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Insecticide resistance compromises the control of *Aedes aegypti* in Bangladesh

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Abstract

BACKGROUND: With no effective drugs or widely available vaccines, dengue control in Bangladesh is dependent on targeting the primary vector *Aedes aegypti* with insecticides and larval source management. Despite these interventions, the dengue burden is increasing in Bangladesh, and the country experienced its worst outbreak in 2019 with 101 354 hospitalized cases. This may be partially facilitated by the presence of intense insecticide resistance in vector populations. Here, we describe the intensity and mechanisms of resistance to insecticides commonly deployed against *Ae. aegypti* in Dhaka, Bangladesh.

RESULTS: Dhaka *Ae. aegypti* colonies exhibited high-intensity resistance to pyrethroids. Using CDC bottle assays, we recorded 2–24% mortality (recorded at 24 h) to permethrin and 48–94% mortality to deltamethrin, at 10× the diagnostic dose. Bioassays conducted using insecticide–synergist combinations suggested that metabolic mechanisms were contributing to pyrethroid

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DISCLAIMER

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

CONFLICT OF INTEREST

There is no interest to declare with this study.

resistance, specifically multi-function oxidases, esterases, and glutathione S-transferases. In addition, *kdr* alleles were detected, with a high frequency (78–98%) of homozygotes for the V1016G mutation. A large proportion (≤ 74%) of free-flying and resting mosquitoes from Dhaka colonies survived exposure to standard applications of pyrethroid aerosols in an experimental free-flight room. Although that exposure affected the immediate host-seeking behavior of *Ae. aegypti*, the effect was transient in surviving mosquitoes.

CONCLUSION: The intense resistance characterized in this study is likely compromising the operational effectiveness of pyrethroids against *Ae. aegypti* in Dhaka. Switching to alternative chemical classes may offer a medium-term solution, but ultimately a more sustainable and effective approach to controlling dengue vectors is required.

Keywords

Aedes aegypti ; insecticide resistance; metabolic resistance; *kdr* ; pyrethroids; operational control

1 INTRODUCTION

Aedes (*Stegomyia*) *aegypti* (Linnaeus, 1762) is the most important vector of arboviruses affecting humans, including dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) viruses. Globally, there are an estimated 390 million annual DENV infections, of which 96 million have clinical manifestations.[1] Many thousands of dengue cases occur annually in Bangladesh, especially during the monsoon months from June to September.[2,3] Dhaka, the capital city, accounts for more than 80% of annual reported dengue cases.[4] A recent study reported that inhabitants of Dhaka exhibited 36–85% dengue seropositivity.[5] Although there is limited testing outside the capital, the same study reported seropositivity ranging from 3% in the rural northeast to 88% in the urban southeast of Bangladesh.[5] In 2019, Bangladesh experienced its largest dengue outbreak with 101 354 hospitalized cases and 179 deaths.[2] Other *Aedes*-borne diseases are also present in the country: sporadic outbreaks of CHIKV have been reported since 2011 with the largest outbreak in 2017 estimated to have infected between 46 and 824 persons per thousand throughout Dhaka.[6–8] The presence of ZIKV in Bangladesh has also been noted.[9,10] Significant human movement between rural and urban areas of Bangladesh[8] and the ubiquitous distribution of the vector place the nation at risk of outbreaks of these *Aedes*-borne diseases.[5]

A recent entomological survey in Dhaka during the monsoon month of August 2021 reported that the Breteau Index (BI) was > 20 in most areas (i.e., > 20 *Ae. aegypti* positive containers per 100 houses inspected) and reached 73 in one location.[11,12] In Dhaka, *Aedes* abundance is strongly associated with favorable climatic factors including rainfall, temperature, and humidity.[13]

In the absence of effective vaccines or specific therapeutics for DENV, CHIKV, or ZIKV infections, control of *Ae. aegypti* is a primary tool for reducing virus transmission.[14] The insecticide classes commonly used to control *Ae. aegypti* include pyrethroids, organophosphates, and carbamates.[15] Of these, the most commonly deployed are pyrethroids because of their effectiveness against insects and their safety profile in mammals,[16]

however, resistance to pyrethroids is now widespread in *Ae. aegypti* and this threatens the effectiveness of insecticide-based control programs.[17–23]

The pyrethroids act by binding to the voltage-gated sodium channel (VGSC) of nerve cells, disrupting normal ion discharge, and depolarization, and causing insect paralysis ("knockdown" or KD) and death.[24,25] In resistant mosquitoes, the selection of non-synonymous point mutations in the VGSC gene prevents the binding of pyrethroids to the VGSC. This protective mechanism is referred to as "knockdown resistance" (*kdr*) and the mutations involved are referred to as *kdr* mutations.[26,27] In *Ae. aegypti*, five mutations have been associated with resistance to type-I (e.g., permethrin) and type-II (e.g., deltamethrin) pyrethroids: S989P, I1011M, V1016G/I, F15340C, and V410L.[28] Their frequency varies geographically, and they can occur independently or together, sometimes with additive effects on pyrethroid resistance.

Metabolic resistance occurs through increased insecticide degradation or sequestration by detoxification enzymes before insecticides exert toxic effects. Enzyme families that detoxify insecticides include cytochrome P450-dependent multi-function oxidases (CYP450 MFO), esterases, and glutathione S-transferases (GST).[29] These "detoxification enzymes" have roles in the metabolism of pyrethroids and other insecticides in *Ae. aegypti*.[30,31] Over-expression of *Ae. aegypti* CYP450 genes, especially members of the CYP6 and CYP9 subfamilies have been linked to pyrethroid resistance. Among the CYP genes proved to metabolize pyrethroids or confer resistance when expressed in *Drosophila*, CYP9J10, CYP6BB2, CYP9J26, and CYP6J28 were most consistent and found in Asia and the Americas.[20,32–34]

In Bangladesh, insecticidal interventions against disease vectors are used for the control of malaria (including the use of long-lasting insecticidal nets [LLINs], and indoor residual spraying [IRS])[35] and to manage the biting nuisance caused by *Culex quinquefasciatus* in major urban centers (thermal fogging with pyrethroid adulticides and larviciding with the organophosphate temephos). Bangladesh had no targeted vector control program for *Ae. aegypti* until 2016, when periodic *Aedes* surveys were initiated, as well as regular thermal fogging, source reduction with community engagement, and larviciding during dengue outbreaks (personal communication: Mohammad Rajaul Karim, Senior Entomologist, Communicable Disease Control, Directorate General of Health Services, DGHS, Bangladesh).[36] As is common for dengue control programs everywhere, Dhaka also faces logistical challenges related to a complex urban environment, human resources, program evaluation, and budget.[36] Recently, Dhaka city corporations have increased insecticide applications in response to nuisance biting by *Cx. quinquefasciatus* and several DENV and CHIKV outbreaks. This may have increased selection pressure for insecticide resistance in *Ae. aegypti*.[37]

The increasing number of reported cases of *Aedes*-borne viral diseases indicates that vector control programs are having little impact. The development of resistance against commonly used insecticides in local *Aedes* populations was detailed in a recent baseline study, however, detailed studies on the intensity of that resistance were lacking.[23] The World Health Organization (WHO) recommends if resistance is confirmed in susceptibility assays

using a discriminating concentration (1×), intensity of resistance has to be determined with higher discriminating concentrations using 5× dose and if < 98% mortality achieved in that dose, susceptibility tests needed to be conducted with 10× dose of the discriminating concentration.[38] In the present study, we aimed to characterize the intensity of pyrethroid resistance in *Ae. aegypti* from Dhaka following WHO recommendations, and explore the presence of metabolic and target-site mediated resistance mechanisms using synergists and by measuring the frequency of *kdr* genotypes. We used an indoor room delivering environmental temperature and humidity conditions that provided unrestricted mosquito movement to examine the operational and behavioral consequences of resistance by exposing free-flying and resting mosquitoes to standardized quantities of domestic and public health insecticide aerosols.

2 MATERIALS AND METHODS

2.1 Dengue case and rainfall data

The number of dengue cases was initially collected from a webpage maintained by the Institute of Epidemiology, Disease Control and Research (IEDCR), Bangladesh until December 31, 2019.[2] When this webpage was discontinued in 2020, dengue data were collected through personal communications and online media releases. Recently, another webpage was opened by Health Emergency Operation Center & Control Room (HEOC) under the Management Information System (MIS), Directorate General of Health Services (DGHS), Bangladesh from where the 2021 data were collected.[3] Rainfall data were collected from the Bangladesh Meteorological Department through an online data purchase system.

2.2 *Aedes aegypti* colonies

Mosquito eggs were collected during the dengue outbreak in June 2019 from five areas of Dhaka, Bangladesh which were selected based on dengue prevalence, population density, and housing type (Fig. 1). The Khilgaon (23.748455 N, 90.423419 E) and Mirpur (23.821743 N, 90.369485 E) areas are characterized by shared houses and small apartments with little vegetation. The highest BI (73) recorded in 2021 was from a neighborhood adjacent to Khilgaon.[12] In contrast, the Uttara (23.863050 N, 90.402950 E), Dhanmondi (23.745307 N, 90.379936 E), and Bashundhara (23.816028 N, 90.432046 E) areas are less densely populated with privately owned houses, larger apartments, and more green space. *Aedes* eggs were collected using oviposition traps as per the baseline study,[23] with one trap installed per property. With a distance of ~200 m from each other, at least 50 traps were installed at each site and left for 5 days before retrieval. Traps were installed indoors to protect from damage, exposure to rainwater, or loss/removal from the spot. Eggs were air-dried and shipped to the Mosquito Control Laboratory, QIMR Berghofer Medical Research Institute (QIMR Berghofer), Australia (import permit: 0002569896).

Eggs were flooded in July 2019 and hatched in trays of reverse osmosis (RO) water at varying densities. The number of eggs collected from Khilgaon was too low to initiate a colony, so all resulting larvae from that site were killed at the fourth instar stage (n = 40) and stored for *kdr* characterization. *Aedes aegypti* was the only species identified

from the traps based on morphological identification undertaken on a sample (n = 20/stage/colony) of larval and adult specimens (except Khilgaon) from each colony following standard taxonomic keys.[39,40] All colonies except Khilgaon were reared to adults, blood-fed, and allowed to oviposit. Parents were killed and stored at −20°C, and F1 eggs were used to continue the colonies. Adult mosquitoes were provided with 10% sucrose solution *ad libitum*. Defibrinated sheep blood (Serum Australis) was provided through parafilm membrane (Bemis™ Parafilm™) for blood feeding and maintenance over generations. Approximately 20 mL of warm blood (37°C) was taken in a pre-sterile plastic Petri dish (92 mm × 16 mm) (Sarstedt Inc.), a piece of parafilm was scrubbed on human skin and stretched about twice its original size and placed over the Petri dish with blood.[41] The Petri dish was then placed upside down on top of the cage net and a 25 cm^2 cell culture flask (Corning Inc.) filled with hot water was kept on top of the Petri dish to keep it warm. Colonies were maintained at ±27°C, 70% relative humidity (RH), and 12 h:12 h day/night light cycling with 30 min dawn and dusk periods.

2.3 Determination of phenotypic resistance (bottle bioassays)

Phenotypic characterization of insecticide resistance was conducted using an adaptation of the CDC bottle bioassay on 3- to 5-day-old, non-blood-fed female F1 mosquitoes.[42] A fully susceptible Australian *Ae. aegypti* strain (Cairns) was used as a control.[43] The KD phenotype (where mosquitoes are unable to stand or fly) was assessed 30 min post-exposure to prescribed diagnostic doses of technical grade insecticide (μg/bottle). Each bioassay included ≥ 100 mosquitoes (≥ 25 mosquitoes per bottle with four bottles as biological replicates). Recent literature indicate that the full impacts of resistance can be better characterized by adding a subsequent observation to score recovery or mortality, usually at 24 h post-exposure.[44–46] Therefore, at 30 min post-exposure, after scoring KD, mosquitoes were removed from the bottles and held in clean plastic cups with sugar solution provided *ad libitum*. Mortality was scored at 24 h. Mosquitoes were considered dead if either they were unable to stand or were immobile.[38] A total of four insecticides belonging to three major insecticide classes were tested by the bottle bioassays (Table 1).

If resistance was detected at the prescribed diagnostic dose, multiples of that dose (2×, 5×, and 10×) were tested to determine the intensity of resistance. Resistance is identified as moderate when < 98% of test insects die at 5× the diagnostic dose and high when < 98% die at 10× the diagnostic dose. The WHO suggests that this is a predictor of operational control failure.[38]

To understand the contribution of metabolic resistance to the observed resistance phenotype, synergists–insecticide combinations were applied using standard bottle bioassay protocols.[42] Insecticide synergists inhibit the enzymes that metabolize insecticides, and if any of these enzymes play a role in the resistance phenotype, their addition should increase mortality.

Synergists used in the bottle bioassays were[42]:

- Piperonyl butoxide (PBO) (400 μg/bottle), which inhibits oxidase activity.
- S,S,S-Tributlyphosphorotrithioate (DEF) (80 μg/bottle), which inhibits esterase activity.

- Ethacrynic acid (EA) (80 μg/bottle), which inhibits GST activity.

For synergist assays, ≥ 125 mosquitoes were introduced into a synergist-treated bottle and a control bottle (acetone only).[42] After 1 h, groups of those mosquitoes (25) were introduced into insecticide-treated bottles. Synergists were combined with 10× the diagnostic dose for permethrin and 1× and 2× the diagnostic dose for deltamethrin.

2.4 Screening for *kdr* alleles

DNA extractions were carried out with an in-house protocol using Proteinase K (Qiagen, Hilden, Germany) and extraction buffer (0.01 mol/L Tris, 0.05 mol/L NaCl, 0.001 mol/L EDTA), and ultrapure water (UPW). Individual mosquitoes were placed in 0.2 mL thin-wall polymerase chain reaction (PCR) tubes and mixed with 100 μL of extraction buffer and 0.5 mg/mL Proteinase K. Tubes were incubated for 5 min at 56°C in a thermal cycler followed by heat deactivation at 98°C for 10 min. The DNA concentration of random samples was determined using a NanoDrop 2000C spectrophotometer (NanoDropTIM, Thermo Fisher Scientific, Waltham, MA, USA). All samples were diluted 10-fold with UPW before PCR.

A total of 253 unexposed F0 mosquitoes (Bashundhara, $n = 52$; Mirpur, $n = 54$; Dhanmondi, $n = 54$, Uttara, $n = 39$; Khilgaon, $n = 54$) and 448 unexposed F1 mosquitoes ($n = 112$) from each colony (except Khilgaon) were screened for the mutations V1016G, F1534C, and V410L.

To identify correlations between each mosquito phenotype after the insecticide exposure (unaffected, recovered, or dead) to a *kdr* genotype, we used a total of 500 F1 mosquitoes exposed to a 10× dose of permethrin and 524 F1 mosquitoes exposed to a 10× dose of deltamethrin from four Dhaka colonies (Bashundhara, Mirpur, Dhanmondi, and Uttara).

We used a quantitative PCR protocol for the detection of V1016G,[26] F1534C,[47] and V410L[28] mutations with minor modifications. Briefly, for V1016G and V410L, each reaction contained 10 μL of iTaq-SYBR Green Supermix (Bio-Rad Laboratories), 0.45 μL of each primer, 1 μL of template DNA and double-distilled water (ddH_2O) for a final reaction volume of 20 μL. Thermal cycling conditions were: 95°C for 3 min; 39 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 30 s; 95°C for 10 s, and a ramp from 65°C to 95°C at a rate of 0.2°C/10 s for melting curve analysis. For F1534C, each reaction contained 9 μL of iTaq-SYBR Green Supermix (Bio-Rad Laboratories), 0.65 μL of each primer, 2 μL of template DNA, and ddH_2O for a final reaction volume of 20 μL. Thermal cycling conditions were: 95°C for 3 min; 37 cycles of 95°C for 10 s, 57°C for 10 s, 72°C for 30 s; 95°C for 10 s, and a ramp from 65°C to 95°C at a rate of 0.2°C/10 s for melting curve analysis.

To confirm the presence of V1016G and F1534C homozygous mutations, 15 samples determined as homozygous for the V1016G allele and 10 for the F1534C allele were sequenced by Sanger amplicon sequencing. We included samples from a susceptible colony (from Cairns, Australia) as controls for the detection of wild-type alleles,[43] and samples from a well-characterized insecticide resistance phenotype, Timor-Leste colony (import permit: 11002978)[43] as a control for the V1016G mutation, and the samples from Mexico colony (import permit: 0001871610)[48] as a control for the F1534C mutation. For V1016G in the DIIS6 region, the forward primer (5′-ACAATGTG-GATCGCTTCCC) and

reverse primer (5′-GTTGATGTGCGATG-GAAATG) were designed by Martins *et al.*[49] For F1534C in the DIIIS6 region, the forward primer (5′-CAGCCGATTCGCGAGACC) and reverse primer (5′-GCGGGAGGTAAGTTATTGTGAAATCG), designed in-house, amplifies a 262 bp region. PCRs were conducted in a 25 μL reaction, consisting of 2.5 U of Phire polymerase, 5 μL 5× reaction buffer, 0.4 μL 10 mmol/L dNTPs, and 0.5 μmol/L forward and reverse primers (Thermo Fisher Scientific). Cycling conditions were: 98°C for 1 min, followed by 40 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 1 min and 30 s, and 72°C for 2 min. PCR amplicons were verified to have the correct molecular weight by gel electrophoresis, purified using a PCR Purification kit (Qiagen), quantified using a Nanodrop 2000, and sequenced by Sanger sequencing using 3.4 pmol of the reverse primer.

Chromatograms were analyzed with Chromas software version 2.6.6 (Technelysium Pty. Ltd). Sequence analysis was performed with BLAST NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Benchling software (https://www.benchling.com/).[50] All nucleotide sequences of DIIS6 and DIIIS6 were aligned using the sequences of the pyrethroid susceptible *Musca domestica* (GenBank accession number U38813.1), *Ae. aegypti* clone S2.2 voltage-sensitive sodium channel (VSSC) messenger RNA (mRNA), partial coding sequence (GenBank accession number MN365031.1) and *Aedes albopictus* sodium channel protein para (LOC109421922), mRNA (GenBank accession number XM_029865152.1) for annotation.

2.5 “Free-flight” assays in an experimental room

To understand the operational impact of insecticide resistance, we tested the effect of public health formulations and commercially available aerosol products from Dhaka under semi-field conditions (free-flight room). We used an internationally available disinsection product as a comparative standard. Tests were carried out using 3- to 5-day-old, non-blood-fed female F1 mosquitoes from the Mirpur, Dhanmondi, and Uttara colonies and a fully susceptible Australian strain (Cairns) as a control.[43] The Bashundhara colony was not included in this experiment as there were insufficient F1 mosquitoes. Historically in Dhaka, the City Corporations have used products containing a combination of permethrin, tetramethrin, and prallethrin applied as thermal fogs. We obtained two public health formulations currently used in Dhaka. The mortality caused by aerosols of those products was compared to domestic (household) aerosols used in Dhaka. The tested chemistries are listed in Table 2. When testing the efficacy of household aerosols, the WHO recommends inclusion of a reference insecticide for which there are data or which is in common use. For that purpose, we used a recommended standard dose of 0.7 g active ingredient (a.i.)/100 m^3 (35 g/100 m^3) for an internationally recognized disinsection aerosol (2% a.i. permethrin), namely “Top of Descent” (TOD) (Callington Haven Pty) as a reference insecticide.[51–53] The same dose was used for all aerosol mixtures calibrated to our 15.7 m^3 free-flight experimental room (26°C ± 1°C, 75% RH, 12.5 air exchanges per hour) against pyrethroid-susceptible and resistant mosquitoes. We released 5.5 g of insecticide by spraying products for a defined period into the free-flight room. The requisite discharge period was determined by weighing the aerosol canister before and after a series of preliminary timed sprays inside a fume hood (Table 2). Consumer products were applied from their original canisters but the public health formulations were collected in glass bottles from the manufacturing companies and were sprayed using Preval sprayer kit (PREVAL Complete Sprayer Kit;

Nakoma Products LLC). Three replicates, each containing > 25 mosquitoes, were performed for each test. The replicates were separated by a 2-h time interval to ensure that the air exchange system had exhausted the room of aerosol droplets. Public health insecticides from Bangladesh were oil-based, so the room was also cleaned with detergent after each aerosol release. In order to check that there were no insecticide residues remaining from prior tests, 15 susceptible mosquitoes were released into the free flight room before each test and 30-min survival was determined. Tests proceeded if there was no mortality.

2.5.1. Sub-lethal/behavioral impact of aerosols on host-seeking mosquitoes

—*Aedes aegypti* females were released into the room and allowed 15 min to acclimatize. An observer entered and sat in the room and counted the number of mosquitoes landing on one leg exposed from knee to toe for 5 min. The observer was otherwise fully protected. As mosquitoes landed, they were counted and waved away. This qualitative measure of host landing prevents biting and does not confound results by the sequential removal of mosquitoes during the observation.[48] The observer then exited the room and the insecticide was released. After 30 min, the observer re-entered the room and mosquito landing events were recorded again for a further 5 min.

2.5.2 Lethal impact of aerosols—All KD and live mosquitoes were collected and kept separately in cups for 24 h with 10% sucrose solution supplied *ad libitum*. After 24 h, alive and recovered mosquitoes were counted and transferred to a cage, left for 5 min then allowed to bite on the forearm of a volunteer for 5 min. Mosquitoes were considered fed if any amount of blood was detected in the midguts (partially or fully engorged). For any other situation (if mosquitoes did not land or landed but did not probe or landed and probed but did not feed), the mosquitoes were classified as unfed.

2.6 Data analysis

Resistance was classified using WHO guidelines.[38] For diagnostic dose (1×), ≥ 98% mortality: susceptible, < 98% to 90% mortality: developing resistance, and < 90% mortality: resistant. For intensity assays (5×), ≥ 98% mortality: low-intensity resistance, < 98% mortality: moderate to high-intensity resistance. For intensity assays (10×), ≥ 98% mortality: moderate-intensity resistance and < 98% mortality: high-intensity resistance. Student's *t*-test was used to test the difference in the mean KD and mortality within and between groups for all bioassays, synergist assays, and free-flight assays. Fisher's exact test was performed to test the association between genotypes of two generations (F0 and F1). Logistic regression was conducted to determine the odds of mosquitoes with different V1016G allele frequencies surviving exposure to permethrin 10× and deltamethrin 10× doses. One sample test of proportions was used to test for the difference between certain genotype combinations over others. These calculations were made using Stata 15 (StataCorp LLC, College Station, TX, USA).

3. RESULTS

3.1 Seasonal dengue trends in Bangladesh

A comprehensive analysis of historical data on dengue showcases a distinct pattern of seasonality. The monsoon months of June to September are characterized by the highest levels of rainfall in the country, which consistently accompany a surge of dengue cases (Fig. 2). This trend has been observed repeatedly and provides strong evidence of the seasonal nature of dengue in Bangladesh.

3.2 Insecticide bioassays

A total of 12 713 female F1 mosquitoes belonging to the four Dhaka colonies and 1119 susceptible Cairns *Ae. aegypti* as control were tested across the bioassays.

Bioassays with permethrin confirmed that the susceptible control strain (Cairns) exhibited 100% KD after 30 min of exposure to the diagnostic dose (1×, 15 μg/bottle). These mosquitoes did not recover, and all were scored dead after a further 24 h. High levels of resistance to permethrin were recorded across all Dhaka colonies. Percentage KD following 30-min exposure time to the diagnostic dose (1×) was 0% in all four colonies except Mirpur (KD < 1%). Even at 10× of the diagnostic dose, KD remained low for all colonies, ranging from 17.6% to 50.8% for Uttara and Bashundhara, respectively. After exposure to 10× the diagnostic dose of permethrin, the highest mortality after a 24-h holding period was only 23.9% ($P = 0.031$) in the Bashundhara mosquito colony, reflecting significant recovery from KD (Fig. 3).

Bioassays with deltamethrin revealed that the susceptible control strain (Cairns) showed 100% KD after 30 min of exposure to the diagnostic dose of deltamethrin (1×, 10 μg/bottle). These mosquitoes did not recover, and all were scored dead after a further 24 h. Substantial deltamethrin resistance was observed across all colonies. In response to the diagnostic dose (1×), only the Bashundhara colony exhibited complete KD, however, this was followed by significant recovery (only 32.8% mortality, $P = 0.00002$) after 24 h. Otherwise, KD ranged from 58% (95% confidence interval [CI] ± 5.92) in the Uttara mosquito colony to 83.4% (95% CI ± 3.73) in the Mirpur mosquito colony. All mosquito colonies exhibited high levels of KD at higher doses but significantly ($P < 0.05$) higher recovery rates (low mortality) after 24 h except the Uttara mosquito colony at 10× (Fig. 4).

All colonies were fully susceptible to the diagnostic dose of malathion (1×, 50 μg/bottle) and bendiocarb (1×, 12.5 μg/bottle). No recovery was observed from KD mosquitoes.

3.3 Impact of synergists on insecticide resistance

Using Student's *t*-test it was demonstrated that the synergists PBO and DEF applied prior to the exposure to 10× the diagnostic dose of permethrin caused significant ($P < 0.05$) increases in KD and mortality (Fig. 5). For instance, in the Uttara colony, KD and mortality rates were increased by 52% ($P = 0.038$) and 31% ($P = 0.013$) after exposure to PBO and by 33% ($P = 0.002$) and 19% ($P = 0.002$) after exposure to DEF. In one colony, susceptibility was fully restored by the addition of the oxidase inhibitor PBO (Bashundhara, Fig. 5). This suggests

that both oxidases and esterases are major contributors to permethrin-resistant phenotypes in *Ae. aegypti* from Dhaka. The addition of GST synergist (EA) had a significantly higher (P = 0.002) effect on the mortality of the Bashundhara colony compared to the mortality without EA pre-exposure (Fig. 5).

The KD and mortality responses to deltamethrin following exposure to synergists varied widely between the colonies (Fig. 6). The Bashundhara and Mirpur colonies were exposed to synergists prior to the diagnostic dose (1×) of deltamethrin. The Dhanmondi and Uttara colonies were more insecticide resistant and were therefore tested at 2× the diagnostic dose. In almost all cases, mortality at 24 h post-exposure was significantly higher ($P < 0.05$) when pre-exposed to PBO or DEF. For instance, in Uttara, the KD and mortality rates increased by 12% ($P = 0.053$) and 43% ($P = 0.011$) after exposure to PBO and by 10% ($P = 0.082$) and 36% ($P = 0.041$) after exposure to DEF. This suggests that oxidases and esterases are also important contributors to the deltamethrin-resistant phenotype in *Ae. aegypti* from Dhaka. Except for the Uttara colony, EA had less impact overall (Fig. 6).

3.4 Frequency of *kdr* alleles

Mutant homozygotes at position 1016 (GG) were detected 78% from Mirpur and Dhanmondi (F0) and 98% from Khilgaon (F0) female individuals. The decrease in the frequency of GG mutant homozygotes from F0 to F1 was not statistically significant (P = 0.245, Fisher's exact test) (Fig. 7(A)). However, there was approximately a 20% increase in the wild-type allele (VV) from F0 to F1 for Mirpur and Dhanmondi. In the case of F1534C, the majority of the mosquitoes were homozygous wild-type (FF) in both F0 and F1 generations ranging from 78% in Mirpur F0 to 97% in Uttara F0 (Fig. 7(B)). No mosquitoes carried the V410L mutation (i.e., all individuals were homozygous for the wild-type allele).

Using the partial sequencing of the VGSC gene, we confirmed that all V1016G mutant homozygotes had the valine to glycine substitution of GTA to GGA. In the case of F1534C, nine out of 10 samples had a homozygous substitution of TTC to TGC, which changes phenylalanine to cysteine, while the other sample had a heterozygous genotype.

One-sample test of proportions confirmed a significantly higher frequency of the V1016G mutant homozygous genotype with homozygous wild-type genotypes of F1534F and V410V ($GG_{V1016G}/FF_{F1534F}/VV_{V410V}$) when compared to other genotype combinations (mean = 0.794, 95% CI 0.694–0.894, $P < 0.0001$).

3.5 Correlation of *kdr* genotype frequencies with the phenotype of mosquitoes surviving pyrethroid exposure

The presence of V1016G homozygous mutant alleles (GG) was associated with survival following permethrin exposure. Almost all individuals with that genotype were F1534F homozygous wild-type (FF) (Supporting Information, Fig. S1(A)), but the results from synergist assays suggest that metabolic mechanisms are also likely to have contributed to survival. Logistic regression analysis revealed that permethrin 10× survived mosquitoes had mutant homozygotes at position 1016 (GG) 2.3 times (95% CI: 1.16–4.71, $P = 0.017$) more than VV and VG genotypes (Table 3). This GG genotype was present 1.6 times more among

deltamethrin 10× surviving mosquitoes but it was not statistically significant (Table 3, Fig. S1(B)).

3.6 Free-flight assays: lethal and sub-lethal impacts

In contrast to bottle assays, the free-flight assays exposed mosquitoes to insecticides while they exhibited typical flight, resting, and host-seeking behaviors. In these tests, three insecticide formulations (A1, P1, and P2) were fully effective against the susceptible (Cairns) strain causing 100% KD and 100% mortality (Fig. 8). Household aerosols failed to KD 100% of susceptible mosquitoes, although after 24 h, mortality ranged from 95% to 98%. Notably, the formulation P1, used for operational control in Bangladesh was the most effective aerosol in terms of KD (82–91%) and mortality (76–98%) of the resistant colonies (Fig. 8). In comparison, the international standard (A1) caused 27–60% and 53–81% KD and mortality respectively.

In all colonies, a significant ($P < 0.05$) reduction in mosquito landing was observed immediately after insecticide exposure (Fig. 9). However, 24 h post-exposure, a large percentage of surviving mosquitoes from the Dhaka colonies (39–88%) continued to feed on a human volunteer (Fig. 10). The few ($n = 7$) fully susceptible mosquitoes (Cairns) that survived also took a blood meal (data not shown), which suggests that any sub-lethal effects of pyrethroids are transient.

4 DISCUSSION

We report very high levels of pyrethroid resistance in *Ae. aegypti* from Dhaka, supported by the results of bioassays that employed a diagnostic dose and multiples of that dose (2×, 5×, and 10×). The very high frequency of the V1016G *kdr* allele suggests that the population is under selective pressure and that *kdr* is contributing to the phenotype, particularly for permethrin. Survivors of permethrin exposure were significantly more likely to have the homozygous V1016G mutation. Tests with synergists also indicated an important role of metabolic mechanisms of resistance in *Ae. aegypti* from Dhaka. Importantly, results from the assays that exposed free-flying and resting mosquitoes to semi-field exposures of standard aerosols suggested a strong operational impact of the resistance on the effectiveness of commercial products containing pyrethroids.

Prolonged use of type-I pyrethroids by thermal fogging campaigns and domestic aerosols has played an important role in the development of high-intensity resistance in *Ae. aegypti* from Dhaka. Although most formulations used in Dhaka contain type-I pyrethroids (i.e., permethrin, phenothrin, prallethrin), it is likely that the selected resistance mechanisms have also contributed to the observed resistance against a type-II molecule, deltamethrin.[56] Using the WHO criteria for resistance classification, populations of *Ae. aegypti* from across Dhaka demonstrated “high intensity” resistance to both type-I (permethrin) and type-II (deltamethrin) pyrethroids (showing < 98% mortality at 10× the dose that kills 100% of susceptible insects). Standard bottle assays using the recommended diagnostic dose and time of 30 min showed 2.4–23.8% mortality for permethrin, and 48.9–94.3% mortality for deltamethrin, across Dhaka. Overall, the Dhaka colonies exhibited expected dose–response (i.e., higher mortality at higher doses for any given insecticide and colony). However,

both the Bashundhara and Mirpur colonies exhibited higher mortality at 2× the diagnostic dose of deltamethrin when compared to 5× the dose. The effect did not persist at 10× the deltamethrin dose and the relationship did not hold true for permethrin. Such anomalies are not uncommon in studies where a large number of insecticide bioassays were conducted[57–60] and may be due to hormesis, a phenomenon in which sublethal insecticide exposure may increase inset survival via a range of mechanisms including stimulating insecticide metabolism.[61,62]

Recently, some researchers have recommended that a bottle assay's measure of KD over time could be augmented with a second endpoint to determine mortality.[44–46] In this study we held mosquitoes for 24 h after insecticide exposure to determine final mortality and to monitor recovery from KD. Our analyses on the correlation between percentage KD and mortality data yielded moderate ($r^2 = 0.73$, $P = 0.0166$) and low ($r^2 = 0.29$, $P = 0.0001$) correlation for permethrin and deltamethrin, respectively (Fig. S2), indicating much recovery after KD that the relationship between KD and mortality is poor. That means that while the traditional CDC bottle assay (KD, 30 min) may be effective at classifying "resistance" in mosquito populations, it is not as effective at predicting mortality, nor was it designed to do so.

Pyrethroids act on the nervous system of insects and many have a rapid "knockdown" effect. Whether insects with mutant *kdr* alleles recover from KD depends on the insecticide dose, the degree to which the mutation prevents binding, and the rate of insecticide metabolism. In this study, the substantial recovery seen after KD suggests poor binding of the pyrethroid to the mutated VGSC and a key role for metabolic mechanisms in "mopping up" the pyrethroids.[27,63]

The resistance phenotypes of Dhaka colonies were associated with a high frequency of the *kdr* mutation V1016G and with metabolic mechanisms. A significant increase in KD and mortality after exposure to the oxidase inhibitor PBO and esterase inhibitor DEF demonstrates that metabolic resistance, mediated by oxidases and esterases play an important role. This is widely reported for *Ae. aegypti*.[64] In this study, the susceptibility of one colony, Bashundhara, was fully restored following the application of PBO prior to permethrin exposure at 10× dose. For other colonies, PBO pre-exposure also caused significant increases in the mortality caused by permethrin and deltamethrin exposure. Type-I (permethrin) and type-II (deltamethrin) pyrethroids are both bound and degraded by P450 oxidases.[65] Our results revealed that oxidative metabolism plays a major role in pyrethroid resistance in *Ae. aegypti* in Dhaka, and that synergized pyrethroids might be used to overcome some of that resistance. In addition, the contribution of esterases (as assessed by DEF pre-exposure) and GSTs (as assessed by EA pre-exposure) was also detected by some degree of recovered susceptibility, further highlighting the importance of additional metabolic resistance mechanisms in these colonies. Synergist assays have advantages over biochemical methods for implicating metabolic mechanisms of resistance because we can observe the direct impacts of synergism on the resistant phenotype. In contrast, biochemical assays, which were used to characterize resistance mechanisms in the baseline Bangladesh study only documented changes in the titre of non-specific enzymes without any indication of impact.[23]

In the previous Bangladesh study, the *kdr* mutations V1016G and F1534C were found at frequencies of 37.8% and 54.1%, respectively, in *Ae. aegypti* collected in 2017 from Dhaka.[23] Our current study reports higher frequencies (78–98%) of V1016G homozygotes but lower frequencies (~2%) of F1534C homozygotes. Such changes in the allele frequencies could not be correlated with phenotypic resistance between these two studies because the baseline study only included exposure to diagnostic doses.[23] It is noteworthy that the largest CHIKV outbreak took place in Dhaka during 2017.[7] From that year, city corporations strengthened control measures by increasing thermal fogging and spraying larvicides.[66,67] The use of household aerosols and mosquito coils was also thought to have increased during this time.[68] These factors could have increased selection pressure resulting in the higher frequency of V1016G we observed. Rapid increases in *kdr* allele frequency among pyrethroid resistant mosquitoes in response to vector control interventions, including the application of insecticide-treated nets (ITN), IRS, and pyrethroid sprays are common. In Asembo and Seme, western Kenya, the frequency of L1014S increased in *Anopheles gambiae* population from near zero to 100% and > 80% between 1996 and 2010 and between 2000 and 2008, respectively, with significant increases in each year. Within those years, major changes in mosquito control took place, for example, in 1997 permethrin-based ITNs were provided to half of the villages in Asembo and by 2003 there was full coverage of ITNs. In Seme, ITN ownership increased between 2000 to 2003 followed by major ITN distribution campaigns in 2004 and 2006.[69] Similar observations have been made from other studies following pyrethroid-based interventions.[70–72] A sudden increase in a *kdr* allele was observed in Colombo, Sri Lanka where the F1534C mutant allele increased from 17.5% to 80.2% in just 2 years.[73] A rapid increase was also observed in *Anopheles coluzzii* in Malabo, Equatorial Guinea, where the L1014F mutant allele increased from 28.5% to 100% between 2011 and 2015 after installation of pyrethroid-based IRS in 2012.[74] Rapid changes over time and high spatial variation in the *kdr* allele frequency has also been recorded from Mexico.[28,75,76] Changes among genotypes within a couple of years were also observed in *Ae. aegypti* populations in Brazil.[77]

The V1016G and F1534C *kdr* mutations have been widely reported in Asia[47,78,79] and both have been implicated in reducing the sensitivity of VGSC to permethrin and deltamethrin.[27] An additional mutation, V410L, has also been reported in association with pyrethroid resistance in the Americas, but its presence in Asia has not been well studied[28] and we confirm that it is not yet present in Dhaka.[23] The V1016G mutation, in isolation, is known to exert considerable protection against both type-I (permethrin) and type-II (deltamethrin) pyrethroids, while the F1534C mutation is thought to confer lower-level resistance to type-I pyrethroids.[27,80,81] It is notable that the active ingredients in all domestic aerosols and public health space sprays identified for this current study are type-I pyrethroids (Table 2). The selection pressures exerted by this chemistry are known to play major roles in the selection of local mosquito genotypes.,[82,83] therefore, it is not surprising that an allele that exerts protection against type-I pyrethroids (V1016G) has been selected in populations from Dhaka.

Routine monitoring by bioassay alone is generally considered an inadequate proxy for predicting insecticide effectiveness in the field, and options to rotate or replace insecticides are limited.[84] The operational implications of resistance identified in bioassays or by

screening for resistance-associated alleles must therefore be carefully considered. Recently, adaptations to bioassays that measure the intensity of resistance using multiples of the diagnostic dose are thought useful for predicting vector control failure, but even these intensity assays have not yet defined a threshold at which operational campaigns are threatened.[85] Our "free-flight" assays provided a valuable connection between bioassays, molecular screening, and mosquito responses to commercial insecticides in "semi-field" settings. Our free-flight tests exposed mosquitoes exhibiting natural behaviors to standard applications of formulated aerosols. The volumes of aerosols allied per cubic meter were derived from WHO recommendations and validated using an international disinsection product. Subsequent observations on mortality allowed us to examine the impacts of domestic and public health formulations of insecticides on resistant Dhaka mosquitoes, in comparison to a fully susceptible colony. Our results demonstrated that the level of permethrin resistance in the Dhaka colonies compromised the effectiveness of both public health and household pyrethroid aerosols. Most formulations, including the standard, had limited effects on the Dhaka population (25–81% mortality), with the exception of one of the public health formulations (P1), causing 76–99% mortality. It is not clear why P1 retained efficacy as its constituents are also type-I pyrethroids, formulated at similar doses to the other tested formulations. Designed for thermal fogging, this product is formulated in an oil-based carrier (kerosene), which can increase toxicity,[86] but a similarly formulated product (P2) showed much lower efficacy (40–79% mortality). It is notable that the most effective products (P1. P2, and A1) are also those with the highest pyrethroid content (Table 2). Also, the time elapsed for releasing the appropriate amount of chemicals for this insecticide was highest among all the test formulations, so it was potentially more evenly distributed in the free-flight assay room than other formulations tested.

Free-flight experiments also allowed us to assess host-seeking and biting behaviors in sub-lethally exposed mosquitoes.[81] Sub-lethal exposure to neurotoxic compounds like type-I pyrethroids has been shown to negatively impact these behaviors, sometimes for pro-longed periods.[81,87,88] This has obvious implications for disease transmission. Although we detected a significant reduction in landing attempts and host location immediately following insecticide exposure both susceptible and resistant survivors recovered their feeding behavior after 24 h. This corroborated some observations on resistant mosquitoes[81] that indicate a very transient effect of sub-lethal insecticide exposure on key behaviors.

5 CONCLUSION

Intensive use of pyrethroids in Dhaka has selected for highly resistant mosquito populations, which was confirmed through bioassays. This pyrethroid resistance is associated with high frequencies of the V1016G *kdr* mutation and activities of detoxifying enzymes. As evidenced from our free-flight experiments, the effective operational use of pyrethroids in control programs is compromised and therefore, requires reconsideration. Despite some malathion resistance reported from one Dhaka colony in the baseline study,[23] other colonies were shown to be susceptible. Here, we also recorded full susceptibility of *Ae. aegypti* colonies to malathion and bendiocarb. Therefore, switching to either of these chemicals could serve as an interim measure.[89] If other additional techniques can be adopted (e.g., targeted indoor residual spraying,[90,91] or the deployment of spatial repellents[48,92]) then

the choice of insecticides might be expanded. Ultimately, scalable and sustainable non-insecticide-based approaches such as *Wolbachia*-based population replacement could have an important role in curbing *Aedes*-borne diseases in Bangladesh.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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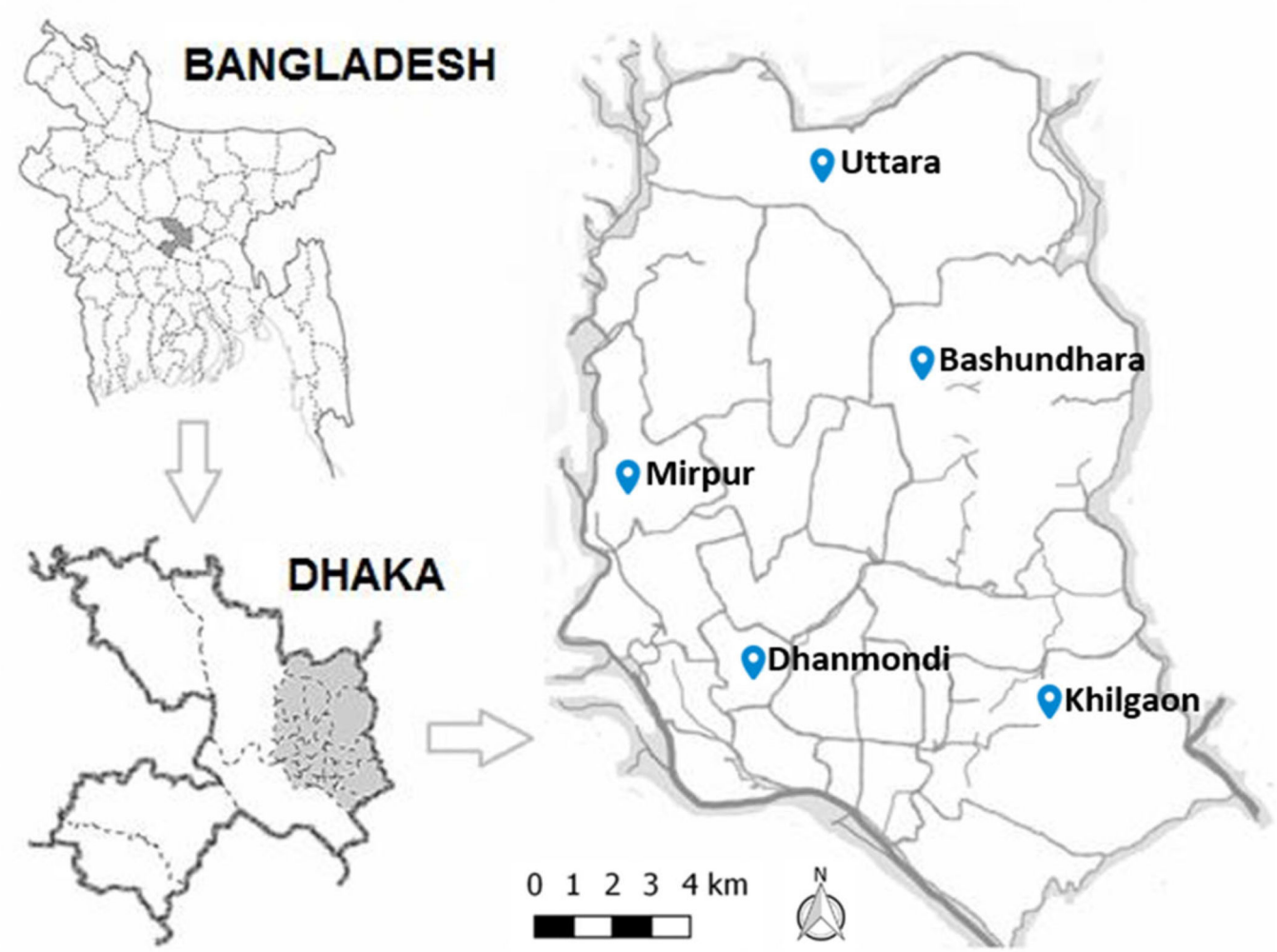

Figure 1.
Aedes egg collection areas in Dhaka, Bangladesh.

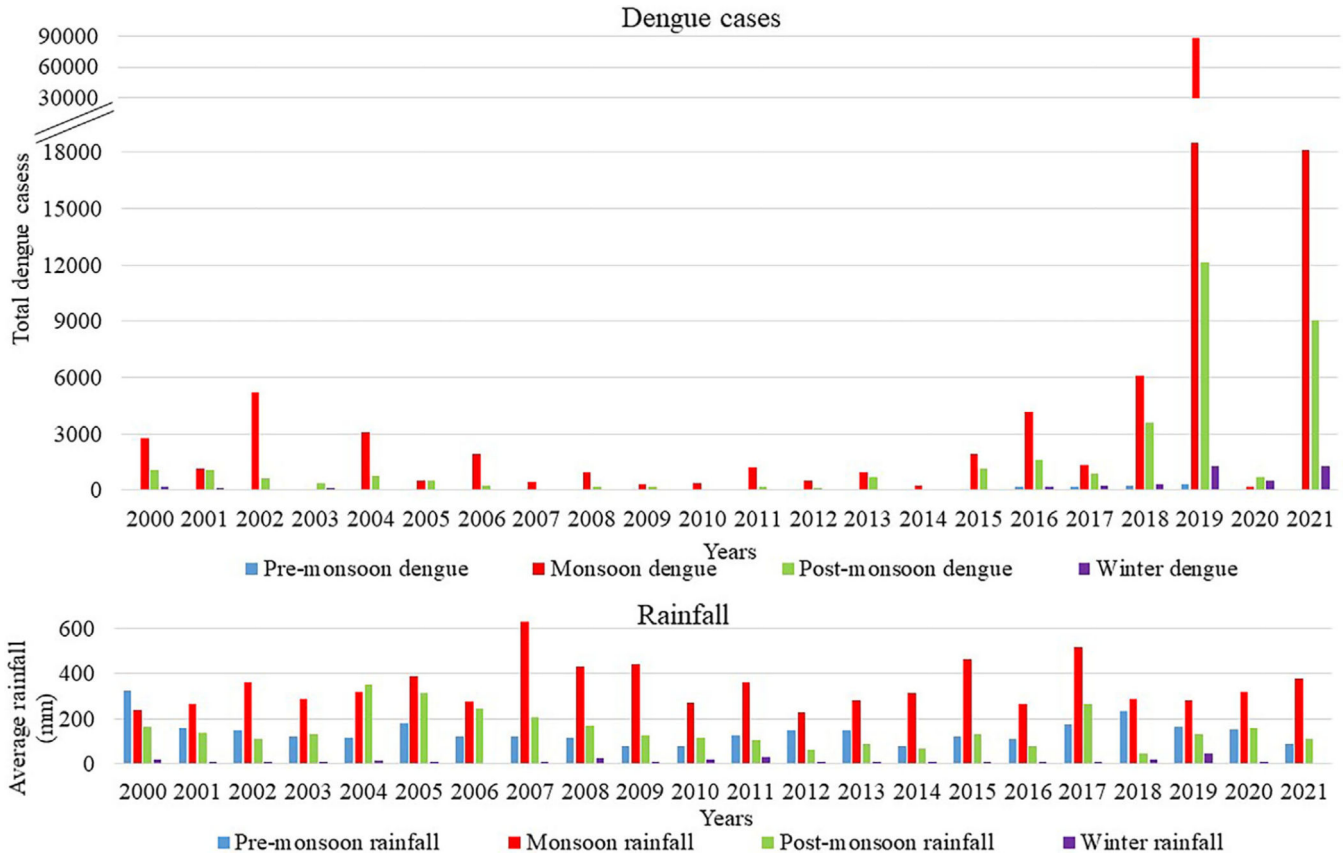

Figure 2.

Total reported dengue cases and seasonal average rainfall (mm) from 2000 to 2021 in Bangladesh. Pre-monsoon = March–May; Monsoon = June–September; Post-monsoon = October–November; Winter = December–February.

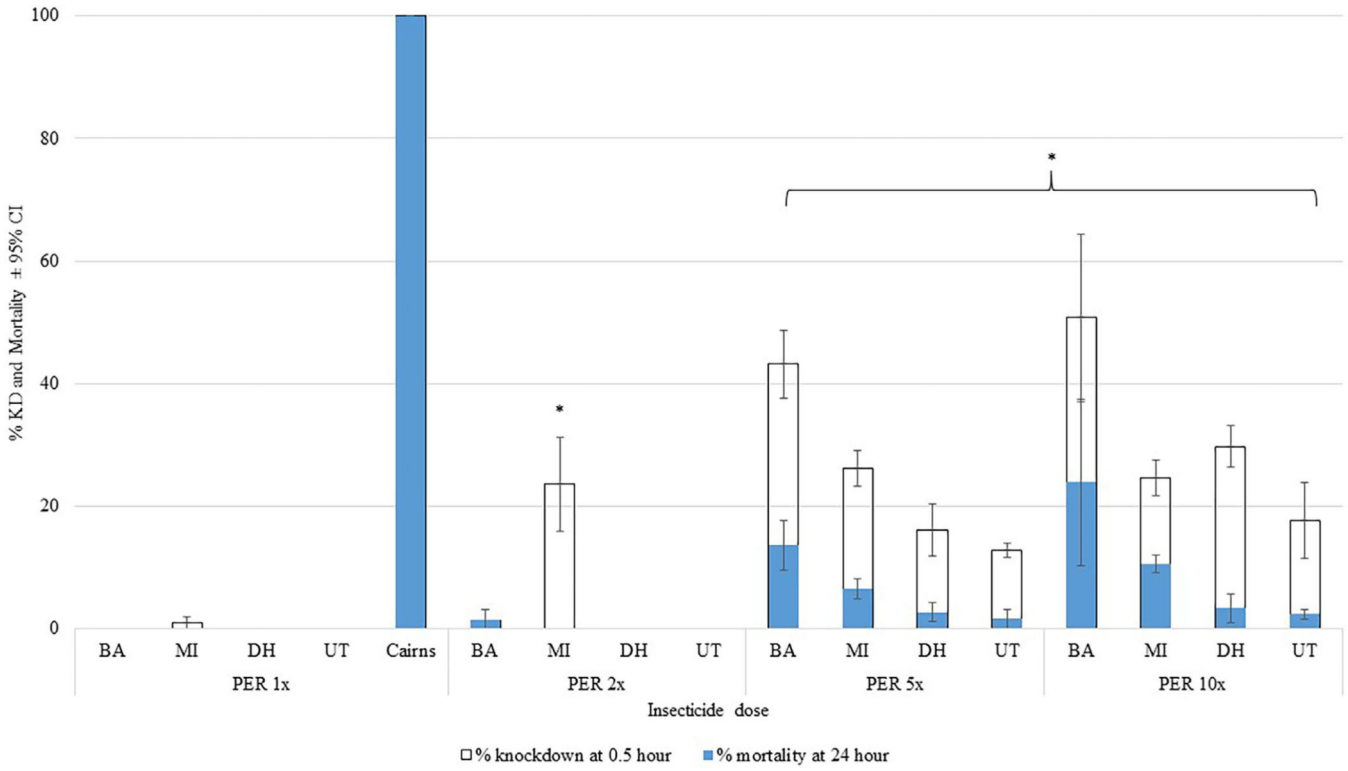

Figure 3.

Aedes aegypti bioassay results (mean knockdown [KD] and mortality ±95% confidence interval [CI]) from four colonies exposed to permethrin. Percent KD (white) at 30-min and 24-h mortality (blue) are shown for mosquitoes exposed to a diagnostic dose (1×, 15 μg/bottle) and to multiples of that dose (2×, 5×, and 10×). BA, Bashundhara; DH, Dhanmondi; MI, Mirpur; PER, Permethrin; UT, Uttara. *Significant recovery from KD for each colony.

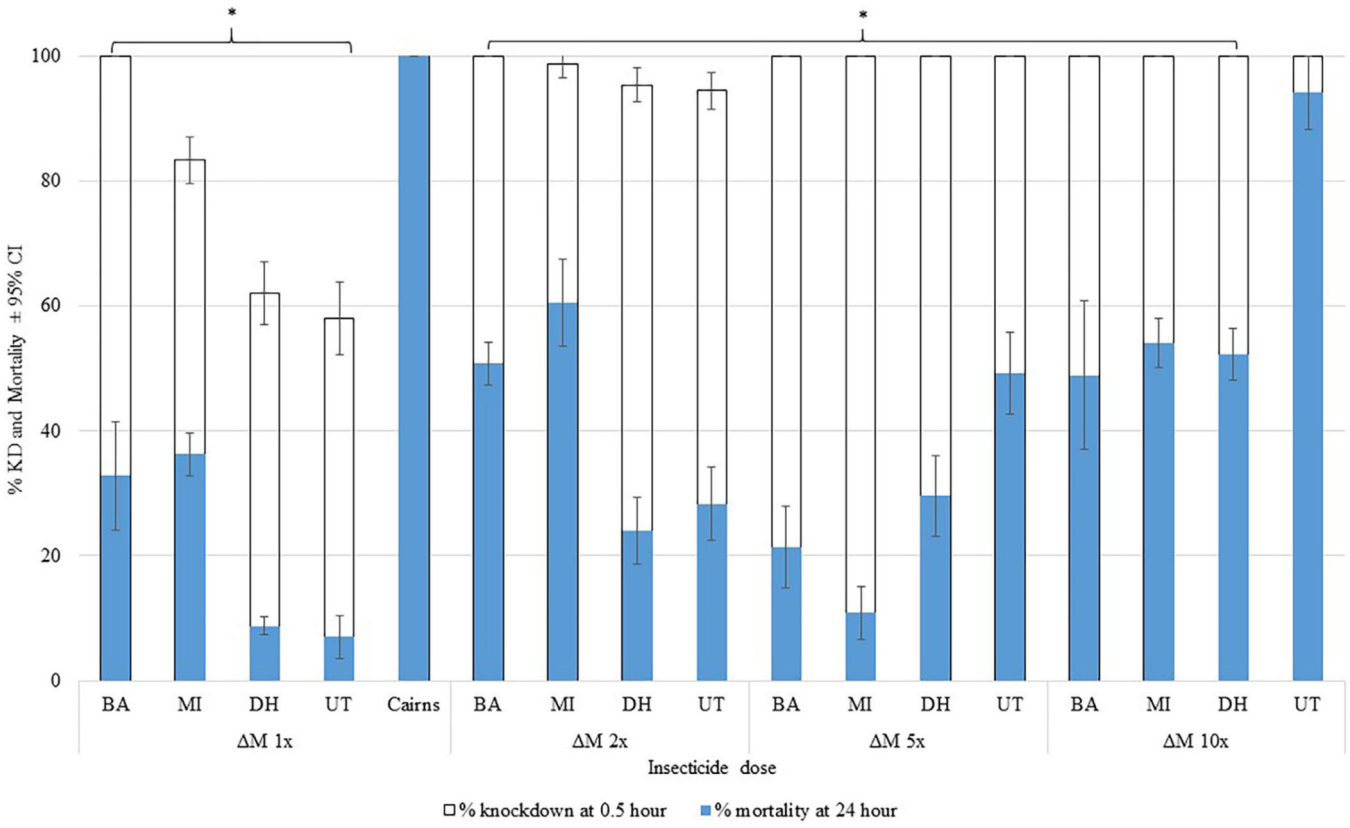

Figure 4.

Aedes aegypti bioassay results (mean knockdown [KD] and mortality ±95% confidence interval [CI]) from four colonies exposed to deltamethrin. Percent KD (white) at 30-min and 24-h mortality (blue) are shown for mosquitoes exposed to a diagnostic dose (1×, 10 μg/bottle) and to multiples of that dose (2×, 5×, and 10×). ΔM, Deltamethrin; BA, Bashundhara; DH, Dhanmondi; MI, Mirpur; UT, Uttara. *Significant recovery from KD for each colony.

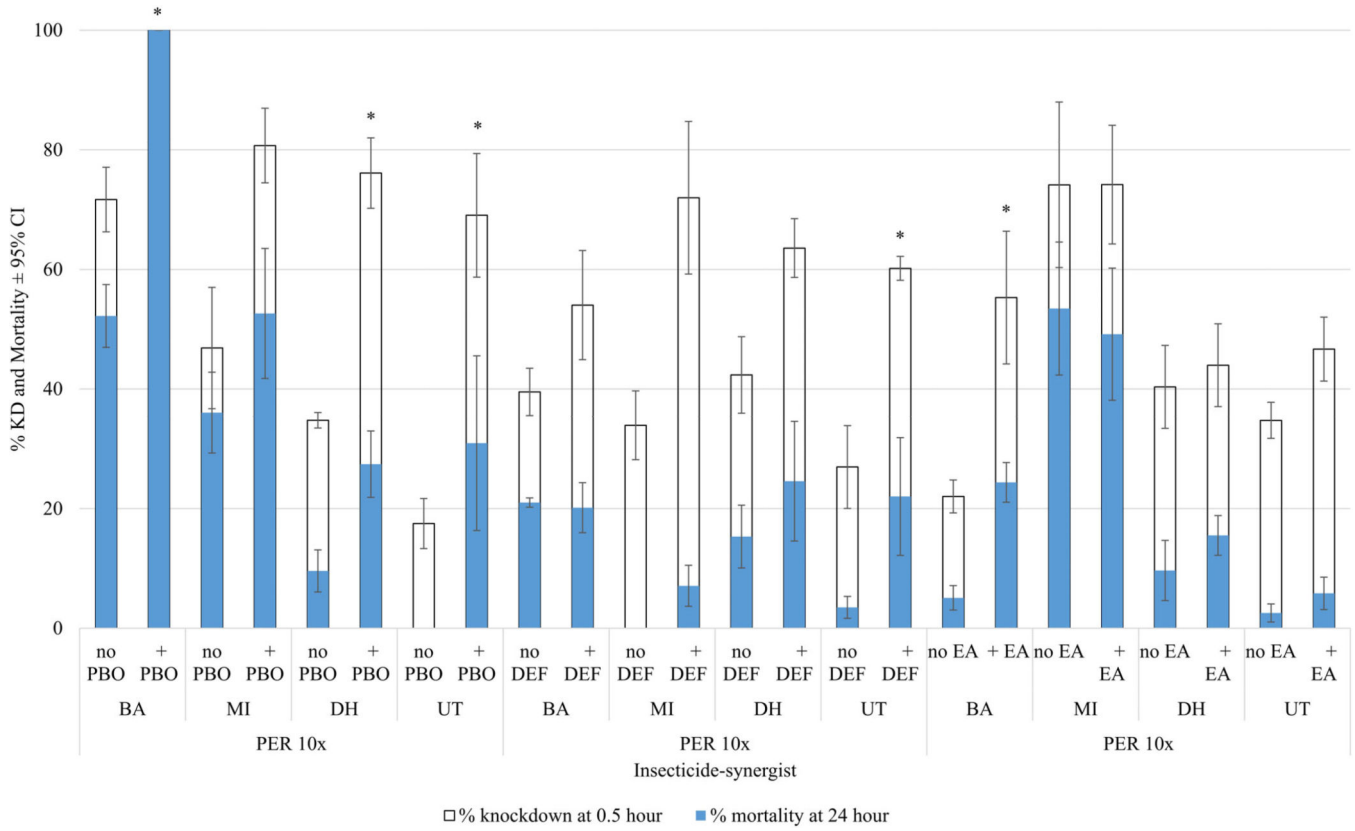

Figure 5.

Aedes aegypti synergist bioassay results (mean knockdown [KD] and mortality ±95% confidence interval [CI]) for four colonies exposed to synergists followed by 10× the diagnostic dose of permethrin. Percent KD (white) and mortality (blue) are shown. BA, Bashundhara; DH, Dhanmondi; MI, Mirpur; PER, Permethrin; UT, Uttara. *Significant increase in KD and mortality after adding synergist.

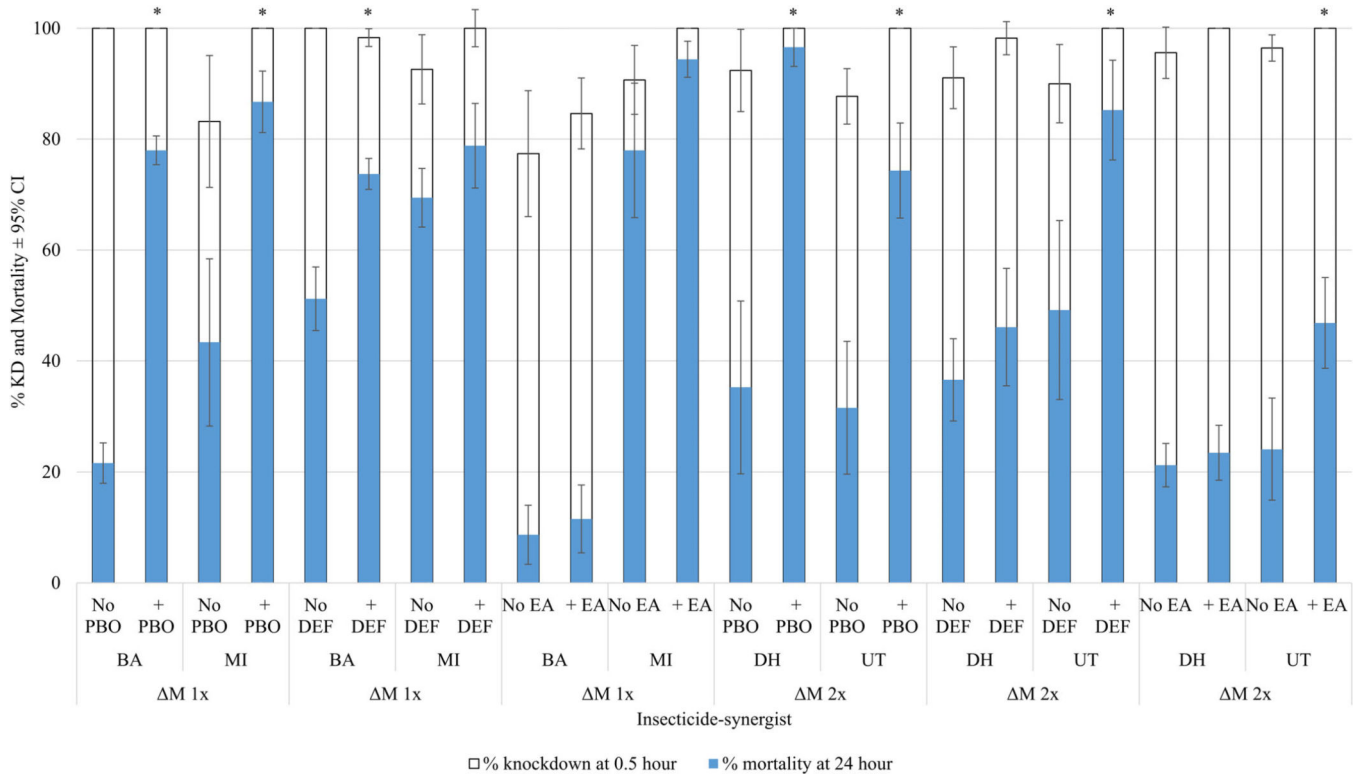

Figure 6.

Aedes aegypti synergist bioassay results (mean knockdown [KD] and mortality ±95% confidence interval [CI]) for colonies pre-exposed to synergists followed by either the diagnostic dose of deltamethrin or 2× the diagnostic dose. Percent KD (white) and mortality (blue) are shown. ΔM, Deltamethrin; BA, Bashundhara; DH, Dhanmondi; MI, Mirpur; UT, Uttara. *Significant increase in mortality after adding synergist.

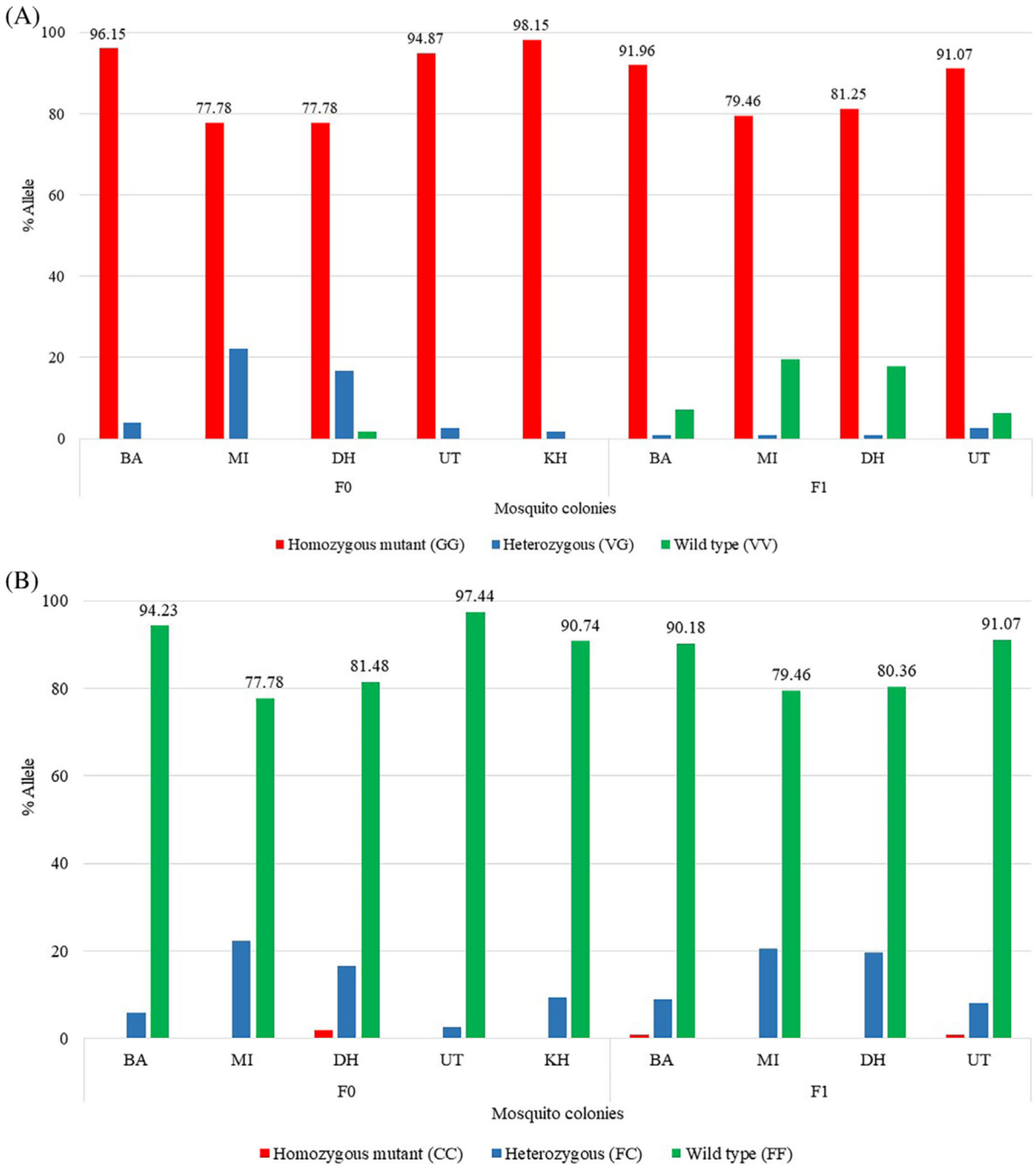

Figure 7.

Frequency of (A) V1016G and (B) F1534C *kdr* genotypes across all *Aedes aegypti* colonies for F0 and F1 generations. BA, Bashundhara; DH, Dhanmondi; KH, Khilgaon; MI, Mirpur; UT, Uttara.

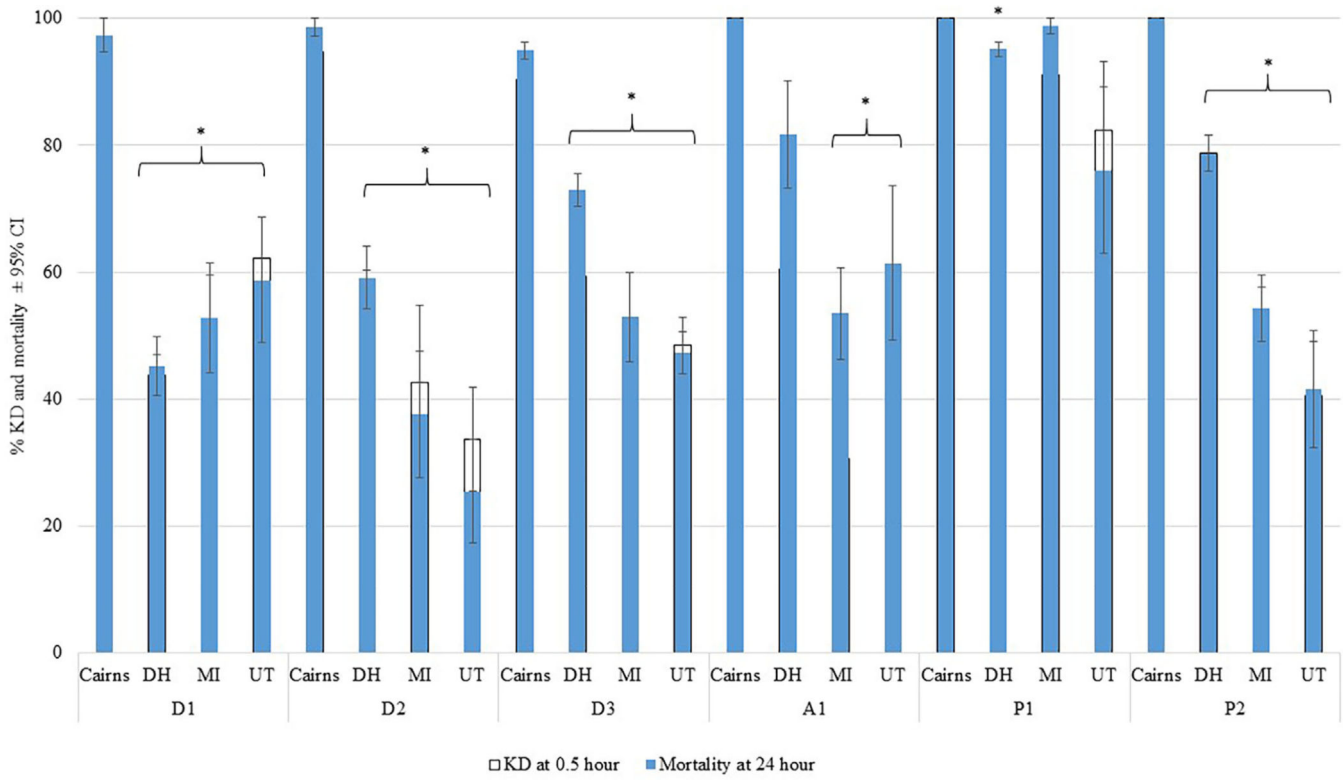

Figure 8.

Aedes aegypti free-flight test results (±95% confidence interval [CI]). Percent knockdown (KD) (white) at 30-min and 24-h mortality (blue) are shown. Each group of *x*-axis label includes mosquito colonies and tested aerosol. DH, Dhanmondi; MI, Mirpur; UT, Uttara. *Significantly lower mortality than the susceptible Cairns strain.

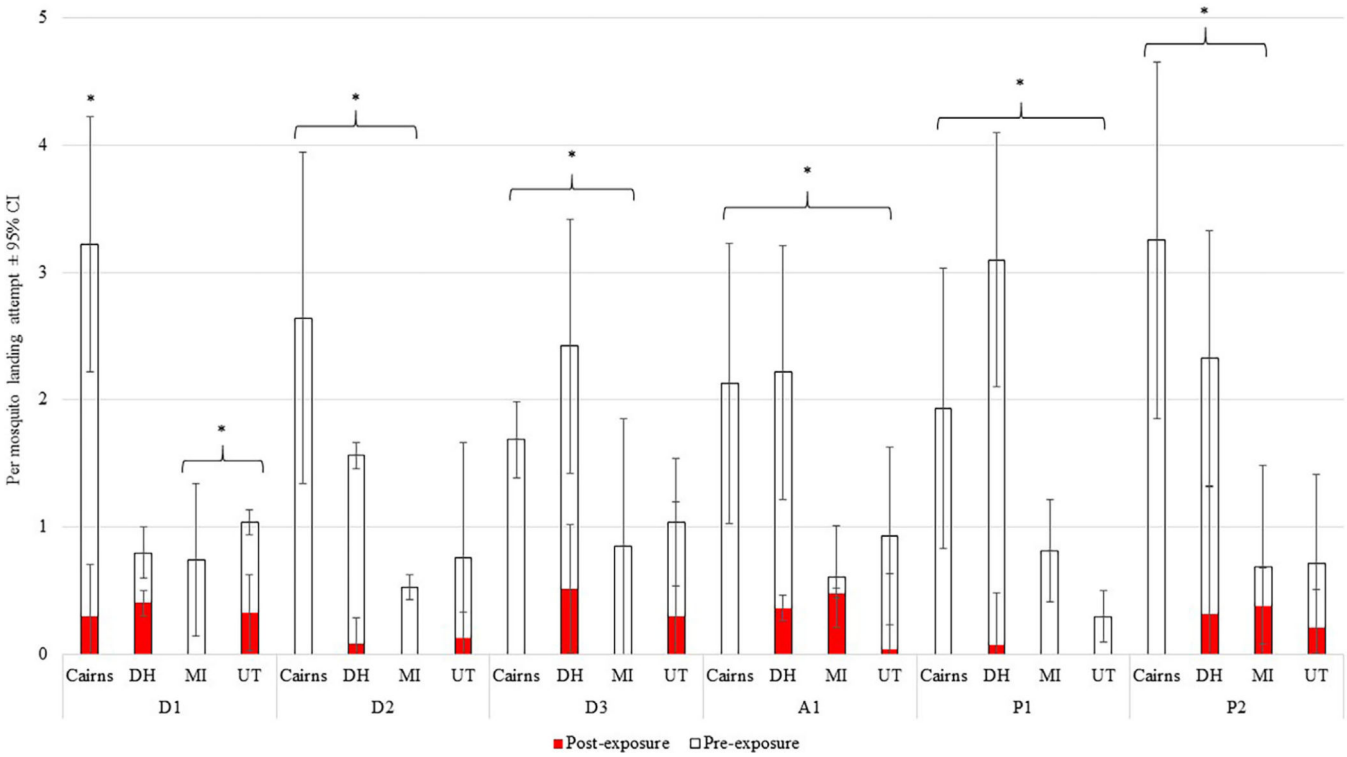

Figure 9.

Per mosquito landing attempts, pre- and post-exposure of insecticides. Each group of *x*-axis label includes mosquito colonies and tested aerosol. DH, Dhanmondi; MI, Mirpur; UT, Uttara. *Significant difference between pre- and post-exposure per mosquito landing attempts within each colony for each insecticide.

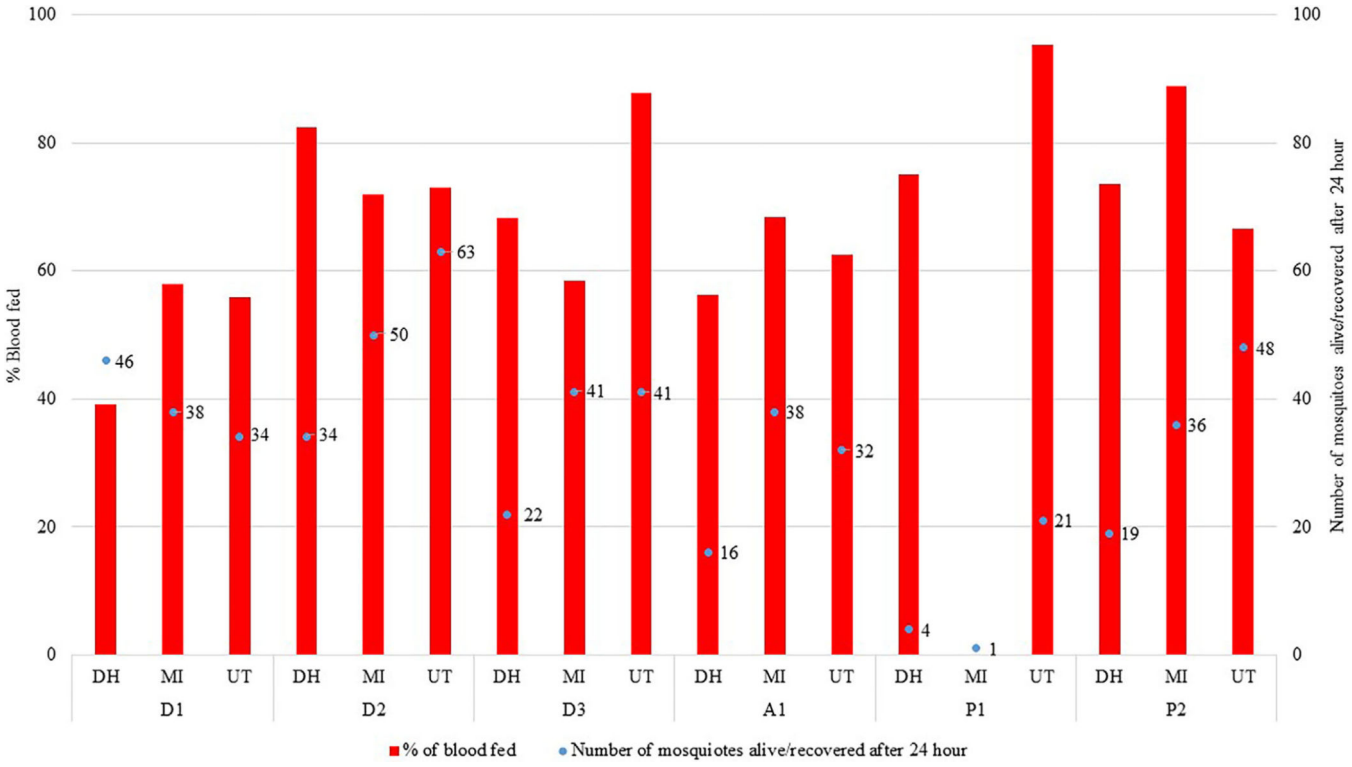

Figure 10.

The number of surviving mosquitoes and the percentage that blood-fed 24 h after insecticide exposure. Each group of *x*-axis label includes mosquito colonies and tested aerosol. DH, Dhanmondi; MI, Mirpur; UT, Uttara. Cairns data are not shown because so few ($n = 7$) mosquitoes survived.

Table 1.

Selected insecticides with diagnostic concentrations and exposure times for Aedes mosquitoes used in the study[42]

Insecticide	Insecticide class	Insecticide concentration (μg/bottle)	Diagnostic/discriminating dose	Exposure time (min)
Permethrin	Pyrethroid	15	1×	30
Deltamethrin	Pyrethroid	10	1×	30
Malathion	Organophosphate	50	1×	30
Bendiocarb	Carbamate	12.5	1×	30

Table 2.

Selected insecticides for the free-flight tests with *Aedes aegypti*

Insecticides	Commercial Name	Company	Purpose	Origin	Time chemical released (second)	Code name used in the manuscript
0.20% *w/w* Prallethrin and 0.15% *w/w* d-phenothrin	ACI Aerosol (ACI)	ACI Limited	Domestic	Bangladesh	4.2	D1
0.075% *w/w* Prallethrin and 0.05% *w/w* d-phenothrin	XPEL Aerosol (XPEL)	Square Toiletries Limited	Domestic	Bangladesh	4.2	D2
0.25% *w/w* d-*trans*-Allethrin and 0.50% *w/w* piperonyl butoxide	HIT Aerosol (HIT)	Godrej Consumer Products Limited	Domestic	India	2.3	D3
0.50% *w/w* Permethrin, 0.20% *w/w* tetramethrin, 0.10% *w/w* es-bioallethrin	PHP-331[a]		Public health	Bangladesh	9.4	P1
0.20% *w/w* Permethrin, 0.20% *w/w* tetramethrin, 0.20% *w/w* prallethrin	PHP-205[a]		Public health	Bangladesh	7.2	P2
2% Permethrin	Top of Descent (TOD)	Callington Haven Pty	Aircraft disinsection	Australia	5.2	A1

[a]To anonymize, only Bangladeshi registration numbers were used.[54]

Note: all insecticides are type-I pyrethroids (molecules without an α-cyano group).[55]

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Table 3.

Association between V1016G genotypes and mosquito resistance phenotypes

Variable[a]	Exposed insecticide	Genotypes	Logistic regression (odds ratio)	95% Confidence interval	*P*-Value
Surviving phenotype	Permethrin 10×	VV	1		
		VG	0.68	0.23–2.03	0.492
		GG	2.34	1.16–4.71	0.017
	Deltamethrin 10×	VV	1		
		VG	1.02	0.35–2.95	0.974
		GG	1.61	0.79–3.22	0.184

[a]Baseline variable: dead phenotype.

Note: VV, wild-type; VG, heterozygous; GG, homozygous mutant.