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EVALUATION OF TOXORHYNCHITES SPLENDENS AS A BIOASSAY HOST FOR D--ETC(U)
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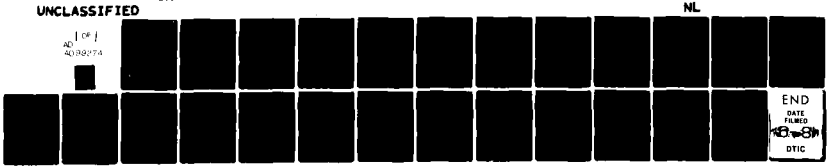

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Toxorhynchites spendens, a non-hematophagous mosquito was evaluated as a bioassay host for the detection and propagation of dengue viruses. All dengue virus serotypes and strains attained titers in T. splendens comparable to those observed for 2 strains of Aedes aegypti. Peak virus titers occurred in Tx. splendens approximately 6 days postinoculation; however, specific fluorescence for all viruses was not observed in 100% of mosquito heads until 12 days postinoculation. A 100% correlation was noted between specific fluorescence in Tx. spendens heads and the recovery of virus from correspondin

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thorax-abdomens. The volume of inoculum tolerated by Tx. splendens was approximately 5 times greater than that injected into Ae. aegypti. Thus, for a given volume of inoculum, the number of Tx. splendens required for virus assays was appreciably less than that needed for Ae. aegypti. The overall survival rate for Tx. splendens following intrathoracic inoculation with dengue viruses was 92%, compared to 41 and 42% for 2 strains of male Ae. aegypti. These findings imply that Tx. splendens would be more efficient than Ae. aegypti as a laboratory assay host for detecting dengue viruses in blood of infected patients and for use in experimental investigations.

EVALUATION OF TOXORHYNCHITES SPLENDENS AS A BIOASSAY HOST FOR DENGUE VIRUSES

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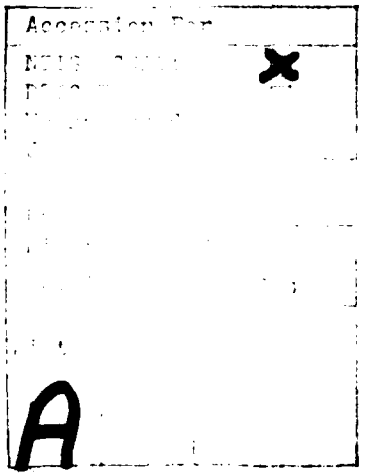
Abstract: Toxorhynchites splendens, a non-hematophagous mosquito was evaluated as a bioassay host for the detection and propagation of dengue viruses. All dengue virus serotypes and strains attained titers in T. splendens comparable to those observed for 2 strains of Aedes aegypti. Peak virus titers occurred in Tx. splendens approximately 6 days postinoculation; however, specific fluorescence for all viruses was not observed in 100% of mosquito heads until 12 days postinoculation. A 100% correlation was noted between specific fluorescence in Tx. splendens heads and the recovery of virus from corresponding thorax-abdomens. The volume of inoculum tolerated by Tx. splendens was approximately 5 times greater than that injected into Ae. aegypti. Thus, for a given volume of inoculum, the number of Tx. splendens required for virus assays was appreciably less than that needed for Ae. aegypti. The overall survival rate for Tx. splendens following intrathoracic inoculation with dengue viruses was 92%, compared to 41 and 42% for 2 strains of male Ae. aegypti. These findings imply that Tx. splendens would be more efficient than Ae. aegypti as a laboratory assay host for detecting dengue viruses in blood of infected patients and for use in experimental investigations.



At least 2 species of the Aedes subgenus Stegomyia have been tested as bioassay hosts for dengue viruses. Aedes albopictus (Skuse) has been shown to be susceptible to infection with all 4 dengue virus serotypes following intrathoraic inoculation (Rosen & Gubler 1974, Kuberski & Rosen 1977). Infectivity titers attained in this species were higher than those observed in the LLC-MK_2 cell plaque assay of Yuill et al. (1968), the technique most commonly used to isolate these viruses. In addition, dengue viruses were isolated more frequently from sera of dengue patients by using Ae. albopictus as compared with LLC-MK_2 cells (Rosen & Gubler 1974). Subsequently, Woodall et al. (1979) and Gubler et al. (1979) found that Ae. aegypti (Linnaeus), was also a sensitive and reliable host for isolating dengue viruses from patients. Virus isolation rates from dengue patients in Southeast Asia obtained by using Ae. aegypti and Ae. albopictus (Gubler et al. 1978, 1979, Kuberski et al. 1977) were consistently higher than rates previously reported using other assay techniques (Russell et al. 1968, Winter et al. 1969, Halstead et al. 1969a, b, Nimmannitya et al. 1969).

In addition to the above studies demonstrating the suitability of Ae. aegypti and Ae. albopictus as assay hosts for dengue viruses, Kuberski & Rosen (1977) suggested that Toxorhynchites amboinensis (Doleschall) was also a suitable host. Furthermore, Burton & Rudnick (1979) reported that Toxorhynchites splendens (Wiedemann) was susceptible to dengue virus infection following intrathoracic innoculation. Although these 4 species appear to be suitable assay hosts, Rosen & Gubler (1974) reported that a Culex species and an Armigeres species were resistant to dengue virus infection following parenteral inoculation. These differences in susceptibility indicate that mosquito species vary in their efficiency as

assay hosts for dengue viruses; however, comparative studies documenting this have not been published.

This study was conducted to evaluate Tx. splendens, a common Southeast Asian mosquito, as a bioassay host for dengue viruses, and to compare its susceptibility to dengue virus with that of different Ae. aegypti strains. Additional evaluations were based on the size of the 2 species, the postinoculation survival and the time required to detect virus specific fluorescence in the 2 species.

MATERIALS AND METHODS

Ae. aegypti mosquitoes were obtained from colonies maintained in the Department of Medical Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. Colony #1, of unknown generations, originated from Koh Samui Island, Thailand, June-July 1968. Colony #3, 10th to 12th generation, was established from larvae collected in Bangkok, Thailand, during August and September 1977. The Tx. splendens colony, of unknown generations, was established from larvae collected in Bangkok during June to August 1976. Mosquitoes of each species were 3 to 10 days old when used in experiments. Immature Ae. aegypti were reared on ground mouse chow and adults were fed a 5% sucrose solution. Immature Tx. splendens were fed larvae of Ae. aegypti and Culex quinquefasciatus (Say); adults were maintained on honey and/or a dextrose solution. Immature Tx. splendens were reared individually in 9-dram vials.

The history of dengue virus serotypes and strains used in these experiments is presented in Table 1. Stock viruses were prepared as 20% suspensions of dengue virus-infected mouse brain tissue in RPMI 1640 medium-20% fetal calf serum (FCS). Infectivity titers of viruses were determined by plaque assays on LLC-MK$_2$ cells (Yuill et al. 1968). Virus

identity was determined by plaque reduction neutralization tests (PRNT) in LLC-MK_2 cells employing dengue virus types 1, 2, 3 and 4 monospecific antisera (Russell & Nisalak 1967). Antisera were produced by injecting rhesus monkeys intramuscularly and subcutaneously with $10^{3.5}$ plaque forming units PFU/ml of each dengue virus serotype. After approximately one month, blood was obtained from monkeys; sera were obtained by centrifugation at 270 x g for 30 min.

Mosquitoes were immobilized for inoculation in 50-ml test tubes in an ice water bath. Inoculation of mosquitoes with virus was performed with a needle and apparatus similar to that described by Rosen & Gubler (1974). Five or more Tx. splendens and 20 to 40 Ae. aegypti were inoculated with each virus dilution, 0.85 µl per Tx. splendens and 0.17 µl per Ae. aegypti. Viruses were diluted in medium as described above and supplemented with 500 units/ml of penicillin and 500 µg/ml of streptomycin. Mosquitoes were incubated at 32° for various time intervals, sacrificed and stored at -70° for virus assay.

Mosquito heads were severed from the thorax and head-squashes were prepared and assayed for dengue virus antigen by the direct fluorescent antibody technique as described by Kuberski & Rosen (1977). Anti-dengue virus sera employed were obtained from humans, 14 days or more after acute dengue virus infections. Sera with hemagglutination-inhibition (HI) titers of 1:640 or greater to all 4 dengue virus antigens were pooled and the gamma globulin fraction was obtained by ammonium sulfate precipitation. Estimation of protein concentration was determined by the Biuret method (Gornall et al. 1949). Antisera were conjugated with fluorescein isothiocyanate (FITC) as described by Goldman (1968). The titer of the conjugate was capable of detecting dengue virus antigen in mosquito heads at a 1:16 dilution; however, a 1:4 dilution was used

in all experiments. Head-squashes from virus-inoculated and uninoculated mosquitoes were examined with the 10x and 25x objectives (dry power) of a Leitz Orthoplan microscope.

The thorax-abdomens (Th-Abd) of test mosquitoes were assayed as pools and/or as individual specimens by the direct plaque technique in LLC-MK_2 cells. Pooled specimens were placed in 1.5 ml and individual specimens, in 1.0 ml of RPMI 1640 medium, supplemented as described above, and disrupted by sonification. Suspensions were centrifuged for 30 min at 12,000 x g at 4°. Replicate cultures of LLC-MK_2 cells were inoculated with each suspension, 0.3 ml per culture.

Survival rates for male Ae. aegypti and both sexes of Tx. splendens were determined by recording the number of dead mosquitoes each day postinoculation. Mosquito size was based on the mean weights of 10 individual mosquitoes of each species.

RESULTS

Comparative results of the propagation of low and high mouse brain-passaged dengue viruses in Ae. aegypti are presented in Table 2. The dilution of virus inoculum at which fluorescence was detected in Ae. aegypti was comparable for the different virus strains, regardless of the passage level of the virus and/or the generation of the mosquitoes. Virus-specific fluorescence was usually detected in 2 or more Ae. aegypti heads up through 10^{-4} dilutions of dengue virus types 1, 2 and 3, while the dilutions of dengue virus type 4 that produced fluorescence were considerably lower.

All 4 dengue viruses replicated to high titers in Tx. splendens (Table 3). Except for strain 050 of dengue virus type 4, virus-specific

fluorescence was detected in one or more Tx. splendens heads up through the 10^{-5} dilution for each virus.

Comparative titers for dengue viruses in LLC-MK_2 cells, and in Ae. aegypti and Tx. splendens are summarized in Table 4. All viruses, except the prototype strains of dengue virus types 3 and 4, attained appreciably higher titers in both mosquito species than in LLC-MK_2 cells.

Table 5 shows the infectivity titers attained by dengue viruses in individual Ae. aegypti and in Tx. splendens after 14 days incubation at 32°. On the basis of mean titers, the amount of each virus type recovered was higher for Tx. splendens than for Ae. aegypti.

FIG. 1 shows the perinuclear pattern of fluorescence most commonly observed in brain ganglia cells of Tx. splendens infected with dengue viruses. Fluorescence was usually more intense and more prevalent in heads of Tx. splendens inoculated with dengue virus types 1 and 2 than those inoculated with dengue types 3 and 4.

Dengue viruses were recovered in LLC-MK_2 cells from pooled Th-Abd suspensions that corresponded to one or more fluorescence-positive heads of Ae. aegypti and Tx. splendens for each virus dilution. Virus was not recovered from Th-Abd suspensions for dilutions of inoculum that did not produce fluorescence in mosquito heads. No evidence of virus-specific fluorescence was observed in heads of control mosquitoes, nor was virus recovered from their Th-Abd.

Since the initial experiment found Tx. splendens as susceptible to infection with dengue viruses as Ae. aegypti, further experiments were not conducted on Ae. aegypti, except for survival studies.

Table 6 shows the time required for dengue virus-specific fluorescence to appear in heads of Tx. splendens and the recovery rate of these viruses from corresponding individual Th-Abd following inoculation of mosquitoes

with 0.85 ml of $10^{4.5}$ to $10^{5.0}$ PFU/ml of each virus. Except for strain 001 of dengue virus type 1, specific fluorescence was not observed in Tx. splendens heads on day 6 postinoculation. On day 9 specific fluorescence was present in most mosquitoes inoculated with all viruses except strain 2877 of dengue virus type 3. Specific fluorescence was observed in all Tx. splendens inoculated with the latter virus on day 12. Dengue viruses were recovered from 100% of Th-Abd suspensions prepared from individual mosquitoes that were positive by head-squash. Most viruses attained peak titers on day 6. Thereafter, titers tended to decrease, especially for dengue virus type 1. In general, dengue viruses type 2 and 4 exhibited the highest titers.

The survival rates for male Ae. aegypti and male and female Tx. splendens, during the 14-day period after inoculation with dengue viruses, are presented in Table 7. Survival rates ranged from 25 to 63% for Ae. aegypti, and from 75 to 100% for Tx. splendens.

The mean weight of 10 individual Tx. splendens was 6.76 mg for males and 7.65 mg for females, in comparison to 1.38 mg for male Ae. aegypti.

DISCUSSION

Dengue virus-specific fluorescence was detected only in heads of mosquitoes whose corresponding Th-Abd contained virus. In a previous report, specific fluorescence was apparently observed in heads of Ae. albopictus, but virus was not recovered from corresponding Th-Abd suspensions (Rosen & Gubler 1974). However, the frequency of these observations was exceptionally low and associated with dengue virus types 3 and 4. On the contrary, the recovery of virus from Th-Abd in the absence of detectable fluorescence in mosquito heads was not uncommon (Rosen & Gubler 1974). Since Th-Abd from virus susceptibility experiments

were pooled, corresponding observations were not made in the present investigation. However, in the experiment designed to determine when fluorescence appeared in heads of Tx. splendens, dengue viruses were recovered from Th-Abd in the absence of fluorescence in the heads of corresponding mosquitoes on days 3 and 6 for all virus serotypes and on day 9 for dengue virus type 3 and 4. Apparently, this is related to the time required for dengue viruses to spread to the head of mosquitoes, as indicated by the recovery of virus from corresponding Th-Abd and the subsequent detection of specific fluorescence in mosquito heads on days 9, 12, 15 and 18 postinoculation.

Dengue viruses attained peak virus titers in Th-Abd of Tx. splendens approximately 6 days postinoculation. However, at this time specific fluorescence was detected in the head of only one mosquito. A comparable period of time was required for dengue viruses to reach maximum titers in the Th-Abd of Ae. albopictus; however, specific fluorescence was present in approximately 50% of the corresponding mosquito heads (Rosen & Gubler, 1974). Otherwise they did not find all Ae. albopictus fluorescence-positive until 10 days postinoculation, which approximates our findings for Tx. splendens. A slightly longer period of time was required for fluorescence to appear in Tx. splendens inoculated with dengue virus types 3 and 4 in comparison to the other serotypes. Similar findings were observed for these serotypes in Ae. albopictus (Rosen & Gubler 1974).

Tx. splendens were found to be approximately 5 times heavier (mean weight) than male Ae. aegypti. Accordingly, the larger Tx. splendens, allowed for the use of a volume of inoculum approximately 5 times the amount injected into a single Ae. aegypti. As a result, the number of mosquitoes required for virus assay was reduced substantially. The larger volume of inoculum tolerated by Tx. splendens in comparison

with Ae. aegypti will be advantageous, especially for detecting low concentrations of dengue virus. Cell culture assays offer an advantage over mosquitoes in regard to the volume of inoculum that can be used; however, titers attained by dengue viruses in LLC-MK$_2$ cells were considerably less than those noted in Ae. aegypti and Tx. splendens.

Ae. aegypti and Tx. splendens were readily infected with all 4 serotypes as well as different strains of dengue viruses. Virus titers were usually higher in Tx. splendens; other findings indicated that this species was a more efficient assay host. Since females of Tx. splendens do not take blood meals (Bates 1949), both sexes can be used in the laboratory without risk of human infection by mosquito bite. The larger size of Tx. splendens in comparison with Aedes species facilitates inoculation, allows the use of a larger volume of inoculum, and results in the replication of larger quantities of virus. Also, the number of Tx. splendens required was lower due to a higher survival rate. Although Ae. aegypti can be reared much faster than Tx. splendens, the findings implied that the latter species would be a more efficient host for diagnosing human dengue virus infection and for use in experimental studies.

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TABLE 1. History of dengue viruses employed in the mosquito bioassay evaluations.

VIRUS	SEROTYPE	HOST/NO. OF PASSAGES	ORIGIN	DATE
DEN-1	77-001	Mouse/3*	Human	1975
	Hawaii**	Mouse/16	Human	1944
DEN-2	74-3379	Mouse/5	Human	1974
	78-189-18A	Tx. splendens/1	Ae. aegypti	1978
	New Guinea C**	Mouse/29	Human	1944
DEN-3	77-2797	Mouse/5	Human	1977
	77-2877	Mouse/5	Human	1977
	H-87**	Mouse/25	Human	1956
DEN-4	77-050	Mouse/3	Human	1977
	H-241**	Mouse/32	Human	1956

*Suckling mouse brain passage.

**Prototype viruses.

TABLE 2. Dengue virus-specific fluorescence in heads of male Ae. aegypti.

VIRUS	SEROTYPE	PASSAGE	AE. AEGYPTI	NO. POSITIVE HEAD SQUASHES/NO. EXAMINED						
				UNDIL.	1*	2	3	4	5	6
DEN-1	001	smb-2	Colony-3	6/6	6/6	6/6	6/6	3/6	1/6	0/6
			Colony-1	6/6	6/6	6/6	6/6	3/6	0/6	0/6
	Hawaii	smb-16	Colony-3	5/6	6/6	5/6	6/6	5/6	0/6	0/6
			Colony-1	5/6	6/6	6/6	6/6	4/6	0/6	0/6
DEN-2	3379	smb-5	Colony-3	6/6	6/6	6/6	6/6	6/6	0/6	0/6
			Colony-1	6/6	6/6	6/6	6/6	1/6	0/6	0/6
	New Guinea C	smb-29	Colony-3	6/6	6/6	6/6	6/6	4/6	0/6	0/6
			Colony-1	6/6	5/6	6/6	6/6	3/6	0/6	0/6
DEN-3	2797	smb-5	Colony-3	5/6	6/6	6/6	6/6	3/6	0/6	0/6
			Colony-1	5/6	6/6	6/6	6/6	2/6	0/6	0/6
	H-87	smb-29	Colony-3	6/6	6/6	6/6	6/6	6/6	0/6	0/6
			Colony-1	6/6	6/6	6/6	6/6	2/6	0/6	0/6

DIN-1	100		sub-3	colony-3	6/6	6/6	6/6	5/6	1/6	0/6	0/6
				colony-1	6/6	5/6	5/5	1/5	0/5	0/5	0/5
		1-2-1	sub-32	colony-3	6/6	6/6	5/5	2/6	0/6	0/6	0/6
				colony-1	6/6	6/6	5/5	3/6	1/6	0/6	NT
	controls				0/16	0/16	0/15	0/16	0/15	0/15	0/16

*$\log_{10}$ dilutions.

**Not tested.

TABLE 3. Dengue virus-specific fluorescence in heads of Tx. splendens.

VIRUS	SEROTYPE	PASSAGE	NO. POSITIVE HEAD SQUASHES/NO. EXAMINED						
			UNDIL.	1*	2	3	4	5	6
DEN-1	001	3	3/3*	3/3	4/4	4/4	4/4	2/4	NT**
	Hawaii	16	4/4	4/4	4/4	4/4	4/4	2/4	NT
DEN-2	3379	5	NT	NT	4/4	4/4	4/4	2/4	0/4
	New Guinea C	29	NT	NT	4/4	4/4	4/4	3/4	0/4
DEN-3	2797	3	NT	5/5	4/4	5/5	3/4	1/4	0/4
	H87	25	4/4	4/4	4/4	3/4	3/4	NT	NT
DEN-4	050	3	4/4	4/4	3/4	4/4	0/4	0/4	NT
	H241	32	3/4	4/4	4/4	3/4	3/4	3/4	NT
	Controls		0/3	0/6	0/8	0/8	0/8	0/8	0/3

*Log_{10} dilutions.

**Not tested.

TABLE 4. Comparative dengue virus titers for mosquitoes and cell culture.

		FLUORESCENCE DOSE$_{50}$/ml*			Log_{10} PFU/ml IN
		AE. AEGYPTI			
VIRUS	PASSAGE	COLONY 1	COLONY 3	TX. SPLENDENS	LLC-MK$_2$ CELLS
DEN-1	smb-2	7.6	7.9	8.1	4.9**
	smb-16	7.7	8.0	8.1	6.7
DEN-2	smb-5	7.8	7.2	8.1	6.2
	smb-29	7.5	8.0	8.4	6.8
DEN-3	smb-5	7.9	7.7	7.6	4.9
	smb-25	7.1	7.7	7.1	7.0
DEN-4	smb-3	6.2	7.2	6.4	5.0
	smb-32	6.7	6.8	7.6	6.7

*Based on fluorescent antibody assay of mosquito head-squashes.

TABLE 5. Dengue virus infectivity titers attained in mosquitoes following intrathoracic inoculation with 0.85 µl of $10^{4.5}$ to $10^{5.0}$ PFU/ml of each virus.

		MEAN LOG_{10} PFU/ml (RANGE)			
VIRUS	SEROTYPE	N	TX. SPLENDENS	N	AL. AEGYPTI
DEN-1	001	10	4.7 (4.6-4.9)	6	4.0 (3.4-4.5)
DEN-2	3379	10	6.1 (5.8-6.3)	10	5.4 (4.8-6.5)
DEN-3	2797	10	4.4 (3.9-5.0)	10	3.3 (2.1-4.0)
DEN-4	050	10	4.3 (3.5-4.9)	10	3.3 (2.5-4.8)

TABLE 6. Specific fluorescence in heads of Tx. splendens and recovery of dengue viruses from corresponding thorax-abdomens by LLC-MK_2 plaque assay.

VIRUS	SEROTYPE	TX. SPLENDENS	RESPONSE BY DAYS					
			3	6	9	12	15	18
DEN-1	001	Heads*	0/4	1/4	4/4	4/4	4/4	4/4
		Th-Abd**	4/4(3.0)	4/4(4.3)	4/4(1.8)	4/4(2.5)	4/4(2.5)	4/4(2.5)
DEN-2	3379	Heads	0/3	0/4	4/4	4/4	4/4	4/4
		Th-Abd	4/4(4.2)	4/4(6.0)	4/4(5.5)	4/4(5.2)	4/4(5.0)	4/4(5.3)
	189-18A	Heads	0/4	0/4	4/4	4/4	4/4	4/4
		Th-Abd	4/4(1.6)	4/4(5.0)	4/4(5.3)	4/4(4.2)	4/4(3.3)	4/4(4.0)
DEN-3	2797	Heads	0/4	0/4	3/4	4/4	4/4	4/4
		Th-Abd	4/4(1.7)	4/4(4.0)	4/4(2.5)	4/4(3.4)	4/4(2.8)	4/4(3.0)
	2877	Heads	0/4	0/4	0/4	4/4	4/4	4/4
		Th-Abd	4/4(2.8)	4/4(1.8)	4/4(4.3)	4/4(3.2)	4/4(3.6)	4/4(3.5)
DEN-4	050	Heads	0/4	0/4	2/4	4/4	4/4	4/4
		Th-Abd	4/4(3.9)	4/4(4.8)	4/4(2.5)	4/4(3.7)	4/4(3.4)	4/4(4.2)

Controls	Heads	0/16	0/16	0/16	0/16	0/16	0/16
	Th-Abd	0/ 6	0/ 6	0/ 6	0/ 6	0/ 6	0/ 6

*No. fluorescent positive/total.

**No. virus-positive/total; ( ) = mean infectivity titer, $\log_{10}$ PFU/ml, for individually infected Th-Abd portions.

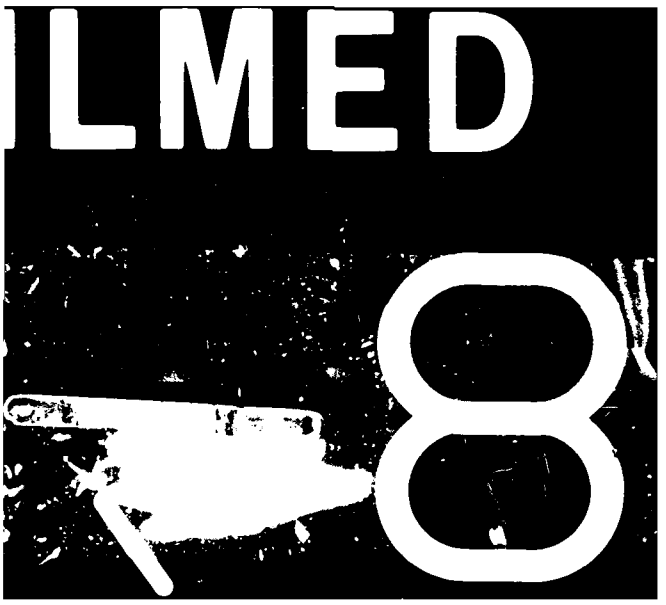
TABLE 7. Survival rates at 14 days of Ae. aegypti and Tx. splendens after intrathoracic inoculation with dengue viruses.

			% SURVIVAL (NO./TOTAL)		
			AE. AEGYPTI		TX. SPLENDENS
VIRUS	SEROTYPE	PASSAGE	COLONY 1	COLONY 3	
DEN-1	001	2	48 (105/ 217)	47 (101/ 216)	75 (36/ 48)
	Hawaii	16	43 (100/ 230)	42 (97/ 230)	95 (59/ 62)
DEN-2	3379	5	25 (48/ 175)	43 (76/ 175)	90 (38/ 42)
	New Guinea C	29	43 (77/ 180)	36 (64/ 180)	94 (51/ 54)
DEN-3	2797	5	44 (91/ 209)	38 (53/ 139)	100 (48/ 48)
	H-87	25	63 (111/ 180)	26 (47/ 180)	93 (28/ 30)
DEN-4	050	3	27 (47/ 175)	55 (97/ 175)	94 (34/ 36)
	H-241	32	31 (67/ 219)	35 (78/ 220)	94 (45/ 48)
	Total		42 (579/1366)	41 (535/1295)	92 (294/320)

FIGURE LEGENDS

FIG. 1. Perinuclear pattern of specific fluorescence in head squash of Fg. splendens infected with Fanae 2 virus.

FIG. 2. Head squash of uninfected Fg. splendens.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