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Autosomal inheritance of alphamethrin, a synthetic pyrethroid, resistance in *Anopheles stephensi* – Liston, a malaria mosquito

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Abstract

Anopheles stephensi – Liston (Culicidae: Diptera) is an important urban malarial vector in the Indian sub-continent, accounting for about 15% of the total annual malaria incidence. Chemical control represents a key strategy in the management of this insect vector. However, owing to erratic and continuous application of insecticides, resistance has become a common phenomenon among them and their control has become an uphill task. The genetics of alphamethrin, a synthetic pyrethroid resistance was studied to determine its mode of inheritance. The late third instar larvae were selectively inbred for 27 and ten generations to synthesize homozygous resistant (R) and susceptible (S) stocks, respectively, to the diagnostic dose of 0.12 mg l^{-1}. The log-dosage probit mortality relationships and degree of dominance (*D*) were calculated. Resistance was observed in both sexes, the dosage-mortality (d-m) line of F_1 was towards the resistant parent and the '*D*' value was found to be 0.8 indicating alphamethrin resistant (*amr*) gene to be autosomal and incompletely dominant. The d-m lines of F_2/backcross exhibited a clear plateau of mortality across a range of doses indicating monogenic resistance. The null hypothesis for monogenic resistance was tested from mortality data of backcross progeny compared with theoretical expectations using the χ^2 test and was found to be non-significant. Understanding genetics of insecticide resistance is significant in prediction and management of resistant insects. The *amr* genes can be used as genetic marker in *A. stephensi*, which can be used in several applications in conducting basic and applied genetic research.

Keywords: *Anopheles stephensi*, alphamethrin, resistance, monofactorial, autosomal, incompletely dominant

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Introduction

Insecticides form the most important component in the global mosquito control effort (Najera & Zaim, 2001; McCarroll & Hemingway, 2002). The wide-spread use of chemical insecticides to control mosquito vectors is largely a result of their effectiveness, simplicity, flexibility and economy. Continuous and chaotic application of these insecticides has lead to various problems such as environmental pollution,

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affecting non-target organisms and most importantly the phenomenon of resistance development among the vectors. Resistance is a genetic phenomenon, with mutations affecting insecticide target proteins and metabolism (Ffrench-Constant *et al*., 2004; Li *et al*., 2007). The development of resistance to all classes of insecticides has posed serious dilemma in vector control programmes. Insecticide resistance is expected to directly and profoundly affect the emergence and re-emergence of vector-borne infectious diseases (Krogstad, 1996). Malaria vector control is currently very much dependent on a single class of insecticides, the pyrethroids (Zaim *et al*., 2000), but there has been dramatic increase in pyrethroid resistance in the malaria vectors (Santolamazza *et al*., 2008).

According to the latest estimate, there were about 219 million cases of malaria in 2010 and an approximately 6,60,000 deaths world-wide (WHO, 2011). In India, annually 2,00,000 (in the range of 1,25,000–2,77,000) deaths occur because of malaria (Dhingra *et al*., 2010). *Anopheles stephensi* is one of the important urban malarial vectors in the Indian subcontinent, accounting for about 15% of the total annual malaria incidence (Shetty, 2002). It is one such medically important vector species, which also possess a number of favourable characteristics such as easy sampling and maintenance, shorter life span with high reproductive potential and polytene chromosomes. The said species has been selected for the present investigation.

The acquisition of insecticide resistance among such medically important vector species could also be used to assess the microevolution processes, because, in response to this strong selection pressure, evolution is quicker (Bouvier *et al*., 2001). As resistance reflects changes in the genotypic architecture of natural population, a full understanding of the evolution of this phenomenon requires an accurate and adequate knowledge of its genetic basis (Roush & Daly, 1990). Beyond the evolutionary approach, genetic data constitute a vital tool for investigating resistance mechanism and for predicting the behaviour of resistance genes, thus leading to a better understanding of resistance risks in populations. This information may be useful in pesticide resistance management for the implementation of appropriate control decisions (Georghiou & Taylor, 1986). Identifying insecticide resistance mechanisms is of paramount importance for pest insect control, as the understandings that underpin insect control strategies must provide ways of detecting and managing resistance and these studies rely heavily on detailed biochemical and genetic analyses (Perry *et al*., 2011). The present paper describes the genetic basis of laboratory developed alphamethrin resistance/susceptible in *A. stephensi*.

Material and methods

Insecticide

Alphamethrin (=alpha-cypermethrin) (96.7% TC), a synthetic pyrethroid, is used in malaria prevention and control. This insecticide is effective against range of pests in agriculture, public health and animal husbandry. The IUPAC nomenclature is, a racemic mixture of (*S*)-α-cyano-3-phenoxybenzyl-(1*R*,3*R*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate and (*R*)-α-cyano-3-phenoxybenzyl-(1*S*, 3*S*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate with the molecular formula $C_{22}H_{19}Cl_2NO_3$. It is a non-systemic insecticide with contact and stomach action, and also acts as nerve poison. Alphamethrin-treated insects show restless behaviour, hyper-excitability, become uncoordinated and are then paralysed; flying insects are generally rapidly knocked down (peripheral nervous intoxification).

Mosquito rearing

Colonies of adult mosquitoes were maintained in cages of iron frames covered with nylon mosquito net and fed with 10% sucrose solution soaked in sterilized cotton. Females were provided with the blood meal from restrained mice upon maturation. Plastic vials (3″ diameter) lined with filter paper were placed inside the cage for oviposition. The laid eggs were kept for 72 h to ensure complete hatching. The hatched larvae were reared in white enamel pans containing filtered tap water and were fed with powdered yeast tablets on regular schedule, throughout their larval period. To avoid scum formation, the water in the pan was changed daily. Pupation begins between 8 and 10 days after hatching. The pupae were transferred into wide-mouthed bottles and emerging adults were released into the cages. These stocks were maintained at a temperature of 25 ± 1°C with relative humidity of 75 ± 5% and 10 h of photoperiod throughout the course of investigations (Shetty, 1983).

Larval bioassay

Test concentrations for bioassay were prepared by adding 1 ml of the insecticide from different stock solutions in 249 ml water. Twenty-five late third instar larvae were introduced to these test solutions. The tests were performed in four replicates for each concentration. A control was set up by adding 1 ml of denatured alcohol (98 ml of absolute alcohol + 2 ml ethyl methyl ketone) in 249 ml water. Mortality was observed after 24 h of exposure and was converted to percent mortality (WHO, 2005).

Selection of diagnostic dose for alphamethrin

To select the diagnostic dose for alphamethrin, LC_{99} was initially calculated from the bioassay of the experimental strain – Goruguntepalya (GGP) using the regression line method (Finney, 1971). Twice the value of LC_{99} was fixed as the diagnostic dose to synthesize homozygous resistant (RR) and susceptible (SS) strains for alphamethrin (WHO, 2006).

Development of alphamethrin resistance and susceptible strains

Alphamethrin resistant (AMR) strain: GGP strain from Bangalore, South India was used to synthesize the homozygous resistant strain. The late third instar larvae from the iso-females of GGP were treated with the diagnostic dose of 0.12 mg l^{-1} separately. Twenty-four hours later the surviving larvae from the test showing lowest mortality were collected, maintained separately and used for inbreeding. Mass treatment was followed to treat the larvae of successive generations and the surviving ones were inbred to obtain further generations. The process of selective inbreeding was repeated for 27 generations by gradually increasing the dose from sub-diagnostic concentrations to 0.12 mg l^{-1} until a pure homozygous resistant (100% survival) strain was established.

Alphamethrin susceptible strain (AMS): GGP strain was used to synthesize homozygous susceptible stock. About 50% of the larvae obtained from the iso-females of GGP were treated to the diagnostic dose of 0.12 mg l^{-1}. The untreated larvae of the line showing the highest percentage of mortality

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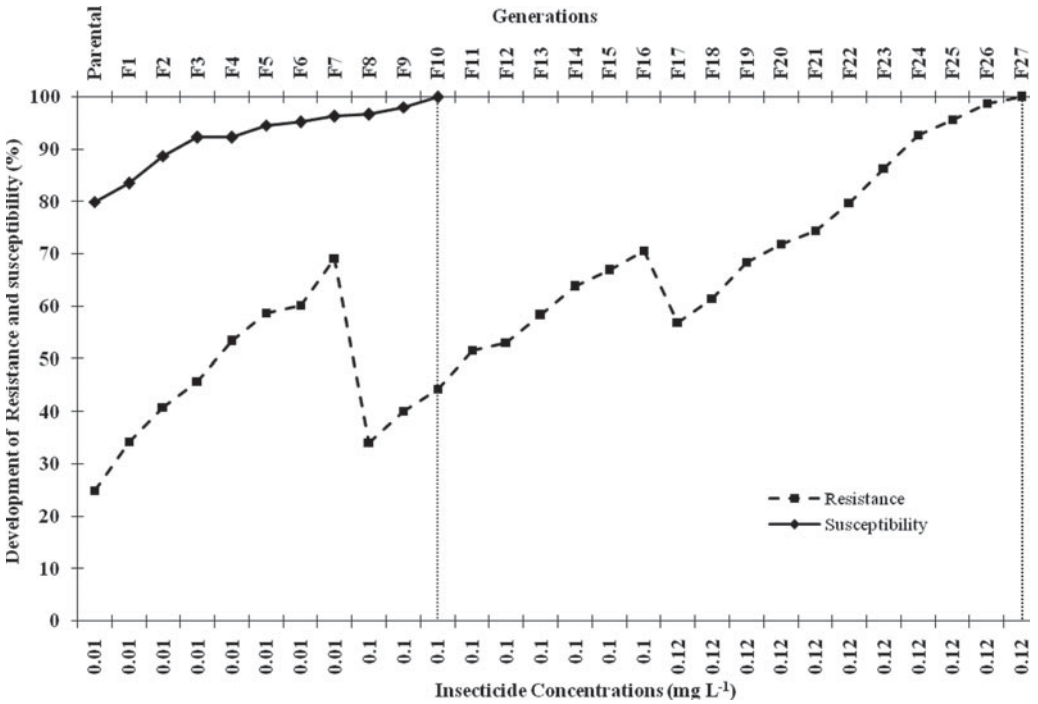

Fig. 1. Development of AMR and susceptibility in *A. stephensi*. The late third instar larvae of GGP strain were initially treated with the sub-diagnostic dose as the diagnostic dose is too high or nearly fatal. The parental generation was treated with 0.01 mg l^{-1}, which exhibited 25% resistance. The surviving larvae were collected and reared separately. The larvae from the F_1 generation were also treated with the same dose. This process of selection, inbreeding and treating to the same dose was carried up to F_7 where the resistance increased to 69.17%. F_8 generation was exposed to 0.1 mg l^{-1}. The resistance level dropped to 34.15% with increase in dose. The same dose was continued up to F_{16} where the resistance increased to 70.71%. F_{17} onwards, the diagnostic dose (0.12 mg l^{-1}) was used for treating which showed 57% resistance. The selection, inbreeding and treating to the diagnostic dose was continued up to F_{27} where the strain showed 100% resistance (homozygous resistance). Similarly late third instar larvae from the iso-female of GGP strain which showed highest percent of susceptibility was reared as separate stock. About 50% of the larvae of the parental generation were treated with 0.01 mg l^{-1}, which showed 80% susceptibility. The remaining larvae were reared as susceptible stock. At each generation, iso-females were separated and 50% of the larvae from each female were treated separately to the sub diagnostic dose. The line showing highest susceptibility was reared as susceptible stock. 0.01 mg l^{-1} was used for treatment for seven generations, where the F_7 was 96.32% susceptible. The dose was gradually increased to 0.1 mg l^{-1} from F_8 onwards up to F_{10} where a 100% homozygous susceptible stock was established.

was selected for inbreeding and the selection procedure was repeated for ten generations to synthesize a pure homozygous susceptible strain.

Genetic studies of AMR

Reciprocal genetic crosses were carried out between the freshly emerged males and females of resistant (R) and susceptible (S) strain (R♂×S♀ and R♀×S♂). A part of the F_1 individuals were backcrossed (F_1×S) to parental type (S) and the remaining were inbred to get F_2 generations. Apart from this, larval bioassay was carried out for late third instar larvae from all the crosses. Simultaneously, male and female larvae from the progeny of all the crosses were subjected to the diagnostic dose (0.12 mg l^{-1}) of alphamethrin to assess sex linkage, if any. The log-dosage probit mortality relationships were recorded for all the genetic crosses (Georghiou, 1969; Finney, 1971; Priester & Georghiou, 1980; Mazzari & Georghiou, 1995) and the degree of dominance was calculated using Stone's formula (Stone, 1968).

Degree of dominance, $D=(2X_2-X_1-X_3)/X_1-X_3$, where X_1, X_2 and X_3 are the logarithms of LC_{50} (concentration for 50% lethality) values for resistant, F_1 hybrid and susceptible strains, respectively. The value of D varies from −1 to 1; $D=1$ indicates complete dominance, $0<D<1$ incomplete dominance, $-1<D<0$ incomplete recessivity and $D=-1$ complete recessivity (Stone, 1968).

Data analysis

LC_{50} and LC_{90} values were calculated according to the method of Finney (1971) and dosage-mortality (d-m; regression) lines were obtained by using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA. Mortality data from bioassays were corrected by natural control mortality using Abbott's formula (Abbott, 1925) and χ^2 values were calculated following the procedure of Bailey (1959).

Results

The homozygous resistant and susceptible strains of *A. stephensi* to alphamethrin were established for the diagnostic dose of 0.12 mg l^{-1} by selective inbreeding for 27 and ten generations, respectively (fig. 1). The data from various crosses for resistance and susceptibility are presented in table 1.

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Table 1. Inheritance pattern of alphamethrin resistance in *A. Stephensi*.

Cross No.	Genetic crosses	No. of ♀'s	No. of larvae tested**	Resistant ♂	♀	Total	%	Susceptible ♂	♀	Total	%	χ^2
	Parental											
1	R♂×R♀	25	1605	813	792	1605	100	–	–	–	–	–
2	S♂×S♀	25	1624	–	–	–	–	841	783	1624	100	–
	F_1 Generation											
3	R♂×S♀	25	1692	543	482	1025	60.50	335	311	646	39.50	1.102*
4	S♂×R♀	25	1790	591	534	1125	62.80	389	376	665	37.20	1.638*
	Back Crosses											
5	S♀×F_1♂ (Cross 3)	25	1726	528	469	997	57.70	382	347	729	42.30	0.59*
6	S♂×F_1♀ (Cross 3)	25	1196	418	423	841	64.80	256	199	455	35.20	2.19*
7	S♀×F_1♂ (Cross 4)	25	1074	269	295	564	52.51	279	231	510	47.49	0.046*
8	S♂×F_1♀ (Cross 4)	25	1636	425	415	840	51.30	423	373	796	48.66	0.017*
	F_2 Generation											
9	F_1♂×F_1♀ (Cross 3)	25	1522	429	486	915	60.20	331	276	607	39.80	1.040*
10	F_1♂×F_1♀ (Cross 4)	25	1706	497	453	950	55.60	410	346	756	44.40	0.313*

R, resistant; *S*, susceptible.
* Non-significant ($P<0.05$).
** Third instar larvae exposed to 0.12 mg l^{-1} for 24 h.
The expected percent mortality for cross 1 is zero, cross 2 is 100 and crosses 3–10 is 50%.

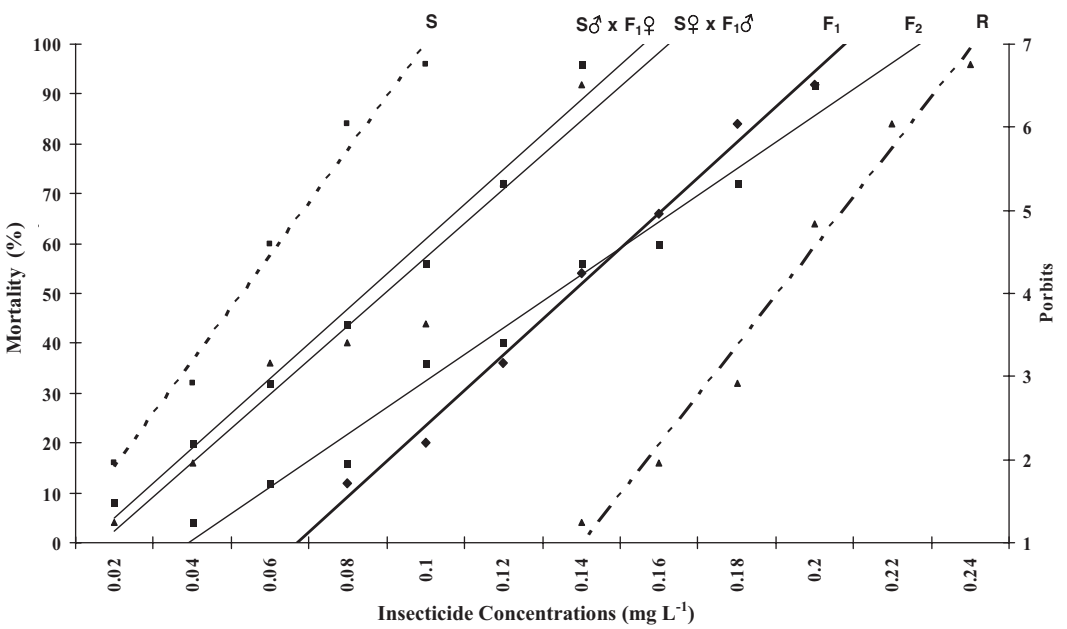

Fig. 2. Dosage mortality relationships of AMR in *A. Stephensi*. The dosage mortality lines were constructed for the larvae from all the crosses including parental, reciprocal, back crosses and also for F_2 generation. The position of F_1 line is slightly towards the dominance line, which indicates incomplete dominance.

The crosses R♂×R♀ (cross 1) and S♂×S♀ (cross 2) showed homozygosity to resistance and susceptibility, respectively. F_1 hybrids from the crosses 3 and 4 (reciprocal crosses between the resistant and susceptible strains) showed 60.50% and 62.80% resistance, respectively. The results of the backcrosses to homozygous susceptible parent showed 57.70%, 64.80%, 52.40% and 51.30% resistance, respectively (crosses 5, 6, 7 and 8). The crosses 9 and 10, i.e., F_2 progeny showed 60.20% and 55.60% resistance, respectively. The log d-m lines (fig. 2) were constructed for resistant, susceptible, F_1 hybrids and F_2 progeny. The d-m line of F_1 was found to be towards the resistant line (fig. 2). '*D*' value was found to be 0.8. 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 response of parental strains were characterized by straight lines, indicating the homogenous nature for resistant and susceptibility. The F_1 offspring also displayed a straight d-m line, confirming homozygosity of the resistance and susceptible genes involved (Raymond *et al.*, 1987). Both the reciprocal crosses (crosses 3 and 4) exhibited a slightly higher percent of resistance than 50% and the position of the log dose

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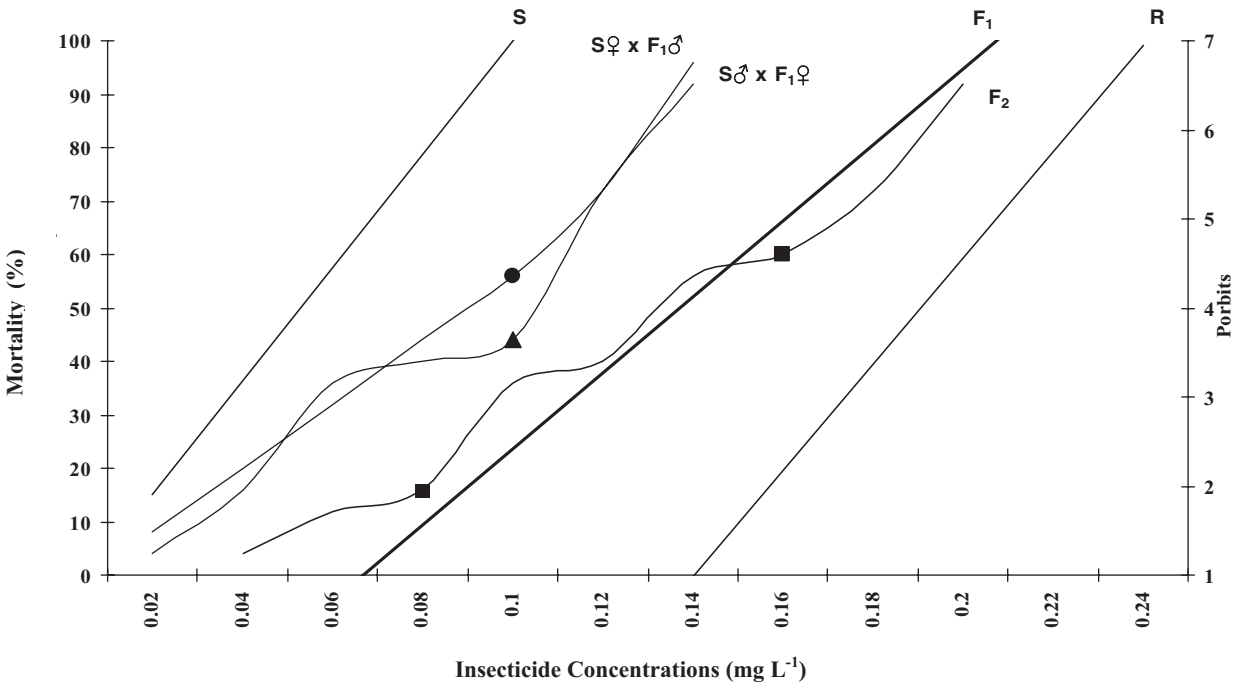

Fig. 3. Dosage mortality relationships of AMR in *A. stephensi* showing break/inflection points on d-m lines of F_2 and backcrosses. The F_2 line showed two inflection points at 0.08 and 0.16 mg l^{-1}, indicating the cessation of S individuals and RS individuals, respectively. Further the backcross lines ($F_1♂ \times S♀$; $F_1♀ \times S♂$) also showed one inflection point each at 0.1 mg l^{-1} indicating complete kill of S individuals leaving behind only RS individuals signifying monogenic inheritance.

probit line was also towards the resistant parent indicating incomplete dominance.

The expected segregation of the backcross of the RS (hybrid) to the S strain for single-factor Mendelian inheritance was calculated by the formula, $x(BC) = ½a_1(RS) + ½a_2(S)$, where x is expected response of backcross at a dose, a_1 and a_2 are observed responses of RS and S populations at that dose (Georghiou & Garber, 1965). The expected response at the diagnostic dose would be 50% kill/resistant, as all the S individuals would perish leaving behind 50% RS individuals. The observed resistance for the four back crosses (crosses 5, 6, 7 and 8) conducted were 57.70%, 64.80%, 52.40% and 51.30% with deviations from 50% being non-significant at $P < 0.05$. Similarly, the expected F_2 segregation was calculated by the formula: $x(F_2) = ¼\ a_1(R) + ½a_2(RS) + ¼a_3(S)$, where x is the expected response of F_2 to a given dose, and a_1, a_2 and a_3 are the observed responses of R, RS (hybrid) and S populations to that dose (Georghiou & Garber, 1965). The expected $x(F_2)$ response would be 50% kill/resistance. The observed resistance in both the F_2 crosses (9 and 10) were 60.20% and 55.60% with deviations from 50% resistance showing no significance at $P < 0.05$. Monogenic control of resistance is indicated when the d-m line of F_2 or backcross exhibit a clear plateau of mortality is across a range of doses. The F_2 curve shows two inflection points: one at 0.08 mg l^{-1} indicating the kill of S individuals and the other at 0.16 mg l^{-1} indicating the kill of RS individuals. The BC lines show one inflection point each at 0.1 mg l^{-1}, indicating the kill of S individuals leaving behind RS individuals (fig. 3). A careful scrutiny of the resistance/susceptible values (table 1), i.e. d-m lines (fig. 2), D (0.8) and the location of F_1 line towards the resistant line does reveal monogenic inheritance and does not reveal any consistent association of resistance through either sex (crosses 3 and 4). Therefore, possibility of sex linkage and cytoplasmic inheritance is ruled out, indicating that the *amr* gene is autosomal, monogenic and incompletely dominant in nature.

Discussion

Insecticides have played an important role in the control of insect vectors especially, mosquitoes. However, the remarkable ability of insect populations to evolve resistance often leaves the control programmes with few options (Ferrari, 1996). Insecticide resistance has been a problem in all insect groups that serve as vectors of emerging diseases. Innumerable genetic, biologic and operational factors influence the development of resistance. In many respects, resistance is a chaotic problem, with different outcomes possible in a particular area, depending on the influence of diverse factors on initial conditions (Brogdon & McAllister, 1998; Andreev *et al*., 1999). The two major forms of resistance are target-site knockdown resistance (*kdr*), which occurs when the insecticide no longer binds to its target, and detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of esterases, cytochrome P450 oxidases or glutathione S-transferases (GST) prevent the insecticide from reaching its site of action (Brogdon & Mcallister, 1998). Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. The target of synthetic pyrethroids is the sodium channels of the nerve sheath (Williamson *et al*., 1996).

As of 1992, the list of insecticide-resistant vector species included 56 *Anophelines* and 39 *Culicines* including *Anopheline*

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examples, *A. stephensi* resistance to DDT, malathion, fenitrothion, temephos, fenthion; *Anopheles culicifacies* complex to DDT, malathion, fenitrothion, fenthion, carbamates, etc. (WHO, 1992). Pyrethroid resistance has been emerging despite early optimism that because of its rapid toxicologic action this newest large class of insecticides would not produce resistance (Malcolm, 1988). High-level pyrethroid resistance has been reported to permethrin, deltamethrin, lambda-cyhalothrin in *Anopheles funestus* from Mozambique (Cuamba *et al*., 2010); deltamethrin, cyfluthrin, alphacypermethrin, lambdacyhalothrin and permethrin in *A. stephensi* from Mangalore, India (Tiwari *et al*., 2010); permethrin in *Anopheles gambiae* from Ghana (Muller *et al*., 2008). *kdr* in *A. stephensi* to fenvalerate was shown to be very quick and of a high degree where in the 12th generation itself the *kdr* was 3.03 (KD_{50}) which warrants caution in the use of synthetic pyrethroids (Verma & Rahman, 1986). *kdr* mechanisms in *Aedes aegypti* from Puerto Rico and Indonesia and *Culex quinquefasciatus* from Louisiana (Brogdon & Mcallister, 1998) and in *A. gambiae* (Elissa *et al*., 1993) have been detected. In Guatemala, pyrethroid resistance was first reported in an *Anopheles albimanus* population resistant to fenitrothion and also deltamethrin cross-resistance (Brogdon & Barber, 1990). *kdr* in insects, resulting from mutation(s) in the voltage-gated sodium channel (*vgsc*) gene is one of the mechanisms of resistance against pyrethroid-group of insecticides and the most common mutation(s) associated with *kdr* in insects, including *Anophelines,* has been reported to be present at residue Leu1014 in the IIS6 transmembrane segment of the *vgsc* gene (Davies & Williamson, 2009; Singh *et al*., 2011). In *Anophelines*, the most common mutation conferring *kdr*, L1014 F (Enayati *et al*., 2003), L1014 S (Singh *et al*., 2011) in *A. stephensi*; L1014F, L1014S and V1010L in *A. culicifacies* (Singh *et al*., 2010) have been identified. Confirming electrophysiological evidence for reduced neuronal sensitivity to pyrethroids has been reported in insects including, *C. quinquefasciatus* and *A. stephensi* (Bloomquist, 1988, 1993). Synergist and electrophysiological studies suggested that *A. stephensi* has a site-insensitivity type of pyrethroid-resistance mechanism (Omer *et al*., 1980; Magesa *et al*., 1994).

Our study showed that the gene *amr* is autosomal, monogenic and incompletely dominant in nature. Such a pattern of inheritance to pyrethroids has been reported in other pyrethroids insecticides such as deltamethrin (Rajashree & Shetty, 1998*a*) and cyfluthrin (Chandrakala & Shetty, 2006). Resistance controlled by single gene develops (Tabashnik, 1986) and spread (Roush & McKenzie, 1987) rapidly compared with that of polygenic resistance. It extends quickly to new areas through migration of resistant insect (Denholm *et al*., 2002). Based on the inheritance studies, one could generate information on specific genetic factors that dictate the evolutionary divergence of discrete resistant populations (Meinke *et al*., 1998). Studies on the nature of insecticide resistance have shown that the phenomenon is due to pre-adaptations which usually involve single gene alleles and that the emergence of insecticide resistant strains is thus a consequence of Darwinian selection (WHO, 1964). Conclusions reported from the present experiment could lead to a better understanding of the rate of resistance development and inheritance mode of resistant gene involved.

It is concluded from the foregoing that the character of induced alphamethrin resistance in the laboratory maintained strain of *A. stephensi* is inherited as a partially dominant (incompletely dominant) single factor without sex linkage or appreciable cytoplasmic influence. The evidence for simple Mendelism, is based on log-dosage probit curves and agreement of the observed responses to those that may be expected in case of monofactorial inheritance.

A better understanding of resistance mechanisms will be extremely important for developing novel strategies to circumvent and to delay resistance development, controlling resistant mosquitoes and thus ultimately reducing the prevalence of mosquito-borne diseases. Characterization of the genes and regulation mechanisms involved in resistance may open the way to entirely new approaches to the study of resistance, eventually leading to the identification of the causative genes in insecticide resistance (Liu *et al*., 2006).

The *amr* gene has several applications in conducting basic and applied research. It can be used as a marker in constructing linkage maps and molecular mapping. This can also be used in synthesizing genetic sexing strains (as a conditional lethal) for the preferential elimination of females in the larval stages; can be used in the genetic control programme through sterile insect technique (SIT) (Shetty, 2002). Such a genetic sexing system has been developed in a few species of mosquitoes by using insecticide resistant genes as a conditional lethal in *A. gambiae*, *A. stephensi* and *C. quinquefasciatus* (Curtis *et al*., 1976; Robinson, 1986; Shetty, 1987). The expression of certain enzymes that are involved in two different types of resistance mechanisms namely metabolite resistance (esterases, phosphatases, dehydrogenases, etc.) and target site resistance (acetylcholine esterases) are very specific in their expressions in each one of the insecticide-resistant strains and also vary within different life stages in a deltamethrin-resistant strain of *A. stephensi* (Rajashree & Shetty, 1998*b*). Similarly, cytological studies carried out from the polytene chromosomes of the ovarian nurse cells in various insecticide-resistant strains are characterized by the presence of specific inversion (s) which could be used as genetic markers (Ghosh & Shetty, 2004).

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