INSECTICIDE SUSCEPTIBILITY STATUS AND BIOCHEMICAL MECHANISMS INVOLVED IN RESISTANCE DEVELOPMENT OF MAJOR DENGUE VECTOR FROM SUB-HIMALAYAN WEST BENGAL, INDIA

A THESIS SUBMITTED TO THE UNIVERSITY OF NORTH BENGAL FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) IN ZOOLOGY

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DEDICATION

Dedicated to My Loving Parents

Mr. Braj Kishore Sahu
&
Mrs. Sushama Sahu

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I declare that the thesis entitled "***Insecticide susceptibility status and biochemical mechanisms involved in resistance development of major dengue vector from sub-Himalayan West Bengal, India***" has been prepared by me under the guidance of Dr. Dhiraj Saha, Associate Professor of Zoology University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

*I certify that Minu Bharati, M.Sc., has prepared the thesis entitled "**Insecticide susceptibility status and biochemical mechanisms involved in resistance development of major dengue vector from sub-Himalayan West Bengal, India**" for the award of Ph.D. degree of the University of North Bengal, under my guidance.*

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PREFACE

I started my research work in 2014 which has been documented in this thesis entitled **"INSECTICIDE SUSCEPTIBILITY STATUS AND BIOCHEMICAL MECHANISMS INVOLVED IN RESISTANCE DEVELOPMENT OF MAJOR DENGUE VECTOR FROM SUB-HIMALAYAN WEST BENGAL INDIA**" under the supervision of Dr. Dhiraj Saha, Department of Zoology, University of North Bengal, Darjeeling.

Dengue fever caused by a flavivirus, is mainly transmitted by *Aedes aegypti and Aedes albopictus* and is an increasing serious public health problem in over 100 countries putting about 2.5 billion people at the risk of infection. *Aedes aegypti* occurs primarily in the tropical and subtropical regions of the world. Asian countries harbor 75% of the world's total *Aedes albopictus* population with the greatest population in the south-east Asia. In India, *Aedes aegypti* has been found to be endemic in the eastern plains (Bihar and Bengal basin), Assam Valley, Western plains (Thar desert), Northern plains (Punjab and Haryana), Indo-Gangetic plains and the coastal areas of Orissa. Sub-Himalayan West Bengal possesses the ambient environment (temperature and relative humidity) for the growth and proliferation of *Aedes* mosquitoes and the associated diseases.

The key strategy to reduce mosquito-borne diseases depends on efficient vector management due to the absence of protective vaccines and medicines for the treatment of the same. In India, insecticides like DDT, malathion, deltamethrin, cyhalothrin, cyfluthrin, alpha-cypermethrin, bifenthrin and bendiocarb are used as adulticides while temephos and mosquito larvicidal oil as larvicides. The extensive use of these

insecticides has led to the development of insecticide resistance of the target insect species.

The efficient vector control therefore needs detailed knowledge about the factors underlying the development of resistance mechanism and formulation of sustainable resistance management strategies. Therefore, there is a need for country–wide regular survey to monitor the insecticide susceptibility of mosquito vectors.

In this context, we aimed to study the insecticide susceptibility status of two dengue vectors *Aedes aegypti* and *Aedes albopictus* from different districts of Sub-Himalayan West Bengal, India and to find out the role of major detoxifying enzymes in the development of resistance to insecticides. The study showed moderate to high level of resistance in the field populations of the dengue vectors against six adulticides and a larvicide which are commonly used either to control the vectors or in the tea plantation areas. The finding of our study also reveals the involvement of major detoxifying enzymes like Carboxylesterases, Cytochrome P450 monooxygenases and Glutathione-S-transferases. Amongst the insecticides tested, Type II synthetic pyrethroids seem to be the most effective in controlling the *Aedes* mosquitoes of sub-Himalayan West Bengal followed by malathion and then temephos. In future, molecular studies can be performed to enrich the biochemical mechanism involved in the insecticide resistance development in the dengue vectors as well as other mosquito vectors.

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I express my sincere appreciation to Dr. Dhiraj Saha, Insect Biochemistry and Molecular Biology Laboratory, Department of Zoology, University of North Bengal for being my supervisor. I am indebted to Sir for his expert guidance, valuable suggestions and continuous encouragements throughout the course of Ph.D. programme.

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Minu Bharati

ABSTRACT

Hematophagous arthropods pose danger to humans owing to their ability of ingesting the disease causing pathogens from an infected individual and inject it into a healthy being causing the transmission of the disease/infection. Of the total diseases suffered globally, around 16.6% are due to vector borne diseases. Globally, more than 1 billion people get infected by vector borne diseases and 1% die primarily by Malaria, Dengue, Leishmaniasis, Chagas Disease, Yellow Fever, Filariasis and Onchocerciasis. Globally, climatic changes are increasing the numbers and distribution of many disease vectors. Additionally, poverty related issues, *i.e.* lack of access to adequate housing, malnutrition, lack of proper sanitation and drainage and unavailability of safe drinking water also contribute towards the increased risk of such diseases.

Mosquitoes, one of the most successful hematophagous arthropods are responsible for the transmission of numerous dreadful diseases, such as Malaria, Dengue, Japanese encephalitis, Yellow fever, West Nile fever, Zika, Chikungunya, Filariasis causing several million deaths throughout the world annually. The most rapidly spreading mosquito borne viral disease is Dengue which has expanded to previously unexplored regions increasing its incidence rate approximately 30 fold in the last 50 years. Another mosquito borne viral disease affecting Africa, Asia and the Indian subcontinent is Chikungunya. Recently this disease resurgence occurred in Asia, Africa, Europe as well as North America.

India owing to its subtropical climate is subjected to regular infections of various vector borne diseases. It is estimated that approximately 3.9 billion people residing through 128 countries inhabit Dengue risk areas. An estimated 390 million infections of

Dengue occur every year, out of which only 25% show clinical manifestations. Around 1.9 million Chikungunya infections have been reported to occur in five Asian countries viz., India, Indonesia, Maldives, Myanmar and Thailand.

India is reported to acquire the largest number of Dengue infections annually with approximately 100 million asymptomatic and 33 million clinically manifested infections). The presence of both the urban vectors and ideal climatic conditions aggravate the factors for major outbreak of Dengue. More than 0.1 million cases of Dengue occur every year in India, the trend towards an increase in infection rates every year.

In 2017, among the Indian states the highest numbers of Dengue infections were reported in West Bengal. The warm and humid temperature and climatic conditions, rapid urbanisation, high vegetation cover, lack of sanitation, hygiene and drainage in majority of the state together provide the ideal ambience for *Aedes* and other mosquito growth and proliferation. Additionally, the high population density of West Bengal supports the efficient circulation of disease causing pathogens.

Aedes mosquitoes pose severe threat to human race because of its capability to transmit several arboviruses, *i.e.* Dengue virus (DENV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Yellow fever virus, *etc*. These diseases have increased severely in the past five decades and expanded itself many folds and spread throughout the globe. *Aedes* mosquitoes are closely associated with the human colonies and occur near such dwellings, commonly laying eggs in and around human houses. Additionally, human made products, *i.e.* tyres, tanks, plastic containers *etc* serve as egg laying sites and

becoming its breeding and proliferating site, thereby increasing their abundance and establishing colonies in human inhabiting areas.

For none of the arboviruses transmitted by *Aedes* mosquitoes, any treatment or vaccination exist. In absence of specific medications and vaccines for all the above mentioned disease, the prevention becomes the only option to restrict disease transmission in humans. Disease prevention for *Aedes* transmitted arbovirus mainly involves vector control and personal prophylactic measures to minimise mosquito biting.

Since the discovery of insecticides, they have been used heavily for mosquito control. In India, Organochlorines (DDT, Dieldrin, Aldrin *etc*), Organophosphates (Malathion, Temephos, Dichlorvos, Chlorpyrifos), Synthetic pyrethroids (Lambdacyhalothrin, Deltamethrin, Permethrin, Cypermethrin and its derivatives, Cyfluthrin) and Carbamates (Propoxur, Bendiocarb) have been widely used for both mosquito control as well as agricultural pest control.

But due to the uncontrolled heavy use of these chemicals/ insecticides, both target as well as non-target species have evolved to resist the actions of those chemicals in their body through different mechanism. This phenomenon interrupting the chemicals to manifest their planned actions is known as Insecticide resistance. Insecticide resistance results in the failure of mosquito control programmes to achieve their planned targets, thereby increasing the risk of DENV infection even after insecticide spray during severe disease outbreaks.

Resistance to insecticides can be caused by an range of modifications within a mosquito, such as behavioural alteration, physiological modifications within the cuticle reducing the insecticide penetration, biochemical changes within the activity of major

insecticide detoxifying enzymes or structural modification within the target of the insecticide thereby blocking the insecticide binding and subsequent action. Metabolic detoxification of insecticides refers to the degradation of the chemicals into non-toxic and water soluble forms by the action of gut enzymes. The enzymes carrying on the task of xenobiotic detoxification generally belong to large families of multigenes, the most notable being the Carboxylesterases (CCEs), Cytochrome P450s (CYP450s) and Glutathione S-transferase (GSTs). Increased synthesis of one such enzyme, *i.e.* CCEs through gene amplification have been reported to confer resistance against organophosphate, carbamates and pyrethroid insecticides in insects .

Target site modification refers to the loss of sensitivity of the active site of the protein targeted by the insecticide. The most notable and commonly found is insensitivity of voltage gated sodium channel gene (VGSC) by synthetic Pyrethroids (SPs) or Organochlorines (OCs), thereby providing resistance against these insecticides, commonly called as knockdown resistance (kdr). Two of the most commonly detected point mutations in resistant *Ae. aegypti* are V1016G/I and F1534C in the IIS6 segment of VGSC.

So, in an attempt to gain knowledge on the prevalence of different species of *Aedes* mosquitoes, their levels of insecticide susceptibility and underlying mechanisms, *Aedes* mosquitoes were randomly sampled from five districts of northern West Bengal. The collected mosquitoes were subjected to insecticide susceptibility testing against 0.0200 and 0.0125 ppm of Temephos, 4% DDT, 5% Malathion, 0.05% Deltamethrin, 0.05% Lambdacyhalothrin, 0.75% Permethrin, and 0.1% Propoxur. The mosquitoes were assayed for the activity of major insecticide detoxifying enzymes *i.e.* Carboxylesterases

(CCEs), Cytochrome P450s (CYP450S) and Glutathione S-transferase (GSTs). Qualitative study for the presence of Carboxylesterase isozymes were also performed. Moreover, synergistic assay with the use of 4% PBO and 10%TPP were also performed to confirm the role of Cytochrome P450s and Carboxylesterases respectively behind the observed resistance. Additionally, the mosquitoes were also screened for two kdr mutations, *i.e.* V1016G/I and F1534C.

It was observed that, throughout the study region, a dominance of *Ae. albopictus* over *Ae. aegypti* was noticed. It was also revealed that for both the *Aedes* species, discarded tyres were the most preferred breeding habitat followed by uncovered cemented tanks. Majority of the studied *Ae. aegypti* populations possessed low resistance levels against temephos but one population NDP^{ae} was found to possess altered susceptibility against 0.02ppm and 0.0125ppm temephos. All the tested populations of *Ae. aegypti,* possessed widespread resistance against DDT with the lowest mortality recorded 47.9% for DAR^{ae} population followed by APD^{ae} (55.4%), NDP^{ae} (56.6%), COB^{ae} (70%) and JPG^{ae} (72.0%). One population of *Ae. aegypti* possessed moderate resistance against malathion, *i.e.* APD^{ae} with 72.5% mortality. Most of the studied population were revealed to be susceptible or incipiently resistant to lambdacyhalothrin and deltamethrin with the mortality ranging from 80.9-100% and 89.2-100% respectively. Against permethrin, very low mortality percentage *i.e.* 50% for NDP^{ae} to incipiently resistant for APD^{ae}, 83.3% were reported. Three of the tested *Ae. aegypti* populations were found to be severely to moderately resistant against propoxur, DAR^{ae}, JPG^{ae} and NDP^{ae}:

From the results of synergistic assay, it was observed that, prior exposure to 4% PBO before DDT was found to increase susceptibility to it in APD^{ae} population, restoring the mortality rate partially, thus a part of the observed resistance might be conferred by detoxification through Cytochrome P450s. In the same population, Carboxylesterases were revealed to drive the partial resistance against malathion, in APD^{ae} population restoring the mortality from 72.5% to 94.0% when exposed to 10% TPP. In APD^{ae}, JPG^{ae} and NDP^{ae}., Cytochrome P450s were revealed to be accountable for partial resistance against deltamethrin and lambdacyhalothrin. In NDP^{ae}, Carboxylesterase linked pathways were revealed to be involved in propoxur resistance, as use of 10% TPP could restore its mortality from 45.4 to 70.4%. Through kdr genotyping, both susceptible and mutant kdr allele were revealed to be present amongst the wild populations of *Ae. aegypti* indicating the possible role of these mutations behind observed resistance against permethrin and DDT. The study of qualitative analysis of Carboxylesterase in *Ae. aegypti* mosquitoes revealed the presence of around five different isozymes of α-Carboxylesterase (Rf values 0.62, 0.68, 0.73, 0.82, 0.97) and three isozymes of β-Carboxylesterase (Rf values 0.62, 0.80 and 0.96) were found. The intensity of the band izozymes depicting the overexpression of enzyme were found to be linked with resistance against the tested organophosphate in some of the field collected *Ae. aegypti* mosquitoes.

Similar pattern of resistance was also noted in *Ae. albopictus* mosquitoes, one population NGK^{al} exhibited incipient resistance against temephos at 0.0200 ppm dosage and two populations, NGK^{al} and SLG^{al} .possessed incipient resistance against 0.0125 ppm temephos. Severe to moderate resistance against DDT was revealed in the tested *Ae. albopictus* mosquitoes, namely SLG^{al}, JPG^{al} and NGK^{al}. However, complete

susceptibility was recorded among the wild *Ae. albopictus* mosquito populations against malathion, deltamethrin and lambdacyhalothrin. In two of the tested *Ae. albopictus* population moderate level of resistance against permethrin was found with mortality percentages 75.4 (APD^{al}) and 75.0 (JPG^{al}). Severely resistant population of Indian *Ae. albopictus* against propoxur was revealed for the first time in this study with very low mortality rate, 42.5%.

Populations NGK^{al}, JPG^{al} and SLG^{al} were reported to possess Cytochrome P450s linked resistance mechanism against DDT, since prior exposure to PBO restored the mortality/susceptibility in these populations. CytochromeP450s were also revealed to confer resistance against permethrin in APD^{al} and JPG^{al}. The results of kdr genotyping revealed that, all but one (SLG^{al}) tested *Ae. albopictus* population were found to possess the 1534C mutant allele, which might be linked to the resistance against permethrin and DDT.

From the study of qualitative analysis of Carboxylesterases, two different isozymes for both α- Carboxylesterases (Rf values 0.81, 0.91) and β- Carboxylesterases (Rf values 0.63 and 0.95) were found amongst the different field caught mosquito populations. The presence of more than one band and the higher intensity of the expressed isozyme were found to be linked with resistance against organophosphates in the field collected *Ae. albopictus* population.

The knowledge gained through this study will help the personnel engaged in dengue vector control for designing of an effective strategy throughout the study region. As a part of habitat destruction, safe disposal of tyres and covering of open cemented tank or water holding containers should be aimed throughout the studied region. For

successful control of *Ae. aegypti* throughout the districts of northern part of West Bengal, deltamethrin and lambdacyhalothrin seem to be the most effective. Similarly for *Ae. albopictus*, deltamethrin and lambdacyhalothrin and malathion were found to be the most potent for dengue vector control. Temephos can also be used throughout the region for *Aedes* vector control with exception for North Dinajpur and Nagrakata where use of synergist along with the larvicide seems promising. A detailed knowledge on the prevalence of other kdr mutations might also provide an insight for effective dengue vector control.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AchE: Acetylcholinisterase,

CB: Carbamate,

CCE: Carboxylesterase,

CDNB: 1-Chloro-2, 4-dinitrobenzene

CHIKV: Chikungunya Virus,

CL: Confidence Limits,

CYP450s: Cytochrome P450s,

DDE: Dichloro Diphenyl Dichloro Ethylene,

DDT: Dichloro Diphenyl Trichloroethane,

DENV: Dengue Virus,

DHF: Dengue Haemorhhagic Fever,

DSS: Dengue Shock Syndrome,

GSH: Glutathione, reduced form,

GST: Glutathione S-Transferase,

h: hour

IGR: Insect Growth Regulator,

KDR: Knockdown Resistance,

KDT_{50}: Timetaken (in minutes) by a pesticide to knockdown 50 per cent of the experimental population

KDT_{95}: Timetaken (in minutes) by a pesticide to knockdown 95 per cent of the experimental population

l: Litre

LC_{50}: Lethal Concentration of a pesticide required to kill 50 per cent of the experimental population

LC_{99}: Lethal Concentration of a pesticide required to kill 99 per cent of the experimental population L

MFO: Mixed Function Oxidases,

mg: Milligram

min: Minutes

ml: Millilitre

MT: Metric Ton

NVBDCP: National Vector Borne Disease Programme,

OC: Organochlorine,

OP: Organophsphate,

PAGE: Polyacrylamide Gel Electrophoresis

PBO: Piperonylbutoxide

PCR: Polymerase chain reaction

ppm: parts per million

Rf: Retardation factor

SD: Standard deviation

SE: Standard error

SP: Synthetic Pyrethroid,

TPP: Triphenyl phosphate

ULV: Ultra Low Volume,

VBD: Vector Borne Disease

VGSC: Voltage Gated Sodium Channel

WP: Wettable Powder

WHO: World Health Organisation

CONTENTS

INTRODUCTION

1. INTRODUCTION:

1.1 Global scenario of vector borne diseases:

Vector borne diseases impose a significant burden on public health and global socio-economic indices. The superiority of disease causing pathogens and transmitting vectors over the human population is well established causing the sustenance, resurgence as well as new emergence of various diseases of public health importance. Vector populations have efficiently adapted themselves for successful pathogen transfer. One such major adaptation is the evolution of hematophagy (blood feeding) in arthropod vectors enabling themselves transmission of numerous disease causing pathogens such as, viruses, protozoans, bacterias and helminths in humans (Gubler, 1998). Hematophagous arthropods ingest the disease causing pathogens from an infected individual and later inject the same into a healthy being causing the successful transmission of the infection. Some of the commonly occurring vector borne diseases are Malaria, Dengue, Chikungunya, Zika, Yellow fever, Lymphatic filariasis, Chagas disease, Onchocerciasis, Loiasis *etc.*

Of the total diseases suffered globally, around 16.6% are due to vector borne diseases (WHO, 2014). Globally, more than 1 billion people get infected by vector borne diseases and 1% die primarily by malaria, Dengue, leishmaniasis, chagas disease, yellow fever, filariasis and onchocerciasis (WHO, 2014). Since, the first discovery of vector mediated disease transfer in humans in 1877, these diseases have stood as a barrier to the development of countries present in the tropic areas (Gubler, 1998).

One of the most historically marked vector borne disease pandemic episode remains the 'Black death' *i.e.* plague in the 14th century followed by the yellow fever

epidemic (Gubler, 2009). Historically, these diseases remained confined to specific areas but later on the scenario changed drastically. Nowadays these diseases have become cosmopolitan owing to the easy transport of vectors mainly due to globalisation, increased human mobility throughout different parts of the world, rapid unplanned urbanisation *etc*. (WHO, 2014) creating opportunities for easy transfer and distribution of vectors and diseases (Figure 1).

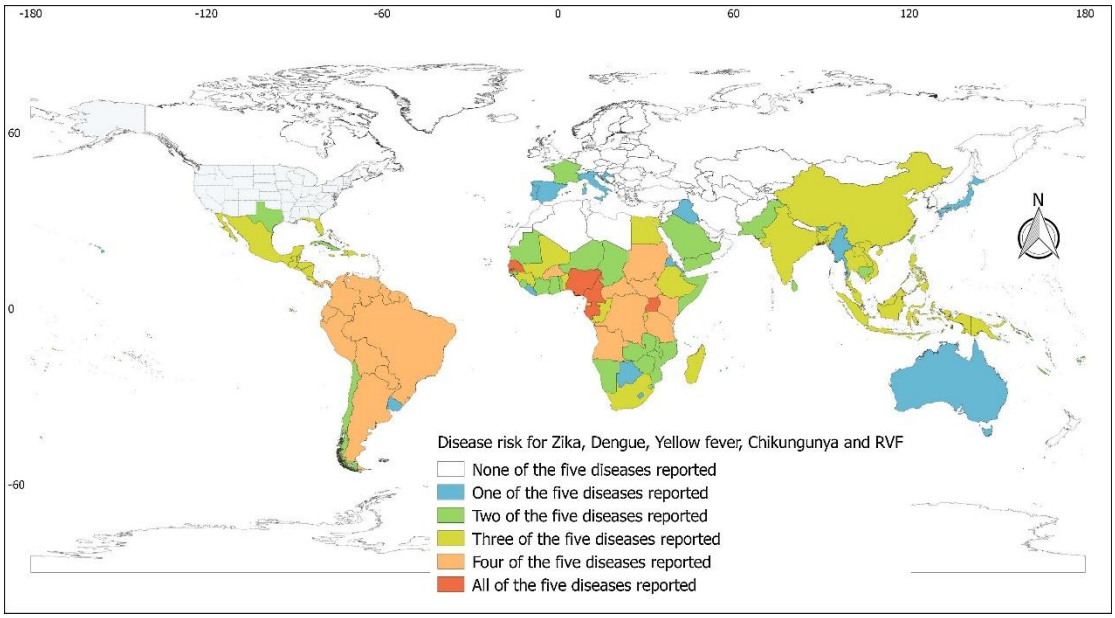


Figure 1: Global mapping of major vector borne diseases Zika, Dengue, Yellow fever, Chikungunya and Rift valley fever (Source: Leta *et al.*, 2018)

Globally, climatic changes are increasing the numbers and distribution of many disease vectors (WHO, 2014). Additionally, poverty related issues, *i.e.* lack of access to adequate housing, malnutrition, lack of proper sanitation and drainage and unavailability of safe drinking water also contribute towards the increased risk of such diseases. The efficiency of a disease agent transfer by a vector is dependent on many factors, particularly on the extent of contact with the host and on feeding behaviour (Gubler, 2009). In this regard, flies and mosquitoes may be assumed to be efficient vectors as evident from their close association with vertebrate hosts.

Mosquitoes, one of the most successful hematophagous arthropods are responsible for the transmission of numerous dreadful diseases, such as malaria, Dengue, Japanese encephalitis, Yellow fever, West Nile fever, Zika, Chikungunya, Filariasis causing several million deaths throughout the world annually (WHO, 2017). Mosquitoes are responsible for the greatest number of infections among other vector borne diseases (Figure 2).

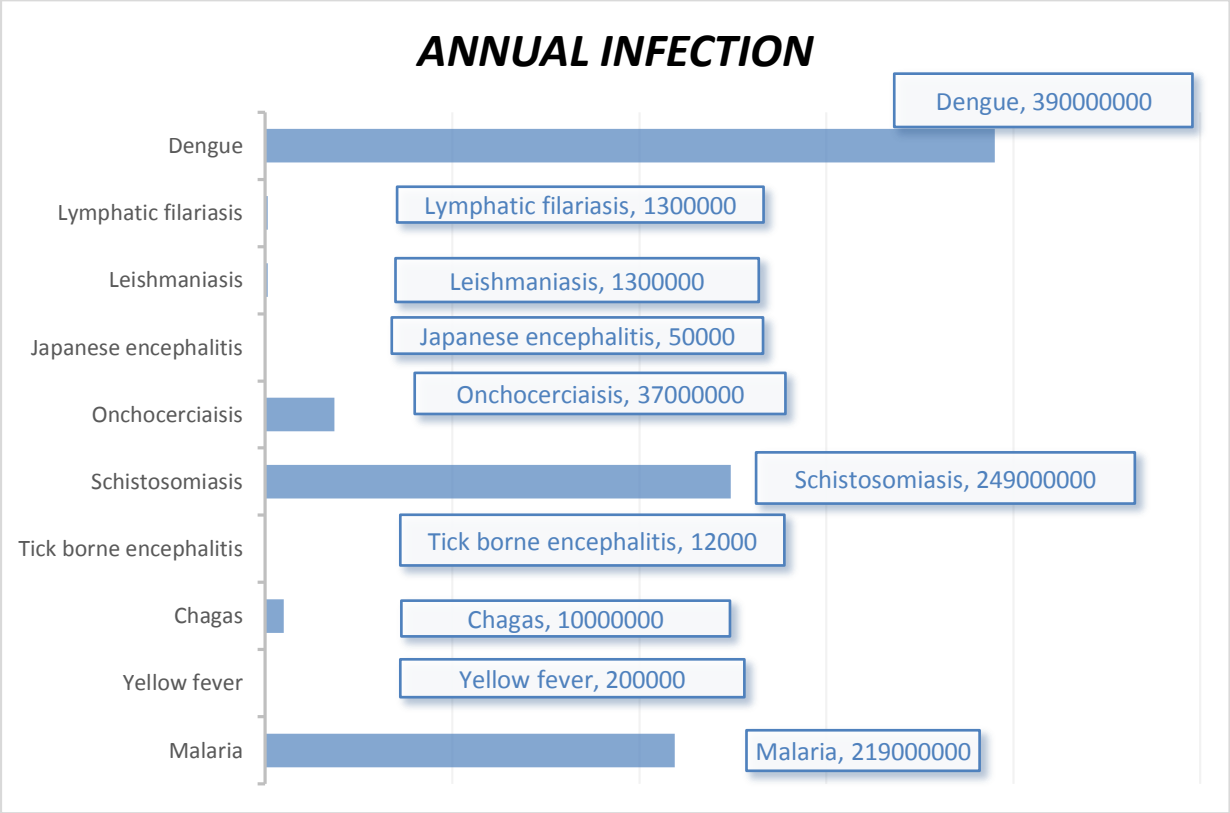


Figure 2: Vector borne diseases' proportion in global scenario

Majority of the tropical and subtropical countries are greatly affected by mosquito borne diseases. Globally, 97 countries have reported malaria transmission posing risk to 3.4 billion people (WHO, 2014). The most adversely affected region is the sub-Saharan region and the population at risk consist of young children, older aged individuals, pregnant women and non immune travellers to disease endemic regions (WHO, 2014). The most rapidly spreading mosquito borne viral disease is

Dengue which has expanded to previously unexplored regions increasing its incidence rate approximately 30 fold in the last 50 years (WHO, 2014). Another mosquito borne viral disease affecting Africa, Asia and the Indian subcontinent is Chikungunya. Recently this disease resurgence occurred in Asia, Africa, Europe as well as North America (WHO, 2014). India owing to its subtropical climate is subjected to regular infections of various vector borne diseases (Figure 3).

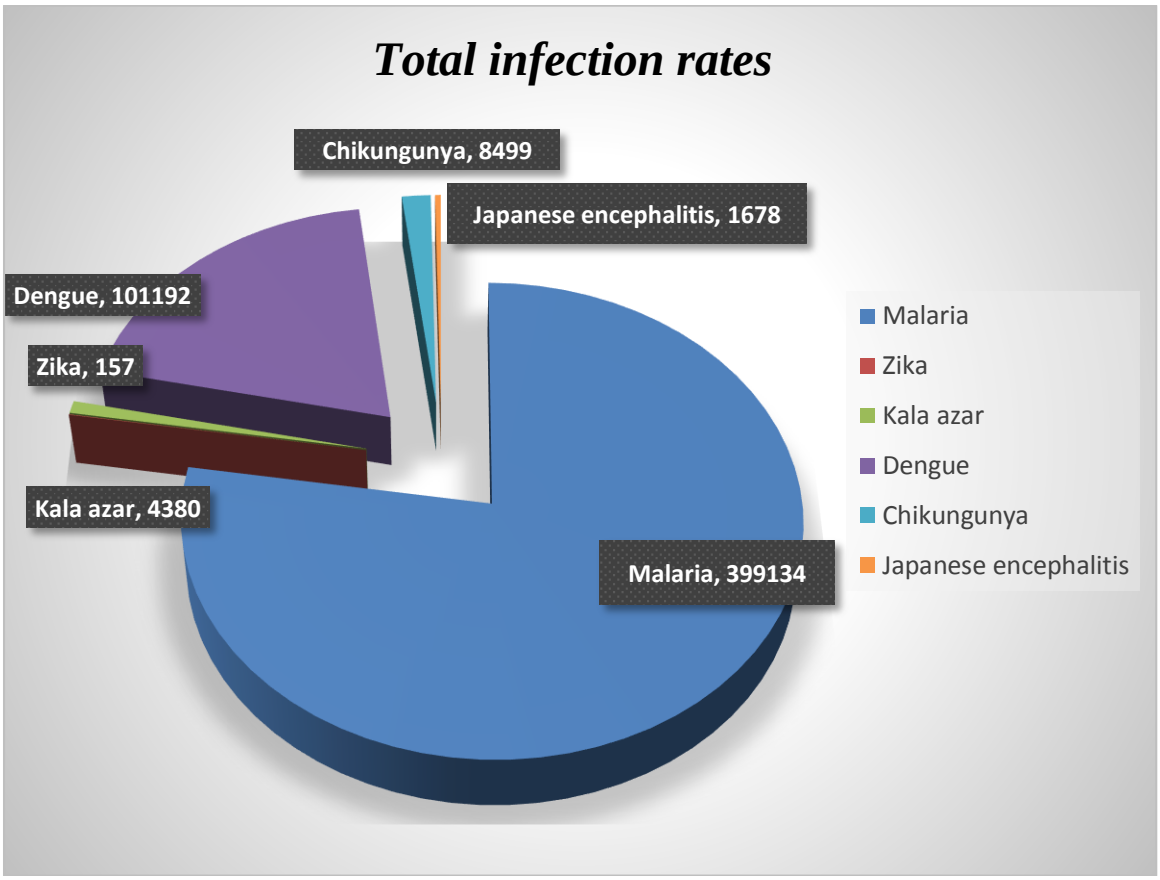


Figure 3: Annual proportion of vector borne disease transmitted by different vectors in India in 2018 (Source: NVBDCP, 2019a)

1.2 Dengue: a global burden

Dengue and Dengue hemorrhagic fever (DHF) are caused by virus belonging to family Flaviviridae, genus *Flavivirus*. *Flavivirus* is a medically very important genus of viruses, members of which can cause Dengue, yellow fever, west Nile

diseases, tick borne encephalitis and Japanese Encephalitis (Kuhn *et al.*, 2002). Dengue virus remains one of the most dreadful human pathogen transmitted by *Aedes* mosquitoes infecting humans worldwide (Figure 4).

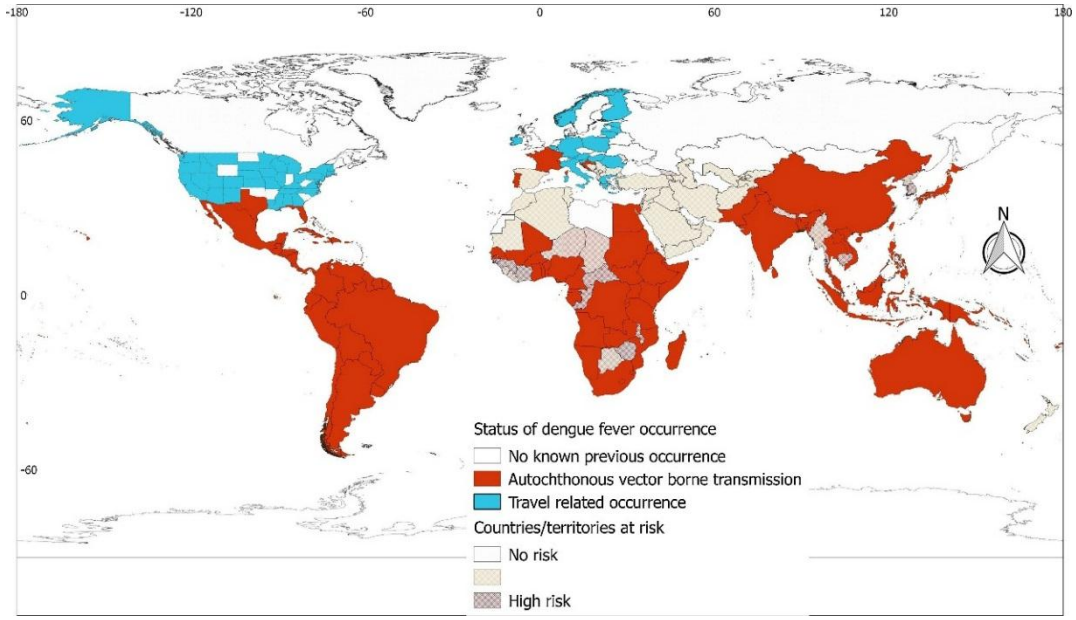


Figure 4: Dengue fever occurrence in global perspective (Source: Leta *et al.*, 2018)

There are four antigenically different yet closely related Dengue virus, generally termed as serotypes, *i.e.* DEN1, DEN2, DEN3 and DEN4. The presence of four different Dengue serotype creates additional complexity to the infection scenario and the serotypes only provide short term cross immunity against each other (Reich *et al.*, 2013). However, infection with one serotype does provide lifetime immunity against the specific serotype. DEN virus is endemic in around 100 countries of the world mainly situated in the tropical and subtropical regions (Sun and Kochel, 2013).The general DENV genome consists of a single chain of RNA (approx. 10,700 nucleotides) with its nucleocapsid embedded into three structural (core, membrane and envelope) and several non structural proteins (Kuhn *et al.*, 2002; Kurane, 2007). The interaction between the Dengue virus and the host immune system is mediated by

a non structural protein, known as NS-1, evoking an adaptive immunity mediated through T cell (John and Rathore, 2019).

The virus is transmitted to humans through the bite of *Aedes* mosquitoes namely *Ae. aegypti* and *Ae. albopictus*. The viruses circulates in the host blood and multiplies extensively for about 2-7 days. After this incubation period, the first clinical symptom is observed, *i.e.* fever (WHO, 2019a). Consequently more symptoms appear and the severity of the disease increases. Infected patients can transmit the virus via an *Aedes* mosquito vector to healthy person usually after the appearance of first symptom.

Reports also exist on the mucocutaneous mode of virus transmission (Chen and Wilson, 2004) as well as transmission from asymptomatic individuals or before the appearance of clinical manifestations (Duong *et al.*, 2015). They also report that asymptomatic individuals are more infectious to mosquitoes than people with symptomatic infections (Duong *et al.*, 2015).

Dengue fever is generally marked by high fever along with joint pain. The so called "breakbone fever" and frontal headache, however rash, nausea and lymphadenopathy may also develop (Kurane, 2007). The fever occurs after an incubation period of 2-7 days. More complex and severe is the Dengue haemorrhagic fever (DHF), that occurs in around 5,00,000 individuals (Gubler 1998). DHF arises due to the leakage of plasma into interstitial spaces leading to low platelet count and hemorrhagic symptoms (Kurane, 2007).

DHF is characterised by four main symptoms, fever, hemorhhages, hepatomegaly and failure of the circulatory system (WHO, 1997). DHF starts similarly as Dengue fever, but extreme weakening and collapse may occur within 2-5

days. The ultimate haematological symptoms of DHF are thrombocytopenia (low blood platelet count), increased RBC volume in the blood (hematocrit), elevated prothrombin time and bleeding time (Kurane, 2007). DHF has been characterised by WHO into four grades, of which most severe are the last two grades (grade 3 and 4) where plasma leakage is so extreme that onset of hypovolemic shock occurs, this is termed as Dengue shock syndrome (DSS) (WHO, 1997).

Dengue being one of the most rapidly spreading disease has increased its incidence rates in the past decades (WHO, 2019b). It is estimated that approximately 3.9 billion people residing through 128 countries inhabit Dengue risk areas (Brady *et al.,* 2012). An estimated 390 million infections of Dengue occur every year, out of which only 25% show clinical manifestations (WHO, 2019a). DHF is marked in tropical and subtropical countries of Asia and Latin Americas (WHO, 2019b). Annually around 2.5% of the infected individuals die due to the occurrence of severe form of Dengue (WHO, 2019a).

Severe Dengue epidemic was first noticed in 1950s in Phillipines (WHO, 2019a). However, now it has spread to almost every human inhabiting continent (Guzman and Isturiz, 2010). This is endemic in Africa, Southeast Asia, Eastern Mediterranean, America and the Western Pacific (WHO, 2019a). In 2015, around 2.35 million cases of Dengue infections were recorded in the Americas with 1181 deaths (WHO, 2019a). Similar epidemics were also noted in Portugal (2012), India (2015), Hawaii island (2015-2016) (WHO, 2019a). Worldwide severe Dengue outbreaks occurred in 2016 with the regions of Americas and western Pacific reporting the greatest disease burden rates (WHO, 2019a). In disease endemic countries, the Dengue burden is around 1300 DALY (Disability adjusted life years) per million population (Bhatt *et al.,* 2013).

1.3 Global burden of Chikungunya:

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus*, family Togoviridae and is responsible for an acute fever along with joint pain and weakness symptom (WHO, 2019c). CHIKV was first identified in 1952 in Tanzania and its outbreaks are recorded in Africa and Asia since then (WHO, 2014) (Figure 5). A peculiarity about Chikungunya outbreak is that, their outbreaks are periodic, often recurring after a period above a decade (WHO, 2019c).

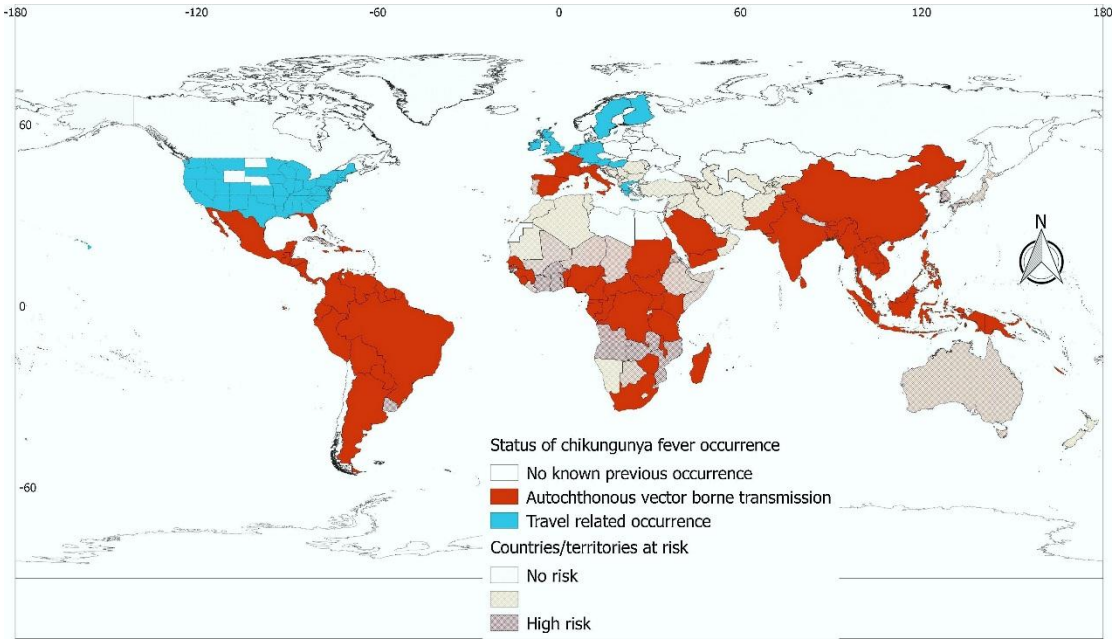


Figure 5: Global spread of Chikungunya fever (Source: Leta *et al.*, 2018)

Since 1982, Chikungunya epidemic have been reported from seven tropical Asian countries. Major epidemics have been reported to occur after 2000 in many countries, *i.e.* Congo (1999-2000), Gabon (2007), countries around Indian ocean (2005), India (2006-2007), Pakistan (2016-2017). Around 1.9 million Chikungunya infections have been reported to occur in five Asian countries viz., India, Indonesia, Maldives, Myanmar and Thailand (WHO, 2019c). Though Africa and Asia are the prime Chikungunya endemic continents, sporadic cases also occur in Europe, mainly

due to imported cases. Infections commonly localized in Europe have been reported in Italy (2007) and France (2013-2014) (WHO, 2019c). Over 43 countries of the Americas have been reported to have local Chikungunya transmissions. In 2015, approx 1.37 million CHIKV infection were reported in Carribean island, USA and Latin America countries (WHO, 2019c). In the Americas, highest infection rates were recorded for Brazil, Colombia and Bolivia in 2016 (WHO, 2019c).

The CHIKV consists of a genome of single stranded RNA (≈12000bp) with an icosahedral capsid (60-70 nm) enclosed within a lipid envelope (Thiberville *et al.*, 2013). Till now three distinct lineages of CHIKV have been identified each with specific genotype and antigenic determinants: i) Asian phylogroup, ii) East, central and southern African and iii) West African phylogroup (Powers *et al.*, 2000).

Two distinct cycles of transmission have been reported for CHIKV urban cycle and sylvatic/enzootic cycle (Singh and Unni, 2011). The transmission of CHIKV in forests (in Africa) with arboreal mosquitoes taking over the function of vector, mainly species of *Aedes* mosquitoes is termed enzootic cycle. In such cycles, non human primates basically serve as the virus reservoir (and thus serving as the virus amplification site). Such a cycle may sometimes infect human colonies inhabiting areas near the forest, initiating the urban cycle (Singh and Unni, 2011). Once the virus is introduced into the urban areas, the task of vectorial transmission of the virus is taken over by the urban anthropophilic *Aedes* mosquitoes, namely, *Ae. aegypti* and *Ae. albopictus* (Chhabra *et al.*, 2008). However, reports also suggest the inter-human transmission of CHIKV from sylvatic mosquito vectors (Tsetsarkin *et al.*, 2016). Common sylvatic vectors include *Ae. furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. africanus* and *Ae. neoafricanus* (Chhabra *et al.*, 2008). So, in general the transmission can be summarised as:

Onset of CHIKV infection in the non human primates in the African arboreal forest > occasional spilling of the infection to humans dwelling nearby > anthropophilic mosquitoes carry on the viral transfer to more human > initiation of human – *Ae. aegypti*/ *Ae. albopictus*- human transmission in the urban areas > established Chikungunya epidemic

Since these two *Aedes* sp. lie in close proximity to human colonies they cause a high exposure rate of humans to the pathogen virus. No sylvatic transmission has been noted to occur outside Africa thus implying that Chikungunya arose in Africa (Chhabra *et al.*, 2008).

The clinical manifestation of Chikungunya starts 2 days after the introduction of the virus in the human body, *i.e.* when the patients have highest viraemia; however it declines afterward (Shah *et al.*, 1964), The symptoms start with fever, which may persist upto two weeks (Staples *et al.*, 2009). In majority of the patients, shortly after the fever, joint paint develops which mostly occurs in wrists, elbows, fingers, ankles and knees (Chhabra *et al.,* 2008). Sometimes maculopapular rash may develop spreading through the extremities and trunk, however, rashes on palms and the face have also been reported (Staples *et al.,* 2009). Although Chikungunya infections rarely result in deaths, yet elderly people (suffering from other medical conditions) and patients with co-infection generally acquire complications which may prove fatal (Economopoulou *et al.,* 2009). Due to similar symptoms and similar vectors, Chikugunya and Dengue is often confusing to differentiate, however Dengue is characterised by haemorrhages whereas Chikungunya is basically identified by the multiple joint anthralgias (Staples *et al.,* 2009).

1.4 Zika statistics and possible future scenario:

Zika is the first major infectious disease responsible for alarming rates of human birth defects (Petersen *et al.,* 2016). The Zika virus (ZIKV) also belongs to genus *Flavivirus* as DENV. It was first isolated in 1947 in Uganda from a rhesus macaque (Kirya, 1977). However, ZIKV causing human diseases was recognised for the first time in Nigeria 6 years later (Macnamara, 1954). Since Zika is vectored by *Aedes* mosquitoes, it poses an immediate danger to the regions suitable for *Aedes* habitation (Figure 6).

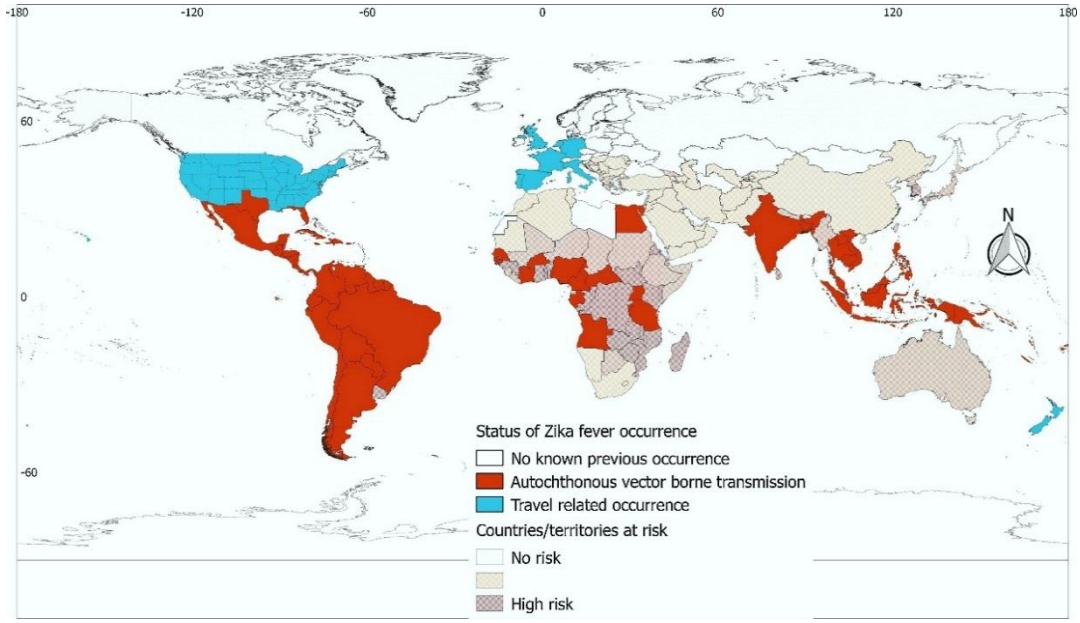


Figure 6: Global status for Zika fever incidence (Source: Leta *et al.,* 2018)

Though sporadic events of Zika were reported in Asia and Africa, the large outbreaks were reported in 2007 and 2013 (French Polynesia) (Musso *et al.*, 2014). Only 13 cases of Zika were reported till 2007, when a major outbreak took place in federated states of Micronesia causing an infection of 74.6% among the total residing population (Duffy *et al.,* 2009). Later outbreaks were reported from French Polynesia and other pacific islands (Petersen *et al.,* 2016). In India, 157 laboratory confirmed cases of Zika have been reported till November, 2018 caused by virus endemic to

Asia (Yadav *et al.,* 2019). Similar to CHIKV transmission, ZIKV has a sylvatic transmission cycle in Africa with similar reservoir and vectors. Moreover, the urban cycle is maintained by the same two *Aedes* species, *i.e. Ae. aegypti* and *Ae. albopictus* as CHIKV. However, *Ae. hensilli* and *Ae. polyniensis* were found to be the vectors of recent outbreaks in YAP and French Polynesia (Petersen *et al.,* 2016).

ZIKV has been found in many species of *Aedes* mosquitoes, *i.e. Ae. aegypti*, *Ae. albopictus, Ae. vittatus, Ae. luteocephalus, Ae. apucoargentus* and *Ae. furcifer* (Marcondes and Ximenes, 2016). Other Aedine mosquitoes have also been reported to carry ZIKV belonging to *Anopheles, Culex* and *Mansonia* genera (Benelli and Romano, 2017). Reports also point on the sexual mode of transmission (Foy *et al.,* 2011) and perinatal transmission of ZIKV (Besnard *et al.,* 2014).

The clinical symptoms associated with Zika is acute febrile illness with fever, conjunctivitis, arthralgia, rash or a combination of these. Other common symptoms include arthritis, headache, retro-orbital pain, vomiting, edema, hematospemia, subcutaneous bleeding, swelling of extremities, *i.e.* hands and ankles (Petersen *et al.,* 2016). However the most adverse effect of ZIKV is on the pregnant ladies causing microcephaly and other congenital malformations in the foetus (Petersen *et al.,* 2016). Microcephaly refers to reduced head size for the gestational size indicating the reduced growth of brain (Woods and Parker, 2013). In adults and children ZIKV can cause some neurologic complications such as myelitis, neuropathy and Guillain-Barre syndrome (WHO, 2019d). Central for disease control and prevention (CDC) has confirmed 69 countries and territories with active ZIKV transmission since 2007 (WHO, 2016).

`1.5 India's burden of Dengue, Chikungunya and Zika:

Dengue virus was first isolated for the first time in 1943 in Japan and in 1944 in India (Gupta *et al.,* 2012). The first virologically proved outbreak of Dengue occurred in during 1963, 1964 in the eastern coasts of India, however first Dengue like epidemic (not virologically proved) can be dated back to 1780s (in Chennai). In India, the onset of DHF was observed to occur in 1988 (Kabra *et al.,* 1992). However, since 1996, Dengue has become a more or less regular phenomenon causing substantial morbidity as well as mortality throughout the Indian country. All the four serotypes of Dengue have been noted to be present in India causing major epidemic episodes (Gupta *et al.,* 2012).

India is reported to acquire the largest number of Dengue infections annually with approximately 100 million asymptomatic and 33 million clinically manifested infections (Bhatt *et al.,* 2013). The presence of both the urban vectors and ideal climatic conditions aggravate the factors for major outbreak of Dengue. More than 0.1 million cases of Dengue occur every year in India, the trend towards an increase in infection rates every year (Table 1) (NVBDCP, 2019a).

Table 1: Infection rates of Dengue and Chikungunya in India during last five years (NVBDCP, 2019a,b)

Year	**Dengue**		**Chikungunya**	
	Case	**Death**	**No. of suspected cases**	**No. of confirmed cases**
2019	5504*	5	--	--
2018	101192#	172	47208	8499
2017	188401	325	67769	12548
2016	129116	245	64057	26364
2015	99913	220	27553	3342

*****Provisional data, #Except data from West Bengal

In India, major Chikungunya epidemic was reported in 1963 in Kolkata. Successively Pondicherry, Tamilnadu, Andhra Pradesh, Madhya Pradesh and Maharashtra reported Chikununya epidemic in 1965 and again in Maharasthra in 1973 (Sudeep and Parashar, 2008). The virus them had a resurgence in 2006 affecting 1.5 million people in thirteen India states (Cecilia, 2004). In 2011 again a major epidemic of Dengue affected every state and territory state of India except Punjab (in states) and Dadra-Nagar Haveli and Pondicherry (in territories) (Cecilia, 2004). The rate of mortality due to CHIKV is rare in India. Since 2015, around 1.39 million cases of infection have been reported throughout the country affecting approx this 213 districts in 15 states (Krishnamoorthy *et al.,* 2009).

Since majority of vector borne diseases adversely affect the Southeast Asian countries, it has been estimated that three region comprising of all the tropical and subtropical countries are at high risk of Zika infections (Messina *et al.,* 2016). Since eleven Southeast Asian countries have reported small/occasional Zika outbreaks, India is at a very high risk of this disease (Tilak *et al.,* 2016). It has been reported that co-circulation of ZIKV along with CHIKV and DENV is very likely in countries where both latter infections are common posing additionally risk on the country (Musso and Gubler, 2016). Moreover, a trade relation with ZIKV affected countries is also a risk factor in this context (Tilak *et al.,* 2016).

1.6 West Bengal and its burden of VBDs:

In 2017, among the Indian states the highest numbers of Dengue infections were reported in West Bengal (Table 2 and 3). Not only Dengue, West Bengal also records high incidences of other VBDs. The prevalence of these VBDs in W.B. may be pertained to many vectors. The warm and humid temperature and climatic

conditions, rapid urbanisation, high vegetation cover, lack of sanitation, hygiene and drainage in majority of the state together provide the ideal ambience for *Aedes* and other mosquito growth and proliferation. Additionally, the high population density of West Bengal (highest in India) along with above mentioned factors support the efficient circulation of disease causing pathogens.

Table 2: Prevalence of Dengue and Chikungunya in West Bengal (Source: NVBDCP, 2019a)

Year	***Dengue***		***Chikungunya***	
	Case	***Death***	***No. of suspected cases***	***No. of confirmed cases***
2019	*	*	*	*
2018	*	*	52	23
2017	37746	46	2103	577
2016	22865	45	1071	117
2015	8516	14	1013	61

*Data not available

Table 3: Statistics of major vector borne diseases and population at risk in northern districts of West Bengal (Source: State Vector Borne Diseases Control and Seasonal Influenza Plan, 2018)

District	***Dengue infection (2017)***	***Malaria infection (2017)***	***Population at high risk***
Alipurduar	74	1404	1024671
Coochbehar	217	129	1011047
Jalpaiguri	855	115	236588
Darjeeling	1266	58	286439
North Dinajpur	283	138	*

*Data not available

1.7 *Aedes*- the main culprit or "vector":

Aedes mosquitoes pose severe threat to human race because of its capability to transmit several arboviruses, *i.e.* DENV, CHIKV, ZIKV, Yellow fever virus, *etc* (Kraemer *et al.,* 2015). These diseases have increased severely in the past five decades and expanded itself many folds and spread throughout the globe. The total global burden put up by these mosquitoes is huge as calculated by the diseases they transmit. *Ae. aegypti* is also a key vector for the transmission of a serious emerging zoonotic disease, the rift valley fever (Pepin *et al.,* 2010). The habitat suitability range for these mosquitoes covers all the habitable continents of the world (Kraemer *et al.,* 2015). In a study, it was reported that of the 250 studied countries/territories 86% were suitable for the sustenance of *Ae. aegypti* and *Ae. albopictus* (Leta *et al.,* 2018). Furthermore the same was found to be more varied for *Ae. albopictus* than *Ae. aegypti*.

The most suitable regions for both the species were found to be the USA, South America, Indian subcontinent, Carribean, sub-Saharan Africa, Southeast Asia and few Pacific countries. In some regions of Asia and Western Africa, the distribution of both the *Aedes* species overlap, whereas in East Africa, Europe, United States, and Australia their distribution varies considerably (Kamal *et al.,* 2018). The ideal ambience for *Ae. aegypti* were mainly spread over the tropical and subtropical regions, whereas that of *Ae. albopictus* were greater str*etc*hing to the temperate regions too, *i.e.* Central USA and Southeast Europe (Leta *et al.,* 2018). This may be related to the ability of *Ae. albopictus* to survive in colder, dry and hardy environments unlike *Ae. aegypti* (Figure 7).

About 50% of the world has more than one arboviral disease (Leta *et al.,* 2018). Around 111 countries are endemic for Dengue, 106 for Chikungunya and 85

for Zika, 43 for yellow fever and 39 for rift valley fever (Leta *et al.*, 2018). Among the continents of the world, Autochthonous transmission of Dengue and Chikungunya have been reported from all the continents, Zika from all continents except Europe; yellow fever from Africa and America and rift valley fever from Africa and Asia only (Leta *et al.*, 2018).

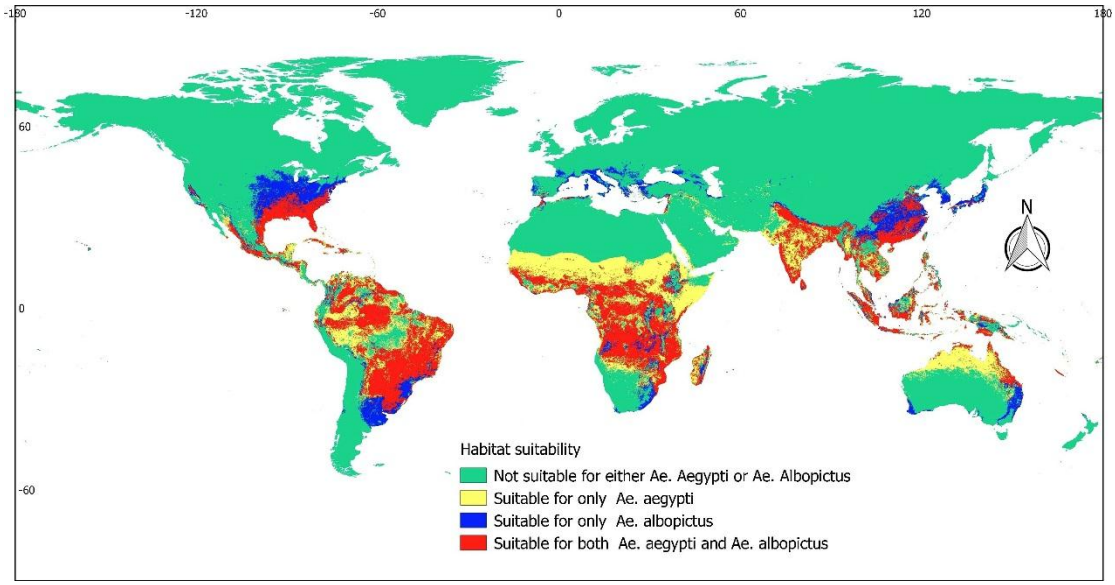


Figure 7: Habitat suitability for *Ae. aegypti* and *Ae. albopictus* throughout the world (Source: Leta *et. al.*, 2018)

Aedes mosquitoes are closely associated with the human colonies and occur near such dwellings, commonly laying eggs in and around human houses. Additionally, human made products, *i.e.* tyres, tanks, plastic containers *etc* serve as egg laying sites and becoming its breeding and proliferating site, thereby increasing their abundance and establishing colonies in human inhabiting areas. With the recent re-emergence of arboviruses, *Ae. albopictus* seems to become more efficient in virus transmission such as CHIKV because of minor mutations, the combination of which may increase the vector competence in this species (Tsetsarkin *et al.*, 2014).

1.7.1 Ae. aegypti:

Ae. aegypti, also known as yellow fever mosquito, is one of the most medically significant mosquito species responsible primarily for the transmission of Dengue, Chikungunya and yellow fever viruses worldwide (Powell and Tabachnick, 2013). *Ae. aegypti* originated in the African continent particularly the sub-saharan Africa region. Though, ancestral population of these mosquitoes were mainly non human feeders and tree holes served the main site of larval growth and habitation (Powell and Tabachnick, 2013). These population still represented by the sub species formosus is comparatively darker than the more recent subspecies adapted with human habitation *i.e. Ae. aegypti aegypti*. The latter lightly colored and domesticated species generally prefer feeding on human blood and breeding on man-made artificial containers. One more subspecies of *Ae. aegypti, i.e. Ae. aegypti queenslandensis* was present in Mediterranean region but doubt remains on its existence (Mattingly, 1967). Soon after domestication, *Ae. aegypti* expanded its distribution throughout different continents as a result of trade through ships. All the tropical and subtropical population of *Ae. aegypti* existing outside Africa is believed to form a monophyletic group (Brown *et al.*, 2014). However *Ae. aegypti aegypti* and *Ae. aegypti formosa* seem to show sympatry though they remain genetically separate yet they randomly mate to produce fertile offsprings when brought together (Moore, 1979). Moreover, these behavioural characters are not strictly maintained by these mosquito subspecies, since these are highly flexible and opportunistic and can respond quickly to environmental disturbances and changes.

Ae. aegypti are widely distributed in Asian and American continents with only five countries recording to carry 55% of the total *Ae. aegypti*, namely India, Thailand, United States of America, Mexico and Brazil (Kamal *et al.*, 2018). In Asian

continent, *Ae. aegypti* have been found to be distributed throughout major regions along with the Central and Southern part. This species has been recorded in Western Saudi Arabia, Western coasts of Arabian gulf, Lebanon, Israel and Syria (Kamal *et al.*, 2018). The *Ae. aegypti* is predicted to spread to Northern Australia, Eastern and Western coasts of Australia, Oceania and New Zealand. In Americas, Southeast USA, Caribbean islands, a minor region in the Pacific coasts of Canada and USA serve as the habitable regions for *Ae. aegypti* (Kamal *et al.*, 2018). In Europe, *Ae. aegypti* is believed to occur in Albania, Cyprus, Croatia, France, Greece, Italy, Spain and the coasts of Turkey and Portugal (Kamal *et al.*, 2018). In African continent, *Ae. aegypti* is maximally distributed across the sub-Saharan countries.

Domestication of *Aedes* mosquitoes determines its vector competence, as *Ae. aegypti aegypti* is more competent to transmit the disease carrying arboviruses than *Ae. aegypti formosus* (Tabachnick, 2013). This may be either due to the virus' adaptation to mosquito or the mosquito's bodily function increasing their competence (Powell and Tabachnick, 2013). The first instance of *Ae. aegypti* as disease vector in human, dates back to 1881 associated with yellow fever by Carbs Juan Fulay (Strode, 1951). Since then, these species have been documented to transmit human diseases throughout the world.

With an average flight range of 400 metres, most female *Ae. aegypti* are reported to spend their lifetime inside or in the vicinity of the human dwellings, where their development to adult has occurred, thus people are responsible for the rapid movement of the virus between the populations (WHO, 2019e). This indoor habitat increases the *Ae. aegypti* mosquitoes' lifespan, since these habitat are not generally susceptible to weather related variations (WHO, 2019e)

1.7.2 Ae. albopictus:

The second major disease transmitting *Aedes* species is *Ae. albopictus* commonly known as "Asian tiger mosquito". As the name goes, it has its origin in Asian continent, first identified in Kolkata as banded mosquito of Bengal by Skuse 1894 (Huang, 1968). Owing to its plasticity and elasticity it can invade regions throughout the world where other mosquitoes cannot thrive, thus known as one of the most invading mosquito species. It was originally a sylvatic mosquito species that became exposed to the human habitats as a result of deforestation, human habitat expansion in the vicinity of forests and ecotourism activities.

Unlike *Ae. aegypti* which is an anthropophilic mosquito, this species mainly prefers to feed on wild animals, however owing to its wide geographical distribution and behavioural plasticity now also efficiently adapted to feed on human blood and transmit diseases of public health importance, even more effectively in some instances than the primary vector, *Ae. aegypti* (Kraemer *et al.*, 2015). Spread of this species is also believed to be an effect of globalisation through trade of tyres, lucky bamboos (potted ornamental), containers carrying dormant mosquito eggs from one region to another, sometimes to previously un-invaded regions. As of now, *Ae. albopictus* has spread through Africa, middle east Europe to the Americas (Gratz, 2004).

Ae. albopictus, a native of Southeastern Asia, is now present in all the five habitable continents during the past four decades (Kamgang *et al.*, 2018). *Ae. albopictus* have been reported to be dominant over *Ae. aegypti* wherever both species co-exist owing to its higher mating competitiveness over *Ae. aegypti (*Bellini *et al.*, 2013). Moreover, wherever these two species show sympatry, *Ae. albopictus* shows preference to habitats/containers surrounded by vegetations.

In Asia, *Ae. albopictus* mainly occurs in rural and suburban regions, mainly surrounded with forests and vegetations. This species have been recorded to be distributed in Cambodia, China, India, Japan, Malaysia, Pakistan, Myanmar, Thailand, Vietnam *etc* (Gratz, 2004; Vontas *et al.,* 2012) In the Americas, the geographical str*etc*h of *Ae. albopictus* extends from Southeast to North USA and South Canadian border with its distribution widely in continental USA but low across the South American continent. Now, *Ae. albopictus* can be found in many American countries spread from USA upto Argentina; Hawaii, the Solomon Islands and Fiji among the Pacific Islands (Paupy *et al.,* 2009). In Africa, the suitable habitats for *Ae. albopictus* has been noted across the red sea coast and the mediterannean coast from Morocco to Egypt, and across the Eastern region. *Ae. albopictus* after its first detection in South Africa (in 1989) was later recorded from other African countries, namely Cameroon, Gabon, Equatorial Guinea and Nigeria. In Central Africa, *Ae. albopictus* was first reported in early 2000 and now it is present in much of the central African countries (Paupy *et al.,* 2009). In European continent, this species shows broad distribution through most of the Western countries and the Balkan region (Kamal *et al.,* 2018).With the first ever detection in Albania(in 1979) amongst the European countries, it has been since then reported in this continent in other countries too, *i.e.* Bosnia and Herzegovina, Croatia, France, Greece, Italy, Montenegro, Netherlands, Serbia, Slovenia, Spain and Switzerland. *Ae. albopictus* have also been reported in the Australian continent. The vector status of this species remained questionable till epidemic Dengue outbreaks in absence of *Ae. aegypti* but in presence of this species occurred (Gratz, 2004).

1.8 Available treatments for *Aedes* transmitted disease:

For none of the arboviruses transmitted by *Aedes* mosquitoes, any treatment or vaccination exist. So, the patients are treated of their symptoms not of the viral infection. Dengue fever is generally treated through early detection and proper case management, thereby reducing the deaths associated with the infection (NVBDCP, 2019a). For uncomplicated Dengue, administration of an analgesics and antipyretic such as paracetamol, oral rehydration and maintenance of body fluids and proper rest is advised (Khetarpal and Khanna, 2016). However, patients are monitored for adverse symptoms and different blood tests till three days of fever onset. Whenever the symptom of decreasing platelet count ($\leq$100,000/mm^3) or rising hematocrit is noticed, immediate hospitalization is recommended and the patients are treated with intravenous fluid, to maintain the plasma volume. However, other signs of Dengue shock syndrome are thoroughly monitored such as, hematocrit, platelet count, pulse rate, blood pressure, temperature *etc*. Mostly within 12-48 hours of fluid therapy, patients recover. Rarely, internal hemorrhage is noted, in such cases blood transfusion becomes necessary (Khetrapal and Khanna, 2016).

Patients infected with Chikungunya are basically advised to get proper rest and adequate hydration and nutrition. For management of the infection, analgesics, antipyretics and fluid supplementation is administered. For the peripheral arthritis, physiotherapy, short term corticosteroid administration or long term anti-inflammatory therapy is advised. However patients who develop complex symptoms such as renal failure, multiorgan system failure, refractory thrombocytopenia, encephalitis or acute infectious polyneuritis *etc* are generally hospitalised and treatment for the condition developed in a patient is provided (NVBDCP, 2019b). For

Zika symptoms are treated with acetaminophen for fever and pain and rest and rehydration is advised (CDC, 2019).

1.9 Disease prevention – the sole method:

In absence of specific medications and vaccines for all the above mentioned disease, the prevention becomes the only option to restrict disease transmission in humans. Disease prevention for *Aedes* transmitted arbovirus mainly involves vector control and personal prophylactic measures to minimise mosquito biting (NVBDCP, 2019a). Personal prophylactic measures consist of use of mosquito repellent tools and mosquito nets to prevent mosquito bites along with covering the whole body by wearing full sleeve clothes with socks (NVBDCP, 2019a).

The second part of disease prevention is vector control which can be done by the strategies mentioned below:

i) Environment management and source reduction strategy,

ii) Mechanical control,

iii) Chemical control,

iv) Health education and mass participation

Under environmental management, identification and subsequent deletion of mosquito breeding habitats, proper management and sealing of water storage containers *etc* are done to minimise mosquito breeding in these vessels. Use of biological agents to minimise the mosquito population is the aim of biological control, conventionally done with the use of larvivorous fish such as Guppy (*Lebistes reticularis*), Gambusia (*Gambusia affinis*) and recently with bacterial formulations *i.e.* *Bacillus thuringiensis* and *Bacillus sphaericus*.

Mechanical control of mosquito is done through fencing or screening the windows and doors, drilling of holes in artificial containers and disposed tyres (so that water is drained), safe disposal of scrap *etc*. The most common method of mosquito control through the use of larvicides and adulticides comes under the chemical control of mosquito. Adult mosquito control consists of fogging, aerosol spray, indoor residual spray, long lasting insecticide treated nets, outdoor barrier spraying *etc* (NVBDCP, 2019a). Moreover, oils or monomolecular films are also used to disrupt air breathing ion mosquito larvae.

Since the discovery of insecticides, they have been used heavily for mosquito control. In India, Organochlorines (DDT, Dieldrin, Aldrin *etc*), Organophosphates (malathion, temephos, dichlorvos, chlorpyrifos), Synthetic pyrethroids (Lambdacyhalothrin, Deltamethrin, Permethrin, cypermethrin and its derivatives, cyfluthrin) and Carbamates (Propoxur, bendiocarb) have been widely used for both mosquito control as well as agricultural pest control (NVBDCP, 2019c). The insecticides and larvicides recommended for mosquito control in India are provided in Table 4 and 5.

Mosquito control intervention makes the heavy use of insecticide at both household as well as higher levels. Insecticide treatment of bednet, curtains, windows, water storage cans *etc* have been reported to be highly effective at minimising the household *Aedes* mosquito infestation (Deming *et al.*, 2016). Similarly Ultra low volume (ULV) spray and thermal fogging have also been shown to be effective at reducing the risk of Dengue virus transmission throughout the world (Karunaratne *et al.*, 2013).

Table 4: List of insecticides recommended for indoor residual spray (IRS) for mosquito control in India (Source: NVBDCP, 2019c)

S. no.	*Name of insecticide*	*Insecticide class*	*Insecticide requirement per million population (MT)*
1.	DDT (50% WP*)	Organochlorine	150.00
2.	Malathion (25% WP)	Organophosphate	900.00
3.	Deltamethrin (2.5%WP)	Synthetic pyrethroid	60.00
4.	Cyfluthrin (10%WP)	Synthetic pyrethroid	18.75
5.	Lambdacyhalothrin (10% WP)	Synthetic pyrethroid	18.75
6.	Alphacypermethrin (5% WP)	Synthetic pyrethroid	37.50
7.	Bifenthrin (10%WP)	Synthetic pyrethroid	18.75

*WP: Wettable powder

However, use of any of the above vector control method is ineffective without the education of the community and their active participation. The knowledge about the vectors and their common breeding habitats along with its control/ management methods should be provided to common mob for efficient prevention of these diseases. Furthermore mass programmes aiming to detect *Aedes* breeding habitats and their elimination through proper disposal, sealing, drilling and proper drainage practices can help immensely in reducing the *Aedes* transmitted arboviruses transmission (NVBDCP, 2019a).

Table 5: Larvicide formulation and dosages recommended for mosquito larvae control in India (Source: NVBDCP, 2019c)

S.no.	*Name of insecticide*	*Class of insecticide*	*Frequency of application*	*Application on*
1.	Mixed larvicidal oil	--	Weekly	Shore of water body
2.	Temephos	Organophosphate	Weekly	Clean water
3.	*Bacillus thuringiensis* var *israelensis* 5% (strain- 164 Serotype H-14)	Biolarvicide	Fortnightly	Both clean and polluted water
4.	*Bacillus thuringiensis* var *israelensis* 5% WP (strain-ABIL Serotype H-14)	Biolarvicide	Weekly	Both clean and polluted water
5.	*Bacillus thuringiensis* var *israelensis* 12 Aqueous suspension	Biolarvicide	Weekly	Both clean and polluted water
6.	Diflubenzuron 25% WP	Insect growth regulator	Weekly	Both clean and polluted water
7.	Pyriproxifen	Insect growth regulator	3 Weekly	Both clean and polluted water

1.10 Constraints of vector control- Insecticide resistance:

The discovery of DDT initiated the use of chemicals in vector control. Shortly after its introduction the potency and efficacy of DDT as both larvicide and adulticide began a new era in vector control. However, soon was observed the ill effects of DDT including environment degradation and insecticide resistance in target species.

But due to the uncontrolled heavy use of these chemicals/ insecticides, both target as well as non-target species have evolved to resist the actions of those chemicals in their body through different mechanism. This phenomenon interrupting the chemicals to manifest their planned actions is known as Insecticide resistance (Corbel and N'Guessan, 2013). Insecticide resistance can be defined as the inability of the insecticide to manifest its planned action at the effective dosage against insects.

Insecticide resistance results in the failure of mosquito control programmes to achieve their planned targets, thereby increasing the risk of DENV infection even after insecticide spray during severe disease outbreaks (Corbel and N'Guessan, 2013). This phenomenon of resistance is widespread among majority of the insects exposed to insecticide in agriculture sector, public health sector or household region. Most of the insecticide used at household level are targeted against mosquitoes in the form of fumigants, coils, sprays, creams *etc*. Moreover when the breeding habitat of mosquito is situated around gardens, agricultural land, they get cross exposure to insecticide sprayed on those regions too thereby increasing the intensity of resistance in them.

Mosquitoes have developed insecticide resistance both as a direct effect of insecticides targeted on them as well as an indirect exposure of insecticide sprayed on agricultural field (Nkya *et al.,* 2013; Overgaard *et al.,* 2005). In tropical and subtropical countries, the high human population, congested area of living, small farming lands, poor sanitation practices, presence of farming land in the vicinity of living areas result in the cross contamination by agricultural run offs containing pesticide residue to mosquito breeding sites, thereby contributing towards the onset of insecticide resistance. Also, the household prophylactic measures *i.e.* use of mosquito repellent coils, creams, lotions, fumigants contain formulations of Synthetic pyrethroids (recent compounds contain transallethrin) also result in insecticide resistance development (Class and Kintrup, 1991).

Resistance to insecticides can be caused by an array of modifications within a mosquito, such as, behavioural alteration, physiological modifications within the cuticle reducing the insecticide penetration, biochemical changes within the activity of major insecticide detoxifying enzymes or structural modification within the target of the insecticide thereby blocking the insecticide binding and subsequent action (Yu,

2008). All the above mentioned mechanisms have been noted to occur in field populations of *Aedes* mosquitoes throughout the world. Also, varying degrees of insecticide resistance have been reported in both *Aedes*

Insecticide resistance in the insect body can be classified among four main mechanisms:

1.10.1 Behavioral resistance/ avoidance:

Behavioral avoidance also called as "deterrence" refers to the act of escape of an insect in response to insecticide treatment on an area. Mosquitoes have been reported to avoid DDTs and pyrethroid insecticides showing moderate to strong irritancy. It may be of two different types:

a. Contact excitation/irritancy: when an insect escapes the insecticide treated areas after making an initial contact with the insecticide.
b. Non contact/spatial repellency: when the insect moves away without making any contact with the toxic chemicals.

1.10.2 Cuticular resistance/ reduced penetration:

Insecticides manifest their action once they bind to their target site. However, if the toxic chemical fails to reach its target, its action will be hindered. In this type of resistance, there is a check on insecticide entry into the insect body. This may be brought by increasing the cuticular covering/ diameter or increasing the fat layer present immediately after the cuticle, thereby restricting the entry of the xenobiotic into the insect body (Yu, 2008).

1.10.3 Metabolic resistance/ insecticide detoxification:

Metabolic detoxification of insecticides refers to the degradation of the chemicals into non toxic and water soluble forms by the action of gut enzymes. During insecticide stress period, the production of these enzymes may get increased thus sequestering more and more insecticide residue. This enzyme production is generally mediated by up-regulation through mutation in trans or cis acting regulatory locus or through the duplication/amplification of the gene coding two techniques increases the quantitative levels of these enzymes. However, modification can also occur in the qualitative proportion of an enzyme, *i.e.* increased ability to detoxify the insecticide resulting from minor change in the coding sequence of the gene, thus in the amino acid sequence.

The enzymes carrying on the task of xenobiotic detoxification generally belong to large families of multigenes, the most notable being the carboxylesterases (CCEs), Cytochrome P450s (CYP450S) and Glutathione S-transferase (GSTs) (Corbel and N'Guessan, 2013; David *et al.*, 2013; Ranson and Hemingway, 2005; Hemingway and Karunartane, 1998). These enzyme families have been reported to provide resistance against different groups of insecticides throughout different insects. Over-expression of insecticide detoxifying enzymes have been found to confer resistance against insecticides in many mosquitoes.

Increased synthesis of one such enzyme, *i.e.* CCEs through gene amplification have been reported to confer resistance against organophosphate, carbamates and pyrethroid insecticides in dipteran insects (Bass and field, 2011). This may provide protection against insecticides by sequestration or slow turnover rate. Similarly, the elevation of isozymes of detoxification enzymes have also found to provide resistance. Metabolic detoxification has more severe outcomes than target site

resistance because these elevated enzyme activity may also provide cross resistance against insecticides belong to different classes unlike target site resistance which can provide resistance against only specific insecticide group.

Over expression of enzyme classes, Carboxylesterases (CCEs), Glutathione S-transferases (GSTs) and Cytochrome P450s (CYP450s) or Mixed Function Oxidases (MFOs) have been reported to confer insecticide resistance in many populations of insecticide resistant *Ae. aegypti* and *Ae. albopictus* population worldwide (Vontas *et al.,* 2012; Ranson *et al.,* 2010). Through advanced studies incorporating transcriptome studies and Detox chip analysis, all the three above mentioned enzyme classes namely, CYP450s, CCEs, GSTs have been implicated in conferring insecticide resistance against insecticides.

1.10.3.1 Carboxylesterases (CCEs):

Carboxylesterases enzyme, one of the major insecticide detoxifying enzyme belong to the Carboxylesterase gene family within the alpha/beta hydrolase superfamily which is one of the most commonly occurring protein folds in nature. This superfamily also contains lipases, dehalogenases, peroxidases, proteases *etc* (Montella *et al.,* 2012). These CCEs carry on the hydrolysis of esters, *i.e.* carboxylesters, splitting it into corresponding carboxylic acid and alcohol. These enzymes have been identified in all living species (Hatfield *et al.,* 2016). These esterases can hydrolyse a varied sort of substrates driving different actions. CCEs are omnipresent and play vital roles in the metabolism of several exogenous compounds, mainly ester carrying xenobiotics (Montella *et al.,* 2012). In insect life-cycle, these enzymes control a wide range of vital functions and behaviour, such as development, reproduction, digestion, odorant degradation, pheromone and other semiochemical hydrolysation (Montella *et al.,* 2012).

CCEs are mainly classified based on the interaction of esterases with organophosphates (Aldridge, 1953a; Aldridge, 1953b), those that hydrolyse them are categorized as esterases A (Est-A), those that are inhibited by them are termed esterases B (Est-B) and those that do not interact with organophosphate are categorized as esterases C (Est-C). The difference in the nature of Est-A and Est-B is due to the susceptibility of the serine residue present in the catalytic site of the enzyme to phosphrylation in Est-B but not in Est-A (Walker & Mackness, 1983).

CCEs are given much importance in insecticide chemistry since, many insecticides contain ester bonds rendering it susceptible to hydrolysis by enzyme activity (Sogorb & Vilanova, 2002). Of the commonly used insecticides, CCEs primarily detoxify OP insecticides, such as temephos, malathion, chlorpyriphos *etc* and secondarily carbamate insecticides such as bendiocarb, propoxur (Hemingway and Karunaratne, 1998). The mechanism of metabolic resistance involves insecticide hydrolysis or sequestration (Montella *et al.,* 2012).

Metabolic resistance to insecticides may arise by multifaceted mechanisms and insecticide resistant populations develop distinctive mechanisms for the efficient degradation of xenobiotic, *i.e.* insecticide (Saavedra-Rodriguez *et al.,* 2012, Poupardin *et al.,* 2008, Strode *et al.,* 2008). Insecticide detoxifying enzymes evolve rapidly by accumulation of mutations that doesn't affecting the original function rather may provide a selectivity advantage (Aharoni *et al.,* 2005). Resistance against OPs driven by over-expression of enzyme and/or insecticide sequestration resulting from CCE gene amplification have been reported in insects particularly, mosquito species (Grigoraki *et al.,* 2017).

Globally, very few CCEs gene have been reported to be amplified to confer resistance in insects. In *Culex* mosquitoes, resistance against OPs has been reported to be conferred by the elevated expression of two loci *i.e.* Est-2 and Est-3 as a result of gene amplification, which may be co-amplified as allelic pairs or amplified individually *e.g.* estβ1 gene in resistant mosquitoes (Bass and Field, 2011; Raymond *et al.,* 2001). Such amplified CCE alleles can get distributed to outlying regions by migration, as from different continents, the same common haplotypes have been noted in mosquitoes providing resistance against insecticides (Grigoraki *et al.,* 2017). Those alleles or combinations might get widely distributed than others owing to the higher fitness possessed by individuals carrying such alleles (Labbe *et al.,* 2009). This is the scenario for Ester2 which occurs in majority of the insecticide resistant populations, similar is true for the co-amplicon estα2-estβ2 (Li *et al.,* 2014).

CCEs have been also be implicated in conferring resistance against other insecticide groups, however the mechanisms of such action is not yet clear. Against temephos an OP insecticide, resistance has been shown to be conferred by the overexpression of CCE through amplification of *CCEae3a* transcript (Poupardin *et al.,* 2014). In other population co-upregulation of two transcripts belonging to different gene family, *i.e. CYP6Z8* and *CCEae3a* was found to confer resistance against OPs and synthetic pyrethroids suggesting the synergistic action by these transcripts (Marcombe *et al.,* 2009). In insects, the differences in the insecticide detoxification profile by enzymes might be due to the high rate of diversification as a result of species-specific evolution of detoxification gene families.

1.10.3.2 Cytochrome P450s (CYP450s) / monooxygenases:

The cytochrome P450-dependent monooxygenases (monooxygenases) are a vital group of enzymes involved in regulating the concentration of endogenous compounds as well as in the anabolism and catabolism of xenobiotics such as pesticides, drugs and plant derived allelochemicals. CYP450s monooxygenases are present in almost all aerobic organisms, ranging from bacteria to animals (Stegeman and Livingstone, 1998). These are the enzymes belonging to the largest superfamily of genes and bring upon the detoxification of xenobiotic compound as well as the metabolism of endogenous molecules such as hormones, steroids, fatty acids *etc* (Scott, 1999). These are found in all aerobic organisms ranging from bacteria to humans (Stegeman and Livingstone, 1998). These are basically heme proteins and can oxidise diverse substrates and this acts as the terminal oxidase in monoxygenase system (Scott, 1999). There appears to be 100 insect P450s (Nelson, 1998) however the first detection was made in 1967 (Ray, 1967). In insect apart from resistance, these play many crucial roles *i.e.* growth and development, tolerance to plant toxins, synthesis and catabolism of insect pheromones and hormones (Scott, 1999). Generally monoxygenase mediated resistance is provided against pyrethroids, imidacloprid, they are also crucial in activation of organophosphates (Hodgson *et al.,* 1991).

The main CYP450s associated with insecticide resistance are P450 reductase and b5 (Scott, 1999). Several P450s have been implicated in conferring resistance against synthetic pyrethroids, Over-expresssed P450s may also confer resistance against carbamates (Brooks *et al.,* 2001). In *Anopheles* mosquitoes CYP6Z1, CYP6Z2, CYP6M2, CYP6P3 and CYP325A3 have been shown to be involved in proving resistance (David *et al.,* 2013). In resistant wild mosquitoes, CYP6P3 and CYP6M2 have been noted to be over transcribed owing to their ability to degrade

permethrin and deltamethrin (Djouaka *et al.,* 2008). Similarly, CYP6Z1 has also been shown to detoxify DDT (Muller *et al.,* 2008) and CYP6M2 has also been found to be elevated in DDT resistance and CYP6Z1 in permethrin and DDT detoxification (Mitchell *et al.,* 2012; Chiu *et al.,* 2008). CYP6P9a and CYP69b have also been reported to be capable of detoxifying permethrin and deltamethrin (Stevenson *et al.,* 2011), thus providing resistance against them. *In Ae. aegypti,* CYP9J subfamily have the potency to metabolize pyrethroid insecticides (David *et al.,* 2013). CYP9J24, CYP9J28, CYP304C1, CYP6CB1, CYP6M10, CYP6M11 have been shown to provide resistance against permethrin, CYP6M6 and CYP6Z6 against deltamethrin and CYP9J32 against both permethrin and deltamethrin (Bingham *et al.,* 2011; Marcombe *et al.,* 2012; Marcombe *et al.,* 2009; Strode *et al.,* 2008). In *Culex quinquefasciatus,* mosquitoes, CYP450s have been implicated in permethrin resistance (David *et al.,* 2013).

1.10.3.3 Glutathione S-transferases (GSTs):

These soluble dimeric proteins are crucial in detoxification and subsequent excretion of many different endogenous as well as exogenous compounds. GST enzymes are encoded by genes belonging to two different supergene families (Hayes and Strange, 2000). These two families code for soluble and microsomal enzymes that provide protection against endogenous stress, *i.e.* reactive oxygen species and exogenous compounds *i.e.* xenobiotics, playing a key role in biotransformation of insecticides and drugs. GSTs drive the conjugation of electrophilic compounds with reduced glutathione (GSH) forming the thioester, rendering the resulting products water soluble which can be excreted out easily (Habig *et al.,* 1974).

In insects, two distinct classes of GSTs have been identified as class I and class II (Fournier *et al.,* 1992). A multigene family encodes the class I insect GSTs whereas a

single gene codes for all class II insect GSTs in majority of the insects (Enayati *et al.*, 2005). In *A. gambiae,* alternative splicing of the class II gene have been shown to produce two transcripts (Ding *et al.*, 2003).

Increased activity of GSTs have been shown to confer resistance against insecticides (Vontas *et al.*, 2012). Insecticide resistance can be conferred by elevation of one or more GST enzymes as a result of either increases in transcriptional rate or gene amplification; modification in qualitative properties of individual enzymes may also increase resistance levels but it less common (Ranson *et al.*, 2001).

GSTs have been implicated in resistance against organochlorines, such as lindane are conjugated to glutathione for detoxification and by dehydrochlorination of DDT moiety into DDE is catalysed by GSTs (Clark and Shamaan, 1984). Such dehydrochlorination of DDT have been shown to provide resistance to DDT in many insect species including mosquitoes belonging to *Aedes* and *Anopheles* genera (Enayati *et al.*, 2005). Amongst GST classes, Epsilon class have been found to be over-expressed in response to DDT selection pressure in *A. gambiae* resistant strain, with the highest dehydrochlorinase activity reported by *GSTe2* (Ortelli *et al.*, 2003). Reports also indicate on the involvement of GSTs in resistance against synthetic pyrethroids. GSTs have not yet been implicated in the direct metabolism of pyrethroid insecticides. Nevertheless, they may play an important role in conferring resistance to this insecticide class either by insecticide sequesteration (Kostaropoulos *et al.*, 2001) or detoxification of pyrethroid induced lipid peroxidation products (Vontas *et al.*, 2001). GSTs have also been implicated in resistance against organophosphates by conjugation of glutathione to organophosphate insecticides either by O-dealkylation (as in tetrachlorvinphos) or O-dearylation (in parathion and methyl parathion) (Hayes

and Wolf, 1988; Enayati *et al.*, 2005). No such detoxifications against carbamates have yet been noted by GSTs.

1.10.4 Target site resistance/ insensitivity:

Target site modification refers to the loss of sensitivity of the active site of the protein targeted by the insecticide. The most notable and commonly found is insensitivity of voltage gated sodium channel gene (VGSC) by synthetic pyrethroids (SPs) or organochlorines (OCs), thereby providing resistance against these insecticides, commonly called as knockdown resistance (kdr) (Kasai *et al.*, 2011). Modifications in the targeted site of insecticides are mainly brought upon by point mutations in the target gene. Several point mutations in VGSC gene have been reported in many mosquito vectors, *i.e. Anopheles, Aedes, Culex etc*. So far more than 50 sodium channel mutations have been identified in pyrethroid resistant insect pests and human disease vectors and many have been functionally confirmed to confer pyrethroid resistance (Du *et al.*, 2016).

Many point mutations conferring target site alteration in voltage gated sodium channel gene and acetylcholinisterase (AchE) gene have been identified in *Ae. aegypti* mosquitoes (Vontas *et al.*, 2012). In *Ae. aegypti* mosquitoes, around ten resistance related mutations have been identified in VGSC gene. Two of the most commonly detected point mutations in resistant *Ae. aegypti* are V1016G/I and F1534C in the IIS6 segment of VGSC (Hamid *et al.*, 2017; Li *et al.*, 2015). Other kdr mutations found in resistant *Ae. aegypti* population are G923V, L982W, I1011M, D1763Y (Du *et al.*, 2016). Double or triple mutations are also found in many insecticide resistant *Ae. aegypti* populations.

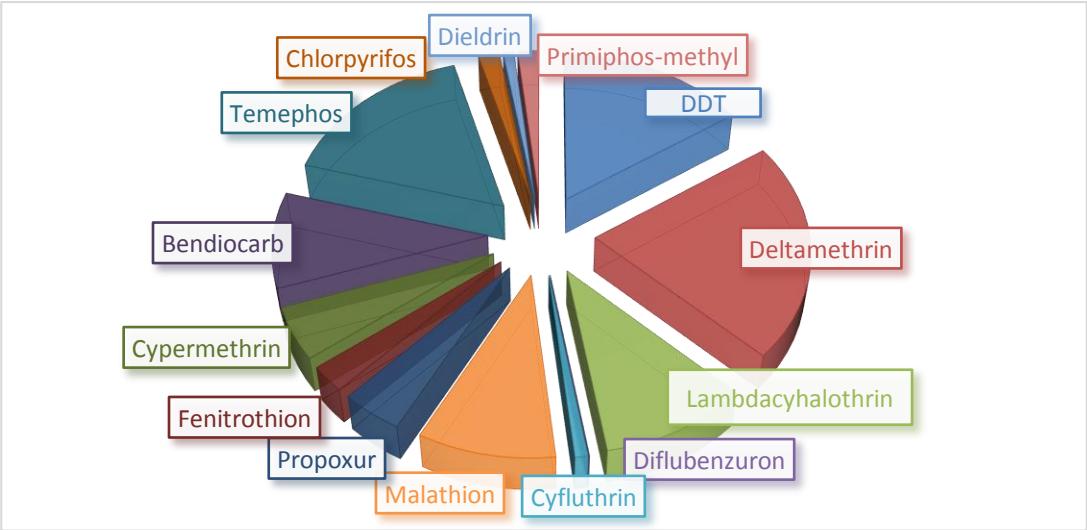
Knockdown resistance (kdr) mutation are widespread in different *Aedes* population. Presence of F1534C, V1016I/G *etc* have been shown to provide varying degrees of selective advantage under insecticide pressure in many populations of *Ae. aegypti* (Alvarez *et al.*, 2015; Plernsub *et al.*, 2016). Reports of kdr mutation in *Aedes albopictus* are very scanty as

compared to *Ae. aegypti*. However, there is a report of presence of kdr mutation (F1534C) in wild *Ae. albopictus* populations in Singapore resistant to permethrin (Kasai *et al.,* 2011). Till date, none of the examined *Ae. albopictus* population has been found to be positive for the presence of kdr mutations in India (kushwah *et al.,* 2015; Chatterjee *et al.,* 2018).

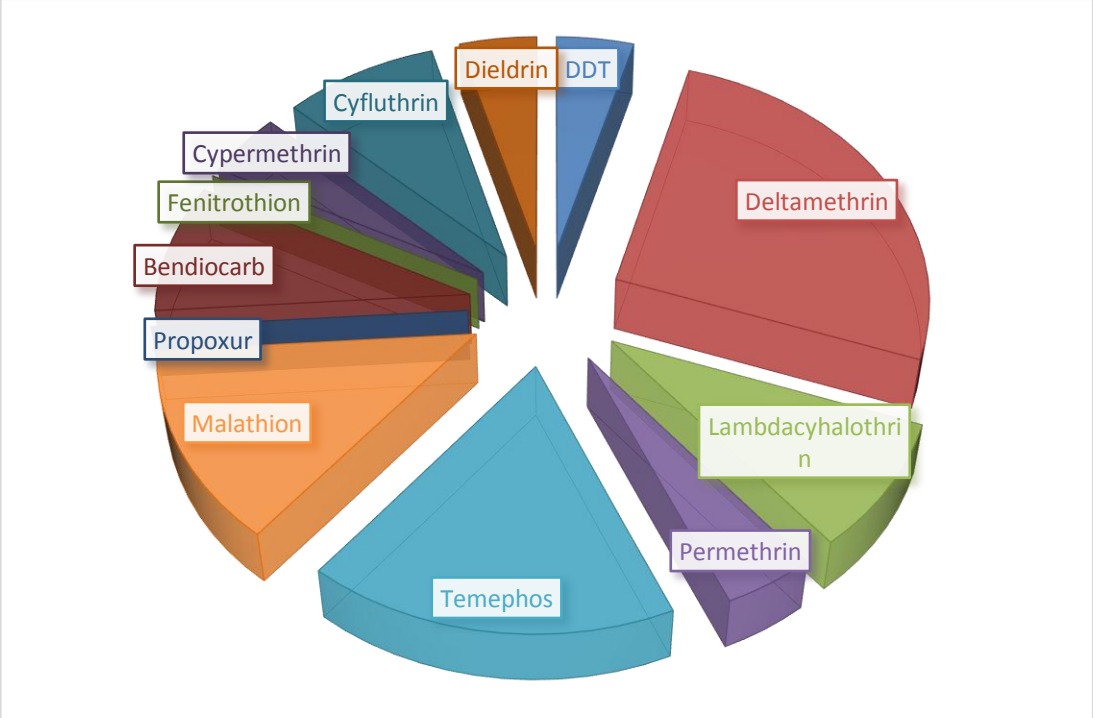
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE:

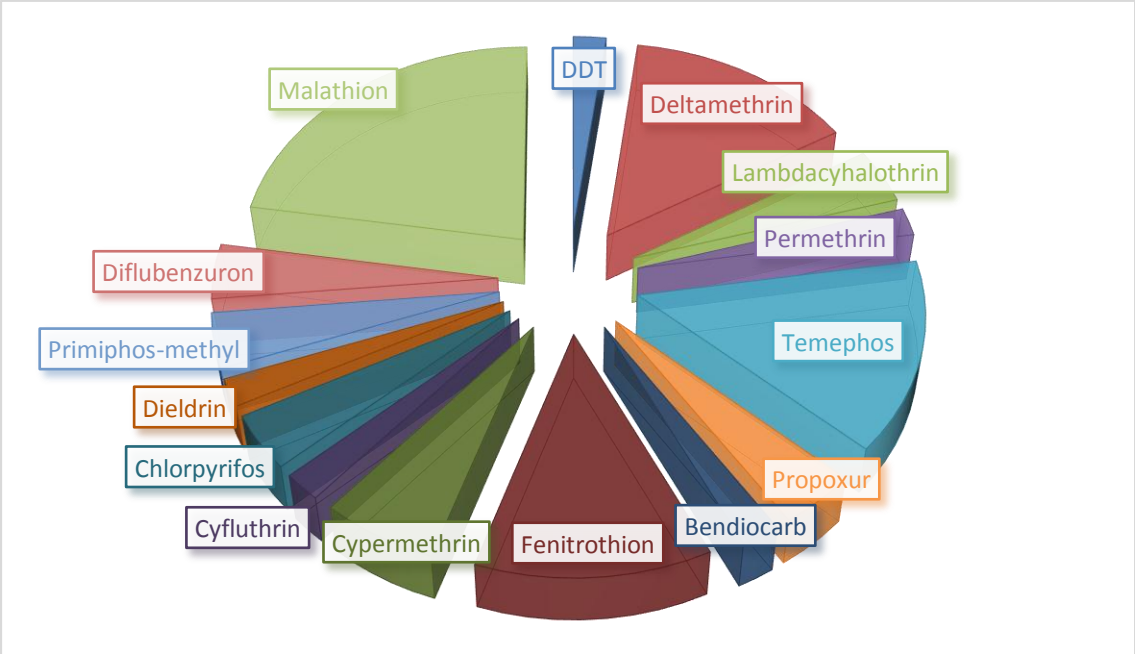
Insecticide resistance is a major problem throughout the world occurring in pests of both agricultural and medical importance. Unchecked usage of insecticides through a long span of time has resulted in the spread of this phenomenon throughout different insect species. The phenomenon of insecticide resistance in mosquitoes has been studied in great detail throughout this decade. Advancements in the form of more sophisticated and precise instruments along with discovery of novel molecular techniques have resulted in increased knowledge on the mechanisms of insecticide resistance occurring in nature. However, the phenomenon of insecticide resistance seems to be mediated by a combination of different mechanisms rather than any single or specific mechanism. Metabolic detoxification through different enzyme groups (CCEs, CYP450s and GSTs) have been found to be the major resistance conferring mechanism against all the four insecticide groups in *Ae. albopictus*. In *Ae. aegypti* for Organochlorines, Organophosphates and Carbamates, it was detoxification system that provided resistance whereas for synthetic Pyrethroids, it was mainly a combination of both detoxification and kdr mutations (Figure 8). The following presents a brief on the status of insecticide resistance and the underlying mechanisms in *Aedes* mosquitoes.



a. Resistant



b. Incipient resistance



c. Susceptible

Figure 8: Depiction of insecticide susceptibility status against various insecticides in *Aedes aegypti* mosquitoes

2.1 Insecticide resistance in Ae. aegypti:

Present status of insecticide resistance in *Ae. aegypti* has been summarised in Table 6. In many studies, both the resistance status as well as the mechanisms involved behind resistance phenomenon have been studied. Resistance against four groups of insecticides namely, Organochlorines (OCs), Organophosphates (OPs), Synthetic Pyrethroids (SPs) and Carbamates (CBs) have been studied in majority of the conducted studies (Table 6). However, in majority of the studies the mechanism conferring resistance against different insecticide group was found to be either metabolic detoxification or target site alteration or both (Figure 9).

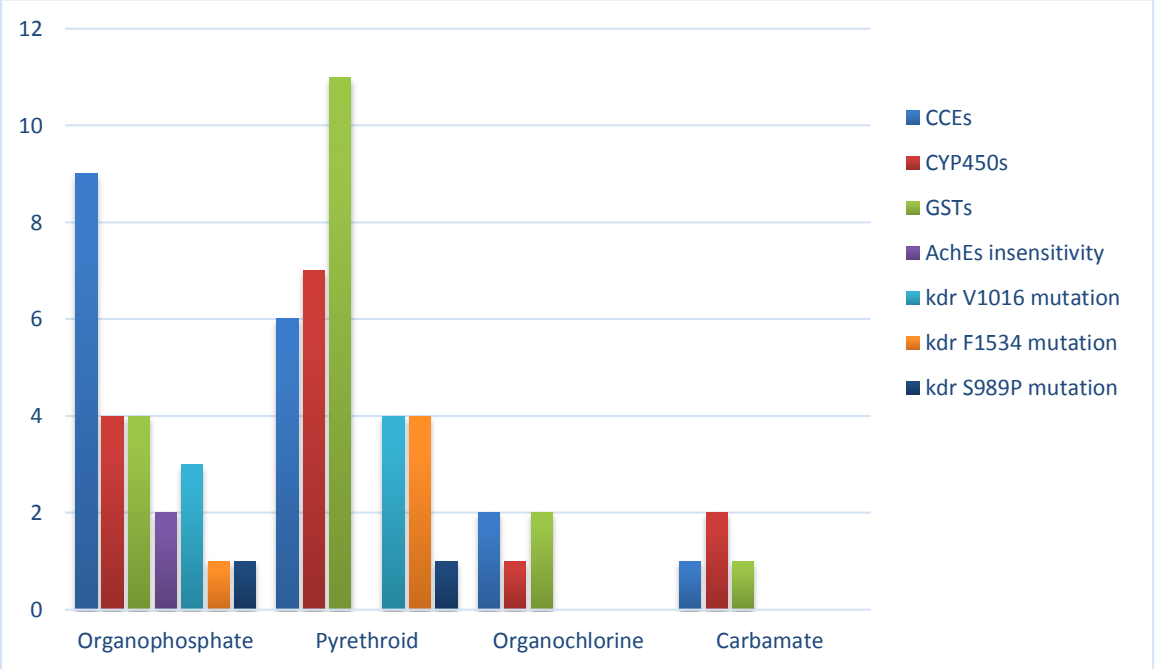


Figure 9: Mechanisms conferring resistance against major groups of insecticide from worldwide studies in *Ae. aegypti*

Worldwide studies have been conducted to unveil the resistance status of *Ae. aegypti* mosquitoes and resistance to all the groups of insecticides have been noted in this species (Table 6). However, widespread resistance have been observed against OCs, SPs and OPs.

2.1.1 Resistance against Organophosphates:

Organophosphates used worldwide for insecticide resistance studies include temephos, chlorpyrifos, fenitrothion, dichlorvos and malathion for larvae and only malathion for adults. Temephos has been the most commonly used mosquito larvicide throughout the world since the late 1960s (Grisales *et al.*, 2013). Also, resistance to this larvicide has also been observed throughout different strains of *Ae. aegypti* from different corners of the world (Table 6). Advanced studies have been made to identify the major underlying mechanism behind temephos resistance. The resistance to temephos has mostly been correlated with metabolic detoxification, either through biochemical CCEs assay or more advanced tools like microarray or detox chip studies. Enhanced detoxification enzymes have been reported to provide resistance against temephos in advanced studies (Bellinato *et al.*, 2016; Saavedra-Rodriguez *et al.*, 2014).

Clear correlation between α- and β- CCEs and GSTs and temephos resistance have been found in *Ae. aegypti* in Brazil with no role of altered AchEs behind resistance (Bellinato *et al.*, 2016). In some α- and β- CCEs and CYP450s have been found to provide resistance against temephos (Putra *et al.*, 2016). In most of the studies resistance to temephos has been linked mainly with CCEs. Microarray studies have reported two CCE genes namely, *CCEae3A* and *CCEae6a* to be the main candidate genes behind resistance to temephos in *Ae. aegypti* (Poupardin *et al.*, 2014). It was also reported that the above mentioned genes may have undergone duplication to provide resistance in *Ae. aegypti* (Poupardin *et al.*, 2014). However, the role of amino acid substitution in the gene may not be rejected. Similar observations were also made by other authors (Gelasse *et al.*, 2017; Yougang *et al.*, 2017), where both CYP450s and CCEs were noticed to be up-regulated in temephos resistant strains of

Ae. aegypti. *CYP6M11* have also been found to be highly over-expressed during temephos resistance (McAllister *et al.,* 2012; Yougang *et al.,* 2017); other members of CYP450 family *CYP6F3, CYP6N12, CYP9M9* were also found related with temephos resistance in *Ae. aegypti* (McAllister *et al.,* 2012; Yougang *et al.,* 2017).

In some other studies, somewhat different pattern of resistance mechanism was observed. Through synergistic studies, CCEs were reported to be the main enzyme group conferring resistance against temephos in *Ae aegypti,* however no up-regulated CCE gene could be observed in the same strains (McAllister *et al.,* 2012; Gelasse *et al.,* 2017). Rather, CYP450s were reported to be up-regulated in those temephos resistant strains of *Ae aegypti* (McAllister *et al.,* 2012; Gelasse *et al.,* 2017). Such findings question on the specificity of enzyme inhibitors and on the sequence similarities of different detoxifying enzyme groups.

Many studies claim to confirm the role of CCEs in temephos resistant *Ae. aegypti* populations. In a temephos resistant *Ae. aegypti* population from India, the observed resistance was conferred by both metabolic detoxification through α- and β-CCEs and target site mutation, G119S in ace-1 gene (Muthusamy and Shivakumar, 2015).A deeper insight revealed that all the three major detoxifying enzyme groups get up-regulated to provide resistance against temephos in *Ae. aegypti,* however CCEs do have a predominant role (Saavedra-Rodriguez *et al.,* 2014).

Through an indirect study incorporating Quantitative Trait Loci (QTL) mapping to identify the resistance genes, a single QTL, rtt1 (resistance to temephos) was identified in chromosome 2 of *Ae. aegypti* (Paiva *et al.,* 2016). This QTL was later identified as a cluster of CCE gene, supporting the strong correlation between CCEs and temephos resistance in *Ae. aegypti* (Paiva *et al.,* 2016).

Though, target site resistance has not been found to be linked to temephos resistance, yet a temephos resistant strain of *Ae aegypti* with a high prevalence of Val1016Ile mutation was reported from Brazil (Aguirre-Obando *et al.,* 2016). In this context, it may be noted that there are more evidences of metabolic detoxification playing the major role than target site alteration in temephos resistance in *Ae. aegypti*.

Many field strains of *Ae. aegypti* mosquito have been reported to exhibit varying pattern of resistance against other Organophosphates. Some studies support the phenomenon of cross resistance between temephos and malathion (Putra *et al.,* 2016), however some others strongly reject it (Gelasse *et al.,* 2017). *Ae. aegypti* strains have been found to be resistant to temephos yet susceptible to malathion, fenitrothion or another organophosphate (Gelasse *et al.,* 2017). This may be pertained to the fact that open chain OPs (malathion) may have different resistance mechanisms than other OPs (temephos) (Hemingway and Ranson, 2000). Resistance against malathion may be conferred by some completely different mechanism other than that against temephos (Gelasse *et al.,* 2017). However, like temephos, resistance against malathion has also been correlated with enhanced activity levels of CCEs (Widiastuti *et al.,* 2016). In other studies, not a single detoxifying enzyme rather a mixture of different enzyme groups, *i.e.* both CCEs and CYP450s were reported to provide resistance against malathion (Putra *et al.,* 2016). Few studies strongly reject the involvement of GSTs in resistance against malathion (Sundari *et al.,* 2016), whereas some other support it (Choovattanapakorn *et al.,* 2017). Through advanced studies it was revealed that CCEs and GSTs may play vital roles in conferring resistance against malathion (Choovattanapakorn *et al.,* 2017). Similarly for resistance against other OPs, *i.e.* fenitrothion and dichlorvos, mixture of different groups of detoxification enzymes were found to be responsible (Muthuswamy *et al.,* 2014;

Seixas *et al.,* 2017). Enhanced activity levels of both CCEs and AchEs were shown to provide resistance against dichlorvos in a strain of *Ae. aegypti* from India (Muthuswamy *et al.,* 2014). GSTs may also prove to be strong candidate for detoxification of OPs (Choovattanapakorn *et al.,* 2017).

2.1.2 Resistance against Organochlorines:

DDT resistant strains of *Ae. aegypti* have been reported from Nigeria, Central African Republic, Senegal, Malaysia, Srilanka, Pakistan, Saudi Arabia, Mexico, Colombia, India and Japan (Table 6). Highly resistant strains (with corrected mortality: 0-2%) have been observed in Malaysia and Columbia. Rest of the reported populations showed low to moderate resistance against DDT. Moreover, no DDT susceptible strain of *Ae. aegypti* could be reported from anywhere in the world.

Resistance against DDT may be contributed either by increased activity of insecticide detoxifying enzymes or by target site mutations, *i.e.* knockdown resistant (KDR) mutations or by a combination of both. In some studies, resistance to DDT was found to be a multifactorial phenomenon, *i.e.* both elevated CCEs and GSTs along with a prevalence of kdr mutations, namely Val1016L (on IIS6 domain of sodium channel gene) and F1534C (on same gene and domain), were shown to contribute to resistance phenomenon (Aponte *et al.,* 2013). However in other studies, either metabolic detoxification alone *i.e.* enhanced α- and β- CCEs, CYP450 MFOs, GST (Ngoagauni *et al.,* 2016)or kdr mutation, *i.e.* I1016 (Aguirre-Obando *et al.,* 2016) was found to be the underlying mechanism. In another such study, kdr F1534C (frequency 0.41-0.79) was found to be associated to DDT resistance in *Ae. aegypti* (Kushwah *et al.,* 2015). Moreover, some other authors have pointed on sex based/ sex limited resistance mechanisms. In one such study, differences were noted between male and female

resistance mechanism, *i.e.* in male *Ae. aegypti* mosquitoes, detoxification through CYP450 was found to confer the observed resistance against DDT whereas in females, this enzyme group could not account for the total observed resistance (Ishak *et al.,* 2015). The exact mechanism or group of mechanisms conferring resistance against DDT still remains unsolved.

2.1.3 Resistance against synthetic Pyrethroids:

Synthetic Pyrethroids are the newest among the insecticide groups, however, most frequent resistance has been observed in both type I and type II pyrethroid insecticides (Table 6). Deltamethrin was the first synthetic pyrethroid (SP) insecticide to be used in mosquito control in the late 1980s. Subsequently, other SP compounds were discovered and brought into use. SP insecticides target the voltage gated sodium channel to manifest their action, *i.e.* same as DDT (Corbel and N'Guessan, 2013). Moreover, SP insecticide were reported to develop resistance at a very fast rate which may be due to cross resistance, *i.e.* moderate to high resistance against DDT is already widespread in *Ae. aegypti* and both SPs and DDT share the same target site.

SP resistance is thought to be conferred mainly by metabolic detoxification and target site mutation. Many past reports have been made on the promising role of detoxifying enzymes, *i.e.* CYP450 (mainly), CCEs and GSTs (lesser extent) (Vontas *et al.,* 2012). However, since 2012, very few reports have claimed CYP450 to be the main mechanism providing resistance against type I and II pyrethroids (Ishak *et al.*, 2015). In other studies, CYP450s along with other detoxifying enzyme classes *i.e.* GSTs and/or CCEs are reported to confer reduced susceptibilities against SPs (Marcombe *et al.,* 2012). Enhanced activities of all the above mentioned enzyme classes have been found to provide resistance against deltamethrin (Marcombe *et al.*,

2012); similar inferences were made on deltamethrin resistant population of *Ae. aegypti* from Chile, where CYP450s, CCEs and GSTs provided the resistance (Table 6). In an Indonesian population of *Ae. aegypti*, CYP450s and β-CCEs provided significant level of resistance against permethrin (Putra *et al.*, 2016). Most frequently, deltamethrin resistance is conferred by GSTs and CYP450s (Alvarez *et al.*, 2013).

Different classes of detoxifying enzyme groups together or alone have been shown to provide pyrethroid resistance against different populations of *Ae. aegypti*. In many studies, CCEs were shown to provide varying patterns of resistance against pyrethroid insecticides, *i.e.* deltamethrin (Bisset *et al.*, 2013), lambdacyhalothrin (Muthuswamy *et al.*, 2014). In another study CCEs along with GSTs were reported to be elevated in pyrethroid resistant populations of *Ae. aegypti* (Muthuswamy and Shivakumar, 2015). The same combination of enzymes were also involved in cypermethrin resistance in wild populations of *Ae. aegypti* (Li *et al.*, 2015). However, some reports completely discourage the involvement of metabolic detoxification in resistance against synthetic pyrethroids (Koou *et al.*, 2014).

Although, metabolic detoxification has been actively linked to SP resistant populations, yet the role of target site resistance is more pronounced than the former in *Ae. aegypti*. Target site alteration alone or along with metabolic detoxification have been shown to confer pyrethroid resistance in *Ae. aegypti*. In a deltamethrin and permethrin resistant population of *Ae. aegypti* both elevated activities of GSTs and CCEs along with a frequent V1016I (80% prevalent) and F1534C (lesser) kdr mutation were observed (Aponte *et al.*, 2013).

Some advanced studies have summarised that in *Ae. aegypti*, resistance against deltamethrin is provided metabolically through an overexpressed GST (specifically GSTe2) and genetically through spread of kdr mutations ,*i.e.* V1016I and

F1534C (Gelasse *et al.,* 2017). Moreover, CYP450s were reported to provide OP resistance only (Gelasse *et al.,* 2017). In a population of *Ae. aegypti* from Chile, kdr mutation I1016 was found to provide resistance against four pyrethroid insecticides, deltamethrin, lambdacyhalothrin, permethrin and cyfluthrin (Maestre-Serrano *et al.,* 2014). Strong correlation have also been observed on kdr mutations Ile1016 and Cys1534 with high to moderate deltamethrin resistance (Deming *et al.,* 2016).

Against permethrin, the sole type I pyrethroid still in use, kdr mutation and elevated activities of α- CCEs played the main role (Alvarez *et al.,* 2016). Prevalence of kdr F1534C have been reported to play the major role in a permethrin resistant *Ae. aegypti* population from India (Muthusamy and Shivakumar, 2015). Some studies report that in majority of *Ae. aegypti* population, metabolic detoxification has a very minor role in pyrethroid detoxification, rather it is kdr mutation that has the main role (Muthusamy and Shivakumar, 2015; Choovattanapakorn *et al.,* 2017). Similar observations have been made on the efficacy of kdr mutation F1534C and V1016G in imparting permethrin resistance in *Ae. aegypti* (Plernsub *et al.,* 2016).

Studies have also been made on the extent of resistance made by different kdr mutations. Kdr mutation F1534C was found to be predominant than V1016I in a deltamethrin resistant population of *Ae. aegypti* (Alvarez *et al.,* 2015). The scenario may be that the deltamethin exposure may have provided a strong selection pressure and individuals with F1534C mutation possessed survival advantage over individuals with V1016I. In another study by the same author, opposite results were observed (Alvarez *et al.,* 2014). The kdr F1534C have been shown to provide moderate resistance against deltamethrin (Ishak *et al.,* 2015; Kushwah *et al.,* 2015) and the presence of V1016G along with the above mutation had an additive effect on pyrethroid resistant population of *Ae. aegypti* in Malaysia (Ishak *et al.,* 2015).

However, populations of *Ae. aegypti* mosquitoes with similar prevalence of kdr mutations yet varying degrees of insecticide resistance have been observed in nature, which indicate the dependency of observed resistance on metabolic detoxification (by GSTs, CYP450s and CCEs or in combination) (Viana-medeiros *et al.*, 2017). Through expression of CYP450 proteins in *E.coli*, it was found that CYP9J32, CYP9J24, CYP9J20 and CYP9J28 proteins have the ability to detoxify deltamethrin (Stevenson *et al.*, 2012). Moreover, CYP9J32 showed the highest ability to metabolize deltamethrin but low permethrin detoxification (Stevenson *et al.*, 2012). The other three CYP450 enzymes also possessed the ability of pyrethroid detoxification but at a lower rate (Stevenson *et al.*, 2012). In other such study, deltamethrin resistant *Ae. aegypti* population exhibited an up regulated CYP9M9 and GSTE7 (Marcombe *et al.*, 2012).

The role of both the above mentioned mechanisms alone or in combination have been found to provide resistance against synthetic pyrethroids in different wild population of *Ae. aegypti*. Although the exact mechanism is not known, the best theory seems to be the role of both the mechanisms in different field population *of Ae. aegypti* as has been observed by Marcombe *et al.*, 2012, where both metabolic detoxification (through CYP450, CCEs and GSTS) along with kdr mutations, *i.e.* V1016I jointly provided resistance against pyrethroids (Marcombe *et al.*, 2012).

2.1.4 Resistance against Carbamates:

Ae. aegypti populations resistant against carbamates have been reported throughout Asian and African continent (Table 6). Resistance against bendiocarb were reported from Pakistan, Malaysia, Nigeria, Mexico, China and Saudi Arabia, whereas, propoxur resistance were reported from India, Cot d'ivore, Nigeria,

Malaysia, Senegal and China. Very few studies have been conducted since 2012 to unveil the mechanism conferring resistance against carbamates. CCEs have long been reported to have a secondary role in conferring resistance against carbamates (Hemingway and Karunaratne, 1998). Bendiocarb resistance was found to be provided by enhanced activities of CYP450s in Malaysian *Ae. aegypti* population (Ishak *et al.*, 2015). However, the CYP450 could account only for a proportion of total observed resistance against bendiocarb (Ishak *et al.*, 2015). Lowered levels of AchE were found to provide resistance against propoxur in another population of *Ae. aegypti* (Ngoagauni *et al.*, 2016). Though the exact mechanism providing carbamates resistance is not yet revealed, but the role of metabolic detoxification seems to be the dominant mechanism owing to the availability of more such reports throughout the world.

Table 6: Insecticide susceptibility/ resistance status and involved biochemical mechanisms in *Aedes aegypti* mosquitoes

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
1.	Temephos	R*	--	Indonesia	Mulyatno *et al.*, 2012
2.	Temephos	R	• Elevated CCEae3A, CYP6M11, CYP9M9 • Elevated CYP9M9 and GSTE7	Martinique island	Marcombe *et al.*, 2012
	Deltamethrin	R			
3.	DDT	R	--	Central Africa	Dia *et al.*, 2012
	Propoxur	R, S			
	Deltamethrin	R, S			
	Lambdacyhalothrtin	R, S			
	Permethrin	S			
	Fenitrothion	S			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
4.	Deltamethrin	R	--	Latin America	Bona *et al.*, 2012
	Cypermethrin	R			
	Temephos	R			
5.	Deltamethrin	S	--	Cot d'ivore	Konan *et al.*, 2012
	Permethrin	S			
	Lambdacyhalothrin	S			
	Propoxur	IR, R			
6.	Deltamethrin	S	--	Haiti	McAllister *et al.*, 2012
	Permethrin	S, IR			
	Malathion	S			
7.	DDT	R	--	Malaysia	Rong *et al.*, 2012
	Propoxur	R			
	Bendiocarb	R			
	Permethrin	R			
	Cyfluthrin	IR			
	Fenitrothion	S			
	Malathion	S			
8.	Temephos	S	--	India	Shetty *et al.*, 2013
	Propoxur	R			
9.	Deltamethrin	S	--	Yemen	Alhag, 2013
	Lambdacyhalothrin	R			
	Permethrin	S			
	Cyfluthrin	S			
	Malathion	R			
	Fenitrothion	R			
10.	DDT	R	• Resistance against malathion related to CCEs	Srilanka	Karunaratne *et al.*, 2013
	Propoxur	S, R			
	Malathion	S, R			
11.	Deltamethrin	R	• Resistance linked with CCE activity but not GSTs or CYPs.	Costarica	Bisset *et al.*, 2013
	Temephos	R			
	Bendiocarb	S			
	Chlorpyrifos	S			
	Cypermethrin	S			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
12.	DDT	R	• Elevated CCEs and GSTs • Kdr mutations V10161 and F1534C prevalent.	Mexico	Aponte *et al.*, 2013
	Deltamethrin	R			
	Permethrin	R			
13.	Deltamethrin	R	• Enhanced GST and CYP activity linked to Deltamethrin resistance. • Enhanced activity of CCEs linked to OP resistance	Venezuela	Alvarez *et al.*, 2013
	Malathion	R			
14.	Deltamethrin	R	--	Brazil	Freitas *et al.*,2014
	Temephos	IR			
15.	Deltamethrin	R	• Metabolic detoxification not involved	Singapore	Koou *et al.*, 2014
	Permethrin	R			
	Cypermethrin	R			
	Etofenprox	R			
	Primiphos methyl	S			
16.	DDT	R	• No correlation with biochemical enzymes. • Kdr L1016 mutation prevalent in all population.	Colombia	Maestre-Serrano *et al.*, 2014
	Deltamethrin	R			
	Permethrin	R			
	Lambda cyhalothrin	R			
	Cyfluthrin	R			
	Primiphos methyl	R			
	Temephos	S, R			
	Malathion	S			
17.	Dichlorvos	R	• Association of AchE and GSTs with OP resistance • CCEs associated with SP resistance.	India	Muthusamy *et al.*, 2014
	lambdacyhalothrin	R			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
18.	Permethrin	R		Brazil	Macoris *et al.*, 2014
	Cypermethrin	R			
	Temephos	R			
	Malathion	S			
19.	Temephos	S, IR, R	--	India	Singh *et al.*, 2014
20.	Permethrin	R	• Metabolic detoxification not involved in resistance.	Singapore	Koou *et al.*, 2014
	Temephos	IR			
	Etofenprox	R			
21.	Temephos (larva)	S		Argentina	Bisset Lazcano *et al.*, 2014
	Fenitrothion (larva)	S			
	Permethrin (larva)	S			
	Cypermethrin (larva)	S			
	Deltamethrin (adult)	S			
	Lambdacyhalothrin (adult)	S			
	Cypermethrin (adult)	S			
	Chlorpyrifos (adult)	S			
22.	Deltamethrin	R	• Mechanism could not be identified	Chilie	Rocha *et al.*, 2015
	Cypermethrin	R			
	Temephos	R			
	Malathion	S			
	Diflubenzuron	S			
23.	Permethrin	S	--	Nigeria	Ayorinde *et al.*, 2015
	Deltamethrin	S, IR			
	DDT	R, IR			
24.	Temephos	R	• Metabolic detoxification through enzymes. • Two kdr mutation also found prevalent	Malaysia	Ishak *et al.*, 2015
	DDT	R			
	Deltamethrin	R			
	Permethrin	R			
	Bendiocarb	R			
	Malathion	R, S			
	Dieldrin	R, S			
25.	Temephos	IR, R	• Kdr mutation Val1016Ile prevalent	Colombia	Aguirre-Obando *et al.*, 2015

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
26.	Cypermethrin	IR, R	--	China	Li *et al.*, 2015
	Lambdacyhalothrin	IR, R			
27.	Deltamethrin	S	--	Colombia	Conde *et al.*, 2015
	Temephos	R			
	Malathion	S			
	Fenitrothion	S			
	Primiphos-methyl	R			
28.	Deltamethrin	IR, R	--	Srilanka	Janaki *et al.*, 2015
	Temephos	R			
29.	DDT	R	--	India	Sivan *et al.*, 2015
	Deltamethrin	S			
	Lambdacyhalothrin	IR			
	Permethrin	IR			
	Cyfluthrin	IR			
	Bendiocarb	R			
	Propoxur	S			
	Dieldrin	S			
	Fenitrothion (adult)	R			
	Malathion (adult)	IR			
	Fenitrothion (larva)	S			
	Malathion (larva)	S			
	Temephos	S			
30.	Deltamethrin	IR	--	Thailand	Thongwat *et al.*, 2015
	Permethrin	IR, R			
	Temephos	S			
31.	DDT	R	--	India	Yadav *et al.*, 2015
	Deltamethrin	S			
	Temephos	R			
	Malathion	S			
32.	DDT	R	• High frequency of kdr F1534C associated with DDT and Deltamethrin resistance	India	Kushwah *et al.*, 2015
	Deltamethrin	R			
	Permethrin	R			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
33.	Lambda cyhalothrin	R	--	Malaysia	Hasan *et al.*, 2016
	Primiphos methyl	S	--		
34.	Temephos	S	• Elevated activity of GST and CCEs	Central Africa	Ngoagauni *et al.*, 2016
	DDT	R			
	Deltamethrin	S, IR			
	Propoxur	S			
	Fenitrothion	S			
35.	Temephos	R	• Involvement of CCEs, CYP450s and GSTs	Brazil	Bellinato *et al.*, 2016
	Deltamethrin	R			
	Diflubenzuron	S			
36.	Temephos	R	--	Indonesia	Hardjanti *et al.*, 2016
	Malathion	R			
37.	DDT	R	--	Cameroon	Yougang *et al.*, 2017
	Deltamethrin	IR			
	Permethrin	S			
38.	Malathion	R	--	Indonesia	Widiastuti *et al.*, 2016
39.	Permethrin	S	--	Nigeria	Oduola *et al.*, 2016
	Bendiocarb	R			
	DDT	S			
40.	DDT	R	--	Pakistan	Khan *et al.*, 2016
	Deltamethrin	R			
	Lambdacyhalothrin	R			
	Bendiocarb	IR			
	Malathion	R			
41.	Permethrin	R	--	Indonesia	Mantolu *et al.*, 2016
42.	DDT	IR	--	Ghana	Suzuki *et al.*, 2016
	Deltamethrin	IR			
	Lambdacyhalothrin	R			
	Permethrin	S			
43.	Temephos	R	• Involvement of EST-β and MFO	Indonesia	Putra *et al.*, 2016
	Malathion	S			
	Permethrin	R			
44.	Temephos	R	--	Indonesia	Prasetyowati *et al.*, 2016
	Malathion	R			

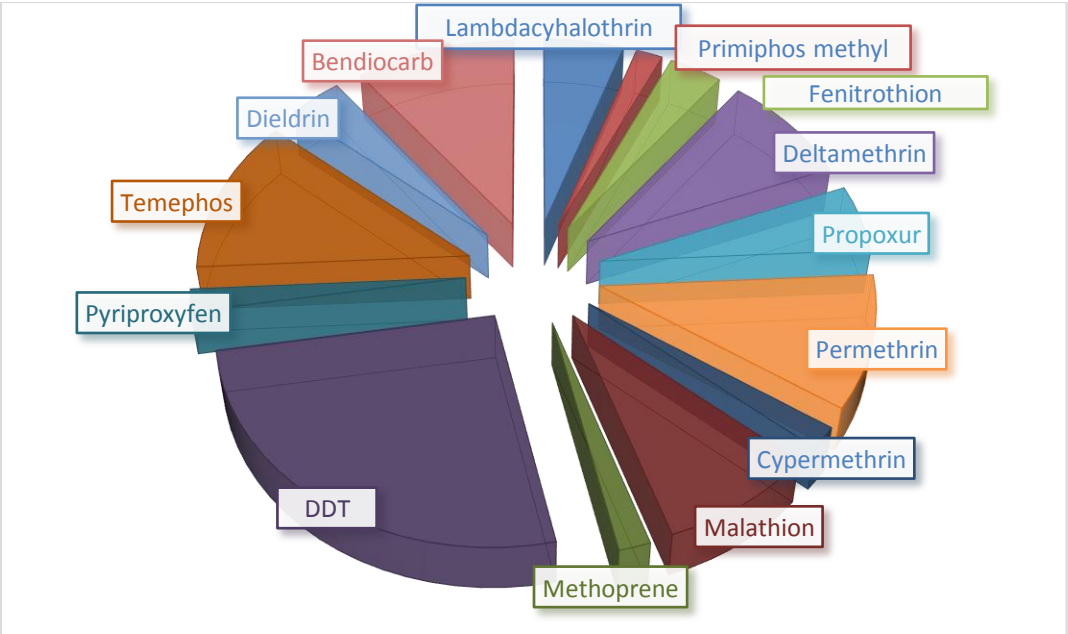
S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
45	DDT	R	--	Saudi Arabia	Alsheikh *et al.*, 2016
	Deltamethrin	R			
	Lambdacyhalothrin	R			
	Permethrin	R			
	Bendiocarb	R			
	Fenitrothion	R			
	Cyfluthrin	S			
46.	Malathion	R	• No involvement of GSTs.	Malaysia	Sundari *et al.*, 2016
47.	Temephos	S	--	Puerto Rico	Del Rio-Galvan *et al.*, 2016
48.	Temephos	IR	• Kdr mutation Val1016Le prevalent in tested population	Brazil	Aguirre-Obando *et al.*, 2016
49.	DDT	R		Pakistan	Arslan *et al.*, 2016
	Permethrin	R			
	Bendiocarb	R			
	Temephos	IR			
	Malathion	R			
50.	Deltamethrin	R	--	Mexico	Deming *et al.*, 2016
	Bendiocarb	IR, S			
	Chlorpyrifos ethyl	R			
51.	Deltamethrin	R	--	Mexico	Flores-Suarez *et al.*, 2016
	Permethrin	R			
	Cypermethrin	R			
	α-cypermethrin	R			
	z-cypermethrin	R			
	Lambdacyhalothrin	R			
	Bifenthrin	R			
	Chlorpyrifos	R			
52.	Deltamethrin	R	--	U.S.A.	Cornel *et al.*, 2016
	Sumithrin	R			
	Malathion	R			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
53.	Deltamethrin	IR	--	Tanzania	Mathias *et al.*, 2017
	Lambdacyhalothrin	IR			
	Permethrin	IR			
54.	Temephos	R	• Deltamethrin resistance related to high allele frequency of kdr mutation V1016L, F1534L • Enhanced GSTe2, CCEae3a, CYP014614, CYP 6B02, CYP6M11, CYP 9J23 • Highly resistant AchE	French West Indies	Gelasse *et al.*, 2017
	Malathion	R			
	Deltamethrin	R			
55.	Temephos (larva)	S	• Metabolic resistance through CYP450s against permethrin and deltamethrin • F1534C fixed in the population • Increased CYP450 expression in resistant populations	Burkina Faso	Badolo *et al.*, 2018
	Fenitrothion (Larva)	S			
	Malathion (Larva)	S			
	Deltamethrin	IR,R			
	Permethrin	IR,R			
	Fenitrothion	S			
	Malathion	S			
	Bendiocarb	R,IR,S			
56.	DDT	R	• Kdr mutations (V1016G and S989P) conferring pyrethroid resistance	Papua New Guinea	Demok *et al.*, 2019
	Deltamethrin	R			
	Lambdacyhalothrin	R			
	Malathion	IR,S			
	Bendiocarb	IR,R			

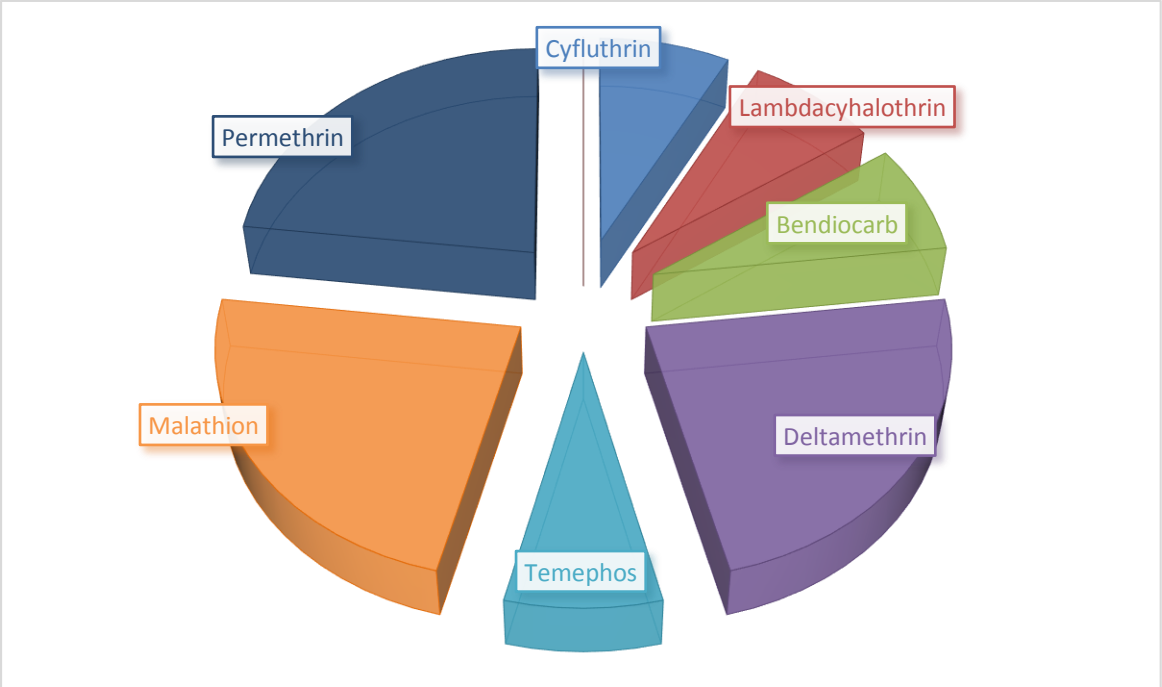
*S: Susceptible, IR: Incipiently resistant, R: Resistant

2.2. Insecticide resistance in Ae. albopictus:

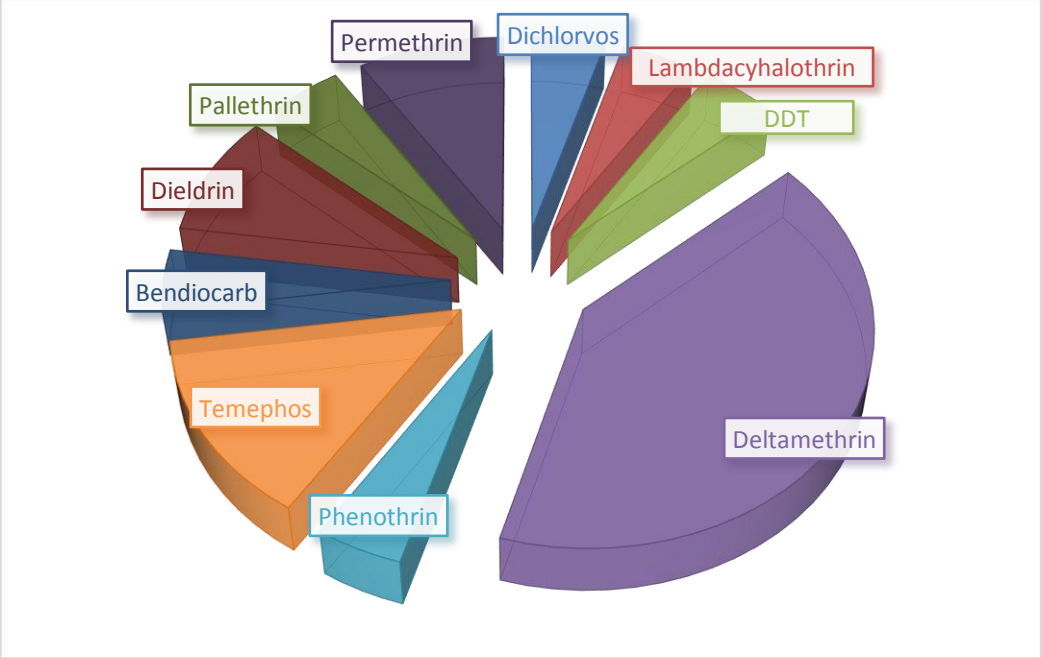
Ae. albopictus has very recently emerged as the potential dengue and chikungunya vector (Kraemer *et al.*, 2015). Increased worldwide expansion of *Ae. albopictus* due to globalisation have increased the risk of disease transmission through this vector (Kraemer *et al.,* 2015). The ability to survive under harsh conditions provide this species a selective advantage over other mosquito vectors. However, few studies have focused on the status and mechanisms of insecticide resistance in *Ae. albopictus*. In most of the studies, it has been found to be more susceptible against insecticides as compared to *Ae. aegypti*. However, DDT resistance was found to be omnipresent throughout different wild population of *Ae. albopictus* (Table 7). This mosquito by nature is an anthrophobic and exophilic species having differential trends of insecticide resistance than *Ae. aegypti* due to differences in their preferential resting sites (Kawada *et al.,* 2010). *Ae. albopictus* populations susceptible, incipiently resistant and resistant to various insecticides have been reported throughout the world (Figure 10).



a. Resistance



b. Incipient resistance



c. Susceptible

Figure 10: Proportion of resistant, susceptible and incipiently resistant *Aedes albopictus* mosquitoes reported throughout the world

DDT resistant strains of *Ae. albopictus* have been seen to occur in Srilanka (Karunaratne *et al.*, 2013), India (Yadav *et al.*, 2015; Das and Dutta, 2014; Dhiman *et al.*, 2014) Cameroon (Yougang *et al.*, 2017), United States of America (Marcombe *et al.*, 2014) Pakistan (Arslan *et al.*, 2016; Mohsin *et al.*, 2016) and Nigeria (Ngoagauni *et al.*, 2016). In *Ae. albopictus*, detoxification through enhanced activities of GSTs alone (Marcombe *et al.*, 2014) or by a combination of both GSTs and α-, β- CCEs may be accounted for resistance against DDT (Das and Dutta, 2014). Moderate resistance to Dieldrin have also been observed in Malaysian population of *Ae. albopictus* (Ishak *et al.*, 2015).

Both temephos resistant and susceptible strains of *Ae. albopictus* have been found throughout the world. Temephos have been found to be very efficient in control of some of the *Ae. albopictus* larvae in many studies (Dhiman *et al.*, 2014; Marcombe *et*

al., 2014). However, low to moderate resistance have been noticed against temephos in different field populations of *Ae. albopictus* (Arslan *et al.,* 2014; Yadav *et al.,* 2015). Enhanced activity of CCEs alone or GSTs and CCEs together (Ngoagauni *et al.,* 2016) have been found to be the underlying mechanism providing altered susceptibility to temephos in *Ae. albopictus*. Resistance to temephos have also been shown to be mediated through the up-regulation of two CCEs, *i.e.* CCEae3a and CCEae6a through gene amplification (Grigoraki *et al.,* 2015; Grigoraki *et al.,* 2016; Grigoraki *et al.,* 2017). Same candidate genes have also been shown to perform similar functions in *Ae. aegypti*. Malathion has been used since a long time throughout the world as a part of mosquito control programmes. This fact is well reflected in the reports of malathion resistant wild populations *Ae. albopictus* (Table 7). Metabolic detoxification through β-CCEs were found to be the main mechanism providing malathion resistance (Marcombe *et al.,* 2014).

Very few reports have been made on pyrethroid resistance in *Ae. albopictus*. Many studies showed the prevalence of high susceptibility levels of different field populations of *Ae. albopictus* to synthetic pyrethroids (Marcombe *et al.,* 2014; Das and Dutta, 2014; Dhiman *et al.,* 2014; Ishak *et al.,* 2015; Yadav *et al.,* 2015). Still *Ae. albopictus* possessing an altered susceptibility to permethrin have been reported from many parts of world (Karunaratne *et al.,* 2013; Arslan *et al.,* 2016). However, *Ae. albopictus* population moderately resistant to permethrin was recorded in Malaysia where part of the observed resistance was contributed by detoxification through CYP450s (Ishak *et al.,* 2015). Wild populations of *Ae. albopictus* resistant to majority of the commonly used synthetic pyrethroid insecticides, *i.e.* deltamethrin, lambdacyhalothrin and permethrin was found in Pakistan (Mohsin *et al.,* 2016). Elevated activity of enzymes have been correlated with resistance against

deltamethrin (Ngoagauni *et al.,* 2016). More reports have focused on metabolic detoxification of insecticides than target site mutations, *i.e.* presence of kdr mutations for *Ae. albopictus*.

Carbamate resistant strains of *Ae. albopictus* have been found in Malaysia, Pakistan and Nigeria (Table 7). Resistance to bendiocarb has been found to be mediated through the detoxifying activities of CYP450s (Ishak *et al.,* 2015). However resistance to propoxur has been indicated to be provided by altered AchEs in other population of *Ae. albopictus* (Ngoagauni *et al.,* 2016).*Ae. albopictus* Larvae significantly resistant to propoxur have also been noted in field populations of Martinique island (Marcombe *et al.,* 2014). More advanced studies and worldwide surveys on the level of insecticide resistance prevailing in different populations of *Ae. albopictus* should be conducted for a deeper understanding of insecticide resistance and mechanisms in *Ae. albopictus*. Different mechanisms have been identified to confer resistance against insecticide groups in *Ae. albopictus* from the studies conducted throughout the world (Figure 11).

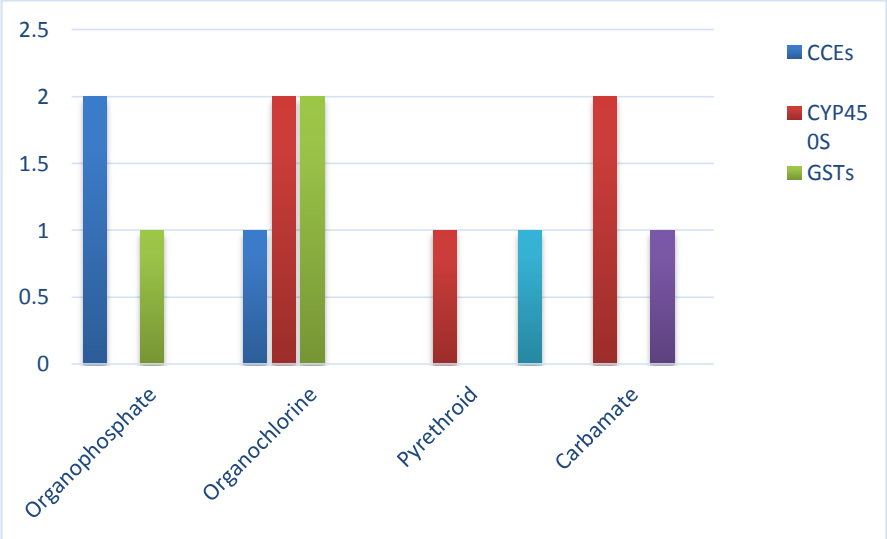


Figure 11: Mechanisms conferring resistance against major groups of insecticide in worldwide studies in *Ae. albopictus*

Table 7: Insecticide resistance status and involved biochemical mechanisms in *Aedes albopictus* mosquitoes

S. no.	Tested insecticide	Susceptibility status	Mechanism implicated	Location	Reference
1.	Deltamethrin	S*	--	Haiti	McAllister *et al.*, 2012
	Permethrin	S			
	Malathion	S			
2.	DDT	R	• A kdr based resistance mechanism may be involved in pyrethroid resistance.	Srilanka	Karunaratne *et al.*, 2013
	Maltahion	S, IR			
	Permethrin	R			
	Propoxur	S			
3.	DDT	R	• Enhanced CCEs and GSTs linked to DDT resistance	India	Das and Dutta, 2014
	Deltamethrin	S			
4.	DDT	R	• GST up regulation in DDT resistant individuals. • β-CCEs instigated to provide malathion resistance.	U.S.A.	Marcombe *et al.*, 2014
	Deltamethrin	S			
	Phenothrin	S			
	Pallethrin	S			
	Malathion	IR			
	Temephos (larva)	S			
	Propoxur (larva)	S, R			
	Methoprene (larva)	S, R			
	Pyriproxyfen (larva)	S, R			
5.	DDT	R	--	India	Dhiman *et al.*, 2014
	Deltamethrin	S			
	Malathion	S			
	Temephos	S			
6.	DDT	R	--	India	Yadav *et al.*, 2015
	Deltamethrin	S			
	Malathion	IR, S			
	Temephos	R			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
7.	DDT	R, IR, S	• CYP450s may be partially involved in providing resistance to DDT and bendiocarb.	Malaysia	Ishak *et al.*, 2015
	Deltamethrin	IR, S			
	Permethrin	IR, S			
	Bendiocarb	R, IR, S			
	Dieldrin	R, IR, S			
	Malathion	R, IR, S			
	Temephos	R			
8.	Deltamethrin	S	--	Srilanka	Janaki *et al.*, 2015
	Temephos	IR, R			
9.	DDT	R	--	India	Sivan *et al.*, 2015
	Deltamethrin	S			
	Lambda cyhalothrin	IR			
	Permethrin	R			
	Cyfluthrin	IR			
	Bendiocarb	R			
	Propoxur	S			
	Dieldrin	S			
	Fenitrothion (adult)	R			
	Malathion (adult)	S			
	Fenitrothion (larva)	S			
	Malathion (larva)	S			
	Temephos	R			
10.	Lambda cyhalothrin	R	--	Malaysia	Hasan *et al.*, 2016
	Primiphos methyl	R			
11.	DDT	R	• Elevated activity of GST and CCEs linked to temephos resistance. • Lower AchE activity linked to propoxur resistance	Central Africa	Ngoagauni *et al.*, 2016
	Deltamethrin	R, S			
	Propoxur	R, S			
	Fenitrothion	R			
	Temephos	R, S			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
12.	DDT	R	--	Pakistan	Mohsin *et al.,* 2016
	Deltamethrin	R			
	Lambdacyhalothrin	IR, R			
	Permethrin	R			
	Bendiocarb	IR, R			
	Malathion	S			
13.	DDT	R	--	Pakistan	Arslan *et al.,* 2016
	Deltamethrin	S			
	Lambdacyhalothrin	S			
	Permethrin	R			
	Bendiocarb	R			
	Malathion	R			
	Temephos	IR			
14.	DDT	S, IR, R	• Role of CYP450s in DDT and Bendiocarb resistance	Malaysia	Ishak *et al.,* 2016
	Deltamethrin	IR, S			
	Permethrin	IR, S			
	Dieldrin	S, IR, R			
	Bendiocarb	S, IR, R			
	Malathion	S, IR, R			
15.	DDT	R	--	China	Yiguan *et al.,* 2016
	Deltamethrin	R			
	Cypermethrin	R			
	Permethrin	R			
	Propoxur	R			
	Temephos	R			
	Dichlorvos	S			
16.	DDT	R	--	Cameroon	Yougang *et al.,* 2017
	Deltamethrin	IR			
	Permethrin	S			
	Bendiocarb	IR			
	Malathion	S			
17.	DDT	S	• CYP450s may be involved behind permethrin resistance	Malaysia	Ishak *et al.,* 2017
	Deltamethrin	S, R			
	Permethrin	S, R			
	Bendiocarb	S			
	Malathion	S			
	Dieldrin	S			
	Temephos	S			

S. no.	Tested insecticide	Susceptibility status	Mechanism of resistance	Country	Reference
18.	DDT	R			Tangena *et al.*, 2018
	Malathion	R, S			
	Deltamethrin	S			
	Permethrin	IR, S			
	DDT (Larva)	R			
	Temephos (Larva)	R, IR, S			
	Deltamethrin (Larva)	R, IR, S			
	Permethrin (Larva)	IR, S			
	Malathion (Larva)	S			
19.	DDT	R, IR	• Absence of kdr mutations	Papua New Guinea	Demok *et al.*, 2019
	Deltamethrin	R, IR			
	Lambdacyhalothrin	R, IR			
	Malathion	R, IR			
	Bendiocarb	R, IR			

*S: Susceptible, IR: Incipiently resistant, R: Resistant

2.3 Conclusion:

More research experiments should be targeted on the accurate identification of up regulated detoxification genes in insecticide resistant populations of *Aedes* mosquitoes. Scientific studies should also be carried out to identify the known as well as novel kdr mutations imparting resistance phenomenon to field populations of *Aedes* mosquitoes worldwide. In this context, data on *Ae. albopictus* is very limited, so more and more experiments should be directed on *Ae. albopictus* for identification of mechanisms conferring resistance to them. The concept of a fixed recommended insecticide dose for mosquitoes throughout different geographical location seem misleading, so the prevailing level of susceptibility/resistance to insecticides should be brought into use to devise the area specific dosage of insecticides for effective

vector control. Also, the knowledge gained through the study of mechanisms conferring insecticide resistance should be used to halt, delay or manage the level of insecticide resistance. Scientific studies to find out the ways to delay the onset of resistance should be conducted. Also, search for ideas and concepts for environmentally safe vector control strategies should be devised, such as mosquito control through botanicals, use of sterile male mosquito technology, discovery of novel group of mosquitocidal compounds with different targets of actions. Lastly, for effective prevention of the disease, involvement of mass/community in source reduction activities and environmental management is inevitable. The mass should be aware of the consequences of unchecked use of insecticides and they should be encouraged to take part in vector control programmes for efficient mosquito management.

MATERIALS AND METHODS

3. MATERIALS AND METHODS:

3.1 Selection of mosquito sampling districts:

Five different sampling districts were selected based on the dengue prevalence in northern part of West Bengal, namely, Alipurduar, Jalpaiguri, Darjeeling, Coochbehar and North Dinajpur. The mosquito samples collected from each sampling site were named as per site abbreviations. Altogether five different populations of *Aedes aegypti* were collected APD^{ae}, JPG^{ae}, DAR^{ae}, COB^{ae} and NDP^{ae} each representing five different districts namely Alipurduar, Jalpaiguri, Darjeeling, Coochbehar and North Dinajpur. The geographical coordinates and other relevant biotic and abiotic factors of the sampling districts are tabulated in Table 8.

Table 8: Details of the Sampling Districts

Districts	**Population abbreviated name**	**Geographical coordinates**	**Disease endemicity**	**Total infection in 2017 (DEN + CHIK)**	***Mosquito* Generation used in Experiments**
Alipurduar	APD^{ae}	26.69° N 89.47° E	Dengue, Malaria, JE	74	F1
Coochbehar	COB^{ae}	26.34° N 89.46° E	Dengue, Malaria, JE, Filariasis	217	F1
Jalpaiguri	JPG^{ae}	26.52° N 88.73° E	Dengue, Malaria, JE, AES	855	F1
Darjeeling	DAR^{ae}	26.71° N 88.43° E	Dengue, Malaria, JE, AES	1266	F1
North Dinajpur	NDP^{ae}	26.27° N 88.20° E	Dengue, Malaria, JE, AES	284	F1

*JE: Japanese Encephalitis, AES: Acute Encephalitis syndrome, F1: Fillial 1 generation

As abundant sample of *Aedes albopictus* could be collected so more than one population were sampled from some districts covering different blocks. Altogether eleven different populations of *Ae. albopictus* were collected namely APD[al], HAS[al], KMG[al] from Alipurduar district, JPG[al], NGK[al], NMZ[al] from Jalpaiguri district, SLG[al], NBU[al], KHR[al] from Darjeeling district, COB[al] from Coochbehar district and ISL[al] from North Dinajpur district. The demographic and disease prevalence details about the collected sites of *Ae. albopictus* have been provided in table 9.

Table 9: Epidemiological data of the study sites on vector borne diseases with special emphasis on dengue and chikungunya.

District	Site	Prevalent vector borne disease	Why considered at high risk	Population at risk	No. of Dengue cases			No. of Chikungunya cases		
					2015	2016	2017	2015	2016	2017
Darjeeling	SLG[al]	Dengue	High infection rate in with 1 death in 2017	1,44,607	65	165	1266	5	0	0
	NBU[al]	Dengue	High rate	5,405						
	KHR[al]	JE	High rate	-						
Alipurduar	APD[al]	Malaria, AES/JE	High rate	-	0	32	74	0	0	0
	HAS[al]	Dengue, Malaria, JE	High rate	2,52,776						
	KMG[al]	Malaria, JE	High rate	-						
Jalpaiguri	JPG[al]	Dengue	High rate	23,424	58	191	855	7	0	0
	NGK[al]	Dengue, JE	High rate	11,580						
	NMZ[al]	Dengue, Malaria	High rate	6,726						
Uttar Dinajpur	ISL[al]	Dengue	High rate	2,900	45	259	283	0	0	1
Coochbehar	COB[al]	Dengue	High rate	31,979	26	37	217	1	0	0

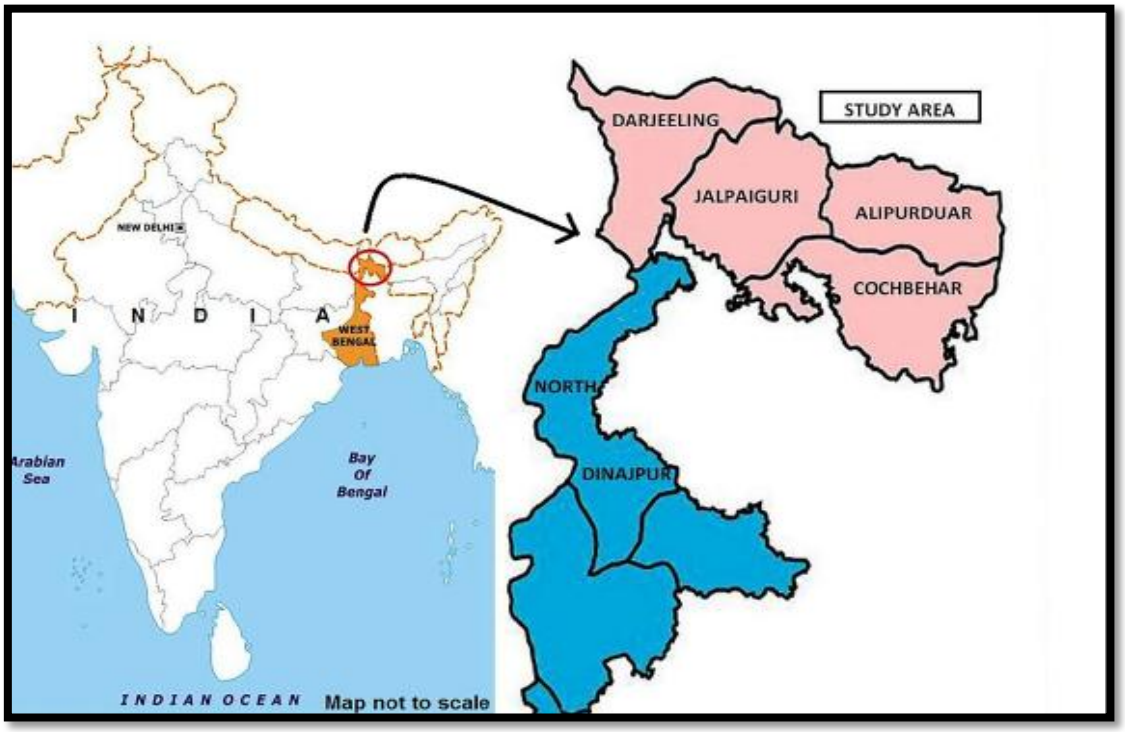
*JE: Japanese encephalitis and AES: Acute encephalitis syndrome

3.2 Collection of larva and adult mosquitoes:

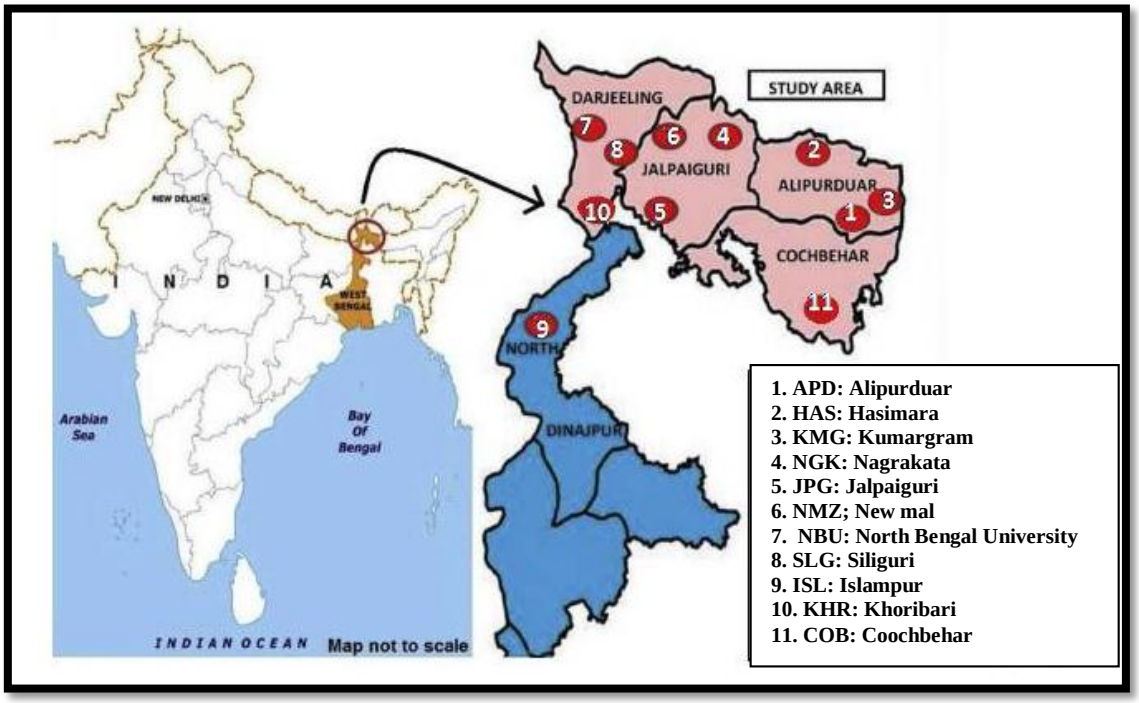
The selected sampling sites (Figure 12) were screened for the possible mosquito breeding environments, *i.e.* shady and cool environments without heavy sunlight. Such environments were critically screened for water containing larva and pupa of *Aedes* mosquitoes. Mosquito larvae or pupae were collected from different wild habitats such as artificial containers, discarded automobile tyres, earthen pots, water holding tanks, discarded buckets, aloevera plantations, tree holes, pots, discarded coconut shells, dry banana leaf containing water *etc*. After initial identification the larvae and pupae were collected and transferred to plastic containers. The samplings were done during March 2015 to August 2019. All the samplings were performed from private and governmental lands and prior permission was taken from the land owner in case of private land and head of the governmental institution in case of governmental land for mosquito collection.

3.3 Rearing of field caught population of mosquitoes:

In the laboratory, the larvae were confirmed as *Aedes* and then again upto subspecies level following standard larval identification keys and then cross checked with adult identification keys (Farajollahi *et al.*, 2013; Tyagi *et al.*, 2014). All the collected mosquitoes were identified either to be *Aedes aegypti* or *Aedes albopictus*. The rearing protocol for these mosquitoes are provided in the next section.



a. Aedes aegypti



b. Aedes albopictus

Figure 12: Sampling sites of *Aedes* mosquitoes distributed in five districts of northern part of West Bengal

Figure 13: Sampling performed for collection of *Aedes* mosquitoes from prospective breeding habitats.

3.3.1 Rearing of susceptible reference population:

The field collected *Aedes* larvae (F_0) were then reared at controlled temperature 25±2^{0}C and 70-80% relative humidity for successive generations. The larvae were reared to F_1 generation upto adults to ensure the homogeneity of the field collected populations. The emerged adults were cross checked with adult identification keys (Farajollhi and Price, 2013; Tyagi *et al.*, 2014). To setup a susceptible laboratory culture, mosquito samples were collected randomly from areas with no insecticide exposure possibilities. The mosquito colonies after collection were reared to F1 generation and were subsequently tested for insecticide susceptibility bioassays. The mosquito population reporting the lowest level of resistance (collected from the Medicinal garden of North Bengal University campus, Siliguri, India) was chosen to be reared for successive generations (Figure 14) without any exposure to insecticides. The collected larvae (F_0) were then subjected to rearing in the laboratory maintaining the same physical factors as mentioned above. The larvae were provided with ground fish feed powder as nutrition source for all the four larval instars. Once the larvae grew into pupae, they were shifted to beakers. Emerging adults were provided with 5% glucose soaked in cotton balls for two days. Then the adults were starved for one day and then provided with anesthetised albino rat (collected from animal rearing centre, North Bengal University campus, Siliguri, India) as a source of blood for the female. After two days of blood feeding, the set up was provided with blotting papers soaked in water containing beakers to serve as the egg laying apparatus for the female mosquitoes. The laid eggs (Figure 15a) were shifted to enamel trays filled with water. The whole setup was covered with mosquito net (Figure 15b). The presence of hatched larvae in the trays marked the onset of next generation. The whole cycle (Figure 14) was repeated for successive generations to be used as the laboratory reared control/ susceptible

population (SP) after at least ten generations. The F1 larvae and adults were used for bioassays and detoxifying enzyme activity studies.

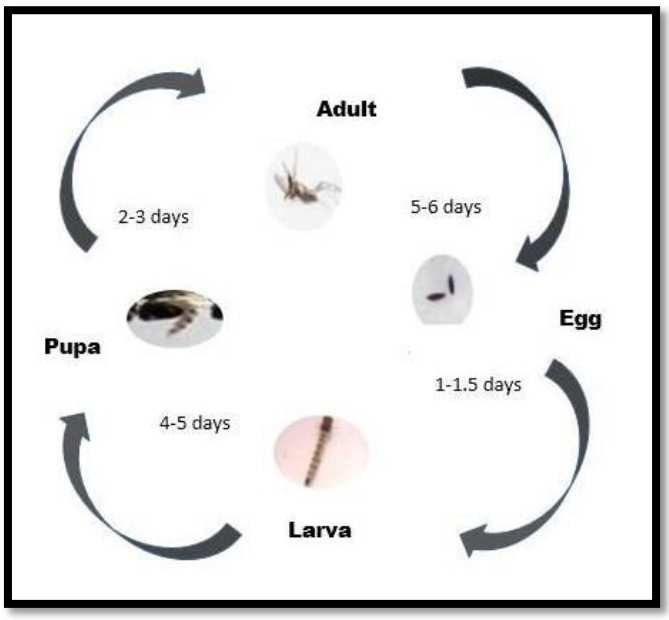


Figure 14: Life cycle of *Aedes* mosquito studied under laboratory condition

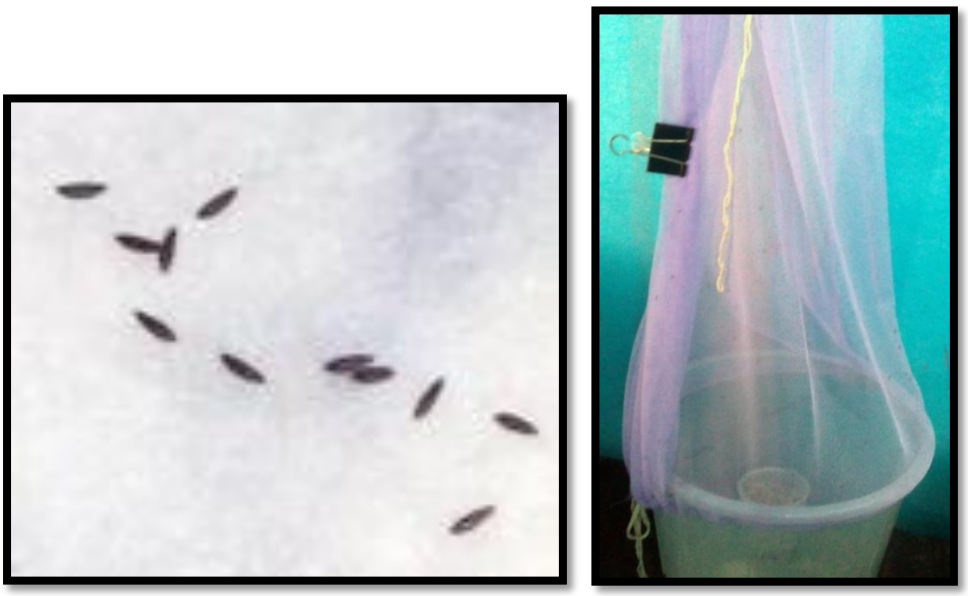


Figure 15: a. *Aedes* eggs laid in laboratory **b.** Laboratory adult mosquito chamber

3.4 Surveying of larvae:

All the sites were screened once in a two month interval for larval stages of mosquitoes belonging to genus *Aedes.* Both private as well as public sector lands/premises were investigated for the study. At least 50 house/premises were selected randomly and screened for presence of possible *Aedes* mosquitoes breeding habitats (both indoor and outdoor). Mosquito breeding habitats such as, Tyres, cemented tanks, artificial containers, thermocol boxes, plant axils, tree holes, refrigerator trays, discarded buckets *etc* were scanned thoroughly for the presence of larval stages. Larvae were collected through random strokes in the breeding habitat and were collected in plastic containers and brought to the laboratory. Entomological indices were determined in this study that helps to assess the risk posed by these mosquitoes in dengue infection throughout the area. Indices such as, Percentage positivity, *i.e.* percentage of total number of *Aedes* infested breeding habitat type, Larva density index, *i.e.* total number of larvae / total number of premises examined, mean no. of larvae per habitat per positive *Aedes* breeding habitat and the preferred habitat for each species were determined.

3.5 Insecticide source:

Single larvicide namely temephos was used in this study. Amongst the adulticide, an organophosphate: 5% Malathion; Synthetic pyrethroid: 0.05% Deltamethrin, 0.05% Lambda cyhalothrin and 0.75% Permethrin; an organochlorine: 4% DDT; a carbamate: 0.1% Propoxur were used. All the insecticides were purchased from Vector control unit, Universiti sains Malaysia (Penang, Malaysia, a WHO Collaborating Centre) as 156.25g/ml solution of temephos and insecticide impregnated papers of adulticides.

***3.6 Larval bioassay**:*

The susceptibility of *Ae. aegypti* and *Ae. albopictus* larvae against an organophosphate larvicide temephos was tested following the WHO guidelines. For larval bioassays, two different doses were selected: 1) 0. 020 mg/L, *i.e.* WHO diagnostic dose and 2) 0.0125 mg/L, *i.e.* National Vector Borne Disease Control Programme (prime governmental vector control organisation in India) recommended dose. Twenty-thirty early fourth instar or late third instar larvae of each *Aedes* population (field caught and laboratory reared susceptible population) were exposed to test vials containing both dosages of temephos in water (Figure 16a). The bioassays were set in triplicate along with a set of control (using equal volume of solvent only) under standard laboratory parameters. Mortality percentages were calculated after 24 hours exposure to temephos. When the larvae failed to evoke any response when stimulated/touched they were considered dead/ moribund (WHO, 2005).

Each population were also tested for the mortality percentages against six dosages lower than the above two for the determination of lethal concentrations LC_{50} and LC_{90} (Concentration at which 50% or 90% larvae are found dead) for each population. Around 20-25 larvae from each population (including susceptible) were exposed to different concentrations of temephos in a vial containing the doses (Marcombe *et al.*, 2014).

Against each concentration three to four replicates and 4-6 serial concentrations selected through random trial (0.0001 - 0.01 mg/L) in the range of the insecticide causing 10% - 90% mortality were set to determine LC_{50} and LC_{90} values. Two sets of control containing the pure solvent (*i.e.* ethanol) only in water were also run. Larval mortality were calculated after 24 hours of temephos exposure following the same criteria for

discrimination between dead and live larvae. If greater than 10% mortality was recorded in controls, the whole set was discarded and set again. Whenever mortality in controls were 0-10%, Abotts correction was applied. If after 24 hours, any larvae moulted into pupae, it was not considered for the calculation of mortality percentages.

***3.7 Adult bioassay**:*

Adult bioassays were also performed following standard WHO protocol (WHO, 2006). The method is similar to larval bioassay except for the fact in adult bioassays insecticide is exposed for 1 hour only. Around 2-3 days old non blood-fed adults, twenty-thirty in number, were introduced in the experimental tubes and exposed to insecticide impregnated papers with WHO recommended diagnostic dose of insecticide (5% malathion, 0.05% deltamethrin and 0.05% lambda cyhalothrin, 4% DDT, 0.1% propoxur and 0.75% permethrin) for 1 hour. After the stipulated time, the mosquitoes were transferred to another tube carrying cotton balls soaked in 10% glucose solution (Figure 16b). The whole set was maintained at laboratory conditions for 24 hours. Mortality percentages were recorded 24 hours post-exposure. For control, mosquitoes were placed in tubes containing papers impregnated with acetone (in case of OC and SP insecticides) and ethanol (for OP and CB insecticides). The whole set was performed along with three replicates. Mortality percentages were calculated as the mean of all set of insecticidal assays.

a.

b.

Figure 16: Set up for testing **a.** larval and **b**. adult insecticide susceptibility

3.8 Synergism test:

For the evaluation of the role of insecticide detoxifying enzymes conferring insecticide resistance, synergism tests were conducted for the field populations using enzyme inhibitors. Two types of inhibitors were used namely, 1. Piperonyl butoxide (PBO, 90%, Sigma from Sigma-Aldrich, Switzerland), a CYP450s inhibitor and 2. Triphenyl phosphate (TPP, 99%, from Sigma-Aldrich, Germany), a CCE inhibitor. In this test, the sub-lethal doses (doses which effectively inhibit the corresponding enzymes

without hampering the suvivality of the mosquitoes) of the synergists *i.e.*4% and 10% for PBO and TPP respectively were used. In case of larval synergism test, the protocol was quite similar to the larval insecticidal bioassays, except that the insecticide was mixed with synergist prior to the test and the larvae were exposed to this mixture for 24 hours. In case of adult synergism test, each population was exposed for one hour to the enzyme blocker prior to insecticide exposure. After PBO/TPP exposure the mosquito populations were exposed to insecticides for 1 hour. Insecticide susceptibility bioassays in WHO bioassays section (exposure to insecticide only) served as positive control for the synergistic assay while bioassays without insecticide were used as negative control.

3.9 Major insecticide detoxifying enzymes' activity:

Single adult non blood fed *Aedes* mosquitoes were homogenized in 100 µL of 0.1M sodium phosphate buffer (pH 7.2) with a teflon micro-pestle in a 1.5 mL centrifuge tube. The pestle was washed with another100 µL of 0.1M sodium phosphate buffer (pH 7.2) to make the whole solution 200 µL. The homogenate was then subjected to centrifugation at 12,000 rpm (revolutions per minute) for 15 minutes in a centrifuge (Sigma 3K30, Sigma United Kingdom). The supernatant to be used as enzyme source for detoxifying enzyme activity assays was collected and stored at -20^{0}C and was used freshly (within 3-4 days).

For every enzyme assay, a minimum of thirty mosquito individuals were used. The whole enzyme assays were run in two technical replicates. In this study, for each enzyme class, a single substrate (two for CCEs) was used for evaluation of the enzyme activity levels. Since, an enzyme group may have many substrates, the substrates used were chosen as per the substrates used in standard protocols (WHO, 1998).

3.9.1 Non-specific esterase (Carboxylesterase) assay:

The activity of Carboxylesterases (CCE) were assayed using α- and β- napthyl acetate as the substrate (α- napthyl acetate α- CCEs and β- napthyl acetate for β-CCEs) for hydrolysation by these enzyme group following the method of van Asperen, 1972 (van Asperen, 1972) with few modifications for use in 96 well microplates (WHO, 1998). Twenty (20) µl of the enzyme source was mixed with 200 µl of substrate working solution and incubated for 15 minutes. Fifty (50) µl of Fast blue B salt (staining agent) mixed with 5% SDS solution (reaction stopping agent) was then poured onto the wells. After 15 minutes, the absorbance was recorded at 540 nm using microplate reader (Biotek, model: ELx800, United States of America) (Figure 17a, b). Blanks were also set following the same method only the enzyme source was replaced with 0.1M sodium phosphate buffer.

Standard curves of α- and β- napthol were prepared for the estimation of CCE activity. Standard solutions of α- and β- napthol were prepared (0.1µM – 1 µM) and their absorbance were recorded at 540nm. A standard curve was prepared from the absorbance values plotted against the known concentrations. Unknown concentrations were then determined plotting the values of absorbance on the standard curves.

3.9.2 CYP450s assay:

The activity of CYP450 monoxygenases were also measured according to standard protocol estimating the approximate heme peroxidise activities (Brogdon *et al.*, 1998) using 3,3',5,5'-Tetramethyl benzidine (TMBZ) as a substrate and H_2O_2 as the peroxidising agent. 20µl of the enzyme was mixed with working solution of TMBZ in sodium acetate buffer. Then 25µl of 3% H_2O_2 solution was added to each well and the whole set was incubated for 2 hours (Figure 17c). Absorbance was then recorded at

630nm using the microplate reader. Blanks were set replacing the enzyme source with 0.1M sodium phosphate buffer. A standard curve for the heme peroxidase activity was prepared using different concentrations of cytochrome c (0.0025 nM to 0.0200 nM) for horse heart type VI (Sigma Aldrich). The total CYP450 was expressed as CYP450 equivalent units (EUs) in mg protein.

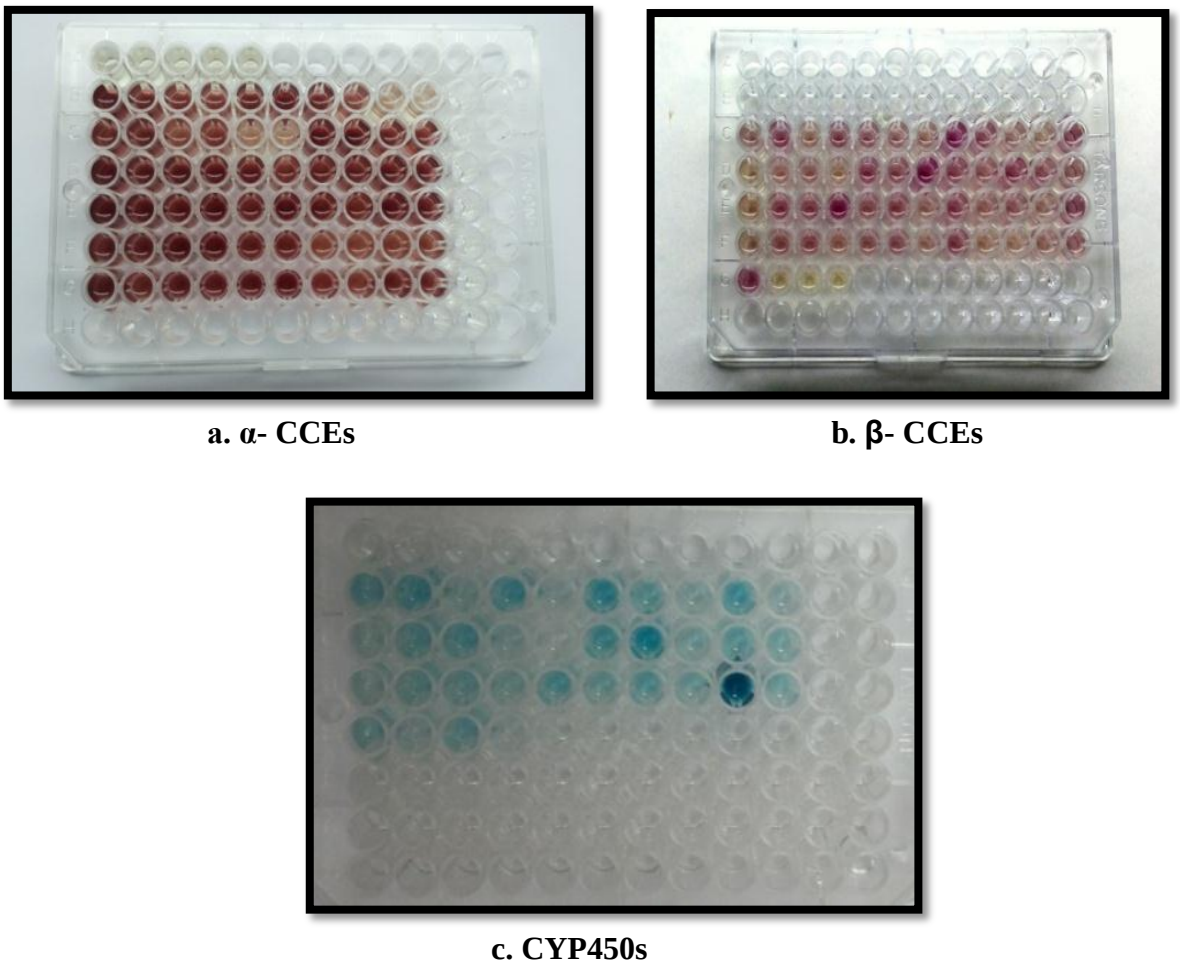
3.9.3 Glutathione S-transferase (GST) assay:

GST activity was assessed following the standard protocol using CDNB and GSH conjugate as the working solution (Habig *et al.,* 1974; WHO, 1998). To 10 µL of homogenate, 200 µL of CDNB-GSH working solution was added in a quartz cuvette. Blanks were set with 10 µL of distilled water mixed with with 200 µL of working solution. 2.7 ml of distilled water was added to the cuvette to make the final volume 2.91 ml. The cuvette absorbance was read at 340 nm continuously for 5 minutes. An extinction coefficient 9.6 $mM^{-1}cm^{-1}$ was used to calculate the change in absorbance per minute to rate of GSTs activity (µM mg protein-^{1} min^{-1}).

3.9.4 Total soluble protein content:

To negate any size differences amongst the mosquito individuals and for the precise estimation of enzyme activity, total protein of each individual was determined according to Lowry *et al.,* 1951 and the activities of the detoxifying enzymes were expressed as per mg protein. To 20 µl of the enzyme source 250 µl of freshly prepared alkaline solution {2% sodium carbonate in 0.1N sodium hydroxide (A) mixed with 0.5% copper sulphate in 1% potassium sodium tartarate (B) in 50:1 ratio, (v:v)} was added. After 10 mins of incubation, 50 µl of folin ciocalteau reagent was added. Absorbance was read after 30 minutes at 630 nm. Blanks were set using 0.1M sodium

phosphate buffer. Standard curve was prepared using different concentrations of bovine serum albumin.



a. α- CCEs **b. β- CCEs**

c. CYP450s

Figure 17: Microplates showing the end point of insecticide detoxifying enzyme's assay

3.10 Isolation of DNA and kdr genotyping:

Genomic DNA was extracted following the SDS extraction method (Barik *et al.*, 2013). DNA concentration was measured using a spectrophotometer at 260 nm. Stock solutions were prepared at a concentration of 25 ng/µl and used for Allele specific-PCR (AS-PCR) genotyping. The primers used and their annealing temperature is provided in Table 10. Each reaction was performed in a 25 µl volume consisting of 1.5 mM MgCl2, 1x PCR buffer (Promega, USA), 0.25 µM common primer, 0.125 µM each mutation specific primer, 200 µM

dNTP mixture (Promega, USA), 0.2 units Taq polymerase (Promega, USA) and 25 ng genomic DNA. The thermal cycling condition was set with an initial DNA denaturation step for two minutes at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at mentioned temperature (Table 10) at and extension at 30 sec at 72°C. PCR amplification products were loaded onto a 3% agarose gel and run for 1 hr at 100 V in TAE buffer. Since, the primers used had GC-rich tails of varying lengths, amplified products could be differentiated by size, 93 bp, 113 bp for F1534C mutation and 60 bp, 80 bp for V1016G (Yanola *et al.,* 2011; Stenhouse *et al.,* 2013; Aguirre-Obando *et al.,* 2017) and the results could be accordingly interpreted. Allele frequency calculations were done following the standard methods (Aguirre-Obando *et al.,* 2017).

Table 10: Details of primers used for kdr genotyping of F1534C and V1016G mutations

Species	Kdr mutation	Primer sequence	Annealing temperature	Product size (bp)	Reference
Ae. aegypti	F1534 (Reverse)	5'-TCTGCTCGTTGAAGTTGTCGAT-3'	60°C	--	Yanola *et al.*, 2011
	F1534F (Forward 1)	5'-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3'		93	
	F1534C (Forward2)	5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3'		113	
Ae. aegypti	V1016 (Forward)	5′-ACCGACAAATTGTTTCCC-3′	55°C	--	Stenhouse *et al.*, 2013
	1016V (Reverse1)	5′- GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA-3′		80	
	1016G (Reverse2)	5′-GCGGGCAGGGCGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-3'		60	
Ae. albopictus	F1534 (Reverse)	5'-TCTGCTCGTTGAAGTTGTCGAT-3'	60°C	--	Aguirre-Obando *et al*., 2017
	F1534F (Forward 1)	5'-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3'		93	
	F1534C (Forward 2)	5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3'		113	

3.11 Electrophoretic analysis of α- and β-esterases:

Native Polyacrylamide Gel Electrophoresis (PAGE) of different *Ae. aegypti* populations *i.e.* field collected as well as from laboratory reared control populations were carried out in 8% gels in tris-glycine (pH 8.3) buffer system. Field collected *Aedes* population as well as laboratory reared control mosquitoes were homogenized freshly in 100 µl of 0.1 M sodium phosphate buffer. To run the gels equal amounts of protein were loaded onto the gel in tris–glycine (pH 8.3) at 100 V for 5–6 h at 4°C.

To stain the gels for α –CCEs, standard staining protocol was followed with minor modifications (Steiner and Johnson 1973; Carvalho *et al.* 2003). The gels were first incubated in sodium phosphate buffer (0.1M, pH 7.2) for 15 mins in dark staining box. Then the gels were transferred to a solution containing 20 mg of α –Napthyl acetate in 5 ml of acetone mixed with 50 mL of sodium phosphate buffer (0.1M, pH 7.2). After 15 mins of incubation, the gels were provided with 5% solution of fast blue B salt for 30 minutes. Same procedure was followed for staining of β-CCEs replacing the substrate with β-Napthyl acetate. The gels were then preserved between transparent plastic sheets for future reference. Bands designated as isozymes of α-CCEs and β-CCEs were designated as Est I, II, III, IV, V based on mobility from anode to cathode and the Rf (Retardation factor) values were calculated.

3.11 Calculation and statistical analyses of the data:

In the bioassays, $LC_{50,}$ LC_{90} and LC_{99} were estimated at 95% confidence interval by putting log dose against probit in SPSS 16.0 software and the obtained linear regression coefficient (r^2) was used to assess the linearity of the whole data set. Double the extrapolated value of LC_{99} (2 X LC_{99}) was taken as the recommended discriminating dose/ diagnostic dose of the insecticide for that specific region/area. The population

with mortality percentages when > 98 is said to be susceptible, 80-97 is assessed as resistance not confirmed (= incipient resistance) and <80 as resistant (WHO, 2006). Resistance ratio 50 *i.e.* RR_{50}, which is an indirect measurement of insecticide resistance development was also determined as the LC_{50} of sampling site divided by the LC_{50} of the susceptible population. Calculation of the average values and standard errors and the comparisons of the mean of different data sets were performed using Graphpad Instat 3.05 at p=0.05.

RESULTS

4. RESULTS:

4.1 Surveying of larva:

Throughout the sampling districts, eleven sites were found to report the presence of *Aedes* mosquitoes. It was found that there was a clear abundance of *Ae. albopictus* over *Ae. aegypti* with its presence recorded in all the sampling sites (Table 11). Whereas, only five out of the eleven sites reported the presence of *Ae. aegypti*, one from each sampling district.

Table 11: Details of the *Aedes* species collected from different sampling sites.

Sl. No.	Site	Abbreviated Population Name	Geographical Coordinates	Type	*Aedes* mosquitoes collected
1.	Alipurduar	ALP	26.49°N 89.52°E	Mixed	*Ae. albopictus and Ae. aegypti*
2.	Hasimara	HAS	26.75°N 89.35°E	Mixed	*Ae. albopictus*
3.	Kumargram	KMG	26.66°N 89.83°E	Rural	*Ae. albopictus*
4.	Nagrakata	NGK	26.88°N 88.91°E	Rural	*Ae. albopictus*
5.	Jalpaiguri	JPG	26.52°N 88.73°E	Urban	*Ae. albopictus and Ae. aegypti*
6.	New mal	NMZ	26.85°N 88.75°E	Rural	*Ae. albopictus*
7.	NBU	NBU	26.71°N 88.35°E	Mixed	*Ae. albopictus*
8.	Siliguri	SLG	26.71°N 88.43°E	Urban	*Ae. albopictus and Ae. aegypti*
9.	Islampur	ISL	26.27°N 88.20°E	Rural	*Ae. albopictus and Ae. aegypti*
10.	Khoribari	KHR	26.55°N, 88.19°E	Rural	*Ae. albopictus*
11.	Coochbehar	COB	26.34°N, 89.46°E	Urban	*Ae. albopictus and Ae. aegypti*

Among the different breeding habitats, tyres were found to be the most preferred habitat for *Ae. albopictus* followed by discarded containers and bamboo stumps (Table 12). Similarly for *Ae. aegypti* both tyres and cemented tanks were found to be preferred followed by discarded containers. Rest of the breeding habitats showed an abundance of *Ae. albopictus* specially the natural ones such as leaf axils, flower pots and bamboo stumps. In majority of the sites where both the species were found to coexist the larval density index were higher for *Ae. albopictus* (Table 13). In NMZ and SLG, the larval densities were higher for *Ae. aegypti*. The highest larval density for *Ae. aegypti* was recorded in ISL, 41.07 and that for *Ae. albopictus* in NGK, 43.84.Similar trend was noted in the results of mean number of larvae per habitat with the same two populations reporting the highest values for *Ae. Aegypti* and *Ae. albopictus* (Table 13).

Table 12: Percentage positivity of breeding habitats for the collected collected *Aedes* mosquitoes throughout the northern districts of West Bengal, India

Sites	Tyres		Cemented tank		Water storage tank		Leaf axils		Discarded containers		Flower pots		Bamboo stump	
	AE	AL	AE	AL	AE	AL	AE	AL	AE	AL	AE	AL	AE	AL
APD	62.50	36.36	25.00	0.00	0.00	0.00	0.00	24.24	12.50	30.30	0.00	9.09	0.00	0.00
HAS	18.18	57.14	54.54	0.00	0.00	9.50	0.00	19.04	27.27	9.52	0.00	4.76	0.00	0.00
KMG	0.00	40.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.00	0.00	0.00	0.00	48.00
NGK	0.00	23.80	0.00	4.76	0.00	16.22	0.00	12.34	0.00	8.48	0.00	23.80	0.00	10.56
JPG	91.06	78.57	0.00	0.00	0.00	0.00	0.00	0.00	9.94	7.14	0.00	14.28	0.00	0.00
NMZ	30.76	36.39	61.50	0.00	0.00	0.00	0.00	0.00	7.69	63.61	0.00	0.00	0.00	0.00
NBU	28.57	0.00	0.00	0.00	0.00	0.00	5.71	0.00	45.70	45.49	11.42	0.00	8.57	54.55
SLG	66.67	18.92	22.92	0.00	0.00	0.00	0.00	5.40	10.41	8.11	0.00	5.40	0.00	62.16
ISL	0.00	65.42	66.66	0.00	2.97	0.00	0.00	0.00	30.06	34.58	0.00	0.00	0.00	0.00
KHR	0.00	100.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
COB	45.45	60.07	36.33	0.00	0.00	0.00	0.00	0.00	0.00	23.21	0.00	16.71	0.00	0.00

AE= *Ae. aegypti*, AL= *Ae. albopictus

Table 13: Entomological indices tested for the collected *Aedes* mosquitoes throughout the northern districts of West Bengal, India

Sites	**Larval density index**		**Mean no. of larvae per habitat**	
	Ae. aegypti	***Ae. albopictus***	***Ae. aegypti***	***Ae. albopictus***
APD	4.09	32.75	31.80	53.44
HAS	6.48	32.90	18.27	48.57
KMG	0.00	14.53	0.00	28.48
NGK	0.00	43.84	0.00	104.38
JPG	2.66	8.94	21.28	17.89
NMZ	17.05	5.72	70.84	28.09
NBU	3.53	34.12	18.10	54.60
SLG	12.48	10.84	69.33	14.65
ISL	41.07	0.00	133.51	0.00
KHR	0.00	11.21	0.00	28.11
COB	12.17	0.00	28.23	0.00

4.2 Aedes aegypti:

4.2.1 Larval bioassay:

Of the tested five populations, only one, *i.e.* NDP^{ae} was found to possess resistance against temephos, with the mortality 92.15% at 0.0200 ppm and 79.45% at 0.0125ppm revealing incipient resistance and resistance respectively. Other populations exhibited complete susceptibility against temephos (Table 14). RR_{50} value, which gives an indication on the extent of underlying resistance development, showed a value greater than 2 in four of the five tested population. The highest RR_{50} value was found for NDP ≈ 35.09, followed by JPG^{ae}, DAR^{ae} and APD^{ae} populations. Similar trend was noticed in LC_{50} and LC_{99} values and NDP populations showed the highest value for both (Table 14). The recommended diagnostic dose were calculated to be ranging from2.6×10^{-1} to 3.2×10^{-4} ppm.

Table 14: Susceptibility status against temephos and corresponding lethal concentration values in *Ae.aegypti* larvae collected from five districts of northern Bengal

Site	WHO dosage mortality (%±S.D.)	Resistance status	NVBDCP dosage mortality (%±S.D.)	Resistance status	LC50 dose (ppm) ±S.E. (95%C.I.)	LC99 dose (ppm) ±S.E. (95%C.I.)	r^2	Recommended dosage (ppm)	RR_{50}
APD[ae]	100±0.0	S*	100±0.0	S	$1.71 \times 10^{-4} \pm 1.7 \times 10^{-5}$	$2.9 \times 10^{-3} \pm 1.9 \times 10^{-4}$	0.89	5.8×10^{-3}	3.00
COB[ae]	100±0.0	S	100±0.0	S	$9.4 \times 10^{-5} \pm 1.1 \times 10^{-6}$	$3.2 \times 10^{-4} \pm 1.6 \times 10^{-5}$	0.92	6.4×10^{-4}	1.65
JPG[ae]	100±0.0	S	100±0.0	S	$5.3 \times 10^{-4} \pm 3.2 \times 10^{-5}$	$8.1 \times 10^{-3} \pm 1.2 \times 10^{-4}$	0.91	1.6×10^{-2}	9.30
DAR[ae]	100±0.0	S	100±0.0	S	$3.1 \times 10^{-4} \pm 2.1 \times 10^{-5}$	$4.3 \times 10^{-3} \pm 3.9 \times 10^{-4}$	0.94	8.6×10^{-3}	5.43
NDP[ae]	92.15±0.9	***IR***	79.45±1.6	***R***	$2.0 \times 10^{-3} \pm 1.2 \times 10^{-4}$	$2.6 \times 10^{-1} \pm 5.4 \times 10^{-2}$	0.90	5.2×10^{-1}	35.09
SP[ae]	100±0.0	S	100±0.0	S	$5.7 \times 10^{-5} \pm 1.1 \times 10^{-6}$	$1.1 \times 10^{-4} \pm 2.9 \times 10^{-5}$	0.87	--	--

*S: Susceptible, IR: Incipient resistance, R: Resistance

4.2.2 Adult bioassay:

Against the sole Organochlorine insecticide, DDT, widespread resistance was noted in all the tested populations with the mortality percentage ranging from 47.9 to 72.0% (Table 15). The DAR^{ae} population exhibited the most severe resistance against DDT followed by APD^{ae} and NDP^{ae}. Two populations COB^{ae} and JPG^{ae} were found possessing moderate resistance against DDT. Against, malathion, one population APD^{ae} possessed moderate resistance with 72.5% mortality and other incipiently resistant DAR^{ae} with mortality percentage, 92.6%. Rest of the populations were completely susceptible to it. Amongst synthetic pyrethroids, the greatest resistance profile was recorded against permethrin with four out of five population resistant and one incipiently resistant (Figure 18). The mortality percentage ranged from as low as 50% for NDP^{ae} to 83.3% for APD^{ae} population. Two of the populations namely COB^{ae} and DAR^{ae} were completely resistance against the rest synthetic pyrethroid insecticide tested. Against both deltamethrin and lambdacyhalothrin, the rest three populations were incipiently resistant with the lowest mortalities recorded in APD^{ae} population. Against propoxur, three of the populations were found to be severely to moderately resistant and the rest incipiently resistant. The mortality percentage recorded against propoxur was noted to be as low as 75.4 (NDP^{ae}) to 97.2 (COB^{ae}).

Table 15: Mortality percentages and insecticide susceptibility status of adult *Ae. aegypti* populations against six insecticides collected from five different districts of northern Bengal

Population	DDT (M%*± S.D.)	St	Malathion (M%± S.D.)	St	Deltamethrin (M%± S.D.)	St	Lambdacyhalothrin (M%± S.D.)	St	Permethrin (M%± S.D.)	St	Propoxur (M%± S.D.)	St
APD^{ae}	55.4 ± 1.42	R	72.5 ± 1.26	R	89.2 ± 0.81	IR	80.9 ± 0.76	IR	83.3 ± 0.88	IR	91.3 ± 0.71	IR
COB^{ae}	70.0± 1.02	R	100.0± 0.00	S	100.0± 0.00	S	100.0± 0.00	S	76.5 ± 0.99	R	97.2 ± 0.72	IR
JPG^{ae}	72.0±1.15	R	99.0± 0.33	S	91.9±0.56	IR	81.5±0.33	IR	64.5± 1.41	R	77.2± 1.21	R
DAR^{ae}	47.9 ±1.41	R	92.6± 0.89	IR	100.0±0.00	S	100.0±0.00	S	60.0±1.03	R	50.0±1.33	R
NDP^{ae}	56.6± 1.32	R	100.0 ± 0.00	S	90.3± 1.01	IR	85.0± 1.13	IR	50.0± 0.71	R	75.4± 1.13	R
SP^{ae}	98.2±0.77	S	100.0±0.00	S	100.0±0.00	S	100.0±0.00	S	100.0±0.00	S	100.0±0.00	S

M%: Mortality percentage, St: Status, S: Susceptible, IR: Incipient resistance, R: Resistance

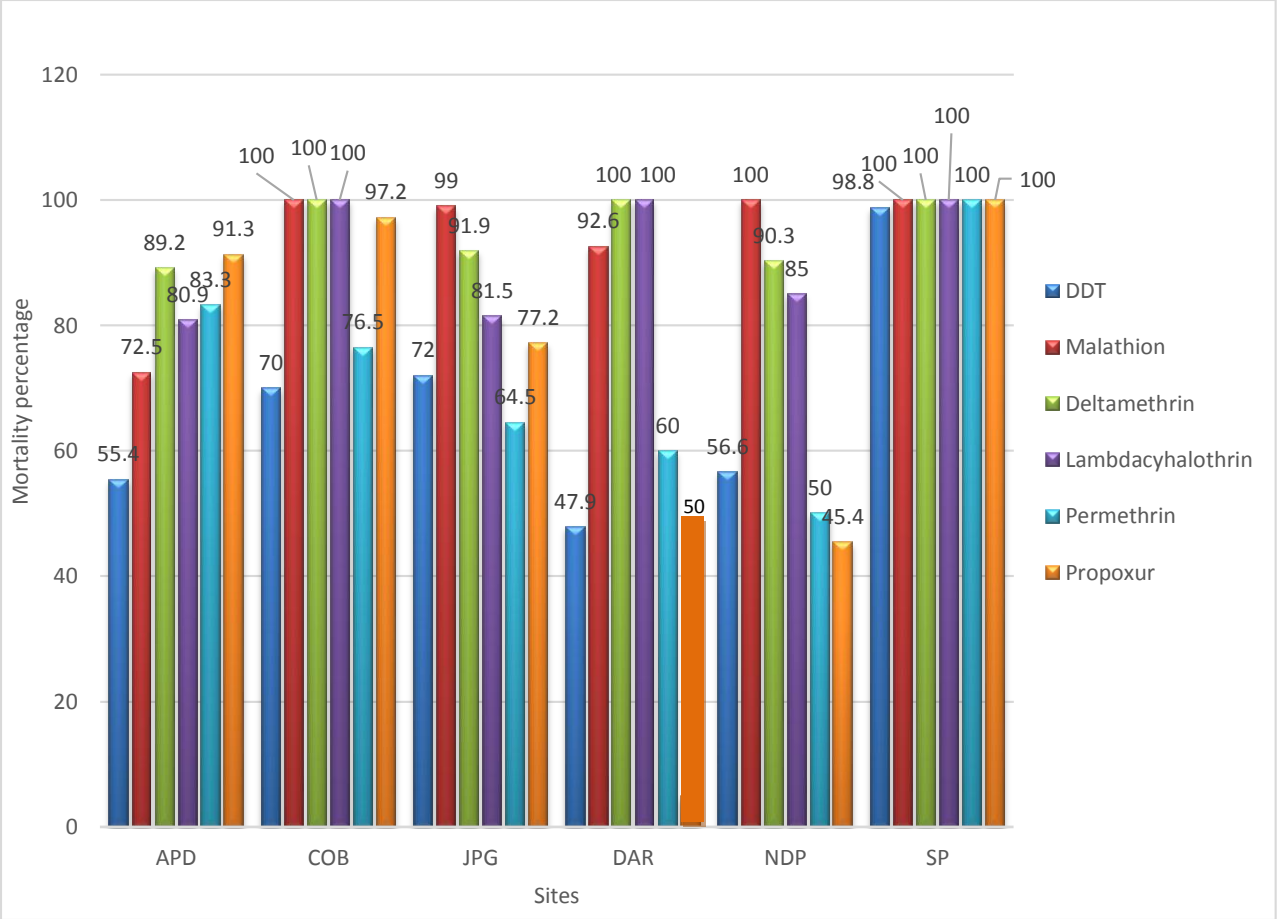


Figure 18: Mortality percentages of *Ae. aegypti* against six adulticides collected from fivedistricts of northern Bengal

4.2.3 Knockdown times:

Against DDT most of the populations showed high values of both KDT_{50} and KDT_{95}. The highest values of both KDT_{50} and KDT_{95}were recorded for DAR^{ae} followed by APD^{ae} and NDP^{ae} (Figure 19).The highest recorded KDT_{50} value was 182.26 mins whereas the highest KDT_{95} value was 239.31 mins. Amongst the three tested pyrethroid insecticides, the pattern of high KDT values wererecorded against permethrin, with the KDT_{50} values less than 60 mins for three of the populations. The highest KDT_{50} value, 78.98 minsand KDT95 value 192.11 mins was noted for NDP^{ae} population followed by

DAR^{ae}. Against lambdacyhalothrin theKDT_{50} values were less than 1hour for all the tested mosquito population and againstdeltamethrin only one population possessed the value greater than that. The highest $KDT_{50}$and KDT_{95} values against deltamethrin were recorded in JPG^{ae}, whereas against lambdacyhalothrin, APD^{ae} exhibited the highest KDT_{50} value and JPG^{ae} the highest KDT_{95} values.

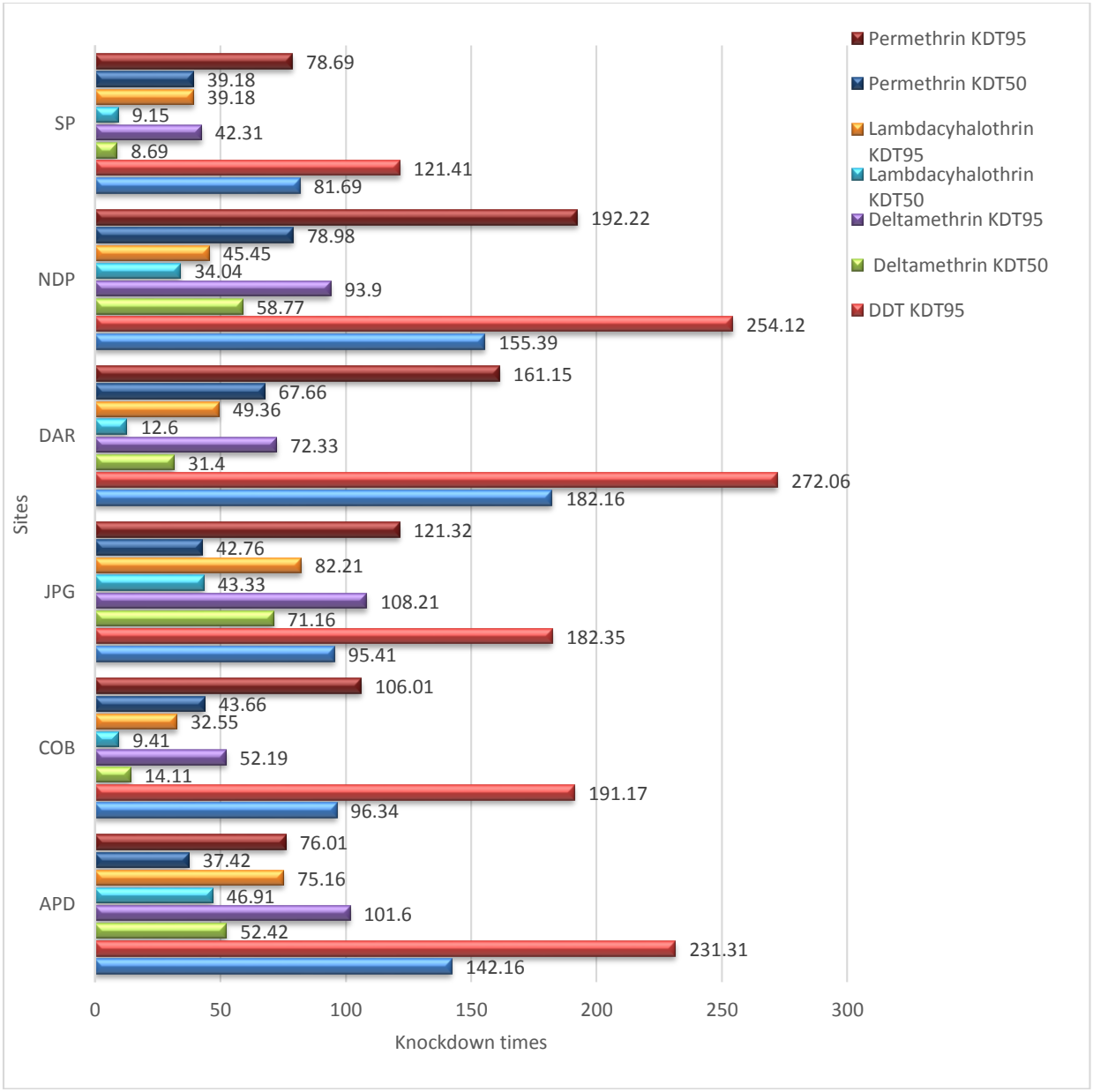


Figure 19: Knockdown rates (KDT_{50} and KDT_{95}) of different field populations of *Ae. aegypti*against organochlorine and synthetic pyrethroid insecticide

4.2.4 Synergism test:

The exposure to enzyme blockers before insecticide susceptibility testing could restore the susceptibility in some of the populations against insecticides. Against DDT, the use of 4% PBO was found to restore the mortality percentage partially in APD^{ae} population elevating the mortality rate from 55.4% without PBO to 80.0% with prior exposure to it (Table 15-16). Against malathion, also APD^{ae} population was noted to partially restore its susceptibility when exposed to 10%TPP, *i.e.* 72.5% to 94.0%. Minor restoration of susceptibility was also noted in DAR^{ae} population against malathion, with the status of incipient resistant restored to susceptible levels. Against deltamethrin and lambdacyhalothrin, PBO was found to increase the susceptibilities in APD^{ae}, JPG^{ae} and NDP^{ae}. Against permethrin, minor elevation in mortality percentage, 8.8% was noted with PBO in NDP^{ae} population. Against propoxur, NDP^{ae} population was found to restore its mortality from 45.4 to 70.4% with exposure to 10% TPP.

4.2.5 Major insecticide detoxifying enzymes' activity:

The activity of α-CCEs were noted to range 1.07 fold to 3.11 fold among the field populations than that of SP^{ae}. NDP^{ae} exhibited the highest activity levels for both α-CCEs and β-CCEs, 0.672 and 0.414 µmoles mg $protein^{-1}$ min^{-} respectively(Table 17). The activity of β-CCEs were noted to range from 1.19 to 2.46 folds than SP^{ae}. JPG^{ae} population was recorded to possess the highest activity for both CYP450s monoxygeneases and GSTs. The activity of CYP450 monoxygenases was highest for JPG^{ae} followed by DAR^{ae} and APD^{ae}, 0.063, 0.061 and 0.058 nM mg $protein^{-1}$ min^{-1} respectively.The activity of GSTs were noted to be ranging from 0.33 to 0.43 µMmg $protein^{-1}$ min^{-1} amongst thefield caught populations of *Ae. aegypti.*

Table 16: Mortality percentages of the field caught *Ae. aegypti*mosquitoes tested against insecticidesafter prior exposure to synergists, 4% PBO and 10% TPP

	Sampling sites (Mortality % ± S.D.)					
	APD[ae]	**COB**[ae]	**JPG**[ae]	**DAR**[ae]	**NDP**[ae]	**SP**[ae]
Temephos+ PBO	100±0.0	100±0.0	100±0.0	100±0.0	93.5±0.0	100±0.0
Temephos+ TPP	100±0.0	100±0.0	100±0.0	100±0.0	99.4±0.0	100±0.0
DDT+PBO	80.0±0.9	67.4±1.4	69.1±1.9	43.8±3.1	60.1±1.9	99.2±0.0
DDT+TPP	43.3±3.5	70.3±2.1	71.1±2.1	33.3±2.2	55.9±0.8	98.0±0.3
Malathion+PBO	65.0±1.7	100.0±0.0	99.0±0.2	93.4±0.5	100.0±0.0	100.0±0.0
Malathion+TPP	94.0±0.4	100.0±0.0	100.0±0.0	96.8±0.0	100.0±0.0	100.0±0.0
Deltamethrin+PBO	99.1±0.0	100.0±0.0	98.3±0.2	100.0±0.0	98.9±0.2	100.0±0.0
Deltamethrin +TPP	89.1±0.7	100.0±0.0	92.7±0.3	100.0±0.0	89.1±0.7	100.0±0.0
Lambdacyhalothrin+PBO	93.6±0.8	100.0±0.0	88.7±0.7	100.0±0.0	95.1±0.7	100.0±0.0
Lambdacyhalothrin+TPP	84.2±0.9	100.0±0.0	85.4±1.0	100.0	83.3±1.1	100.0±0.0
Permethrin+PBO	87.2±1.1	78.2±1.1	69.7±1.2	43.3±2.9	58.8±2.4	98.7±0.2
Permethrin+TPP	88.9±0.8	79.9±0.8	66.6±1.6	52.7±2.1	55.9±1.8	100.0±0.0
Propoxur+PBO	92.7±0.6	96.7±0.4	60.0±1.8	42.3±1.9	50.3±2.3	100.0±0.0
Propoxur+TPP	94.2±0.6	97.2±0.3	65.0±2.3	31.6±3.1	70.4±1.1	100.0±0.0

Table 17: Activities of major insecticide detoxifying enzymes in field caught *Ae. aegypti* mosquitoes collected from districts of northern Bengal

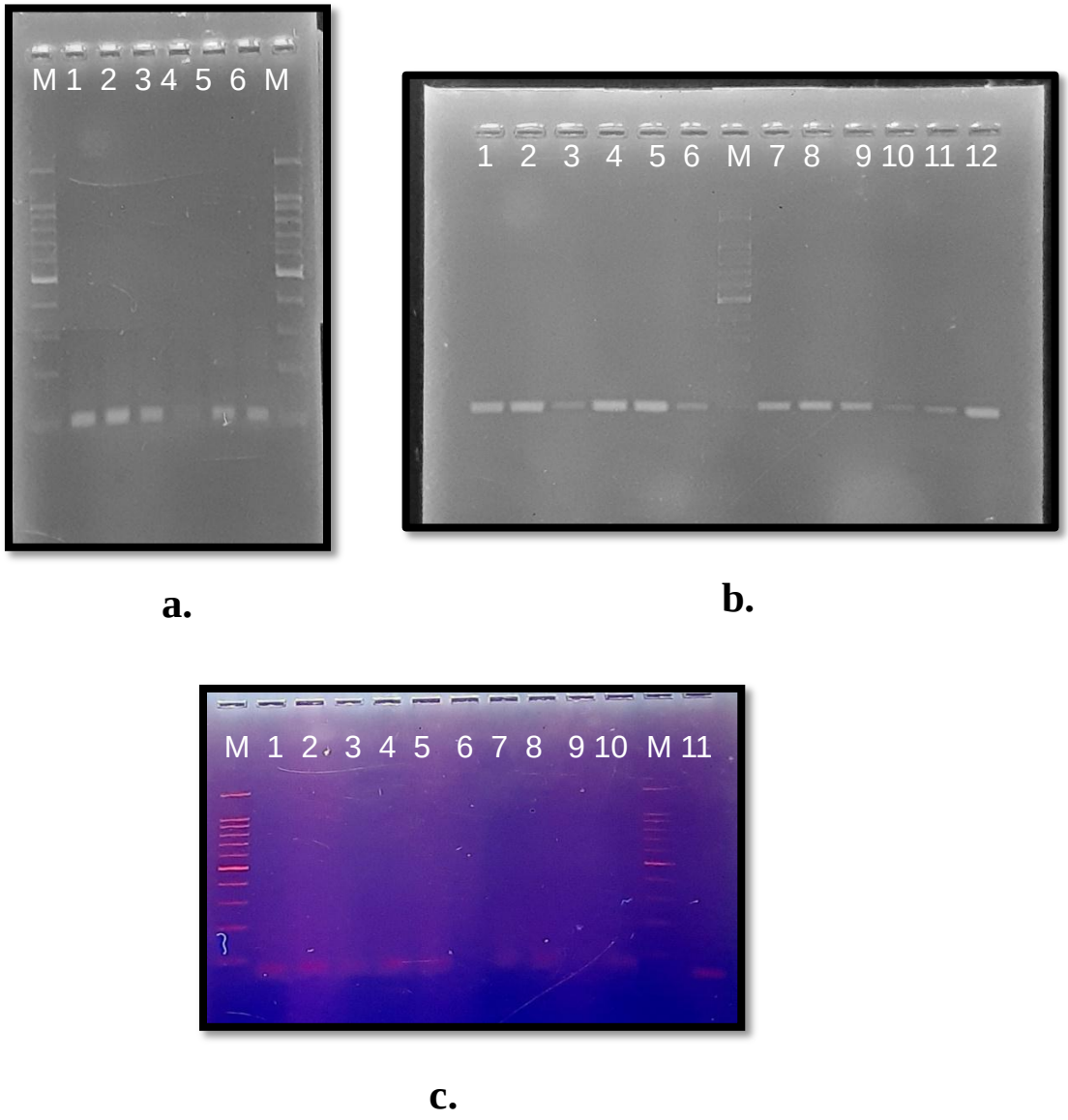
Sites	α-CCEs (μM mg protein^{-1} min^{-1}) ± S.E.	β-CCEs (μM mg protein^{-1} min^{-1}) ± S.E.	CYP_{450}Monoxygenase (nM mg protein^{-1} min^{-1}) ± S.E.	GSTs (μMmg protein^{-1} min^{-1}) ± S.E.
APD^{ae}	0.322 ± 0.007^{b*}	0.231 ± 0.004^{b}	0.058± 0.0017^{b}	0.37 ± 0.002^{a}
COB^{ae}	0.231 ± 0.004^{a}	0.187 ± 0.002^{b}	0.047 ± 0.0006^{a}	0.33 ± 0.003^{a}
JPG^{ae}	0.276 ± 0.003^{b}	0.201 ± 0.006^{b}	0.063± 0.0021^{b}	0.43 ± 0.007^{a}
DAR^{ae}	0.367 ± 0.009^{b}	0.211 ± 0.008^{b}	0.061 ± 0.0019^{b}	0.39 ± 0.004^{a}
NDP^{ae}	0.672 ±0.018^{c}	0.414 ± 0.009^{c}	0.052 ± 0.0011^{b}	0.34 ± 0.006^{a}
SP^{ae}	0.216 ± 0.003^{a}	0.168 ± 0.004^{a}	0.041 ± 0.0007^{a}	0.31 ± 0.004^{a}

*Within columns, means followed by the same letter do not differ significantly (P=0.05) in Tukey's multiple comparison test (HSDa).

4.2.6 Kdr genotyping:

A total of 120 mosquitoes were randomly chosen and genotyped for F1534C gene. Both susceptible and mutant kdr allele was amplified in majority of the tested samples (Figure 20). The frequency of the 1534C allele was 50%. The frequencies of homozygote CC and homozygote FF were found to be 0% since all the examined population were heterozygote FC (frequency = 100%). Allele containing C was found in heterozygotes and CC allele was not encountered inthe tested populations (Table 18). In case of V1016G genotyping also, both the alleles were present in the wild population of *Ae. aegypti* mosquitoes (Table 18). The frequency of the 1016G allele was 45%. Allele containing G was mostly found in heterozygotes but GG genotype was prevalent in one

of the population, ISL^{ae}. Moreover, homozygote VV was also found to be present in the APD^{ae} and SLG^{ae} populations.



a. **b.**

c.

Figure 20: 3% Agarose gel loaded with 100 bp DNA marker (M) and allele specific PCR products a. F1534F susceptible 108 bp (lane 1-6), b. F1534C mutant 93 bp (Lane 1-12) and c. V1016F/G [Lane 1-10 loaded with V1016V(80 bp) and Lane 11 with V1016G mutant (60 bp)]

Table 18: Results of F1534C and V1016G kdr genotyping from randomly selected populations of *Ae. aegypti*

Mutation screened	Population	Total samples	FF	FC	CC	Frequency of C allele (%)
F1534C	APD^{ae}	24	0	24	0	50
	COB^{ae}	24	0	24	0	50
	JPG^{ae}	24	0	24	0	50
	DAR^{ae}	24	0	24	0	50
	NDP^{ae}	24	0	24	0	50

V1016G	Population	Total samples	VV	VG	GG	Frequency of G allele (%)
	APD^{ae}	24	12	12	0	25
	COB^{ae}	20	0	20	0	50
	JPG^{ae}	24	0	24	0	50
	DAR^{ae}	24	8	16	0	33
	NDP^{ae}	24	0	14	10	70

4.2.7 Qualitative analysis of α- and β- Carboxylesterases:

Around five different bands corresponding to isozymes were noted amongst the different field caught mosquito populations (Figure 21) with the Rf values 0.62,0.68, 0.73, 0.82, 0.97 (Table 19). All the five bands were revealed in NDP^{ae} population, whereas the rest possessed single isozyme, *i.e.*α-Est V. The band intensity of α-Est V was noted to be highest in APD^{ae} followedby NDP^{ae} and then the rest (Figure 21). In NDP^{ae}population the intensity of all the bandsother than α-Est V, were very high.

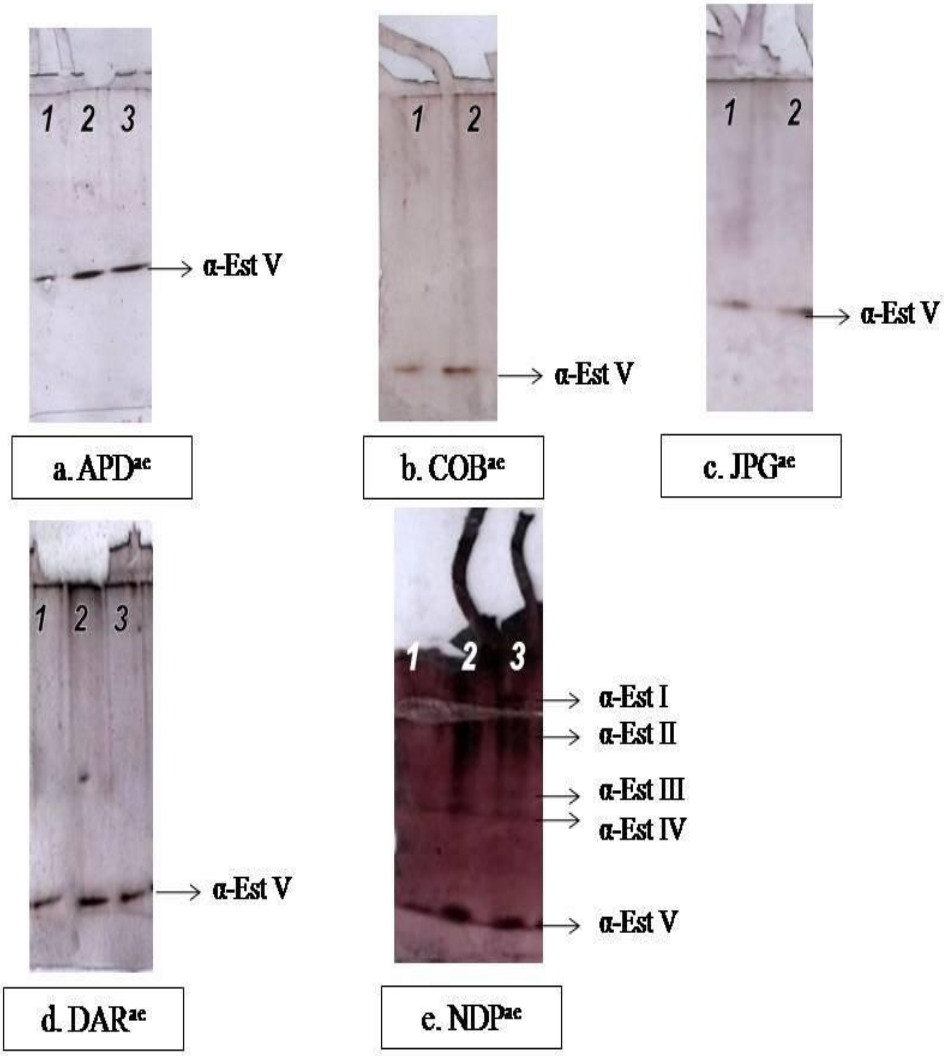


Figure 21: Electrophoregram of α-Carboxylesteraseisozymes in*Aedes aegypti* mosquitoes collected from northern districts of West Bengal. Lane 1 in all the gels are loaded withSP^{ae}.

Table 19: The summarized report of the electrophoregram of α-Carboxylesterase isozymes in *Aedes aegypti* mosquitoes collected from northern districts of West Bengal.

Sites	Band number	Intensity	Rf value
APD^{ae}	α-Est V	+++*	0.96
COB^{ae}	α-Est V	+	0.95
JPG^{ae}	α-Est V	+	0.95
DAR^{ae}	α-Est V	++	0.95
NDP^{ae}	α-Est I	+++	0.62
	α-Est II	+++	0.68
	α-Est III	+++	0.73
	α-Est IV	+++	0.82
	α-Est V	++	0.97
SP^{ae}	α-Est V	+	0.95

*+ to +++ represents low toseverity of the expression of isozymes of CCEs.

The electrophoretic study of β-CCEs revealed the presence of three distinct bands (Figure 22) with the Rf values 0.62, 0.80 and 0.96 (Table 20). Similar to the electrophoregram of α-CCEs, the highest number of isozymes wereexpressed in NDP^{ae} population, with the rest populations expressing a single band *i.e.*β-Est III (Figure 22). The predominant isozyme among the studied populations, *i.e.*β-Est III was intensely expressedin APD^{ae} population as evident from the band thickness, than the rest populations exhibiting the low expression. In NDP^{ae}, both β-Est I and β-Est II were expressed withhigh band intensity followed by β-Est III with moderate band thickness.

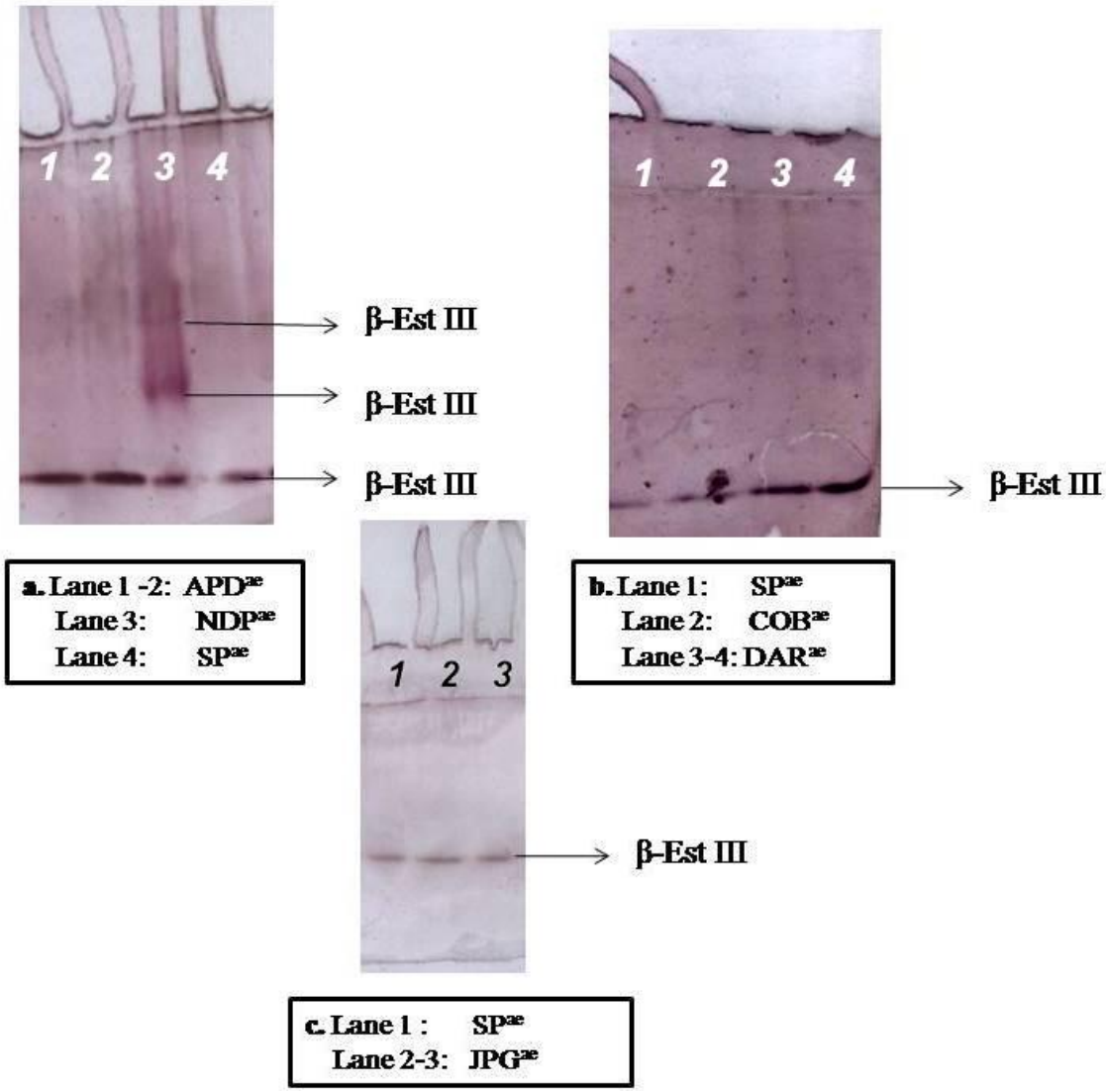


Figure22: Electrophoregram of β-Carboxylesteraseisozymes in*Aedes aegypti* mosquitoes collected from northern districts of West Bengal.

Table 20: The summarized report of the electrophoregram of β-Carboxylesterase isozymes*Aedes aegypti* mosquitoes collected from northern districts of West Bengal.

Sites	Band name	Intensity	Rf value
APD^{ae}	β-Est III	+++*	0.96
COB^{ae}	β-Est III	+	0.96
JPG^{ae}	β-Est III	+	0.97
DAR^{ae}	β-Est III	++	0.96
NDP^{ae}	β-Est I	+++	0.62
	β-Est II	+++	0.80
	β-Est III	++	0.97
SP^{ae}	β-Est III	+	0.96

*+ to +++ represents low toseverity of the expression of isozymes of CCEs.

4.3 Aedes albopictus

4.3.1 Larval bioassay:

Only one of the eleven tested population, *i.e.* NGK^{al} was found to possess incipient resistance against temephos at WHO recommended dosage and two populations, NGK^{al} and SLG^{al}at Indian Govt. recommended dose (Table 21). Rest of the populations were recorded to possess mortality percentages within susceptible range. Similarly, the RR_{50} values greater than 2 were noted in NGK^{al},SLG^{al} and JPG^{al} population (Table 21) amongst the field populations, the LC_{50} values ranged from 0.0001 to 0.0047 ppm, whereas the LC_{99} values were noted to be ranging from 0.038 to 0.081 ppm.

Table 21: Susceptibility status against temephos and corresponding lethal concentration values in *Ae. albopictus* larvae collected from districts of northern part of west Bengal

SITES	Mortality (%±S.D.)				LC_{50} (ppm)±SE (95% C.L.)	LC_{99}(ppm)±SE (95% C.L.)	r^2	RR_{50}	RR_{99}
	0.02mg/L	Status	0.0125mg/L	Status					
APD^{al}	100±0.0	S*	100±0.0	S	0.0027±0.0004^{b*}	0.058±0.0003^{b}	0.91	1.68	1.38
HAS^{al}	100±0.0	S	100±0.0	S	0.0013 ±0.0003^{a}	0.044±0.0001^{a}	0.86	0.81	1.04
KMG^{al}	100±0.0	S	100±0.0	S	0.0014±0.0003^{a}	0.040±0.0001^{a}	0.89	0.87	0.95
NGK^{al}	97±0.6	IR	94±0.8	IR	0.0047±0.0001^{c}	0.081±0.0020^{d}	0.94	2.93	1.92
JPG^{al}	100±0.0	S	100±0.0	S	0.0034±0.0002^{b}	0.046±0.0030^{a}	0.82	2.12	1.09
NMZ^{al}	100±0.0	S	100±0.0	S	0.0032±0.0010^{b}	0.049±0.0040^{a}	0.89	2.00	1.16
NBU^{al}	100±0.0	S	100±0.0	S	0.0001±0.0001^{a}	0.038±0.0005^{a}	0.87	0.06	0.90
SLG^{al}	98±0.3	S	96±0.3	IR	0.0042±0.0003^{c}	0.074±0.0006^{c}	0.94	2.62	1.8
ISL^{al}	100±0.0	S	100±0.0	S	0.0024±0.0002^{b}	0.049±0.0001^{b}	0.91	1.5	1.16
KHR^{al}	100±0.0	S	100±0.0	S	0.0017±0.0001^{a}	0.042±0.0001^{a}	0.90	1.06	1.00
COB^{al}	100±0.0	S	100±0.0	S	0.0021±0.0004^{a}	0.046±0.0003^{a}	0.87	1.31	1.09
SP^{al}	100±0.0	S	100±0.0	S	0.0016±0.0002 ^{a}	0.042±0.0030^{a}	0.95		-

*Mortality percentage <80%: resistant, 80-98%: Intermediate resistance, >98% susceptible, C.L.: Confidence limits, within columns, means followed by the same letter do not differ significantly(P=0.05) in Tukey's multiple comparison test (HSDa)

4.3.2 Adult bioassay:

In field caught *Ae. albopictus* mosquitoes, widespread resistance against DDT was noted with the mortality percentages ranging from 40.0 to 96.0% (Table 22). Severe resistance against DDT was noted in SLG^{al}, moderate in JPG^{al} and NGK^{al} and incipient in rest of the population. Against malathion, deltamethrin and lambdacyhalothrin, complete susceptibility was revealed among the wild mosquito populations (Figure 23). Against permethrin, two of the population, *i.e.* APD^{al} and JPG^{al} possessed moderate resistance with mortality percentages 75.4 and 75.0 respectively. Four of the tested populations, HAS^{al}, NGK^{al}, NMZ^{al} and COB^{al} were incipiently resistant to permethrin. A single population severely resistant to propoxur, *i.e.* JPG^{al} was revealed in this study with a very low mortality, 42.5%. Six other populations too showed altered susceptibility to propoxur with the mortality percentages lying within unconfirmed resistance status.

Table 22: Mortality percentages and insecticide resistance status against six insecticides in adult *Ae. albopictus* collected from districts of northern part of West Bengal

Sites	DDT		Malathion		Deltamethrin		Lambdacyhalothrin		Permethrin		Propoxur	
	M%*	Status	M%	Status	M%	Status	M%	Status	M%	Status	M%	Status
APD^{al}	92.8±0.3	IR	100±0.0	S	100±0.0	S	100±0.0	S	75.4±1.3	R	100±0.0	S
HAS^{al}	88.9±0.5	IR	100±0.0	S	100±0.0	S	100±0.0	S	96.2±0.4	IR	100±0.0	S
KMG^{al}	94.3±0.8	IR	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S
NGK^{al}	63.1±2.6	R	100±0.0	S	100±0.0	S	100±0.0	S	92.1±0.6	IR	99.3±0.0	S
JPG^{al}	75.4±1.7	R	100±0.0	S	100±0.0	S	100±0.0	S	75.0±1.9	R	42.5±3.4	R
NMZ^{al}	#	#	100±0.0	S	100±0.0	S	100±0.0	S	95.2±0.9	IR	89.7±0.9	IR
NBU^{al}	96.0±0.4	IR	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	92.0±0.5	IR
SLG^{al}	40.0±3.5	R	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	92.3±0.5	IR
ISL^{al}	93.3±0.3	IR	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	80.1±0.9	IR
KHR^{al}	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	98.9±0.3	S
COB^{al}	90.0±0.7	IR	100±0.0	S	100±0.0	S	100±0.0	S	96.4±0.5	IR	90.9±0.6	IR
SP^{al}	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S	100±0.0	S

*M%: Mortality percentage, S: Susceptible, IR: Incipient resistance, R: Resistance

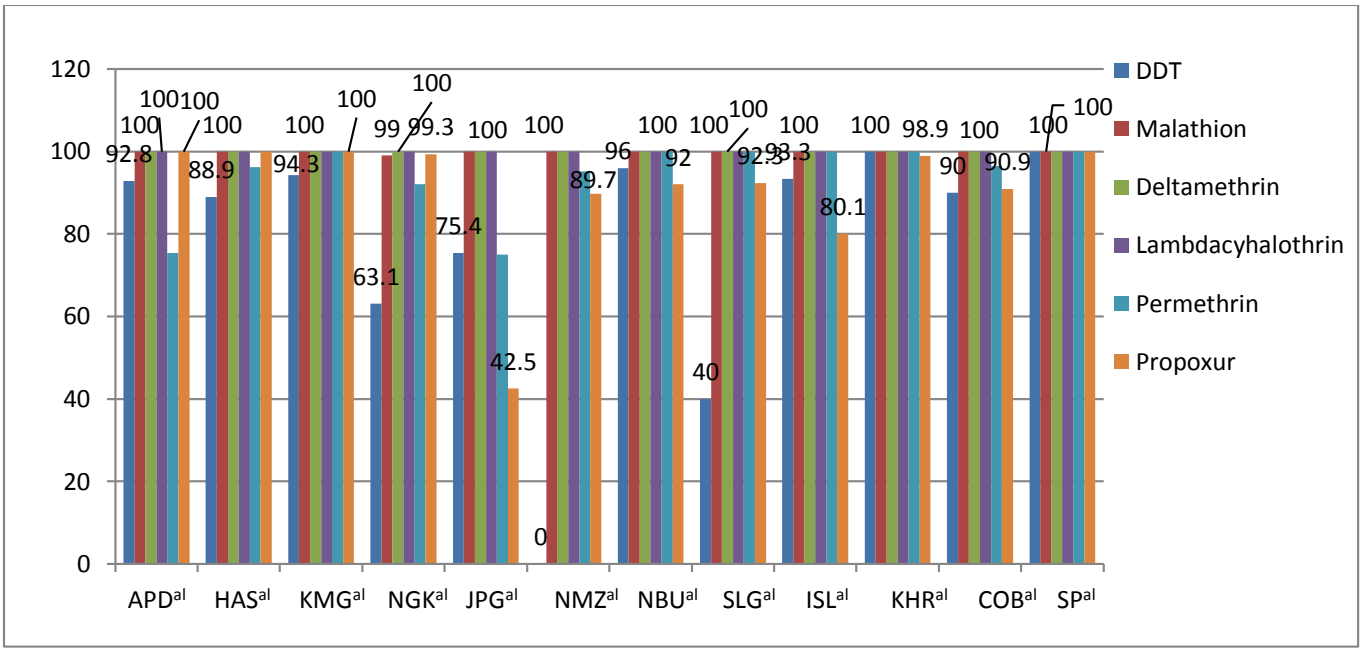
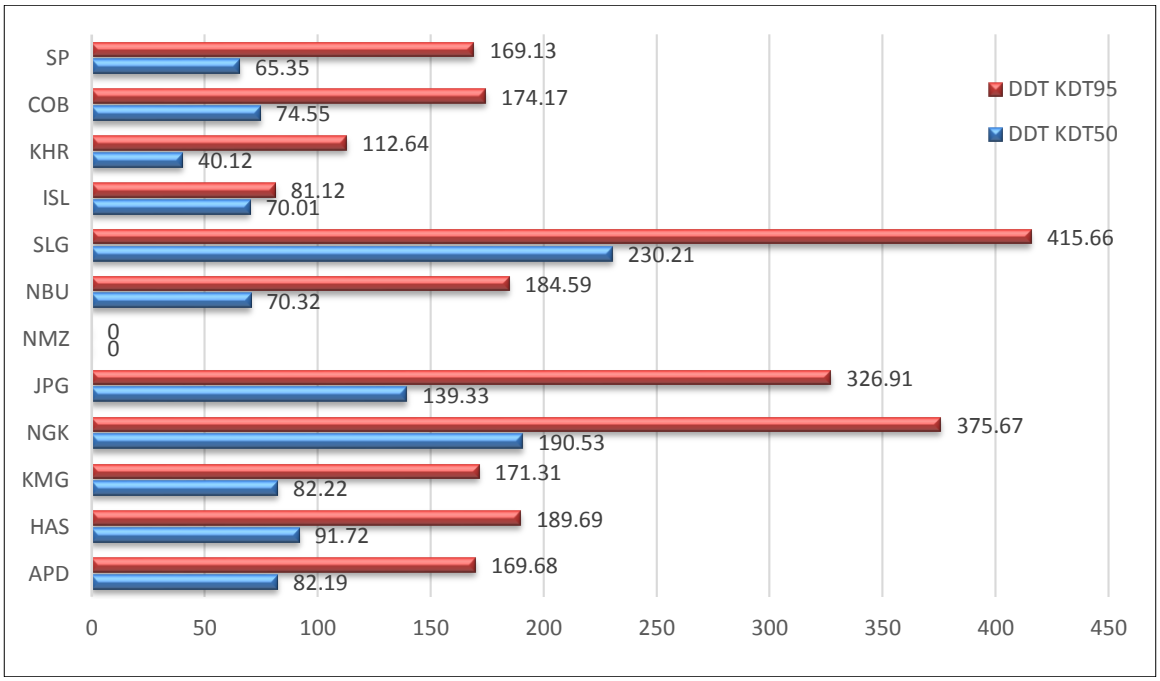


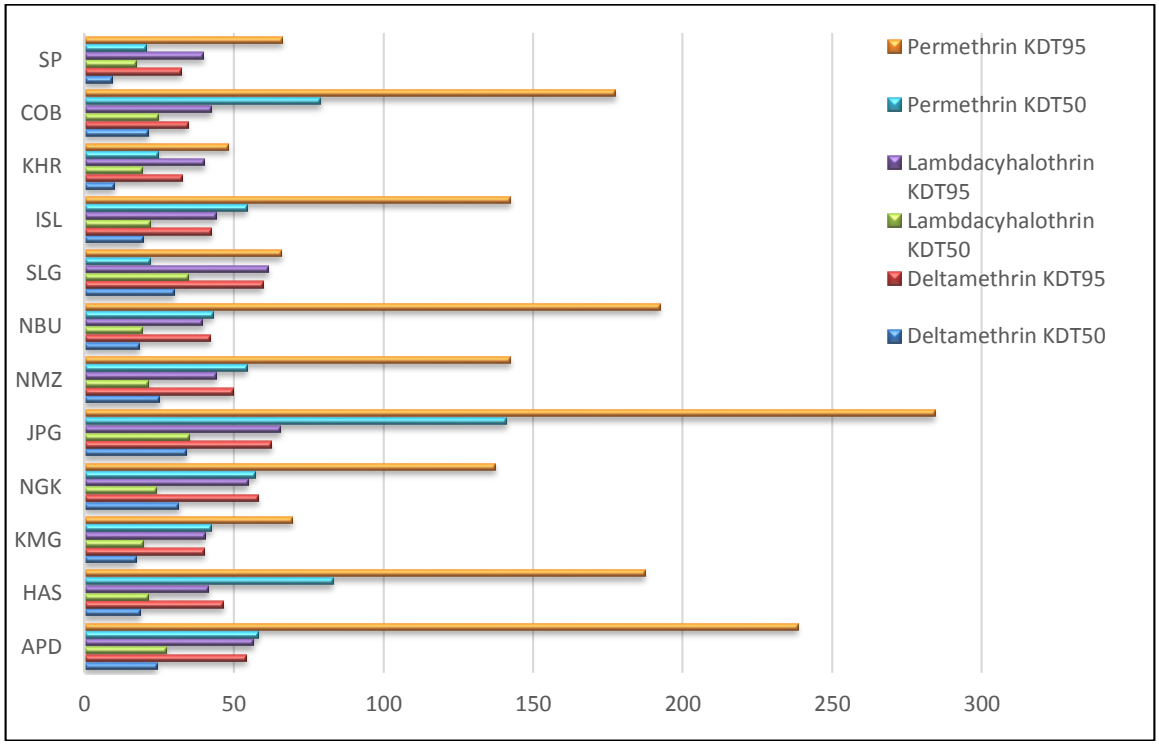
Figure 23: Mortality percentages against six insecticides in *Ae. albopictus* collected from districts of northern part of west Bengal

4.3.3 Knockdown times:

In most of the field caught *Ae. albopictus* mosquitoes, greater than 60 min KDT_{50} values were noted. The highest KDT_{50} and KDT_{95} values against DDT were exhibited by SLG^{al} population, 230.21 and 415.66 respectively (Figure 24). Very low knockdown times were noted amongst the mosquitoes against deltamethrin and lambdacyhalothrin. Against permethrin, JPG^{al} population recorded very high knockdown times, *i.e.* KDT_{50} of 141.12 and KDT_{95} value of 284.38 respectively.



a.



b.

Figure 24: Knockdown rates (KDT_{50} and KDT_{95}) of different field populations of *Ae. albopictus* against a. Organochlorine and b. synthetic Pyrethroid insecticides

4.3.4 Synergism test:

Use of TPP along with propoxur helped in restoring the mortality to susceptibile level in NGK^{al} population. Against DDT, the prior exposure to PBO restored the susceptibility to some extent in NGK^{al} (increasing mortality from 63.1% to 78.2), JPG^{al} (from 75.4% to 94.1%) and SLG^{al} (from 40.0% to 81.25%) (Table 23). Minor restoration in susceptibility to DDT was also noted when the mosquitoes were exposed to TPP in some of the tested populations. Against permethrin, inhibiting CYP450s through PBO could enhance the mortality levels from resistant to incipiently resistant (80%) in both APD^{al} and JPG^{al}. However, against propoxur none of the synergist could increase the susceptibility considerably in any of the field population of *Ae. albopictus*.

Table 23: Susceptibilities against insecticide along with synergists, 4% PBO and 10% TPP in different *Ae. albopictus* mosquitoes

	Sampling sites											
	APD[al]	**HAS**[al]	**KMG**[al]	**NGK**[al]	**JPG**[al]	**NMZ**[al]	**NBU**[al]	**SLG**[al]	**ISL**[al]	**KHR**[al]	**COB**[al]	**SP**[al]
Temephos+ PBO	100±0.0	100±0.0	100±0.0	97.0±0.4	100±0.0	100±0.0	100±0.0	98.2±0.3	100±0.0	100±0.0	100±0.0	100±0.0
Temephos + TPP	100±0.0	100±0.0	100±0.0	100.0±0.0	100±0.0	100±0.0	100±0.0	99.2±0.2	100±0.0	100±0.0	100±0.0	100±0.0
DDT+PBO	97.0±0.3	90.9±0.5	96.2±0.3	78.2±0.6	94.1±0.3	#	96.6±0.4	81.25±0.7	96.1±0.3	100±0.0	94.5±0.4	100±0.0
DDT+TPP	90.9±0.5	92.1±0.4	94.9±0.5	69.7±0.9	79.8±0.6	#	97.1±0.2	59.0±1.1	93.4±0.6	100±0.0	92.1±0.3	100±0.0
Deltamethrin + PBO	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
Deltamethrin+ TPP	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
Lambdacyhalothrin + PBO	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
Lambdacyhalothrin+ TPP	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
Permethrin + PBO	93.6±0.3	100±0.0	100±0.0	95.6±0.3	88.3±0.6	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
Permethrin + TPP	81.16±0.6	97.2±0.3	100±0.0	91.5±0.3	72.4±0.8	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	95.8±0.3	100±0.0
Propoxur + PBO	100±0.0	100±0.0	100±0.0	100±0.0	44.1±1.2	88.4±0.5	92.4±0.4	91.6±0.3	81.1±0.8	100±0.0	87.6±0.5	100±0.0
Propoxur + TPP	100±0.0	100±0.0	100±0.0	100±0.0	50.2±0.9	90.5±0.4	93.7±0.2	95.1±0.3	83.2±0.7	100±0.0	91.4±0.5	100±0.0

4.3.5 Major insecticide detoxifying enzyme assay:

As compared to the susceptible population, significantly higher activity of α-CCEs were noted in NGK^{al}, SLG^{al}, JPG^{al} and NMZ^{al} population. NGK^{al} population was found to possess the highest activity of α-CCE, *i.e.* 2.87 µmoles mg protein^{-1} min^{-1} followed by SLG^{al}, 0.82 µmoles mg protein^{-1} min^{-1} (Table 24). For β-CCEs higher activities were noted for NGK^{al}, SLG^{al} and APD^{al} population, *i.e.* 3.16, 2.83 and 2.74 folds than SP^{al} respectively. The activity of CYP450s monoxygenases were recorded to range from 1.03 to 1.94 times SP^{al}, with the highest activity noted in JPG^{al} population, *i.e.* 0.62 nM mg protein^{-1} min^{-1}. The activity of GSTs ranged from 0.305 to 0.385 µM mg protein^{-1} min^{-1}.

Table 24: Activities of major insecticide detoxifying enzymes in field caught *Ae. albopictus* mosquitoes collected from districts of northern Bengal

Sites	α-CCEs (µmoles mg protein^{-1} min^{-1})	β-CCEs (µmoles mg protein^{-1} min^{-1})	CYP_{450s} (nmoles mg protein^{-1} min^{-1})	GSTs (nM mg protein^{-1} min^{-1})
APD^{al}	0.49±0.003^{b*}	0.85±0.005^{c}	0.056±0.0011^{b}	0.341±0.0013^{a}
HAS^{al}	0.84±0.007^{c}	0.49±0.006^{b}	0.036±0.0005^{a}	0.331±0.0011^{a}
KMG^{al}	0.42±0.003^{b}	0.39±0.002^{a}	0.033±0.0006^{a}	0.311±0.0009^{a}
NGK^{al}	2.87±0.012^{d}	0.98±0.008^{c}	0.051±0.0007^{b}	0.385±0.0015^{a}
JPG^{al}	0.66±0.005^{c}	0.43±0.002^{b}	0.062±0.0009^{b}	0.328±0.0015^{a}
NMZ^{al}	0.69±0.007^{c}	0.48±0.005^{b}	0.039±0.0005^{a}	0.330±0.0016^{a}
NBU^{al}	0.38±0.002^{a}	0.36±0.004^{a}	0.034±0.0007a	0.318±0.0009^{a}
SLG^{al}	0.82±0.005^{c}	0.88±0.007^{c}	0.034±0.0009^{a}	0.372±0.0022^{a}
ISL^{al}	0.39±0.003^{a}	0.38±0.002^{a}	0.036±0.0007^{a}	0.321±0.0012^{a}
KHR^{al}	0.37±0.007^{a}	0.35±0.004^{a}	0.033±0.0004^{a}	0.305±0.0017^{a}
COB^{al}	0.51±0.007^{b}	0.48±0.007^{b}	0.038±0.0009^{a}	0.338±0.0014^{a}
SP^{al}	0.34±0.001^{a}	0.31±0.004^{a}	0.032±0.0006^{a}	0.289±0.0012^{a}

*Within columns, means followed by the same letter do not differ significantly (P=0.05) in Tukey's multiple comparison test (HSDa).

4.3.6 Kdr genotyping:

Altogether, 242 *Ae. albopictus* mosquitoes were genotyped for F1534C gene. Except for one field population, *i.e.* SLG^{al}, rest were found to carry both the alleles. The frequencies of homozygote CC were found to be 3.3%, heterozygote FC, 50.4% and homozygote FF 46.3% (Table 25) Moreover, the frequency of the 1534C allele amongst the wild population was 29.8%.

Table 25: Results of F1534C kdr genotyping from randomly selected populations of *Ae. albopictus*

Mutation screened	Population	Total samples	FF	FC	CC	Frequency of C allele (%)
F1534C	APD^{al}	24	4	20	0	41.6
	HAS^{al}	24	18	6	0	12.5
	COB^{al}	20	8	12	0	30.0
	JPG^{al}	24	0	22	2	54.1
	NGK^{al}	28	2	22	4	62.5
	NMZ^{al}	22	0	20	2	54.5
	SLG^{al}	26	26	0	0	0
	NBU^{al}	26	24	2	0	3.8
	KHR^{al}	22	8	14	0	31.8
	ISL^{al}	26	22	4	0	7.6

4.3.7 Qualitative analysis of α- and β- Carboxylesterases:

Around two distinct different bands corresponding to isozymes were noted (Figure 25) amongst the different field caught mosquito populations with the Rf values 0.81, 0.91 (Table 26). Both the bands were expressed in NGK^{al} and JPG^{al} population, whereas the rest possessed single isozyme, *i.e.*α-Est II. The band intensity of α-Est I was noted to be more in NGK^{al} than JPG^{al} (Figure 25). Similarly, intensity of α-Est II was highest in APD^{al} and SLG^{al}.

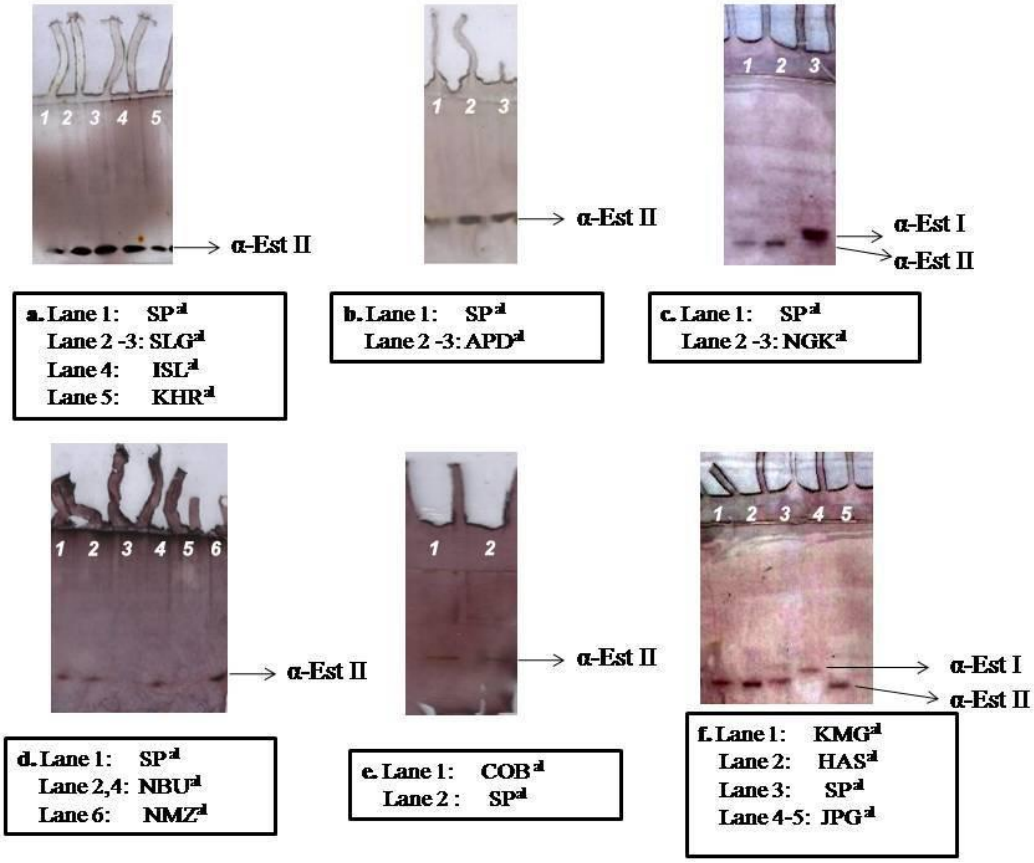


Figure 25: Electrophoregram of α-Carboxylesterase isozymes in *Aedes albopictus* mosquitoes collected from northern districts of West Bengal.

Table 26: The summarized report of the electrophoregram of α-Carboxylesterase isozymes in *Aedes albopictus* mosquitoes collected from northern districts of West Bengal.

Sites	Band number	Intensity	Rf value
APD^{al}	α-Est II	+++*	0.90
HAS^{al}	α-Est II	++	0.91
KMG^{al}	α-Est II	+	0.91
NGK^{al}	α-Est II	+++	0.91
	α-Est II	++	0.80
JPG^{al}	α-Est I	++	0.81
	α-Est II	++	0.92
NMZ^{al}	α-Est II	++	0.90
NBU^{al}	α-Est II	++	0.92
SLG^{al}	α-Est II	+++	0.91
ISL^{al}	α-Est II	++	0.91
KHR^{al}	α-Est II	++	0.91
COB^{al}	α-Est II	+	0.91
SP^{al}	α-Est II	+	0.91

*+ to +++ represents low to severity of the expression of isozymes of CCEs.

Altogether two distinct isozymes were observed in the electrophoretic study of β-CCEs (Figure 26) with the Rf values 0.63 and 0.95 (Table 27). The highest number of isozymes were expressed in SLG^{al} population, with the rest populations expressing a single band β-Est II (Figure 26). The prevalent isozyme *i.e.* β-Est II was intensely expressed in NGK^{al} population followed by APD^{al}, JPG^{al}, SLG^{al}.

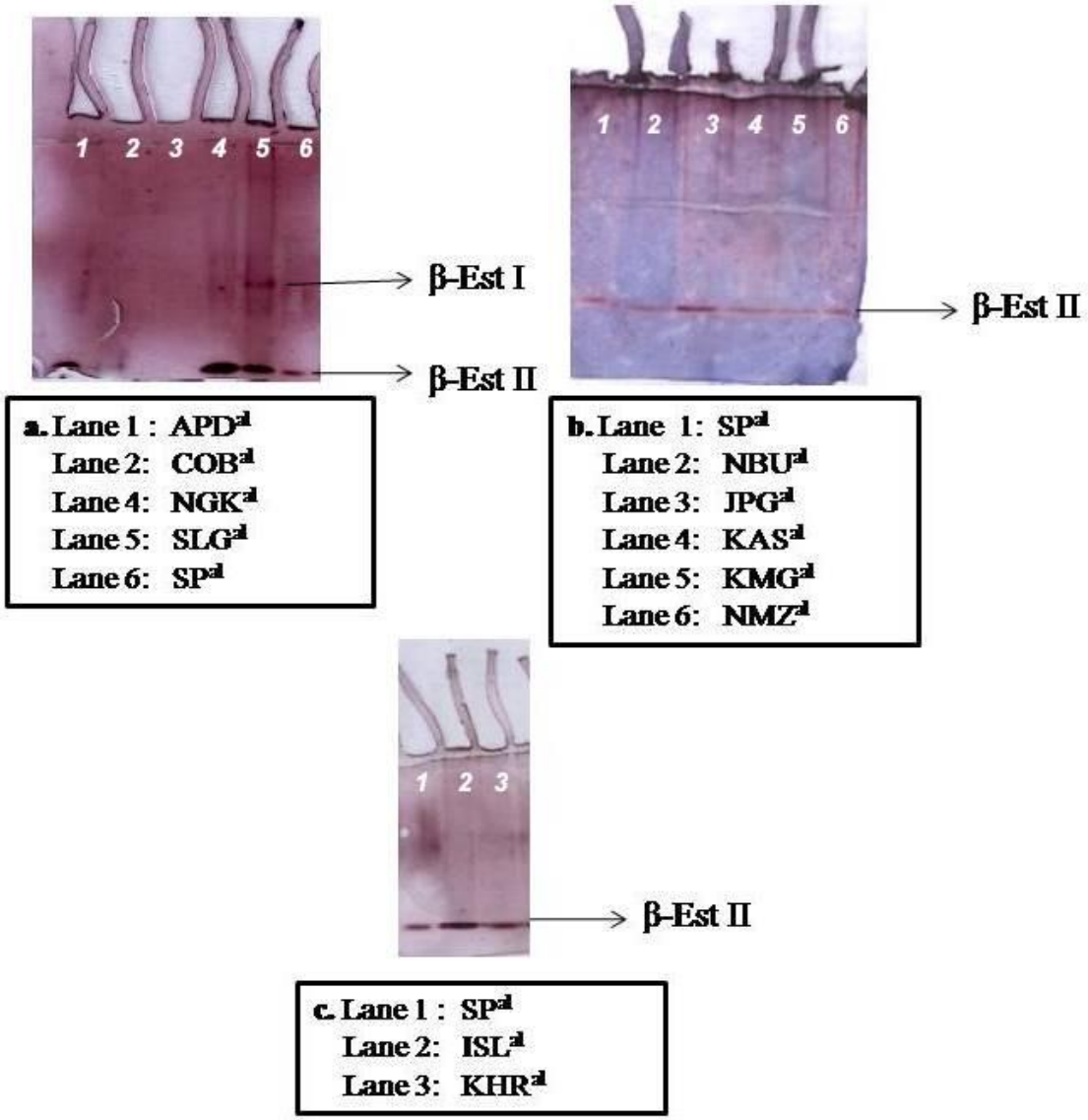


Figure 26: Electrophoregram of β-Carboxylesterase isozymes in *Aedes albopictus* mosquitoes collected from northern districts of West Bengal.

Table 27: The summarized report of the electrophoregram of β-Carboxylesterase isozymes in *Aedes albopictus* mosquitoes collected from northern districts of West Bengal.

Sites	Band number	Intensity	Rf value
APD^{al}	β-Est II	++*	0.95
HAS^{al}	β-Est II	+	0.95
KMG^{al}	β-Est II	+	0.95
NGK^{al}	β-Est II	+++	0.95
JPG^{al}	β-Est II	++	0.95
NMZ^{al}	β-Est II	+	0.95
NBU^{al}	β-Est II	+	0.95
SLG^{al}	β-Est I	+	0.63
	β-Est II	++	0.95
ISL^{al}	β-Est II	++	0.95
KHR^{al}	β-Est II	++	0.95
COB^{al}	β-Est II	++	0.95
SP^{al}	β-Est II	+	0.95

*+ to +++ represents low to severity of the expression of isozymes of CCEs.

DISCUSSIONS

5. DISCUSSION:

5.1 Surveying of Aedes mosquitoes:

Throughout the study region, a dominance of *Ae. albopictus* over *Ae. aegypti* was noticed. Majority of the part of the studied area had been rural region covered with dense vegetation for a long time span. The urbanisation in this region is very recent in this region which might explain the abundance of rural vector of dengue throughout the sampling area (Gratz, 2004). It might also be the case that some kind of competition might be occurring between these two *Aedes* species since both occupy similar niche resulting in the establishment of one species with considerable competitive advantage over the other in the studied area. *Ae. albopictus* have been reported to outcompete *Ae. aegypti* occupying same habitat owing to its higher mating competitiveness over *Ae. aegypti* (Bellini *et al.*, 2013).

In this study, it was found that for both the *Aedes* species, discarded tyres were the most preferred breeding habitat resulting highest positivity indices (Table 12). Similar inferences have been observed in other such studies (Vijayakumar *et al.*, 2014; Ferede *et al.*, 2018). The advantage of tyres used as breeding habitats by mosquitoes lie on the fact that the water inside it is not easily observable and thus is less prone to climatic disturbances, maintaining ideal environmental conditions for *Aedes* development (Ferede *et al.*, 2018). Owing to the structure of tyres, some amount of water is always carried in it and even if it dries completely, it may serve as a good egg preserving surface, which when refilled with water may again become the mosquito breeding habitat (Rao *et al.*, 2010). The second most preferred habitat for *Ae. aegypti* in this study was revealed to be uncovered cemented tanks, mainly used by garage workers to check tyre leakage. In India, cemented tanks and plastic

containers are regarded as the major breeding habitats of *Ae. aegypti* (Balakrishnan *et al.*, 2006). Thus use of discarded tyres for *Ae. aegypti* breeding might have taken place fairly recently in India. Other breeding habitats such as bamboo stumps and plant axils were also found to be preferred mainly by *Ae. albopictus*, owing to its behaviour and history of being the sylvatic mosquito thus preferring the natural habitats (Gratz, 2004).

From the result of larval density index, it was found that higher densities were noted for *Ae. aegypti* in ISL followed by NMZ and SLG. Similarly, for *Ae. albopictus* very high larval densities were observed for NGK followed by NBU, HAS and APD. This points on the risk of dengue transmission in and around this sites during disease outbreak. To minimise the disease risk, proper control measures should be taken ahead of the disease outbreak season by destroying the mosquito breeding habitats and treatment with larvicide in water storage containers.

5.2. Insecticide susceptibility status and underlying mechanism in Aedes aegypti mosquitoes:

5.2.1 Insecticide susceptibility status of Aedes aegypti mosquitoes:

In this study, insecticide susceptibility was tested against few commonly used insecticides (adulticides and larvicide) belonging to four different groups of insecticides. For larvicides, an organophosphate insecticide, *i.e.* temephos was chosen owing to the frequent usage of it by both governmental and non-governmental organisation in mosquito larvae control. Amongst the adulticides, one Organochlorine- DDT, one Organophospahate- malathion, three synthetic Pyrethroids- type I, permethrin; type II, deltamethrin and lambdacyhalothrin and one Carbamate, propoxur were tested.

5.2.1.1 Insecticide susceptibility status against organophosphates:

- *Temephos:*

Temephos or O,O,O',O'- tetramethyl O,O'-thiodi-p-phenylenebis (Phosphorothioate), is widely used throughout the world for mosquito larvae control owing to its cost effectiveness as well as efficiency (Grisales *et al.,* 2013). However the wide usage has resulted in widespread resistance against this insecticide in majority of the mosquito vectors particularly dengue vectors. Majority of the sites from our sampling region have shown low resistance levels but higher resistance ratios. Moreover, one population NDP^{ae} was found to possess incipient resistance against 0.02ppm temephos, *i.e.* WHO recommended diagnostic dose and resistance against 0.0125ppm, *i.e.* NVBDCP recommended diagnostic dose . The increased RR_{50} values for most of the sites along with the presence of one resistant *Ae. aegypti* population from this dengue endemic region indicates that in near future temephos may become ineffective in dengue control throughout the region. For dengue vector, temephos is generally recommended for water storing sources and vessels at a dose not exceeding 1mg/L. Four of the sites were noted to possess trend towards resistance development with an RR_{50} value above 2 for APD^{ae}, DAR^{ae}, JPG^{ae} and NDP^{ae} (Table 14).

In India, moderately resistant *Ae. aegypti* have been noted to be widespread throughout the Indian capital, New Delhi with mortality percentages ranging from 66.22 to 95.11 (Singh *et al.,* 2014). Similarly, high level of resistance against temephos with mortality percentages ranging from 4% to 10% at 0.02 ppm were noted in Andaman and Nicobar islands (Sivan e*t al.,* 2015). Severe level of resistance to temephos have also been recorded in wild *Ae. aegypti* population from Assam (Yadav *et al.,* 2015) with RR_{99} values of 2.5-4.5. Even higher resistance ratio levels were also

exhibited by mosquito population from Karnataka with LC_{90} values 1.00 ppm to 17.03 ppm (Shetty *et al.,* 2013). Throughout the world, the prime mosquito control organisations of most of the countries recommend and rely on the use of temephos for disease prevention such as America, Carribean, India, Srilanka *etc* (Rawlins, 1998; Karunaratne *et al.,* 2013; NVBDCP, 2019c). So resistance against temephos was noted in *Aedes aegypti* successively after a long period of its use worldwide, Carribean (Georghiou, 1987; Rawlins and Wan, 1995), French Polynesia (Failloux *et al.,* 1994) and other parts of the world (Vontas *et al.,* 2012). Field caught population of *Ae. aegypti* from Thailand have been reported to exhibit mortality percentages ranging from 50.5 to 96.0 (Jirakanjanakit *et al.,* 2014). Similarly *Ae. aegypti* have been recorded to possess severe to moderate resistance with mortality percent as low as 16% (Chen *et al.,* 2005) to moderate resistance with RR_{50} value ~ 2 (Ishak *et al.,* 2015; Rohani *et al.,* 2001). Severe resistance against this oraganophosphate has also been recorded in another Southeast Asian country *i.e.,* Indonesia with mortality percent of 22% to 605 and LT_{50} in the range of 2.2 to 8.5 (Mulyatno *et al.,* 2012). In Cambodia, incipiently resistant population of temephos have been recorded with LC_{95} value of 0.03mg/l (Polson *et al.,* 2001). From the neighbouring country of Pakistan, incipiently resistance *Aedes aegypti* mosquitoes have been reported, mortality percent 81.25 to 86.67% (Arslan *et al.,* 2016). Similar instance of temephos resistant *Ae.aegypti* mosquitoes have also been recorded, highly resistant; El salvador (Lazcano *et al.,* 2009) and incipiently resistant from Argentina (Llinas *et al.* 2010).

The LC_{50} value of the tested mosquitoes against temephos were found to be low for majority of the population ranging from 0.000057 to 0.002 ppm (Table 14). Similar trend of LC_{50} value of Temephos were also noted for mosquitoes collected

from Assam region, (LC_{50}: 0.002 to 0.0029 ppm) (Yadav *et al.* 2015), Mumbai (LC_{50}: 0.0012- 0.010) and Delhi (LC_{50}:0.0014- 0.0125) population (Tikar *et al.* 2008).

Similarly the L_{C99} dosages of different field population were found to be ranging from 0.26 to 0.00011 ppm. In nearby state of Assam, similar LC_{99} values were observed for one population collected from Sotia *i.e.* LC_{99}-0.12, whereas higher for rest of the population (Yadav *et al.,* 2015). In our study, the recommended dosage for only one population was found to be above the WHO diagonostic dose *i.e.* for NDP^{ae}, 0.52 ppm and one population above NVBDCP range, JPG^{ae} 0.016ppm.Such a scenario indicates the silent mode of resistance development throughout these sites which may result in the failure of vector control approach.

Temephos is recommended not only for *Aedes* control but also for the control of other disease causing mosquitoes such as *Anopheles* sp., *Culex* sp., *etc* (WHO, 2009*)*. Apart from mosquito control, it is also used in control of blackfly, midge, flea and other insects of public health as well as on agricultural fields for cutworms, thrips, *Lygus etc* (WHO, 2009). Owing to the wide usage of temephos in different mosquito species, resistance against it is also common (Chakraborti and Tandon, 2000; Shetty *et al.*, 2013; Soltani *et al.*, 2015). Resistance has also been noted against temephos in African population of blackfly (Hemingway *et al.,* 1991). Prevalence of resistance in all these vector species may have even more drastic outcome to the human public health disrupting the efficiency of all the vector control approaches in action.

There is an immediate need of assessing the insecticide resistance levels in other mosquito vectors against temephos and if resistance is noted then successive replacement of temephos by other effective insecticide.

- *Malathion:*

After mosquito population were found to possess resistance against DDT, it was replaced by hexachlorocyclohexane which was also effective for a very short time span and then was launched an organophosphate insecticide "Malathion" (Raghavendra *et al.,* 2010). However, initially providing efficient results, vector mosquito started developing resistance against this insecticide also. In our study, only one out of the five tested population was found to possess moderate resistance, *i.e*. mortality percentage 72.5 against malathion, APD population (Table 15). Another population, DAR^{ae} was reported to exhibit incipient resistance against it with mortality percentage 92.6. Rest of the population were completely susceptible to malathion as was the lab reared susceptible population. Malathion is being used in Alipurduar district (Personal communication) under which APD^{ae} site is located which explains the reason behind the resistance possessed by this population. However, the presence of insecticide resistance in DAR^{ae} site may be because of the cross exposure of insecticides sprayed on agricultural lands contaminating the mosquito breeding habitats as run offs containing the toxic moiety as have been reported in other mosquito vectors (Philbert *et al.,* 2014). Darjeeling district is known for tea cultivation and malathion being one of the key insecticide sprayed on tea fields apart from synthetic pyrethroids justifies involvement of above scenario and implying that similar might be the case for DAR^{ae} population (Saha and Mukhopadhyay, 2013). Malathion when used for mosquito control is used in 5% solution and similar or higher dosages are recommended for insect pests of agricultural importance (Anon., 2002a). The toxicity of this OP to non-target organisms has led to the withdrawal of its usage in majority of the agricultural pest control as well as mosquito control programmes.

Three of the population, *i.e.* JPG^{ae}, COB^{ae} and NDP^{ae} were recorded to be fully susceptible to malathion, thereby indicating that this OP can still be used during intense dengue outbreaks in the districts from which these population were collected. Throughout the country, both malathion resistant as well as susceptible *Aedes aegypti* population have been reported. Malathion resistant strains have been reported from Jharkhand, Andaman and Nicobar islands and many major cities of India possessing low level of resistance *i.e.* mortality percentage between 90-98% (Singh *et al.*, 2011; Sivan *et al.*, 2015; Tikar *et al.*, 2008). Whereas susceptible populations were recorded from Delhi (Katyal, 2001), Goa (Thavaselvam *et al.*, 1993), Assam (Yadav *et al.*, 2015) and Jharkhand (Singh *et al.*, 2011). Resistance against malathion in *Anopheles* sp. have been observed since 1987 with mortality as low as 12%` (Raghavendra *et al.*, 2010), whereas amongst the northern states of India the mortality have been noted to range from 68.46 to 95.43 % (Tikar *et al.*, 2011). Mosquito population with similar level of resistance, incipient resistance against malathion have been reported from Malaysia, mortality 91% (Ishak *et al.*, 2015), Thailand with RR_{90} ranging 2.2-6.6 and also other regions of the world (Vontas *et al.*, 2012).

5.2.1.2 Insecticide susceptibility status against Organochlorine:

- *DDT:*

DDT has been used throughout the world in both agricultural and public health sector in both malaria control and dengue prevention tactics since the 20^{th} century, as a result of it, majority of the regions of the world, intense DDT pressure exists (Vontas *et al.*, 2012). Throughout the studied region also, widespread resistance against DDT was noted with the mortality percentage noted to be as low as 47.9% for DAR^{ae} to 72.0% for JPG^{ae}. Severe to moderate resistance levels against DDT brings

into notice the dominance of DDT based mosquito control programmes for a large time duration throughout the area. Similar pattern of DDT resistance in wild population of *Aedes* mosquito have also been noted in nearby regions, i) Assam, with mortality percentage in the range of 65.0 to 70.5 (Yadav *et al.*, 2015), ii) Delhi, with 74% mortality percentage (Katyal *et al.*, 2011), iii) Jharkhand with corrected mortality percentage 54.68 to 63.88 (Singh *et al.*, 2011), iv) Andaman and Nicobar island with 74% mortality implying moderate resistance in *Ae. aegypti* mosquitoes (Sivan *et al.*, 2015). Stoppage of DDT usage by most of the governmental and non-governmental mosquito control agencies (Raghavendra *et al.*, 2010) seems to be a good approach as evident through the level of resistance prevailing among the mosquitoes against this insecticide. Many species of malaria vector, *Anopheles spp.* are also resistant to DDT with corrected mortality percentage less than 10 since 1985 (Das *et al.*, 1986) with the trend of resistance still being followed in *Anopheles culicifacies* (Raghavendra *et al.*, 2010; Mishra *et al.*, 2012). The first report to record the resistance against DDT in *Ae. aegypti* dates back to 1967(Azeez, 1967).

Similar to the resistance levels, the KDT_{50} and KDT_{95} values of DDT were also higher for most of the tested populations. Amongst the field caught populations, the lowest KDT_{50} was 95.41 (JPG population) whereas the highest was recorded, 182.16 for DAR^{ae} population, the population possessing the greatest resistance amongst others (Figure 19). Similar values were obtained for KDT_{95} with the lowest time recorded for JPG^{ae} whereas the highest for DAR^{ae}. As compared to SP^{ae}, all the field population possessed higher values of both KDT_{50} and KDT_{95}, implying the pattern of resistance development in the populations. Similar pattern of KDT_{50} values were observed for JPG^{ae} and COB^{ae} population with that of the neighbouring state of Assam with KDT_{50} values of 99.5 min and 83.2 min for Serajuli and Kusumtola

population respectively (Yadav *et al.*, 2015). In this study, even in the lab reared population the corrected mortality percentage against DDT was not equal to 100%, which again imparts light on the intensity of DDT resistance prevailing in the field population of dengue vectors.

5.2.1.3 Insecticide susceptibility status against synthetic Pyrethroids:

- *Deltamethrin, Lambdacyhalothrin and Permethrin:*

Synthetic pyrethroids are the most recent amongst the insecticide classes. In this study the mosquitoes were tested for their susceptibility against one type I pyrethroid *i.e.*, permethrin and two type II commonly used & recommended synthetic pyrethroid insecticide for mosquito control *i.e.*, deltamethrin and lambdacyhalothrin. The mosquitoes exhibited a higher resistance to the Type I pyrethroid than that of Type II. Against permethrin, three of the populations were resistant with mortality 60.0 to 76.5%, one incipiently resistant population, APD^{ae} (mortality percentage 83.3). The insecticide treated mosquito nets distributed throughout the districts by anganwadi centers as a part of national vector control programme contain either permethrin or deltamethrin (NVBDCP, 2019d) which maybe the reason behind the development of resistance against permethrin in these mosquitoes. Furthermore, none of the tested mosquitoes were found to be susceptible to this insecticide, this might have dreadful consequences in the near future for vector control programme using permethrin. The similarity between the resistance pattern between DDT and permethrin indicates the extent of cross-resistance occurring between the two insecticides (Table 15).

The KDT_{50} and KDT_{90} values against permethrin, though higher than other tested synthetic pyrethroids was significantly lower than that of DDT. The KDT_{50}

values were lower than 60 minutes for three of the populations *i.e.*, APD^{ae}, COB^{ae} and JPG^{ae} ranging from 37.40 to 52.76 min. Higher than 60 min KDT50 values were recorded for DAR^{ae} and NDP^{ae}, 67.66 to 78.98 min. Very few studies have been conducted to reveal the KDT_{50} and KDT_{90} values against permethrin in *Ae. aegypti* mosquitoes. Once such study from Nigeria reported very low KDT values with the KDT_{95} value ranging between 23.4 to 43.2 (Ayorinde *et al.,* 2015), however in another such study very low KDT_{50} and KDT_{95} values were recorded for susceptible population of *Ae. aegypti* from the same region (Ndams *et al.*, 2006), Senegal, KDT_{95} value 20.5 to 21.8 mins (Dia *et al.,* 2012).

Throughout the country resistant populations of *Ae. aegypti* against permethrin have been reported from Delhi-mortality percent 66.8-82.3 (Kushwah *et al.,* 2015), South Andaman district- mortality percent 84.76 (Sivan *et al.,* 2015), Southern India-RR- 5.1-6 (Muthusamy *et al.,* 2014). However susceptibility has also been noted against 0.75% permethrin (as used in this study) from Kerala (Sharma *et al.,* 2004), Jharkhand (Singh *et al.,* 2011); or against lower dose *i.e.*0.25% by Katyal *et al.,* 2001 with 100% mortality throughout the country. Mortality percentages have mostly been found greater than 60% for the tested field caught *Ae. aegypti* populations. Permethrin resistant wild population of *Ae. aegypti* have been reported in Malaysia (Othman-wan *et al.,* 2010; Ishak *et al.,* 2015), Columbia (Gonzalez *et al.,* 2011), Thailand (Somboon *et al.,* 2003) and other countries of the world (Moyes *et al.,* 2017). Altered susceptibility against permethrin have also been noted in *Anopheles* (Mittal *et al.,* 2004), *Culex* (Kumar *et al.,* 2011) or even in other insect vectors (Hemingway and Ranson, 2000).

Against other two synthetic pyrethroid insecticides, three population were incipiently resistant against both deltamethrin and lambdacyhalothrin. These

insecticide resistant populations recorded the mortality percentage ranging between 80.9 to 85.0% for lambdacyhalothrin and 89.2 to 91.9 for deltamethrin (Table 15). Rest of the two tested population, *i.e.* DAR^{ae} and COB^{ae} reported complete susceptibility against both the synthetic pyrethroid insecticides. As was the scenario in case of permethrin resistance, same seems to hold true since LLITNs contain either permethrin or deltamethrin, thereby providing the exposure to deltamethrin in most of the population (Table 15). Also, these two synthetic pyrethroids are extensively used in agricultural fields for insect pest control (Gurusubramanian *et al.,* 2008). APD^{ae} and JPG^{ae} sites are located in tea belts of northern Bengal with dominance of intensive tea gardens, whereas NDP^{ae} has intense pineapple cultivations (Saha and Mukhhopdhyay, 2013; Das *et al.,* 2011). In both the tea and pineapple cultivating fields synthetic pyrethroids and Organophosphates are sprayed, which may again provide the cross resistance to the mosquito populations residing in the vicinity of the agricultural fields (Nkya *et al.,* 2013). The 100% mortality as recorded in COB^{ae} and DAR^{ae} leads to the recommendation that during intense dengue or chikungunya outbreaks in these districts usage of permethrin may help reduce the infection rates.

Ae. aegypti is an anthropophilic mosquito *i.e.* its habitat is placed near human dwellings, the altered susceptibilities against synthetic pyrethroid insecticides seem to be due to the direct exposure to household mosquitocidal tools such as mosquito repellent coils, fumigant spray and creams which chiefly contain pyrethroid derivatives (Yadav *et al.*, 2015). In India, a household spray sold under the brand name "Hit" mainly contains transallethrin whereas another commonly used tool, repellent coil sold under the brand name "Mortein" contains a cocktail of permethrin and transallethrin and fumigant sold under the brand name "all-out" contains allethrin.

Throughout India, majority of the studied population have been recorded to be susceptible to deltamethrin in Assam with mortality percentage 98.8-100 (Yadav *et al.,* 2015), Jharkhand with mortality percentage 98.26-100 (Singh *et al.,* 2011), Kerala (Sharma *et al.,* 2004), Southern Andaman, mortality percentage (Sivan *et al.,* 2015), Delhi with 100% mortality (Katyal *et al.,* 2001). Similar is the scenario for lambdacyhalothrin, however incipient resistance and resistance have been reported in *Ae. aegypti* population from Andaman island with 80.95% mortality (Sivan *et al.,* 2013), Jharkhand with onset of IR *i.e.* mortality percentage: 97.33-97.8 (Singh *et al.,* 2011). Rest of the studies have reported complete susceptibility of *Ae. aegypti* against lambdacyhalothrin (Katyal *et al.,* 2001; Sharma *et al.,* 2004)

Although few reports exist on the pyrethroid resistance in Indian *Ae. aegypti* populations, worldwide many such studies have been conducted reporting pyrethroid resistance such as in Thailand (resistant against deltamethrin with 61.2-73.9%) (Somboon *et al.,* 2003); South American countries, namely Cuba, Jamaica, Costarica, Peru and Venezuela (mortality percentages 56.3-96.4 against lambdacyhalothrin; 72.7-97.2% against deltamethrin (Rodriguez *et al.,* 2007); Senegal with mortality percentage 94.5 against deltamethrin and 81.6% for lambdacyhalothrin (Dia *et al.,* 2012); Vietnam with mortality 19.33-97.00% against deltamethrin and 25.0-96.0% against lamdacyhalothrin (Huong *et al.,* 2004).

Amongst the mosquitoes that result in knockdown, through closure of voltage gated sodium channel, the lowest KDT_{50} and KDT_{95} values were noted for these two synthetic pyrethroids. The KDT_{50} value for deltamethrin was lowest for COB^{ae} (14.11) and highest for NDP^{ae} (58.77), and all the KDT_{50} values were less than 60 minutes (Figure 19). The pattern of KDT_{95} was also similar as the KDT_{50} with the values ranging between 52.19-108.21 mins for the field caught populations of *Ae.*

aegypti. Similarly for lambdacyhalothrin, the KDT_{50} values were lower than 60 min ranging between 9.41- 43.33, with the lowest and highest values exhibited by the same population as in case of deltamethrin. Apart from two populations, *i.e.* APD^{ae} and JPG^{ae}, the rest population reported very low KDT_{95} value *i.e.* below 60 mins (Figure 19). However, two populations exhibited higher values of KDT_{95}indicating the onset of a resistance phenomenon which may be revealed in near future by the failure of dengue control efforts using lambdacyhalothrin. Lower susceptibility and knockdown rates have been recorded in an Senegal population of *Ae.aegypti* with KDT95 value of 27-30.3 min for deltamethrin and 26.9-34.2 for lamdacyhalothrin (Dia *et al.,* 2012), Central Africa with KDT95 as 11.4 –24.6 min (Kamgang *et al.*, 2011), central African republic with KDT95 values 23.5-42.0 min (Ngoagauni *et al.*, 2016), *etc*. Most of the studies with reports of higher resistance against these two synthetic pyrethroids did not present the data of knockdown times as comparatively few studies have till date been conducted on the KDT_{50} and KDT_{95} values of synthetic pyrethroids.

5.2.1.4 Insecticide susceptibility status against carbamate

- *Propoxur*

In India, the only carbamate insecticide that is recommended for *Aedes* mosquito control is Bendiocarb. However, in this study, we have tested the susceptibility against other carbamate insecticide *i.e*, propoxur which is used throughout different countries of the world and also recommended by food and agriculture organisation of the United nations for both dengue and malaria vector control (FAO, 2002). This insecticide was tested to assess whether this insecticide serve as an alternate vector control in India by assessing the prevailing resistance or

intermediate resistance among different field population of *Ae. aegypti*. Throughout the study sites, none of the *Aedes* population was found to possess susceptibility to propoxur. Three populations were found resistant against propoxur, of which one population DAR^{ae} was found to possess severe resistance with mortality 50.0% and two had moderate to low resistance with mortality percentage 77.2 and 75.4 for JPG^{ae} and NDP^{ae} (Table 15). Rest of the populations were found to possess incipient/unconfirmed resistance with mortality percentages of 91.3 to 97.2 for APD^{ae} and COB^{ae} respectively. The districts from where APD^{ae} and COB^{ae} were collected are neighbouring districts and the similar resistance phenomenon observed for propoxur and also other tested insecticides seems to be a result of similar mosquito control practices at both individual level or organization level in both the districts. As already stated, propoxur is not used or recommended for mosquito control in India, yet the observed resistance phenomenon operating in all the tested *Aedes aegypti* population may be pertained to the usage of household insect repellents containing propoxur in a very high dose for larger insects such as termites, bed bugs *i.e* in Baygon spray and many other repellent chalks manufactured by local companies.

Field populations of *Aedes aegypti* with resistance against 0.1% propoxur have been reported in Delhi *i.e* mortality 85% (Katyal *et al.,* 2001), Madurai *i.e*. mortality 96% (Marriapan *et al.,* 2017), Karnataka in larval population (Shetty *et al.,* 2013). Throughout the country, propoxur susceptible population of *Aedes aegypti* were noted in Andaman island (Sivan *et al.,* 2015) and in Kerala (Sharma *et al.,* 2004). Around the globe, propoxur resistant *Ae. aegypti* population have been reported from Senegal (Dia *et al.,* 2012), Thailand (Pethuan *et al.,* 2007) Malaysia (Rong *et al.,* 2012), Cot D ivore (Konan *et al.,* 2012) *etc.*

Resistance have also been noted against another more frequently used carbamate, *i.e* bendiocarb (0.1%) in Andaman in India (Sivan *et al.,* 2015), Cameroon (Yougang *et al.*, 2017), Mexico (Deming *et al.*, 2006), Pakistan (Mohsin *et al.*, 2016; Arslan *et al.*, 2016), Malaysia (Rong *et al.*, 2012) *etc*.

5.2.1.5 Overall view on resistance in Aedes aegypti:

Amongst all the tested mosquito population namely APD^{ae} and JPG^{ae} showed resistance ranging from low to severe resistance against all the tested adulticides. Highest resistance data in case of APD^{ae} population was noted against DDT (mortality percentage 55.4), whereas that for JPG^{ae} population, it was recorded against permethrin. If we arrange the toxicity of the tested insecticides in order of descending potency/efficacy it would be

Deltamethrin>lambdacyhalothrin>temephos>malathion>propoxur>permethrin> DDT

Throughout the studied region, during intense dengue and chikungunya outbreak deltamethrin usage may help in efficient disease prevention. Except for two Alipurduar and Darjeeling, all other districts may also get good dengue prevention with the use of malathion (5%) amongst the adulticide. Moreover, temephos also seems to be the safest choice for dengue vector control throughout the studied region at WHO dose for NDP^{ae} and at NVBDCP recommended dose for all other sites.

5.2.2. Mechanisms of resistance:

5.2.2.1 Mechanism of insecticide resistance against Organophosphate:

- *Temephos:*

In mosquitoes, the CCE based resistance mechanism forms the primary mechanism of OP resistance and secondary mechanism of CB resistance (Hemingway

and Karanaratne, 1998). It also has been found to confer resistance against synthetic pyrethroid in other insect species. Acetylcholine Esterases (AchEs) are the common target site for both OPs and CBs, so insensitive AchEs may also give rise to resistance against these insecticides.

In this study most of the studied mosquito populations were completely susceptible against temephos (Table 14). However, one population *i.e.,* NDP recorded incipient resistance against 0.02ppm (WHO dose) and moderate resistance against 0.0125ppm (NVBDCP dose). Significantly higher activities of both α-CCE and β-CCE compared to other tested population were noted in NDP^{ae} populations implying the role of CCEs based mechanisms behind resistance against temephos. The result of native PAGE also indicated similar involvement of CCEs in case of NDP^{ae} population. In this population, comparatively higher number of isozymes were noted *i.e.*, 5 in case of α-CCE and 3 in β-CCE. Moreover, the presence of intense bands in NDP^{ae} population indicates the elevated rate of temephos detoxification by over expression of CCE isozymes (Lima-catelani *et al.,* 2004). Similar involvement of CCEs isozymal variation conferring resistance against temephos in *Aedes aegypti* have been reported from India in Tamil Nadu (Muthusamy and Shivakumar, 2015) and beyond India (Bisset *et al.,* 2007; Grigoraki *et al.,* 2016). Mechanisms mediated by over-expression of CCEs activity have also been reported in *Aedes aegypti* populations resistant to temephos and other Organophosphate insecticide (Bellinato *et al.,* 2016). In an artificially selected population of *Aedes aegypti*, only after two generation of selection with temephos, CCE over-expression were noted governing the onset of resistance against it (Saavedra-Rodriguez *et al.,* 2014). Molecular investigation have identified the main CCE genes over-expressed in temephos

resistant mosquito as *ccae3A* and *ccae6A* (Poupardin *et al.,* 2014; Grigoraki *et al.,* 2015).

From the result of the LC_{50} and LC_{99}, it was found that for one population that is JPG, the recommended dose of temephos was reported to be higher than the India Government recommended dose (NVBDCP, 2019a), thereby indicating the onset of undetected resistance which will be detected by the bioassays in near future.

Higher values of recommended dose (as calculated by 2 times of LC_{99} dose) serve as an indirect indicator of resistance development in mosquito population (Yadav *et al.,* 2015). For NDP^{ae} population the recommended dose was calculated to be higher than both the WHO and India Government doses. The extent of temephos resistance development could impart a significant negative effect on the dengue prevention strategies since this is the primary and preferable effort for dengue control (Bisset *et al.,* 2013). The rotation of larvicides with *Bacillus thuringiensis israleansis* or pyriproxyfen may help in minimizing the mosquito population as well as the resistance development as this have been approved for usage in drinking water (Bisset *et al.,* 2013).

The results of synergistic assays confirmed the role of CCEs based mechanisms behind the observed resistance against temephos in NDP populations as the exposure to TPP (CCEs inhibitor) was found to restore the susceptibility of the larvae to it (Table 16) whereas the role of CYP450 was ruled out as PBO could not increase the mortality percentages in the test mosquito populations. Different CCE genes have been pinpointed responsible for resistance against temephos (Poupardin *et al.,* 2014).

- *Malathion:*

Resistance against malathion have been associated with elevated activity levels of CCE enzymes of field population of *Aedes aegypti* (Hemingway and Karunaratne, 1998). Against malathion, only two populations, APD^{ae} and DAR^{ae} showed altered susceptibility. Same populations also reported significantly higher activities of α- and β-CCEs, indicating the role of CCEs behind the observed resistance. However, NDP^{ae} population was found to exhibit the highest activity of both the CCEs among the field population, yet there was complete susceptibility against malathion. This may be pertained to the fact that resistance against malathion are brought upon by some malathion specific esterases which is a separate group of esterase enzymes (Ziegler *et al.,* 1987; Hemingway and Karunaratne, 1998). Moreover, malathion is a OP insecticide with an open chain and CCEs mediated mechanisms governing resistance against such compound may involve a separate group of esterase with no relation to other organophosphates such as temephos (Rodriguez *et al.,* 2001; Gelasse *et al.,* 2017). Since in this study, activity level of CCEs as a whole was measured, it may be the scenario here that in NDP^{ae} the activities of other esterases are high but these of malathion specific ones are low thus resulting in no resistance against it (Table 17). Opposite occurs in case of APD^{ae} and DAR^{ae} population where specifically malathion specific CCEs may have elevated giving rise to moderate resistance and incipient resistance respectively. Similar instances of elevated levels of CCEs have been found to be linked to malathion resistance (Alvarez *et al.,* 2013; Francis *et al.,* 2017).

From the results of synergist assay, it was revealed that the use of TPP which inhibits CCEs could significantly restore the susceptibility partially in APD^{ae} population and to a lower extent in DAR^{ae} population. This confirms the involvement

of CCEs linked pathways in providing resistance against malathion. However in no case was full susceptibility restoration recorded so mechanisms other than CCEs might also be associated with the observed resistance such as an insensitive AchE.

The results of native PAGE too gave a clearer insight into the observed resistance pattern against malathion. Single and darker bands with Rf values = 0.95-0.96 of α- and β- CCEs were observed in both APD^{ae} and DAR^{ae} with the band intensity more intense in the former (Figure 20-21). When equal amount of protein are loaded on to the gel, the intensity of bands in native PAGE gives an indication of the elevation/ overexpression of the particular isozyme (Lima-Catelani *et al.*, 2004). It may be stated that higher detoxification of malathion as a result of higher expression of α-ESTV and β- EST III may contribute to the levels of resistance occurring in APD^{ae} and DAR^{ae} populations.

The role of AchE cannot be ruled out behind the observed resistance against malathion in the field population of *Ae. aegypti* since the use of TPP could not increase the mortality to 100%. It seems that a combination of both elevated CCE levels and insensitive AchE might be governing the observed resistance pattern. However, the involvement of AchE insensitivity in malathion resistance is not common (Moyes *et al.*, 2017).

5.2.2.2 Mechanism of insecticide resistance against Organochlorine:

- *DDT:*

Resistance against DDT in mosquitoes can mainly be conferred by the elevated actions of Insecticide detoxifying enzymes (quantitatively or qualitatively),

mainly GSTs (Hemingway *et al.,* 2004; Marcombe *et al.,* 2014), CYP450s (Ishak *et al.,* 2015) and CCEs (Ngoagauni *et al.,* 2016) or through the mutations in voltage gated sodium channel, *i.e.* knockdown resistance (Vontas *et al.,* 2012) or by a combination of both (Aponte *et al.,* 2013). From the results of enzyme activities it is seen that the population with higher resistance levels namely APD^{ae}, DAR^{ae} and NDP^{ae} possess significantly higher activities of both α and β-CCEs (Table 17). Similar association were also noted between the resistance levels and CYP450s monoxygenase level. Elevated activity levels of GSTs, *i.e.* the prime DDT detoxifying enzyme were recorded throughout different mosquito populations, however the elevation was statistically insignificant. The associations of elevated activities CCEs with DDT resistance have been recorded in a central Africa republican population of *Ae. aegypti* (Ngoagauni *et al.,* 2016). Some studies have also pinpointed on the partial role of CYP450 mediated pathways in DDT degradation (Ishak *et al.,* 2017; Prapanthadara *et al.,* 2002).

However the use of enzyme blockers throughout the mosquito populations presented a different scenario. Only in one population, PBO was found to restore the susceptibility against DDT, *i.e.* elevating the mortality percentage from 58.2 to 80%, thus implying that CYP450 might be involved partially behind the insecticide resistance against DDT in *Ae. aegypti* population (Table 16). Ability of CYP450S in restoring the susceptibilities in *Ae. aegypti* against DDT have also been recorded in Malaysia (Ishak *et al.,* 2015), in one population from Cameroon (Kamgang *et al.,* 2017), Thailand (Choovattanapakorn *et al.,* 2017).

Since, GSTs over-expression is regarded to be the prime DDT metabolising mechanism particularly, GSTe2 has been implicated in *Ae. aegypti* to confer DDT resistance (Lumjuan *et al.,* 2005). DDT hydrochlorinase activity has been implicated

to be exhibited by GSTe2, GSTe5 and GSTe7 and the elevated level of epsilon class of GSTs have been shown to result in resistance against DDT (Lumjuan *et al.*, 2011; Marcombe *et al.,* 2009). But in this study, similar levels of GSTs activity was recorded throughout the mosquito population, so there may be the involvement of kdr mutations giving rise to DDT resistant populations.

In this study, the prevalence of two kdr mutations F1534C and V1016G was also tested, both of which though are mainly linked with synthetic pyrethroid resistance have also been shown to provide cross resistance against DDT (Davies *et al.,* 2007). The association between these mutations have been noted to be linked with DDT and synthetic pyrethroids resistance (Kushwah *et al.,* 2015). We have noted the presence of F1534c mutation in all the studied population (Table 18) which may be a factor behind the prevalence of DDT resistance throughout the studied *Ae. aegypti* population. The frequency of C allele was same throughout the test population, *i.e.* 50%, yet the variation between the DDT resistance levels may be because of the additive effect of another DDT resistance mechanism along with the kdr mutation (Aponte *et al.,* 2013). Out of the three populations recording the highest resistance against DDT, only one population NDP^{ae} was found to exhibit the mutated allele frequency of 70% along with presence of GG individuals, whereas the other two DAR^{ae} and APD^{ae} had the allele frequency of 33 and 25% respectively. So, no significant association could be made between the V1016G allele and DDT resistance which may be because V1016G is directly concerned with pyrethroid resistance (Du *et al.,* 2013). However, the presence of these kdr mutations might be the reason behind the elevated knockdown times recorded against DDT and a part of the observed resistance.

5.2.2.3 Mechanism of insecticide resistance against synthetic Pyrethroids:

- *Deltamethrin, Lambdacyhalothrin and Permethrin:*

Two mechanisms are generally linked to confer resistance against pyrethroid insecticides, *i.e.* increased activity of metabolic enzymes mainly CYP450s; partly CCEs and GSTs and the presence of kdr mutations contributing to target site insensitivity (Marcombe *et al.,* 2012; Vontas *et al.,* 2012). In this study we have found higher resistance against permethrin and lower levels for deltamethrin and lambdacyhalothrin. The CYP450s monoxygenases activity levels were higher than the SP^{ae} for most of the field population except COB^{ae} which was also recorded to possess 100% susceptibility to both the tested Type II pyrethroid. Two *Ae. aegypti* populations recording the highest resistance (though IR) against deltamethrin and lambdacyhalothrin were found to exhibit significantly higher activities of CYP450s than the SP^{ae}. Moreover, the same population were also found to possess elevated activity of CCEs too which leads to assume that both CYP450s and CCEs may be involved in degrading the pyrethroid molecules in the tested mosquitoes thus giving rise to resistance (Polson *et al.,* 2011).

The use of synergists revealed that in majority of the population PBO could restore the susceptibility towards deltamethrin and lambdacyhalothrin, thereby confirming the role of CYP450s associated pathways in conferring resistance observed in APD^{ae}, JPG^{ae} and NDP^{ae} population supplementing the results of enzyme activity levels. Moreover, the use of PBO was found to restore the mortality percentages to susceptible ranges in case of deltamethrin thereby confirming that detoxification by CYP450s enzymes alone conferred resistance against deltamethrin in these population. Whereas in case of lambdacyhalothrin, the use of PBO could only

partially restore the susceptibility to an extent within incipient resistance range, thereby indicating that mechanisms other than CYP450s could be contributing towards the observed resistance. *Ae. aegypti* populations have been reported to possess resistance against synthetic pyrethroids mainly by the elevated activity of CYP9 family such as CYP9J26, CYP9J28 and CYP9M6 (Ishak *et al.,* 2017). The use of TPP, however was found to decrease the resistance levels (though slightly) in most of the tested population except JPG^{ae}, where a 2.2 % increase in mortality was noted against deltamethrin when used along with TPP. The reduced susceptibilities with the use of TPP may be explained by the fact that synergists have been found to sometimes suppress the insecticide entry into the mosquito body thereby increasing the resistance level (Kasai *et al.,* 2014). Many such populations of *Ae. aegypti* possessing CYP450s mediated resistance against deltamethrin and lamdacyhalothrin have been found throughout the world (Gonzalez *et al.,* 2011; Marcombe *et al.,* 2012).

Similarly involvement of metabolic enzymes behind permethrin resistance was also indicated by the results of enzyme bioassays. Involvement of CYP450s monoxygenases in permethrin resistance was noted in APD^{ae}, JPG^{ae}, DAR^{ae} and NDP^{ae} populations since they exhibited significantly higher activity of CYP450s. Whereas significantly elevated quantitative levels of α- and β- CCEs in NDP^{ae} and moderately in APD^{ae} were revealed implying the involvement of this family of insecticide detoxifying enzymes providing resistance against permethrin in these population. The detoxification of synthetic pyrethroids is generally linked with CYP450s family yet CCEs may play a minor role in providing a partial resistance against synthetic pyrethroids (Bisset *et al.,* 2013).

The results of synergist assays revealed that only one population, *i.e.* NDP^{ae} the susceptibility to permethrin increased partially with the use of PBO and TPP (8%

and 5% respectively), whereas in rest of the population negligible or negative differences were noted. It may however be stated that in NDP^{ae} population showing the highest resistance against permethrin among other tested population, CYP450s mediated reactions may provide a part of the observed resistance. Moreover a combination of both CYP450s and PBO may be involved and responsible for some of the observed resistance in NDP^{ae} population. But the prevalence of widespread resistance against DDT as well as permethrin amongst the tested mosquito populations implies the importance of underlying kdr mutation throughout the studied region.

The high frequency of F1534C allele in all the studied population brings into focus the role played by these enzymes behind the observed synthetic pyrethroid resistance. This mutation has been shown to be strongly correlated with pyrethroid resistance in *Ae. aegypti* (Kawada *et al.,* 2009; Chen *et al.,* 2019). Although this mutated allele was noted in all the population at same frequency yet widespread resistance was not noted against all the tested synthetic pyrethroids but only to permethrin. This may be pertained to the fact that F1534C mutation plays a key role in providing resistance against type I pyrethroids only and not type II pyrethroids (Li *et al.,* 2015). Similar scenario seems to be true in our study. This mutation has been shown to confer resistance against permethrin, NRD57 and biosemithrin whereas against cypermethrin and lambdacyhalothrin, this mutation has been revealed to reduce the resistance (Li *et al.,* 2015). So it may be stated that the observed permethrin resistance may be a result of the presence of F1534C kdr mutation. Whereas that of deltamethrin and lambdacyhalothrin are mainly because of elevated activity of insecticide detoxifying enzymes and other kdr mutation but not F1534C. Involvement of F1534C based resistance mechanisms against permethrin have been

documented in Thailand (Yanola *et al.,* 2011). Kdr mutation, V1016G is generally revealed to confer resistance against many synthetic pyrethroids such as cypermethrin, lambdacyhalothrin, transfluthrin, d-allethrin, parallethrin *etc* (Li *et al.,*2015). Individuals with a V1016G mutation in their vgsc have been reported to have a lower susceptibility to permethrin than 1534C mutation (Maestre-Serrano *et al.,* 2019).This seems to holds true in some of the mosquito population in this study also. As the population with highest frequency of 1016G allele, *i.e.* NDP^{ae} (allele frequency= 70%) along with the presence of GG individuals was also found to record the highest resistance level (mortality 50%). The involvement of Kdr mutations behind IR is evident in COB^{ae} population with significantly lower enzyme activity levels but resistance against DDT and permethrin. Presence of V1016G providing resistance against permethrin have been reported in Indonesia (Brengues *et al.,* 2003) and Taiwan (Chung *et al.,* 2019).

There are also reports revealing the additive effect of two kdr mutations with V1016G increasing the resistance in individuals with F1534C (Plernsub *et al*., 2016). The same may be occurring in the tested population as both the mutated alleles have been found to be present in them. No individual with double homozygote mutation, *i.e.* GG and CC were found in this study which seems to be because of the fitness cost associated with such a haplotype as suggested in other such studies (Ishak *et al.,* 2015). This study goes well with reports suggesting the dominant role of metabolic detoxification in deltamethrin resistance and kdr mutations mainly F1534C in permethrin resistance (Ishak *et al.,* 2017). The variability in *Ae. aegypti* population against permethrin resistance may be imparted to the additive effect of both the mechanisms; insecticide detoxifying enzymes *i.e.* CYP450s and CCEs along with kdr mutation (Aponte *et al.,* 2013). Presence of both V1016G and F1534C in *Ae. aegypti*

population with resistance against permethrin or DDT have been found in different regions of world (Harris *et al.,* 2010; Aponte *et al.,* 2013; Plernsub *et al.,* 2016).

5.2.2.4 Mechanism of insecticide resistance against Carbamate:

- *Propoxur:*

Generally detoxification of CBs have been studied to be conferred by the same mechanism as that of OP resistance (Karanaratne and Hemingway, 1998). However, involvement of CYP450 monooxygenases mediated pathway have also been inferred to provide partial resistance against CB (Ishak *et al.,* 2015). Here, severe resistance was found against propoxur in NDP^{ae} and DAR^{ae}, the same population with significantly higher CCEs activity. Elevated CCEs activity have been shown to provide resistance against Carbamates in *Ae. aegypti* (Seixas *et al.,* 2017). One population, JPG^{ae} was shown to possess moderate resistance with mortality 77.2%, with significantly higher activities of CCEs than SP^{ae} but lower than APD^{ae} which was found to possess IR against propoxur. Significantly higher activity of CYP450s monooxygenase seem to be also involved in providing resistance against propoxur in this population. Thus the combination of both CCEs and CYP450s might provide resistance against propoxur in the tested population of *Ae. aegypti.*

However, in most of the population except NDP^{ae}, the use of enzyme inhibition had negative impact on the mortality percentage. Synergistic assay confirm the partial role of CCEs in conferring resistance against propoxur. NDP^{ae} population has been reported to possess resistance against both temephos and propoxur, implying the possibility that detoxification via CCEs might provide cross resistance between temephos and propoxur in this population. Since in DAR^{ae} and APD^{ae} population partial resistance mechanism against malathion was recorded, it may be due to altered

insensitive AchE, providing cross resistance between propoxur and malathion since it is the common target site for both the insecticide. Synergistic assay could not confirm the role of metabolic enzymes in JPG^{ae} population, it might be the case for JPG^{ae} that some different mechanism is operating in the population providing severe propoxur resistance which is yet to be explored.

The result of native PAGE also implies the involvement of CCEs isoforms behind propoxur resistance as maximum number of CCEs isoforms were observed in these populations. Isoforms other than α- Est-V and β- Est-III might be playing a key role in propoxur detoxification in NDP^{ae}. Since the involvement of CCEs in APD^{ae} and DAR^{ae} have been ruled out by synergist assay which exhibited high intense bands for the above mentioned two isoforms. However, the involvement of α- and β-CCEs isoforms in propoxur resistance is rare (Karunaratne and Hemingway,1998).

5.3. Insecticide susceptibility status and underlying mechanism in Aedes albopictus mosquitoes:

5.3.1 Insecticide susceptibility status of Aedes albopictus mosquitoes:

It was observed that throughout the study sites, there was an abundance of *Ae albopictus* (Table 11 and 13). This rural vector of dengue poses an immediate danger to region under study since majority of the studied district is composed of rural areas. Incidences of DENV and CHIKV infections are higher in areas where proper sanitation, waste disposal& drainage facilities are unavailable thereby increasing the disease statistics.

The demography of the region under study along with the common practices of livelihood, makes the assessment of insecticides susceptibility in this rural vector

of Dengue and Chikungunya inevitable for effective dengue prevention. So, the susceptibility of eleven populations of *Ae. albopictus* were tested against one larvicide and six adulticides same as that of *Ae aegypti*.

5.3.1.1. *Insecticide susceptibility status against organophosphates:*

- *Temephos:*

Through the study of susceptibility against temephos, one of the field populations of *Ae. albopictus*, NGK^{al} was reported to be incipiently resistant against it through the WHO bioassay protocol using 0.02ppm of insecticide with the mortality 97%. Similar trend of incipient resistance were also noted for the NVBDCP dosage of temephos in two populations with the mortality percentage of 94 and 96 for SLG^{al} and NGK^{al} respectively. Rest of the nine populations were found to possess complete susceptibility against temephos with 100% mortality.

The LC_{50} values were lower for all the tested mosquitoes, however the LC_{99} values were calculated to be above WHO recommended dosage. The calculated RR_{99} value were low for majority of the populations however for two of the population the values were ≈2, the same population reporting incipient resistance (Table 21). The RR_{99} value gives an indication on the trend of insecticide resistance development that is not yet revealed by the bioassays. The population that recorded high RR_{99} values thus indicate that soon these may develop higher level of resistance against temephos, the prime larvicide. So, the authorities engaged in vector control should make prior strategy incorporating the knowledge of the resistance in progress against this larvicide in *Ae. albopictus*.

As compared to *Ae. aegypti*, very few reports exist on the insecticide susceptibility of *Ae. albopictus*. After the recent report proving the key role of this

vector behind major Dengue and Chikungunya outbreaks, focus has been put on the control and thus insecticide resistance status of this species. Many Indian populations of *Aedes aegypti* have been reported to possess resistance against temephos from Andaman Island (Sivan *et al.,* 2015) with mortality percentages as low as 0% to 6%, Assam (RR_{99} values 2.5-5.4) (Yadav *et al.,* 2015) Whereas susceptible strain were reported from Karnataka (Shetty *et al.,* 2013), Kerala (Sharma *et al.,* 2004), Jharkhand (Singh *et al.,* 2011) and Assam (Dhiman *et al.,* 2014).

Similarly higher RR_{99} values have been reported in *Ae. albopictus* with values both greater and less than 2. Higher RR_{99} values *i.e.,* 2.5-5.4 were reported from Assam (Yadav *et al.,* 2015). The values of RR_{50}> 1 were noted in American strain of *Ae. albopictus* with the values ranging from 1.13-1.41 (Marcombe *et al.,* 2012).

Lower values of RR_{90} were 1.4-1.7 ppm, reported from military station of Assam (Dhiman *et al.,* 2014), Malaysia with RR_{95} values 1.15- 1.17 ppm (Mohiddin *et al.,* 2016) and 0.75-1.45 ppm (Chen *et al.,* 2013). From Orissa *Ae. albopictus* population with RR_{50} values as great as 15.3 ppm have also been reported (Rath *et al.,* 2018).

- *Malathion:*

All the tested mosquito populations resulted in susceptibility against malathion with the mortality rate ranging between 99-100%.Similar scenario was observed for *Ae. aegypti,* where three of the tested population were susceptible to malathion. This results go well with the other populations of *Ae. albopictus* tested against malathion throughout India.

In the neighbouring state of Assam also, field caught population of *Ae. albopictus* were found to be susceptible to 5% malathion (Yadav *et al.,* 2015; Dhiman *et al.,* 2016). Also from other parts of the country such as South Andaman district (Sivan *et al.,* 2015) and southern part of India (Sharma *et al.,* 2004), the wild population of *Ae. albopictus* were reported possess susceptibility to this adulticide. Many strains of this species have shown similar levels of susceptibility against malathion throughout the world (Duong *et al.,* 2016, Liu *et al.,* 2004; Srisawat *et al.,* 2011). The results suggest that malathion may be chosen during severe Dengue and Chikungunya outbreaks for *Ae. Albopictus* control in all the tested districts to minimize the transmission at the peak disease season.

5.3.1.2. Insecticide susceptibility status against Organochlorine

- *DDT:*

Amongst the adulticides tested, widespread resistance was noted against DDT (Table 22). Only one population was completely susceptible against DDT, *i.e.*, KHR^{al}, whereas six of the population possessed incipient resistance (mortality percentage 88.9-96.0) and three resistant to it. SLG^{al} with 40% mortality was severely resistant whereas other two JPG^{al} and NGK^{al} were moderately resistant to DDT with mortality percentage 75.4 and 63.1 respectively. Both JPG^{al} and NGK^{al} sites belong to same district and similar resistance pattern observed for both the sites imparts light on the similar mosquito control practice throughout the district. The high resistance observed in DAR seems to be a combined result of both the direct exposure to DDT as it was the chief control agent in the past and an indirect exposure through other vector control or pest control approaches of either agricultural or public health sector (Anon., 2002b). Altered susceptibilities against DDT in majority of the tested mosquitoes and

also in *Aedes aegypti* confirm the level of DDT selection pressure prevailing in the studied region.

The complete susceptibility of KHR^{al} to DDT seems to be because of the absence of any government or non-government mosquito control organizations throughout the site and the conventional practices of the rural people for mosquito control rather than mass vector control using insecticides.

Most of the *Ae. albopictus* tested for DDT susceptibility have reported widespread resistance against it. In India, DDT resistance with mortality percentages as low as 35.76 has been reported from Jharkhand (Singh *et al.,* 2011), 78.09% in Andaman (Sivan *et al.,* 2015), 40.2% Northern Assam (Das and Dutta), 45.2% in Assam (Yadav *et al.,* 2015) Orissa (Rath *et al.,* 2018). Similarly, *Ae. albopictus* population from different countries have also exhibited resistance against this organochlorine such as in Pakistan (Arslan *et al.,* 2016), USA (Marcombe *et al.,* 2014), Central African Republic (Ngoagauni *et al.,* 2016), Malaysia (Ishak *et al.,* 2015), China (Li *et al.,* 2018)

The KDT_{50} and KDT_{95} values for DDT were found to be very high for most of the population (Figure 23). KDT_{50} values below 60 minutes was noted for a single population, KHR^{al} and for the rest of the field populations, range of KDT_{50} was 70.01- 230.21 mins. For KDT_{90} values, none of the population reported value less than 60 minutes. Even the most susceptible of all and the lab reared control reported a value of 42.64 and 169.13 mins respectively. Such a high value even for these two resistant population indicates the onset of inefficacy of DDT in the mosquito populations which as a result of the persisting condition and intense selection pressure may lean towards resistance in near future. Similar scenario of elevated KDT_{50} and

KDT_{95} values against 4% DDT have been reported throughout the world (Kamgang *et al.,* 2011; Marcombe *et al.,* 2014; Li *et al.,* 2018).

5.3.1.3 Insecticide susceptibility status against synthetic Pyrethroids:

- *Deltamethrin, Lambdacyhalothrin and Permethrin:*

Of the three synthetic pyrethroid insecticides, one showed variable pattern of resistance whereas the other two showed complete susceptibility in all the tested population. For permethrin, two resistant, four incipiently resistant and five susceptible population were recorded (Table 22). This variability in resistance pattern may be pertained to the variability in usage of personal mosquito protection tool, some of which contain formulations and cocktails of permethrin with other insecticide (Ponlawat *et al.,* 2005). Generally these tools are used inside human houses and in the vicinity of human dwellings but since *Ae. albopictus* is basically a forest dwelling mosquito, the exposure to such human tools might have occurred in only some of the population thereby causing variable pattern of susceptibility or resistance against permethrin throughout the eleven study sites ranging from susceptible to resistant.

Both permethrin resistant and susceptible population of *Ae. albopictus* have been reported throughout India. Many resistant or incipiently resistant field population of *Ae. albopictus* such as from Andaman with 73.3% mortality (Sivan *et al.,* 2013), Kerala and Delhi (Kushwah *et al.,* 2015) have been reported. Moreover permethrin resistance in *Ae. albopictus* is common throughout the world *i.e.* Srilanka (Karunaratne *et al.,* 2013; Pakistan (Mohsin *et al.,* 2016); China (Yiguan *et al.,* 2016), Malaysia (Ishak *et al.,* 2015; Rahim *et al.,* 2017).

Two of the tested population reported very high values of KDT_{50} and KDT_{90} which were also found to possess resistance against permethrin. However, some of the population that showed lower resistance levels reported comparatively higher values of KDT_{50} and KDT_{95}. Populations HAS^{al} and COB^{al} with mortality percentage 96.2 and 96.4 exhibited KDT_{50} value of >60 min *i.e.* 83.03 and 78.96 min respectively. Similarly NBU^{al} and COB^{al} with mortality percentage100 and 96.4 showed the KDT_{95} value to be 192.28 and 177.57 mins. Such high values of KDT_{50} and KDT_{95} associated with a low level of resistant or total susceptibility provides light on the inefficacy and delay in the manifestation of insecticide action and thus the scope of severe resistance development in the near future. Similar instance of high KDT_{95} values, *i.e.* 23-154 mins in Italian *Ae. albopictus* population (Pichler *et al.*, 2018) and higher values of KDT_{95} were also obtained in a Califiornian population of *Ae. albopictus* (Cornel *et al.*, 2016).

Complete susceptibility against deltamethrin and lambdacyhalothrin with mortality percentage 100 was recorded in the *Ae. albopictus* from northern districts of West Bengal. The populations were also found to possess KDT_{50} values below 60 min and KDT_{95} value around 60 mins. Similar susceptibility profile against deltamethrin has been noted in *Ae. albopictus* population from Assam (Dhiman *et al.*, 2014; Yadav *et al.*, 2015; Das and Dutta, 2014). Insecticides permethrin, deltamethrin and lambdacyhalothrin belong to the same insecticide group yet variability was noted in their susceptibility pattern. This may partly be explained by the fact that permethrin has been in use in mosquito control earlier than the other two which were brought into mosquito control programmes later.

As compared to *Ae. aegypti* resistance profile, against the synthetic pyrethroids, comparatively lower resistance was found in the sibling species *Ae.*

albopictus This may be because of the behaviour of these mosquitoes, *Ae. aegypti* being anthropophilic thus getting regular exposure to the personal protection measures against mosquito containing derivatives of synthetic pyrethroid while the zoophilic *Ae. albopictus* only gets occasional exposure to the pyrethroids targeted against mosquitoes. Populations of mosquito with higher resistance against permethrin but lower or none against other synthetic pyrethroids have also been reported in other studies (Sivan *et al.,* 2015; Ishak *et al.,* 2015; Arslan *et al.,* 2016). However, in other *Ae. albopictus* populations exhibiting resistance / incipient resistance against deltamethrin and/or lambdacyhalothrin have also been noted to occur (Kamgang *et al.,* 2011; Ishak *et al.,* 2015; Kushwah *et al.,* 2015; Ngoagauni *et al.,* 2016; Hasan *et al.,* 2016; Rahim *et al.,* 2017).

5.3.1.4 Insecticide susceptibility status against Carbamate:

- *Propoxur:*

Against the sole carbamate insecticide tested a pattern from susceptible to incipient resistant was noted. One severely resistant population, five incipient resistant and rest fully susceptible population was recorded. Such a pattern may be result of either direct exposure to propoxur through the household insect repellent tools containing this carbamate or through indirect exposure through agricultural sector. A severely propoxur resistant population, JPG^{al} with mortality 42.5% was reported for the first time from this study area. Reports on the prevalence of propoxur resistant *Ae. albopictus* population are few, some being noted in Central African republic, mortality 94% (Ngoagauni *et al*., 2016) and in China (Yiguan *et al.,* 2016; Li *et al.,* 2018). However, other studies conducted throughout India have reported, propoxur susceptible population of *Ae. albopictus* (Sharma *et al.,* 2014; Sivan *et al.,* 2015).

5.3.1.5 Overall view on resistance in Aedes albopictus:

Arranging the insecticides in the descending order of their toxicity in *Ae. albopictus* throughout the region will be in following order:

Deltamethrin and lambdacyhalothrin>malathion> temephos>propoxur>permethrin>DDT

Amongst the tested mosquito population, KHR^{al} was the only population that possessed susceptibility to all the tested insecticides followed by KMG^{al} (incipient resistance against DDT only) and then NBU^{al} (incipient resistance against DDT and propoxur). Rest of the population possessed variable pattern of resistance against different groups of insecticide.

5.3.2. Mechanisms of resistance:

5.3.2.1 Mechanism of insecticide resistance against Organophosphate:

- *Temephos:*

From the results of larval bioassay against temephos, most of the tested *Ae. albopictus* population was found to be resistant to it except NGK^{al} (which possessed incipient resistance against both WHO and NVBDCP doses) and SLG^{al} that possessed incipient resistance against NVBDCP dose. Similar results were also noted from the results of RR_{99} values, where these two population were found to possess significantly higher values ≈ 2 than other tested population. An RR_{95} value of 2 and above suggests the onset of resistance against insecticides in the mosquito population exhibiting it. Moreover a high variability was noted in the LC_{50} and LC_{99} values among different field populations of *Ae. albopictus.* This basically differs either due to the inherent variations occurring in geographically isolated populations or due to exposure to similar insecticide or allelochemicals (Wesson, 1990)

The site from where SLG^{al} population was collected is a site undergoing rapid urbanization and temephos is being used by different pest control agencies throughout the locality which may explain the altered susceptibility in this population (personal communication) whereas NGK^{al} site is located in a rural locality and no history of temephos usage yet the presence of incipient resistance against it seems to be the result of cross exposure to the other insecticides belonging to same group which is being used extensively in agricultural field such as tea, vegetable crops throughout the region (Gurusubramanian *et al.,* 2007). Similar instances of insecticide sprayed in agricultural sector *i.e.* cotton, vegetable have been found to contaminate mosquito breeding habitat (Kamgang *et al.,* 2011; Arslan *et al.,* 2016).

As already discussed, generally CCEs based mechanisms are involved in resistance against OPs in mosquitoes. Moreover, NGK^{al} and SLG^{al} population were found to exhibit significantly higher activity than SP for both α- and β-CCEs *i.e.* approximately 9.7, 5.1 and 2.3, 3.7 times respectively. The results of native PAGE also supplemented the above findings since more than one (two isozymes of α- CCEs in NGK^{al} and two isozymes of β-CCEs in SLG^{al}) and moderately intense bands of α- and β-CCEs were noted in these two populations implying that CCEs based mechanisms might be driving the detoxification of temephos in these populations (Lima-Catelani *et al.,* 2004). Significantly higher activities of CYP450s were also noted in ISL^{al} population indicating that a minor role might be played by this enzyme in conferring temephos resistance. CYP450s have been reported to provide resistance against temephos in *Aedes* mosquitoes (Grisales *et al.,* 2013).

However, the use of synergists provided a clearer insight into the scenario. Use of TPP along with temephos helped to restore susceptibility to temephos in NGK^{al} population at WHO dosage conferring the role of CCEs behind the observed

resistance. Whereas in SLG^{al} population complete susceptibility could not be achieved with the use of TPP, though partial restoration in insecticide susceptibility in the mosquito population was noted. This brings into focus that apart from CCEs mediated mechanism, other detoxifying enzymes might also be involved in conferring a proportion of the observed resistance in SLG^{al} population. Detoxification mediated by GSTs may also provide resistance against temephos in SLG^{al} population as significantly higher activities of GSTs were noted in this population. Genes of GSTs mainly epsilon GSTs have been found to be over-expressed in temephos resistant colonies of *Aedes* mosquitoes (Saavedra-Rodriguez *et al.,* 2014). However, the role of insensitive AchE cannot be ruled out which also forms an important mechanism of resistance against temephos. Moreover tools addressing all these mosquitoes might help in the identification of exact mechanism of temephos resistance in SLG^{al}population. As in this study, in NGK^{al} population, similar involvement of CCEs behind temephos resistance in *Ae. albopictus* have been reported in (Ngoagauni *et al.,* 2016; Grigoraki *et al.,* 2016). Insensitive AchE providing such resistance has not yet been noted in *Ae. albopictus* mosquitoes throughout the world.

- *Malathion:*

As complete susceptibility was noted against malathion, it seems that malathion specific esterases which are generally noted behind resistance against malathion are very low or not present throughout the tested mosquito samples. Though malathion has been used for a large period of time in India for mosquito control yet 0% resistance against it seems to be because in India most of the mosquito control efforts target mosquito breeding habitats of other mosquito species such as drains but not that of *Ae. albopictus*. Moreover, since *Ae. albopictus* is a zoophilic mosquito and has recently as a result of deforestation migrated to human dwellings,

the earlier interventions seem to have not provided malathion exposure to these mosquitoes. These mosquitoes also prefer to colonize breeding habitats outside human houses thereby again not getting exposure to household mosquito control tools in the past when it contained formulations of malathion (Anon., 2002b).

5.3.2.2 Mechanism of insecticide resistance against Organochlorine

- *DDT:*

Resistance against DDT is generally dependent on GSTs mediated mechanism and in few cases other detoxifying enzyme groups *i.e.* CCEs or CYP450s or kdr mutations providing cross resistance between DDT and synthetic pyrethroids (Vontas *et al.,* 2012). Throughout the mosquito population similar activities of GSTs were noted. However, the values were higher in case of SLG^{al} and NGK^{al} population, which were also found to possess severe resistance against DDT with mortality 40% and 63.1 % respectively. So, GSTs might be involved in these mosquito populations providing resistance against DDTs. Similar involvement of GSTs in DDT resistance have been reported in *Ae. albopictus* from India (Das and Dutta, 2014), U.S.A. (Marcombe *et al.*, 2014), China (Li *et al.,* 2018) *etc.*

These two populations were also recorded with higher activities of α- and β-CCEs which have also been implicated in conferring resistance against DDT in some mosquito colonies (Aponte *et al.,* 2013). Similarly, JPG^{al} and NGK^{al} were noted to express significantly higher activity of CYP450s which may also play a minor role behind DDT resistance (Ishak *et al.,* 2015).

The use of synergists also supplemented the above findings as use of CYP450s inhibitor was found to restore the susceptibilities against DDT moderately in SLG^{al},

JPG^{al}, NGK^{al}and to a lower extent in APD^{al}, HAS^{al}, KMG^{al}, ISL^{al} and COB^{al}; thereby affirming the role of CYP450S in a part of the observed resistance against DDT. Similarly use of CCEs inhibitor was found to moderately restore the susceptibility in SLG^{al} population and faintly in HAS^{al}, JPG^{al} and NGK^{al}. Thus similar partial involvement of CCEs can be confirmed in these mosquito population conferring DDT resistance. Or it may be stated that a combination of all the major insecticide detoxifying enzymes may be driving the detoxification of DDT in these *Ae. albopictus* population.

However, in no case complete susceptibility could be restored in any of the mosquito population, thereby putting into focus the role of target site insensitivity through kdr mutations in these mosquito population. In this study, presence of a major kdr mutation, *i.e.* F1534C was revealed in most of the mosquito population except SLG. From the results of kdr mutations genotyping it seems that in JPG^{al} and NGK^{al} population, these mutations might be playing a key role behind the altered susceptibilities of these population against DDT. So in JPG population, the combined predominant effect of GSTs, F1534C mutated allele along with minor role of CCE might be governing the observed resistance pattern. Similarly in ISL population, predominant role of all the detoxifying enzymes along with the kdr mutations might have resulted in severe resistance against DDT. Whereas in SLG population, only the role of CYP450s, CCEs and GSTs seem to be the driving factor of DDT resistance. Synergist assays with GST inhibitor and assessment of other kdr mutations would have helped in locating the exact mechanism of DDT resistance in SLG^{al} and other *Ae. albopictus* populations.

5.3.2.3 Mechanism of insecticide resistance against synthetic Pyrethroids:

- *Deltamethrin, Lambdacyhalothrin and Permethrin:*

Against permethrin also widespread resistance was observed with the most severe resistance observed in JPG^{al} and APD^{al}population. Both the population were recorded to express significantly higher activities of CYP450 monoxygenases than that of the SP^{al} and some other populations thereby indicating the involvement of this enzyme family behind the observed resistance against permethrin. In *Ae. albopictus* resistance to permethrin have been reported to be drawn through with elevated activities of CYP450s, particularly through the up-regulation of CYP6 and CYP9 subfamily (Ishak *et al.,* 2017). Similar detoxification mechanism may also be operating in these two field populations, however to confirm the involvement of specific subfamily, studies incorporating sophisticated molecular tools should be performed. CCEs may also detoxify synthetic pyrethroids in mosquitoes (Sahgal *et al.,* 1994; Aponte *et al.,* 2013; Chareonviriyaphap *et al.,* 2013). Similar associations have also been noted in JPG population where significantly higher activity of α-CCEs were noted and in APD population where significantly higher levels of β- CCEs activity were recorded implying the involvement CCEs based mechanisms behind the observed resistance.

The use of PBO before permethrin was found to increase the susceptibility percentages moderately in both APD^{al} and JPG^{al} population confirming the role of CYP450s in permethrin resistance. Lower levels of susceptibility restoration was also noted in NBU^{al}, HAS^{al}, NGK^{al}, NMZ^{al}, ISL^{al} and COB^{al} with complete susceptibilities in some of them, thus implying the similar inferences in this population behind the incipient resistance against permethrin. The partial association of CCEs was also confirmed in APD^{al}population through the result of synergistic assays. In NBU^{al}

population complete susceptibilities was restored with the use of both the enzyme inhibitors indicating the role of both the detoxifying enzymes in the resistance pattern.

The mutated allele 1534C have been linked with permethrin resistance in *Ae. aegypti*. Similar seems to hold true for *Ae. albopictus* too as evident in this study. Since 100% mortality against deltamethrin and lambdacyhalothrin amongst the tested mosquito population were recorded although the mutated allele was found to be present in most of the populations. Against permethrin, this allele seems to provide protection against it predominantly in JPG^{al} with C allele frequency of 54.1 APD^{al}, 41.6% and minorly in NGK^{al} and NMZ^{al} with very high allele frequencies.

Similar associations between pyrethroid resistance and F1534C mutation have been studied in *Ae. albopictus* (Li *et al.,* 2018; Auteri *et al.,* 2018). The low frequencies of these allele in HAS^{al}, NBU^{al}, ISL^{al} also correlate with the low level of resistance against permethrin. Moreover SLG^{al} population with complete susceptibility against all the tested synthetic pyrethroids insecticides, no mutant allele was recorded. Comparing the resistance against permethrin with DDT, the involvement of kdr mutation seem to govern DDT resistance in all the *Ae. albopictus* population except SLG^{al}. Interestingly, cross resistance between these two insecticides seem to be operating in JPG^{al} population with similar mortality % against both the insecticide.

5.3.2.4 Mechanism of insecticide resistance against Carbamate

- *Propoxur:*

Severe resistance in JPG^{al} population against carbamate insecticide in this study might be partially driven by the over-expression of CCEs as evident from the elevated levels of α- CCE activities. Similar involvement of CCEs also seem to hold

true in NGK^{al} and APD^{al} population reporting significantly higher CCEs activity, both α- CCEs, β- CCEs and only β- CCEs respectively along with incipient resistance against propoxur. However role of CYP450s have also been noted to provide resistance against carbamates in larvae and adult of *Ae. albopictus* (Ishak *et al.,* 2015). Highest activities of CYP450s was recorded in the severely propoxur resistant mosquito population JPG indicating similar involvement of this detoxifying enzyme in detoxification of propoxur.

However, the results of synergist assay confirmed a partial role of CCEs behind propoxur resistance in JPG^{al}population restoring insecticide susceptibility to ≈8% only and even lower in case of SLG^{al}, NBU^{al}, NMZ^{al} and ISL^{al}. From the results of native page no clear association could be drawn between any CCE isoform and propoxur resistance. The incomplete restoration of susceptibility by the two enzyme blockers suggest the possible prevalence of insensitive AchE operating in the tested population behind propoxur resistance. However very few studies have reported the involvement of AchE alteration behind propoxur resistance (Ngoagauni *et al.,* 2016). Since few studies have been conducted to identify the specific and exact mechanism of resistance against propoxur in *Aedes* mosquitoes some different mechanisms other than that for other insecticides could also be involved. Studies concerned with mechanisms identification might help in filling the gaps for propoxur or other carbamate resistance in mosquitoes.

RESEARCH HIGHLIGHTS

6. RESEARCH HIGHLIGHTS:

- Throughout the study region, a dominance of *Ae. albopictus* over *Ae. aegypti* was noticed.
- In this study, it was found that for both the *Aedes* species, discarded tyres were the most preferred breeding habitat resulting highest positivity indices followed by uncovered cemented tanks.
- For *Ae. albopictus*,the natural habitats were also preferred such as bamboo stumps and plant axils.
- Higher larval densities were recorded for *Ae. aegypti* in Islampur, New Mal and Siliguri. Similarly, for *Ae.albopictus* very high larval densities were observed for Nagrakata, North Bengal University, Hasimara and Alipurduar.
- To minimise the disease risk in areas where high larval densities were noted, proper control measures should be planned before the disease outbreaks season by mosquito breeding habitat destruction.
- Majority of the studied *Ae. aegypti* populations possessed low resistance levels against temephos but higher resistance ratios. One population NDP^{ae} was found to possess incipient resistance against 0.02ppm and resistance against 0.0125ppm temephos.
- Widespread resistance against DDT was revealed in all the tested populations of *Ae. aegypti* with the mortality 47.9% for DAR^{ae} population, 55.4% for APD^{ae}, 56.6% for NDP^{ae}, 70.0 % for COB^{ae} and 72.0% for JPG^{ae}.
- The population APD^{ae} reported moderate resistance against malathion with 72.5% mortality followed by incipiently resistant DAR^{ae} with mortality 92.6%.

- Most of the studied population were revealed to be susceptible or incipiently resistant to lambdacyhalothrin and deltamethrin with the mortality ranging from 80.9-100% and 89.2-100% respectively.
- Wide spectrum of resistance was noted against permethrin, with mortality as low as 50% for NDP^{ae} to 83.3% for APD^{ae} population.
- Three of the tested *Ae. aegypti* populations were found to be severely (DAR^{ae}: 50.0% mortality)to moderately resistant (NDP^{ae}: 75.4%mortality) against propoxur.
- Against DDT most of the populations showed high values of both KDT_{50} and KDT_{95}were recorded in majority of the tested populations of *Ae. aegypti* against DDT indicating its inefficacy in mosquito control.
- Amongst the pyrethroid insecticides, high KDT values were recorded against permethrin, with the highest KDT_{95} value for NDP^{ae} population, *i.e*. 192.11 mins.
- Prior exposure to 4% PBO before DDT was found to increase susceptibility to it in APD^{ae} population, restoring the mortality rate 24.6%.Thus a part of the observed resistance might be conferred by detoxification through Cytochome P450s.
- Against malathion, Carboxylesterases were revealed to drive the resistance (though partially) in APD^{ae} population elevating the mortality from 72.5% to 94.0% when exposed to 10%TPP,.
- Against deltamethrin and lambdacyhalothrin, Cytochrome P450s were recorded to be responsible for partial resistance in APD^{ae}, JPG^{ae} and NDP^{ae}.
- Carboxylesterase linked pathways were revealed to be involved in propoxur resistance in NDP^{ae} , as use of 10% TPP could restore its mortality from 45.4 to 70.4% .

- The activity of α-CCEs and β-CCEs were noted to range 1.07 fold to 3.11and 1.19 to 2.46 folds respectively among the field populations than the control population, *i.e.* SP^{ae}. The activity of CYP450 monoxygenases were noted to be 1.14 to 1.53 fold than SP^{ae}. The activity of GSTs were uniform amongst the field caught populations of *Ae. aegypti* ranging from 1.06 to 1.39 times higher than that of SP^{ae}.
- Through kdr genotyping, both susceptible and mutant kdr allele were revealed to be present amongst the wild populations of *Ae. aegypti*. The frequency of the 1534C mutantallele was 50, whereas the frequency of the 1016G mutant allele was 45%.
- Throughout the tested field populations of *Ae. aegypti* around five different isozymes of α-Carboxylesterase (Rf values 0.62, 0.68, 0.73, 0.82, 0.97) and three isozymes of β-Carboxylesterase (Rf values 0.62, 0.80 and 0.96) were found.
- The highest number of isozymes of both the Carboxylesterases were recorded in NDP^{ae} population, whereas the rest possessed a single isozyme (with varying intensities) in both the electrophoregrams.
- Amongst the tested field caught populations of *Ae. albopictus*, only one of the eleven tested population (NGK^{al}) exhibited incipient resistance against temephos at 0.02 ppm dosage. For the India Government recommended dosage of 0.0125 ppm, two populations possessed incipient resistance, NGK^{al} and SLG^{al} .
- Amongst the field populations of *Ae. albopictus*, the LC_{50} values ranged from 0.0001 to 0.0047 ppm. Similarly, the LC_{99} values were found to be in the range of 0.038 to 0.081 ppm.
- Severe to moderate resistance against DDT was revealed in the tested *Ae. albopictus* mosquitoes, namely SLG^{al}, JPG^{al} and NGK^{al}.

- However, complete susceptibility was recorded among the wild *Ae. albopictus* mosquito populations against malathion, deltamethrin and lambdacyhalothrin.
- Moderate level of resistance against permethrin was found in two of the *Ae. albopictus* population with mortality percentages 75.4 (APD^{al}) and 75.0 (JPG^{al}).
- Severely resistant population of Indian *Ae. albopictus* against propoxur was revealed in this study for the first time with very low mortality, 42.5%.
- As in *Ae. aegypti*, similar high values of KDT_{50} and KDT_{95}were noted against DDT, whereas low knockdown times were noted amongst the same mosquitoes population against deltamethrin and lambdacyhalothrin.
- Populations NGK^{al}, JPG^{al} and SLG^{al} were reported to possess Cytochrome P450 linked resistance against DDT, since prior exposure to PBO restored the mortality/susceptibility in these populations 15.1%, 19.7% and 41.25 % respectively. Similarly, in APD^{al} and JPG^{al}, CytochromeP450s were revealed to confer resistance against permethrin.
- Significantly higher activity of α-CCEs were noted in NGK^{al}, SLG^{al}, $JPG^{al}$and NMZ^{al} population. Similarly, for β-CCEs higher activities were noted for NGK^{al}, SLG^{al} and APD^{al} population, *i.e.* 3.16, 2.83 and 2.74 folds than SP^{al} respectively. The activity of CYP450s monoxygenases were recorded to range from 1.03 to 1.94 times SP^{al}. The activity of GSTs were found to be ranging from 0.305 to 0.385 µM mg $protein^{-1}$ min^{-1} .
- The results of kdr genotyping revealed that, all but one (SLG^{al}) tested Ae. albopictus population were found positive for 1534C mutant allele reporting the frequency of this allele to be 29.8%.
- Two different isozymes for both α- carboxylesterases (Rf values 0.81, 0.91) and β-carboxylesterases (Rf values 0.63 and 0.95) were found amongst the different field

caught mosquito populations. Isozymes α-Est II and β-Est II were more prevalent than the other isozyme. In case of α- carboxylesterases, NGK^{al} and JPG^{al} exhibited the presence of both the isozymes, whereas in were expressed in β- carboxylesterases SLG^{al} possessed both the isozymes.

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7. REFERENCES:

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8. PUBLICATIONS OUT OF RESEARCH WORK

Original Research Articles:

1. **Bharati, M.,** Rai, P. and Saha, D., 2019. Insecticide resistance in *Aedes albopictus* Skuse from sub-Himalayan districts of West Bengal, India. *Acta tropica*. *192*: 104-111.
2. **Bharati, M.** and Saha, D., 2018. Multiple insecticide resistance mechanisms in primary dengue vector, *Aedes aegypti* (Linn.) From dengue endemic districts of sub-Himalayan West Bengal, India. *Plos One*. 13(9): e0203207.
3. **Bharati, M.** and Saha, D., 2018. Assessment of insecticide resistance in primary dengue vector, *Aedes aegypti* (Linn.) from Northern Districts of West Bengal, India. *Acta tropica*. 187: 78-86.
4. **Bharati, M.,** Saha, P. and Saha, D., 2018. Variation in esterase activity among different *Aedes aegypti* L. populations from the Dooars and Terai Regions of West Bengal, India. In *Proceedings of the Zoological Society*. 71(3): 239-247.
5. **Bharati, M.** and Saha, D., 2017. Insecticide susceptibility status and major detoxifying enzymes' activity in *Aedes albopictus* (Skuse), vector of dengue and chikungunya in Northern part of West Bengal, India. *Acta tropica*. 170: 112-119.

Book Chapters:

1. **Bharati, M.** and Saha, D., 2017. Mosquito Borne Diseases: Current Status and Control Approach in India. In: Vector-Borne Diseases & Treatment. Openaccess ebooks. ISBN: 978-93-87500-10-5. Las Vegas, USA.

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9. *LIST OF PAPER PRESENTATIONS AT SEMINARS/ CONFERENCES*

- Presented a research paper entitled "*Insecticide Susceptibility Status and Underlying Biochemical Mechanisms Among Different Aedes Albopictus S. Populations from Three Sub-Himalayan Districts of West Bengal, India*" in International Zoology Seminar On "Concepts on Conservation and Propagation of Indigenous Life forms in Eastern Himalayan Region" held at Darjeeling govt. college, Darjeeling on 12th May, 2017.

- Presented a research paper entitled "*Spatiotemporal Variation In Abundance and Habitat Preference of Aedes Mosquitoes in Northern Part of West Bengal, India*" in "2nd Regional Science Congress 2017" held at Siliguri college, Siliguri on 8th December, 2017.

- Presented a research paper entitled "*Multiple Insecticide Resistance in Primary Dengue Vector, Aedes aegypti (Linn.) From Dengue Endemic Districts of sub-Himalayan West Bengal*" in "National Conference on Recent Trends in Zoological Research in North East India" held at North Eastern Hill University, Shillong on 20th April, 2018.

- Presented a research paper entitled "*Insecticide Resistance Profiling in Wild Populations of Aedes albopictus Skuse from Sub-Himalayan West Bengal, India*" in "India International Science Festival 2018" held at Indira Gandhi Pratisthan, Lucknow on 06th October 2018.

- Presented a research paper entitled "*Spatial Variation and Insecticide Susceptibility Status of Aedes albopictus against Three Major Groups of Insecticide in Sub-Himalayan West Bengal, India*" in "Golden Jubilee International Conference on Trends in Zoology" at Burdwan University, Burdwan on 3rd January, 2019.

- Presented a research paper entitled "*Assessment of Insecticide Resistance in Wild Populations of Aedes albopictus Skuse from Sub-Himalayan West Bengal, India*" in "West Bengal 26th State Science Congress" held at Science city, Kolkata on 28th Feb, 2019.

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Insecticide susceptibility status and major detoxifying enzymes' activity in *Aedes albopictus* (Skuse), vector of dengue and chikungunya in Northern part of West Bengal, India

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ABSTRACT

Mosquitoes belonging to *Aedes* genus, *Aedes aegypti* and *Aedes albopictus* transmit many globally important arboviruses including Dengue (DENV) and Chikungunya (CHIKV). Vector control with the use of insecticide remains the suitable method of choice to stop the transmission of these diseases. However, vector control throughout the world is failing to achieve its target results because of the worldwide development of insecticide resistance in mosquitoes. To assess the insecticide susceptibility status of *Aedes albopictus* from northern part of West Bengal, the susceptibility of eight different *Aedes albopictus* populations were tested against a commonly used larvicide (temephos) and some adulticides (malathion, deltamethrin and lambda cyhalothrin) along with the major insecticide detoxifying enzymes' activity in them. Through this study, it was revealed that most of the populations were found susceptible to temephos except Nagrakata (NGK) and Siliguri (SLG), which showed both a higher resistance ratio (RR_{99}) and a lower susceptibility, thereby reflecting the development of resistance against temephos in them. However, all tested adulticides caused 100% mortality in all the population implying their potency in control of this mosquito in this region of India. Through the study of carboxylesterase activity, it was revealed that the NGK population showed a 9.6 fold higher level of activity than susceptible population. The same population also showed a lower level of susceptibility and a higher resistance ratio (RR_{99}), indicating a clear correlation between susceptibility to temephos and carboxylesterase enzymes' activity in this population. This preliminary data reflects that the NGK population is showing a trend towards resistance development and with time, there is possibility that this resistance phenomenon will spread to other populations. With the recurrence of dengue and chikungunya, this data on insecticide susceptibility status of *Aedes albopictus* could help the authorities engaged in vector control programmes to formulate effective measures against this mosquito in this region.

1. Introduction

Dengue, dengue haemorrhagic fever and chikungunya caused by *Aedes aegypti* and *A. albopictus* have become a seasonal phenomenon in India and its recurrence has become very much regular than it was ever. India experienced its first virologically proved outbreak of dengue in 1963 (Gupta et al., 2012). Tropical and subtropical countries like India, because of its high temperature and relative humidity, lack of proper drainage system and water stagnation and large vegetation cover provides a congenial microclimate for mosquito breeding (Hamdan et al., 2005). Annually, about 50 million people get infected by dengue with 0.5 million cases of dengue haemorrhagic fever (Guzman et al., 2010). In 2016, 1,11,880 cases of dengue were reported from India causing 227 deaths {National Vector Borne Disease Control Programme (NVBDCP), India, 2016}. 17,702 cases of dengue infection were reported from the state of West Bengal causing 34 deaths, which was the highest among any other Indian state (NVBDCP India, 2016). This state has always remained a hotspot for mosquito borne diseases.

A. albopictus, was first reported from Kolkata by Skuse (1894) as "the banded mosquito of Bengal" (Huang, 1968; Knight and Stone, 1977). It harbours a number of medically important arboviruses. DEN-4 (Dengue serotype 4) virus has been isolated from this mosquito in West Bengal, proving its role as a vector in this region (Gratz, 2004). Now, it is playing the role of secondary vector of

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dengue causing infections in rural and suburban areas. It is expanding rapidly, pertaining to its nature of high survivability in a broad range of physical environments (Hawley, 1988).

In the absence of vaccines and medications for dengue and chikungunya, the only approach for prevention of these diseases is to stop the transmission of these diseases by way of vector control (Kongmee et al., 2004; Bharati et al., 2016). Vector control is mainly done by destroying the mosquito breeding sites and using insecticides against adults and larvae of mosquito. In India, the recommended insecticides for mosquito management are DDT (Dichlorodiphenyltrichloroethane), malathion, deltamethrin, lambda cyhalothrin, cyfluthrin, alpha-cypermethrin, bifenthrin and bendiocarb for Indoor Residual Spray (IRS), whereas temephos, mosquito larvicidal oil (MLO) are used as larvicides (NVBDCP India, 2016). Following its discovery by Paul Muller in 1939, DDT has been used both in public health as well as agricultural sector. The commonly used household protections against mosquito (insecticide based repellent creams and formulations) mainly contain synthetic pyrethroids (Chareonviriyahpap et al., 1999).

Recurrent use of insecticide causes development of insecticide resistance in the target organism. Insect contains a plethora of enzymes involved in development of insecticide resistance. Metabolic resistance develops by way of higher level of activity of α- and β-carboxylesterases (CCEs) and acetylcholinesterases (AchEs), Cytochrome P450 (CYP_{450}) and Glutathione *S*-transferases (GSTs). These enzymes show higher level of activity during recurrent exposure to insecticide (continuous selection pressure) through the altered expression of related genes. These genes spread very rapidly in the population through successive generations and because of the advantage this resistance gene provides, these genes establish dominance within the population (Karunaratne et al., 2013). This scenario indicates failure of vector control measures in achieving its target.

Carboxylesterases have been implicated in detoxifying Organophosphate (OP), carbamate and to a lesser extent synthetic pyrethroids and hence providing metabolic resistance against these insecticides (Hemingway et al., 2004). Additionally, CYP_{450} mediated monoxygenases are involved in conferring pyrethroid resistance (David et al., 2013; Scott et al., 1998) and GSTs are involved in the development of DDT resistance (Hemingway et al., 2004).

This report presents the baseline susceptibility status of *A. albopictus* to some of the commonly used and government recommended insecticides for vector control in this dengue hotspot of India along with the activity of detoxifying enzymes generally involved in resistance mechanisms. Understanding the mechanism involved in insecticide resistance is very vital for prevention of onset of resistance phenomenon (Karunaratne et al., 2013). In addition, the lethal concentration causing 50% and 90% mortality, i.e. LC_{50} and LC_{90} of a larvicide (temephos) and some insecticides (malathion, deltamethrin and lambda cyhalothrin) have been determined. These data would be helpful in the planning of effective strategies for control of this mosquito vector in this region.

2. Materials and methods

2.1. Selection of sampling site

Nine different sampling sites were selected based on the dengue prevalence within three districts of northern part of West Bengal, namely, Alipurduar, Jalpaiguri and Darjeeling. The sampling sites were Alipurduar Junction area (APD), Old Hasimara area (HAS), Kumargram block area (KMG) (Alipurduar district), Jalpaiguri town area (JPG), Nagrakata block area (NGK), Malbazar railway station area (NMZ), Binnaguri cantontment area (BIN) (Jalpaiguri district), Siliguri town area (SLG) and North Bengal University campus (NBU) (Darjeeling district). The sampling sites were selected based on the following criteria: sites near or far from human dwellings; agricultural land or bare land area; rural and urban area. The geographical coordinates and other relevant environmental factors during sampling of the sampling sites are recorded in Table 1.

Table 1
Details of sampling sites.

Site	Coordinates	Elevation (m)	Type	Dengue Prevalent
APD	26.49°N 89.52°E	53	Semi urban	Yes
HAS	26.75°N 89.35°E	104	Rural	Yes
KMG	26.66°N 89.83°E	48	Rural	Yes
JPG	26.52°N 88.73°E	89	Urban	Yes
NGK	26.88° N 88.91°E	214	Rural	Yes
NMZ	26.85°N 88.75°E	187	Rural	Yes
SLG	26.71°N 88.43°E	120	Urban	Yes
NBU	26.71°N 88.35°E	116	Rural	Yes

2.2. Collection of larva and adult mosquitoes

The selected sampling sites were screened for the larva and adult mosquitoes. Mosquito larvae were collected from different field habitats such as discarded automobile tyres, earthen pots, artificial containers, water holding tanks, discarded buckets, aloevera plantations, tree holes, pots etc. The collected larvae, pupae and adults were brought to the laboratory and screened for *A. albopictus* by using standard larval and adult identification keys (Farajollahi and Price, 2013; Tyagi et al., 2015) and then transferred to marked plastic containers. The sampling was done during March to November 2016, pre-monsoon, monsoon and post-monsoon seasons. No Aedes mosquitoes could be collected from Binnaguri cantonment area during the sampling period.

2.3. Rearing of field caught population of mosquitoes

In the laboratory, the field collected larvae (F_0) were reared at temperature 25 ± 2 °C and 70–80% relative humidity. The rearing was done based on the standard method following Bharati et al., 2016 for successive generations. The larvae were reared to F_1 generation upto adults to ensure the homogeniety of the field collected populations and were cross checked with adult identification keys (Tyagi et al., 2015). The F1 adults were used for bioassays and detoxifying enzyme activity studies. The field caught populations were allowed to rear for successive generations without any exposure to insecticides in the laboratory maintaining the same physical factors as mentioned earlier and provided with anesthesised rat as a source of blood for the females (for the completion of gonotrophic cycle) in each generation. The tenth generation larvae and adults were taken as susceptible population (SP).

2.4. Insecticide ***source***

Temephos (90%) was purchased from Heranba Chemicals (Mumbai, India) and analytical grade malathion (≥98%), deltamethrin (≥98%) and lambda cyhalothrin (≥98%) were obtained from Sigma Aldrich (Bangalore, India).

2.5. Larval bioassay

The susceptibility of *A. albopictus* larvae against temephos (an organophosphate) was tested following the WHO guidelines (WHO, 2005). Two discriminating doses were selected: 1. WHO recommended dose (0.020 mg/L) and 2. India government recommended dose (0.0125 mg/L). Thirty (30) late third instar or early fourth instar larvae of each population (field caught and laboratory reared sus-

ceptible population) were exposed to test vials containing 99 mL tap water with 1 mL of temephos concentration in ethanol so as to provide a 0.020 mg/L and a 0.0125 mg/L solution. The bioassay was done in triplicate with one set of control (using 1 mL ethanol) in laboratory conditions. Mortality percentage was recorded after 24 h of exposure to temephos. Larvae were considered dead or moribund when they failed to evoke any response when touched (WHO, 2005).

For the determination of lethal concentrations (LC_{50} and LC_{90}) of temephos, 60 larvae from each population (including susceptible) were transferred to plastic cups containing 99 mL of distilled water with 1 mL of appropriate concentration of temephos (from stock solution) to obtain the desired dosage, starting with the lowest concentration (Marcombe et al., 2014). Triplicates for each concentration and 4–6 successively higher concentrations (0.0001 – 0.01 mg/L) in the range of the insecticide causing 10% – 90% mortality were set to determine LC_{50} and LC_{90} values. Control test contained 99 mL of distilled water and 1 mL of pure ethanol. Larval mortality was recorded after 24 h of insecticide exposure and the criteria for discriminating between dead and live larvae remained the same.

2.6. Preparation of insecticide impregnated papers for adult bioassays

Insecticide treated papers were prepared for performing adult bioassays (Karunaratne and Hemingway, 2001). Desired concentrations (diagnostic dose) of insecticides were made in acetone and applied evenly on to rectangles of Whatman No.1 filter paper (12 cm × 18 cm) by using potter spray. Papers were left at room temperature until the acetone had completely evaporated. The papers were then wrapped in foil and stored at −20 °C for future use.

2.7. Adult bioassay

Adult bioassays were also performed following WHO guidelines (WHO, 2006). Thirty (30) non blood-fed adults were exposed to insecticide impregnated papers with WHO recommended diagnostic dose of insecticide (5% malathion, 0.05% deltamethrin and 0.05% lambda cyhalothrin) placed in tubes for 1 h. After the stipulated time, the mosquitoes were transferred to another tube called retention tube containing cotton balls soaked in 10% glucose solution and maintained at laboratory conditions. Mortality was recorded 24 h post-exposure. For control, mosquitoes were placed in tubes containing papers impregnated with silicone oil and acetone.

For LC_{50} and LC_{90} values determination, different concentrations of insecticides were prepared in acetone from the stock solution as mentioned above. Insecticide impregnated papers were prepared for a range of concentration of insecticide for each insecticide. Two tubes for each concentration with 50 adults in each and 4–6 concentrations of the insecticides in the range causing 10% – 90% mortality were used to determine LC_{50} and LC_{90} values. Mosquitoes were exposed to different concentrations of insecticides for 1 h, then they were transferred to retention tubes containing cotton balls soaked in 10% glucose solution and mortality rates were recorded 24 h after exposure. Controls were set as mentioned above.

2.8. Insecticide detoxifying enzymes' ***activity***

Single adult *A. albopictus* was homogenized in 100 μL of 0.1 M sodium phosphate buffer (pH 7.2) with a teflon micro-pestle in a 1.5 mL centrifuge tube. The pestle was washed with another100 μL of 0.1 M sodium phosphate buffer (pH 7.2). The homogenate was centrifuged at 12000 rpm (revolutions per minute) for 15 min in a centrifuge (Sigma 3K30, Sigma,U.K.) (Bharati et al., 2016; Raymond et al., 1986). The supernatant was stored at −20 °C to be used as enzyme source for detoxifying enzyme activity assays.

2.8.1. Non-specific esterase (carboxylesterase) assay

The activity of carboxylesterases (CCE) hydrolyzing α- and β-napthyl acetate as substrate were assayed according to Van Asperen (1962) with minor modifications for using in microplate. Fast blue B salt was used as the staining agent. Absorbance was recorded at 540 nm using microplate reader (Biotek ELx800, USA). Standard curves of α- and β-napthol were prepared for estimation of CCE activity.

2.8.2. Cytochrome P450 Monooxygenase (CYP_{450}) assay

The activity of CYP_{450} was measured according to Brogdon et al. (1998) using 3,3′,5,5′-Tetramethyl benzidine (TMBZ) as a substrate and H_2O_2 as the peroxidising agent. Absorbance was recorded at 630 nm using the microplate reader. A standard curve for the heme peroxidase activity was prepared using different concentrations of cytochrome c (0.0025 nM to 0.0200 nM) for horse heart type VI (Sigma Aldrich). The total CYP_{450} was expressed as CYP_{450} equivalent units (EUs) in mg protein.

2.8.3. Total protein content

Total protein of each individual of *A. albopictus* was determined according to Lowry et al., 1951 to negate out any size differences among individuals and for the correct expression of enzyme activity.

3. Calculation

In the bioassays, control mortalities were never found above 5%, so no calculation of corrected mortality was needed. LC_{50}, LC_{90} and LC_{99} were estimated at 95% confidence interval by putting log dose against probit in SPSS 16.0 software and the obtained linear regression coefficient (r^2) was used to assess the linearity of the data set. The resistance ratio, RR_{99} was calculated as LC_{99} of respective field population divided by the LC_{99} of SP. A RR_{99} value >2 implies a resistant population whereas a value <2 is said as susceptible. The population with mortality percentages when >98 is said to be susceptible, 80–97 is assessed as incipient resistance and <80 as resistant (WHO, 2005).

4. Results

4.1. Larval bioassay

Most of the populations were found to be 100% susceptible to both the WHO recommended diagnostic dosage as well as government recommended dose. However, NGK population was found to have the mortality percentage below 98 at WHO recommended discriminating dose and in NGK and SLG populations the mortality percentages were found to be, 94 and 97 respectively for government recommended dose i.e. 0.0125 m mg/L i.e. below 98% but above 90%, thereby in the range of incipient resistance status (Table 2). The LC_{50} values ranged from 0.00098 to 0.00420 ppm (parts per million) and the LC_{90} values had a range of 0.038–0.081 ppm. RR_{99} value was found to be between 0.90-1.90. The lowest value was found for NBU and KMG followed by HAS, JPG, NMZ and APD, implying that all these population were susceptible to temephos. NGK and SLG populations were found to have a higher RR_{99} (≈2) than other popu-

Table 2
Temephos bioassay in field caught and susceptible *A.albopictus* larvae.

SITES	Mortality (%age) after 24 h exposure		LC_{50} (ppm) ±SE (95% CI)	LC_{99}(ppm) ±SE (95% CI)	r^2	RR_{99}	Status
	0.02 mg/L	0.0125 mg/L					
APD	100	100	0.0027 ± 0.0004	0.058 ± 0.0003	0.91	1.38	S
HAS	100	100	0.0013 ±0.0003	0.044 ± 0.0001	0.86	1.04	S
KMG	100	100	0.0014 ± 0.0003	0.040 ± 0.0001	0.89	0.95	S
NGK	97	94	0.0047 ± 0.0001	0.081 ± 0.0020	0.94	1.92	IR
JPG	100	100	0.0034 ± 0.0002	0.046 ± 0.0030	0.82	1.09	S
NMZ	100	100	0.0032 ± 0.0010	0.049 ± 0.0040	0.89	1.16	S
NBU	100	100	0.0001 ± 0.0001	0.038 ± 0.0005	0.87	0.90	S
SLG	98	96	0.0042 ± 0.0003	0.074 ± 0.0006	0.94	1.8	IR
SP	100	100	0.0016 ± 0.0002	0.042 ± 0.0030	0.95	–	

S: Susceptible, IR: Incipient resistance.

Table 3
Susceptibility status of different field caught adult *A. albopictus* populations against adulticides.

Sites	Malathion			Status	Deltamethrin			Status	Lambda-cyhalothrin			Status
	Mortality%	LC_{50} (ppm)	LC_{90} (ppm)		Mortality%	$LC_{50(ppm)}$	LC_{90} (ppm)		Mortality%	LC_{50} (ppm)	LC_{90} (ppm)	
APD	100	0.063	0.130	S	100	1.74×10^{-3}	1.20×10^{-2}	S	100	2.50×10^{-5}	1.10×10^{-4}	S
HAS	100	0.068	0.140	S	100	1.40×10^{-3}	7.70×10^{-3}	S	100	1.10×10^{-5}	0.71×10^{-4}	S
KMG	100	0.047	0.092	S	100	5.62×10^{-4}	4.20×10^{-3}	S	100	4.30×10^{-5}	2.50×10^{-4}	S
NGK	99	0.091	0.210	S	100	3.10×10^{-3}	1.12×10^{-2}	S	100	2.00×10^{-4}	1.00×10^{-3}	S
JPG	100	0.072	0.110	S	100	1.12×10^{-3}	7.10×10^{-3}	S	100	2.43×10^{-5}	0.90×10^{-3}	S
NMZ	100	0.054	0.090	S	100	1.01×10^{-3}	9.42×10^{-3}	S	100	3.76×10^{-5}	6.67×10^{-4}	S
NBU	100	0.046	0.090	S	100	0.97×10^{-4}	0.82×10^{-3}	S	100	1.21×10^{-5}	2.32×10^{-4}	S
SLG	100	0.078	0.170	S	100	1.41×10^{-3}	9.60×10^{-3}	S	100	6.91×10^{-5}	3.20×10^{-4}	S

S: Susceptible.

lations, 1.9 and 1.8 respectively, reflecting their incipient resistance status.

4.2. Adult bioassay

All the tested populations were found fully susceptible to all the tested insecticides at WHO recommended discriminating doses (5% Malathion, 0.05% Deltamethrin and 0.05% Lambda cyhalothrin). The LC_{50} values for malathion ranged from 0.046 ppm to 0.091 ppm whereas the LC_{90} values were observed between 0.092 – 0.21 ppm (Table 3). The LC_{50} and LC_{90} values of deltamethrin and lambda cyhalothrin were found to be very low. LC_{90} values ranged from as low as 0.000071 ppm to as high as 0.001 for lambda cyhalothrin and from 0.00082 to 0.012 ppm for deltamethrin (Table 3).

4.3. Detoxifying enzyme assays

The carboxylesterase (α- and β-) and CYP_{450} activity among the field populations of mosquitoes showed considerable variations when compared to SP (Table 4). The α-carboxylesterase activity ranged from 0.42 to 3.04 μ moles mg protein^{-1} min^{-1}. When compared with the SP, 1.1 – 9.6 times higher level of α-carboxylesterase activity was observed. Similar trend of variation of β–carboxylesterase activity was also observed in the field populations. The higher level of activity of β–carboxylesterase ranged from 0.23–5.11 folds in comparison to SP. The activity ranged from as low as 0.32 μ moles mg protein^{-1} min^{-1} to as high as 1.33 μ moles mg protein^{-1} min^{-1}. In both the assays, the highest activity was observed in NGK population followed by SLG population (Table 4). The results of CYP_{450} assay revealed that all the populations had similar range of CYP_{450} activity, ranging from 0.029 to 0.098 n moles mg protein^{-1}. In comparison to SP, CYP_{450} activity ranged from 1.03 to 3.50 fold in the tested populations. Among the tested populations, NGK population showed the highest level of activity.

5. Discussion

Through the above study we have obtained the knowledge on the level of Insecticide susceptibility status in this dengue endemic part of India. It was found that none of the studied population of *A. albopictus* was found resistant to temephos, which is the mainly used insecticide against larval forms of the dengue vectors in India (NVBDCP, 2016). The mortality were found above 98% for most of the tested populations, indicating that the populations are susceptible. However, SLG population which was sampled near human dwellings and domestic areas and NGK population from rural area surrounded by tea gardens, showed the mortality less than 98% along with a higher resistance ratio. The larval bioassays showed the variation between the LC_{50} and LC_{90} values of temephos against *A. albopictus* and on comparing the LC values of the field population with that of the susceptible control population, the RR_{99} value was found to be around 1 for most of the tested population except SLG and NGK populations, where the resistance ratio was recorded ≈2 which stands to be the borderline value, above which the population is said to be at the verge of resistance development (Yadav et al., 2015). However, most of the population can be said to be fully susceptible and such susceptible strains of *A. albopictus* have also been found throughout India (Dhiman et al., 2014; Sharma et al., 2004; Sivan et al., 2015; Yadav et al., 2015) South Asia, i.e. Thailand (Ponlawat et al., 2005; Jirakanjanakit et al., 2007), Malaysia (Chen et al., 2005; Mohiddin et al., 2016; Nazni et al., 2000), Srilanka (Karunaratne et al., 2013) and throughout the world (Kamgang et al., 2011; Marcombe et al., 2014; Neng et al., 1992; Romi et al., 2003; Toma et al., 1992). Though in its close relative, *A. aegypti* the occurrence of moderate to highly resistant population has been shown in India (Tikar et al., 2008; Yadav et al., 2015) and also throughout the world (Alsheikh et al., 2016; Bellinato et al., 2016; Bisset et al., 2013; Chediak et al., 2016; Grisales et al., 2013; Macoris et al., 2003; Melo-Santos et al., 2010; Putra et al., 2016; Rawlins and Wan, 1995). The different sampling sites also showed tremendous variation in the LC_{50} and LC_{90} values, though all below the WHO recommended diagnostic doses. This may be ascertained to the fact

Table 4
Activity of insecticide detoxifying enzymes in different field caught populations of *A. albopictus* along with susceptible population.

SITES	α-Carboxylesterase activity (μ moles mg protein^{-1} min^{-1} ± SE)	β −Carboxylesterase activity (μmoles mg protein^{-1} min^{-1} ± SE)	Monoxygenase activity (n moles mg protein^{-1} ± SE)
APD	0.53 ± 0.07	0.62 ± 0.02	0.035 ± 0.003
HAS	0.81 ± 0.43	0.45 ± 0.02	0.039 ± 0.007
KMG	0.48 ±0.01	0.32 ± 0.01	0.030 ± 0.004
NGK	3.64 ± 0.32	1.33 ± 0.38	0.098 ± 0.006
JPG	0.52 ± 0.08	0.41 ± 0.03	0.039 ± 0.001
NMZ	0.56 ± 0.06	0.51 ± 0.01	0.029 ± 0.004
NBU	0.42 ± 0.03	0.39 ± 0.01	0.032 ± 0.001
SLG	0.88 ± 0.04	0.97 ± 0.23	0.041 ± 0.002
SP	0.38 ± 0.02	0.26 ± 0.04	0.028 ± 0.003

that considerable inherent variations occur between population belonging to separated geographical locations rather than due to the influence of insecticide application/spray (Wesson, 1990). SLG population sampling site is an urban site with a very recent history of temephos usage as a mosquito larvicide by various pest control organizations (Personal communication), this may have lead to the onset of incipient resistance growth as the continuous insecticide exposure in an area is a vital tool for the development of insecticide resistance (Hemingway and Ranson, 2000). However NGK population sampling site has no such history of temephos usage but the use of similar insecticides belonging to the same group, i.e. organophosphate such as chlorpyrifos, dichlorvos etc against tea, jute, vegetable pest management through the mode of agricultural runoff could have cross contaminated the sites of *Aedes* breeding and hence may have provoked the development of insecticide resistance in the population inhabiting this area. Many earlier authors have also emphasised on the role of agricultural sector and the ability of the insecticide residue to move into mosquito breeding sites and thereby building up of insecticide development (Nkya et al., 2013; Nkya et al., 2014; Overgaard et al., 2005; Philbert et al., 2014). Similarly insecticides used actively in cotton, vegetable fields have also been anticipated in contaminating the mosquito breeding sites (Arslan et al., 2016; Kamgang et al., 2011) resulting in selection of DDT and pyrethroid resistance in many species of malaria causing mosquito genera, *Anopheles* (Antonio-Nkondjio et al., 2011; Corbel et al., 2007; Chandre et al., 1999; Chouaïbou et al., 2008; Djouaka et al., 2008; Diabate et al., 2002; Kamgang et al., 2011; Luc et al., 2016; Mueller et al., 2008; Reid and McKenzie, 2016; Yadouleton et al., 2011).

The carboxylesterase (CCE) activity supported the bioassays result to some extent as the NGK population was found to exhibit the highest level of activity of carboxylesterases among tested populations, with a level of ≈9.6 fold higher activity than the susceptible control population. CCEs have been claimed to play a role in conferring resistance to OPs through metabolic detoxification in *Culex* mosquitoes (Mouches et al., 1986; Raymond et al., 1989). The positive correlation between the increased CCEs activity and increased lethal concentration values in NGK population may bring the conclusion that in this population the major mechanism of resistance (therefore, higher LC values) may be the metabolic detoxification of the insecticide by CCEs within the insect body. Conversely, the increased enzyme activity could also have arose due to the continuous alternative insecticide exposure (Pethuan et al., 2007). The sampling site of SLG population inspite of showing a comparable LC_{90} and RR_{90} values to NGK population, exhibited a very low CCE activity range similar to other field populations. The underlying reason behind this may be that in this population, altered AChE (target site for OP), another major mechanism of resistance to OP may be the reason behind resistance (Fournier and Mutero, 1994) rather than metabolic detoxification as in NGK population.

The results of adult bioassay exhibited complete susceptibility of all the field caught populations of *A. albopictus* to all the tested insecticides at WHO recommended diagnostic dosages. Similar to the results of larval bioassay, the LC_{50} and LC_{90} values of NGK population was found to be substantially higher than other tested population though below the recommended diagnostic dose. The underlying reason may be the same as discussed earlier. Corrected mortalities were found to be >98% in all the tested population, so it may be concluded that all the studied population were susceptible to malathion. In the nearby state of Assam, similar susceptibility levels have been found in field caught *A. albopictus* (Dhiman et al., 2014; Yadav et al., 2015). This mosquito has been found to be susceptible to malathion also in other parts of India namely Andaman and Nicobar islands (Sivan et al., 2015), South India (Sharma et al., 2015) and the throughout the world (Dorta et al., 1993; Duong et al., 2016; Karunaratne and Hemingway, 2001; Liu et al., 2004; Raweewan et al., 2011; Sames et al., 1996; Wesson, 1990).

The synthetic pyrethroids, deltamethrin and lambda cyhalothrin caused 100% mortalities in all the tested populations, thereby inferring the population fully susceptible to these two insecticides. Though most of the sites were set near human dwellings and the household personal mosquito protection measures in India include mosquito repellent coils, creams, long lasting insecticide treated nets [supplied throughout the concerned areas by "Integrated Child Development Services" (ICDS) an Government organization], all of which carry the active insecticide belonging to synthetic pyrethroids, yet the mosquitoes were found to be fully susceptible to this group. This may be explained by the fact that *A. albopictus* are exophilic and anthrophobic i.e. they prefer to remain outdoors rather than indoors (Kawada et al., 2010) where this protection measures are employed unlike its near relative *A. aegypti*, (the primary vector of dengue) which prefer indoors (Ponlawat et al., 2005). Pertaining to this fact, majority of the *A. albopictus* population tested against pyrethroid throughout the world have reported full susceptibility (Das and Dutta, 2014; Kamgang et al., 2011; Marcombe et al., 2014; Pethuan et al., 2007; Raweewan et al., 2011; Yadav et al., 2015). Converse being the reports from Pakistan (Arslan et al., 2016), Japan (Kawada et al., 2010), Srilanka (Karunaratne et al., 2013), Malaysia (Hasan et al., 2016) and Thailand (Chuaycharoensuk et al., 2011; Ponlawat et al., 2005). In contrast to this, field populations of *A. aegypti* have shown considerable pyrethroid resistance in nearby areas within India (Yadav et al., 2015) as well as throughout different parts of world (Vontas et al., 2012).

The biochemical assay concerning the CYP_{450} activity was also found in support of the above inference. As most of the population showed very low activity of CYP_{450} ranging from 1.3–3.6 fold that of control population. The highest activity was recorded in NGK population, though the difference is insignificant in comparison to other populations. The LC values against pyrethroid also showed a similar trend and all the LC_{90} values were very much lower than that of the recommended diagnostic dosages. The highest LC_{90} value

was exhibited by NGK, which can be because of the higher CYP_{450} which are believed to play active role in pyrethroid metabolism (David et al., 2013) and CCE activities which is also claimed to metabolise pyrethroid, though to a lesser extent as compared to OPs and carbamates (Hemingway et al., 2004). However, it cannot be confirmed that whether the higher LC_{90} value is due to higher level of enzyme activity (metabolic resistance) or increased frequency of KDR (Knock down rate) mutations (target site resistance).

6. Conclusion

From the above results it can be concluded that NGK population is at borderline resistance ratio for temephos with a higher LC_{90} values for other tested insecticides. In addition to that, NGK population also showed a higher level of detoxifying enzyme activity, indicating the population is at the verge of resistance development in the near future. The higher activities of CCE as compared to CYP_{450} in all the studied populations may be due to the continuous use of OPs in India since 1960s till now in the studied area (Personal communication). Temephos can still be used in India for mosquito larvae control during severe dengue outbreaks as most of the populations were found completely susceptible to it. Malathion and synthetic pyrethroids seem to be a good approach for adult *A. albopictus* control in mosquito management practices. This study seems to be the first such surveillance study concerning the insecticide susceptibility status of the recently expanded secondary vector of dengue in this part of India. The endemicity of this region for malaria and dengue needs a regular planned surveillance throughout the region for the implementation of effective vector control strategies. And, the absence of data on insecticide resistance status limits the success of vector control strategies in this country. These observations can also help the concerned authorities to select and formulate the most effective insecticides and their dosages against dengue vector in this area. Amongst the insecticides tested, synthetic pyrethroids seem to be the most effective in controlling this mosquito followed by malathion and then temephos. In future, molecular studies could be performed in the direction of biochemical mechanisms involved in the development of insecticide resistance in *A. albopictus* along with the search of novel tools and molecules to manipulate those mechanisms for prevention of insecticide resistance development in these dengue vectors as well as other mosquito vectors and integrated mosquito management. Other strategies such as use of botanicals (Ghosh et al., 2012) or genetically modified sterile male technology (Alphey et al., 2010; Harris et al., 2011) can also contribute immense in this field being environment friendly as well as long term effective approach.

Declaration of interest

The authors declare that they have no conflict of interest.

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PLOS ONE

RESEARCH ARTICLE

Multiple insecticide resistance mechanisms in primary dengue vector, *Aedes aegypti* (Linn.) from dengue endemic districts of sub-Himalayan West Bengal, India

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Abstract

Background

Mosquitoes belonging to genus *Aedes* are the prime vectors of several arboviral diseases such as Dengue, Zika and Chikungunya worldwide. Every year numerous cases of dengue infections occur throughout the world, proper control of which depends on efficient vector control. However the onset of insecticide resistance has resulted in failure of vector control approaches.

Principal findings

This study was carried out to unveil the degree of prevailing insecticide resistance along with its underlying mechanisms among the primary dengue vector in dengue endemic districts of West Bengal, India through standard WHO protocol. It was observed that, the majority of the tested populations were found to possess resistance to more than one insecticide. In adult bioassay, the toxicity levels of the six tested insecticides was found to decrease in the following order: deltamethrin > lambdacyhalothrin > malathion > propoxur > permethrin > DDT. In larval bioassay, one of the tested populations was found to possess moderate resistance against temephos, mortality percentage 92.5% and 79.8% for WHO (0.0200 ppm) and National Vector Borne disease Programme, India recommended dose (0.0125 ppm) respectively. Carboxylesterases were found to be involved in conferring resistance as revealed in synergistic and quantitative assay against temephos in North Dinajpur (NDP) population and malathion in Alipurduar (APD) and Darjeeling (DAR) populations. Similar correlations were also observed in the majority of the tested populations between reduced susceptibilities against pyrethroid insecticides and Cytochrome P_{450}s activity.

Conclusion

Efficient disease management in this region can only be achieved through proper integrated vector management along with tools to minimize insecticide resistance. This study may help

the concerned authorities in the formulation of an effective vector control strategy throughout this region incorporating the knowledge gained through this study.

Introduction

Mosquitoes transmit diseases of public health importance such as dengue, chikungunya, malaria, filariasis *etc*, thus presenting a threat to human health. *Aedes* mosquitoes namely, *Ae*. *aegypti* and *Ae*. *albopictus*, key vectors of dengue virus (DENV) and chikungunya virus (CHIKV) [1–2] have recently invaded different geographical regions throughout the world [3]. Annually, dengue, chikungunya and yellow fever collectively infect around 75 million people, with 25,000 deaths [1]. Recently, a new dengue serotype has appeared in the Asian continent that follows the sylvatic cycle unlike the other four serotypes which follow the human cycle [4–5]. Emergence and spread of such new serotypes may enhance the severity of the disease.

Since last few years, annually more than one lakh cases of dengue infections occur in India [6] resulting in substantial rates of mortality and morbidity. In the state of West Bengal, 10,697 people were infected with dengue in 2017 with 19 deaths [6]. This state provides an ideal *Aedes* mosquito breeding environment owing to the presence of large vegetation cover and high rainfall [7].

In absence of specific medications against dengue the sole method of disease prevention relies on control of vector mosquitoes. The prevention and control of dengue in India is followed through integrated vector management which includes entomological surveillance; following source reduction, use of larvicides and larvivouros fish, environment management as anti larval measures; and following regular anti adult measures through either indoor residual spray by 2% pyrethrum extract or fogging by 5% malathion during disease outbreaks [6]. Additionally, some commercially available mosquito control/repellant tools are also widely used in India by the general public (for personal protection) which contain compounds mainly belonging to pyrethroid group of insecticides.

Due to indiscriminate use of insecticides, mosquitoes have evolved strategies to resist the planned actions of insecticides in their bodies, this phenomenon is known as insecticide resistance [8]. Mosquitoes have developed insecticide resistance both as a direct effect of insecticides targeted on them as well as an indirect exposure of insecticide sprayed on agricultural field [7,9–10]. Insecticide resistance is the major obstacle nowadays in efficient vector/pest control approaches. Altered susceptibilities of *Aedes* species to insecticides could be either governed by metabolic detoxification through enzyme systems present in the body or through altered target site in field populations. Over expression or gene amplification of enzyme families/classes, Carboxylesterases (CCEs), Glutathione S-transferases (GSTs) and Cytochrome P_{450}s (CYP_{450}s) or Mixed Function Oxidases (MFOs) have been shown to confer insecticide resistance in many populations of insecticide resistant *Aedes* mosquito population worldwide [1,11]. Moreover, target site alteration either as a result of point mutations in voltage gated sodium channel gene or an insensitive AchE mechanisms have been identified in vector mosquitoes [1,11]. Knockdown resistance (kdr) mutations, *i.e.* mutation in voltage gated sodium channel are widespread in *Aedes* population and have been shown to provide selective advantage over pyrethroid and organochlorine insecticide pressure in many populations of *Aedes aegypti* [1,12].

Identification of prevailing level of insecticide resistance along with its underlying mechanisms have important implications for vector control. The findings of this study may be helpful

in designing efficient integrated vector control strategies along with tools to combat insecticide resistance during intense disease outbreaks.

Materials and methods

Selection of sampling districts and mosquito collection

Five different sampling districts were selected in northern part of West Bengal, namely, Alipurduar, Jalpaiguri, Darjeeling, Coochbehar and North Dinajpur. The relevant biotic and abiotic factors of the sampling sites are provided in Table 1. The selected sampling sites (Fig 1) were screened for the larva and pupa of *Aedes* mosquitoes. Mosquito larvae/pupae were collected from different wild habitats only such as discarded automobile tyres, earthen pots, artificial containers, water holding tanks, discarded buckets, aloevera plantations, tree holes, pots *etc*. The larvae initially identified as *Aedes* were collected and transferred to plastic containers and brought to the laboratory. The sampling was done during March to November 2017 and March 2018 to April 2018, pre-monsoon, monsoon and post-monsoon seasons and the details of total collection (sampling site and season wise) is provided in Table 1. Since all the sampling was done from private land, prior permission was taken from the land owner for mosquito collection.

Selection and rearing of susceptible and field caught population of mosquitoes

In the laboratory, the larvae were identified upto subspecies level following standard identification keys [13–14]. All the collected mosquitoes were identified to be *Aedes aegypti aegypti*. The field collected larvae (F_0) were then reared at temperature 25±2˚C and 70–80% relative

Table 1. Details of the sampling sites.

Districts	Population name	Geographical coordinates	Total numbers of mosquito (larvae and pupae) sampled	*Mosquito* Generation used in Experiments	Disease endemicity	Last season of dengue outbreak	Total infection in 2016
Alipurduar	APD	26.69˚ N 89.47˚ E	2018: Pr M-1067 2017: Pr M-1258 M-907 Po M-1103	F1	Dengue, Malaria, JE	2017	16
Coochbehar	COB	26.34˚ N 89.46˚ E	2018: Pr M-965 Pr M-1141 M-701 Po M-922	F1	Dengue, Malaria, JE, Filariasis	2017	37
Jalpaiguri	JPG	26.52˚ N 88.73˚ E	2018: Pr M-694 Pr M-967M-1231 Po M-709	F1	Dengue, Malaria, JE, AES	2017	168
Darjeeling	DAR	26.71˚ N 88.43˚ E	2018: Pr M- 1165 Pr M-1032M-971 Po M-1121	F1	Dengue, Malaria, JE, AES	2017	165
North Dinajpur	NDP	26.27˚ N 88.20˚ E	Pr M- 754 Pr M- 1948M-852 Po M-768	F1	Dengue, Malaria, JE, AES	2017	87
Susceptible population	SP	--	--	F10	--	--	

JE: Japanese Encephalitis, AES: Acute Encephalitis syndrome, Pr M: Pre-monsoon, M: Monsoon,

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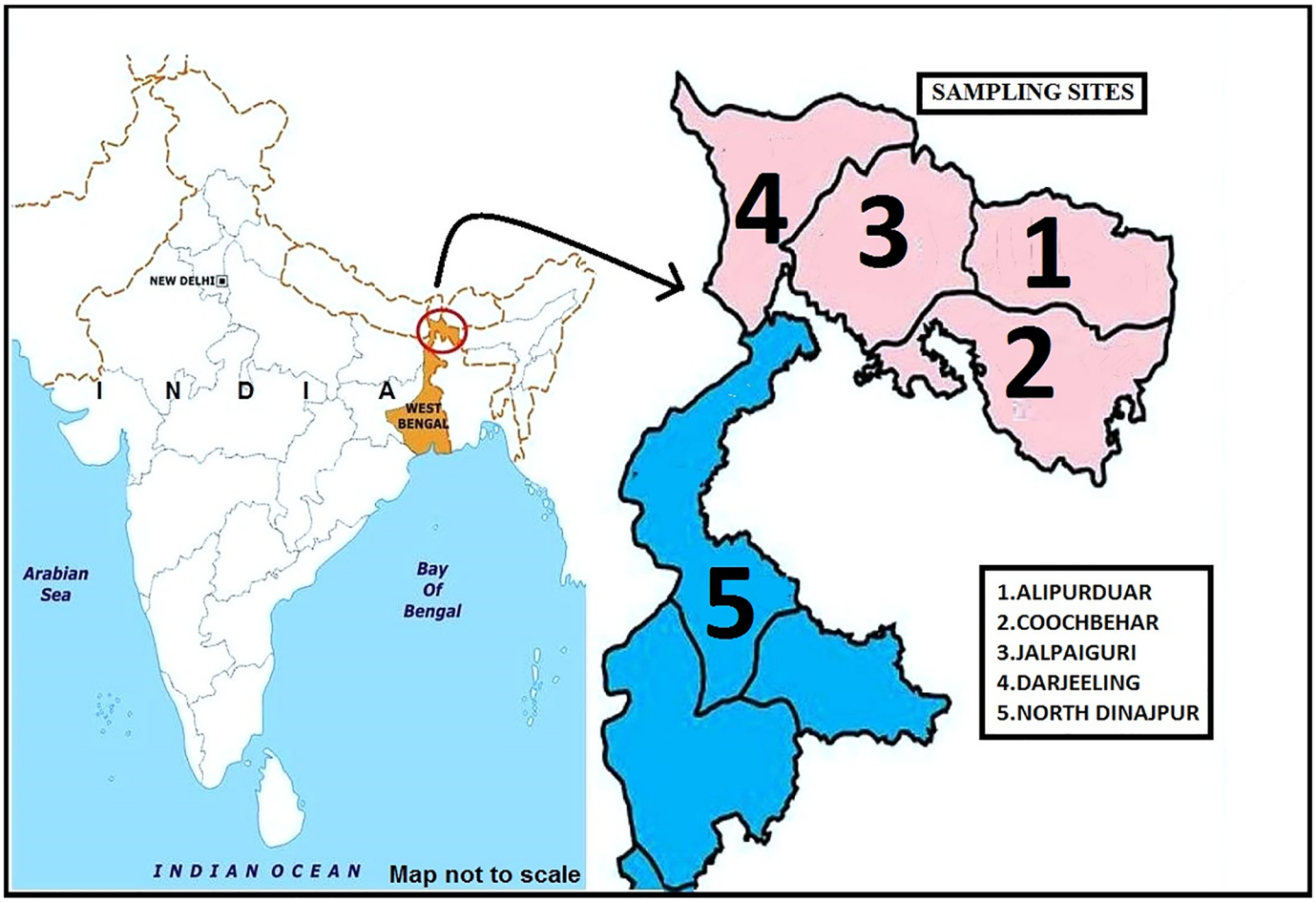

Fig 1. Map of the sampling sites.

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humidity. The rearing was done based on the standard method [7] for successive generations. The larvae were reared to F_1 generation upto adults to ensure the homogeneity of the field collected populations. The emerged adults were cross checked with adult identification keys [14]. The F1 larvae and adults were used for bioassays and detoxifying enzyme activity studies. To setup a susceptible laboratory culture, mosquito samples were collected randomly from five organically managed areas with lowest insecticide exposure possibilities. The mosquito colonies after collection were reared to F1 generation and were subsequently tested for insecticide susceptibility bioassays. The mosquito population that was recorded to possess the lowest level of resistance (collected from the Medicinal garden of North Bengal University campus, Siliguri, India) was chosen from the rest to be reared for ten additional generations without any exposure to insecticides in the laboratory maintaining the same physical factors as mentioned earlier and provided with anaesthetised rat as a source of blood for the females in each generation to be used as the laboratory reared control/ susceptible population (SP).

Insecticide

Temephos solution (156.25 mg/L) and insecticide impregnated papers 4% DDT, 0.05% deltamethrin, 0.05% lambdacyhalothrin, 0.75% permethrin, 5% malathion and 0.1% Propoxur were purchased from Vector control unit, Universiti sains Malaysia.

WHO bioassay

To assess the susceptibility status of adult mosquitoes, thirty (30) 2–3 days non blood-fed adults were exposed to insecticide impregnated papers with WHO recommended diagnostic dose of insecticide (4% DDT, 0.05% deltamethrin and 0.05% lambdacyhalothrin, 0.75% permethrin, 5% malathion and, 0.1% Propoxur) placed in tubes for 1 hour [15]. After one hour, the mosquitoes were transferred to retention tube containing cotton balls soaked in 10% glucose solution. Mortality percentages were recorded 24 hours post-exposure. For control, mosquitoes were place in tubes containing papers impregnated with silicone oil and acetone. For synthetic pyrethroids and organochlorine insecticides, number of knocked down mosquitoes were counted for every ten minutes, to determine the knockdown time, *i.e.* KDT_{50} and KDT_{95}.

To assess the susceptibility of *Ae. aegypti* larvae against temephos standard WHO guidelines were followed [16]. Thirty (30) late third instar or early fourth instar larvae of each population were exposed to test vials containing two different discriminating doses: 1. WHO recommended dose (0.0200 mg/L) and 2. India government recommended dose (0.0125 mg/L) of temephos in water. One set of control (using solvent instead of insecticide solution) was also set under laboratory conditions. Mortality percentage was recorded post 24 hours of temephos exposure. Larvae were considered dead or moribund if they failed to evoke any response when touched [16].

For the determination of resistance ratio, *i.e.* RR50 of temephos, standard methodology was followed [7]. Both adult and larval assays were performed in triplicates and the mortality percentages were taken as the average of the three assays.

Synergism tests

Synergism tests were conducted using the field populations to evaluate the effectiveness of synergists on detoxification of insecticides. Piperonyl butoxide (PBO) (90%, Sigma from Sigma-Aldrich, Singapore), a CYP_{450}s inhibitor and triphenyl phosphate (TPP) (99%, from Sigma-Aldrich, Singapore), a CCE inhibitor were used. The sub-lethal doses of both the synergists *i.e.* 4% and 10% for PBO and TPP respectively were used in synergism tests. The protocol for the synergism tests were similar to the larval bioassays described above, except that the insecticide was mixed with synergist prior to the test. For adult bioassays, each population was exposed to synergist for one hour prior to insecticide exposure. Diagnostic tests in WHO bioassays section (exposure to insecticide only) served as positive control while bioassays without insecticide were used as negative control.

Insecticide detoxifying enzymes' activity

Single adult *Ae. aegypti* were homogenized in 100 μL of 0.1M sodium phosphate buffer (pH 7.2) with a teflon micro-pestle in a 1.5 mL centrifuge tube and the whole solution was made 200 μL with 0.1 sodium phosphate buffer. The homogenate was centrifuged at 12,000 rpm (revolutions per minute) for 15 minutes in a centrifuge (Sigma 3K30, Sigma,U.K.) [7]. The supernatant was stored at -20˚C and was used within 3–4 days as enzyme source for detoxifying enzyme activity assays. For each biochemical test, a minimum of thirty individuals were assayed. A duplicate set was also run for each enzyme assay. In this study, a single substrate for each enzyme group (two for CCEs) has been used for assessing the enzyme activity levels. Though, an enzyme group may have many substrates, yet the substrates used are identical to substrates used in standard protocols [17].

Non-specific esterase (carboxylesterase) assay. The activity of carboxylesterases (CCEs) hydrolyzing α- and β- naphthyl acetate as substrate were assayed according to standard WHO guidelines[17] with minor modifications for using in microplate [7].

CYP_{450} assay. The activity of CYP_{450} was also measured according to standard WHO guidelines [17] using 3,3',5,5'-Tetramethyl benzidine (TMBZ) as a substrate and H_2O_2 as the peroxidising agent. The total CYP_{450} was expressed as CYP_{450} equivalent units (EUs) in mg protein.

Glutathione S-transferase (GST) assay. GST activity was assessed following the WHO protocol [17] using CDNB/GSH as the working solution in wells of microtitre plate.

Total protein content. Total protein of each individual of *Ae. aegypti* was determined according to standard WHO guidelines [17] to cancel out any size differences among individuals and for the correct expression of enzyme activity.

Calculation

In the insecticide susceptibility bioassays, no calculation of corrected mortality was needed because control mortalities were below 5%. The population with mortality percentages when $>$ 98 is said to be susceptible, 80–97 is assessed as resistance not confirmed (= unconfirmed resistance = incipient status) and $<$80 as resistant [15–16]. LC_{50} was estimated at 95% confidence interval by putting log dose against probit in SPSS 16.0 software and the obtained linear regression coefficient (r^2) was used to assess the linearity of the data set. Resistance ratio 50 *i.e.* RR50, which is an indirect measurement of insecticide resistance development was also determined as the LC_{50} of sampling site divided by the LC_{50} of the SP Similarly the knockdown time for 50% (KDT_{50}) and 95% (KDT_{95}) of tested mosquitoes were calculated using probit analysis.

Results

WHO bioassays and synergistic tests

The study of adult bioassays revealed that multiple resistance was prevalent among the tested populations against an array of insecticides (Fig 2). All the tested populations were reported to exhibit reduced mortalities against DDT with the highest mortality percentage of 70.2%. Neither CCEs nor CYP_{450}s could be assigned as the detoxifying enzyme governing the resistance against DDT, since in only one population (*i.e.* APD), PBO exposure was found to enhance susceptibility to DDT whereas in others no such involvement could be noted (Fig 2). Against synthetic pyrethroids, the lowest mortality percentages were recorded against permethrin, *i.e.* 50% to 87.6%. Against deltamethrin and lambdacyhalothrin, three of the tested populations *i.e.* APD, JPG, NDP possessed unconfirmed/incipient resistance, whereas rest were found to be susceptible. In APD, JPG and NDP population, susceptibility was found to be restored when prior exposure to PBO was done thereby indicating the role of CYP_{450}s in resistance against deltamethrin and lambdacyhalothrin. Two out of six tested populations showed mortality percentages below susceptible level against Malathion (Fig 2). In one population, *i.e.* APD, use of TPP was found to restore the susceptibility against malathion, enhancing the mortality rate from 70.40% to 94% (S1 Table). Against propoxur, mortality percentages were noted to range from 45.45% to 97.70%, and probable role of CCEs could be assigned to confer such resistance in NDP population as evident through synergism study.

All the tested populations were completely susceptible to both the concentrations of temephos except, one population, *i.e.* NDP with mortality percentages 92.5% (0.0200 ppm) and 79.8% (0.0125 ppm) respectively (Table 2). The use of TPP along with temephos could restore the mortality percentage to susceptible levels (Fig 2). All the tested populations exhibited their respective RR50 values ranging from 1.65 to 35.09. The highest RR50 value, *i.e.* 35.09 was exhibited by NDP population followed by JPG population with RR50 value of 9.30.

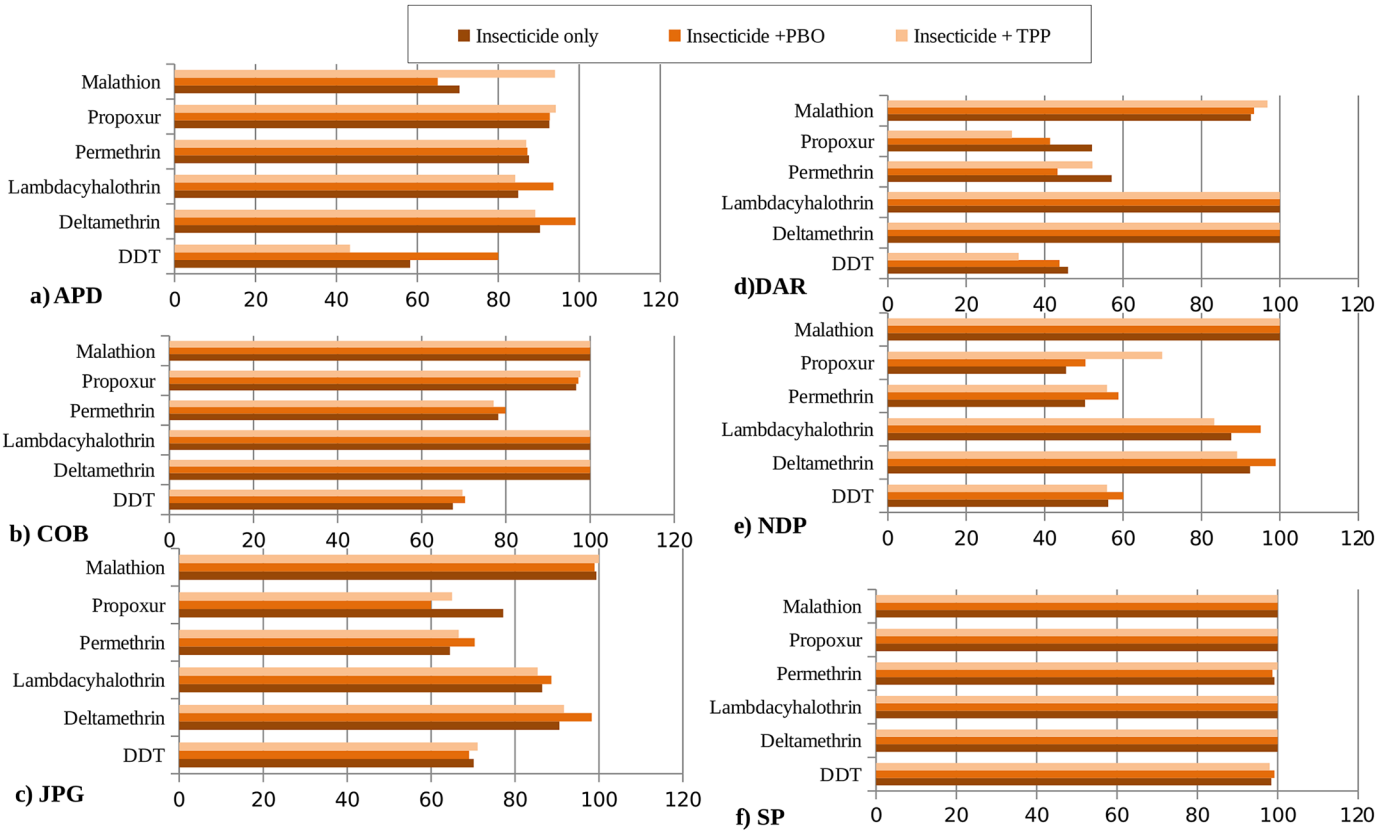

Fig 2. Insecticide susceptibility status against six adulticides among wild *Ae. aegypti* populations from northern districts of West Bengal.

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Knockdown rates

The lowest KDT_{95} value against DDT was noted from SP, *i.e.* 121.41, whereas DAR population recorded the highest KDT_{95} value (Table 3). Against, deltamethrin, JPG population recorded the highest KDT_{95} value of 108.21. COB and APD population reported the greatest KDT_{95} value, 32.55 and 76.01 against lambdacyhalothrin and permethrin respectively.

Biochemical enzyme assay

The activity of major detoxifying enzyme groups were varying among different field caught populations of *Ae. aegypti* (Table 4). The activity of α-CCEs ranged from 1.12 to 3.12 times that of the SP, *i.e.* 0.241 to 0.668 μmoles mg protein^{-1} min^{-1}. Similarly, the activity of β-CCEs among the field populations of *Ae. aegypti* ranged from 0.181 to 0.406 μmoles mg protein^{-1}

Table 2. Susceptibility to temephos in larval *Ae. aegypti* collected from districts of northern Bengal.

Sites	Mortality %age (0.0200 ppm)	Mortality %age (0.0125 ppm)	RR50
APD	100	100	3.00
COB	100	100	1.65
JPG	100	100	9.30
DAR	100	100	5.43
NDP	92.5	79.8	35.09
SP	100	100	--

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Table 3. Knockdown rates (KDT_{50} and KDT_{95}) of different *Ae. aegypti* populations against tested organochlorine and synthetic pyrethroid insecticides.

Sampling site	DDT		Deltamethrin		Lambdacyhalothrin		Permethrin	
	KDT_{50} (min)	KDT_{95} (min)	KDT_{50} (min)	KDT_{95} (min)	KDT_{50} (min)	KDT_{95} (min)	KDT_{50} (min)	KDT_{95} (min)
APD	142.16	239.31	52.42	101.6	46.91	75.16	37.42	76.01
COB	96.34	191.17	14.11	52.19	9.41	32.55	43.66	106.01
JPG	95.41	182.35	71.16	108.21	43.33	82.21	52.76	121.32
DAR	182.16	272.06	31.40	72.33	12.60	49.36	67.66	161.15
NDP	155.39	254.12	58.77	93.9	34.04	45.45	78.98	192.22
SP	81.69	121.41	8.69	42.31	9.15	39.18	39.18	78.69

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min^{-1}. The level of CYP_{450} monoxygenase activity and GST activity were similar throughout the tested mosquito populations, ranging from 0.044 to 0.063 nmoles mg $protein^{-1}$ min^{-1} and 0.32 to 0.42 GSH-CDNB conjugate μM mg $protein^{-1}$ min^{-1} respectively.

Discussion

The objective of this study was to reveal the mechanism of prevailing insecticide resistance in the wild populations of *Aedes aegypti* in five dengue endemic districts of sub-Himalayan West Bengal. To determine the underlying mechanisms of insecticide resistance, detoxifying enzymes' activity, synergism assays and determination of knockdown times, *i.e.* KDT_{50} and KDT_{95} were assessed.

In this study, none of the tested *Ae. aegypti* populations were found to be susceptible to DDT (Fig 2), with mortality percentages ranging from as low as 46% to 70.2% and 98.4% for SP (Fig 2). The KDT_{50} and KDT_{95} values were also significantly higher than the SP for the field populations of *Ae. aegypti* indicating the inefficacy of DDT in dengue vector control. The *Ae. albopictus* populations from nearby regions of West Bengal have also been found to possess similar levels of resistance against DDT [18], This result points on the existing DDT selection pressure on *Ae. aegypti* and other mosquito vector populations throughout the study region, which may be pertained to the widespread use of DDT in both agriculture and public health sector throughout the world since the past 70 years [1]. DDT resistance have been linked either by sodium channel mutations leading to target site insensitivity [19] or through enhanced detoxification by insecticide detoxifying enzymes, *i.e.* GSTs [20], CYP_{450}s [1] or CCEs [21]. However, the use of CCE and CYP_{450} inhibitors before DDT exposure showed no significant change in mortality percentage in all populations except APD. The partial recovery of susceptibility to DDT in APD population using PBO, suggests the possible role of CYP_{450}s in

Table 4. Activities of major detoxifying enzymes in different field caught populations of *Ae. Aegypti*.

Sites	α-CCEs (μmoles mg $protein^{-1}$ min^{-1}) ± S.E.	β-CCEs (μmoles mg $protein^{-1}$ min^{-1}) ± S.E.	CYP_{450s} (nmoles mg $protein^{-1}$ min^{-1}) ± S.E.	GSTs (μMmg $protein^{-1}$ min^{-1}) ± S.E.
APD	$0.313 \pm 0.008^{b*}$	0.226 ± 0.008^{b}	0.056 ± 0.0002^{b}	0.39 ± 0.006^{a}
COB	0.241 ± 0.004^{a}	0.181 ± 0.001^{b}	0.044 ± 0.0003^{a}	0.32 ± 0.002^{a}
JPG	0.279 ± 0.001^{b}	0.217 ± 0.007^{b}	0.063 ± 0.0011^{b}	0.42 ± 0.001^{a}
DAR	0.359 ± 0.007^{b}	0.224 ± 0.006^{b}	0.059 ± 0.0009^{b}	0.41 ± 0.009^{a}
NDP	0.668 ± 0.021^{c}	0.406 ± 0.007^{c}	0.057 ± 0.0021^{b}	0.33 ± 0.009^{a}
SP	0.214 ± 0.002^{a}	0.161 ± 0.009^{a}	0.040 ± 0.0005^{a}	0.31 ± 0.002^{a}

*Within columns, means followed by the same letter do not differ significantly (P = 0.05) in Tukey's multiple comparison test (HSDa).

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metabolisation of DDT as in other mosquito vectors such as CYP6M2 gene in *An. gambiae* [22]. The involvement of GSTs in the observed resistance against DDT could not be studied, since no significant difference was found in GST activity among the field populations. However, the presence of target site insensitivity, *i.e.* kdr mutations needs to be explored to characterize the exact mechanism/s of resistance against DDT [19].

Most of the tested populations were found to be susceptible or incipiently resistant to deltamethrin and lambdacyhalothrin In some of the populations, namely, APD, JPG and NDP the KDT_{50} and KDT_{95} values were also greater implying the onset of resistance against these two pyrethroid insecticides [23]. Since, long lasting insecticide treated nets mainly use deltamethrin as the active insecticide in India [6], the progress of such resistance needs regular monitoring. Against permethrin, varied pattern of resistance was noted ranging from resistant to susceptible levels. In India, synthetic pyrethroids are widely used throughout different agricultural fields to manage the pest populations. The observed resistance against synthetic pyrethroids could be a result of cross exposure or contamination of mosquito habitats by pyrethroids sprayed on agricultural fields [7,9–10]. Against synthetic pyrethroids either metabolic detoxification by CYP_{450}s (or other detoxifying enzyme classes) or presence of kdr mutations are known to confer resistance in *Ae. aegypti* [24,25–26]. Results of synergism tests revealed that in majority of the populations incipiently resistant to deltamethrin or lambdacyhalothrin, PBO exposure was found to restore (either completely or partially) the susceptibility to these two insecticides, thereby suggesting the role of CYP_{450}s behind the altered susceptibility. Moreover, the CYP_{450} activity levels were also significantly higher in APD, JPG and NDP populations, thereby supplementing the results of synergistic study. Many populations of *Ae. aegypti* have been found to possess CYP_{450}s mediated resistance against deltamethrin or lambdacyhalothrin worldwide [1,26,27]. However, in case of resistance against permethrin, though the detoxifying enzyme activity indicated the possible role of CYP_{450}s in JPGand DAR population and CCEs in DAR and NDP population, yet the inefficacy of enzyme inhibitors in enhancing the toxicity of permethrin on any of the field populations of *Ae. aegypti* strikes out the involvement of metabolic detoxification behind the observed resistance. Moreover, the similar pattern of resistance against both permethrin and DDT imparts light on the possible role of kdr mutations [19, 25–26] in conferring resistance against both the insecticides having same target site of action. Presence of kdr mutation providing resistance against both pyrethroid and organochlorine insecticides have been found in different mosquito vector species such as *Ae. aegypti* [19] and *An. gambiae [28]*. Insecticide susceptibility test against malathion revealed the presence of two resistant or possibly resistant population, *i.e.* APD and DAR amongst the six tested populations. Against organophosphate insecticides the prime mechanisms of resistance have been found to be either through enhanced detoxification by enzymes, mainly CCEs [29] or through insensitive AchE [21]. In both APD and DAR populations, pre exposure to CCE inhibitor TPP could moderately enhance the mortality percentages from 70.4% to 94% and 92.6% to 96.8% respectively. Moreover, the significantly higher activities of both α- and β-CCEs also points on the presence of malathion specific CCE mechanism mediated resistance to be prevalent in these populations [30].

Resistance against one more insecticide was tested, *i.e.* propoxur, a carbamate insecticide, three field populations (JPG, DAR and NDP) were found to be resistant, whereas remaining (APD and COB) were found to possess unconfirmed resistance against propoxur (Fig 2). Propoxur is not used in India for mosquito control [6], so the presence of propoxur resistant (or incipiently resistant) populations of *Ae. aegypti* seems to be a result of accidental exposure to propoxur (via pest control tools) or cross resistance to other xenobiotics [31]. The resistance mechanisms providing resistance against propoxur are generally similar to mechanisms of organophosphate resistance. In one of the tested population, *i.e.* NDP resistance against both

propoxur and temephos was noted along with an increased activity of CCEs. Furthermore, pre exposure to TPP, was found to restore the susceptibility against both the insectides, implying the possibility that CCEs mediated detoxification may be governing the cross resistance between propoxur and temephos in NDP population.

Majority of the studied larval *Ae*. *aegypti* populations were found to be susceptible to temephos except one population *i.e*. NDP population. The NDP population was reported to possess the highest RR50 value, *i.e*. 35.09, as well as the lowest mortality percentages among the tested populations (incipient resistance against 0.0200 ppm and resistance against 0.0125 ppm of temephos (Table 2). The NDP mosquito population were collected from areas around the ASEAN trade network highway, the consequences of possessing such insecticide resistance thus appears dangerous to not only India but to neighbouring countries also. The presence of mosquito population resistant to temephos seems to be an obvious result of regular spray of temephos as the choice of larvicide against both dengue and malaria vector control in Governmental and corporate sectors of India [6]. Since, temephos is the widest used larvicide in India [6], development of resistance against this larvicide may have serious implications in dengue prevention efforts [32].

Mainly, the insecticide detoxification enzyme groups associated with resistance against temephos are CCEs [33–34], however some studies also suggest the role of other detoxifying enzymes such as CYP_{450}s and GSTs [35]. The use of synergist TPP but not PBO was found to restore the susceptibility to temephos in NDP population, thus pinpointing the mechanism of temephos resistance in this population to be CCE mediated metabolic detoxification. Through the results of detoxifying enzyme activity also, similar inference could be made since the activity of α- and β- CCEs were noted to be significantly higher in NDP population compared to other tested populations (Table 4). Involvement of CCEs in development of resistance against temephos have also been noted in many populations of *Ae*. *aegypti* throughout the world [33–34, 36].

Conclusion

For an efficient vector control, the instance of insecticide resistance against such a multiple group of insecticides needs proper attention and action. In that context, regular monitoring throughout the study area is inevitable. Furthermore, to gain a complete knowledge of prevailing insecticide resistance mechanism, the mapping of kdr mutations throughout the study region must be done. In some of the *Ae*. *aegypti* populations, where use of synergists along with insecticide could enhance the potency of insecticide must be taken into account when devising an *Aede*s control strategy. From this study, the use of deltamethrin and lambdacyhalothrin seem to be the choice of insecticide for *Ae*. *aegypti* control throughout the study region. Indian Government may introduce newer strategies for integrated vector management such as newer compounds *i.e*. etofenprox, neonecotenoids, azadirachtin *etc* or techniques such as or dopamine receptor antagonists as insecticides or introduction of sterile male mosquito seem to hold potential for mosquito control in future. Advanced studies focusing at gene level may also help gain detailed knowledge about the resistance phenomenon.

Supporting information

S1 Table. Insecticide susceptibility status of adult *Ae. aegypti* against six adulticides.
(DOCX)

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Insecticide resistance in *Aedes albopictus* Skuse from sub-Himalayan districts of West Bengal, India

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ABSTRACT

Dengue is one of the most rapidly spreading infectious diseases prevalent throughout the tropical and sub-tropical regions of the world. In absence of specific medications and vaccines, the sole method of disease prevention relies on vector control mainly using insecticides. But with the advent of Insecticide resistance, worldwide vector control programs are facing failure. In this study, eleven different *Ae. albopictus* population from sub-Himalayan districts of West Bengal, India were investigated as per WHO protocols to find out the current status of insecticide susceptibility against DDT, permethrin and propoxur. Also the role of three insecticide detoxifying enzymes underlying observed resistance was investigated through quantitative and synergistic assays to unveil the mechanism of insecticide resistance. It was found that majority of studied populations were resistant to 4% DDT. Two populations, namely Alipurduar (APD) and Jalpaiguri (JPG) were severely resistant to 0.75% permethrin, whereas only JPG population was found to exhibit severe resistance against 0.1% propoxur. Moreover, the involvement of detoxifying enzymes was also noted in conferring resistance against DDT and Permethrin. This study indicates the inefficacy of DDT in controlling *Ae. albopictus* populations in the study region. This study may help in implementation of an efficient vector control and insecticide resistance management strategies.

1. Introduction

Aedes albopictus Skuse, the Asian tiger mosquito is one of the most invasive insect species transmitting numerous diseases of public health concern (Badieritakis et al., 2018). Originating from Southeast Asian countries, this species has successfully established itself throughout varying and challenging environments in different countries as an after effect of globalization. Like it's closely related species, *Ae. aegypti*, this species is also responsible for transmission of infective viruses of several mosquito borne disease such as dengue, chikungunya, yellow fever, Zika *etc*. Earlier, only *Ae. aegypti* was known to act as a vector of several diseases. Since the detection of dengue virus (DENV) in *Ae. albopictus*, this species is now considered potential disease vector throughout the world. Recent reports indicate its role as primary vector in disease outbreaks (Bonizzoni et al., 2013) along with susceptibility to infection and competitive exclusion of *Ae. aegypti*, thereby increasing the mass awareness along with control approaches targeting this species (Wong et al., 2013). In India, both the disease transmitting species of *Aedes* mosquitoes are widely distributed and have been reported for outbreaks of dengue and chikungunya (Gratz, 2004). In India, *Ae. albopictus* has been found to carry the DENV acquired either through vertical transmission (Tewari et al., 2004) or transovarian transmission and sometimes through a combination of both (Kumari et al., 2011).

DENV is the most dangerous flavivirus in terms of human morbidity and mortality (Neupane et al., 2014). Dengue is now endemic to 100 countries with approximately 2.5 billion people inhabiting such areas (Guzman et al., 2010). Moreover, annually around 2,50,000–5,00,000 cases of DHF occur worldwide (Guzman et al., 2010). Annually, around 100,000 infections of dengue occur in India. During last two years, infection rates were recorded even higher than that (NVBDCP Dengue, 2018). Chikungunya caused by an Alphavirus, has been reported to occur in around 40 countries (WHO, 2018). In 2006, around 1.5 million infections of chikungunya occurred in India alone (WHO, 2018). Recent re-emergence of Zika virus, also poses a risk to human population throughout the world (Singh, 2017).

Till date, the efficient management of *Aedes* mosquito borne diseases depends on prevention and treatment of the disease symptoms (NVBDCP Dengue, 2018). A major part of disease prevention is fulfilled by chemical vector control with the use of synthetic adulticides and larvicides. Since, its first discovery and usage, the phenomenon of

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Insecticide resistance has become widespread among difference arthropods transmitting diseases to humans. Insecticide resistance may be defined as a measure to withstand the effects of insecticide by developing some kind of tolerance in the insect body against the applied chemicals (Corbel and N'Guessan, 2013). Insecticide resistance through metabolic detoxification makes use of the enzyme groups present inside the insect body to detoxify the insecticide molecules, whereas in target site alteration, minor changes in the target site gene renders the insecticide molecules incapable of binding to its supposed target. Major enzyme systems involved in conferring insecticide resistance belong to enzyme groups such as Carboxylesterases (CCEs), Cytochrome P450 s (CYP_{450s})/ monoxygenases, Glutathione S-transferases (GST), Acetylcholinisterases (AchEs) *etc*. The common sites of target site mutations are either sodium channel gene (common target for Synthetic pyrethroid and Organochlorine insecticides) or Acetylcholinisterase gene (target site for Organophosphates and Carbamates) (Corbel and N'Guessan, 2013).

Development of Insecticide resistance results in failure in achieving the targeted aims of any vector control approach, so insecticide resistance management (IRM) has become an inevitable part of it. Monitoring of prevailing levels of insecticide resistance throughout different regional population of *Aedes* mosquitoes may help in implementation of effective and sustainable arbovirus vector control approaches (Singh et al., 2014). This study focuses on the prevailing insecticide susceptibility levels among wild population of *Ae. albopictus* from districts of sub-Himalayan west Bengal, India against three groups of insecticide, *i.e.* Organochlorine, Synthetic pyrethroid and Carbamate. The results of this study may prove helpful to the officials engaged in designing of vector control programmes for an effective strategy incorporating methods to combat/ manage the phenomenon of insecticide resistance.

2. Materials and methods

2.1. Selection of sampling sites and collection of mosquitoes

Eleven different sampling sites of West Bengal, namely, Siliguri town (SLG), North Bengal University (NBU), Alipurduar (APD), Hasimara (HAS), Kumargram (KMG), Jalpaiguri (JPG), Nagrakata (NGK), Newmalbazar (NMZ), Islampur (ISL), Khoribari (KHR) and Coochbehar (COB). All the selected sites belonged to five districts of northern part of West Bengal *i.e.* Darjeeling, Alipurduar, Jalpaiguri, North Dinajpur and Coochbehar. The geographical map of the sampling site is provided in Fig. 1. These sites were selected based on the prevalence of a very high number of vector borne diseases particularly dengue and malaria. The details of the selected sampling sites and related disease epidemiology have been recorded in supplementary Table S1. Moreover, the selected area lacks any scientific study helpful for prevention of these diseases. All the selected sampling sites were monitored critically for different life stages of mosquitoes. Wide range of mosquito breeding habitats such as cemented tanks, tyres, coconut shells, plant axils, plantation pots, tree holes *etc* were searched for the presence of *Aedes* mosquitoes. Once initial identification as *Aedes* was done, the immature life stages of *Aedes* mosquitoes were collected in plastic containers and brought to the laboratory. The sampling was done during March to November 2017, pre-monsoon, monsoon and post-monsoon seasons.

2.2. Rearing of field caught population of mosquitoes

In the laboratory, the larvae were identified upto species level following standard identification keys (Farajollahi and Price, 2013). The field collected larvae (F_0) were then reared at temperature 25 ± 2 °C and 70–80% relative humidity. The rearing was done based on the standard method (Bharati et al., 2017) for successive generations. The larvae were reared to F_1 generation upto adults to ensure the homogeneity of the field collected populations. The emerged adults were cross checked with adult identification keys (Tyagi et al., 2015). The emerged F1 adults were used for bioassays and biochemical enzyme activity studies for the field collected populations. For setting up the susceptible culture, different mosquito populations were collected and tested for insecticide susceptibility status. One population collected from an organically managed garden was found to possess the highest insecticide susceptibility levels and thus was selected to be reared (upto F10) for use as control culture (CC). This population was allowed to grow for successive generations without any exposure to insecticides in the laboratory maintaining the same physical factors as mentioned above. A mixture of yeast powder and ground fish feed powder was provided as food source for larvae, whereas 5% sucrose solution was provided to the adults. Additionally, anesthetized rat was provided as a source of blood meal for the completion of gonotrophic cycle of females in each generation. Three days old female mosquitoes were fed blood for two days (six hours each day). The tenth generation larvae and adults (F10) were used as Culture Control (CC).

2.3. Insecticide source

Insecticide impregnated papers, *i.e.* DDT (4%), Propoxur (0.1%) and Permethrin (0.75%) were purchased from Vector Control Research Unit, Universiti sains Malaysia, Malaysia. These insecticides were selected based on the history of insecticide usage in India. DDT represents the past usage, permethrin represents the present and propoxur represents an insecticide that may be used in the country in near future for chemical control of mosquitoes.

2.4. Adult bioassay

Adult bioassays were performed following the standard WHO protocols (WHO, 2006) with minor modifications. Around 20–25 non blood-fed adult mosquitoes were exposed to insecticide impregnated papers with WHO recommended diagnostic dose of insecticide (4% DDT, 0.1% Propoxur and 0.75% permethrin) for 1 h. At an interval of ten minutes, *i.e.* 10, 20, 30, 40, 50, 60 min the total number of mosquitoes knocked down were noted. After an hour, the mosquitoes were transferred to a tube that carried cotton balls soaked in 10% glucose solution. The whole experimental set up was maintained at laboratory conditions for 24 h. Mortality was recorded 24 h after the exposure to insecticides. For control, mosquitoes were place in tubes containing papers impregnated with silicone oil and acetone. The whole experiment was set along with three replicates. Mortality percentages were calculated as the mean of all set of assays.

2.5. Synergism studies

Synergism tests were conducted to evaluate the role of insecticide detoxifying enzymes on degradation of insecticides. Piperonyl butoxide (PBO) (CYP_{450s} inhibitor, 90% Sigma), and triphenyl phosphate (TPP) (CCE inhibitor, 99% Sigma), were used. Sub-lethal doses of both the synergists, *i.e.* 4% and 10% for PBO and TPP were used in synergism tests. Each population was exposed for one hour to synergist (PBO/ TPP) and then subjected to insecticide exposure. This study was conducted in triplicate.

2.6. Insecticide detoxifying enzymes' activity

Single adult *Ae. albopictus* were homogenized in 100 µL of 0.1 M sodium phosphate buffer (pH 7.2) with a teflon micro-pestle in a 1.5 mL centrifuge tube. The pestle was washed with another100 µL of 0.1 M sodium phosphate buffer (pH 7.2) making the whole solution 200 µL. The homogenate thus obtained was centrifuged at 12,000 rpm (revolutions per minute) for 15 min in a table top centrifuge (Eppendorf centrifuge 5417R, Eppendorf, Germany.) (Bharati et al., 2016). The

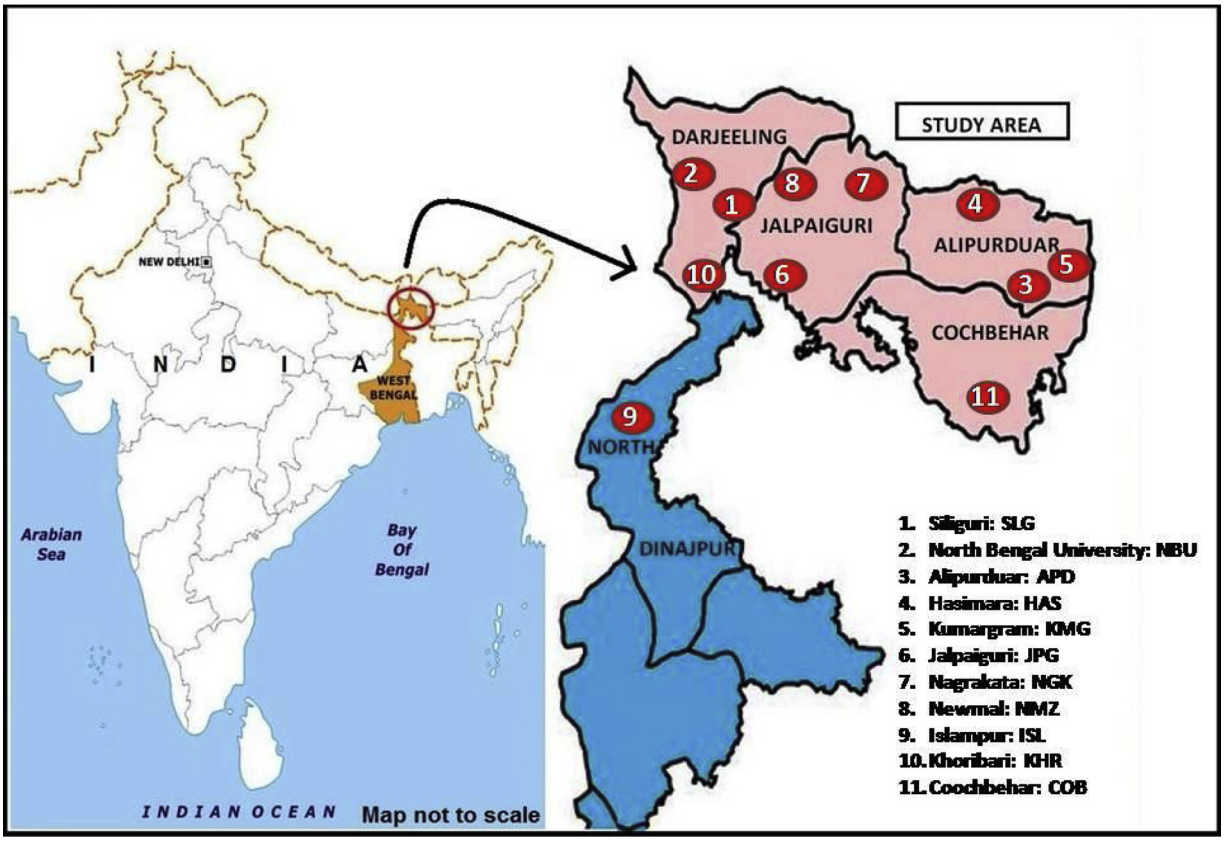

Fig. 1. Different sampling sites (1–11) in the study area spread over five different districts of West Bengal, India.

supernatant was stored at -20 ± C and was used freshly as enzyme source for detoxifying enzyme activity assays. For each biochemical test, a minimum of thirty individuals were assayed along with a duplicate set for each enzyme assay.

2.6.1. Carboxylesterases (non-specific esterase) assay

The activity of carboxylesterases (CCE) hydrolyzing α- and β- napthyl acetate as substrate were assayed according to van Asperen (1962) with minor modifications for using in microplate. Fast blue B salt was used as the staining agent. Absorbance was recorded at 540 nm using microplate reader (Biotek ELx800, USA). Standard curves of α- and β-napthol were prepared and the CCE activity were estimated.

2.6.2. CYP_{450s} assay

The activity of CYP_{450s} were measured according to Brogdon and Janet, 1997 using the substrate 3,3′,5,5′-Tetramethyl Benzidine (TMBZ) and peroxidising agent H_2O_2. Absorbance was recorded at 630 nm using the microplate reader (Biotek Elx800, USA). A standard curve for the heme peroxidase activity was also prepared using serial concentrations of cytochrome c (0.0025 nM to 0.0200 nM) for horse heart type VI (Sigma Aldrich). The absorbance values thus observed were converted to CYP_{450s} equivalent units (EUs) in mg protein with the help of standard curve.

2.6.3. Glutathione S-transferase (GST) assay

GST activity was assessed following the standard protocol (Habig et al., 1974), 10 µL of enzyme was mixed with 200 µL of CDNB/GSH (working solution) in wells of microtitre plate. Blanks were set with 10 µL of distilled water along with 200 µL of working solution. The plate was read continuously for 5 min at 340 nm in spectrophotometer (Rayleigh UV-2601, Beijing Rayleigh Analytical Instrument Corporation) and the GST activity (µmoles mg protein^{-1} min^{-1}) was calculated.

2.6.4. Total protein content

Total protein of each individual of *Ae. aegypti* was determined according to Lowry et al. (Lowry et al., 1951) to cancel out any size differences among individuals and for the correct expression of enzyme activity.

2.7. Calculation

In the bioassays, control mortalities were below 5%, so no calculation of corrected mortality was needed. The population with mortality percentages when > 98 is said to be susceptible, 80–97 is assessed as resistance not confirmed (= unconfirmed resistance = incipient status) and < 80 as resistant (WHO, 2006). For the determination of knock-down rates, a log-probit software (SPSS 16.0) was used.

3. Results

Widespread resistance was observed amongst the tested mosquito populations against DDT, *i.e.* mortality percentages were noted to range from 40.0 to 100.0%. Three of the populations, *i.e.* SLG, JPG and NGK were found to possess severe resistance against DDT (mortality percentages < 80%), whereas rest of the populations were found to be incipiently resistant or susceptible to 4% DDT (Table 1). Against permethrin, two of the populations *i.e.* APD and JPG were found to be severely resistant with mortality percentages 75.4 and 75.0% respectively. However, against propoxur, only one population *i.e.* JPG was found to be severely resistant with mortality rate 42.5%, whereas five of the tested population were susceptible and rest had unconfirmed resistance. In majority of the population both PBO and TPP could increase the potentiality of DDT, though partially (Table 2). Exposure to PBO increased the mortality percentage against DDT from 40% to 81.2% in SLG population. Similar elevation of mortality percentages were also noted in some other populations. Against permethrin, PBO

Table 1
Insecticide susceptibility/resistance profile amongst field collected *Ae. albopictus* mosquitoes (n = 100) against DDT (4%), Permethrin (0.75%) and Propoxur (0.1%).

Sampling site	Mortality percentages								
	DDT (4%)			Permethrin (0.75%)			Propoxur (0.1%)		
	Test	Control	Status	Test	Control	Status	Test	Control	Status
SLG	40.0 ± 1.24	0.0	R	100 ± 0.00	0.0	S	92.3 ± 0.81	0.0	IR
NBU	96.0 ± 0.72	0.0	IR	100 ± 0.00	0.0	S	92.0 ± 0.72	0.0	IR
APD	92.8 ± 0.88	1.9	IR	75.4 ± 1.31	0.0	R	100.0 ± 0.00	0.0	S
HAS	88.9 ± 1.92	0.0	IR	96.2 ± 0.66	0.0	IR	100.0 ± 0.00	0.0	S
KMG	94.3 ± 0.92	0.0	IR	100 ± 0.00	0.0	S	100.0 ± 0.00	1.6	S
JPG	75.4 ± 1.66	2.3	R	75.0 ± 1.46	2.3	R	42.5 ± 2.12	0.0	R
NGK	63.1 ± 2.10	0.0	R	92.1 ± 1.10	1.2	IR	99.3 ± 0.64	0.0	S
NMZ	#	#	–	95.2 ± 0.66	0.0	IR	89.7 ± 0.91	0.0	IR
ISL	93.3 ± 1.1	0.0	IR	100.0 ± 0.00	0.0	S	80.1 ± 1.7	0.0	IR
KHR	100 ± 0.00	0.0	S	100 ± 0.00	0.0	S	98.9 ± 0.80	0.0	S
COB	90.0 ± 0.94	0.0	IR	96.4 ± 0.72	0.0	IR	90.9 ± 0.91	1.2	IR
CC	100 ± 0.00	0.0	S	100 ± 0.00	0.0	S	100 ± 0.00	2.4	S

# denotes experiment couldn't be performed because of low sample volume, S: susceptible, IR: Incipient resistance/ Resistance not confirmed, R: Resistance.

treatment prior to insecticide exposure increased the mortality from 75.4% to 93.6 and 75.0 to 88.3 for APD and JPG population respectively. Against propoxur, only JPG was found resistant and using the synergists could not elevate the potentiality of insecticide in that population. However no strict conclusion can be made on the involvement of metabolic detoxification in resistance against propoxur.

The KDT_{50} and KDT_{95} values were noted to be very high amongst the tested field populations for DDT, *i.e.* the KDT_{50} values ranged from 70.32 to 230.21, whereas KDT_{95} values ranged from 84.59 to 415.66 (Table 3). However, the knockdown rates against permethrin were low for most of the tested populations except one, *i.e.* JPG (Table 3). The KDT_{50} and KDT_{95} values against permethrin were 141.12 and 248.38 respectively for JPG population. Against propoxur, the lowest values were observed for both KDT_{50} and KDT_{95}, *i.e.* the KDT_{50} values ranged from as low as 7.84 to 130.25 and KDT_{95} values ranged from 20.20 to 379.76 min. Against propoxur also, the highest KDT_{50} and KDT_{95} values were observed for JPG population as observed against other tested insecticides.

Varying levels of detoxification enzyme activity were noted amongst different field collected populations of *Ae. albopictus* (Table 4). The activity of α-CCEs ranged from 0.38 to 2.87 μmoles mg protein^{-1} min^{-1}, *i.e.* 1.11 to 8.44 times than CC whereas that of β-CCEs, ranged from 0.36 to 0.98 μmoles mg protein^{-1} min$^{-1,}$ *i.e.* 1.16 to 3.16 times amongst examined field populations. The highest values for both α-CCEs and β-CCEs were noted for NGK population. The activity of CYP_{450s} were noted to be 1.06 to 1.93 times than the activity of CC for field populations. Similarly, the activity of GSTs were noted to be 0.289 to 0.372 μmoles mg protein^{-1} min^{-1} The highest activity of GSTs was noted for SLG population, *i.e.* 1.28 times higher than the activity of CC.

4. Discussion

The aim of this study was to assess the prevailing insecticide resistance status and its mechanism among different field caught populations of *Ae. albopictus* in dengue endemic districts of Northern West Bengal, India. DDT, an Organochlorine insecticide has been used in India since its discovery though not specifically to dengue vectors rather against malarial vectors. In this study, only one, *i.e.,* KHR population was found to be completely susceptible to DDT (Table 1). Mortality percentages against DDT varied from as low as 40.0% to 96.0% among other tested populations (Table 1). DDT has been used continuously since last six decades throughout the country. The property of low degradability in the environment, its history of heavy usage throughout the study site along with the results of this study impart light on the insecticide selection pressure prevailing throughout the study sites against DDT (Vieira et al., 2001). Similar studies on *Ae. aegypti* from nearby sites also exhibited similar pattern of resistance against DDT (Bharati and Saha, 2018). DDT resistant colonies of *Ae. albopictus* are widespread throughout India, *i.e.* Assam (Das and Dutta, 2014; Dhiman et al., 2014; Yadav et al., 2015), Orissa (Rath et al., 2017), Andaman- Nicobar islands (Sivan et al., 2015); other Southeast Asian countries, Pakistan (Arslan et al., 2016), Malaysia (Ishak et al., 2015) and also recently invaded countries, United States of America (Marcombe et al., 2014), Central African Republic (Kamgang et al., 2011; Ngoagouni et al., 2016). The KDT50 and KDT95 values against DDT were also comparatively higher than that of the culture control. Even in the least resistant population amongst the tested population, *i.e.* NBU, the value of KDT50 was 70.32 which again indicate the reduced potential of DDT in control of these mosquitoes (Dia et al., 2012).

Through the synergistic study, it was found that both CYP_{450s} and CCEs could partially restore the susceptibility of the field collected *Ae. albopictus* to DDT in majority of the tested population particularly in SLG, APD, JPG and NGK. Moreover, through the study of detoxifying enzymes activity, it was found that, all the field caught population exhibited similar levels (< 2 fold) of GST and CYP_{450s} activity (Higher than CC), which is in conjunction with the absence of DDT susceptible population of *Ae. albopictus* in most of the tested area. GST is generally implicated to carry out the detoxification of DDT to DDE, thereby resulting in resistance against it (Lumjuan et al., 2011). Some study also point on the role of other detoxifying enzyme groups, such as CCEs or CYP_{450s} in conferring resistance against DDT in *Ae. albopictus* (Das and Dutta, 2014; Ishak et al., 2015). Field strains of many *Aedes* mosquitoes have been found to express moderate to severe resistance against DDT driven by an elevated GST activity (Aponte et al., 2013; Marcombe et al., 2014; Das and Dutta, 2014) or CYP_{450s} activity (Ishak et al., 2015). Similar inferences can also be drawn in our study. Target site mutations, commonly known as kdr mutation (for DDT and SP) also form an equally important resistance providing mechanism against DDT and SPs in *Aedes* mosquitoes (Aponte et al., 2013; Maestre-Serrano et al., 2014; Kushwah et al., 2015; Goindin et al., 2017). So, the role of kdr mutations in providing resistance against DDT cannot be ruled out, however, a recent study from nearby area reported the absence of any major kdr mutation in *Ae. albopictus* populations (Chatterjee et al., 2018). So, the major mechanism underlying DDT resistance amongst the tested field populations can be inferred to be through enhanced detoxification by insecticide detoxifying enzymes, namely GSTs and CYP_{450s}. Moreover, throughout the world, very few studies have reported the presence of major kdr muations in *Ae. albopictus* (Kasai et al., 2011; Obando et al., 2017; Chen et al., 2016; Xu et al., 2016), whereas other such studies couldn't find any such mutation in this mosquito (Ishak et al., 2015). So, it seems that in *Ae. albopictus*, the role of insecticide detoxifying enzymes seem to be the predominant mechanism

Table 2
Susceptibility against insecticide along with synergists (piperonyl butaoxide and triphenylphosphate) of different adult *Ae. aegypti* populations (n ≥70).

Sampling sites												
	SLG	NBU	APD	HAS	KMG	JPG	NGK	NMZ	ISL	KHR	COB	CC
DDT + PBO	81.25 ± 0.7	96.6 ± 0.4	97.0 ± 0.3	90.9 ± 0.5	96.2 ± 0.3	94.1 ± 0.3	78.2 ± 0.6	#	96.1 ± 0.3	100 ± 0.0	94.5 ± 0.4	100 ± 0.0
DDT + TPP	59.0 ± 1.1	97.1 ± 0.2	90.90 ± 0.5	92.1 ± 0.4	94.9 ± 0.5	79.8 ± 0.6	69.7 ± 0.9	#	93.4 ± 0.6	100 ± 0.0	92.1 ± 0.3	100 ± 0.0
Permethrin + PBO	100 ± 0.0	100 ± 0.0	93.6 ± 0.3	100	100 ± 0.0	88.3 ± 0.6	95.6 ± 0.3	100 ± 0.0	100	100 ± 0.0	100 ± 0.0	100 ± 0.0
Permethrin + TPP	100 ± 0.0	100 ± 0.0	81.16 ± 0.6	97.2 ± 0.3	100 ± 0.0	72.4 ± 0.8	91.5 ± 0.03	95.1 ± 0.3	100	100 ± 0.0	95.8 ± 0.3	100 ± 0.0
Propoxur + PBO	91.6 ± 0.3	92.4 ± 0.4	100 ± 0.0	100 ± 0.0	100 ± 0.0	44.1 ± 1.2	100 ± 0.0	88.4 ± 0.5	81.1 ± 0.8	100 ± 0.0	87.6 ± 0.5	100 ± 0.0
Propoxur + TPP	95.1 ± 0.3	93.7 ± 0.2	100 ± 0.0	100 ± 0.0	100 ± 0.0	50.2 ± 0.9	100 ± 0.0	90.5 ± 0.4	83.2 ± 0.7	100 ± 0.0	91.4 ± 0.5	100 ± 0.0

# denotes experiment couldn't be performed because of low sample volume.

of resistance against DDT and Pyrethroids.

Two out of the eleven tested populations were found to exhibit severe resistance against permethrin, whereas, other five populations were found to be susceptible and another four with unconfirmed resistance. Resistance against pyrethroid insecticides are generally believed to be carried out either by metabolic detoxification by mainly CYP_{450s} and partly by CCEs and GSTs or through target site mutations, *i.e.* kdr as against DDT (Corbel and N'Guessan, 2013). Permethrin resistant populations of *Ae. albopictus* have been reported from Srilanka (Karunaratne et al., 2013). In this study, we found that pre-exposure to PBO resulted in increased mortality percentages for APD population, from 75.4 to 93.6 and for JPG population 75.0 to 83.3 against permethrin. Also, the population with highest level of permethrin resistance, i.e. JPG and APD possessede the highest activity for CYP_{450s} enzyme (Table 4). Similar correlation between elevated CYP_{450s} activity and permethrin resistance have also been found to occur in Malaysia (Ishak et al., 2015), USA (Marcombe et al., 2014), Central African Republic (Ngoagouni et al., 2016). The commonly used mosquito repellent tools contain formulations of permethrin or similar pyrethroid compounds which may have caused a selection in those (resistant) populations resulting in such pattern of resistance (Chareonviriyahpap et al., 1999). In a study from the similar area, *Ae. albopictus* populations completely susceptible to two other pyrethroids (type II) were noted (Bharati and Saha, 2017). This pinpoints on the fact that detoxification of type I and type II pyrethroids can be brought by independent mechanisms. Also some studies have reported that, resistance against pyrethroid insecticides is mainly a result of kdr mutations and partially due to enhanced activities of CYP_{450s} (Choovattanapakorn et al., 2017), yet some others also confirm the role of metabolic detoxification through CYP_{450s} alone in pyrethroid resistance (Ishak et al., 2016). Permethrin is one of the insecticides which is widely used in long lasting treated nets (Gunasekaran et al., 2009) and also to control agricultural pest populations in India (Kumar et al., 2013). The vicinity of human habitable areas with agricultural land in India may also provide an indirect exposure to mosquitoes (as non target species) and thus contribute towards the observed resistance (Overgaard et al., 2005).

We have also tested the susceptibility status of field caught *Ae. albopictus* population against a carbamate insecticide, *i.e.* propoxur, which is not generally recommended in mosquito control approaches in India (NVBDCP, dengue India). But, surprisingly, it was found that, one of the field populations was severely resistant and five were incipiently (unconfirmed) resistant against this carbamate (Table 1). The observed resistance (both severe and intermediate) could be a result of heavy use of some household pest control tools that contain propoxur as the key insecticidal component, such as Baygon (S. C. Johnson & Son, Inc) Flykill (Act Agro Chem Private Limited, India) *etc*. Moreover, propoxur is also heavily used in tea plantations throughout the study area, which may also provide a cross exposure to mosquitoes by contaminating mosquito breeding sites and thereby lead to resistance (Overgaard et al., 2005; Bharati and Saha, 2017). Resistance against carbamates are generally known to be conferred either by detoxification through CCEs or mutations in acetylcholinesterase (ace-1) gene, which is the target site for OP and CCEs insecticides (Hemingway and Karunaratne, 1998; Ngoagouni et al., 2016). In our study, pre-exposure to TPP and PBO couldn't increase the potentiality of propoxur in tested *Ae. albopictus* populations. Moreover, though we have recorded elevated activities of CCEs in most of the propoxur resistant populations, yet no strict correlation could be made between the two. This brings into light the importance of *ace* major mutation in the propoxur resistance, which may be the mechanism in this study too. Since, till date no propoxur resistant *Ae. albopictus* population has been reported, though intermediate resistance was reported (Ngoagaouni et al., 2016; Karunaratne et al., 2013), this study seems to be the first ever report of severely resistant (mortality percentage = 42%) field collected population of *Ae. albopictus* against propoxur. However, resistance against other commonly used carbamate, *i.e.* Bendiocarb has been noted (Ishak et al.,

Table 3
Knockdown rates (KDT_{50} and KDT_{95}) of different *Ae. albopictus* populations (n ≥ 90) against DDT (4%), permethrin (0.75%) and propoxur (0.1%).

Sampling site	DDT		Permethrin		Propoxur	
	KDT_{50} ± S.D.	KDT_{95} ± S.D.	KDT_{50} ± S.D.	KDT_{95} ± S.D.	KDT_{50} ± S.D.	KDT_{95} ± S.D.
SLG	230.21 ± 4.2	415.66 ± 5.3	21.94 ± 1.2	65.86 ± 0.9	41.42 ± 1.1	139.98 ± 1.8
NBU	70.32 ± 2.1	184.59 ± 1.8	42.90 ± 1.4	192.28 ± 1.1	46.44 ± 0.9	178.77 ± 2.2
APD	82.19 ± 1.7	169.68 ± 2.1	58.20 ± 1.2	238.52 ± 2.9	7.84 ± 0.6	20.20 ± 0.9
HAS	91.72 ± 2.0	189.69 ± 1.7	83.03 ± 1.9	187.35 ± 1.8	47.87 ± 1.0	251.69 ± 3.2
KMG	82.22 ± 1.9	171.31 ± 1.9	42.31 ± 1.1	69.53 ± `1.2	23.64 ± 0.9	72.21 ± 1.2
JPG	139.33 ± 3.1	326.91 ± 3.1	141.12 ± 2.0	284.38 ± 2.3	130.25 ± 1.2	379.76 ± 4.3
NGK	190.53 ± 2.1	375.67 ± 2.9	57.19 ± 0.9	137.41 ± 1.2	9.69 ± 0.7	25.30 ± 0.9
NMZ	#	#	54.32 ± 1.2	142.47 ± 1.6	42.29 ± 0.9	129.16 ± 1.6
ISL	70.01 ± 1.9	81.12 ± 2.0	19.34 ± 0.9	55.42 ± 2.1	45.61 ± 1.1	131.04 ± 1.9
KHR	40.12 ± 2.0	112.64 ± 1.8	24.67 ± 1.2	48.14 ± 2.3	16.15 ± 0.7	36.14 ± 1.2
COB	74.55 ± 1.7	174.17 ± 2.4	78.96 ± 1.4	177.57 ± 2.4	48.59 ± 1.2	212.34 ± 2.4
CC	65.35 ± 1.7	169.13 ± 2.1	20.82 ± 1.1	66.12 ± 1.0	6.31 ± 0.7	18.41 ± 0.8

2015). Some reports also suggest the role of CYP_{450s} behind resistance against Bendiocarb in adult (Ishak et al., 2015) and in larva (Marcombe et al., 2014), results of this study also seem to support above inferences since similar correlation has been noted in this study between propoxur resistance and CYP_{450s} activity with JPG population recording the highest values for both. Amongst the tested populations, JPG was found to be resistant to all the three insecticides along with a higher activity of all the detoxifying enzymes assayed.

5. Conclusion

From this study, we have observed that throughout the dengue endemic region of West Bengal, moderate to severe resistance against majority of the tested insecticides exist. With the recent increase in dengue infection rates, this pattern of insecticide resistance may prove to be very dangerous to people inhabiting this region. For an efficient prevention of this disease in next season, a well planned vector control strategy incorporating the results of this study and tools to manage/minimize insecticide resistance in the field population should be implemented. Since, very few resistant populations of *Ae. albopictus* were recorded against permethrin and propoxur, these two insecticides may be used in chemical vector control during intense disease outbreak, but a thorough surveillance and follow-up of insecticide resistance and involved mechanisms must be done. Rotation of insecticides seems to be the most suitable method of vector control to prevent / minimize the development of insecticide resistance in this region. The study of population dynamics of *Aedes* mosquito should be aimed for accurate prediction of the upcoming mosquito proliferation season (=disease outbreak season), so that the vector population could be controlled even before the disease proliferates thereby minimizing infection rates. Also, though very scanty report exists on the presence of kdr mutations in *Ae. albopictus,* yet regular surveillance of kdr mutations should be done to prevent fixation of such mutations (once developed) within a population. Indian government may also introduce newer insecticides for dengue vector control such as Bti (*Bacillus thuringiensis israelensis*) or IGRs (Insect growth regulators), against which no resistance has been recorded till date. In addition, the specificity of Bti to dipterans reduces the risk of harming non target species. Most of the insecticides seem to be dangerous to non target species as well as the environment, so hunt for safer alternatives such as phytochemicals with mosquitocidal or repellent potency is the prime need of today. Indian government should also take initiatives for safe disposal of waste materials, sanitation, regular garbage collection and adequate water supply, along with popular participation and society's encouragement towards the mechanical elimination of breeding sites - which certainly has a very significant impact in the population density without affecting insecticides resistance. Since, the study sites are endemic to only to dengue but to other mosquito borne diseases also such as, malaria, chikungunya, Japanese encephalitis, filariasis *etc*, an integrated vector control strategy should be designed addressing all the disease causing mosquito vector populations in this region. Advanced techniques in identification of key resistance mechanisms may also prove helpful for a proper designing of an integrated mosquito management (IMM) or even an integrated vector management (IVM) approach.

Declaration of interest

The authors declare that they have no conflict of interest.

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Table 4
Activity of major insecticide detoxifying enzymes in different field caught populations of *Ae. albopictus* (n ≥ 50).

Sites	α-CCEs (μmoles mg protein^{-1} min^{-1})	β-CCEs (μmoles mg protein^{-1} min^{-1})	CYP_{450s} (nmoles mg protein^{-1} min^{-1})	GSTs (μmoles mg protein^{-1} min^{-1})
SLG	0.82 ± 0.005^{b*}	0.88 ± 0.007^{b}	0.034 ± 0.0009^{a}	0.372 ± 0.0025^{a}
NBU	0.38 ± 0.002 ^{a}	0.36 ± 0.004^{a}	0.034 ± 0.0007^{a}	0.318 ± 0.0009^{a}
APD	0.49 ± 0.003^{a}	0.85 ± 0.005^{b}	0.056 ± 0.0011^{b}	0.341 ± 0.0013^{a}
HAS	0.84 ± 0.007^{b}	0.49 ± 0.006^{a}	0.036 ± 0.0005^{a}	0.331 ± 0.0011^{a}
KMG	0.42 ± 0.003 ^{a}	0.39 ± 0.002^{a}	0.033 ± 0.0006^{a}	0.311 ± 0.0009^{a}
JPG	0.66 ± 0.005^{b}	0.43 ± 0.002^{a}	0.062 ± 0.0009^{b}	0.328 ± 0.0015^{a}
NGK	2.87 ± 0.012^{c}	0.98 ± 0.008^{b}	0.051 ± 0.0007^{b}	0.385 ± 0.0021^{a}
NMZ	0.69 ± 0.007^{b}	0.48 ± 0.005^{a}	0.039 ± 0.0005^{a}	0.330 ± 0.0016^{a}
ISL	0.39 ± 0.003^{a}	0.38 ± 0.002^{a}	0.036 ± 0.0007^{a}	0.321 ± 0.0022^{a}
KHR	0.37 ± 0.005 ^{a}	0.35 ± 0.004^{a}	0.033 ± 0.0004^{a}	0.305 ± 0.0017^{a}
COB	0.51 ± 0.007	0.48 ± 0.007	0.038 ± 0.0009	0.338 ± 0.0014^{a}
CC	0.34 ± 0.001	0.31 ± 0.004	0.032 ± 0.0006	0.289 ± 0.0012^{a}

* Within columns, means followed by the same letter do not differ significantly (P = 0.05) in Tukey's multiple comparison test (HSDa).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.02.007.

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