EFFECT OF SUBLETHAL DOSAGES OF INSECTICIDES ON CHIKUNGUNYA VIRUS SUSCEPTIBLE AND REFRACTORY STRAINS OF *AEDES AEGYPTI*

DT Mourya, MD Gokhale and K Banerjee

National Institute of Virology, 20A Dr Ambedkar Road, Pune 411 001, India

Abstract. Three strains of *Aedes aegypti* mosquitos viz (i) CRS, refractory to Chikungunya (CHIK) virus by oral route of infection but susceptible to DDT (2) CSS, susceptible to CHIK virus and also susceptible to DDT (3) CSS-DDTR, susceptible to CHIK virus but resistant to DDT, were examined for the effect of sublethal dosages of DDT and deltamethrin on their fecundity.

Biochemical analysis showed that there was an increase in glutathione s-transferase activity in the CSS-DDTR strain which was associated with DDT resistance. There was an increase in acetylcholinesterase activity in the CRS strain, however it was not associated with resistance to all the three insecticides tested.

No significant differences in the fecundity of these three strains were observed, though there was some increase in the number of non layers in CSS-DDTR strain after the treatment of DDT and mean number of eggs laid by CSS and CRS strains was slightly reduced ($0.5 > p < 0.1$) after the treatment with deltamethrin.

INTRODUCTION

In India, dengue (DEN) and chikungunya (CHIK) viruses have been responsible for explosive epidemics in past. Recent studies have shown that refractivity/ susceptibility of *Aedes aegypti* to oral infection of DEN and CHIK viruses is genetically controlled and the refractivity genes are dominant (Miller and Mitchell, 1991; Mourya *et al,* 1994). Any normal population will contain both genotypes. Therefore presence of a high proportion of virus susceptible genotype in the mosquito population is essential for an outbreak of these viruses.

Recent field studies have shown that *Ae. aegypti* is spreading to the rural areas of Maharashtra state and has been responsible for several outbreaks of DEN (Ilkal *et al,* 1991). *Ae. aegypti* populations from the rural areas were found to be resistant to DDT due to an increase in glutathione-s-transferase (GST) activity (Mourya *et al,* 1993), while one such mosquito strain collected from a village during an epidemic of DEN which was resistant to DDT, showed higher susceptibility to CHIK virus than the virus refractory strain.

It is of great importance to know whether the extensive DDT spraying during antimalarial operations in rural areas might have selected for virus susceptible genotypes as well as for the DDT resistant genotype. There is no direct indication stating that, in the rural areas DDT spray might have selected both of the genotypes. Moreover in *Ae. aegypti* mosquitos genes responsible for DDT-resistance due to increased metabolism are situated on chromosome II (Munstarmann, 1990) while CHIK virus susceptibility/ refractivity gene(s) are on linkage group III (Mourya *et al,* 1994). Therefore selection pressure of insecticides would have to work independently on each.

There are two possible mechanisms by which proportion of CHIK virus refractory and susceptible genotypes might change in a population. 1) CHIK virus refractory mosquitos which are susceptible to DDT and CHIK virus susceptible mosquitos which are resistant to DDT may have differential fecundity that would tend to alter the frequency of the CHIK virus susceptible/refractory genotypes in a population; 2) alternatively, insecticidal stress like sublethal dosages of insecticides could have a differential effect on a CHIK virus refractory and CHIK virus susceptible genotype of mosquitos. Since in nature every insecticide degrades with time after its application, hence in the later period of application different genotypes present in a mosquito population would receive sublethal dosages and these may have distinctive effects on their fecundity. The present study was conducted to test these possibilities.

MATERIAL AND METHODS

Mosquitos

CHIK virus susceptible strain (CSS) : An *Ae. aegypti* strain was established from the main colony maintained at the National Institute of Virology, Pune by selecting the progeny of a female having low activity of GST. One hour exposure to 4% DDT impregnated papers showed that it was also susceptible to DDT.

CHIK virus refractory strain (CRS) : Similarly a sub-colony was established from a rosy eye *Ae. aegypti* mutant obtained from main laboratory colony, this strain was refractory to CHIK virus by oral infection as compared to CSS strain, but susceptible to DDT.

DDT resistant strain (CSS-DDTR) : A field collected strain which initially showed resistance to DDT was selected further with DDT for 15 generations, was used in the present study as resistant strain. This strain was susceptible to CHIK virus by oral infection as compared to the CRS mutant strain.

Viruses : The following virus strains of CHIK virus were employed in the study :

(1) Calcutta strain (634029) isolated from a patient during an epidemic occurred at Calcutta, India in 1963, used at 7th mouse passage level; stock used for experiments was prepared in ATC-15 cell line.

(2) Barsi strain (731468) isolated from a patient during an epidemic occurred at Barsi, India in 1973, used at 1st mouse passage level, stock was also prepared in mice.

(3) Senegal strain (8914670). Originally isolated from *Ae. luteocephalus* from Senegal, obtained from Yale University, Connecticut, USA. Used at 3rd mouse passage level; stock was prepared in mice.

The virus strains were assayed in infant mice by the intracerebral route of inoculation.

Infection of mosquitos through membrane : Dilution of the virus was made in defibrinated chicken (white leghorn fowl) blood. Four to five days old female mosquitos were fed on the infected blood through fresh chicken skin as described earlier (Banerjee *et al,* 1988). The stock of feeding suspension was prepared and then distributed into different feeding cups to feed different strains of mosquitos simultaneously.

Detection of virus in mosquitos : Detection of CHIK viral antigen in the head squashes of the mosquitos was done using indirect immunofluo rescence anti-bodies (IFA) technique (Ilkal *et al,* 1984; Mourya *et al,* 1987).

Larvae were reared in enamel pans containing tap water and were fed on a mixture of yeast powder and dog biscuit (1:1). Insectary conditions were kept at 28 ± 2°C, 80 ± 10 RH, and 13:11 LD. Adults were held in 30 × 30 × 30 cm screened cages and fed on 10% glucose solution in moistened cotton pads. Females were fed on white leghorn.

Bio-assay

Larval bio-assay : This was carried out with DDT using a standard kit from WHO. Ethanol concentrations of deltamethrin were prepared from the technical grade of insecticide which was provided by Roussel India Ltd. The WHO method was followed for larval bio-assay (WHO, 1981a). Probit analysis (Finneys, 1971) was applied to the mortalities obtained for the different concentrations of the larvicides to calculate the sublethal concentration LC_{30} and LC_{50} for different mosquito strains.

Adult bioassay : One or two days old, 20-25 unfed female mosquitos were exposed to the insecticide impregnated papers obtained from WHO for one hour. Tests were performed as per the protocol outlined by WHO (1981b). The dosage of DDT and deltamethrin were 4% and 0.025%, respectively. After exposure, the mosquitos were maintained in the insectary at 28 ± 2°C and 80-90% relative humidity (RH). The percent mortality count was done 24 hours after exposure. Cotton pads soaked in 10% glucose solution were provided during the recovery period of 24 hours.

Experiment : Larvae from each strain were divided into 3 batches. Each batch consisting of about 200 I-instar larvae, was placed in 250 ml of water in a 500 ml beaker. The larvae were fed as follows: 25 mg of feed/day/beaker for 4 days, followed by 50 mg for 2 days and 100 mg for the remaining period. Fresh feed was added to each beaker after changing the water every day. When the larvae reached III or IV stage, one batch was treated with ethanol as control. The other two batches were treated with LC_{30} and LC_{50} of the insecticide respectively.

Emerging adults were kept in small cages for 4-5 days to ensure mating. About 30 to 40 female mosquitos were removed from these cages and weighed

individually. After weighing, they were transferred to plastic vials (5 cm height and 3 cm diameter) with a nylon net at the top. The mosquitos were allowed to feed on chickens through the nylon netting. Immediately after feeding, the weight of individual fed females was determined. Each vial was provided with 10% glucose in cotton pads and a piece of wet blotting paper for egg laying. After 3-4 days of feeding, the egg count was made and the misquitos were provided with a second blood meal as per the procedure described above. This process was repeated up to the 3rd gonotropic cycle. Mosquitos which did not lay eggs were scored as non layers and were removed. The eggs thus obtained, were conditioned in dessicators and allowed to hatch separately to assess the effect of sublethal dosages of insecticide on the hatching rates.

Enzyme assays : Assays were performed on female mosquitos. Adults were collected after one day of eclosion and stored at -70°C until assayed. Mosquitos were homogenized in distilled water with the help of plastic pestle (Kontes) in microfuge tubes and centrifuged at 10,000 *g* for 10 minutes. The methods followed for esterase (Est A and B), acetylcholinesterase (AChE) and glutathione s-transferase (GST) was same as mentioned in an earlier communication (Mourya *et al,* 1992). The protein content was estimated in 40 µl of supernatant fluid from each individual homogenate by the method described by Lowry (Lowry *et al,* 1951). A reference standard protein curve was prepared using bovine serum albumin fraction 5.

RESULTS

Results of oral susceptibility of different strains to CHIK virus are given in Table 1. CRS strain was about 5 fold refractory to CHIK virus then the susceptible

Table 1

Comparative susceptibility of CRS, CSS and CSS-DDTR strains of *Aedes aegypti* to CHIK virus after oral infection.

Virus strain	Titer of feeding suspension	Mosquito strain					
		CRS		CSS		CSS-DDTR	
Calcutta strain (634029)	4.2	0/40* (F 3)	(0.00)	18/40	(45.00)	14/32 (F3)	(40.00)
Calcutta strain (634029)	4.2	3/64 (F 5)	(4.68)	9/40	(22.5)	ND	
Calcutta strain (634029)	4.2	1/50 (F 7)	(2.00)	10/69	(14.4)	ND	
Calcutta strain** (634029)	5.5	5/27 (F 13)	(18.57)	11/19	(57.89)	32/62 (F 11)	(51.61)
Senegal (8914670)	5.5	15/77 (F 8)	(19.43)	82/90	(91.11)	ND	
Barsi (731468)	4.8	2/22 (F 10)	(9.00)	10/32	(31.25)	ND	
Barsi (731468)	4.8	6/70 (F 13)	(8.57)	19/70	(27.14)	26/70 (F 16)	(37.14)
Total		32/350	(9.14)	159/360	(44.16)	72/167	(43.11)*

* = Number of head squashes positive/ number examined.
** = Virus stock was prepared in ATC-15 cell line.
Percentages in parenthesis.

Table 2

Comparative susceptibility of adults of different *Aedes aegypti* strains to diagnostic concentrations of insecticides.

Strains	% Mortality				
	DDT (4%)	Deltamethrin (0.025%)	Malathion (5%)	OC control	OP control
CSS	98.88 (89/90)*	100.00 (75/75)	100.00 (50/50)	2% (1/50)	0% (0/21)
CRS	98.57 (69/70)	100.00 (70/70)	100.00 (45/45)	0% (0/25)	0% (0/23)
CSS-DDTR (F1)	58.57 (41/70)	100.00 (67/67)	100.00 (56/56)	4% (1/25)	2.3% (1/43)
CSS-DDTR (F16)	8.00 (4/50)	98.33 (59/60)	100.00 (71/71)	4% (1/25)	0% (0/19)

* = (Number dead / Number tested)

Table 3

Various enzyme activities in different strains of *Aedes aegypti.*

Enzymes	Enzyme activity (SE)		
	Mosquito strain (n = 24)		
	CRS	CSS	CSS-DDTR*
GST[1]	A 39.914 (2.375)	34.752 (1.821)	64.946 (4.270)
	L 20.862 (2.358)	20.197 (1.675)	39.371 (3.558)
AChE[1]	A 93.819 (6.151)	64.816 (5.667)	75.267 (5.078)
	L 39.831 (4.238)	20.101 (1.777)	19.963 (1.777)
EST-A[2]	A 5.330 (0.160)	6.387 (0.251)	5.059 (0.189)
	L 5.528 (0.239)	4.618 (0.221)	3.916 (0.192)
EST-B[2]	A 4.024 (0.164)	3.392 (0.185)	3.989 (0.248)
	L 4.931 (0.346)	4.196 (0.284)	2.908 (0.170)

* : After 15 generations of continues selection pressure.
A : Activity in adults
L : Activity in larvae
1 : (μ mol/min/mg protein)
2 : (n mol/min/mg protein)

Table 4

Effect of sublethal dosages of deltamethrin on fecundity of *Aedes aegypti* mosquitos.

Deltamethrin treatment	Mean No. of eggs laid in different gonotropic cycles			Mean No. of eggs per gonotropic cycle (n)	% Non-layer	% Eggs hatched	Mean blood ingested/ cycle	Ratio of egg out put and blood ingested
	G1	G2	G3					
				Susceptible *Aedes aegypti* (CSS)				
Untreated	41.80	41.00	52.60	49.39 (17)	15.00	88.13	1.15	42.94
LC 30 (0.000411)	22.46	38.71	36.42	32.54 (16)	23.81	75.73	1.46	22.28
LC 50 (0.000511)	17.80	22.37	32.16	24.11 (20)	28.57	69.60	1.41	17.09
				DDT-resistant *Aedes aegypti* (CSS-DDTR)				
Untreated	53.4	51.6	57.85	54.28 (17)	22.72	86.37	1.53	35.47
LC 30 (0.002897)	64.33	70.6	68.8	67.68 (16)	27.27	76.41	1.81	37.20
LC 50 (0.003999)	52.11	45.66	54.25	50.70 (20)	33.33	68.57	1.55	32.70
				Susceptible *Aedes aegypti* (CRS)				
Untreated	37.28	45.00	60.00	47.42 (23)	11.53	94.74	0.92	51.55
LC 30 (0.000399)	22.30	26.20	46.85	31.78 (25)	19.35	84.34	0.97	32.76
LC 50 (0.000499)	15.60	19.00	29.83	38.09 (22)	26.66	71.95	0.88	21.47

CSS and CSS-DDTR strains. Difference in refractoriness of CRS strain was consistent with different filial generations and also with different CHIK virus strains.

Results of bioassays performed on these strains showed that all the three strains were susceptible to the insecticides tested except CSS-DDTR which was resistant to DDT and percent mortality reduced to 8% after 15 generations of selection pressure by DDT (Table 2). Biochemical assays performed on these mosquito strains are given in Table 3. GST activity in the CSS-DDTR strain was 1.868 fold higher in adults and 1.949 fold higher in larvae then the CSS strain, indicating that this strain was resistant to DDT. However, there was no difference in the GST activity of CSS and CRS strains. Similarly, the AChE activity was higher in CRS as compared to the CSS and CSS-DTR strains, though there was no resistance to Malathion. There was no difference in the esterase activity of the three mosquitos strains.

Results show that following insecticide treatment there was an increase in number of mosquitos which did not lay eggs in all the three strains treated with either DDT or deltamethrin (Table 4, 5). Although the increase in number of non layers was very high in CSS-DDTR mosquitos when treated with DDT (Table 5).

None of the mosquito strains showed difference in the mean weight of blood ingested when treated with any of the insecticides. When the ratio of mean blood

Table 5

Effect of sublethal dosages of DDT on fecundity of *Aedes aegypti* mosquitos.

Deltamethrin treatment	Mean No. of eggs laid in different gonotropic cycles			Mean No. of eggs per gono-tropic cycle (n)	% Non-layer	% Eggs hatc-hed	Mean blood inges-ted/ cycle	Ratio of egg out put and blood ingested
	G1	G2	G3					
				Susceptible *Aedes aegypti* (CSS)				
Untreated	43.88	44.62	51.70	46.77 (31)	08.82	88.13	1.01	46.41
LC 30 (0.0079)	65.21	59.81	49.63	58.21 (32)	11.11	92.02	1.32	44.09
LC 50 (0.0176)	57.81	67.44	39.00	54.79 (25)	19.35	93.66	1.23	44.51
				DDT-resistant *Aedes aegypti* (CSS-DDTR)				
Untreated	59.90	75.75	62.50	66.05 (16)	20.00	84.05	1.62	40.77
LC 30 (5.00)	72.00	57.50	76.00	68.50 (7)	65.00	79.04	1.48	46.28
LC 50 (10.00)	56.00	73.00	56.00	59.92 (13)	48.00	88.97	1.75	35.23
				Susceptible *Aedes aegypti* (CRS)				
Untreated	60.00	52.60	50.00	54.20 (19)	13.63	96.08	0.95	57.23
LC 30 (0.008)	46.68	47.37	46.85	46.90 (31)	22.50	87.77	1.03	45.86
LC 50 (0.0161)	55.00	49.83	43.00	49.27 (20)	37.50	92.59	1.01	48.59

ingested and mean number of eggs laid (BI/EL) in each gonotropic cycle were compared, it was low only in the case of CSS ($\chi^2 = 6.35, 0.5 > p < 0.1$, df;2) and CRS ($\chi^2 = 4.35$, $0.5 > p < 0.1$, df;2) when treated with LC_{50} deltamethrin (Table 4). No reduction in BI/EL ratio was seen with DDT treatment (Table 5).

Although the hatching percentage of eggs was reduced in all the three mosquito strains with deltamethrin treatment at LC_{50} doses but it was not significant, no reduction in percent hatching was noticed with DDT treatment in any of the mosquito strains (Table 4, 5).

DISCUSSION

It has been a general practice to determine the effect of insecticides by relating the mean of number of eggs with different concentrations of insecticides, while the mean weight of blood ingested by the mosquitos is not considered to determine the effect of insecticides. Therefore instead of studying the relationship of mean number of eggs with insecticide concentrations we have studied the relationship of BI/EL ratio with insecticide concentrations.

Earlier studies conducted by Firstenberg and Sutherland (1981). On *Ae. aegypti* showed that the egg production increased when the larvae were treated with DDT at 0.1 ppm dose. The present study showed almost no effect on BI/EL ratio with DDT in case of any of our DDT susceptible (CSS and CRS) strains. Perhaps the susceptibility of our strain is greater because at 0.1 ppm there was over 98% mortality. However significant reduction of BI/EL

ratio was observed, only when the DDT-susceptible (CSS and CRS) mosquitos were treated with deltamethrin.

Reyes-Villanueva *et al* (1991), studied the effect of the application of different concentrations of Abate on *Ae. aegypti* and the mean number of eggs laid by the mosquitos. They suggested that by extrapolating the regression line obtained by these parameters it would be possible to arrive at a dose of insecticide which would result in the total suppression of laying of eggs. However from our studies it is apparent that no such exrapolation of dose of DDT and deltamethrin is possible because it would result in 100% mortality of the mosquitos. Therefore determination of the dose which would result in total suppression of eggs is not possible. An alternative strategy of mosquito control by the application of sublethal doses of an insecticide which would result in the total suppression of laying of eggs as suggested by Reyes-Villanueva *et al* (1990) is not applicable in the case of DDT and deltamethrin.

From the present data it appears that there was no appearent difference in the fecundity of CSS, CRS and CSS-DDTR strains, even when these strains were treated with insecticides. However further studies encluding field observations are required to confirm if there is any correlation of susceptibility of *Ae. aegypti* mosquitos to CHIK virus with insecticides resistance.

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