume in the capillary was precisely controlled with the syringe plunger.

Prior to inoculation, each mosquito was held in an individual cotton-stoppered test tube. The tube was immersed in a beaker of wet ice for 5 to 10 minutes to anesthetize the mosquito. The immobilized mosquito was placed in a 100 × 15 mm petri dish on the stage of a dissecting microscope. The inoculating capillary was inserted into the thorax through the suture just anterior to the sternopleuron and below the first thoracic spiracle. After the needle was introduced, the mosquito was positioned so the fluid level in the needle could be observed through the microscope. The inoculum was the amount of fluid in a 2 mm length of the needle, approximately 0.0013 ml.

Mosquitoes inoculated with the same dilution of the same virus were placed in a gauze-covered paper cup containing 5% sucrose and held for 7 to 10 days at 27°C , 85% R.H. Fresh sugar pads were provided daily. Starting 1—2 hours after inoculation, records were taken daily of the number of surviving mosquitoes.

For virus isolation in tissue culture, the surviving mosquitoes were killed by freezing on day 8. Mosquitoes used for indirect fluorescent antibody staining were killed on day 10. Virus isolation in LLC—MK2 cells used the direct plaque technique.

Slides for indirect fluorescent antibody (IFA) staining were cleaned with ethyl alcohol. The head was cut from each mosquito to be tested and squashed on a slide under a coverslip precoated with 1% silicon solution (Siliclad). The coverslip was then discarded and the head preparation allowed to dry in air. After removing any excess chitinous material, the remaining tissue was fixed in cold acetone (4°C) for 10

minutes and dried in air. The slide was flooded with dengue hyperimmune mouse ascitic fluid diluted 1:10 in phosphate buffered saline (PBS) and incubated at 37°C for 45 minutes in a moist chamber. The slide was washed twice for 5 minutes with PBS and dried with a cold air blower. One drop of fluorescein conjugated anti-mouse gamma globulin (Antibodies Incorporated) diluted 1:10 in PBS was added and allowed to incubate at 37°C for 45 minutes. The slide was washed twice with PBS and mounted in buffered glycerol (1 part PBS to 9 parts glycerol) before being examined under a fluorescent microscope.

Initial studies were directed at determining the survival of mosquitoes after inoculation, the optimal age of mosquitoes and the susceptibility of a larger species, *Armigeres subalbatus*. In addition the titers of virus passed through mosquitoes were compared to those of the original seeds.

PROGRESS: Mosquito mortality was evaluated by comparing early and late survivors. Early survivors were the mosquitoes that were alive 24 hours after inoculation. Late survivors were alive 7 days after inoculation. Death during the first 24 hours was assumed to be due to trauma inflicted by inoculation. Later deaths were due to trauma, infection, advanced age or other reasons.

It was found that with experience in inoculation the percentage of early and late survivors increased. (Table 1)

Table 1. Effect of Experience at Inoculating *A. aegypti* Mosquitoes on the Numbers of Early and Late Survivors 1973-1974

Month	No. Inoculated	Early Survivors		Late Survivors	
		No.	(%)	No.	(%)
September	480	316	(66)	258	(54)
October	332	195	(59)	168	(51)
November	299	232	(78)	187	(63)
January	359	249	(69)	230	(64)
February	389	296	(76)	271	(70)
Combined	1,859	1,288	(69)	1,114	(60)

Mosquitoes that were 3 to 5 days old at the time of inoculation survived somewhat better than those younger or older (Table 2); however, mosquitoes up to 13 days old were used successfully. The age of mosquito seems to have little bearing on the success of inoculation.

An attempt was made to improve mosquito survival by trying to infect a larger species. Two hundred fifty-five *Armigeres subalbatus* were inoculated with the same volume as the *A. aegypti*. Survival of *Armigeres* was 85% at day 1 and 70% on day 7 compared to 69% and 60% respectively for *A. aegypti*. Virus was not isolated from any *Armigeres*, so these mosquitoes cannot be used to detect dengue.

The direct plaque titers in tissue culture for viruses passed through mosquitoes were usually within one log of the original seed viruses (Table 3). By calculating the amount of virus actually inoculated into the mosquitoes it was found that the virus multiplied 0.1 to 8,000 times in the insect host. No conclusions can be drawn on the relative sensitivity of the two methods, but the results do suggest that the mosquito inoculation method of virus isolation may detect low concentrations of virus in human sera that might otherwise be missed.

The IFA test gave excellent fluorescence in infected LLC-MK2 cells.

Table 2. Survival of *A. aegypti* Mosquitoes of Different Ages

Age at Inoculation (Days)	No. Inoculated	Early Survivors		Late Survivors	
		No.	(%)	No.	(%)
1	45	25	(56)	24	(53)
2	135	102	(76)	88	(65)
3	233	219	(94)	196	(84)
4	345	244	(71)	193	(56)
5	194	167	(86)	150	(77)
6	216	126	(58)	117	(54)
7	60	39	(65)	29	(48)
8	254	170	(67)	153	(60)
9	75	62	(83)	49	(65)
11	75	59	(79)	55	(73)
13	60	45	(75)	38	(63)
19	48	6	(13)	0	(0)
20	59	24	(41)	22	(37)

Table 3. Multiplication of Viruses in *A. aegypti* Mosquitoes

Virus Strain	Mosquito Pool		Seed Virus Titer (LLC-MK2)	Virus Multiplication Factor (VMF)*
	No. Mosq.	Titer (LLC-MK2)		
Dengue 1				
CH 53432 MK 2-5	10	77 x 10 ⁻³	1 x 10 ⁻²	178
CH 53432 MK 2-5	10	10 x 10 ⁻³	4 x 10 ⁻³	58
CH 53432 <i>A. albo.</i>	4	9 x 10 ⁻¹	4 x 10 ⁻¹	130
Dengue 2				
CH 53544 MK 2-2	9	40 x 10 ⁻⁴	4.4 x 10 ⁻⁴	231
CH 53544 MK 2-3	10	2 x 10 ⁻⁴	12 x 10 ⁻⁴	4
CH 53544 MK 2-3	4	6 x 10 ⁻⁴	37 x 10 ⁻²	0.09
CH 53544 MK 2-3	5	10 x 10 ⁻⁴	4 x 10 ⁻⁴	116
CH 53974 MK 2-3	2	109 x 10 ⁻⁵	2 x 10 ⁻⁵	6295
Dengue 3				
CH 53489 MK 2-3	14	15 x 10 ⁻⁴	1 x 10 ⁻³	25
CH 53489 MK 2-3	5	2 x 10 ⁻¹	4 x 10 ⁻²	231
CH 53875 MK 2-1	11	4 x 10 ⁻⁴	1 x 10 ⁻⁴	84
CH 53875 MK 2-1	3	49 x 10 ⁻⁴	4 x 10 ⁻³	94
Dengue 4				
12 M 374 MK 2-5	1	3 x 10 ⁻⁴	2 x 10 ⁻⁴	347
12 M 374 MK 2-6	5	8 x 10 ⁻⁴	1 x 10 ⁻³	37
Chikungunya				
SM 184	7	25 x 10 ⁻⁴	1 x 10 ⁻⁵	8247

$$* \text{VMF} = \frac{\text{Titer of mosquito pool}}{\text{No. mosquitoes}} \times \frac{1}{\text{Titer of seed virus}} \times \frac{0.3\text{ml}}{0.0013\text{ml}}$$

The failure to find fluorescence in the mosquito heads may be due to (1) inadequate removal of auto-fluorescing chitinous debris and/or (2) absence of salivary gland material from the prothorax. A study is being made of these possibilities. In addition, direct fluorescent antibody testing will be tried in an effort to more closely follow the Rosen technique. Plans are being made to determine the relative sensitivity of the two methods of virus isolation for detecting viremia in hemorrhagic fever patients.

REFERENCE:

1. Rosen, L.: Personal communication.