

EFFECT OF TEMPERATURE AND INSECTICIDE STRESSES ON *Aedes Aegypti* LARVAE AND THEIR INFLUENCE ON THE SUSCEPTIBILITY OF MOSQUITOES TO DENGUE-2 VIRUS

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Abstract. Two major factors, higher temperatures and the application of insecticides, can drastically alter the genetic structure of a vector mosquito population. Due to these two stresses, the majority of the population gets wiped out, but the ones that withstand the stress and survive are likely to pass on survivability, and have an altered physiology. Our study shows that exposures to higher temperatures and DDT during the larval stage affects their susceptibility as adult mosquitoes to the DEN-2 virus. The overall transcription and translation status of heat shock protein (Hsp70) in virus high- and low-susceptible was the same as that in other batches. In the case of a DDT-resistant (R-7) strain two bands were obtained during RT-PCRs after heat shock. These two alleles were obtained only with HY-1 in which R-7 males were used for the crosses, suggesting that the second allele is probably male sex linked. The higher expression of Hsp70 may provide DDT-resistant strains a better chance of survival high temperature environments, particularly in homozygotes and hybrids. It was also interesting to note that these strains have a significantly lower susceptibility to the virus. Wide-spread DDT-resistance and a rise in temperature above the average temperature during summer may result in a population with a low susceptibility to the virus. Several families of heat shock proteins are known to be expressed in mosquitoes, and may have a cumulative role in determining susceptibility to the virus, which itself is governed by several genes.

INTRODUCTION

Aedes aegypti is the main vector of dengue viruses in India. Several gene(s) govern the susceptibility of this mosquito to viruses and it is also known that the refractoriness is recessive (Miller and Mitchell, 1991; Mourya *et al*, 1994a). It has been suggested that presence of a higher proportion of virus-susceptible genotypes in a population are essential to sustain virus transmission in an area. Several intrinsic factors are known to affect this trait, but only two main extrinsic factors (temperature and insecticides) drastically alter the genetic structure of the population in an area. Both these factors are known to cause profound effects on the physiology of the vector species. Earlier studies were conducted to look for an association between virus susceptibility and insecticides (Mourya *et al*, 1994b), however an association between insecticides

selection pressure and increased virus susceptibility was found to be indirect, probably due to the involvement of several gene(s) controlling it. Similarly, recent studies with the Chikungunya virus and the stress of heat on the vector showed that temperature affects up- and down-regulation of certain immunoresponsive genes, which were found to affect susceptibility (Mourya *et al*, 2004b).

Under these two stresses, the majority of the population gets wiped out, but the ones which withstand the stress of a higher temperature or an insecticide and survive are more likely to have an affected physiological status. A lot of work has been done in the past to understand the role of higher temperatures on the vector competence of mosquitoes, but the effect of higher temperatures during larval stages, which may affect virus susceptibility of the resulting adults, has not been studied.

Our study proposed to investigate (a) the effect of exposure to various temperatures and insecticide during the larval stages and its result on the susceptibility of adult mosquitoes to den-

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gue (DEN)-2 virus, which overcame these stresses; (b) the transcription and translation status of a heat shock protein (Hsp70) in virus-susceptible and -refractory strains and DDT-susceptible and -resistant strains after heat shock.

MATERIALS AND METHODS

Mosquitoes

Ae. aegypti mosquitoes employed for the experiment were from a laboratory colonies maintained at this institute. The mosquitoes were reared in an insectary maintained at $28 \pm 2^\circ\text{C}$ and 70-80% RH.

Virus

The DEN-2 virus strain (TR1751) used in our study was originally isolated from the serum of a patient in Trinidad in 1953. It has undergone more than 122 passages in infant mice brains. Stock used for experiments was prepared from infant mice by intracerebral (ic) route.

Infection of mosquitoes through membrane

Four- to five-day old female mosquitoes were orally infected through an artificial membrane, Parafilm (American National Can, Greenwich, USA) as described by Harada *et al* (1996). Post-feed virus titers in the mosquito feeding suspensions were determined in infant mice via the ic route. On the 8th day post-infection, the virus positivity of the mosquitoes was determined by indirect immunofluorescence on head squashes as described earlier (Mourya *et al*, 1994a).

DEN high-susceptible (VS) and low-susceptible (VR) isofemale lines of *Ae. aegypti*

The establishment of these isofemale lines of mosquitoes has been described earlier (Mourya *et al*, 2000). Crossing experiments were carried out with virus-high-susceptible and -low-susceptible strains (F22) in 30 cm³ wooden cages with wire mesh.

DDT-resistant strain (R-7) of *Ae. aegypti*

The DDT-resistance was established from adult mosquitoes collected from Shahjahanpur City, Uttar Pradesh State as described earlier (Mourya *et al*, 1994c), since then this has been maintained by the selection pressure of DDT every alternate generation.

DDT-susceptible strain of *Ae. aegypti*

Laboratory colonies of *Ae. aegypti* maintained at this Institute for over 30 years were used as the reference susceptible strain. This strain has no history of exposure to any insecticides. Crossing experiments were carried out with DDT-susceptible and -resistant strains in 30 cm³ wooden cages with wire mesh.

Heat shock to the larvae

Larvae from the parent strains and hybrids were exposed to heat shock at 45.5°C for 10 minutes. The adults obtained from the larvae exposed to various higher temperatures were fed on fresh chicken blood and the virus mixture. Some of the larvae were used for RT-PCR for determining expression of Hsp70.

Dot blots assays

After heat shock, larval homogenates from both virus-high and -low susceptible strains were prepared (Mourya *et al*, 1998). Protein was estimated and adjusted to equal amounts in both batches; two fold serial dilutions were made and protein dots were deposited on a nitrocellulose (NC) membrane using dot blot equipment. The NC membrane was then washed in 50 mM phosphate buffer saline (PBS) (pH 7.2) and blocked with 4% bovine serum albumin (BSA) and incubated with anti-Hsp70 monoclonal antibodies (Sigma Chemical, USA) for 2 hours at 37°C . The reaction was detected using anti-mouse IgG tagged with alkaline phosphatase (Sigma) and 5-bromo, 4-chloro, 3-indolil phosphate as the substrate.

Western blot assays

To determine the levels of Hsp70 in the larval homogenates of different batches, the homogenates were subjected to SDS PAGE (4% stacking and 12% resolving) and the proteins were transferred to the NC membrane. After blocking with 4% BSA in PBS for one hour, the blots were processed as described above for dot-blot assays.

RNA extraction and RT-PCR

Total RNAs were extracted from controls and surviving larvae at 1, 4, 8, 24 and 72 post-heat-shock hours (PHSH), using Trizol reagent (Gibco-BRL) according to the manufacturer's protocol. RNA from five larvae was dissolved in 100 μl

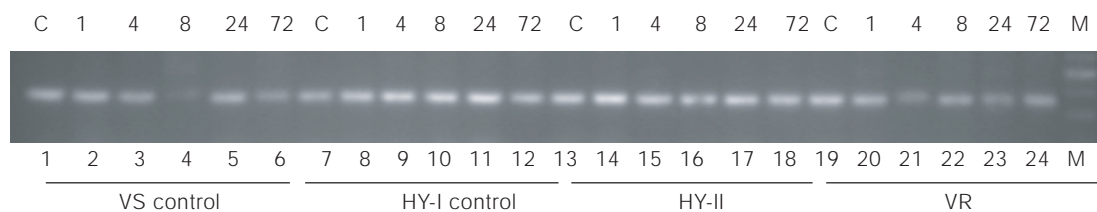


Fig 1—RT-PCR-assays expression profiles for Hsp70 in larvae (VS) (1) Controls, (2) 1 Post heat-shock hour (PHSH), (3) 4 PHSH, (4) 8 PHSH, (5) 24 PHSH, (6) 72 PHSH; larvae (HY-1) (7) Controls, (8) 1 PHSH, (9) 4 PHSH, (10) 8 PHSH, (11) 24 PHSH (12) 72 PHSH; (HY-2) (13) Controls, (14) 1 PHSH, (15) 4 PHSH, (16) 8 PHSH, (17) 24 PHSH, (18) 72 PHSH; (VR) (19) Controls, (20) 1 PHSH, (21) 4 PHSH, (22) 8 PHSH, (23) 24 PHSH, (24) 72 PHSH.

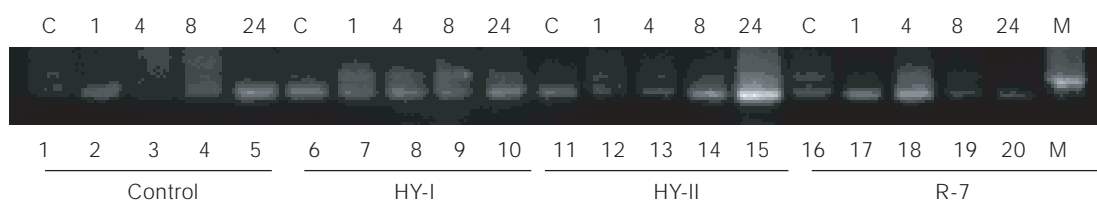


Fig 2—RT-PCR-assays expression profiles for Hsp70 in larvae (VS) (1) Controls, (2) 1 Post heat-shock hour (PHSH), (3) 4 PHSH, (4) 8 PHSH, (5) 24 PHSH; larvae (HY-1) (6) Controls, (7) 1 PHSH, (8) 4 PHSH, (9) 8 PHSH, (10) 24 PHSH; (HY-2) (11) Controls, (12) 1 PHSH, (13) 4 PHSH, (14) 8 PHSH, (15) 24 PHSH; (16) Controls, (17) 1 PHSH, (18) 4 PHSH, (19) 8 PHSH, (20) 24 PHSH.

DEPC treated water. cDNA were prepared using MMLV-Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. A PCR cycling program and primers used for Hsp70 were similar to that described by Fulton *et al* (1997).

RESULTS

Infection of DEN high-susceptible (VS) and low-susceptible (VR) mosquitoes through membrane

Oral susceptibility to DEN virus was studied in the adults, which were reared from larvae (which were given heat shock of 44.5°C temperatures for 10 minutes) of parent strains (VS and VR) and the hybrids from these strains. It was interesting to note that there was an increase in the susceptibility to virus in the VS strain when heat stress was given. The oral susceptibility of hybrids from VS and VR showed the difference between HY-2 as compared to unstressed VS control. This difference was lower in HY-2 compared to HY-1 (Table1).

Infection of DEN high-susceptible (VS) and DDT-resistant strain (R-7) mosquito strains through membrane

Susceptibility to virus in the adults of par-

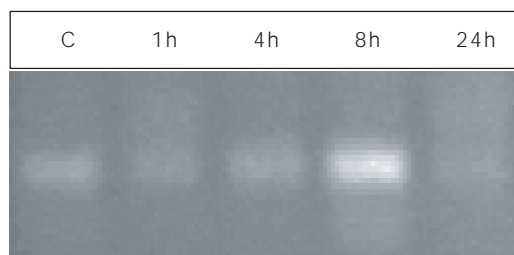


Fig 3—Western-blot analysis of translation of Hsp70 protein: homogenate of larvae (R7) after heat shock at 44.5°C for 10 minutes: (lane-A) normal control; (lane-B) 1 post heat-shock hour (PHSH); (lane-C) 4 PHSH; (lane-D) 8 PHSH; (lane-E) 24 PHSH.

ent strains (VS and R-7) and the crosses performed with both way hybrids from these strains showed that there was a difference of 11% in the unstressed VS and R-7 parents. Similar differences in the susceptibility of the HY-1 and the VS strain were noticed. It was interesting to note that there was a small difference between the susceptibility of the HY-2 and the VS strains. Data obtained from these progeny showed that a significant difference ($p < 0.01$) was noted only with VR, R7 and HY-1, compared to the un-

Table 1

Susceptibility of *Aedes aegypti* mosquitoes to oral infection with DEN-2 virus. Adults were obtained from the larvae, which were given a heat shock at 44.5°C for 10 minutes during the IV instar larval stage.

Mosquito strains	Replicate No.	No. of head squashes positive/tested ^a		ϕ , p-value
Virus high-susceptible (VS)	1	13/39	(33.3)	
Control without any stress	2	14/38	(36.8)	
	3	16/38	(42.1)	
	Total	43/115	Average (37.4)	
Virus high-susceptible (VS)	1	18/40	(45.0)	
	2	15/39	(38.5)	
	3	17/38	(44.7)	
	Total	50/117	Average (42.7)	0.00037 p<0.01
Hybrid-1 (VR ♂ x VS ♀)	1	14/30	(00)	
	2	13/35	(00)	
	3	12/32	(00)	
	Total	39/97	Average (40.4)	0.00002 p<0.01
Hybrid-2 (VR ♀ x VS ♂)	1	13/37	(000)	
	2	16/35	(000)	
	3	12/35	(000)	
	Total	41/107	Average (38.4)	0.000003 p<0.01
Virus low-susceptible (VR)	1	7/30	(00)	
	2	9/40	(00)	
	3	11/40	(00)	
	Total	27/110	Average (24.4)	0.0232 p>0.01
Hybrid-1 (R-7 ♂ x VS ♀)	1	12/50	(00)	
	2	10/40	(00)	
	3	7/40	(00)	
	Total	29/130	Average (22.2)	0.0537 p>0.01
Hybrid-2 (R-7 ♀ x VS ♂)	1	12/37	(000)	
	2	12/35	(000)	
	3	9/35	(000)	
	Total	58/107	Average (30.8)	0.0012 p<0.01
DDT-resistant (R-7)	1	6/30	(000)	
	2	9/35	(000)	
	3	12/38	(000)	
	Total	27/103	Average (25.8)	0.0119 p>0.01

^a= Number of head squashes positive/number examined on the 8th PI day.

Post-feeding virus titer = ranged between 2.0 to 2.3 logs MID₅₀ /0.02ml in three replicates

Percentages in parenthesis.

p-value: Pearson's coefficient; using χ^2 for proportions for head squashes positive and number tested for comparing controls with test groups.

stressed (VS) controls (Table1). In all these cases the susceptibility was decreased significantly.

RNA extraction and RT-PCR

RT-PCR performed on the RNAs extracted from the batches VS, VR and both the hybrids

showed that the overall level of expression of the Hsp70 was same, except in the case of the VS strain where level was decreased on the 8th PHSH. However, the RT-PCR performed on the RNAs extracted from the batches VS, R-7 and hybrids showed that the R-7 strain had an addi-

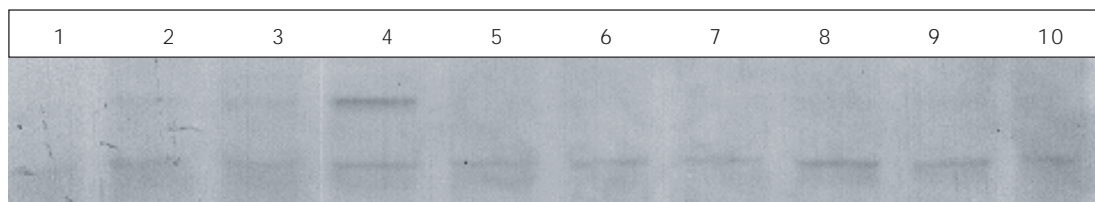


Fig 4—Western-blot analysis of translation of the Hsp70 protein: homogenate of larvae after heat shock at 41°C for 60 minutes: (DDT-resistant strain R-7) (1) 1 post heat-shock hour (PHSH), (2) 4 PSHH, (3) 8 PSHH, (4) 24 PSHH, (5) controls: (DDT-susceptible strain VS) (6) 1 PSHH, (7) 4 PSHH, (8) 8 PSHH, (9) 24 PSHH; (10) Controls.

tional band, and expression was high on the 4th PSHH. It was very interesting to note that the additional allele was observed only in the hybrids (HY-1), where male R-7 mosquitoes were crossed with female VS, while the level of expression was highest in HY-2 on the 24th PSHH after heat shock (Figs 1 and 2).

Western-blot and dot-blot assays

The Western-blot analysis performed on these batches showed that in VS normal control mosquitoes the level of the Hsp70 protein was highest on the 8th PSHH (Fig 3). In all these blots a single band was visible; except for R-7, where two bands were visible (Fig 4). To determine the quantitative changes, dot-blots were performed which showed an increased amount of Hsp70 protein in the heat shocked larvae (Fig 5).

DISCUSSION

For several decades it has been known that higher temperatures directly affect the vector competence of mosquitoes, by shortening the extrinsic incubation period of arboviruses. Only one report by Kay *et al* (1989) has suggested that this can affect vector competence indirectly. They reported that the larval rearing temperatures influence the flavivirus vector competence of adult mosquitoes. Our recent studies on this mosquito species with the CHIK virus also showed a similar phenomenon (Mourya *et al*, 2004).

Our data showed that in the case of normal larvae (VS) after heat shock the translation level of Hsp70 was found to be very high, at 8th PSHH, but the transcription level was low, compared to other batches. In the other batches of VR, neither of the hybrids showed any difference in transcription or translation. In the case of the

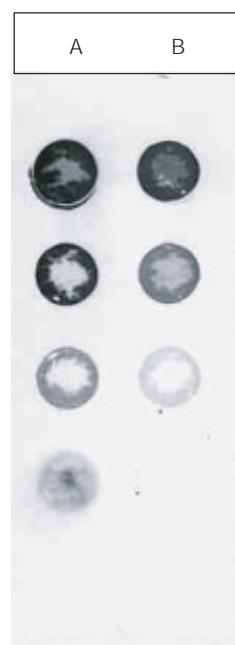


Fig 5—Quantitative dot-blot assays: (A) homogenates of larvae heat shocked at 44.5°C for 10 minutes; (B) homogenates of control larvae. Initial concentration of proteins in both lanes was 5 µg/dot; further dots were diluted 2-fold. They were then reacted with Hsp70 antibodies raised in mice and the binding was examined by anti-mouse antibodies tagged with alkaline phosphatase.

R-7 strain, during RT-PCRs, two bands were obtained after heat shock. It was interesting to note that these two alleles were also obtained only with HY-1, in which the R-7 males were used for the crosses. These findings were further supported by the Western-blot studies. During these experiments Hsp70 expression was studied in the larvae where the sex was not known. When specific sexes were used for the crosses in the

adult stages, the progeny larvae which were subjected to heat shock showed that the second allele was probably male sex linked, since in *Ae. aegypti* the sex determination genes are present on chromosome 1.

It appears that the higher expression of Hsp70 may have provided the DDT-resistant strains with a better survival chances during high temperatures in the environment, particularly in the homozygotes and the hybrids. It is also interesting to note that both these strains had significantly lower susceptibilities to the virus. We hypothesize from the data that in an area with wide-spread DDT-resistance, during a summer when the temperature rises above average, there may be a higher proportion of low-virus susceptible genotypes selected, which could promote increased insecticide resistance.

The association of these stresses on virus susceptibility was apparent since after heat-shock there was an increase in the susceptibility of mosquitoes to dengue virus as seen in the case of the VS strain, which did not express the second allele. Virus susceptibility in *Ae. aegypti* mosquitoes is known to be recessive and governed by several genes (Miller and Mitchell, 1991; Mourya *et al*, 1994a). It is interesting to note that in virus susceptible genotypes, the Hsp70 level was extremely low on at the 8thPHSH. In mosquitoes, the midgut is the primary site of virus replication, and after a few hours the virus disseminates from the midgut to other organs via the hemolymph. The lower expression of Hsp70 indicates that a lower Hsp70 response may help in reducing the host defense, thus indirectly helping to further disseminate the virus.

There are several families of heat shock proteins known to be expressed in mosquitoes. These may have a cumulative role in determining the susceptibility to the virus, which itself is governed by several genes. At this juncture it is difficult to say whether the stress responses due to insecticide and temperature may be interacting synergistically or antagonistically. To understand these mechanisms, a detailed study using microarray is required to see the up- and down-regulation of the genes involved in determining virus susceptibility in mosquitoes during such stresses.

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