Annals of Genetics and Genetic Disorders

Research Article
Published: 05 Sep, 2018

Inheritance Pattern of Cypermethrin Resistance, a Synthetic Pyrethroid Insecticide, in *Aedes aegypti* (L.), Vector for Dengue, Dengue Haemorrhages and Chikungunya

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Abstract

Resistance to the synthetic pyrethroid cypermethrin in *Aedes aegypti* was induced in the laboratory and the genetics of its inheritance is studied and described in this paper. To synthesise a Homozygous Resistant (R) and Susceptible (S) strains, late third instar larvae were exposed to a diagnostic dose of 0.004 mg/L for 33 and 13 generations respectively. The two contrasting strains were crossed for genetic analysis aimed at determining inheritance pattern of cypermethrin resistance (*cyp*). The log-dosage probit mortality relationships and degree of dominance (D) was calculated. The gene *cyp* in *Ae. aegypti* is found to be autosomal, monofactorial with incomplete dominance.

Keywords: Insecticide; Tolerance; Mosquito; Genetics; Susceptibility

Introduction

Aedes aegypti is the common vector of Yellow fever, dengue, chikungunya and zika viruses [1,2]. WHO reports that the year 2016 was witness to large dengue outbreaks worldwide, and estimates that there are around 390 million dengue infections per year worldwide, with 3.9 billion people in 128 countries at risk of infection [3]. Aedes mosquitoes have caused 349936 suspected chikungunya cases in 2016 [4]. Aedes is endemic over large areas of tropics and subtropics and dengue is the most rapidly spreading mosquito-borne viral disease in the world Control of Aedes vectors is the main way to prevent arboviral disease transmission [5-7]. Chemical control programmes are threatened by the development of resistance to insecticides as seen in literature for *Ae. Aegypti* [8-11]. Cypermethrin is a non-systemic, broad spectrum, insecticidal pyrethroid, with rapid knockdown activity and is used in public health programmes to control insect pests, and also as insecticide in agriculture and registered by Ministry of Agriculture, Government of India for house-hold use against insects [12-14].

Voltage-gated sodium channels are the primary targets of pyrethroid insecticides. In various insects including mosquitoes, mutations in the sodium channel known as knockdown resistance (kdr) are responsible for pyrethroid resistance [15]. Insecticide resistance over a period of time would cause failure of effects of the insecticide used, and the implications of this on public health would be disastrous (WHO 2012) To control insecticide resistance, WHO (2012) recommends improvement of understanding of resistance, so as to adapt a suitable strategy for sustainable vector control [1]. The paper describes mode of inheritance of cypermethrin resistance in *Ae. aegypti*.

Materials and Methods

Mosquito rearing

Collection of field population of *Ae. aegypti* larvae was done from Jaya Prakashnarayan Nagar (JPN), Bangaluru, India. They were cultured in an insectary, according to according to method used by Shetty [16], at 25 ± 1°C, relative humidity 75 ± 5%, and a 14-hour photoperiod. Adult mosquitoes were reared in iron-framed cages of cotton net cloth, and were fed with 10% sucrose solution in cotton pads, ad-libitum. Female mosquitoes were regularly provided with blood meal from mice, after maturity to facilitate egg-laying. Plastic cups (3 in. in diameter) containing clean water, lined with filter paper were placed inside the cage for oviposition. Powdered yeast was used as larval diet.

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Received Date: *09 Jun 2018*
Accepted Date: *29 Aug 2018*
Published Date: *05 Sep 2018*

Citation:
Geetha CK, Shetty NJ . Inheritance Pattern of Cypermethrin Resistance, a Synthetic Pyrethroid Insecticide, in Aedes aegypti (L.), Vector for Dengue, Dengue Haemorrhages and Chikungunya. Ann Genet Genet Disord. 2018; 1(1): 1004.

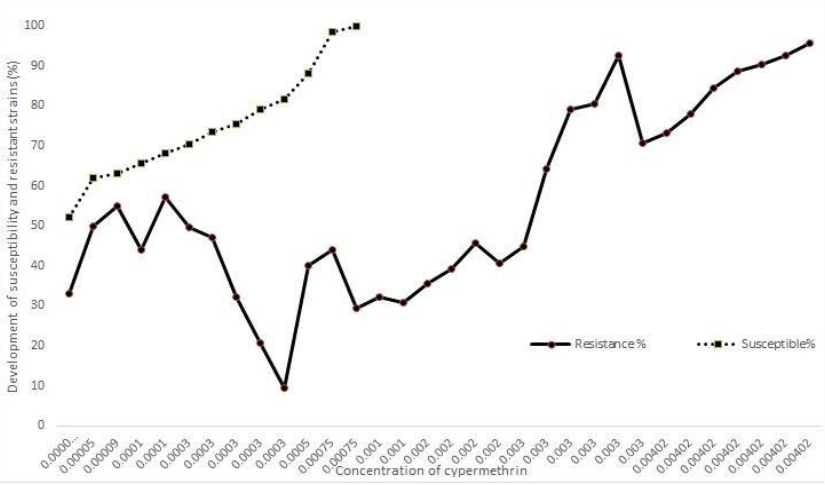

Figure 1: Development of homozygous cypermethrin resistance and susceptible strains of *Ae. aegypti* in each generation 100% susceptible and resistant strains were achieved after inbreeding for 13 and 33 generations respectively.

Insecticide

The International Union for Pure and Applied Chemistry (IUPAC) nomenclature for cypermethrin is [cyano-(3-phenoxyphenyl) methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate (C22H19Cl2NO3).

Larval bioassay

The stock solutions of cypermethrin were prepared using denatured alcohol (98 ml of absolute alcohol+2 ml of methyl ethyl ketone) as a solvent. The stock and a range of working solutions of increasing concentrations were prepared following the procedures described by WHO [17,18]. The initial phase of the study was conducted exposing 25 early fourth instar larvae batches with 4 replicates in 500mL glass beakers with 1mL of the designated concentration of cypermethrin and made up to 250 mL with dechlorinated tap water. Mortality was observed after 24 hr of treatment and percentage mortality was calculated WHO [17,18].

Selection of diagnostic dose for cypermethrin

LC_{99} of cypermethrin was determined from larval bioassay of JPN strain using regression line method, and this was used to calculate the diagnostic dose of cypermethrin [19]. According to WHO [17,18], twice the value of LC_{99} is fixed as diagnostic dose to synthesise homozygous resistant (R) and susceptible (S) strains for insecticide, and 0.004 mg/ L was calculated to be the diagnostic dose of cypermethrin.

Synthesis of cypermethrin resistant and susceptible strains

Cypermethrin Resistant strain: JPN strain from Bengaluru was used as the experimental population, which was subjected to selection by exposure of late third instar larvae to diagnostic dose (i.e1.99 mg/l) of cypermethrin. After 24 hrs, surviving larvae from the test group showing lowest mortality of iso female population were selected, maintained separately and inbred. The larvae of successive generations were mass treated with sub diagnostic doses of cypermethrin and the surviving larvae were inbred to obtain further generations, and this process was repeated till a pure homozygous resistant strain was established for the diagnostic dose of 0.004 mg/l which took 33 generations of inbreeding.

Cypermethrin susceptible strain: JPN strain of *Ae. aegypti* was used to establish susceptible strain. Half the number of larvae from isofemales of JPN were exposed to diagnostic dose of 0.004 mg/l. The remaining half of the batch showing the highest mortality were selected for inbreeding, and selection was continued until a homozygous susceptible strain for cypermethrin was developed. After inbreeding for 13 generations, susceptible strain was developed.

Genetic study of cypermethrin resistance

Twenty five pairs of freshly emerged females and males of homozygous resistant (R) and susceptible (S) strains, were used to carry out reciprocal genetic crosses (S♂xR♀ and R♂xS♀). A part of the F_1 offspring were subjected to inbreeding to obtain F_2 generation, and the remaining F_1 offspring of both sets of genetic crossing in parental generation, of both sexes were backcrossed (F_1xS) with parental type S. Additionally, the third instar larvae from all crosses were subjected to larval bioassays. For all the genetic crosses, the log-dosage probit mortality relationships were recorded [19] and the degree of dominance (D) was calculated using Stone's formula [20] $\boldsymbol{D= 2X_2 - X_1 - X_3 / X_1 - X_3}$ where D is degree of dominance and X_1, X_2 and X_3 are logarithms of the LC_{50} values of the resistant, F_1 hybrid and susceptible strains respectively. The value of D indicates the mode of inheritance of resistance, i.e. if D=1, the trait is completely dominant, if $0<D<1$ incompletely dominant, if $-1<D<0$ incompletely recessive, or completely recessive ($D = -1$).

Data analysis

The LC_{50} and LC_{90} values of cypermethrin were calculated according to the method of Finney [19] and the dosage mortality (d-m) lines were constructed using data of larval bioassays. Data analysis was carried out in Microsoft Office Excel 2010. Mortality data were obtained from the test groups as well as in the control, if any, and Abbot's formula was used to correct the data from the larval bioassays [21]. Chi square (χ^2) values were calculated [22].

Results

Homozygous resistant and susceptible strains of *Ae. aegypti* to 0.004 mg/l of cypermethrin were synthesized by continuous selection and inbreeding for 33 and 13 generations respectively (Figure 1).

Genetic crossing of the two contrasting strains were performed,

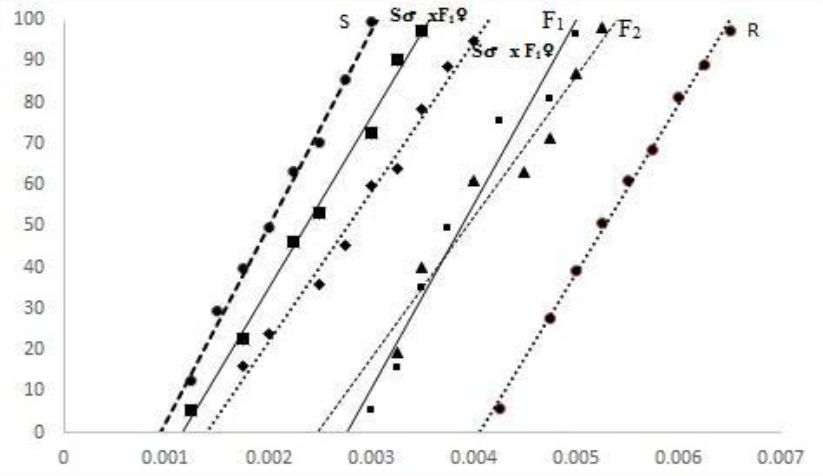

Figure 2: Dosage-mortality relationships of cypermethrin resistant and susceptible strains of *Ae. aegypti*. The crosses involved included Parental (S, R) Reciprocal (F_1) Backcrosses (S♂xF_1♀and S♀xF_1♂) and F_2 generation.

Table 1: Inheritance pattern of cypermethrin resistance in *Aedes aegypti.*

S. No.	GENETIC CROSSES	NO OF FEMALETESTED	NO OF LARVAE TESTED	RESISTANT		SUSCEPTIBLE		χ^2
				ALIVE	%	DEAD	%	
	Parental							
1	S♂xS♀	25	2165		_	2165	100	_
2	R♂xR♀	25	1952	1952	100	_	_	_
	F_1 generation							
3	S♂xR♀	25	1956	1042	53.27	914	46.73	0.214*
4	R♂xS♀	25	1924	1082	56.24	842	43.76	0.779*
	Backcrosses							
5	S♂xF_1♀(Cross 3)	25	1875	974	51.95	901	48.05	0.076*
6	S♀xF_1♂ (cross 3)	25	1921	1022	53.2	899	46.8	0.204*
7	S♂xF_1♀(cross 4)	25	1963	1061	54.04	902	45.96	0.326*
8	S♀xF_1♂ (cross 4)	25	1820	960	52.74	860	47.25	0.15*
	F_2 generation							
9	F_1♂xF_1♀(cross 3)	25	2241	1239	55.29	1002	44.71	0.559*
10	F_1♀xF_1♂ (cross 4)	25	2123	1138	53.6	985	46.4	0.259*

the results of which are presented in Table 1. Crosses 1 and 2 indicated clear homozygosity to resistance (100% survival) and susceptibility (100% mortality) respectively. The expected segregation of the backcross (cross 5-8) of the RS (F_1 hybrid) to the S strain for the monogenic Mendelian inheritance was calculated using the formula [23].

$$x(BC) = \left(\frac{1}{2}\right) a_1 (RS) + \left(\frac{1}{2}\right) a_2 (S)$$

Where x is the expected response of the backcross at a given dose and a_1 and a_2 are observed responses of RS and S lines. 50 % survival is the expected value, since half of the offspring which are susceptible will perish leaving behind 50% heterozygotes which would survive the exposure. Backcrosses were performed with homozygous susceptible parent which resulted in 56.81, 54.2, 53.09 and 50.31 % respectively (Crosses 5,6,7,8), which showed no significant difference from the expected value (P<0.05).

The expected F_2 segregation was calculated by the formula Georghiou and Garber [23].

$$x(F_2) = \left(\frac{1}{4}\right) a_1 (R) + \left(\frac{1}{2}\right) a_2 (RS) + \left(\frac{1}{4}\right) a_3 (S)$$

where, x is the expected F_2 to the diagnostic dose and a_1, a_2 and a_3 are observed responses of R, RS and S population to the dose. The expected response would be 50%. F_2 progeny which are result of crosses 9 and 10 showed 54.8 and 56.72% respectively. D-m lines (Figure 2) of resistant, susceptible lines, F_1 hybrids and F_2 progeny were constructed. The d-m line of F_1 was inclined more towards the resistant line (Figure 2) and D value was calculated to be 0.384. The null hypothesis (P<0.05) of monogenic resistance was tested from mortality data of backcross progeny compared with theoretical expectations using the χ^2 test.

The d-m lines of parental strains as well as that of the F_1 offspring were straight in nature, indicating that resistance and susceptibility to cypermethrin (*cyp*) was homozygous. The D value of 0.384 which lies in between 0 and 1; reciprocal crosses (Crosses 3 and 4) having more than 50% resistance and the position of d-m lines of being towards the resistant parent all suggest that *cyp* is incompletely dominant.

Discussion

This paper discusses the results of study of mode of inheritance of *cyp* in *Ae. Aegypti* in an Indian strain. The d-m lines derived from the parental crosses were straight as characteristic of a homozygous resistance trait. F_1 d-m lines were also derived as straight lines. D-value of 0.384 suggests incomplete dominance of cypermethrin resistance trait. The two crosses involving F_1 hybrid showed similar effects on exposure to cypermethrin, indicating resistance is monofactorial. The backcrosses F_1x S resulted in almost a genetic ratio of 1: 1 of resistant and susceptible individuals. F_2 mortality was in the ratio S: RS: R which is 1:2:1, which was close to the expected ratio. These results suggest that *cyp* follows monogenic mode of inheritance, which is similar to earlier studies on insecticide resistance which exhibit an autosomal monogenic inheritance in *Anopheles stephensi* and similarly in *Aedes aegypti* [24-30]. Insecticide resistance can be monogenic, polygenic or sometimes a single entity is responsible for resistance with cross-resistance to another insecticide.

Resistance to cypermethrin has been discovered and studied in many insect pests including mosquitoes [13,26,31,32]. Beta-cypermethrin resistance in *Musca domestica* was discovered to be caused by a single autosomal gene, which is inherited as an incompletely recessive factor [33]. In *An. stephensi*, cypermethrin resistance is due to a single gene, which is incompletely dominant and autosomal [26]. Also, higher oxidative and hydrolytic detoxification enzyme activities are associated with cypermethrin resistance in insects [32].

Insecticide resistance is viewed as an extremely serious threat to crop protection and vector control, and is considered by many parties, including industry, the WHO, regulatory bodies and the public, to be an issue that needs a proactive approach.

Resistance to pyrethroid insecticides in mosquitoes is mainly conferred by two mechanisms: (1) a mutation in the voltage-gated sodium channel (either by a substitution of the amino acid leucine with phenylalanine [Leu → Phe] or serine [Leu → Ser] at the same position in domain IIS6 of the protein) or (2) by elevated levels of microsomal monooxygenases [34]. Enzymes responsible for neutralising the activity of insecticides in organisms are transcribed by detoxifying genes which are abundant in number in *Ae. aegypti* [35,36] Further studies on insecticide resistance might lead to the discovery of new novel genes responsible for resistance, which would help in vector control.

Insecticide resistance is an extremely serious threat to crop protection and vector control, and is considered by industry, the WHO, regulatory bodies and the public, to be an issue that needs a proactive approach [34]. Resistance has developed to every chemical class of insecticide, including microbial drugs and insect growth regulators and deeper understanding of how resistance arises and maintains itself in populations requires molecular genetics studies [35]. This study can therefore help in expand current knowledge on the mechanism of cypermethrin resistance, and help in providing an insight while framing vector control programmes.

The gene *cyp* established in this study, is a monofactorial, autosomal incompletely dominant gene which will serve as a good marker for *Ae. aegypti.* In *Cx. quinquefasciatus* a sexing strain was synthesized for the preferential elimination of females during early larval stage by using a Malathion resistance gene linked to male determining factor [37]. Similarly *chl* gene could be used to conduct future experiments in basic and applied genetic research like discovery of cross resistance and the causative factors, the synthesis of transgenic strains, synthesis of genetic sexing strains for preferential elimination of females, in future genetic programmes involved to control of the species. Linkage mapping, molecular mapping of resistance genes can also be undertaken, which will go a long way in understanding insecticide resistance.

Acknowledgement

This work was supported by the Research Grants from Department of Science and Technology (DST), New Delhi (No, SERB No.SB/SO/AS-05/2012 Dated 27-05-2013) to Prof N J Shetty.

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