J Vector Borne Dis 52, September 2015, pp. 261–264

Deltamethrin induced functional mortality of *Anopheles stephensi*, the urban malaria vector, in relation to resistance development

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Key words *Anopheles stephensi*; deltamethrin; functional mortality; leg loss; resistance

Anopheles stephensi Liston (Diptera: Culicidae), the urban malaria vector, accounts for about 15% of the total malaria incidence in India[1]. The vector control primarily relies on the use of chemical insecticides like synthetic pyrethroids in the form of insecticide treated nets (ITNs), long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)[2]. In urban areas, IRS is not feasible; hence use of ITNs and LLINs could provide an effective protection against transmission of malaria. Besides LLINs, pyrethroids are popularly used in the form of commercial mosquito repellents such as mats, liquidators and coils.

Deltamethrin is one such synthetic pyrethroid receiving much attention worldwide for its use in the control of mosquito vectors especially malaria vectors due to its known higher efficacy against them[3]. Further, deltamethrin is one among the six pyrethroid insecticides recommended for treating mosquito nets. It acts on voltage gated sodium channel (VGSC) and by interrupting the action potential, it causes the neuron to fire spontaneously leading to eventual death of the target insects. However, selection pressure due to continuous use of synthetic insecticides can result in development of resistance[4].

Continuous insecticide pressure selects an insect population with (i) an altered integument permitting lesser penetration of insecticide, (ii) modifications in the structure or the expression level of detoxifying enzymes, (iii) mutations in the insecticide target sites of insects that prevent interaction with the insecticide, (iv) behavioural changes, such as avoiding contact with the insecticide[5], and (v) increased cuticular thickness[6]. While some modifications or alterations conferring a selective advantage spread quickly in a given population under insecticide pressure, they can be associated with a high fitness cost[5]. In this paper, we present the results of induced leg loss leading to functional mortality of *An. stephensi* under deltamethrin selection pressure in the laboratory over generations.

The laboratory reared strain of *An. stephensi*[7], which is susceptible to deltamethrin, was used for the study. Female mosquitoes were allowed to feed on an artificial membrane (Parafilm 'M') blood feeder (defibrinated bovine blood warmed at 37°C), from the Day 3 of post-emergence. Enamel bowl (22 × 18 × 5 cm) filled with dechlorinated water and lined with Whatman filter paper were used to collect the eggs. The eggs were transferred to rearing trays for hatching. The cyclic colony was maintained in the laboratory at 27 ± 2°C and 70 ± 10% RH with a photoperiod of 14 and 10 h darkness.

Adult susceptibility test kits[8] and deltamethrin impregnated papers with the diagnostic concentration of 0.05% were procured from the University of Sains Malaysia, Penang, Malaysia. Deltamethrin impregnated papers at different sublethal concentrations (ranged from 0.004 to 0.025%) were prepared in the laboratory as per the WHO protocol[8]. Adult mosquito bioassays were conducted using WHO test kits[8]. Three days old, 25 non-blood fed females of *An. stephensi* were released into the holding tube for one hour acclimatization. After acclimatization, only the healthy mosquitoes were allowed to fly from the holding tube to the exposure tube lined with deltamethrin impregnated paper. After exposure to the insecticide for one hour, the alive mosquitoes were allowed to fly back to the holding tubes and the knocked down/dead mosquitoes were gently blown to the holding tube by keeping it to downward position, and held at 27 ± 2°C and 70 ± 5% RH for 24 h. Cotton pads soaked in 10% sugar solution were provided as food source. At the end of the holding period, the tubes were shifted to a clean cloth cage where the surviving mosquitoes were allowed to get released from the tubes by opening their lids. Dead mosquitoes (true mortality) were removed and counted.

The alive mosquitoes were then collected individually using a glass aspirator, observed for the number of legs present in each mosquito and released into a separate clean cloth cage (30 × 30 × 30 cm). Similar procedure was followed for the controls. The survivors were then kept for subsequent blood feeding. Utmost care was taken during handling of mosquitoes to make sure that there was no physical injury/morphological deformity to mosquitoes. Four replicates were maintained for each experiment with parallel controls. When mortality in the

control group ranged from 5 to 20%, the mortality in the exposed group was corrected using Abbott's formula[9]. If the mortality exceeds 20% in the control, the test was repeated.

The susceptible strain of *An. stephensi* was put under selection pressure in the laboratory by exposing adult female mosquitoes to the deltamethrin sublethal concentration of 0.004% (F1–F19), selected initially based on the number of survived female mosquitoes found adequate to raise the next generation. However, in order to increase the insecticide pressure, female mosquitoes were subsequently exposed to 0.005% (F20–F31), 0.007% (F32–F36) and 0.01% (F37–F40). The survivors were used to bring out the next progeny for subsequent generations (F1, F2 and so on). Such selection was continued up to 40 generations. Controls (unexposed) were maintained parallely throughout the study for comparison.

At the end of every 4th generations, *i.e.* F1, F4, F8, F12, F16, F20, F24, F28, F32, F36 and F40, the adult mosquitoes survived with only three legs and/or one wing were included as functionally dead ones (functional mortality). The functional mortality was calculated using the following formula.

$$\text{Functional mortality (\%)} = \frac{\text{No. of mosquitoes lost at least three legs and/or one wing}}{\text{Total number of mosquitoes observed}} \times 100$$

Corresponding to the number of survived mosquitoes in each exposure test, an equal number of mosquitoes were randomly selected from the unexposed/controls and examined for comparison.

For further comparison, field strain of *An. stephensi* was obtained from Thiruvanmiyur (13.00249°N, 80.2561°E), Adayar (13.0063°N, 80.2574°E), George Town (13.0939°N, 80.2839°E) and Besant Nagar (13.0002°N, 80.2668°E) of Chennai City (Tamil Nadu, India), where this species has been the vector of malaria. Larvae collected from these sites were brought to VCRC laboratory and reared. Larval skins were mounted and identified to species[10]. The F1 progeny (3-days-old) were subjected to insecticide adult exposure test[8].

The number of legs lost in survived mosquitoes of the exposed group was compared with that of the control group using the Fisher exact test in EpiCalc 2000 version 1.02. Similar comparison was also made between the exposed and the control mosquitoes of the field strain.

The laboratory maintained parent colony of *An. stephensi* was fully susceptible to deltamethrin 0.05% (diagnostic dosage) as evidenced by the 100% corrected mortality (Table 1). Partial survival (13%) of exposed mosquitoes was first observed at the sublethal dosage of 0.004%. The highest survival (30%) to the sublethal dosage was recorded at generation F28. The increased survival over generations was indicative of an increased tol-

Table 1. Leg loss and functional mortality in *An. stephensi* exposed to sublethal dosages of deltamethrin in comparison to unexposed controls and field strain

Sublethal dosage of deltamethrin (%)	Generations	No. of mosquitoes survived at 24 h after exposure (n =100)	Number of legs lost by mosquitoes									Functional mortality (%)*	
			Forelegs			Middle legs			Hind legs				
			Exposed	Control	p-value	Exposed	Control	p-value	Exposed	Control	p-value	Exposed	Control
0.05**	Parental	0	–	–	–	–	–	–	–	–	–	–	–
0.004	Field†	20	0	0	–	0	0	–	3 (3)	0	0.077	0	0
	F1	13	2 (2)	0	0.149	6 (5)	1 (1)	0.036	15 (10)	0	0.0	23.1 (3/13)	0
	F4	20	2 (2)	0	0.152	3 (3)	0	0.077	14 (10)	0	0.0	10 (2/20)	0
	F8	26	2 (2)	0	0.153	2 (2)	0	0.153	9 (6)	0	0.001	0	0
	F12	26	1 (1)	0	0.314	3 (3)	1 (1)	0.298	6 (5)	0	0.011	0	0
	F16	27	2 (1)	0	0.153	2 (2)	0	0.153	5 (5)	0	0.022	0	0
0.005	Field†	19	0	0	–	2 (2)	0	0.151	5 (3)	1 (1)	0.088	0	0
	F20	16	0	0	–	3 (3)	1 (1)	0.301	7 (6)	1 (1)	0.023	6.25 (1/16)	0
	F24	26	0	0	–	1 (1)	0	0.314	6 (5)	0	0.011	0	0
	F28	30	0	0	–	2 (2)	0	0.153	4 (3)	1 (1)	0.170	0	0
0.007	F32	20	0	0	–	0	0	–	4 (4)	1 (1)	0.165	0	0
	F36	27	0	0	–	0	0	–	4 (3)	1 (1)	0.169	0	0
0.01	F40	17	0	0	–	1(1)	0	0.313	3 (3)	0	0.076	0	0

*There was no loss of wings in both exposed and control groups at all generations in survived mosquitoes; **Diagnostic concentration of deltamethrin; †The field strain (Chennai) of *An. stephensi* was exposed once only to each of the two sublethal dosages of deltamethrin for functional mortality.

erance of the adult *An. stephensi* to deltamethrin.

After exposure to the sublethal concentrations of deltamethrin, there was a loss of forelegs, though in small numbers (1–2), from generations F1–F16 in the exposed group. Subsequently from generation F20 and up to F40, there was no loss of forelegs in the exposed as well as in the controls, of all generations. There was no significant difference ($p>0.05$) in foreleg loss between the exposed and the control. In the case of middle legs, loss was observed in both the exposed and the control groups. In the exposed group, the number of mosquitoes which lost their legs ranged from 1 to 5 from generation F1–F28. In the controls, the middle leg loss was very meagre and recorded only at F1, F12 and F20 generations. The middle leg loss was significantly higher in the exposed group than the controls ($p<0.05$) only at generation F1 and not at other generations. Compared to fore and middle legs, the loss of hind legs was higher among the exposed mosquitoes, ranged from 3 to 10 from generations F1–F40. The maximum number of mosquitoes losing their hind legs (10 nos.) was observed at generation F1 and F4; however, the number gradually reduced in subsequent generations and reached the minimum value of 3 at generation F40. In the controls, one hind leg was found lost in one mosquito each at generation F20, F28, F32 and F36. The hind leg loss was significantly higher among the exposed from generation F1 to F24 than the controls ($p<0.05$), while at generations F28 to F40, the loss was not significantly different between the exposed and the controls ($p>0.05$). There was no loss of wings in survived mosquitoes of both exposed and control group sat all generations. The functional mortality, thus based only on loss of legs, was observed at generations F1 (3 nos.), F4 (2 nos.) and F20 (1 no.).

In the field (Chennai) strain, there was no loss of forelegs on exposure to the sublethal dosages of 0.004 and 0.005%. In the case of middle legs, while no loss was observed on exposure to 0.004%, there was a loss when exposed to 0.005%, but the loss was not significantly different from the control ($p=0.151$). Hind leg loss was observed with both the dosages, but the loss was not significant when compared to the controls ($p>0.05$). There was no loss of wings. Functional mortality was not observed in the field strain on exposure to the sublethal concentrations.

In the current study, the functional mortality of *An. stephensi*, based on loss of legs on exposure to sublethal dosages of deltamethrin was studied. The functional mortality refers to mosquitoes that are disabled and would not survive in the wild; usually this includes mosquitoes that have lost at least three legs and/ or one wing whereas, true mortality refers to those which are not able to move[11]. With the sublethal exposure over generations, the mortality (true mortality) of *An. stephensi* was found decreasing and the survival increasing, indicating its increased tolerance with reduced number of leg loss at later generations. In the experiment, 23.1 and 10% of functional mortality were recorded at generations F1 and F4, respectively, against the sublethal concentration of 0.004%. There was no functional mortality at subsequent generations, F8, F12 and F16. When exposed to the next higher sublethal concentration of 0.005%, the functional mortality of 6.2% was observed at F20 and not at other generations up to F40.

The efficacy of deltamethrin against different life stages of *An. stephensi* has been well documented in India[3]. The mode of action of synthetic pyrethroids on mosquito nervous system closely resembles that of DDT[12], penetrating mainly through legs. Though, the primary target of synthetic pyrethroids is ganglia of insect central nervous system, some pyrethrin-poisoning effects were also observed in detached legs[13], which negatively impact survival-hood of live mosquitoes.

The susceptibility assays conducted for *An. arabiensis* against deltamethrin-treated PermaNet showed 88% mortality, 6% surviving with 1–3 legs and 6% surviving with 4–6 legs[14]. Similarly, sandflies, when exposed to deltamethrin nets in cone bioassays lost some of their legs before dying. However, the surviving and flies were able to fly with the loss of legs, sometimes up to four[15]. When testing synthetic pyrethroids with adult mosquitoes, surviving individuals were commonly found with many legs missing, sometimes up to five[16]. In the current study, though there was a loss of forelegs initially up to generation F16, no loss was noticed subsequently from F20 to F40. In the field strain also, there was no loss of forelegs (Table 1). The reason for this could be due to the fact that in forelegs only claws and extremity of 5th tarsal segment touch the treatedsurface[17]. Middle leg loss was noticed in the exposed group almost at all generations, though it was significant ($p<0.05$) only at generation F1, because the middle legs are also occasionally used by mosquitoes for resting, making the contact with the surface through the joint between 2nd and 3rd tarsal segments[17].

There are no additional reports available regarding significant loss of forelegs and middle legs due to contact with the treated surface. Hind leg loss was significantly higher in the exposed mosquitoes ($p<0.05$) from generation F1 to F24. Since, hind legs touch the surface with tarsal segments 2, 3, 4 and 5 and are always in close contact with the surface, they pick up insecticide particles in

greater quantity. Therefore, removal of hind legs resulted in much reduced mosquito mortality in DDT susceptibility tests[17]. This demonstrated that insecticide particles penetrate primarily through hind legs. Since, high insecticide penetration occurs through hind legs and unusually a high density of synthetic pyrethroid receptors (subset of tarsal Na^+ channels on which synthetic pyrethroids are more active) are present in the tarsi of hind legs[18], pyrethroid treatment would normally cause a high level of mortality to susceptible mosquitoes. This also includes the functional mortality through loss of hind legs as observed in the current study. Such fitness disadvantage was overcome by mosquitoes when they were under continuous selection pressure with insecticides through adopting modifications to their advantage such as increasing cuticular thickness of hind legs that tends to reduce the degree of contact and slow down the penetration of toxic effect of insecticide, and is likely to be a reason for development of resistance also (VCRC unpublished data)[6]. This could be the reason for not losing hind legs significantly ($p<0.05$) at generations F28, F32, F36 and F40 as recorded in the current study.

Loss of legs in the field (Chennai) strain was low and this could be due to the prolonged selection pressure in the field exerted from different forms of insecticides. Thus, a decline in leg loss in a population under selection pressure could be an indication that it started moving towards resistance development. Nevertheless, application of synthetic pyrethroids, where *An. stephensi* occupy a niche that remain unexposed to any insecticide will render an effective control due to both true and functional mortality.

ACKNOWLEDGEMENT

The authors are grateful to Dr P. Jambulingam, Director and Dr A.M. Manonmani, Scientist 'F' and Head, HRD, VCRC for the facilities provided. We thank Dr S. Subramanian, Scientist 'E' for his help in statistical analysis, and Mr. T. Vijayakumar, Mr. R. Hemasankar and Mr. K. Manojkumar, Division of Vector Biology and Control, for their technical support.

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Received: 24 November 2014 *Accepted in revised form:* 25 March 2015