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Status of insecticide resistance and detoxifying enzyme activity of *Aedes albopictus* population in Sonitpur district of Assam, India

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Abstract
Sonitpur district, Assam (India) is well known for malaria incidence since many years. The extensive use of insecticides for the control of malaria and other vector species is being carried out for vector management. The insecticide resistance status of *Aedes albopictus* (Diptera: Culicidae) to DDT (dichloro-diphenyl-trichloroethane) and deltamethrin was studied in the Sonitpur district of Assam. Even today DDT is the most widely used insecticides for public health programs in India. Recent information on the level of resistance to DDT and deltamethrin in an *Aedes* mosquito population of North East India is scarce. Continued monitoring of insecticide resistance status and identification of the underlying mechanism of resistance in the *Aedes* mosquito population is of prime importance. Insecticide susceptibility assays were performed on wild-caught adult female *Aedes albopictus* mosquitoes to the discriminating doses of DDT (4%) and deltamethrin (0. 05%) recommended by the World health organization (WHO). In all study sites, mortality as a result of DDT varied from 40.2 to 80.2% as compared to 94.2% in the susceptible laboratory strain (S-lab), indicating that *Ae. albopictus* is resistant to DDT in all study sites except Gohpur. The species was found to be 100% susceptible to deltamethrin in all study sites. Results from biochemical assays demonstrated increased alpha esterase, beta esterase and glutathione - S-transferase activity of *Aedes albopictus* at all study sites. Therefore, alpha esterase, beta esterase and glutathione - S-transferase activity seems to be associated with mechanisms responsible for insecticide resistance in *Ae. albopictus.* The results presented here provide the first report and baseline information about the insecticide resistance status of *Ae. albopictus* in North East India, and associated information about biochemical mechanisms that are essential for monitoring the development of insecticide resistance in the area.

Keywords: *Aedes albopictus*, DDT, Deltamethrin, Insecticide resistance

1. Introduction
Mosquito borne diseases are the main public health concern in developing countries like India. Dengue and chikungunya are mosquito borne viral diseases that occur in more than 100 countries, placing two-fifths of the world's population at risk [1]. Epidemic outbreak of dengue in 2006 was reported in all tropical regions of the world, out of which India was the most affected nation [2]. The mosquitoes *Aedes aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1894) are important dengue vectors [3] as well as vectors of other numerous diseases. *Ae.* (*Stegomyia*) *albopictus* (Diptera: Culicidae) is an epidemiological important mosquito responsible for the transmission of many viral pathogens [4, 5]. *Ae. albopictus* received considerable attention in India after recent reports indicating its potential role in disease transmission in various parts of the country [4, 6, 7, 8]. Unplanned urbanization, the lack of proper waste management and inadequate vector control measures are the key factors for emergence of dengue virus cases which are widely prevalent in many of the tropical developing countries including India [9, 10].
In India, the first dengue virus was reported in Delhi State in 1996 [11] and subsequently it spread all over India within the last decade. North East India owing to its remoteness and special climatic conditions were free from Dengue and Chikungunya cases until 2009. However, the disease was prevalent in the rest of India waiting to create havoc in Assam. During 2010, Dengue cases were being reported threatening to emerge as an epidemic form in Assam. Till October 2010, 139 cases were reported with one death. The majority of cases were

reported from Kamrup district (113) followed by Golaghat (8), Dibrugarh (4), Sonitpur (3), Jorhat (3), Barpeta (2), Darrang (2) and one each from Nalbari, Nagaon, Dhemaji and Lakhimpur [12].

Vector control is the only known option to interrupt the transmission of the disease as long as an effective, safe and affordable vaccine is not available for these arboviral diseases. A powerful tool in vector control programmes is the application of insecticides. The use of chemical insecticide DDT, an organochlorine was thought to be the great solution for controlling insects of public health concern. India, especially Assam has witnessed widespread and indiscriminate use of insecticides like DDT, deltamethrin, permethrin etc. Unplanned and irrational use of insecticides has contributed to the development of resistance against insecticides in disease vectors like *Culex, Anopheles* [13]. The recent outbreak of dengue cases necessitated the study of insecticide resistance in *Aedes* populations.

This has made dengue becoming one of the most insecticides targeted vector borne disease [14]. The profound use of insecticides for vector control has led to the development of insecticide resistance in several insect vectors including *Ae. albopictus* [15]. The insecticide resistance mechanism includes the detoxification of insecticides and alteration of insecticide target sites [16]. The insects detoxify insecticides by using enzymes, such as cytochrome P450, esterases (α- and β-) or glutathione S-transferases (GSTs) [16] among others GST plays a role in DDT resistance, while non-specific esterase mostly involved in resistance to organophosphates, carbamates and sometime to pyrethroids [17].

The aim of this study was to evaluate the current status of insecticide susceptibility/resistance in populations of *Ae. albopictus* collected from different areas of Sonitpur District including assessment of the underlying resistance mechanisms present in these vector populations. The insecticide resistance was evaluated using standardized bioassay kits (WHO) and biochemical analysis. This study is immensely important to develop future resistance management strategies and also will lead to the selection of suitable insecticide for mosquito control in these areas.

2. Materials and methods

2.1 Mosquito samples

Sonitpur district (92˚20'E-93˚45'E and 26˚20'N-27˚05'N) of Assam, India is having an area of 5324 km^2 is situated on the north bank of the river Brahmaputra. The study was carried out in different areas of Sonitpur District namely Tezpur, Gogra T.E., Sirajuli, Balipara, Rangapara and Gohpur. The larvae collected from different eco-climatic parts of the district during the period of 2012-2013 .Pre-monsoon, monsoon and post monsoon ovitrap survey was conducted in different parts of Sonitpur District. Dark plastic containers (15 cm diameter; 12 cm depth) were used as an ovitrap. Larvae were collected from the natural breeding habitats as well as from the ovitraps set in residential areas. The collected larvae were reared in the mosquito rearing room at the Defence Research Laboratory, Tezpur. The adults were identified as *Ae. albopictus* mosquitoes based on the standard identification keys of Barraud [18]. Mosquitoes were reared to generation F1 for adult bioassay. The mosquitoes were fed rabbit blood meal. The insecticide susceptibility test was performed on F1 generations of field caught larvae of *Aedes* mosquito and from a mosquito colony maintained in the Defence Research Laboratory for approximately 03 years, Tezpur as a susceptible reference strain (S-Lab) to compare against the susceptibility levels of the field populations. The F1 generations were also used for biochemical assays.

2.2 Insecticide susceptibility bioassays

The insecticide susceptibility test was performed on F1 generations of *Aedes albopictus* mosquitoes. Mortality and knock down were measured using WHO test kits [19]. The tests were carried out using 4 % DDT and 0.05% deltamethrin, the diagnostic doses were recommended by WHO. For each of the insecticide tested, mosquitoes were divided into batches of 20-25 per test and exposed to insecticide-treated papers for 1 h in DDT (4%) and deltamethrin (0.05%). The effects of papers treated only with carrier oils were assayed in parallel as a control. At the end of the exposure period, mosquitoes were transferred into tubes with untreated white filter papers (holding tubes) and allowed a 24 h recovery period.

Mosquito exposed to DDT and deltamethrin, the numbers of knock down were recorded every 10 min for up to 1 h during exposure and mortality rates were recorded over the recovery period. The same bioassays were carried out in the laboratory-reared susceptible strain (S-Lab) to compare with the susceptibility levels of the field populations. During the 24 h recovery period, all mosquitoes were provided with 10% sugar water. Corrected mortality was calculated following the Abbott's formula [20].

2.3 Biochemical assays

The adult non-blood fed 2-3 days old female mosquitoes of F1 generation were used for bioassays were subjected to biochemical analysis. Female mosquitoes were individually homogenized in 1.5 ml micro-centrifuge tubes having 50 µl distilled water and then diluted with 150 µl distilled water. Tubes were kept in ice flakes during the whole homogenization. The homogenates were spun at 12000 g for 2 min at 4 °C. The supernatant was used for biochemical analysis. Three microplates with duplicate mosquito homogenates were used for three enzymes and one microplate for total protein. Each biochemical assay was replicated twice with new individuals from the same mosquito population on two different days. Sample sizes for each biochemical assay ranged from 24 to 80 mosquitoes per location. Absorbance was measured using a Bio-Rad Microplate Reader.

2.4 Total Protein Assay

The total protein content of individual *Ae. albopictus* mosquitoes was determined in order to detect the differences in size among individuals using Lowry *et.al* method [21]. The results were compared with bovine serum albumin (BSA) standard curve. The plates were read at 550 nm wavelength.

2.5 Nonspecific esterase assay

The method of Peiris and Hemingway [22] was employed for the determination of alpha esterase and beta esterase assay. The plates were read at 570 nm wavelength.

2.6 Glutathione S-transferase assay

Glutathione S-transferase assay (GST) activity was assayed according to the method described by WHO [23]. Reduced glutathione was used as the substrate and the plate was read at 340 nm after 20 min incubation as the end point.

2.7 Statistical analysis
Mortality percentage was determined between population from different study sites for a particular insecticide, and the WHO criteria for evaluating resistance or susceptibility in a mosquito population was used [19]. Mosquitoes were considered susceptible to insecticide if the percent mortality was >98 %. Incipient insecticide resistance (tolerance) if final mortality ranging between 80 to 97%. Test populations were considered resistant if percent mortality was below 80%. The results of biochemical analysis in different population were expressed as absorbance values. ANOVA (Dunnet Multiple Comparison Test) was used to compare the protein content and enzyme expression levels between population from different study sites.

3. Results
3.1 Susceptibility bioassay
The susceptibility status of *Aedes albopictus* to a diagnostic dose of DDT 4% and deltamethrin (0.05%) is shown in table 1. The data show that *Ae. albopictus* was resistant to DDT in all study sites except Gohpur. The percent mortality range was 40.2- 80.4%, compared with 94.2% for the S-lab control strain. The highest resistance to DDT was found in Tezpur area, and the lower in Gohpur. Adult bioassays were performed in batches of 20-25 mosquitoes per test, with replicates, and the percentage mortality values of replicates are presented in table1. All populations of *Ae. albopictus* were found to be 100% susceptible to deltamethrin (0.05%) in all study sites.

Table 1: Susceptibility and resistance status of *Aedes albopictus* to diagnostic dose of DDT (4%) and deltamethrin (0.05%).

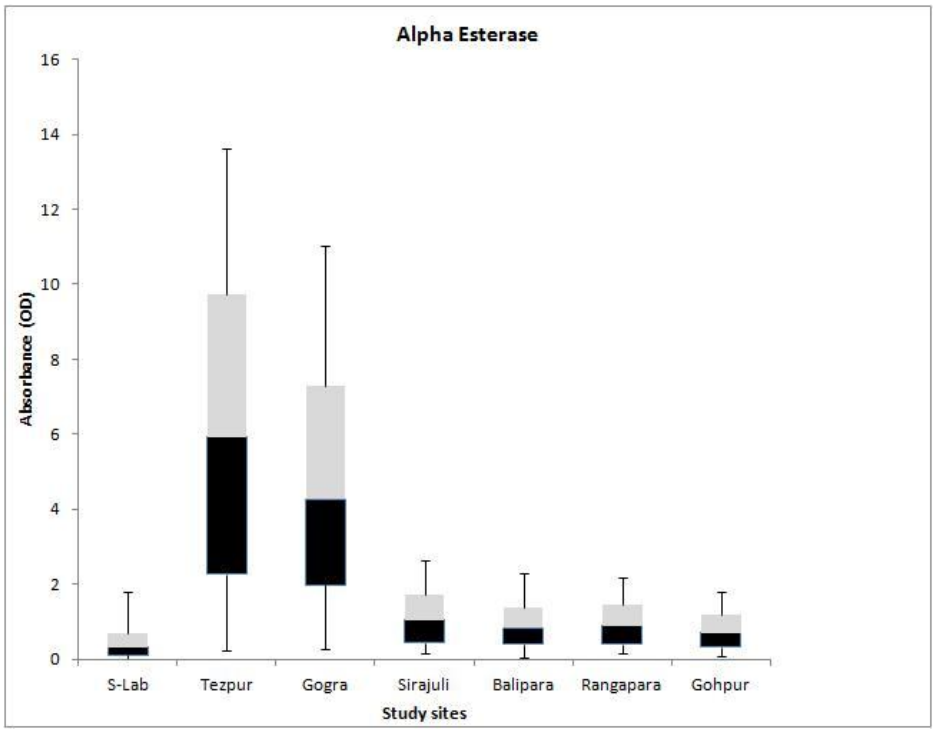
	DDT (4%)		Deltamethrin	
Study sites	**No. of adult mosquitoes exposed**	**% Mortality**	**No. of adult mosquitoes exposed**	**% Mortality**
Tezpur	144	40.2	144	100
Gogra T.E	143	42.9	143	100
Sirajuli	145	50	139	100
Balipara	143	65.7	135	100
Rangapara	145	71.7	140	100
Gohpur	143	80.4	141	100
S-lab	145	94.2	150	100

3.2 Biochemical assay
The results of the biochemical assays of detoxifying enzymes from different study sites are presented in table 2. Total protein content was measured in each mosquito to detect the size differences among individuals that might require correction factors for the enzyme assays. As the amount of protein content in individual mosquito revealed that the average size of individuals from different study sites was not significantly different ($P > 0.05$), and therefore no adjustment was needed for the enzyme analysis to take into account the differences in the size of mosquitoes. The enzyme activity results are presented here as absorbance value (OD value). Enzyme activity levels are significantly different among different populations including the S-lab strain ($P < 0.0001$). The investigations indicated that the esterase and glutathione –S-transferase enzymes had shown a tendency to increase when compared with the laboratory susceptible strain. All populations except Rangapara and Gohpur had 100% increased esterase and GST activity. Tezpur area and Gogra T.E. had the highest activity levels of all the enzymes tested.

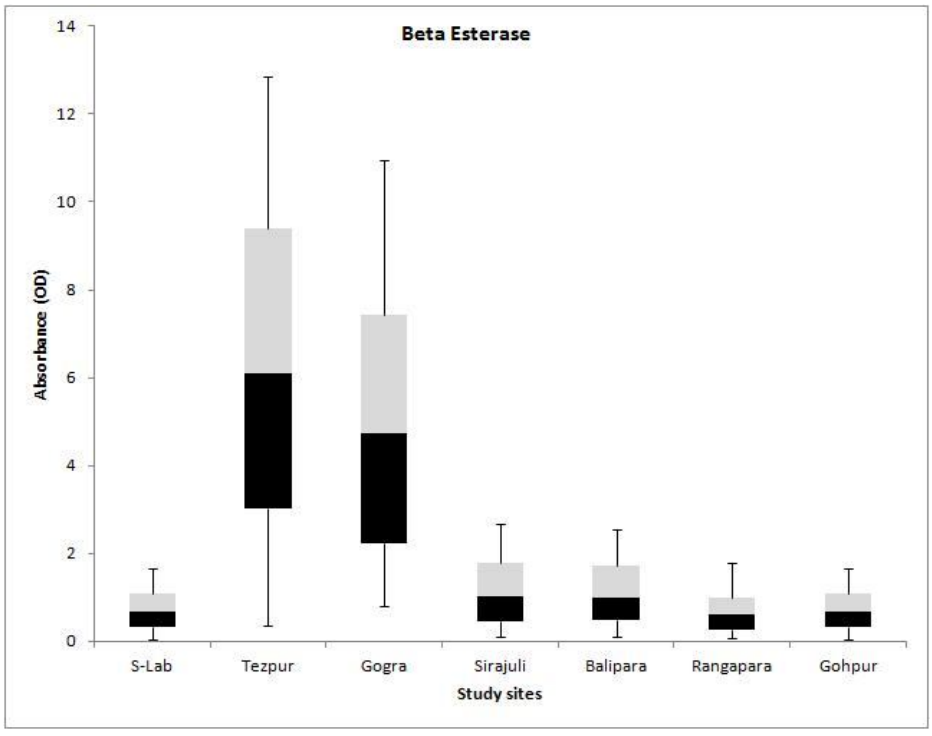
Table 2: Enzyme activity level (mean and standard deviation of mean absorbance) of adult *Aedes albopictus* as measured by absorbance in different study sites

Alpha-esterase				Beta-esterase			Glutathione S-transferase		
Study sites	**Mean (n)**	**SD**	**95% Confidence intervals**	**Mean(n)**	**SD**	**95% Confidence intervals**	**Mean(n)**	**SD**	**95% Confidence intervals**
Tezpur	3.2(24)	0.10	3.1-3.2	3.12(24)	0.09	3.08-3.15	0.20(80)	0.014	0.196-0.203
Gogra T.E	2.6(42)	0.10	2.5-2.6	2.46(42)	0.07	2.44-2.48	0.12(80)	0.017	0.116-0.123
Sirajuli	0.66(24)	0.12	0.60-0.71	0.67(24)	0.06	0.64-0.696	0.11(48)	0.005	0.108-0.111
Balipara	0.56(28)	0.11	0.51-0.60	0.61(28)	0.05	0.59-0.629	0.08(70)	0.007	0.078-0.08
Rangapara	0.51(35)	0.10	0.48-0.54	0.42(35)	0.04	0.405-0.434	0.06(40)	0.016	0.05-0.065
Gohpur	0.41(35)	0.08	0.38-0.44	0.39(42)	0.07	0.37-0.41	0.05(48)	0.007	0.05-0.047
S-lab	0.32(28)	0.08	0.30-0.34	0.35(45)	0.07	0.32-0.37	0.04(36)	0.01	0.04-0.036

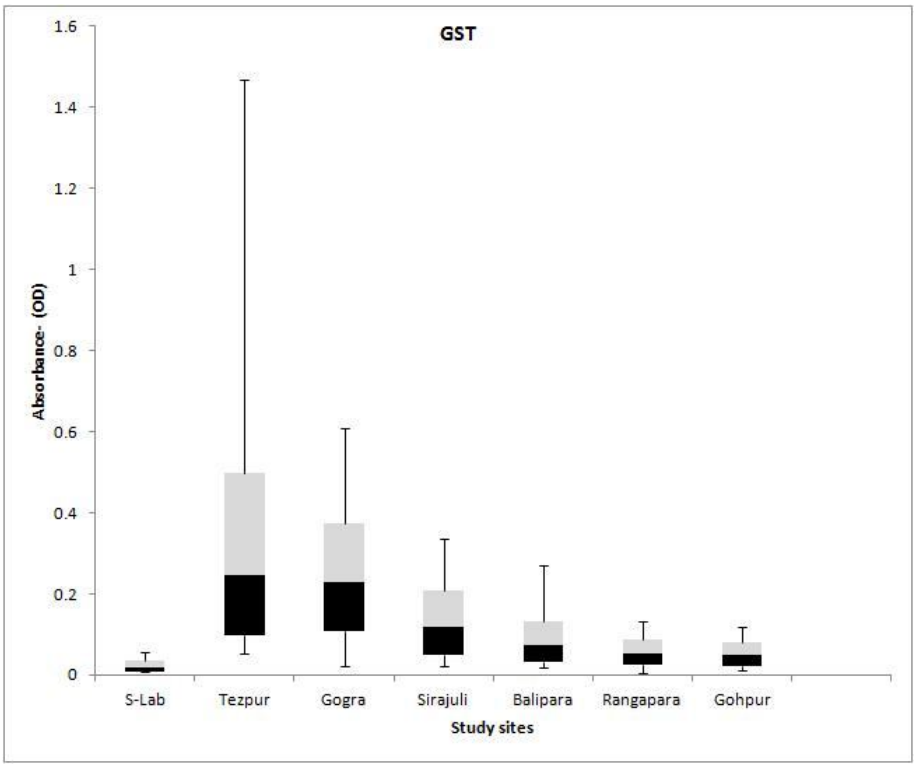
n= Number of mosquito tested


Alpha Esterase
Absorbance (OD)
16
14
12
10
8
6
4
2
0
S-Lab
Tezpur
Gogra
Sirajuli
Balipara
Rangapara
Gohpur
Study sites

(a)


Beta Esterase
Absorbance (OD)
14
12
10
8
6
4
2
0
S-Lab
Tezpur
Gogra
Sirajuli
Balipara
Rangapara
Gohpur
Study sites

(b)



(c)

Fig 1: Box plot distributions of absorbance values for (a) alpha- esterase, (b) beta-esterase and (c) glutathione S- tranferase in field populations of *Aedes albopictus*

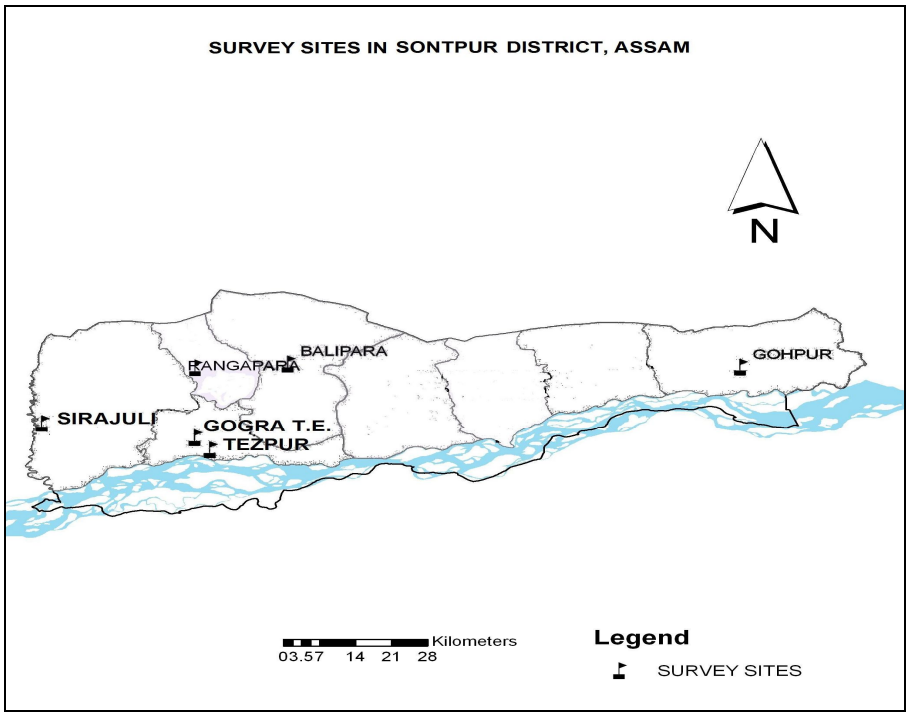


Fig 2: Survey sites in Sonitpur district, Assam

4. Discussion

The resistance status of *Ae. albopictus* was studied at different places in Sonitpur District of Assam, North-East India. WHO criteria for characterizing insecticide resistance/susceptibility was employed, where susceptibility is defined by mortality rates greater than 98% after 24 h post exposure. The investigation has revealed the resistance to DDT found in all study sites except Gohpur (table 1), whereas all individual samples were susceptible to deltamethrin in all study sites. In this study, a high level of DDT resistance was observed in *Ae. albopictus* populations, which can be correlated with the prolonged use of DDT to control malaria in Sonitpur district for many years, as this was a malaria endemic area . This investigation revealed the high level of DDT resistance in *Ae. Albopictus* of Tezpur followed by Gogra T.E., Sirajuli and Balipara respectively. Whereas, the samples from Gohpur showed complete susceptibility to DDT. Due to dwindling cases of malaria, the use of DDT was discontinued in Sonitpur district since last few years. However, persistence of DDT in the environment may have contributed to the emergence of resistant strains. A similar study was carried out in Delhi, India where *Ae. aegypti* was resistant to DDT and dieldrin, but susceptible to organophosphates and pyrethroid insecticides [24]. Insecticide susceptibility test on adult and larvae conducted in Jharkhand, India showed that dengue vectors resistant to DDT and susceptible to other insecticides [25]. There are other reports of high levels of DDT and pyrethroid resistance in *Ae. aegypti* and *Ae. albopictus* in different parts of the world [17, 26, 27].

The degree of resistance can be simply measured as the proportions of adults collected that have enzyme activity levels greater than those of susceptible controls [13]. Based on the results of the biochemical assays employed here, it is most evident that insecticide resistance especially high DDT resistance is mediated by detoxification. The biochemical profiling of candidate detoxification enzyme systems from all study sites showed evidence of alpha esterase, beta-esterase and GST elevation and the presence of clear correlations between enzyme levels and resistance phenotypes across study sites, which amounts to a definite identification of mechanisms controlling resistance.

The increased activity of esterase accounts for resistance to organophosphates, carbamate and pyrethroid insecticides [28, 29, 30]. An elevated GST activity often accounts for DDT and organophosphates resistance [31, 32, 33]. Increased levels of GST activity in all the study sites populations may explain the high levels of DDT resistance among mosquito populations. However, high esterase activity was observed at all study sites but without accompanying pyrethroid resistance. It indicates that this high esterase activity may also be contributing to observed DDT resistance as suggested by Hemingway and Ranson [15]. As high esterase activity correlates mainly with the organophosphate resistance; although malathion and deltamethrin treated nets are used in these areas. Further study on Deltamethrin and malathion susceptibility may reveal the hidden reasons for such a high level of esterase activity in these populations.

Biochemical assays should be used along with conventional bioassays in vector control programmes to improve the surveillance of resistance and monitoring of the efficacy of insecticides. Detection of resistance will help public health personnel to formulate appropriate steps to encounter reductions in effectiveness of control effort that may accompany with the emerging problem of insecticide resistance.

Moreover early detection and knowledge of the resistance status as well as the underlying mechanisms in vector mosquitoes are essential for effective long term control of dengue vectors. Our study suggests that continuous resistance monitoring should be conducted in the North eastern region to identify the efficacy of compounds for dengue control and to facilitate selection of compounds with the greatest promise for halting or minimising dengue infections. Community awareness, cooperation with public health campaigns to reduce larval *Aedes* breeding sites, and well –managed rotation of the effective insecticides are recommended strategies for management of dengue vectors.

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6. Conflict of interest

The authors declare that they have no conflict of interests.

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