BioPublisher®

Journal of Mosquito Research 2015, Vol.5, No.15, 1-15
http://jmr.biopublisher.ca

Research Article Open Access

Insecticide Confrontation in Dengue Vector: Enzymatic Characterization of Tolerance Level in the Mysore Field Populations of *Aedes aegypti*

Vasanth Patil H.B.[1,2], Nithin K.S.[3], Sathish Kumar B.Y.[1]

1. Postgraduate Department of Studies and Research in Bio-technology, University of Mysore, JSS College, Ooty Road, Mysore – 570 025, INDIA
2. JSS Research Foundation (University of Mysore), Sri Jayachamarajendra College of Engineering, Manasagangothri, Mysore, INDIA
3. Postgraduate Department of Studies and Research in Chemistry, University of Mysore, JSS College, Ooty Road, Mysore – 570 025, INDIA

Corresponding author email: bysathish@gmail.com

Journal of Mosquito Research, 2015, Vol.5, No.15 doi: 10.5376/jmr.2015.05.0015
Received: 30 Apr., 2015
Accepted: 02 Jun., 2015
Published: 22 Sep., 2015

Copyright © 2015 Vasanth Patil et al., This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Preferred citation for this article:
Vasanth Patil H.B., Nithin K.S., and Sathish Kumar B.Y., 2015, Insecticide Confrontation in Dengue Vector: Enzymatic Characterization of Tolerance Level in the Mysore Field Populations of *Aedes aegypti*, Journal of Mosquito Research, Vol.5, No.15 1-14 (doi: 10.5376/jmr.2015.05.0015)

Abstract The susceptibility status of an insect population to any insecticide depends on several factors such includes genetic constitution, ecology of breeding place, previous history of insecticide application if any in that area and the cross resistance spectra. In view of the frequent outbreaks of dengue in different parts of the country and Karnataka state, in particular Mysore and surrounding districts, it is felt essential to generate a base line data on the susceptibility of vectors of local importance and their genetic differentiation through biochemical markers. Resistance to insecticides developed by *Aedes aegypti* was biochemically detected among its population collected from five different urban areas of Mysore city and from four rural locations of Mysore, Mandya and Hassan districts. Insect larvae exposed to different concentrations of insecticide - Deltamethrin for 24 hr. Insecticide resistance/tolerance level in terms of LC_{50} and LC_{90} for the insecticide was high in rural population than urban. Correspondingly, the reason for the resistance was detected through qualitative and quantitative analysis of three biochemical marker enzymes viz., A-Esterase, B-Esterase, Dehydrogenase (G6PD), and Phosphtases (Acid, Alkaline). The allelic frequency of Esterases and Phosphatases was more in rural over urban populations and the same was implied in quantitative estimation also. Wherein the allelic frequency of both the Phosphatases remains same in all the populations but the enzyme concentration was elevated in rural over the urban populations. The inspection of the present study reveals that, the Mysore populations of *Ae. aegypti* shows much variation for which their ecology was responsible.

Keywords Deltamethrin; Esterases; G6PD-Glucose 6 phosphate dehydrogenase; Acid Phosphatase; Alkaline Phosphatase

Introduction

Arthropods being haematophagous (blood sucking) have the ability to behave and demeanor as vector; found to transmit human diseases, where many viruses, bacteria, protozoa and helminths parasites make use of such species for transmission between the vertebrate hosts. Historically, major vector born diseases viz., malaria, dengue, chikungunya, yellow fever, and plague were responsible for most life threatening pathological conditions in humans (Gubler, 1991). Not long after the discovery in 1877 that mosquitoes transmitted filariasis from human to human, malaria (1898), yellow fever (1900) and dengue (1903) were shown to have similar transmission cycles. By 1910, other major vector-borne diseases such as African sleeping sickness, plague, Rocky Mountain spotted fever, relapsing fever, chagas disease, sandfly fever and louse-borne typhus had all been shown to require a blood-sucking arthropod vector for transmission to humans (Philip & Rozenboom, 1973).

Vector born Dengue viruses are flaviviruses and its four serotypes DEN-1, 2, 3 and 4 sequentially causes infections and are responsible for Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) in humans. The viruses are transmitted to man by the bite of infective mosquitoes, mainly *Aedes aegypti* and secondarily by *Aedes albopictus*. DHF is characterized by increased vascular permeability, hypovolaemia and abnormal blood clotting mechanisms. Due to rapid growth of urban centers the rainwater harvesting in diverse types of containers resulting in multiple storage practices and provided the place for breeding vector species. Before the advent of DDT in 1939, vectors were controlled by environmental sanitation and with non-synthetic chemicals especially plant

products. However, in the past few decades a galaxy of synthetic residual insecticides flooded the field of public health and agriculture. Later with the development of organophosphorus compounds, a new era of insect and pest control began (Hassal, 1982). Unfortunately, problems and backlashes have emerged; indiscriminate use of insecticides in the control of insect pests has led to resistance development by the target species and also eliminated natural enemies. Large quantities of four classes of insecticides viz., organochlorines, organophosphates and carbamates, are applied annually to fields or indoors in China, directly or indirectly bringing heavy selection pressure on vector populations. One among the seven major species of vector mosquito in China was *Ae. aegypti* wherein all have evolved resistance to all the above types of insecticide except the carbamates (Feng et al., 2006). The degree of resistance varies among mosquito species, insecticide classes and regions. To overcome such problems and in the background of the failure of organochlorides and organophosphates in many foci, search for new compounds have been undertaken throughout the world. With the result, more safe and degradable insecticides were realized by focusing on pyrethroids, which constitute a new generation of highly potent insecticides derived from a group of esters. During 1980's insecticides belonging to synthetic pyrethroid groups were introduced in the public health program in India. The pyrethrin was originally extracted from the flower heads of *Chrysanthemum cinerariaefolium*. Currently, these are important weapons against insect pests of medical importance. They show remarkably high toxicity and rapid action against wide range of insects with relatively low mammalian toxicity. In recent years, several new pyrethroids are available with the structural rearrangement of the stereochemical features of the parental ring. They are recognized as nerve poisons that do not interact with acetyl cholinesterase as organophosphates and carbamates do. Synthetic pyrethroids are effective as contact insecticides and to a lesser extent as stomach poisons as well (Mulla et al., 1980). The larvicidal effects of the pyrethroid have been extensively evaluated on various mosquito species including dengue vectors (Ranvanshi et al., 1982; Verma et al., 1983). The action of pyrethroids on insects, and mammals depends on the optical and geometrical configurations of their acidic and alcoholic compounds. In the natural pyrethrum all the esters are present in most active optical and geometric configurations as against the commercial pyrethroids where two or four or eight isomers are found (Elliot & Janes, 1973). Inspite of the said qualities, many authors have reported development of resistance against pyrethroids as well (Chandre et al., 1988; Ponlawat et al., 2005). Hence, it has become essential to test the pyrethroid susceptibility status of vector populations in different foci.

The susceptibility status of an insect population to any insecticide depends on several factors such as the genetic constitution, ecology of breeding place, previous history of insecticide application if any in that area and the cross resistance spectra. Recent study in African continent indicated that the extensive use of pyrethroid insecticides in malaria vector control has increased dramatically in the past decade through the scale up of insecticide treated net distribution programmes and indoor residual spraying campaigns. Inevitably, the major dengue and malaria vectors have developed resistance to these insecticides and the resistance alleles are spreading at an exceptionally rapid rate throughout Africa (Hilary et al., 2011). In view of the frequent outbreaks of dengue in different parts of the country (India), including Karnataka state, it is felt essential to generate a base line data on the susceptibility of vectors of local importance.

Even today, Liquid formulations of pyrethroids are extensively used in households; especially in urban and city limits to protect themselves from mosquito bites, through repellent, knockdown and killing effects (Jaswanth et al., 2002). However, prolonged exposure to these chemicals due their long term persistence, high toxicity and propensity for accumulation may lead to the development of resistance in the insect populations, and cause resurgence of minor or alternative pests/vectors, creating ecological disasters (Sharma et al., 1992).

A large number of studies have shown that multiple, complex resistance mechanisms in particular, increased metabolic detoxification of insecticides and decreased sensitivity of the target proteins or genes are likely responsible for insecticide resistance (Nannan Liu, 2015). Resistance/tolerance to insecticides can be defined as an inherent ability to tolerate dosage of an

insecticide that would be lethal to majority of individuals in a population. Insecticide tolerance/ resistance is pre-adaptive, that is mutations resulting in survival (resistance genes) do not arise as a consequence of insecticide usage, but spontaneously at a frequency dependent on the natural mutation rate (Callaghan, 1991). Under insecticide selection pressure more individuals with resistant genes will survive than those without these mutations. The development of resistance in the field may be influenced by various factors. The primary routes of resistance in all insects are alteration in the insecticide target sites or the rate at which it is detoxified. Three enzyme systems namely, Glutathione-S-transferases, Esterases and Monooxygenases are involved in detoxification of four major class of insecticides namely organochlorides, organophosphates, carbamates and pyrethroids. These enzymes act by rapidly metabolizing the insecticides to nontoxic products or by binding and very slowly turning over the insecticide (sequestration) (Hemingway & Karunarathne, 1998). It is generally acknowledged that, Esterases play an important role in degrading the organophosphates. Recent studies have shown that pyrethroid resistance is also associated with increased nonspecific Esterase activity in mosquitoes (Ganesh et al., 2002; Urmila et al., 2001).

Development of resistance by vectors has been a major problem in controlling communicable diseases transmitted especially by mosquitoes. Monitoring the resistance development and the mechanisms associated with it is important at each place as it may involve different and complex pattern. In this regard, one of the significant advancement in the mosquito genetics has been the investigation of enzyme variability by electrophoretic technique. The technique involves the electrophoretic separation and specific staining of enzyme bands and allows the examination of variation in protein production by genes. Further, understanding the more applied but analogous problems involved in insecticide resistance management depend on the ability to measure allelic frequency in different populations (Brent, 1986). Isozyme polymorphism as evident from gel electrophoresis could be used as ‘biochemical markers’ in studying the genetics of insecticide resistance in the absence of any visible markers (Chakraborti et al., 1993). Carboxylesterases, Phosphatases and Dehydrogenases are the group of enzymes that take part in the detoxification process. Polymorphism is a notable characteristic of insect Esterases. The former is involved in the detoxification of organophosphates and pyrethroids (Devonshire et al., 1992). Depending on their activity, Carboxylesterases are designated as A-Esterases (Est-A) and B-Esterases (Est-B). Further, Monooxygenase or the mixed function oxidases (MFO) are the enzymes that give protection against a variety of insecticides in arthropods. Metabolic detoxification is associated with changes in Monooxygenase activity, producing pyrethroid-specific resistance (Berge et al., 1998). This complex involves a reductase and one or more cytochrome P-450s and requires NADP as cofactor (Devonshire et al., 1992). Glucose-6-Phosphate Dehydrogenase (G6PD) generates this cofactor for monooxygenases. Hence an increase in the activity of MFO’s will be reflected in the activity of G6PD (Kumar et al., 1991). An increase in the activity of this enzyme is the most versatile mechanisms of resistance in insects. Oxidation mediated by Monooxygenase is considered to be the major pathway for pyrethroid detoxification though to a limited extent esterase hydrolysis is also possible (Ganesh et al., 2002).

Recent field study in Orissa by Swetapadma et al., 2014 has shown that extensive use of insecticides and development in agricultural practices had an impact which led to the changes in mosquito fauna observed from the Orissa coastal area and Chilika lake inspite of ecological changes, industrial development and other natural calamities. Therefore, identification and surveillance of insecticide resistance/tolerance should be an important component of any vector control program at local level. Given the high cost associated with developing, testing and producing new insecticidal compounds, it is imperative to understand how resistance develops in a target species and to design control strategies that eliminates or minimize resistance development.

In light of these informations, the present investigation was taken up to assay and compare the susceptibility status of *Ae. aegypti* larvae collected from different areas of Mysore city and surrounding places against synthetic pyrethroids and to analyze the isozyme variations of Est-A, Est-B, G6PD, Acid Phosphatase (AcPH) and Alkaline Phosphatase (APH) in order to work out the allelic frequencies in the populations of

Ae. aegypti from Mysore city and surrounding places. Further, these enzymes were assayed using spectrophotometer to understand the genetic resistance mechanism involved. These investigations were aimed to learn the genetic differentiation if any, between the populations and also to correlate the insecticide susceptibility difference with the biochemical data.

1 Material and Methods

1.1 Collection of larvae

Aedes agypti larvae were collected from habitats in Mysore urban areas viz., Vishveshwara nagar, Chamundipuram, J.P. Nagar, Agrahara, Kuvempunagar and surrounding rural areas viz., Mandya, Nanjangud, Hunsur, Channarayapatna of Hassan district. They were brought to laboratory and maintained.

1.2 Larval rearing

Aedes aegypti larvae collected from the aforementioned areas were transferred to a clean enamel/plastic bowls and were maintained at 28±2°C and 80±5% humidity, employing dechlorinated water. Hardness and pH of the rearing water were measured periodically and maintained at permissible range. The immature were fed with yeast granules and transferred to fresh enamel bowl on alternate days. Late third instar or early fourth instar larvae were used for bioassay studies.

1.3 Larval Bioassay

Larval susceptibility tests on *Ae. aegypti* lavae were carried out following the standard WHO procedure (WHO, 1981). Synthetic pyrethroid such as Deltamethrin was employed for the larval bioassay. Different test concentrations of the said synthetic pyrethroid were prepared using ethanol as solvent. One ml of the known concentration of this was added to 249 ml of the dechlorinated water in a 500ml glass beaker. Twenty five healthy early fourth instar larvae were released in to three replicates of each concentration. Control tests were done with same volume of dechlorinated water supplemented with one ml of ethanol. Dead/moribund larvae were recorded after 24hr of exposure (larvae were considered to be moribund if they failed to flex their head to siphon, when stimulated). The percent mortality was calculated for each concentration and corrected by using Abbott's (1925) formula; provided the control mortality was less than 20%. All the tests were carried out at room temperature of 28±2°C and 80±5% relative humidity.

1.4 Statistical Analysis

The LC_{50} and LC_{90} values for each population with fiducial limits for the larval bioassay experiments were calculated by probit regression analysis as per Finney (1971); Difference in LC_{50} values between the populations for pyrethroid tested was also determined.

1.5 Enzyme Analysis - Qualitative Assay

Aedes aegypti from different areas were employed for the investigation so as to understand the isozyme profiles. The mosquito immatures were reared in the laboratory. Either larvae or adults were used for enzyme assay as per the stage specific activity of the enzymes. For the present study, five enzymes such as Carboxylesterases Est-A and Est-B (EC 3.1.1.1), G6PD (EC 1.1.1.49), AcPH (EC 3.1.3.2) and APH (EC 3.1.3.1) were analyzed to establish the differential isozyme profiles in the populations of *Aedes aegypti*.

1.5.1 Native polyacrylamide gel electrophoresis

Continuous nondenaturing polyacrylamide gel electrophoresis (Native PAGE) was conducted to detect the isozyme profiles of the urban and rural populations of a dengue/chikungunya vectors according to the procedures of Gopalan et al., (1997) and Bonning et al., (1991). A medium sized dual vertical slab (10 x 12 cm) gel electrophoresis system was used for the current study. Gels of 0.75 mm thickness were cast employing Teflon spacers. Separating and stacking gels were prepared with 8% and 4% acrylamide respectively. The gel buffer with 8.8 and 6.8 pH was prepared with tris (hydroxyl methyl aminomethane) respectively for separating and stacking gels. A 12 slot teflon comb was employed to make the wells for sample loading. A constant power supply unit (Systronics, India, model No. 610) was used as power source for running the experiment.

1.5.2 Sample preparation and running the gels

Early instar or one day old adult mosquitoes were individually homogenized under cold conditions with 25 µl of 40% sucrose solution in an eppendorf tube using a Knot's pestle. Later cell debris was flocculated by spinning at 2400rpm for 5min at 4°C, supernatant obtained were used for enzyme assays. For G6PD enzyme profile, adult mosquitoes were employed. An equal (15-20 µl) volume of supernatant from all the different samples was carefully loaded to each well. Sodium Borate buffer (0.3 M, pH 8.65) was used as

tray buffer for carboxyl esterases, Phosphatases and Tris EDTA-Borate buffer (pH 9.0) for G-6-PD assay. The gels were initially run at 40V for 25-35min and then at 60V for nearly 5hr at 4°C employing a refrigerator.

1.5.3 Staining Techniques

1.5.3.1 Qualitative assay of Est-A and Est-B

Substrate was prepared by amalgamation of A/B naphthyl acetate (1.85 mg/ml) in 0.1M Sodium phosphate buffer (pH 5.9) and Fastblue RR salt (2 mg/ml) in 0.1M phosphate buffer (pH 6.5) collectively incubated with gel containing resolved native Esterase at 37 ^{0}C in dark for 20 min. in order to trace the isozyme patterns (Patricia et al., 1988).

1.5.3.2 Qualitative assay of G6PD

G6PD zymogram was prepared according to the method described by Umberto & Antoniettina (1973); with slight modifications. In brief, Substrate containing 25 mg of each Glucose 6 phosphate and NADP, 75 mg of each $MgCl_2$ and $MnCl_2$, 25 mg NBT (Nitro Blue Tetrazolium) and 5mg PMS (Phenazonium Methosulphate) were primed in 0.05M Tris HCl buffer (pH 8.5) and were incubated with the zymogram having native enzymes at 37°C for 2 hrs in obscurity.

1.5.3.3 Qualitative assay of APH and AcPH

125 mg of polyvinyl pyrrolidone, 25 mg of each fastblue RR salt and sodium-1-Naphthyl phosphate, 15 mg of each $MgCl_2$ and $MnCl_2$ and 500mg NaCl were made ready in 25 ml of 0.05 M tris buffer (pH 8.5) and the gels were stained for nearly 2 hour in gloom at 37°C for APH assay. For AcPH, 125 mM Sodium acetate buffer (pH 5.0) used while other components are alike that of APH assay.

1.5.4 Fixatio**n**

After the appearance of bands, the gels were washed with distilled water and were fixed in 7% glacial acetic acid solution. Zymograms were prepared depending upon the mobility of the bands. The commonest band appeared in both the population of the species was designated as 1.00. Likewise bands with higher mobility were designated with the number greater than 1.00 in an increasing order (*i.e.,* 1.02, 1.04, 1.05 likewise). Similarly the bands which had lesser mobility, were designated with number in a decreasing order (*i.e.,* 0.98, 0.90, 0.89 likewise.). The numbering has been made by considering the distance traveled by individual bands.

1.6 Enzyme activity analysis - Quantitative assay

1.6.1 Sample preparation

Batches of thirty early fourth instar mosquito larvae or one day old adult mosquitoes were individually homogenized in 200 µl of distilled water under cold conditions using a polypropelene pestle and spun at 5000rpm for 2min in a microcentrifuge at 4°C. Replicate of the 20 µl supernatant from each sample was used as enzyme source. For assays viz., Naphthyl acetate esterase assays, assay of both the Phosphatases and G6PD using microtiter plate.

1.6.2 Estimation of Naphthyl acetate esterase

Est-A activity was quantitatively assayed in microtiter plate where, 200 µl of A-naphthyl acetate solution (100 µl of 30 mM A-naphthyl acetate in 10 ml of 0.02 M phosphate buffer pH 7.2) was added to 20 µl of the supernatant sample homogenates. The reaction was carried for 30 min at room temperature before the addition of 50 µl of Fastblue stain solution (22.5 mg fastblue RR salt in 2.25 ml distilled water and 5.25 ml 5% sodium lauryl sulphate diluted in 0.1 M phosphate buffer of pH 7.0) to each well to stop the reaction. Replicate blanks were maintained where enzyme is replaced by distilled water. The enzyme activity was read at 570 nm as end point. Absorbance levels for individual mosquitoes were compared with the help of a standard curve of absorbance for known concentrations of A-naphthol and the results are reported as µg of A-naphthol produced per min per mg of larval protein (Patricia et al., 1988). The methodology followed for Est-B is similar except for the substrate used *i.e.,* instead of A-naphthyl acetate, B-naphthyl acetate was employed. The results were reported as µg of B-naphthol produced per min per mg of larval protein (Patricia et al., 1988).

1.6.3 G6PD Assay

The total activity of G6PD was estimated following the modified method of Kumar et al., (1991). In brief 100 µl of the larval homogenate was taken in 1 ml capacity microcuvette and incubated with 400 µl of 1.5 M Tris HCl (pH 7.5) containing 3.8 x 10^{-4}M NADP, 10 µl of 0.3 M $MgCl_2$, 500 µl of 0.03 M D-Glucose-6-phosphate. It was read at 340 nm with a UV spectrophotometer. The amount of NADP reduced

(μM/mg protein) was calculated from the molar extinction coefficient 6.22 x 10^6. Specific activity was expressed in μMoles of NADH reduced/min/mg of protein.

1.6.4 AcPH and APH assays

Both the Phosphatases were quantified following the method of Rupangi et al., (2013) using spectrophotometer with some modifications. For AcPH assay 0.2 ml of assay mixture (equal volume of 16 mM p-nitrophenyl phosphate and 180 mM Sodium acetate buffer of pH 5.0) was added to 25 μl of the sample homogenate. This sample was incubated at 37°C for 20 min. The reaction was then blocked by adding 0.6 ml of 0.25 M NaOH. The samples were centrifuged at 15,000rpm for 2 min. Replicate control contained 25 μl homogenate and stopping reagent (0.25M NaOH) before the addition of the 0.2 ml of assay mixture. The absorbance values were measured at 410 nm. Total enzyme activity was calculated using the published extinction co-efficient value (920) for a known path length (1 cm). For the assay of APH the assay mixture is replaced by adding equal volume of 16mM p-nitrophenyl phosphate and 250 mM sodium borate NaOH buffer of 9.8 pH. To every 10ml of this assay mixture 20 μl of 1.0 M $MgCl_2$ solution was added at the time of reaction. Other condition was alike that of AcPH assay and activity was expressed in μg/indivisual.

1.6.5 Protein Estimation

Protein assay of the early fourth instar larvae and one day old adult mosquitoes were carried out as per the standard procedures of Lowry et al., (1951).

2 Results

2.1 Insecticide Susceptibility Test

After successful rearing and treatment with synthetic pyrethriods, the insecticide susceptibility test against the dengue vector *Ae. aegypti* from urban and rural area were carried out; where the LC_{50} and LC_{90} values for insecticide Deltamethrin tested was determined in order to check the frequency of vulnerability to Deltamethrin wherein rural area population shows high values lethal dose requirements especially in Hunsur and Channarayapatna populations with the IC_{90} values of 13.9×10^{-4} and 21.7×10^{-4} ppm respectively (Table 1 and Figure 1).

2.2 Qualitative Analysis of Enzymes

Native PAGE was employed for qualitative analysis and to study the allelic frequency of enzymes such as Est-A (Figure 2, Table 2), Est-B (Figure 3, Table 3), G6PD, AcPH and APH (Figure 4, 5, 6, Table 4) which degrades the organophosphorus and pyrethrenoids; a common insecticides used to control the vectors in both urban and rural areas. Qualitative assay revealed the level of enzyme expression which was quantified based on its band intensity and mobility.

2.3 Quantitative Analysis of Enzymes

The quantitative analysis of enzymes such as Est-A (Figure 7), Est-B (Figure 8), G6PD (Figure 9), AcPH (Figure 10) and APH (Figure 11) for *Ae. aegypti* from urban and rural area populations were carried out (Table 5) in spite of qualitative assay in order to understand the genetic resistance mechanism involved and differentiation in resistance genetics and its product expression that supports survival of insects among the populations and also to correlate the insecticide susceptibility difference with the biochemical data.

Table 1 Comparison of Susceptibility status (LC_{50} and LC_{90}) of different populations of *Aedes aegypti* larvae to synthetic pyrethroid deltamethrin

Populations	$LC^{\dagger}_{50}$ (ppm‡)	$LC^{\dagger}_{90}$ (ppm‡)
Urban Area		
1. VISHVESHWARA NAGAR	1.2×10^{-4}	2.2×10^{-4}
2. CHAMUNDIPURAM	2.5×10^{-4}	8.4×10^{-4}
3. J.P NAGAR	2.6×10^{-4}	5.9×10^{-4}
4. AGRAHAR	1.9×10^{-4}	5.7×10^{-4}
5. KUVEMPUNAGAR	1.7×10^{-4}	7.1×10^{-4}
Rural Area		
6. NANJUNGUD	3.6×10^{-4}	9×10^{-4}
7. HUNSUR	3.4×10^{-4}	13.9×10^{-4}
8. CHANNARAYAPATTANNA	13.1×10^{-4}	21.7×10^{-4}
9. MANDYA	3×10^{-4}	9.8×10^{-4}

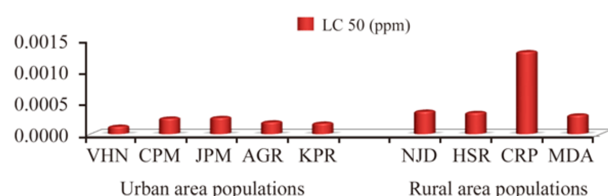

Figure 1 LC50 of Deltamethrin against different poulations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA- Mandya

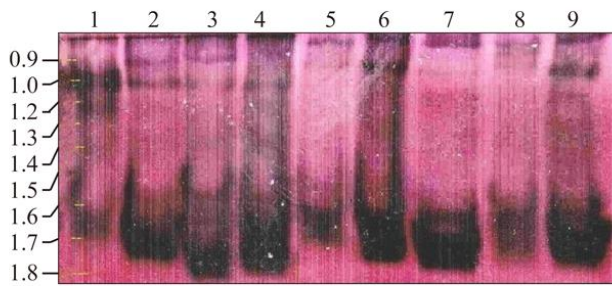

Figure 2 Isoenzyme profile of Est-A in *Aedes aegypti* from different Population
(1) Vishveshwara Nagar (2) Chamundipuram (3) JP Nagar (4) Agrahar (5) Kuvempunagar (6) Nanjangud (7) Hunsur (8) Channarayapattanna (9) Mandya. n=25

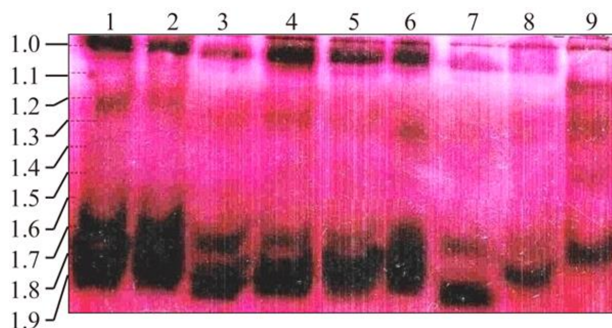

Figure 3 Isoenzyme profile of Est-B in *Aedes aegypti* from different population
(1)Vishveshwara Nagar (2) Chamundipuram (3) JP Nagar (4) Agrahar (5) Kuvempunagar (6) Nanjangud (7) Hunsur (8) Channarayapattanna (9) Mandya. n=25

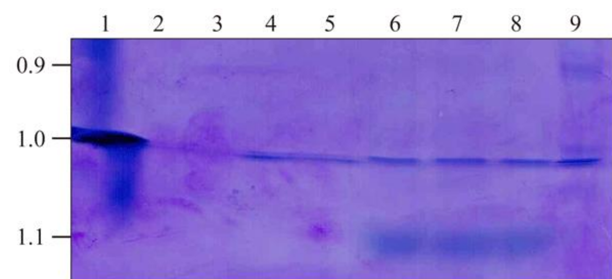

Figure 4 Isoenzyme profile of G6PD in *Aedes aegypti* from different population
(1)Vishveshwara Nagar (2) Chamundipuram (3) JP Nagar (4) Agrahar (5) Kuvempunagar (6) Nanjangud (7) Hunsur (8) Channarayapattanna (9) Mandya. n=25

3 Discussions

Vector control is a very important part of the global strategy for management of mosquito-associated diseases, and insecticide application is the most important component in this effort (Nannan Liu, 2015). Of which Dengue is currently one of the most important arboviral diseases, with 2.5 billion people living in areas of risk and many tens of millions of cases occurring each year (Gubler, 1998). It is one of the most rapidly rising mosquito transmitted infections in the world (Lam, 1993) and has been identified as a re-emerging disease in south east Asia (WHO, 1999). Dengue has been known in India since 1945 (Sabin, 1952), and the classical dengue fever (DF) was mainly associated with febrile illness and joint pains. The severe form of infection manifests as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS); 44% of these cases can be fatal (Rigau et al., 1998). In India, the first outbreak of DHF/DSS was documented in Delhi in 1988 (Kabra et al., 1992). But prior to this, endemic transmission of all four dengue serotypes had been reported (WHO, 1997).

In southern India, dengue was mainly an urban disease in the 1960s and 1970s, associated with the container breeding vector *Ae. aegypti*. In 1993 epidemic of dengue fever was happened in Mangalore, Karnataka state, India (Padbidri et al., 1995). Similarly, in 2001 an epidemic of dengue in Chennai was also reported

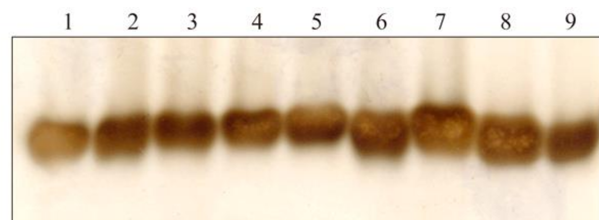

Figure 5 Isoenzyme profile of AcPH in *Aedes aegypti* from different population
(1)Vishveshwara Nagar (2) Chamundipuram (3) JP Nagar (4) Agrahar (5) Kuvempunagar (6) Nanjangud (7) Hunsur (8) Channarayapattanna (9) Mandya. n=25

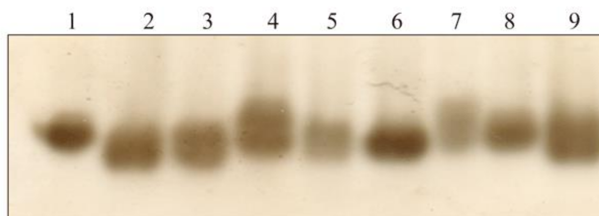

Figure 6 Isoenzyme profile of APH in *Aedes aegypti* from different population
(1) Vishveshwara Nagar (2) Chamundipuram (3) JP Nagar (4) Agrahar (5) Kuvempunagar (6) Nanjangud (7) Hunsur (8) Channarayapattanna (9) Mandya. n=25

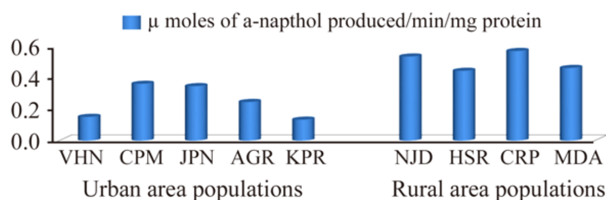

Figure 7 Specific Est-A Activity in different populations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA-Mandya

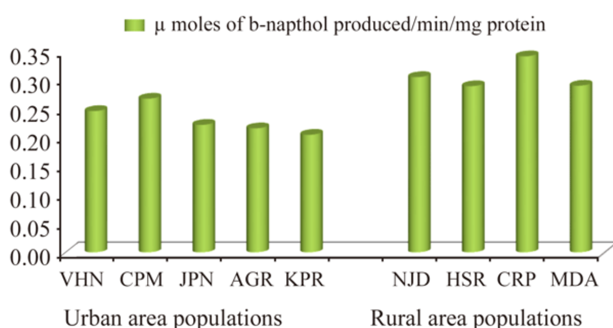

Figure 8 Specific Est-B activity in different populations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA-Mandya

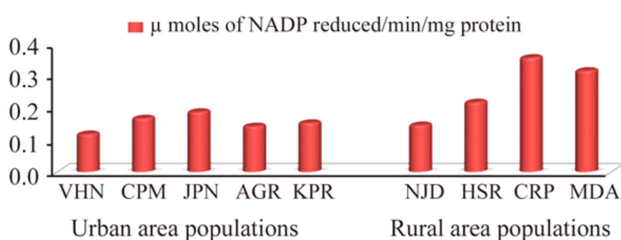

Figure 9 Specific G6PD activity in different populations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA-Mandya

(Kabilan et al., 2005). Much isolation of all the four serotypes of dengue virus (DENV) was made from pools of *Ae. aegypti*; for the first time DEN1 and DEN4 (five isolates) in 1961 (Carey et al., 1964) and DEN2 (two isolates) in 1966 (Myers et al., 1966) and 36 isolates of all the four serotypes in 1968. During that time there was no much breeding of *Aedes* species.

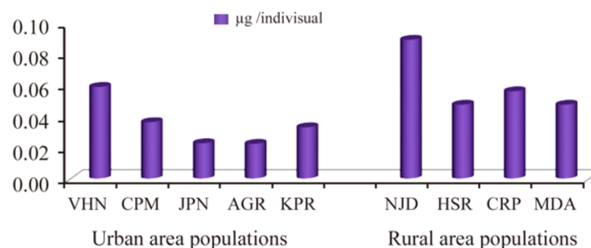

Figure 10 Activity of AcPH in different populations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA-Mandya

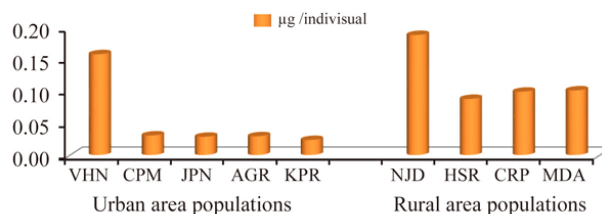

Figure 11 Activity of APH in different populations of *Aedes aegypti*

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA-Mandya

So dengue fever was absent. Subsequently, with the introduction of piped water supply, storage facility, etc., dengue made incursions to rural areas of south India and outbreaks have been reported (Abdul, 1997). In rural areas of India, the role of *Ae. aegypti* as a principal vector had already been well documented (Ilkal & Dhanda, 1991). *Ae. albopictus* also has been considered a potential vector of dengue and several virus isolations have been made in southeast Asia (Hawley, 1988).

Vector control has been the main program to control the communicable diseases. However, continuous and indiscriminate use of pesticides for public health or agriculture purpose, has posed serious problems such as development of insecticide resistance by vectors and pests. Various attempts to overcome this problem have lead to the discovery of more potent insecticides. Among these, synthetic pyrethroids are playing a major role because of their high efficacy, low mammalian toxicity and photostable nature with a

Table 2 Allelic Frequency of A-Esterase (Est-A) in *Aedes aegypti* larvae from different areas

Sl. No.		Frequency of alleles in Urban Area population					Frequency of alleles in Rural Area population			
	EMS	VHN	CPM	JPN	AGN	KPR	NJD	HSR	CRP	MDA
1	0.9	0.92	0.90	0.91	0.89	0.95	0.88	0.85	0.83	0.82
2	1.0	0.97	1.0	1.0	0.95	1.0	1.0	1.0	0.98	0.97
3	1.2	0.57	0.49	0.52	0.40	0.5	0.7	0.72	0.69	0.70
4	1.3	0.19	0.21	0.22	0.30	0.18	0.36	0.40	0.36	0.40
5	1.4	0.28	0.32	0.36	0.36	0.25	0.37	0.37	0.40	0.36
6	1.5	0.11	0.09	0.16	0.20	0.10	0.25	0.31	0.29	0.25
7	1.6	0.88	0.92	0.88	0.90	0.95	0.89	0.95	0.90	0.90
8	1.7	0.86	0.88	0.80	0.92	0.89	0.95	0.93	0.92	0.89
9	1.8	0.74	0.69	0.55	0.70	0.60	0.80	0.87	0.79	0.70

Legend: EMS-Electormorphs, VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR-Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA- Mandya

Table 3 Allelic Frequency of B-Esterase (Est-B) in *Aedes aegypti* larvae from different areas

Sl. No		Frequency of alleles in Urban Area population					Frequency of alleles in Rural Area population			
	EMS	VHN	CPM	JPN	AGN	KPR	NJD	HSR	CRP	MDA
1	1.0	1.0	1.0	1.0	1.0	1.0	0.95	0.92	0.98	0.92
2	1.2	0.97	0.92	0.92	0.95	0.96	0.80	0.81	0.80	0.81
3	1.3	0.57	0.47	0.50	0.42	0.55	0.50	0.72	0.68	0.70
4	1.4	0.13	0.23	0.19	0.32	0.20	0.36	0.46	0.40	0.31
5	1.5	0.27	0.33	0.40	0.37	0.16	0.37	0.37	0.42	0.36
6	1.6	0.16	0.15	0.20	0.29	0.32	0.29	0.39	0.35	0.36
7	1.7	0.89	0.88	0.87	0.87	0.88	0.92	0.95	0.96	0.95
8	1.8	0.80	0.87	0.82	0.95	0.92	0.95	0.95	0.96	0.96
9	1.9	0.72	0.55	0.49	0.55	0.20	0.31	0.88	0.68	0.75

Legend : EMS-Electormorphs, VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR-Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA- Mandya.

Table 4 Allelic Frequency of Glucose 6 phosphate (G6PD), Acid Phosphatase (AcPH) and Alkaline Phosphatase (APH) in *Aedes aegypti* larvae from different areas

Sl. No		Frequency of alleles in Urban Area population					Frequency of alleles in Rural Area population			
	EMS	VHN	CPM	JPN	AGN	KPR	NJD	HSR	CRP	MDA
G6PD										
1	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.58
2	1.0	1.0	1.0	0.98	1.0	1.0	1.0	1.0	1.0	1.0
3	1.1	0.0	0.0	0.0	0.0	0.0	0.50	0.72	0.68	0.30
AcPH										
1	1.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
APH										
1	1.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Legend: EMS-Electormorphs, VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR-Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA- Mandya

potentially valuable residual activity (Mulla et al., 1980; Rajvanshi et al., 1982). Vector control programs all over the world mainly rely on chemical insecticides even today, because of their immediate impact. So it has become very important to monitor various vectors in every endemic area for their ecological status and insecticide susceptibility in the background of the changing environmental and epidemic scenario before

Table 5: Activity of Est-A, Est-B, G6PD, AcPH and APH in different populations of *Aedes aegypti*

Populations	Est-A (μM of a-napthol produced/mg of protein/min)	Est-B (μM of b-napthol produced/mg of protein/min)	G6PD (μM of NADP reduced/mg of protein/min)	AcPH (μg/individual)	APH (μg/individual)
Urban Area	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
VHN	0.1497±0.0703	0.2523±0.0658	0.1204 ±0.1219	0.0593 ±0.0137	0.1583 ±0.0192
CPM	0.3572±0.0579	0.2744±0.0628	0.1702 ±0.0273	0.0365 ±0.0210	0.0307 ±0.0105
JPN	0.3426±0.1091	0.2277±0.0256	0.1907 ±0.0121	0.0229 ±0.0096	0.0287 ±0.0190
AGN	0.2431±0.1018	0.22150±08035	0.1452 ±0.0201	0.0225 ±0.0110	0.0295 ±0.0123
KPR	0.1322±0.0451	0.2100±0.0678	0.1556 ±0.0165	0.0332 ±0.0126	0.0236 ±0.0155
Rural Area	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
NJD	0.5285±0.0598	0.3129±0.0425	0.1487 ±0.1092	0.0898 ±0.0155	0.1885 ±0.0157
HSR	0.4387±O.1017	0.2967±0.0725	0.2211 ±0.0224	0.0478 ±0.0199	0.0881 ±0.0206
CRP	0.5632±0.1041	0.3499±0.2582	0.3622 ±0.0119	0.0565 ±0.0106	0.0995 ±0.0119
MDA	0.4569±0.1304	0.2976±0.0803	0.3210 ±0.0221	0.0478 ±0.0119	0.1015 ±0.0229

Legend: VHN- Vishweshwara nagar, CPM- Chamundipuram, JPN- J.P. Nagar, AGN- Agrahara, KPR- Kuvempunagar, NJD- Nanjangud, HSR- Hunsur, CRP- Channarayapatna, MDA- Mandya. n = 30

the implementation of a control program. Laboratory bioassays are convenient for evaluating the efficacy of chemicals on disease transmitting vectors.

Insecticide susceptibility test is one of the most important aspects to be monitored in public health programs related to vector control. The variation in the susceptibility levels will depend on the breeding habitat, genetic constitution of the mosquito species and the general ecology of the area (Shililu et al., 2003). Thus the larvicidal efficacy of insecticides will vary from place to place and from species to species. Such studies will give us an insight in to the susceptibility status of the local vectors, which will be of immense help in planning out an effective control strategy. Bioassay on *Aedes* species gathers more importance in the current situation as there has been widespread epidemic of Dengue and Chikungunya in Karnataka and many other states (Ravi, 2006).

Bioassay results on this species are available from other places also especially in populations of *Ae. aegypti* larvae form rural areas of Maharashtra state, India and found that, Deltamethrin was more effective than organochlorine and organophosphate tested (Chakraborti et al., 1993). Difference in the LC_{50} values between the populations of *Ae. aegypti* was noticed for the insecticides; temephos, malathion and permethrin in Thailand (Ponlawat et al., 2005). And in line with the present study, it was found that *Ae. aegypti* more resistant/tolerant in rural population. Similarly, susceptibility tests were also carried out on *Ae. aegypti* populations against temephos insecticide in Federal District, Brazil (Carvalho, 2004) and found differences in mortality between the populations tested. Deltamethrin was found to be superior to lambdacyhalothrin and cyfluthrin against *Ae. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* at Delhi (Ansari et al., 1998), which supports the present investigation.

Perusal of the present result indicates that the rural population was found to possess relatively more tolerance against the pyrethroid - deltamethrin tested (Table 1). There have been outbreaks of chikungunya and dengue in Karnataka along with other states. Such epidemics are eye-opener to intensify vector control especially in the rural areas. As far as insecticide resistance mechanisms are concerned in Malaysia, a considerable amount of research indicated that Malaysian mosquitoes have demonstrated variable biochemical mechanisms in resistance to various insecticide classes (Wan et al., 2013). It is in this regard the present study in Karnataka, have gained importance. The scrutiny of the overall results of the bioassay reveals that, the populations of the species may show much variation for which their ecology will also play a major role. In order to classify the genetic differences (responsible for insecticide tolerance) if any, between the populations from urban and rural

area of *Aedes aegypti* species, isozyme profile and enzyme activity studies were carried out.

Esterases, Dehydrogenases and Phosphatases are standard metabolic enzymes which are studied by Native PAGE to gain insight into the species as well as strain variations in mosquitoes. Esterases are complex enzymes acting on a variety of substrates and are capable of hydrolyzing ester bonds. On the other hand Phosphatases work on a limited type of substrates where they will either add or remove the phosphate group. In Dehydrogenases, the reduced NADP or NADPH formed during the reaction passes electrons through an intermediate electron carrier, usually phenozine methosulphate to a tetrazolium compound, resulting in the formation of an insoluble purple diformazen dye at the site of the enzyme activity. In the present study, three types of enzymes viz., Esterases, Phosphatases and Dehydrogenases were employed. These enzymes can be grouped into Group I, Group II and Group III based on their activity. The data obtained has revealed more poplymorphism in esterases in constrast to group II and group III enzymes which are less polymorphic. This also shows that Esterases are highly changeable in nature as evident from the present study on both the populations of *Ae. aegypti*.

Phosphatases are highly active and stable hydrolytic enzymes, used in immunological assay as a label. It is also an excellent marker with potentially useful applications in assessment of variability in natural population (Sakai et al., 1973). These are also responsible for the breakdown of P-S bonds of the insecticides (Callaghan, 1991). Organophosphates, Carbamates and Pyrethroids contain carboxylester and phosphotriester bonds that are subject to attack by enzymes. In addition, Phosphatases are also involved in metamorphic events in insects. In the present study, the populations of *Ae. aegypti* from both urban and rural area have shown one allele for AcPH (Figure 5), so the frequency of the two alleles between the populations was insignificant (P>0.05). In contrast Igbokwe & Milles (1982) have compared 5 inbred strains of *Ae. aegypti* for APH and AcPH and found 7 and 4 bands respectively at Venezuela.

The allelic frequency of the third group of enzymes observed *i.e.*, dehydrogenase (G6PD) (Table 4) for *Ae. aegypti* along with its allelomorphs (Figure 4) of urban and rural populations. G6PD is a NADPH generating enzyme involved in redox reaction. The NADPH generated by G6PD is utilized by monooxygenases. Cytochrome dependent monooxygenases are extremely important metabolic system involved in catabolism and anabolism of xenobiotics and endogenous compound. Monooxygenase mediated detoxification is a common mechanism by which insects become resistant to insecticides. Thus increase in the monooxygenase activity must be reflected in G6PD activity also (Kumar et al., 1991). In the present study, G6PD enzyme has shown variability between the populations of *Ae. aegypti* from urban and rural habitats (Figure 9). The rural population has revealed three bands G6PD$^{0.9,\ 1.00\ \text{and}\ 1.05}$ in contrast to only one in urban population (Figure 4). Kumar et al., (1991) could get only two G6PD isozyme bands each for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* Delhi, India. Pushpalatha & Vijayan (1999); have studied the isozyme patterns of G6PD of two culicine mosquitoes namely *Cx. vishnui* and *Cx. fuscocephala* from Mysore, and could find two bands each in both the species. However, the study of Fakoorziba (2005); revealed that *Cx. tritaeniorhynchus* variant (type B) at Mysore polymorphic with 3 alleles compared to only one allele (monomorphic) in another type A. The present study reveals that the rural populations of the species are polymorphic with 3 alleles each when compared to only one allele in urban populations (Figure 4).

For decades, field studies on resistance problems *insitu* have relied on the WHO bioassay kit. Although much has been learned from these detection systems, it has several inherent limitations. Only one insecticide can be tested per insect without a known discriminating dose, large number of insects is needed to generate probit lines. Using this method, determination of the resistance level or mechanism is not possible. Moreover discriminating dosage that could be applied to all mosquitoes does not exist. Also false positive may occur because of deteriorating filter papers of procedural variables, such as temperature or humidity. Finally, the bioassay methods are ineffective in detecting resistance phenotypes at low frequency (Brogdon et al., 1988). For these reasons, development of quantitative techniques such as spectrophotometric and microtitre plate assay are being used nowadays to

detect resistance levels considering some important detoxifying enzymes as targets. On the other hand, as different isozymes are inherited co-dominantly at individual loci, it is possible to detect the genotype of an individual from the electrophoretic profile. Therefore, it has become apparent that such techniques hold considerable promise in relating specific target enzyme to resistant genotypes rather than phenotypes in a single insect.

Esterases can be divided into carboxylesterase (CaE) and acetyl cholinesterase (AChE), the former is involved in detoxification of organophosphates and pyrethroids. In the present investigation, the results of Spectrophotometric analysis of Est-A (Figure 7) and Est-B (Figure 8) have revealed that in *Ae. aegypti* – rural larvae, there was more activity than that of urban (Table 5). An elevation of non-specific esterases associated with deltamethrin resistance in four out of five field populations of *Ae. aegypti* from Thailand when compared to susceptible strain (Yaicharoen *et al*., 2005). Similarly In eastern Uganda *Anopheles gambiae* adults were exposed to WHO discriminating concentrations of DDT, permethrin, deltamethrin, bendiocarb and malathion. Survival rates to DDT were as high as 85.4%, alongside significant resistance levels to permethrin (38.5%), reduced susceptibility to deltamethrin, but full susceptibility to bendiocarb and malathion (Urvashi et al., 2009).

The quantitative estimation studies on AcPH of *Ae. aegypti* larvae have revealed that in rural population there was more activity than that of urban (Figure 10). APH also shows enhanced activity in rural larvae than that in urban larvae (Figure 11). As phosphatases are involved in breakdown of P-S bonds containing insecticides, significantly higher level of phosphatases in rural populations especially in Nanjangud populations can be correlated with relatively more pyrethroid tolerance noticed (Table 5). Similarly elevated level of Phosphatases was observed in *Helicoverpa armigera* an important cotton pest at Gulbarga, India (Srinivas et al., 2003).

Spectrophotometric analysis of G6PD enzyme of *Ae. aegypti* (Table 5) has registered significant difference in activity in populations from urban and rural area (Figure 9). Thus, *Ae. aegypti* has shown more activity in rural populations especially in Channarayapatna and Mandya populations compared to urban population. The differences in the G6PD activity in normal and treated lines were also noticed earlier (Ganesh et al., 2002; Urmila et al., 2001; Kumar et al., 1991). Thus the higher tolerance level in rural population may be due to more activity of the G6PD enzyme.

In the present investigation a total of five enzymes, which have direct or indirect role in overall fitness were studied. The overall result indicates that all the three groups (I, II & III) of enzymes may have certain role in conferring relatively more fitness in terms of tolerance to insecticide tested in *Ae. aegypti*, rural populations. This reiterates the idea that biochemical approach for the detection of insecticide resistance/ tolerance will help us in Integrated Vector Management (IVM) in rural areas, where these species might have an earlier exposure to agricultural insecticides. The overall profiles of isozyme studies also indicate that, out of five enzyme loci examined in two populations of *Ae. aegypti*, the isozymes of Est-A (Figure 2, Table 2) and Est-B (Figure 3, Table 3) have revealed significant variation in the allelic frequency. On the other hand, certain alleles could be seen only in one population and absent in the other. In addition, monomorphism was found for *Ae. aegypti* - AcPH (Figure 5) and APH (Figure 6) enzymes (Table 4). In the present study susceptibility status of urban populations and Resistance ability of vector in rural populations of Mysore and surrounding areas are biochemically studied quantitatively in addition to qualitative assay's and are documented in order to aid the researchers in designing novel potent molecules that are safe to mammals and have more target specific to the vectors in future vector management strategy.

4 Conclusions

The knowledge on diversity, systematics with a base line data on susceptibility to insecticides along with their biochemical genetics will be helpful while undertaking any control measures in future in rural areas. Identification of resistance mechanisms will be helpful to reveal the cross-resistance spectrum facilitating the choice of alternative insecticides and allows monitoring of foci with endemicity. So these two populations of *Ae. aegypti* from urban and rural habitats have presented differential isozyme profile

and enzyme activity with significant susceptibility variation to deltamethrin.

Acknowledgment

Authors are grateful to University Grants Commission, New Delhi, for the financial assistance (UGC Reference no. MRP(S)-511/09-10/KAMY013/UGC-SWRO) to carry out research work. Authors are also thankful to JSS Mahavidh- yapeetha and the Principal, JSS College, Ooty road, Mysuru for sustain and amenities provided.

Reference

Abbott W.S., 1925, A method for computing the effectiveness of an insecticide, Journal of Economic Entomology, 18: 265-267
http://dx.doi.org/10.1093/jee/18.2.265a

Abdul Kader M.S., Paul Kandaswamy, Appavoo N.C., and Anuradha L., 1997, Outbreak and control of dengue in a village in Dharmapuri, Tamil Nadu, Journal of Communicable Disease, 29(1): 69-71

Ansari M.A., Kapoor N., and Sharma V.P., 1998, Relative efficacy of synthetic pyrethroids-impregnated fabrics against mosquitoes under laboratory conditions, Journal of American Mosquito Control Association, 14: 406-409

Berge J.B., Feyereisen R., and Amichot M., 1998, Cytochrome P-450 monooxygenase and insecticide resistance in insects, Philosophical Transactions of the Royal Society (London) B, 353: 1701-1705
http://dx.doi.org/10.1098/rstb.1998.0321

Bonning B.C., Hemingway J., Romi R., and Majori G., 1991, Interaction of insecticide resistance genes in field populations of *Culex pipiens* (Diptera: Culicidae) from Italy, in response to changing insecticide selection pressure, Bulletin of Entomological Research, 8: 5-11
http://dx.doi.org/10.1017/S0007485300053177

Brent K.J., 1986, Detection and monitoring of resistant forms*:* An overview. In*:* pesticide resistance strategies and tactics for management, (Edited by National Research Council) National Academic Press Washington D. C., 298-312
http://dx.doi.org/10.1126/science.143.3602.131

Brogdon W.G., Beach R.F., Stewert J.M., and Castanaza L., 1988, Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan, *Anopheles albimanus*. Bulletin WHO, 66(3): 339-346

Callaghan A., 1991, Insecticide resistance: mechanism and detection methods, Sci Progress Edinburgh, 75: 423-438

Carey D.E., Myers R.M., and Reuben R., 1964, Dengue types 1 & 4 viruses in wild-caught mosquitoes in south India, Science, 143: 131-132

Carvalho and Maria Do Socorro Laurentino De., 2004, Susceptibility of *Aedes aegypti* larvae to the insecticide temephos in the Federal District, Brazil. Revista de Saude Publica, 38 (5): 623-629
http://dx.doi.org/10.1590/S0034-89102004000500002

Chakraborti S., Mourya D.T., Gokhale M.D., and Banerjee K., 1993, Insecticide susceptibility status and enzyme profile of *Aedes albopictus* populations from different localities of Maharashtra State, India, Journal of Medical Reseach, 97: 37-43

Chandre F., Darriet F., Darder M., Cuany A., Doanniao J.M., Pasteur N., *et al*., 1998, Pyrethroid resistance in *Culex quinquefasciatus* from West Africa, Medical and Veterinary Entomology, 12: 359-366
http://dx.doi.org/10.1046/j.1365-2915.1998.00120.x

Devonshire A.L., Field L.M., and Williams M.S., 1992, *Molecular biology of insecticide resistance, In:* Insect molecular science. Crampton, J.M., Egglesten, P. (eds.). Academic Press, London, 173-183

Elliot M and Janes N.F., 1973, Chemistry of natural pyrethrins (Casida, J. E. ed) Academic Press New York, 56-100
http://dx.doi.org/10.1016/b978-0-12-162950-2.50011-9

Fakoorziba M.R., 2005, Study on genetics (enzymes), pyrethroids bioassay and biocontrol of *Culex tritaeniorhynchus* Giles (Diptera: Culicidae), a Japanese encephalitis vector, [PhD dissertation], University of Mysore, Mysore

Feng Cui, Michel Raymond and Chuan-Ling Qiao, 2006, Insecticide resistance in vector mosquitoes in China, Pest Management Science, 62(11): 1013-1022
http://dx.doi.org/10.1002/ps.1288

Finney D.J., 1971, Probit analysis, Cambridge, Cambridge University Press, 3rd edition

Ganesh K.N., Vijayan V.A., Urmila J., Gopalan N., and Shri Prakash S., 2002, Role of esterases and monooxygenases in the deltamethrin resistance in *Anopheles stephensi* at Mysore, Indian Journal of Experimental Biology, 40: 583-588

Gopalan N., Bhattacharya B.K., Shri Prakash., and Rao K.M., 1997, Characterization of carboxylesterases from malathion-resistant *Culex quinquefasciatus* (Diptera : Culicidae) mosquitoes, Pesticide Biochemistry and Physiology, 57: 99-108
http://dx.doi.org/10.1006/pest.1997.2253

Gubler D.J., 1991, Insects in Disease Transmission, In: Strickland GT, editors. Hunter tropical medicine, Philadelphia (PA): W. B. Saunders, 7th edition

Gubler D.J., 1998, Dengue and dengue haemorrhagic fever, Clinical Microbiology Reviews, 11(3): 480-496

Hassal K.A., 1982, The chemistry of pesticides, The Macmillan Press. London and Basingstoke, 372

Hawley W.A., 1988, The biology of *Aedes albopictus*, Journal of American Mosquito Control Association, 4: 1-39

Hemingway J and Karunarathne S.H.P.P., 1998, Mosquito carboxylesterase: review of the molecular biology and biochemistry of a major insecticide resistance mechanism, Medical Veterinary Entomology, 12: 1-12
http://dx.doi.org/10.1046/j.1365-2915.1998.00082.x

Hilary Ranson, Raphael N'Guessan, Jonathan Lines, Nicolas Moiroux, Zinga Nkuni, and Vincent Corbel, 2011, Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends in Parasitology, 27(2): 91-98
http://dx.doi.org/10.1016/j.pt.2010.08.004

Igbokwe E.C., and Mills M., 1982, Electrophoretic variability in the phosphatase system of the yellow fever mosquito *Aedes aegypti*, Comparitive Biochemistry and Physiology, 73(3): 457-458
http://dx.doi.org/10.1016/0305-0491(82)90058-x

Ilkal M.A., Dhanda V., and Hassan M.M., 1991, Entomological investigations during outbreaks of dengue fever in certain villages in Maharashtra state, Indian Journal of Medical Research, 93: 174–178

Jaswanth A., Ramanathan P., and Ruckmani, 2002, Evaluation of mosquitocidal activity of *Annona sqamosa* leaves against filarial vector mosquito *Culex quinquefasciatus* Say, Indian Journal of Experimental Biology, 40(3): 363-365

Kabilan, L., Balasubramanian, S., Keshava, S.M., and Satyanarayana, K., 2005, The 2001 dengue epidemic in Chennai, Indian Journal of Pediatrics, 72(11): 919-923
http://dx.doi.org/10.1007/BF02731664

Kabra S.K., Verma I.C., Arora N.K., Jain Y., and Kalra V., 1992, Dengue haemorrhagic fever in children in Delhi, Bulletin of the World Health Organization, 70(1): 105-108

Kumar S., Thomas A., Pillai M.K.K., 1991, Involvement of monooxygenases as a major mechanism of deltamethrin resistance in larvae of three species of mosquitoes, Indian Journal of Experimental Biology, 29: 379-384.

Lam, S.K., 1993, Rapid dengue diagnosis and interpretation, Malaysian Journal of Pathology, 15(1): 9-12

Lowry O.H., Rosebrough N.J., Farr A.L., and Randall R.J., 1951, Protein measurement with Folin phenol reagent, Journal of Biological Chemistry, 193(1): 265-275

Mulla M.S., Darwazehk H.A., and Dhillon M.S., 1980, New pyrethroids as mosquito larvicides and their effect on non-target organisms, Mosquito News, 40: 6-11

Myers R.M., Carey D.E., Derantiz C.M., Reuben R., and Bennet B., 1969, Virological investigations of the 1966 outbreak of dengue type III in Vellore, southern India, Indian Journal of Medical Research, 57: 1392-1401

Nannan Liu, 2015, Insecticide Resistance in Mosquitoes: Impact, Mechanisms, and Research Directions, Annual Review of Entomology, 60: 537-559
http://dx.doi.org/10.1146/annurev-ento-010814-020828

Padbidri V.S., Adhikari P., Thakare J.P., Ilkal M.A., Joshi G.D., and Pereira P., 1995, The 1993 epidemic of dengue fever in Mangalore, Karnataka state, India, Southeast Asian Journal of Tropical Medicine and Public Health, 26(4): 699-704

Patricia R Penilla, Americo D Rodriguez, Janet Hemingway, Jose Torres, Juan I Arredondo-Jimenez, and Mario H Rodriguez, 1988, Resistance management strategies in malaria vector mosquito control, Baseline data for a large-scale field trial against *Anopheles albimanus* in Mexico, Medical Veterinary Entomology, 12(3): 217-233

Philip C.B and Rozenboom L.E., 1973, Medico-veterinary entomology: a generation of progress. In: Smith, R. F., Mittler, T. E., Smith, C. N. (eds.), History of entomology, Palo Alto (CA): Annual Reviews Inc

Ponlawat, A., Scott, J.G., and Harrington, L.C., 2005, Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* across Thailand, Journal of Medical Entomology, 42(5): 821-825
http://dx.doi.org/10.1093/jmedent/42.5.821
http://dx.doi.org/10.1603/0022-2585(2005)042[0821:ISOAAA]2.0.CO;2
http://dx.doi.org/10.1093/jmedent/42.5.844
http://dx.doi.org/10.1603/0022-2585(2005)042[0844:BFPOAA]2.0.CO;2

Pushpalatha, N and Vijayan, V.A., 1999, Isozyme profiles in relation to ecological status in two Japanese encephalitis vectors, *Culex vishnui* and *Culex fuscocephala* (Diptera: Culicidae), Entomonology, 24(4): 297-306

Rajvanshi A.C., Wattal B.L., Dass M., and Joshi G.C., 1982, Laboratory evaluation of two new synthetic pyrethroids against larvae of five vector mosquitoes, Journal of Communicable Disease, 14: 52-56

Ravi V., 2006, Re-emergence of Chikungunya virus in India, Indian Journal of Medical of Microbiology, 24(2): 83-84
http://dx.doi.org/10.4103/0255-0857.25175

Rigau-Perez J.G., Clark G.G., Gubler D.J., Reiter P., Sanders E.J., and Vorndam A.V., 1998, Dengue and dengue haemorrhagic fever, Lancet, 352: 971-977
http://dx.doi.org/10.1016/S0140-6736(97)12483-7

Rupangi Verma Puri P., Vineel Reddy, Anil K., Tyagi, 2013, Secreted Acid Phosphatase (SapM) of *Mycobacterium tuberculosis* Is Indispensable for Arresting Phagosomal Maturation and Growth of the Pathogen in Guinea Pig Tissues, PLOS ONE, 8(7): 1-12

Sabin A.B., 1952, Research on dengue during World War II, American Journal of Tropical Medicine and Hygiene, 1: 30-50

Sakai R.K., Iqbal M.P., and Baker R.H., 1973, Genetics of an alkaline phosphatase in a mosquito *Culex tritaeniorhynchus*, Annals of Entomological Society of America, 66: 913-916
http://dx.doi.org/10.1093/aesa/66.4.913

Sharma R.N., Gupta A.S., Patwardan S.A., Hebbalakar D.S., Vrudshali T., and Bhonde, S.B., 1992, Bioactivity of Lamaiaceae plants against insects, Indian Journal of Experimental Biology, 30(3): 244-246

Shililu J.I., Tewolde G. M., Brantly E., Githure J.I., Mbogo C. M., and Beier J.C., 2003, Efficacy of *Bacillus thuringienesis var. israelensis*, *Bacillus sphaericus* and temephos for managing *Anopheles* larvae in Eritrea, Journal of American Mosquito Control Association, 19: 251-258

Srinivas R., Udikeri S.S., Jayalakshmi S.K., and Sreeramulu K., 2003, Identificaiton of factors responsible for insecticide resistance in *Helicoverpa armigera* (Hubner), Resistant Pest Management Newsletter., 13: 59-65

Swetapadma dash, Hazra R.K., and Satpal Singh Bisht, 2014, Investigation on the Mosquito Fauna of Shoreline Habitats of Orissa Coast, India, Journal of Mosquito Research, 4(20):1-5

Umberto Bianchi And Antoniettina Rinaldi, 1973, Tissue specific Glucose 6-Phosphate Dehydrogenase in Anopheline Mosquitoes, Journal of Histochemistry and Cytochemistry, 22(4): 260-265

Urmila J., Vijayan, V.A., Ganesh K.N., Gopalan N., and Shri Prakash., 2001, Deltamethrin tolerance and associated cross-resistance in *Aedes aegypti* from Mysore, Indian Journal of Medical Research, 113: 103-107

Urvashi Ramphul, Thomas Boase, Chris Bass, Loyce M. Okedi, Martin J. Donnelly, and Pie Muller, 2009, Insecticide resistance and its association with target-site mutations in natural populations of *Anopheles gambiae* from eastern Uganda, Transactions of the Royal Society of Tropical Medicine and Hygiene, 103(11): 1121-1126
http://dx.doi.org/10.1016/j.trstmh.2009.02.014

Verma K.V.S., Wattal B.L., and Rajvanshi A.C., 1983, Larvicidal efficacy of synthetic pyrethroids against mosquito, *Journal of Communicable Disease*, 15: 219-221

Wan Norafikah O., Nazni W.A., Lee H.L., Zainol-Ariffin P., and Sofian-Azirun M., 2013, Development of permethrin resistance in *Culex quinquefasciatus* Say in Kuala Lumpur, Malaysia, Saudi Journal of Biological Sciences, 20: 241-250
http://dx.doi.org/10.1016/j.sjbs.2013.02.001

WHO, 1997, Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control, WHO, Geneva, 2nd edition

WHO, 1999, Prevention and Control of Dengue and Dengue Haemorrhagic Fever: Comprehensive Guidelines, WHO, Regional Publications, SEARO No. 29

WHO, 1981, Instruction for determining the susceptibility or resistance of mosquito larvae to insecticides, WHO/VBC, 81-807

Yaicharoen R., Kiatfuengfoo R., Chareonviriyaphap T., and Rongnoparut P., 2005, Characterization of deltamethrin resistance in field populations of *Aedes aegypti* in Thailand, Journal of Vector Ecology, 30(1): 144-150