

Evaluation of *Aedes aegypti*, *Armigeres subalbatus*,  
and *Toxorhynchites splendens* as Bioassay  
Hosts for Dengue Viruses

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OBJECTIVES :

1. To determine the feasibility and effectiveness of employing *Toxorhynchites splendens* as a bioassay host for dengue viruses.
2. To compare *Aedes aegypti* and *Armigeres subalbatus* to *Toxorhynchites splendens* for detection and propagation of dengue viruses.

BACKGROUND : The mosquito species *A. albopictus* has been reported to be a more sensitive bioassay host than conventional cell culture techniques for detecting and propagating dengue viruses (1). Studies conducted at AFRIMS to develop the mosquito assay employing *A. aegypti* have yielded inconsistent results. During 1977, investigation was initiated to assess *T. splendens* as an alternative bioassay host for dengue virus serotypes. The large size of *T. splendens* allows for an increase in the volume of inoculum from .17 U/l for individual *A. aegypti* to .85 U/l for the former species. However, the exceptionally long development period of *T. splendens* has posed a question regarding the feasibility of producing adequate numbers for employment as a bioassay host for dengue viruses.

METHODS : Male *A. aegypti* were obtained from colonies established and maintained in the Department of Entomology, AFRIMS. The new colony, 10th to 12th generation, was established from larvae collected in Bangkok during August and September, 1977. The old colony, unknown generation, originated from Samui Island, Thailand, June-July, 1968. The *T. splendens* colony, unknown generation, was established from larvae that were collected in Bangkok during July, 1976. Male *Armigeres subalbatus*, unknown generation, were obtained from a colony established during 1966 from specimens provided by the U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia.

Mosquitoes of each species were 3 to 5 days old when employed in experiments. *A. aegypti* and *Ar. subalbatus* were reared and maintained according to standard laboratory procedures. *A. aegypti* larvae were provided continuously to *T. splendens* larvae and the diet for adults was honey. The procedures for rearing and maintaining *T. splendens* were modified in an attempt to increase the

yield of this species. The oviposition substrate was changed from bamboo cups to photography trays having a black interior. First instar larvae were transferred to individual 3 dm. vials instead of waiting to the third instar stage of development to avoid losses due to cannibalism. Currently, *T. splendens* are being maintained in 45 x 45 x 45 cm. cages; however, more recent observations suggest that this species will reproduce in smaller cages. The latter is being evaluated in order to increase rearing space.

The origins and passage levels of dengue stock viruses employed are listed in Table 1. Mosquitoes were immobilized and inoculated intrathoracically according to methods described previously (2). Usually five or more *T. splendens* and 20 to 25 *A. aegypti* were inoculated with each virus dilution and/or each human leukocyte suspension, 0.85  $\mu$ l and 0.17  $\mu$ l per individual mosquito, respectively. Virus dilutions and leukocyte suspensions were prepared in RPMI 1640 medium that was supplemented with heat-inactivated fetal calf serum (FCS), final concentration of 10%, 200 units of penicillin/ml., and 150  $\mu$ g of streptomycin per ml. After a 14 day incubation period at 32°C, mosquitoes were stored at -70°C for virus assay. The leukocyte suspensions were obtained from the blood of clinically diagnosed DHF patients of the Children's Hospital as described elsewhere in this report. All leukocyte suspensions had been assayed for dengue viruses in LLC-Mk<sub>2</sub> cell culture; however, the technician was not aware of the results at the time specimens were tested for virus by mosquito inoculation.

Human anti-serum that had a hemagglutinating inhibition titer of 1:640 or greater to all 4 dengue virus serotypes was obtained from patients of the Children's Hospital. Anti-serum shown to be negative for hepatitis B surface antigen by radio immune assay was pooled and labeled with fluorescent isothiocyanate (FITC). Tissue imprints of squashed mosquito heads were prepared and assayed for virus by the fluorescent antibody technique as described previously (2). A 1:2 dilution of conjugated antiserum was used throughout the study.

Tissue smears of mosquito heads were examined for fluorescence with the 10 x and 25 x objective of a Leitz fluorescent microscope equipped with a vertical illuminator. The thorax-abdomen portions of mosquitoes for each virus dilution and for each leukocyte suspension were pooled, and disrupted by sonic energy in the presence of 1.5 ml. of RPMI 1640 medium, 10% fetal calf serum, 500 units of penicillin/ml. and 500  $\mu$ g of streptomycin/ml. Thorax-abdomen suspensions were spun for 30 minutes at 10,000 rpms in a 4°C centrifuge. Each suspension was tested for virus in LLC-Mk<sub>2</sub> cells by direct plaque assay.

**RESULTS :** Comparative results of the propagation of high and low passage dengue viruses in *A. aegypti* and *T. splendens* are presented in Tables 2 and 3. The dilution of low and high passages viruses that yielded fluorescence in each mosquito species was approximately the same, except with high passage dengue 4 virus, which produced fluorescence at a higher dilution in *T. splendens* than did low passage dengue 4 virus. The titers of dengue viruses in each mosquito species varied slightly. Dengue-4 produced lower titers as well as inconsistent FA results in both *A. aegypti* and *T. splendens*. Apparently, the problem was related to the batch of FITC labeled antiserum as virus was recovered in most cases from thorax-abdomen suspensions in the absence of fluorescence. Furthermore, the use of a newly prepared batch of FITC labeled dengue virus antiserum led to a marked

increase in fluorescence. The extent and intensity of fluorescence observed in infected mosquitoes appeared to be related to the quantity of virus in each mosquito species. Suspensions prepared from thorax-abdomen of *T. splendens* consistently yielded more virus than similar *A. aegypti* suspensions. Fluorescence observed in mosquito head smears was virus specific as indicated by the recovery of virus in LLC-Mk<sub>2</sub> cells from corresponding suspensions prepared from pooled thorax-abdomens. Perinuclear staining was the most common type of virus-specific fluorescence observed in mosquito head smears. Occasionally fluorescence was observed as granule-and/or flake-like particles scattered over the surface of tissue smears prepared from infected and uninfected mosquitoes. Virus was not detected in thorax-abdomen suspensions associated with head smears that exhibited the latter type of fluorescence. The head smears prepared from *Ar. subalbatus* that were inoculated with dengue-1, 3 and 4 viruses failed to fluoresce; however, virus was recovered from corresponding thorax-abdomen suspension of these mosquitoes. Dengue-1 and 4 viruses were recovered from mosquitoes through 10<sup>-3</sup> dilutions of the inoculum and dengue-3 virus was recovered through the 10<sup>-4</sup> dilution of the inoculum. Apparently the failure to observe fluorescence was not related to the FITC labeled antiserum as aliquots of the same batch gave specific fluorescence for control smears of all 4 dengue viruses in concurrent experiments involving *A. aegypti* and *T. splendens*.

A total of 127 human leukocyte suspensions were tested for dengue virus by intrathoracic inoculation of *A. aegypti*. Of these suspensions, nine were positive for virus by the F.A. technique. Eight of nine abdomen-thorax suspensions corresponding to the F.A. positive head smears yielded plaques in LLC-Mk<sub>2</sub> cells. In addition, virus was detected in 2 thorax-abdomen suspensions, but no evidence of fluorescence was observed in corresponding head smears. The plaque forming units (PFU) for 2 thorax-abdomen suspensions were too numerous to count, while the count for 8 suspensions ranged from 2 to 89/.3 ml of inoculum. The number of head smears that were F.A. positive for each leukocyte suspension varied from 1 of 5 to 6 of 6. Of the above 127 leukocyte suspensions, 23 dengue viruses were isolated by direct and delayed plaque assay employing LLC-Mk<sub>2</sub> cell cultures. All viruses detected by intrathoracic inoculation of *A. aegypti* were also isolated in LLC-Mk<sub>2</sub> cell cultures.

Of the above 127 leukocyte suspensions that were assayed for virus in *A. aegypti*, 26 have been tested in *T. splendens*. This included the 23 suspensions that were positive for dengue virus by cell culture assay. Fluorescence was observed in head smears for 18 of the 26 suspensions. Twelve of the 26 corresponding thorax-abdomen suspensions yielded virus by cell culture assay. In addition, 2 thorax-abdomen suspensions were positive for virus even though corresponding head smears were F.A. negative. The plaque count for 10 thorax-abdomen suspensions was too numerous to count and 13 and 30 for the other two suspensions that yielded viruses. Evidence of virus infection was not detected in *T. splendens* following inoculation of this species with 3 of the 26 suspensions that were negative for virus by cell culture assay.

Although the same leukocyte suspensions were tested for dengue viruses by cell culture assay and by the mosquito inoculation technique, the findings must be interpreted with caution due to the inconsistencies in the testing and treatment of specimens. Leukocyte suspensions were first assayed in LLC-Mk<sub>2</sub> cells

which required one or more freeze-thaw cycles. The suspensions were then stored at -70 C for one week to 4 months prior to inoculation of mosquitoes. That this may have altered the infectivity properties was suggested by the failure to reisolate dengue viruses by direct and delayed plaque assay from 9 leukocyte suspensions that were to be employed in another study. The presence of virus specific-like fluorescence in mosquito head smears prepared from dengue virus inoculated mosquitoes and the absence of detectable virus in corresponding thorax-abdomen suspensions has been reported previously (2). However, the frequency was exceptionally low compared to that in data obtained for dengue viruses and *T. splendens*. Such findings were not observed in *T. splendens* that were inoculated with low passage mouse brain propagated dengue seed viruses. Nor was perinuclear fluorescence observed in control head smears prepared from *T. splendens*. Further investigations will be required to assess *T. splendens* as a bioassay host for dengue viruses. *A. aegypti* will no longer be considered for dengue virus assay since an adequate number of *T. splendens* are now available. Modification of rearing technique has increased the yield of this species from an average of 60 to 200 per week.

Table 1. Dengue viruses used to inoculate *Aedes aegypti*, *Armigeres subalbatus* and *Toxorhynchites splendens*

Serotype	Date Isolated	Passage Level
Dengue-1 (D75-001)	1975	SMB-03
Dengue-2 (Hawaii)	1944?	SMB-16
Dengue-2 (CH3379)	1974	SMB-05
Dengue-2 (New Guinea)	?	SMB-29
Dengue-3 (77-2797)	1977	SMB-05
Dengue-3 (H87)	1956	SMB-25
Dengue-4 (D77-050)	1977	SMB-03
Dengue-4 (H241)	1956	SMB-32

Table 2. Comparative titration of high and low passage dengue viruses in an *Aedes aegypti* mosquitoes

Dengue Virus Serotype	Passage	<i>A. aegypti</i>	Log <sub>10</sub> Dilutions						
			Undil.	1	2	3	4	5	6
D-1	SMB-16	Old colony	5/6*	6/6	6/6	6/6	4/6	0/6	0/6
D-1	SMB-16	New colony	5/6	6/6	5/6	6/6	5/6	0/6	0/6
D-1	SMB-02	New colony	6/6	6/6	6/6	6/6	3/6	1/6	0/6
D-1	SMB-02	Old colony	6/6	6/6	6/6	6/6	3/6	1/6	0/6
D-2	SMB-29	Old colony	6/6	4/6	6/6	6/6	3/6	0/6	0/6
D-2	SMB-29	New colony	6/6	6/6	6/6	6/6	4/6	0/6	0/6
D-2	SMB-5	New colony	6/6	6/6	6/6	6/6	0/6	0/6	0/6
D-2	SMB-5	Old colony	6/6	6/6	6/6	6/6	1/6	0/6	0/6
D-3	SMB-25	Old colony	4/6	6/6	5/6	5/6	1/6	0/6	0/6
D-3	SMB-25	New colony	6/6	5/6	5/6	5/6	6/6	0/6	0/6
D-3	SMB-5	New colony	ND**	6/6	6/6	6/6	3/6	0/6	0/6
D-3	SMB-5	Old colony	ND	6/6	6/6	6/6	2/6	0/6	0/6
D-4	SMB-32	Old colony	0/6	3/6	0/6	1/6	1/6	0/6	0/6
D-4	SMB-32	New colony	1/6	0/6	0/6	0/6	0/6	0/6	0/6
D-4	SMB-32	Old colony	6/6***	6/6	5/6	3/6	1/6	0/6	ND
D-4	SMB-32	New colony	4/4***	6/6	5/6	2/6	0/6	0/6	0/6
D-4	SMB-03	New colony	6/6***	6/6	6/6	5/6	1/6	0/6	0/6
D-4	SMB-03	Old colony	6/6***	5/6	5/5	1/5	0/5	0/5	0/5

\* Number of mosquito head smears showing fluorescence/number of mosquito head smears assayed for virus.

\*\* Not done

\*\*\* Results of more recent experiments employ a new batch of FITC labeled dengue virus antiserum.

Table 3. Comparative titration of high and low passage dengue viruses in *Toxorhynchites splendens*

Dengue Virus Serotype	Passage	Log <sub>10</sub> Dilutions						
		Undil.	1	2	3	4	5	6
D-1	SM-16	4/4*	4/4	4/4	4/4	4/4	2/4	ND**
D-1	SM-03	3/3	3/3	4/4	4/4	4/4	2/4	ND
D-2	SM-29	ND	ND	4/4	4/4	4/4	3/4	0/4
D-2	SM-05	ND	ND	4/4	4/4	4/4	2/4	0/4
D-3	SM-25	4/4	4/4	4/4	3/4	3/4	ND	ND
D-3	SM-05	ND	5/5	4/4	5/5	3/4	1/4	0/4
D-4	SM-32	1/4	1/4	1/4	0/4	0/4	0/4	ND
D-4	SM-34	1/4	2/4	1/5	0/4	0/4	0/4	0/4
D-4	SM-32	4/4***	3/4	4/4	3/4	3/4	3/4	ND
D-4	SM-34	3/4***	4/4	4/4	3/4	3/4	3/4	ND

\* Number of mosquito head smears showing fluorescence/number of mosquito head smears assayed for virus.

\*\* Not done

\*\*\* Results of more recent experiment employing a new batch of FITC labeled dengue virus antiserum

#### REFERENCES :

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