RESEARCH ARTICLE

Prevalence of *kdr* mutations and insecticide susceptibility among natural population of *Aedes aegypti* in West Bengal

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Abstract

Background

Aedes albopictus and *Aedes aegypti* are the major vectors of arboviral diseases. As effective vaccines are not available for most of the arboviral diseases, vector control by using insecticides play the key role to reduce the disease transmission. The emergence and spread of resistance to different classes of insecticides by the vectors is a major obstacle to control the disease transmission. Information about vector susceptibility to different insecticides and their mechanisms are very important for formulating proper vector control measures. The present study was designed to assess the susceptibility of *Ae. aegypti* against three different classes of adulticides, one larvicidal agent available and polymorphisms in the voltage-gated sodium channel (*VGSC*) gene related to insecticide resistance.

Methods

Immature stages of *Ae. aegypti* were collected from three dengue endemic municipal areas of West Bengal and reared in the laboratory. Larvae and adults (F1 progeny) were used for insecticide bioassay as per WHO protocols. Knock down resistance gene (*kdr*) mutations were assessed by direct sequencing of PCR products.

Results

The *Ae. aegypti* population was found to be susceptible to type II pyrethroids and malathion but highly resistant to DDT. A high rate of polymorphisms in the *VGSC* gene was observed among the collected mosquitoes. A double mutant V1016**G** + F1534**C** was found to be associated with DDT resistance but neither V1016**G** nor F1534**C** alone showed the same association. Association between the *kdr* mutations and the susceptibility status of pyrethroids could not be established due to very small sample size. A low to moderate level of resistance was noticed against temephos among the larval population based on WHO criteria.

Conclusion

The replacement of DDT by type II pyrethroids for the management of dengue vectors is an appropriate decision taken by the national program which is supported by the findings of a higher level of resistance to DDT. Persistence of polymorphisms in the *VGSC* gene might be an indication of emergence of resistance against pyrethroid insecticides that should be monitored at a regular interval. Attempts should be made to determine the effectiveness of other larvicides for replacement of temephos if needed in future. Along with the chemical insecticides different biological vector control methods as well as biopesticides should also be used in vector control programmes.

Introduction

Mosquito-borne arboviral diseases like dengue, chikungunya, yellow fever and Zika are major public health problem with more than 4 million disability adjusted life years globally [1, 2]. The major causes behind emergence and spread of arboviral diseases are demographic changes, massive urbanization, population movement, trade, transport and lack of effective vector control strategies which favour the world-wide distribution of these viruses and vector mosquitoes [3, 4, 5, 6, 7]. During the last decade a higher level of mortality and morbidity has been observed due to dengue and Zika virus infection [8]. Both these diseases are mainly transmitted by *Aedes albopictus* and *Ae. aegypti* mosquitoes [9, 10]. The spread of the vectors was amplified during the Second World War due to rapid human movement and transportation leading to dengue epidemic [6]. After the war, rapid urbanization led to rapid spread of dengue and hyper-endemicity with multiple serotypes in most South East Asian countries, with severe forms of the disease [11]. Urban and sub-urban colonization comes with new man-made breeding sites for mosquitoes such as regular water containers, disposed water-holding vessels, waste disposal areas, small containers, and discarded tyres all that may help *Ae. albopictus* and *Ae. aegypti* to thrive and multiply [4, 12, 13]. *Ae. albopictus* and *Ae. aegypti* are potential vectors for dengue epidemics as they breed preferentially in artificial containers [14, 15, 16, 6]. To date no effective anti-viral agent is recommended against arboviruses including dengue virus. A vaccine against dengue, Dengvaxia® (CYDTDV), has been licensed since 2015, but the overall efficacy of trials has been about 60% and it has not been used on a large scale [17]. Recently the World Health Organization (WHO) does not recommend wide spread vaccination with Dengvaxia® as it increases the rate of dengue haemorrhagic fever in sero-negative individuals [18]. Effective vector control plays the key role for reducing transmission of arboviruses worldwide and is the essential component of the WHO strategy for the prevention, control, and elimination of Neglected Tropical Diseases [19]. However, the emergence and spread of insecticide resistance in vector mosquitoes is becoming a major obstacle to reaching the goals set by WHO. Resistance to different classes of insecticides have been recorded among both the *Aedes* vector species in different parts of the World [20]. The worldwide insecticide resistance network supported by the World Health Organisation is established to track insecticide resistance among the vectors of arboviruses and to evaluate the potential for deployment of alternative vector control interventions [21]. Four mechanisms have been found to be associated with insecticide resistance-metabolic enzyme-based resistance, reduced target site sensitivity due to mutations in target genes, reduced penetration of insecticide due to thickening of the cuticles and behavioural changes [22]. The first two mechanisms are studied extensively [23, 24, 25,

26] but the role of cuticular penetration has not been well explained [20]. Increased production of three metabolic enzymes i.e. cytochrome P450 monooxygenases (P450s), esterases and glutathione S-transferases are principally associated with insecticide resistance [22, 27]. Resistance due to target site insensitivity is associated with mutations at the *VGSC* gene, commonly referred to as knockdown resistance (*kdr*). The VGSC mutations modify the target site of insecticide so that insecticide does not bind and cause the prolonged opening of the sodium channel resulting in rapid paralysis of the insects [28].

In India, vector control measures against *Aedes* mosquitoes are primarily based on use of temephos as a larvicide, thermal fogging and ultra-low volume space spray of malathion to control dengue outbreaks and use of pyrethroid-treated bed nets to reduce human vector contact [29]. Until the recent past DDT was used as an indoor residual spray (IRS), that has been replaced by a synthetic pyrethroid (type II, alpha-cypermethrin). Several reports are available on insecticide resistance status of the dengue vector [30, 31, 32, 33, 34] from India, but such reports from West Bengal are very rare particularly for *Ae. aegypti* [35]. A regular monitoring of insecticide resistance and studies on mechanisms behind it are very important to detect the effectiveness of the used insecticides and newer ones against the prevailing vector population of any geographical region. The present study was undertaken to determine the insecticide susceptibility status of *Ae. aegypti* to three different classes of adulticides, one larvicidal agent and polymorphisms in *VGSC* gene to correlate with observed insecticide susceptibility status.
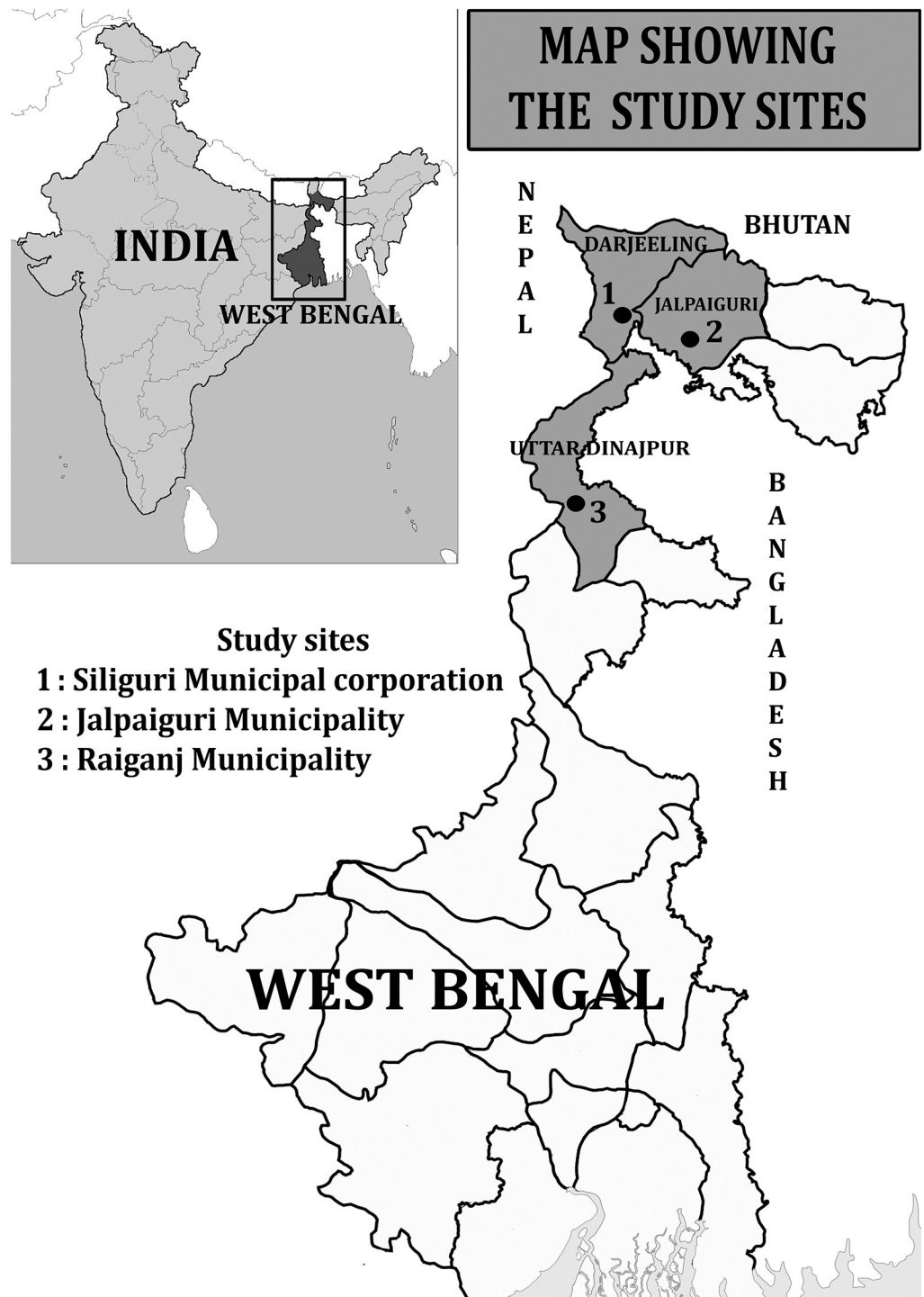
Materials and methods

Study areas and mosquito sampling

The study was conducted in three different urban areas of West Bengal, namely, Siliguri Municipal Corporation (SMC) of Darjeeling (26.720695˚ N, 88.427686˚ E), Jalpaiguri Municipality of Jalpaiguri (26.544386˚ N 88.720568˚ E), and Raiganj Municipality of Uttar Dinajpur district (25.619691˚ N, 88.1256˚ E) (Fig 1). All the study sites were urban or sub-urban in nature. The study was under taken from March 2017 to June, 2018. The climatic conditions were humid and sub-tropical in nature and the temperature varies from 8˚C in winter to 40˚ C in summer.

During the field survey, the study team visited house to house and looked for mosquito larvae and pupae in different natural breeding places present in and around the human dwellings. Different types of breeding sites like storage water tanks, discarded tyres, construction sites, flower pots, plastic cups, coconut shells, discarded containers etc were searched for *Aedes* sp. in all study areas. The larvae and pupae of *Aedes* sp. were collected from the domestic and peri-domestic natural breeding places. The collected immature stages of mosquitoes were gathered in plastic containers containing water from the breeding habitat and transferred to the laboratory.

Mosquito rearing and identification

The collected larvae and pupae were transferred into a larva rearing tray in the laboratory and supplied with food for ornamental fishes and yeast available in the local market. The mosquito larvae and pupae were reared under controlled laboratory conditions such as temperature 25˚C±2˚C and humidity 80%±10%. The adult mosquito cages were supplied with suckling mice as a blood source for feeding the adult female mosquitoes. After emergence, the adult mosquitoes were identified using the standard identification keys of Barraud, 1934 [36] and Tyagi *et al.*, 2012 [37]. The identified *Ae. aegypti* were allowed to breed under laboratory conditions. The larvae and adults of the F1 generation were used for insecticide bioassays.

MAP SHOWING THE STUDY SITES

Study sites
1 : Siliguri Municipal corporation
2 : Jalpaiguri Municipality
3 : Raiganj Municipality

Fig 1. Map showing the study sites in three different districts of West Bengal.

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Larval susceptibility bioassay

The WHO standard bioassay protocol was used for estimation of susceptibility of *Ae. aegypti* larvae to temephos (procured from the Vector Control Research Unit, Universiti Sains

Malaysia, Malaysia) [38]. Seven concentrations of temephos (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 ppm) were prepared from 1 ppm stock temephos solution using 95% ethanol and used for larval bioassay as per WHO recommendations [39, 40]. Twenty to twenty-five late third instar to early fourth instar larvae were placed in each disposable paper cups filled with the required concentration of temephos solution and double distilled water at room temperature (25°C±2°C). Each set of bioassays was replicated at least four times and accompanied with two sets of controls (equal concentration of 95% ethanol). The mortality of larvae was recorded after 24 hours of exposure and was calculated by dividing the number of dead larvae by the total number of larvae tested. A test was considered invalid if pupation rate was greater than 10%, or mortality rate in the control was greater than 20% [38]. Field caught populations were reared for successive generations without any exposure to insecticides in the laboratory maintaining the controlled laboratory conditions mentioned earlier. The twentieth-generation larvae were used as a laboratory strain. The degree of resistance was determined by the resistance ratio (RR), which is calculated by comparing the lethal concentration (LC_{50}) value for a study population with the LC_{50} value for a laboratory-maintained colony. When RR is <5 the field population is considered susceptible (S), when RR is between 5 and 10 mosquitoes are considered to have moderate resistance (MR), and when RR is >10 the mosquitoes are highly resistant (HR) [41].

Adult susceptibility bioassay

The WHO adult bioassay protocol was used for determination of susceptibility status of adult *Ae. aegypti* against four different insecticides e.g.4% DDT, 0.05% deltamethrin, 0.05% alpha cypermethrin (Alpha-cyp) and 5% malathion [42]. Laboratory-emerged (F1 progeny), 2–3 days old unfed female *Ae. aegypti* mosquitoes were used for the bioassay. The adult bioassay kit and insecticide-impregnated papers were procured from the Vector Control Research Unit, Universiti Sains Malaysia, Malaysia. In each set of individual insecticide bioassays four experimental tubes (replicates) were set up and another one or two tubes were used as control. Before the experiment, 20–25 adult female mosquitoes were kept in each holding tube for one hour for acclimatization to experimental conditions. After acclimatization mosquitoes from four such tubes were exposed to insecticide-impregnated papers and one or two tubes to control tubes, respectively. Silicone oil was used as control for deltamethrin and alpha cypermethrin, olive oil, and risella oil for malathion and DDT respectively. Mosquitoes were exposed to insecticides for one hour and cumulative knock down was recorded after 10, 15, 20, 30, 40, 50, and 60 minutes. After exposure, the mosquitoes were transferred to holding tubes and fed on 5% sucrose solution for the next 24 hours. After that time, mortality was scored to determine the susceptibility status as per WHO recommendations [42]. Mosquitoes were considered dead if they were motionless, when they were mechanically stimulated, following the method of Gonzalez Audino [43]. The live and dead mosquitoes resulting from the bioassays were stored at -20°C and used for molecular assays.

Data analysis

Larval bioassay data were analysed using Log dose probit (Ldp) Line computer software (Ehabsoft, Cairo Egypt; available at: http://www.ehabsoft.com/ldpline) according to Finney's method [44]. Lethal concentrations (LC_{10}, LC_{50}, and LC_{99}) along with the slope were estimated at 95% confidence intervals (CI). For adult bioassays, corrected mortality was calculated by using Abbott's formula: Corrected Mortality (CM) (%) = [(% of observed mortality – % of control mortality) / (100 – % of control mortality)] x 100. Mosquitoes were considered susceptible (S) if the corrected mortality (CM) rate was greater than 98%; resistant (R) if

mortality rate was less than 90% and mortality rate between 90–98% was considered as possible resistance (PR) and requiring verification by alternative methods like enzyme bioassay and molecular marker studies, as per WHO recommendation [42]. Knockdown time (KDT_{10}, KDT_{50}, and KDT_{95}) is the time required for knockdown of a particular proportion of mosquitoes following exposure to any insecticide. KDTs were determined using Log dose probit (Ldp) Line computer software (Ehabsoft, Cairo Egypt; available at: http://www.ehabsoft.com/ldpline) programme according to the Finney's method [44]. The association of point mutations with observed insecticide bio-assay was analysed by Fisher's exact test using Graph pad (version 3.06).

DNA isolation and *kdr* mutation detection

Genomic DNA was extracted from both live and dead mosquitoes (individually) by using the DNeasy Blood & Tissue Kit (Qiagen, Germany), as per the manufacturer's instructions. Isolated DNA was stored at -20˚C until further study.

PCR was done using two different primer pairs targeting important amino acid loci of domain II (S989**P**, I1011**M**, I1011**V**, V1016**G**, and V1016**I**) and F1534**C** of domain III of the *VGSC* gene, as described earlier by Kawada et al., 2016 [45]. PCR amplifications were carried out in a final volume of 50μl which include 3μl of genomic DNA as template. The reaction mixture contained PCR buffer, 0.2mM of dNTPs, 2.5 mM MgCl2, 0.3μM of each of the primer and 1.5U of AmpliTaq polymerase (Perkin Elmer, Branchburg, NJ, USA). The PCR reaction were carried out in Applied Biosystem Veriti96 well thermal cycler (Perkin Elmer, Branchburg, NJ, USA) and cycling parameters were an initial denaturation at 94˚ C for 3 minutes followed by 35 cycles of denaturation, 94˚ C for 15 s, annealing 55˚ C for 30 s and extension 72˚ C for 30s. The final elongation was done at 72˚ C for 10 min.

The quality of PCR products was ascertained by 2% agarose gel electrophoresis following ethidium bromide staining. The PCR product was gel purified using the Qiagen gel extraction kit (Qiagen, Germany) and sequencing was outsourced from Chromous Biotech, Bangalore. Two different primers AaSCR6 –CGACTTGATCCAGTTGGAGA (reverse primer for domain II) and AaSCR8– TAGCTTTCAGCGGCTTCTTC (reverse primer for domain III), as described earlier [44], were used for sequencing of the PCR products.

Analysis of sequence

In the present study, we numbered the codon positions of *Ae. aegypti* (996, 1018, 1021, 1023 and 1565) corresponding to the positions of *Musca domestica* (989, 1011,1014,1016 and 1534 respectively) [23]. The sequences were analysed using the software BioEdit Sequence Alignment Editor version 7.0.9.0. The sequences were aligned with the reference sequence for *Ae. aegypti* (GenBank accession no. EU399181.1) using an online multiple sequence alignment (Pairwise sequence alignment) tool.

Ethical statement

The aims and objectives of the study were explained to the local population of the study areas. Permission was taken from the owners of private houses/lands before collection of immature stages of mosquito. The study did not involve with any endangered and protected species. Mosquitoes were maintained under optimal conditions such as temperature, humidity, and adequate food supply in the laboratory. The study protocol was approved by the Institutional Ethics Committee of Calcutta School of Tropical Medicine, Kolkata.

Table 1. Temephos sensitivity status of *Ae. aegypti* larvae in West Bengal.

Values	Study sites			Laboratory/ susceptible strain
	Darjeeling	**Jalpaiguri**	**Uttar Dinajpur**	
	Siliguri MC (n = 320)	**Jalpaiguri Municipality (n = 320)**	**Raiganj Municipality (n = 320)**	
LC_{10} (95% CI) [mg/L]	0.0022 (0.0016–0.0029)	0.0014 (0.0006–0.0022)	0.001 (0.0003-.0.0014)	0.0002
LC_{50} (95% CI) [mg/L]	0.0168 (0.0139–0.0202)	0.0099 (0.0058–0.0157)	0.0079 (0.0038–0.0143)	0.0022
LC_{99} (95% CI) [mg/L]	0.6684 (0.4464–1.0996)	0.3328 (0.2032–1.0385)	0.3601 (0.2643–1.4563)	0.0891
X^2 (p)	5.46 (0.36)	13.47 (0.019)	36.29 (<0.001)	371.42 (<0.0001)
Slope	1.45±0.08	1.52 ± 0.11	1.40 ± 0.07	1.45 ± 0.17
R	0.99	0.96	0.95	0.81
G	0.01	0.09	0.13	6.57
RR_{50}/RR_{99}	7.64/7.5	4.5/3.74	3.59/4.04	
Status[#]	**MR**	**S**	**S**	

n = number; $LC_{10}/LC_{50}/LC_{99}$ = lethal concentration 10%/50%/99%, **RR** = resistance ratio, **g** = 'g' is a factor used for fiducial limit calculations

[#]Classification as per WHO, 2016: **S** = Susceptible (RR <5), **MR** = Moderate Resistance (5 <RR <10), **HR** = High Resistance (>10).

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Results

Larval susceptibility status

The results of larval susceptibility bioassay to temephos are presented in Table 1. The LC_{50} values of Siliguri MC, Jalpaiguri Municipality and Raiganj Municipality was 0.0168 mg/L, 0.0099 mg/L and 0.0079 mg/L, respectively; whereas LC_{99} values were 0.6684 mg/L, 0.3328 mg/L, and 0.3601 mg/L, respectively. The LC_{50} and LC_{99} values of laboratory strain were 0.0022mg/L and 0.0891mg/L, respectively. The calculated RR_{50} and RR_{99} values in Siliguri MC, Jalapiguri Municipality and Raiganj Municipality were 7.64, 7.5, 4.5 and 3.74, 3.59 and 4.04, respectively. So, the calculated RR_{99} values indicated that the *Ae. aegypti* larval population of Siliguri MC was moderately resistant (MR) to temephos, whereas larval population of Jalpaiguri Municipality and Raiganj Municipality were susceptible (S) to temephos.

Susceptibility status of adult *Ae. aegypti* to different insecticides

The results of the adult susceptibility bioassay are presented in Table 2. After 24 hours of exposure, the corrected mortality rates for 4% DDT were 68.20% to 74.70%. The obtained mortality rates were well below the WHO recommended 90% mortality rate for resistance. So, the results suggested that the *Ae. aegypti* population from the study areas were highly resistant to DDT. In all of the study sites, the corrected mortality rate for 0.05% deltamethrin was above 98%, except in SMC where the corrected mortality was 97.72%. The corrected mortality rates for 0.05% alpha cypermethrin and 5% malathion were >98.0% and >99.0% in all the study sites indicating that the natural population of *Ae. aegypti* of all the study areas were susceptible to deltamethrin, alpha cypermethrin and malathion except SMC where a low level of resistance was recorded to only deltamethrin.

The knockdown time (KDT_{10}, KDT_{50}, KDT_{95}) for DDT, deltamethrin, alpha cypermethrin and malathion are given in Table 2. The observed KDT_{50} values were 24.25 to 32.54 mins for DDT, 12.28 to 17.65 mins for deltamethrin, 16.01 to 19.32 mins for malathion and 13.99 to 15.16 mins for alpha cypermethrin. The KDT_{95} values for DDT were 75.86 to 119.21 mins, for deltamethrin 32.52 to 53.76 mins for malathion, 39.78 to 44.94 mins and 42.09 to 46.84 mins

Table 2. Insecticides susceptibility status of *Ae. aegypti* against 4% DDT, 0.05% deltamethrin, 5% malathion and 0.05% alpha cypermethrin in West Bengal.

Insecticides	Districts	Municipality	Mosquito exposed		Mosquito died		Observed Mortality (%)		CM (%)	KDT_{10} (Min) [95% CI]	KDT_{50} (Min) [95% CI]	KDT_{95} (Min) [95% CI]	χ^2 (p)	Slope	Status#
			T*	C*	T*	C*	T*	C*							
4% DDT	Darjeeling	Siliguri MC	194	48	149	4	76.80	8.33	**74.70**	9.97 [8.22–11.58]	24.25 [22.23–26.33]	75.86 [64.89–92.98]	4.09 (0.54)	3.32±0.25	R
	Jalpaiguri	Jalpaiguri Municipality	168	44	117	2	69.64	4.55	**68.20**	11.83 [9.59–13.85]	32.54 [29.71–35.81]	119.21 [96.17–159.91]	1.63 (0.89)	2.92±0.25	R
	U. Dinajpur	Raiganj Municipality	185	45	134	2	72.43	4.44	**71.15**	10.44 [8.42–12.27]	28.15 [25.71–30.82]	100.61 [82.88–130.59]	2.74 (0.74)	2.97±0.24	R
0.05% DEL	Darjeeling	Siliguri MC	184	43	180	2	97.83	4.65	**97.72**	6.25 [4.83–7.55]	15.12 [13.47–16.66]	47.02 [40.85–56.56]	10.25 (0.07)	3.34±0.28	PR
	Jalpaiguri	Jalpaiguri Municipality	164	40	164	3	100.	7.50	**100.0**	5.76 [4.43–6.94]	12.28 [10.87–13.56]	32.52 [28.74–38.33]	1.62 (0.89)	3.89±0.36	S
	U. Dinajpur	Raiganj Municipality	172	48	169	3	98.26	6.25	**98.14**	7.41 [5.96–8.75]	17.65 [15.98–19.26]	53.76 [47.03–63.82]	9.16 (0.10)	3.4±0.26	S
5% MAL	Darjeeling	Siliguri MC	164	40	163	1	99.39	2.50	**99.37**	10.49 [7.98–11.98]	18.81 [15.86–21.68]	39.78 [34.9–50.9]	15.99 (0.007)	5.05±0.33	S
	Jalpaiguri	Jalpaiguri Municipality	170	42	170	2	100.0	4.76	**100.0**	7.24 [5.86–8.5]	16.01 [14.49–17.45]	44.28 [38.98–52.22]	7.71 (0.17)	3.72±0.29	S
	U. Dinajpur	Raiganj Municipality	160	41	160	1	100.0	2.44	**100.0**	10.01 [7.45–11.58]	19.32 [16.3–22.34]	44.94 [38.96–59.37]	13.17 (0.02)	4.49±0.32	S
0.05% ALHA-CYP	Darjeeling	Siliguri MC	162	40	160	1	98.77	2.5	**98.73**	6.10 [4.69–7.41]	14.89 [13.24–16.44]	46.84 [40.64–56.46]	5.99 (0.31)	3.31±0.28	S
	Jalpaiguri	Jalpaiguri Municipality	170	44	167	0	98.24	0	**98.24**	5.93 [4.62–7.16]	13.99 [12.43–15.44]	42.09 [37.21–49.24]	2.38 (0.79)	3.44±0.27	S
	U. Dinajpur	Raiganj Municipality	166	41	166	2	100.0	4.88	**100.0**	6.78 [5.45–8.01]	15.16 [13.66–16.56]	42.53 [37.67–49.65]	8.38 (0.14)	3.67±0.28	S

*T = Test, C = Control, CM = Corrected Mortality

#S = Susceptible (CM ≥98%); R = Confirmed Resistance (CM <90%); PR = Possible Resistance (CM = 90–97%)

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for alpha cypermethrin. The knock down rate of *Ae. aegypti* against DDT, deltamethrin, malathion and alpha cypermethrin over an exposure time of 1 hour is given in Fig 2(A)–2(D).

Prevalence of *kdr* mutations in *Ae. aegypti* and their association with insecticide resistance

Kdr mutations were successfully analysed among 110 mosquitoes, of which 46 were DDT exposed (alive 24, dead 22), 35 were deltamethrin exposed (alive 7, dead 28) and 29 were alpha cypermethrin exposed (alive 5, dead 24). An overall prevalence of V1016**G** (GTA→G**G**A) mutation was detected in 23 (20.91%, 95% CI: 14.36–29.43) samples, of which only 5 were heterozygous (V/**G**1016). Mutant F1534**C** (TTC→T**G**C) was detected in 58 (52.73%, 95% CI: 43.47–61.81) samples of which 9 were heterozygous (F/**C**1534) and 51 (46.36%, 95% CI: 37.32–55.64) samples harboured T1520**I** (ACC→A**T**C) mutation. We did not find heterozygous double mutant (V/**G**1016 + F/**C**1534) in any sample. A synonymous mutation in domain II, T1044T (ACT→AC**G**) was found in all the sample analysed. Two single mutant genotypes 1016**G** + 1534F and 1016V + 1534**C** were prevalent in 10.91% (95% CI: 6.35–18.11) and 42.73% (95% CI: 33.88–52.07) mosquitoes, whereas a double mutant genotype 1016**G** + 1534**C** was observed in 10% (95% CI: 5.68–17.02) mosquitoes (Table 3). The DNA sequences have been submitted to GenBank under accession numbers MK032480 and MK032481.

Regarding distribution of genotype, single mutant 1016**G** + F1534 and V1016 + 1534**C** were recorded among five and fifteen DDT resistant (alive) mosquitoes & one and nine among DDT sensitive (dead) mosquitoes. By analysing with Fisher exact test, double mutant 1016**G** +1534**C** was found to be associated with DDT resistance (OR = 33.0, P = 0.0269), but no such association was recorded for individual point mutations at codon 1016**G** and 1534**C** (Table 3). As the number of tolerant mosquitoes obtained from adult bioassay with deltamethrin and alpha-cypermethrin were very few, we did not attempt to analyse any association of *kdr* mutation with them.

Discussion

Ae. aegypti is highly anthropophilic, aggressive day biter with peak activities during early morning and late afternoon. They prefer to feed indoors and rest outside in close proximity to
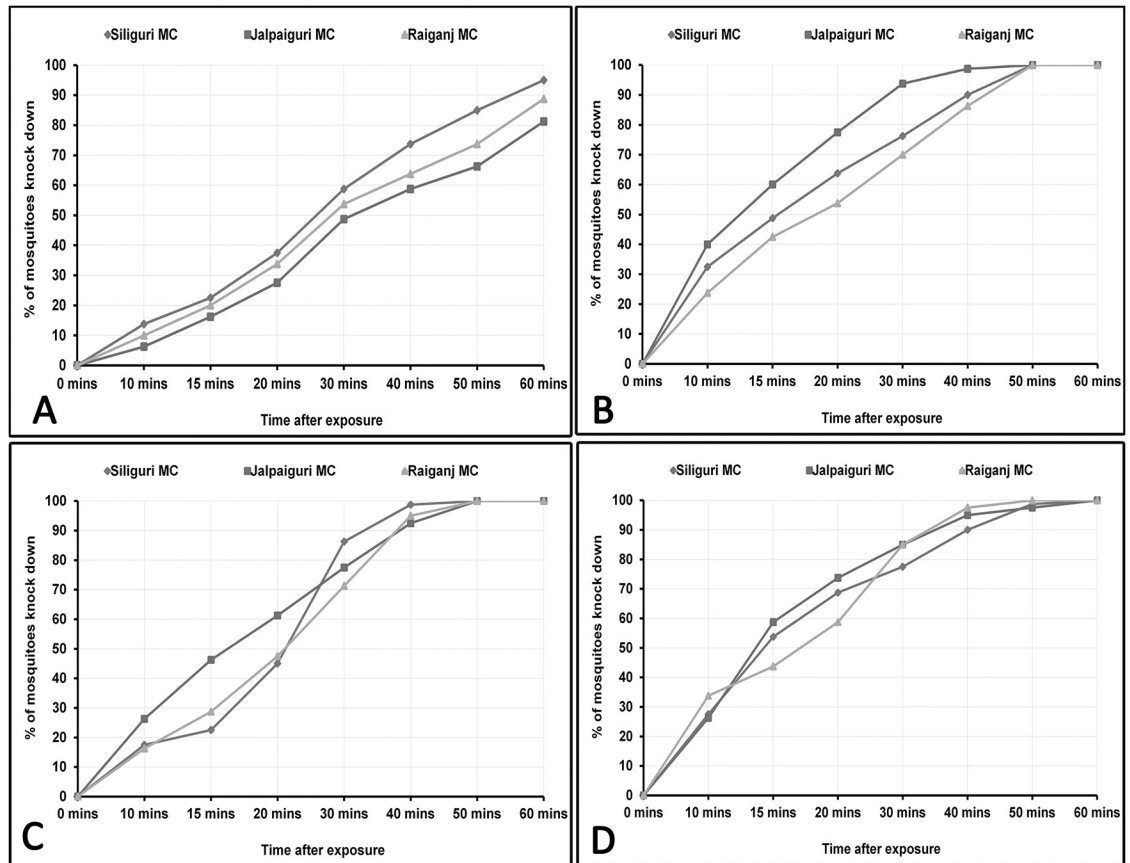
Fig 2. Knock down rate (KDR) of *Ae. aegypti* against 4% DDT. (A), 0.05% deltamethrin (B), 5% malathion (C), 0.05% alpha cypermethrin (D) in West Bengal.

their breeding sites [16]. It is very difficult to control the adult mosquitoes through IRS (indoor residual spray) due to typical feeding and resting behaviour. Use of insecticides as space sprays using thermal fogging and ultra-low volume application are the choice of methods for controlling the *Aedes* population. In general, management of breeding sites with effective larvicides plays an important role for this purpose. The selection of effective insecticidal agents (larvicide and adulticide) is very important. In the present study, we attempted to determine the susceptibility status of *Ae. aegypti* in three different classes of insecticides; DDT (organochlorine), deltamethrin and alpha-cypermethrin (type II pyrethroid), malathion (organophosphate) as adulticide, temephos as larvicide and polymorphisms in *VGSC* gene among the mosquitoes collected from three different districts of West Bengal.

In India DDT was used as an insecticidal agent for a long time. The first case of DDT resistant *Ae. aegypti* was reported from India by Azeez (1967) [46] and then it spread widely across the country [32, 33,47,48,49]. In the present study we also observed a significantly high level of DDT resistance in all study sites with higher KDT and low KDR values. Alongside this problem, DDT has long term toxicity in the environment. So, the replacement of DDT by pyrethroid is an appropriate decision taken by the National Vector Borne Disease Control Programme (NVBDCP). Pyrethroid is a class of insecticide recommended by the World Health Organization for controlling mosquitoes due to its high efficacy against insects and low mammalian toxicity [50].

Table 3. Distribution of point mutations and combined genotypes of VGSC gene among *Ae. aegypti* exposed to DDT, deltamethrin and alpha-cypermethrin.

Phenotypes	N	Occurrence of point mutations n (%, 95% CI)						Occurrence of combined genotypes n (%, 95% CI)			
		1016		1520		1534		1016V+ 1534F	1016G+ 1534F	1016V+ 1534C	1016G + 1534C
		Val (GTA)	Gly (GGA)	Thr (ACC)	Ile (ATC)	Phe (TTC)	Cys (TGC)				
DDT resistant (alive)	24	16 (66.67, 46.71–82.03)	8 (33.33, 17.97–53.29)	12 (50.0, 31.43–68.57)	12 (50.0, 31.43–68.57)	6 (25.0, 12.0–44.9)	18 (75.0, 55.1–88.0)	1 (4.16, 0.74–20.25)	5 (20.83, 9.24–40.47)	15 (62.5, 42.71–78.84)	3 (12.5, 4.34–31.0)
DDT sensitive (dead)	22	20 (90.91, 72.19–97.47)	2 (9.09, 2.53–27.81)	12 (54.55, 34.66–73.08)	10 (45.45, 26.92–65.34)	12 (54.55, 34.66–73.08)	10 (45.46, 26.92–65.34)	11 (50.0, 30.72–69.28)	1 (4.55, 0.81–21.8)	9 (40.9, 23.26–61.27)	1 (4.54, 0.81–21.8)
DEL resistant (alive)	7	3 (42.85, 15.82–74.96)	4 (57.15, 25.04–84.18)	4 (57.14, 25.04–84.18)	3 (42.86, 15.82–74.96)	2 (28.57, 8.22–64.11)	5 (71.43, 35.89–91.78)	1 (14.28, 2.57–51.32)	1 (14.29, 2.57–51.32)	2 (28.57, 8.22–64.11)	3 (42.85, 15.82–74.96)
DEL sensitive (dead)	28	25 (89.28, 72.81–96.29)	3 (10.72, 3.71–27.19)	18 (64.29, 45.83–79.3)	10 (35.71, 20.7–54.17)	18 (64.28, 45.83–79.3)	10 (35.72, 20.7–54.17)	16 (57.14, 39.07–73.49)	2 (7.14, 1.98–22.64)	9 32.14, 17.93–50.66)	1 (3.57, 0.63–17.71)
ALPHA-CYP resistant (alive)	5	2 (40.0, 11.76–76.93)	3 (60.0, 23.07–88.24)	2 (40.0, 11.76–76.93)	3 (60.0, 23.07–88.24)	3 (60.0, 23.07–88.24)	2 (40.0, 11.76–76.93)	1 (20.0, 3.62–62.45)	2 (40.0, 11.76–76.93)	1 (20.0, 3.62–62.45)	1 (20.0, 3.62–62.45)
ALPHA-CYP sensitive (dead)	24	21 (87.5, 69.0–95.66)	3 (12.5, 4.34–31.0)	11 (45.83, 27.89–64.92)	13 (54.17, 35.08–72.11)	11 (45.83, 27.89–64.92)	13 (54.17, 35.08–72.11)	10 (41.67, 24.47–61.17)	1 (4.17, 0.74–20.25)	11 (45.83, 27.89–64.92)	2 (8.33, 2.31–25.84)
TOTAL	110	87 (79.09, 70.57–85.64)	23 (20.91, 14.36–29.43)	59 (53.64, 44.36–62.67)	51 (46.36, 37.32–55.64)	52 (47.27, 38.19–56.53)	58 (52.73, 43.47–61.81)	40 (36.36, 27.97–45.67)	12 (10.91, 6.35–18.11)	47 (42.73, 33.88–52.07)	11 (10.0, 5.68–17.02)

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Emergence and spread of pyrethroid resistance in *Aedes* mosquitoes is a global problem for controlling vector borne arboviral diseases. A substantial geographical variation of pyrethroid resistance has been noted. Generally, a lower level of resistance was noticed in Asia and Africa whereas a higher level of resistance was observed in mosquitoes from the Caribbean, Mexico, and South America [51].

In the present study we observed that the *Ae. aegypti* populations of three study areas were susceptible to both the pyrethroids tested except in SMC of Darjeeling district where the corrected mortality was 97.72% for deltamethrin, slightly lower than the cut off level of 98% recommended by WHO [42]. A Similar observation was also made for *Ae. albopictus* of the same study site [52]. Though the pyrethroids have been introduced recently for control of vectors, the group has been used widely as a pesticide in agriculture. As all the study areas were urban in nature, exposure from agricultural applications of the insecticide was low and that might be the cause behind higher rate of pyrethroid sensitivity among the *Ae. aegypti* population. Unlike our study, pyrethroid resistance has been reported from different parts of India [33, 48] in contrary, pyrethroid sensitivity was also been reported [31, 47,49] from other parts of the country. *Ae. aegypti* from all of the study areas were highly susceptible to malathion. Similar observations have been made from different parts of the country [31, 47 and 48]. In the study areas malathion was used on rare occasions particularly to control dengue and Japanese Encephalitis outbreaks. If needed malathion might be an alternative to pyrethroid insecticides in future.

Along with the chemical insecticides different biological vector control methods as well as bio-pesticides should also be used in vector control programmes.

The larval susceptibility of *Ae. aegypti* to temephos showed moderate resistance in one study area. In contrast temephos susceptibility was recorded from other parts of the country [31, 47,49]. The present study areas were urban and semi-urban in nature and controlled by local municipal authorities. The local authorities used temephos for management of mosquito breeding sites for a long time. This longer exposure might be the cause behind observed moderate resistance among the prevailing *Ae. aegypti* population of Siliguri MC area. So, the mode of use of temephos should be monitored closely or temephos should be replaced by other larvicidal agents if needed in future.

Several mutations in *VGSC* gene of *Ae. aegypti* have been reported, but only a few of them have been confirmed to be associated with pyrethroid resistance. In Asian countries, two *kdr* mutations V1016**G** and F1534**C** are common in *Ae. aegypti* [53, 54]. We also observed a high rate of point mutations at F1534**C** (52.73%) and V1016**G** (20.91%), similar to the observations from other parts of the country [32, 33].

There is a specific relation of these single nucleotide polymorphisms (SNPs) to the insecticide resistance. Mutant V1016**G** is reported to be associated with resistance to type I (permethrin) and type II (deltamethrin) pyrethroids, while F1534**C** with resistance to type I pyrethroids only [55]. In the present study mutant 1016**G** was not found to be associated with observed DDT resistance. Subsequently, the mutation at F1534**C** of S6 subunit of domain III was reported in DDT/ permethrin-resistant *Ae. aegypti* in Thailand and Vietnam [54, 56] but we did not observe any such association. Interestingly a double mutant (1016**G** + 1534**C**) in *VGSC* gene was found to be associated with resistance to DDT. But such correlation for deltamethrin and alpha-cypermethrin could not be established. Results may be confounded by a small sample size. We observed only five resistant mosquitoes of those that were exposed to alpha-cypermethrin and seven for deltamethrin.

A stepwise two additional mutations, S989**P** and D1763**Y** with V1016**G** were reported to be associated with permethrin resistant *Ae. aegypti* from south-east Asian countries [57, 58] but no such additional mutations were found in our study. An additive effect of double heterozygous mutation (V/G1016 + F/C1534) and triple heterozygous mutation (S/P989 + V/G1016 + F/C1534) to pyrethroid resistance have been reported from Thailand [59]. But no such double or triple heterozygous mutation was detected in the present study.

We observed a mutation at T1520**I** with a prevalence of (46.36%) which is similar to that observed by Khuswaha et al, 2015 [33] from India, but the role of this mutation in insecticide resistance is yet to be established.

Conclusion

From the present study it was evident that the *Ae. aegypti* populations from each of the study areas were susceptible to the currently used pyrethroid i.e. 0.05% alpha cypermethrin and also to 0.05% deltamethrin, but highly resistant to DDT. However, presence of a high level of polymorphisms in *VGSC* gene may be an indication of emerging pyrethroid resistance. So, the susceptibility of used pyrethroid and polymorphisms in target genes should be monitored at regular intervals to detect the emergence of pyrethroid resistance among the *Ae. aegypti* population. As malathion is highly sensitive, it might be an alternative in near future if needed. A low to moderate level of resistance to temephos among larval populations was also noticed. Further study is required to observe the larval susceptibility to other larvicides to replace temephos for proper management of *Ae. aegypti*. Along with the chemical insecticides different

biological vector control methods as well as biopesticides should also be used in vector control programmes.

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