

Evaluation of *Toxorhynchites splendens*
(Diptera: Culicidae) as a Bioassay
Host for Dengue Viruses

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OBJECTIVES : To develop a technique for isolating dengue viruses employing *Toxorhynchites splendens* and the direct fluorescent antibody assay.

BACKGROUND : This is a continuation of an investigation that was initiated during 1977 to develop and evaluate the mosquito assay technique for detecting and isolating dengue viruses (1). Previous studies showed *Aedes aegypti* and *Toxorhynchites splendens* to be equally susceptible to dengue virus infection following intrathoracic inoculation. Additional findings, however, indicated that, of the two species, *T. splendens* was a more suitable host. The mortality rate following inoculation of *T. splendens* was substantially lower than that observed for *Ae. aegypti* and the large size of *T. splendens* as compared to *Ae. aegypti* allowed for a 5 fold increase in the volume of inoculum. Thus the number of mosquitoes required to be inoculated per specimen was reduced by 50%. In addition, the virus yield per individual mosquito was consistently higher for *T. splendens* than for *Ae. aegypti*. The only disadvantage of using *T. splendens* was the additional time and personnel that were required to mass rear this species as compared to *Ae. aegypti*.

The specific objective of this study was to determine the pattern of replication of different dengue virus serotypes and/or strains and to determine the earliest post-inoculation day that dengue virus antigen could be detected in the heads of mosquitoes by the direct fluorescent antibody technique.

MATERIALS AND METHODS : The dengue virus serotypes and strains employed are presented in Table 1. Eight or more *T. splendens* were inoculated intrathoracically with each virus dilution, 0.85 ul per mosquito. Viruses were diluted in RPMI 1640 medium that was supplemented with heat inactivated fetal calf serum (FCS) to a final concentration of 10%, 200 units of penicillin/ml, and 150 ug of streptomycin/ml. After various time (days) intervals at 32°C, five or more mosquitoes were stored at -70°C for virus assay. Tissue smears of individual mosquito heads were prepared and examined for virus antigens by the direct fluorescent antibody technique.

The corresponding thorax-abdomen tagmata for the mosquito heads were triturated individually by sonic energy in the presence of 1.5 ml of RPMI 1640

medium, 10% FCS, 500 units of penicillin/ml and 50 ug of streptomycin/ml. Resultant suspensions were centrifuged for 30 minutes at 10,000 rpms in a 4°C centrifuge. Log₁₀ dilutions were prepared of each suspension and assayed for virus in LLC-Mk2 cells by the direct plaque assay technique. Control mosquitoes that received virus diluting medium were assayed as described above. Methods used to assay mosquitoes by the direct fluorescent antibody and by the direct plaque assay for dengue viruses are presented in the Annual Progress Report, AFRIMS, 1977-1978, pp.

RESULTS : The quantity of dengue virus recovered from individual mosquitoes following intrathoracic inoculation is presented in Tables 2, 3, 4 and 5. As shown in these tables, dengue virus antigen could not be detected in mosquito heads until or after virus levels had reached maximum titers. An incubation period of 9 days or less was required for the detection of virus antigen in heads of all mosquitoes inoculated with dengue 1 and 2 serotypes. No difference was noted in the time required for dengue virus antigen to appear in mosquito heads for the 2 strains of dengue-2 virus.

The time required for the appearance of virus antigen in heads of mosquitoes inoculated with dengue-3 and dengue-4 viruses ranged from 9 to 12 days. Of the two dengue-3 virus strains, antigen was detected in 3 of 4 mosquito heads for virus #2797 on day 9 post inoculation, while antigen could not be detected after the same incubation period in heads of mosquitoes that were inoculated with virus #2877. In contrast to dengue virus serotypes 1 and 2, the data indicate that dengue-3 and dengue-4 virus infected *T. splendens* should be retained for 12 or more days in order to insure the presence of virus antigen in the mosquito heads. Figures 1-5 show the perinuclear fluorescence in ganglia cells of *T. splendens* infected with dengue-1, 2, 3 and 4 viruses and the absence of fluorescence in cells of mosquito head that was not inoculated with dengue viruses.

The results of a study conducted during 1978 to compare *T. splendens* with LLC-Mk2 cells for isolating dengue viruses from clinically diagnosed dengue hemorrhagic fever patients are presented in another section of this Annual Progress Report. This is a final report, as the development of the mosquito assay for dengue viruses employing *T. splendens* has been accomplished.

REFERENCES :

1. Watts, D.M., Scott, R.M., Nisalak, A., and Andre, R.A. 1977. Evaluation of *Toxorhynchites splendens* as a Bioassay Host for Isolating dengue viruses. AFRIMS Annual Progress Report. pp. 63-66.
2. Watts, D.M., Nisalak, A., Burke, D.S. and Nimmamitya, S. Comparison of the mosquito inoculation technique with cell culture for isolating dengue viruses from dengue patients.

Table 1. Dengue Viruses Used in Investigations to Evaluate *Toxorhynchites splendens* as a Bioassay Host for these Viruses.

Dengue virus serotype	Origin/Date	Host/No. of passages
Den-1(001)	Human/1975	Mouse/5*
Den-2(3379)	Human/1974	Mouse/5
Den-2(189-18A)	<i>A. aegypti</i> /1978	<i>T. splendens</i> /1
Den-3(2797)	Human/1977	Mouse/5
Den-3(2877)	Human/1977	Mouse/5
Den-4(050)	Human/1977	Mouse/5

* Stock virus prepared from infected suckling mouse brain.

Table 2. Concentrations of Dengue Virus Type-1 in Thorax-abdomen Tagmata of *Toxorhynchites splendens* Determined by Cell Culture Assay and Detection of Virus Specific Fluorescence in the Heads of the Same Mosquitoes Following Intrathoracic Inoculation.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	2.5%	4.8	1.8	3.0	1.0	3.0
	3.1	4.3	2.2	2.5	2.5	2.5
	3.5	4.1	1.5	2.0	2.0	1.9
	2.8	4.0	1.6	2.5	2.7	2.5
Heads	0/4**	1/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* \log_{10} PFU/1.p ml, dengue-1 (001) per individual thorax-abdomen tagmata of *T. splendens*.

** Number positive/number assayed by head squash.

*** Not inoculated.

Table 3. Concentration of Dengue Virus Type 2 Strains in Thorax-abdomen Tagmata of *Toxorhynchites splendens* Determined by Cell Culture Assay and Detection of Virus Specific Fluorescence in the Heads of the Same Mosquitoes Following Intrathoracic Inoculation.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	1.3	4.8	5.6	3.8	3.5	5.0
	0.5	4.5	5.5	4.8	3.5	4.2
	2.0	5.6	4.8	4.5	3.5	4.2
	2.5	5.3	4.5	3.8	2.8	2.8
Heads	0/4**	0/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-2 (189-18A) per individual thorax-abdomen tagmata of *T. splendens*.

** Number positive/number assayed by head squash.

*** Not inoculated.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	4.5*	5.8	5.5	5.1	4.5	5.1
	4.2	5.7	5.5	4.8	5.1	5.2
	4.0	6.2	5.3	5.5	4.8	5.6
	4.1	6.4	5.5	5.3	5.8	5.1
Heads	0/3**	0/4	4/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-2 (3379) per individual Thorax-abdomen tagmata to *T. splendens*.

** Number positive/number assayed by head squash.

*** Not inoculated.

Table 4. Concentrations of Dengue Virus Type-3 Strains in Thorax-abdomen Tagmata of *Toxorhynchites splendens* Determined by Cell Cultures Assay and Detection of Virus Specific Fluorescence in the Head of the Same Mosquitoes Following Intrathoracic Inoculation.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	0.0	4.5	1.8	3.8	2.5	2.5
	2.0*	4.2	1.8	2.5	3.5	2.5
	2.5	3.9	4.0	3.8	2.5	4.0
	0.5	3.5	2.5	3.3	2.8	2.8
Heads	0/4**	0/4	3/4	3/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-3 (2797) per individual thorax-abdomen tagmata of *T. splendens*.

** Number positive/number assayed by head squash.

*** Not inoculated.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	3.5*	1.5	4.0	4.0	4.0	2.5
	2.5	2.8	4.5	3.1	3.2	4.8
	2.0	0.7	4.2	3.3	ND**	2.8
	3.0	2.0	4.3	2.5	ND	3.8
Heads	0/4***	0/4	0/4	4/4	4/4	4/4
Controls****	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-3 (2877) per individual thorax-abdomen tagmata of *T. splendens*.

** Not done.

*** Number positive/number assayed by head squash.

**** Not inoculated.

Table 5. Concentrations of Dengue Virus Type 4 in Thorax-abdomen Tagmata of *Toxorhynchites splendens* Determined by Cell Culture Assay and Detection of Virus Species Fluorescence in the Head of the Same Mosquitoes Following Intrathoracic Inoculation.

<i>T. splendens</i>	Days after inoculation					
	3	6	9	12	15	18
Thorax-abdomens	3.8* 4.3 3.5 3.8	4.7 5.1 4.7 4.6	2.3 2.5 2.5 2.6	3.0 4.1 3.8 3.8	2.5 3.8 3.8 3.3	4.1 5.0 4.1 3.5
Heads	0/4**	0/4	2/4	4/4	4/4	4/4
Controls***	0/4	0/4	0/4	0/4	0/4	0/4

* Log_{10} PFU/1.0 ml, dengue-4 (050) per individual thorax-abdomen tagmata of *T. splendens*.

** Number positive/number assayed by head squash

*** Not inoculated.

Figure 1. Perinuclear Fluorescence in Ganglia Cells of *Toxorhynchites splendens* Inoculated with Dengue Virus Type 1.

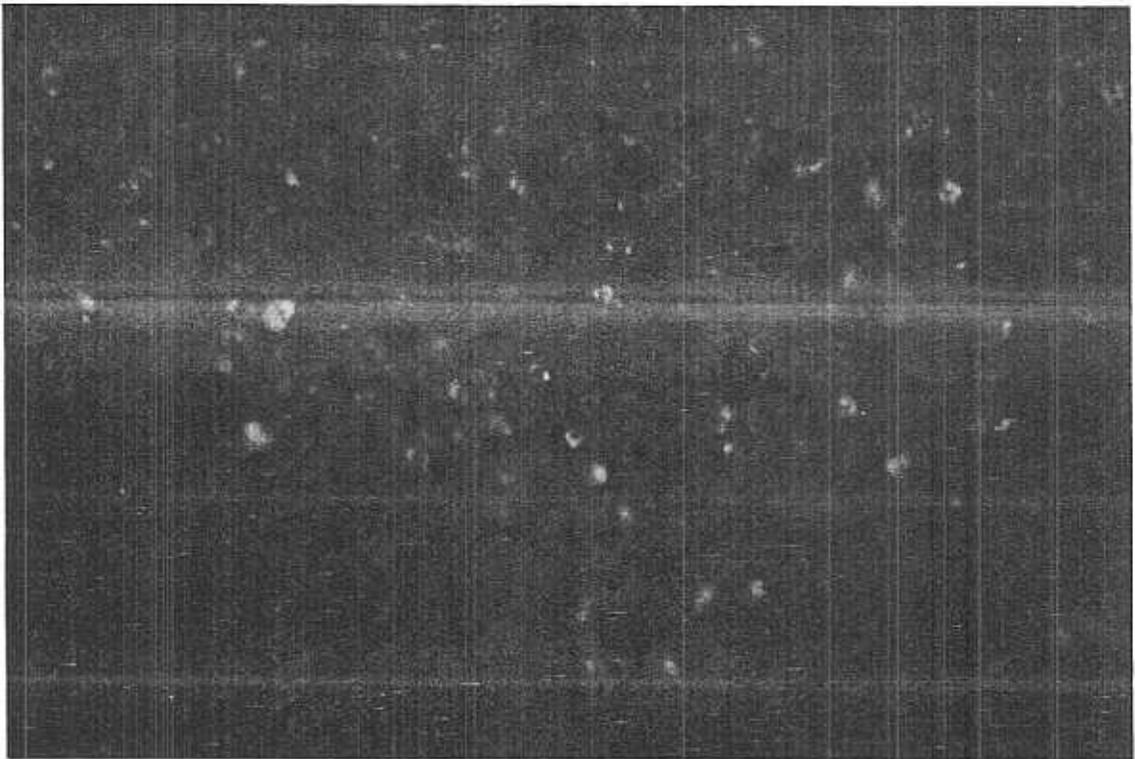


Figure 2. Perinuclear Fluorescence in Ganglia Cells of *Toxorhynchites splendens* Inoculated with Dengue Virus Type 2.

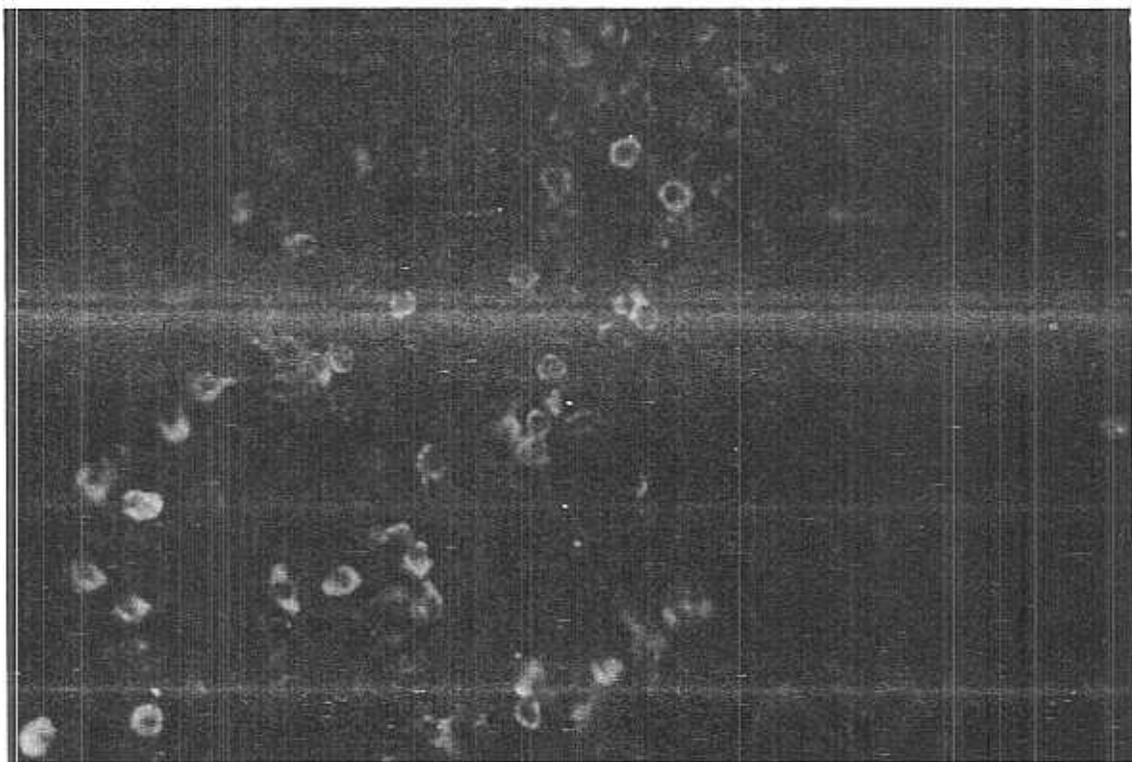


Figure 3. Perinuclear Fluorescence in Ganglia Cells of *Toxorhynchites splendens* Inoculated with Dengue Virus Type 3.

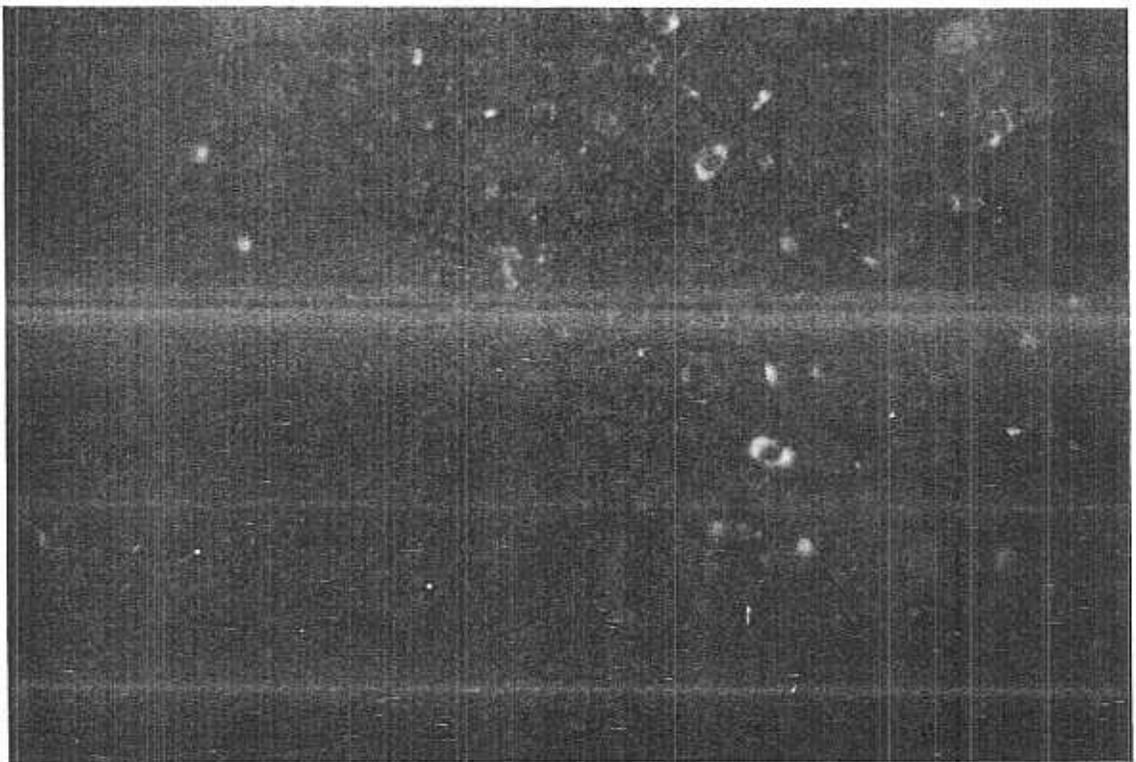


Figure 4. Perinuclear Fluorescence in Ganglia Cells of *Toxorhynchites splendens* Inoculated with Dengue Virus Type 4.

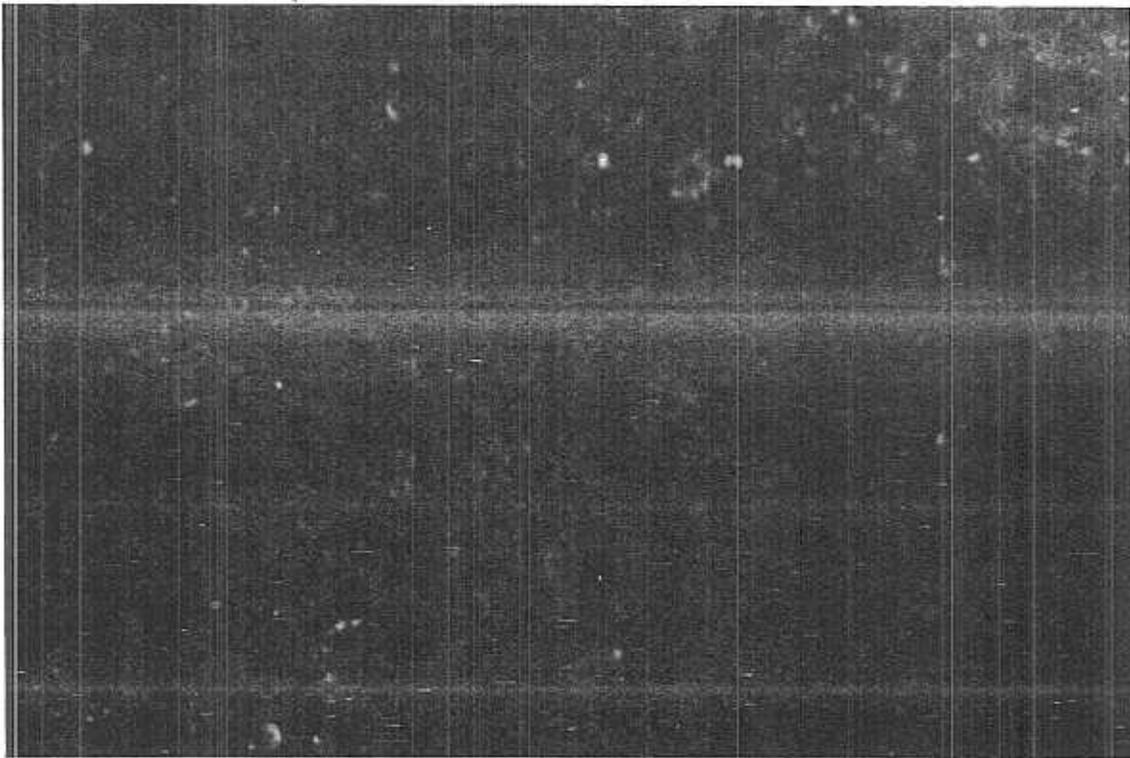


Figure Absence of Perinuclear Fluorescence in Ganglia Cells
Toxorhynchites splendens that was no Inoculated with
Dengue Viruses

