

Evaluation of *Toxorhynchites splenens* as a Bioassay Host for Isolating Dengue Viruses

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OBJECTIVE : To evaluate *Toxorhynchites splenens* as a bioassay host for detecting and replicating dengue viruses.

BACKGROUND : Investigations employing a laboratory colony of *Aedes aegypti* as a bioassay host for dengue viruses have yielded inconsistent results. Available data suggested that the susceptibility to dengue virus infection varied for individual mosquitoes. Pertinent findings include the failure to detect dengue viruses in all *A. aegypti* that were inoculated with $10^{6.0}$ PFU/0.3 ml following a 14 day incubation period. Variation in susceptibility was observed more frequently for dengue 1, 3 and 4 than for dengue 2 virus. In addition, the low infectivity yields associated with mosquitoes after being inoculated with high concentrations of dengue viruses and with dengue virus infected leukocytes failed to provide evidence of virus amplification. These findings were based on plaque assay, complement fixation and fluorescent antibody assay of mosquitoes. This report consists of the preliminary findings of an investigation initiated to evaluate the mosquito bioassay system employing *T. splenens*.

MATERIALS & METHODS : Mosquitoes were obtained from a laboratory colony of *T. splenens* that was established during July 1976 in the Dept of Entomology, AFRIMS. *Culex quinquefasciatus* larvae were provided continuously to *T. splenens* larvae and the diet for the adults consisted of honey. Dengue viruses employed were mouse seeds including type 1, (Hawaiian strain, 16th passage), type 2, (New Guinea c, 29th passage), type 3, (H87, 25th passage), and type 4, (H241, 31st passage). The infectivity titer in LLC-Mk₂ cells were $1.5 \times 10^{6.0}$, $1.9 \times 10^{6.0}$, $3.1 \times 10^{6.0}$ and $1.7 \times 10^{6.0}$ PFU per 0.3 ml for dengue virus type 1, 2, 3 and 4, respectively.

Mosquitoes were inoculated with \log_{10} dilutions of each dengue virus serotype as described by Rosen and Gubler (1). Virus dilutions were prepared in RPMI 1640 medium, and the volume inoculated was 0.85 μ l per mosquito. After a 14 day incubation period at 32°C, mosquitoes were sacrificed for virus assay employing fluorescent antibody (FA) and complement fixation (CF) tests. The FA and CF tests were performed according to methods of Kuberski and Rosen (2, 3). Pooled human dengue virus antisera were labeled with fluorescent isothiocyanate (FITC) as described in the 1975-1976 SEATO Annual Progress Report. Tissue imprints resulting from squashing mosquito heads were flooded with FITC labeled antisera and examined with the 10x objective of a Leitz fluorescent microscope.

The CF antigens were prepared by placing 2 *T. splenens* (thorax-abdomen) in 1.0 ml of chilled buffered saline and disintegrating them with sonic energy employing a S110 model sonifier. The suspension was centrifuged at 3,300 rpm for 15 minutes at 4°C, and the supernatant fluid was used as the CF antigen. Dengue virus hyperimmune antisera used in CF tests were prepared according to methods of Brandt et al. (4). Uninfected mosquitoes were employed as controls in both the FA and CF test. FA observations were made by 2 or more observer.

RESULTS : The data of this study indicate that *T. splenens* was susceptible to infection on parenteral inoculation with dengue viruses. Susceptibility to infection appeared to be lower for dengue 3 and 4 viruses, especially the latter. In addition, the intensity of fluorescence and the CF titers were less for mosquitoes inoculated with dengue 3 and 4 viruses. Similar results have been reported for *Aedes albopictus* and dengue virus (3).

The low CF titers associated with dengue 4 inoculated mosquitoes that failed to yield positive FA results cannot be explained on the basis of data obtained in this study. Evidence based, however, on the low CF titers (1:4 to 1:8) observed for uninfected control mosquitoes suggests that the mosquitoes may not have been infected. Further investigations employing infectivity assays in conjunction with FA tests will be required to address this question.

In regard to the use of CF tests for identifying dengue viruses, the data of the study indicate that dengue 3 and 4 viruses will require additional studies. Such studies will need to consider virus passage level and the specificity of the antisera.

Specific fluorescence was observed in head squashes of *T. splenens* following the inoculation of this mosquito with different concentrations of dengue 1, 2, 3 and 4 viruses (Table 1). The most characteristic form of specific fluorescence was the appearance of rings of fluorescence which were observed in conjunction with well defined areas of background fluorescence. The amount of tissue exhibiting fluorescence and the intensity of fluorescence was substantially lower in mosquitoes inoculated with dengue 3 and 4 viruses. Non-specific fluorescence was observed in tissue smears prepared from control and infected mosquitoes. The more prevalent form was small fluorescent granules that appeared to be associated with the surface, as opposed to being within the mosquito tissue.

After removing the head of *T. splenens* for FA assay, the thorax and abdomen portions of the mosquito were employed as antigens for virus identification studies in CF tests. As shown in Table 2, dengue 1 and 2 viruses could be identified; however, this was not possible for dengue 3 and 4 viruses. CF titers for the latter viruses were also much lower than those observed for dengue 1 and 2 viruses. On the basis of data of this study and of previous findings mentioned regarding *A. aegypti*, the mosquito assay system cannot at this point be considered to be a reliable technique for isolating and identifying dengue viruses. Further studies to perfect the mosquito inoculation technique are underway.

REFERENCES :

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3. Kuberski, T.T., and Rosen, L., 1977. A Simple Technique for the Detection of Dengue Antigen in Mosquitoes by Immunofluorescence. Am. J. Trop. Med. Hyg., 26:533-537.
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Table 1. Specific Fluorescence Detected in Head Tissue of *Toxorhynchites splendens* on Day 14 Following Inoculation with Log₁₀ Dilutions of Dengue Viruses

Virus	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Dengue-1	2/3 ^a	4/4	4/4	4/4	4/4	1/4	0/4
Dengue-2	4/4	4/4	4/4	4/4	4/4	2/4	0/4
Dengue-3	0/2	2/4	4/4	2/4	3/4	0/4	^b
Dengue-3	1/3	3/3	3/3	2/2	1/3	0/3	
Dengue-4	2/4	1/4	0/4	0/4	0/4	0/4	
Dengue-4	0/3	0/4	2/4	2/4	1/3	0/4	
Controls	0/6	0/6	0/6	0/6	0/6	0/6	

^a No. fluorescence positive/no. fluorescence negative mosquitoes

^b Not tested

Table 2. Complement Fixing Titers of Dengue Virus Hyperimmune Antisera with Antigens Prepared from *Toxorhynchites splenens* Infected with Dengue Viruses.

Virus	Antigen Dilution of Inoculum	Dengue Virus Antisera			
		D-1	D-2	D-3	D-4
Dengue-1	10 ⁻¹	512 ^a	128	16	0
	10 ⁻²	1024	256	8	0
	10 ⁻³	512	8	8	0
	10 ⁻⁴	512	32	32	0
			2048	256	16
Dengue-2	10 ⁻¹	128	256	16	32
	10 ⁻³	64	256	16	4
	10 ⁻⁵	128	1024	32	8
Dengue-3	10 ⁻¹	32	32	16	16
	10 ⁻³	32	16	8	8
	10 ⁻⁵	16	16	16	16
Dengue-3	10 ⁻¹	64	16	16	16
	10 ⁻²	32	32	32	32
	10 ⁻³	16	16	16	8
Dengue-4	10 ⁻¹	16	2	4	16
	10 ⁻²	8	8	2	16
	10 ⁻³	16	8	2	8
		8	8	2	4

^a Reciprocal complement fixation titers.