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Pyrethroid resistance in *Aedes albopictus* in Pudong New Area, Shanghai China

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ABSTRACT

Objective: To evaluate the phenotypic resistance profile of *Aedes* (*Ae.*) *albopictus* against multiple insecticides and to delineate the frequency and pyrethroid-resistance association of knockdown resistance (*kdr*) mutations V1016G, I1532T and F1534S in *Ae. albopictus* in Pudong New Area, Shanghai, China.

Methods: From June to July 2024, a total of 1 249 *Ae. albopictus* eggs and 4 732 adults were collected from five towns in the Pudong New Area. These collected specimens were then reared to obtain the F1 generation including 10 235 larvae at the late 3rd to early 4th instar stages, as well as 5 800 non-blood-fed adults aged 3 to 5 days. Larval resistance was assessed *via* the immersion method, and adult resistance *via* the contact tube method. For the larval, 0≤resistance ratio (RR)<3 indicates sensitivity; 3≤RR<10 denotes low resistance; 10≤RR<40 signifies moderate resistance; RR≥40 represents high resistance. While for adults, a mortality rate of ≥98% stands for sensitivity, 80%≤mortality rate<98% indicates suspected resistance, and mortality rate<80% denotes a resistant population. A pooled sequencing strategy (8 sample pools) was used to detect genotype *kdr* mutations including V1016G, I1532T, F1534S in the voltage-gated sodium channel (*VGSC*) gene.

Results: *Ae. albopictus* larvae displayed low resistance to fenthion (RR=5.85), temephos (RR=4.63) and β-cypermethrin (RR=3.20), but remained fully susceptible to propoxur (RR=0.33). Adults showed incipient resistance to β-cypermethrin (mortality: 92.7%), lambda-cyhalothrin (mortality: 96.8%) and permethrin (mortality: 86.6%), while remained sensitive to propoxur (mortality: 100%). For *Ae. albopictus* population in the Pudong New Area, pyrethroid tolerance was primarily driven by F1534S mutation, which was amplified when the mutation was paired with V1016G or I1532T.

Conclusions: These findings highlight the need for integrated resistance monitoring and targeted insecticide rotation—such as prioritizing propoxur for *Ae. albopictus* larval and adult control—to mitigate resistance spread and preserve vector management efficacy.

KEYWORDS: *Aedes albopictus*; Pyrethroid; Resistance; Knockdown resistance gene; Resistance phenotype

Summary

Question: What is the phenotypic resistance profile and *kdr* mutation distribution in *Aedes* (*Ae.*) *albopictus* population in Pudong New Area, Shanghai China?

Finding: F1534S dominates the resistant pools (75% frequency), and its combination with V1016G confers complete resistance to lambda-cyhalothrin, whereas the I1532T+F1534S haplotype produces complete resistance to permethrin. In contrast, propoxur retains full efficacy which showed 100% mortality for *Ae. albopictus* adults and resistance ratio of 0.33 for the larval.

Meaning: We recommend to use propoxur as the first choice in order to prevent vector control failure due to pyrethroid resistance in *Ae. albopictus*. The *kdr* synergistic combination of F1534S+V1016G and I1532T+F1534S, now documented in local populations, can serve as an early molecular marker of impending pyrethroid failure.

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1. Introduction

Chikungunya virus (CHIKV), an alphavirus primarily transmitted by mosquitoes, causes severe acute and persistent joint pain accompanied by inflammatory responses, posing a significant challenge to public health systems worldwide[1]. Notably, CHIKV infection can lead to fatal outcomes, with reported mortality rates ranging from 1 to 2.2 deaths per 1 000 confirmed cases[2]. Once confined to sylvatic cycles in Africa and Asia, CHIKV triggered explosive outbreaks during the 2004–2019 epidemic, spreading to over 100 countries and resulting in instances of autochthonous transmission in Europe and the Americas[3]. In China, imported chikungunya cases have been reported annually since 2010, with local transmission confirmed in Zhejiang (2017) and Yunnan (2019)[4,5]. The cosmopolitan vector *Aedes* (*Ae.*) *albopictus*, prevalent in urban green spaces and residential areas of Shanghai, is now recognized as the primary bridge vector for CHIKV in temperate Asia[6,7]. Consequently, vector control remains the only effective strategy to mitigate the spread of CHIKV; however, increasing insecticides resistance poses a significant threat to its sustainability[8]. *Ae. albopictus* control programs in China primarily rely on integrated strategies combining environmental management with chemical interventions, where insecticides remain the cornerstone of control measures[9,10]. Pyrethroids are the predominant class of insecticides, a status attributed to their potent neurotoxicity in mosquitoes and low mammalian toxicity. Consequently, organophosphates and carbamates function as critical alternatives for resistance management[10,11]. However, *Ae. albopictus* populations have demonstrated rapid development of pyrethroid resistance across Thailand, Italy and Vietnam, and the resistance ratios increased up to 50-fold in some regions[12,13]. Recent surveillance data (2017-2023) from Shanghai's Pudong New Area reveals an alarming trend of increasing pyrethroid resistance among local *Ae. albopictus* populations, underscoring the urgent need for ongoing resistance monitoring[9]. Current resistance assessment protocols primarily utilize bioassays and insecticide contact tube methods standardized by the World Health Organization (WHO), which may not adequately reflect field resistance patterns[9,13]. Although several Chinese research institutions have made efforts to establish diagnostic doses for common insecticides such as deltamethrin, these thresholds remain provisional and lack nationwide standardization[14,15]. Molecular studies have demonstrated a strong association between pyrethroid resistance in Chinese *Ae. albopictus* populations and target-site mutations in voltage-gated sodium channel (*VGSC*) genes, particularly the I1532T and F1534S substitutions within the knockdown resistance (*kdr*) loci[14,16]. These findings highlight the necessity of integrating phenotypic resistance monitoring with molecular characterization of resistance mechanisms to enhance effective vector control[16].

Extensive insecticide application has been conclusively demonstrated to drive resistance evolution in *Ae. albopictus* through both target-site insensitivity and metabolic detoxification mechanisms[16,17]. Pyrethroid resistance in this species is primarily mediated by mutations in *VGSC*, with three codon positions (V1016, I1532, and F1534) identified as major resistance hotspots across global populations[18-20]. The F1534C mutation, first reported in *Ae. albopictus* by Kasai *et al.*, has now widely distributed in Asia and Europe[18-20]. The I1532T mutation was initially characterized in Italian *Ae. albopictus* before being identified in Chinese field populations, demonstrating international spread of this resistance allele[14,21,22]. Similarly, the V1016G mutation exhibits a global distribution pattern, having been documented in Vietnam, Italy and multiple Chinese provinces with varying frequencies that correlate with local insecticide use histories[19,20,22]. Contemporary research has quantitatively established that different *VGSC* mutations confer varying resistance levels, and Kasai *et al.* demonstrated that V1016G produces significantly stronger resistance than F1534C, though the latter remains strongly associated with type Ⅱ pyrethroid and DDT resistance[20]. The resistance contribution of I1532T appears more context-dependent, showing variable effects across different genetic backgrounds[21,23]. Notably, synergistic interactions between multiple *kdr* mutations can dramatically enhance resistance phenotypes. Field populations carrying combined S989P+V1016G+F1534C genotypes exhibit substantially reduced pyrethroid susceptibility compared to single-mutation strains[17,24,25]. However, significant regional variations exist in mutation patterns. For instance, Chen *et al.* found only the F1534S mutation was found in resistant Haikou populations and no double mutants detected. In contrast, Zhou *et al.* reported coexisting I1532T and F1534C mutations in Beijing populations, though these mutations lacked clear phenotypic correlations[22,23]. Despite these global and national insights, there remains a significant lack of systematic integration between local insecticide application practices, resistance phenotypes, and the underlying genotypic mutations in specific regions such as the Pudong New Area. This gap hinders the development of targeted and effective mosquito control strategies. In Pudong New Area, the lack of quantitative data on pyrethroid application rates and contexts has precluded a rigorous assessment of the link between selection pressure and the rising resistance in *Ae. albopictus*. Equally limiting, previous surveillance either measured mortality rates without genotyping the *VGSC* or screened *kdr* mutations without concurrent phenotypic confirmation, leaving a critical genotype–phenotype disconnection. Finally, no standardized, replicable framework exists that translates field-derived insecticide-use data into actionable resistance-management decisions. To address these gaps, we initiated a program that integrates three advancements into a cohesive workflow: (1) town-level quantitative surveys of insecticide use in commercial and public spaces across

five representative towns, conducted in real-time alongside WHO bottle bioassays and larval tests; (2) the establishment of the first joint genotype-phenotype platform for *Ae. albopictus* in the region, which simultaneously assesses resistance phenotypes and *VGSC* mutation frequencies; (3) a three-stage technical framework that commences with mapping actual usage patterns and culminates in evidence-based recommendations for insecticide rotation or alternative interventions. Standardized sampling methods, including ovitraps for larvae and aspirators for adults, coupled with uniform laboratory rearing, ensure data reproducibility. Additionally, targeted PCR amplification combined with Sanger sequencing allows for precise correlation of mutations with resistance phenotypes. This technical framework establishes a model that sets a new standard for mosquito resistance research in the country. Furthermore, it creates the foundational framework for a smart control system. This system uses molecular data to provide an early warning of insecticide resistance, which then will guide the precise application of targeted insecticides.

2. Methods

2.1. Ethical statement

Field collections for this study were conducted without the necessity for permission from local ethical review board, and verbal consent was obtained from the proprietors of all mosquito collection sites. During the survey on insecticide usage, participating companies provided oral consent after receiving a thorough explanation of the research objectives and methodology. This survey was approved by the Ethics Review Committee of Shanghai Pudong New Area Center for Disease Control and Prevention (No.PDCDCLL-20240101-002) on January 1, 2024. This study did not involve the collection of human samples or personal data. Informed consent to participate was obtained from all of the participants in the study. Our study adhered to the Declaration of Helsinki.

2.2. Survey of pesticide usage

Five pest control companies, primarily engaged in vector control activities in the Pudong New Area of Shanghai, were selected to participate in a survey regarding the types and quantities of insecticides used during 2023-2024, building upon historical usage patterns observed from 2017-2022. A questionnaire was administered to document the trade names, pesticide types, and application sites (Supplementary file 1).

Among these entities, Mintai Pest Control Limited Company serves as the principal contractor for vector-related epidemic response and support for major events within these districts. In contrast, the remaining four pest control operator (PCO) companies act as the primary contractors for their respective streets or towns, collectively accounting for a total of 30 questionnaires. After elucidating the research objectives to all participants, we obtained verbal consent. Due to the variability in active ingredient concentrations across different insecticides, the effective dosage varies accordingly. To standardize the comparison of insecticides usage across different application sites, we adopted the formula proposed by Zhang *et al.*[26]. in their study on *Ae. albopictus* resistance surveillance in Putuo District, Shanghai:

$$M = (\sum_{dosage})/(6 \text{ recommended dosages})$$

Here, M represents the standardized treatment area, $\sum_{dosage}$ is the total amount of pesticide applied, and the denominator adjusts for the recommended dosage per unit area. The factor of 6 was empirically derived from historical operational data in urban mosquito control programs to account for field application efficiency and environmental variability[26]. This approach enables consistent cross-comparison of pesticide usage patterns despite differences in formulation concentrations.

2.3. Mosquito sources and rearing

Between June and July 2024, a study was conducted in Pudong New Area, where five towns including Huinan Town, Weifang Xincun Street, Hangtou Town, Puxing Road Street, and Zhangjiang Town were selected as sample collection sites for *Ae. albopictus* based on the area's geographical environment and high incidence of mosquito-borne infectious diseases in the past. Mosquito eggs were collected using larval oviposition traps, while adult mosquitoes were captured with a mechanical aspirator (Figure 1). The climatic conditions during this period included an average daily temperature of 27.86 ℃, a daily relative humidity of 67.82%, and an average daily precipitation of 6.45 mm. The eggs collected from *Ae. albopictus* ovitraps were transferred to enamel trays and reared in the laboratory using dechlorinated tap water and larval diet. Upon reaching adulthood, these mosquitoes, along with adult *Ae. albopictus* collected using electric aspirators, were blood-fed on white mice for three days. Eggs were subsequently collected using ovitraps, and the egg-laden papers were stored in a humidified chamber at 4 ℃. After a 7-day egg collection period, the egg papers were placed in dechlorinated tap water to hatch the F1 larval generation. Late third-instar and early fourth-instar larvae were selected for insecticide resistance testing using the larval immersion method. The remaining F1 larvae were reared to adulthood under controlled conditions: (26±1) ℃, (60±10)% relative humidity, and a 14:10-hour light-dark cycle. Adult mosquitoes were maintained on a 10% sucrose solution provided *via* sponge feeders. For adult resistance phenotyping, 3-5-day-old, non-blood-fed F1

female mosquitoes were subjected to the WHO contact tube assay to identify resistant and susceptible phenotypes for subsequent resistance gene analysis. Rearing mosquitoes to the F1 generation before experimentation ensured developmental uniformity across test cohorts, thereby minimizing individual variability and enhancing the reproducibility of resistance assessments. Eggs collected using ovitraps were morphologically identified as *Ae. albopictus* under a stereomicroscope based on characteristic features, including their solitary, spindle-shaped, black appearance, typically attached to moist surfaces near the waterline or submerged at the bottom. To further confirm species identity, a subset of eggs was reared to adulthood under laboratory conditions, and emerged adults were identified using standardized taxonomic keys[27,28]. Additionally, eggs oviposited by F1-generation adults (blood-fed on laboratory mice) were confirmed as *Ae. albopictus* through identical morphological and rearing protocols.

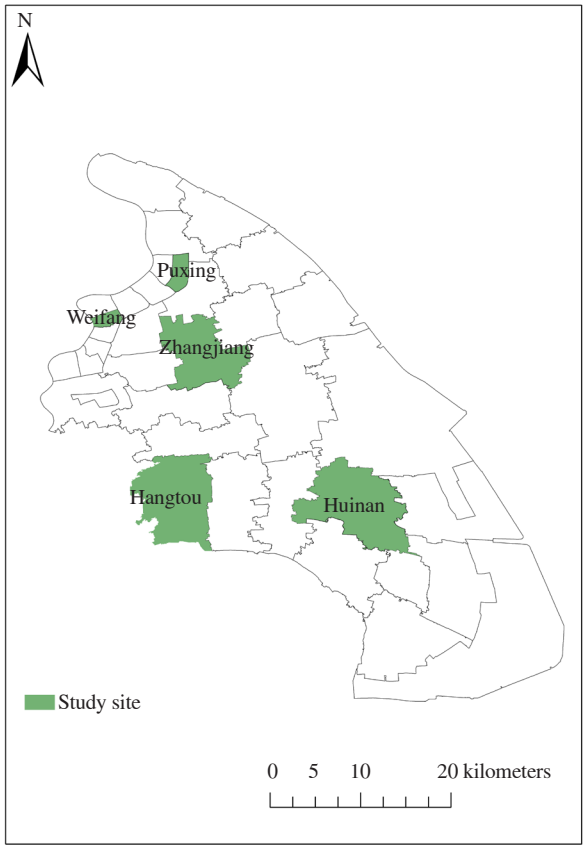

Figure 1. The sampling sites for insecticide resistance monitoring of *Ae. albopictus* in Pudong New Area in 2024.

2.4. Pesticide resistance assessment

2.4.1. Rationale for insecticide selection

The larvicides tested [Temephos and Fenthion (Organophosphates)], β-Cypermethrin (Pyrethroids) are commonly used for breeding site treatment in Pudong New Area, with Propoxur (Carbamates) serving as a susceptible control. Adulticides [Lambda-cyhalothrin, Permethrin (Pyrethroids)] were selected to align with mainstream adult mosquito control practices (space spraying), matching local operational use and enabling *kdr* mutation analysis[29].

All bioassays followed the national standard (GB/T 29566-2013 biological determination methods for insecticide resistance in mosquitoes), with strict synchronization of insect age, temperature, and humidity to minimize variability[30]. Biological testing methods for mosquito resistance to pesticides are standardized bioassays that expose field-collected mosquitoes to a known diagnostic dose or concentration of an insecticide and then record the proportion that die within a specified time, thereby providing a direct measure of phenotypic resistance that can be compared across time and space. Analytical grade acetone was procured from Jiangsu Tongsheng Chemical Reagent Co., Ltd. Genomic extraction and analysis were performed by Shanghai Bojie Medical Technology Co., Ltd.

2.4.2. Resistance testing of *Ae. albopictus* larvae

The pesticides employed in the larval assays included 90.0% Fenthion, 90.0% Temephos, 97% Propoxur, and 95.8% β-Cypermethrin, all supplied by the Vector Control Division of the Shanghai Center for Disease Control and Prevention (CDC).

Detection of insecticide resistance in mosquito larvae was conducted by the immersion method according to the national standard[30]. Experimental conditions were maintained at a temperature of (25±1) ℃ and a relative humidity of 60%-80%. Each experimental unit was prepared with 149 mL of dechlorinated tap water. Pesticides were diluted using acetone to create seven concentration gradients, with the lowest concentration achieving a larval mortality rate of less than 20% and the highest exceeding 80%. A volume of 1 mL of the pesticide solution was introduced into each beaker and homogenized using a glass rod, followed by the addition of 30 selected larvae and 50 mL of dechlorinated tap water, resulting in a final volume of 200 mL. After an incubation period of 24 hours, the number of deceased larvae was recorded.

2.4.3. Resistance testing of adult *Ae. albopictus*

The pesticides employed in the adult assays comprised 0.08% β-Cypermethrin, 0.7% Lambda-cyhalothrin, 0.4% Permethrin, and 0.05% Propoxur membrane, along with a blank control membrane, all supplied by the Vector Control Division of the Shanghai CDC.

According to the national standard[30] using the contact tube method for testing, experimental conditions were maintained at a temperature of (25±1) ℃ and a relative humidity of 60%-80%. A gauze net was positioned over both the red contact tube and the green recovery tube, with the recovery tube connected *via* a partition. An aspirator was utilized to transfer 30 test mosquitoes into a recovery tube, where they were allowed to acclimate for 30 minutes. A plain

A4 sheet of paper was inserted into the contact tube, and a pesticide membrane was affixed; this procedure was repeated six times. The control tube underwent an identical treatment. After the initial 30-minute acclimation period, the test mosquitoes were introduced into the pesticide contact tube, the partition was secured, and the setup was left undisturbed for 1 hour. Subsequently, the mosquitoes were transferred back into the recovery tube, which was maintained in an upright position and covered with cotton soaked in a 10% sugar solution. After 24-hour, mortality rates and total mosquito counts were recorded. Both surviving and deceased mosquitoes were stored at -80 ℃ for subsequent DNA analysis.

2.5. *kdr* gene detection

To verify the association between *kdr* mutations and insecticide resistance phenotypes, this study adopted a pooled sample sequencing strategy. Eight sample pools, each comprising 4–182 phenotypically homogeneous *Ae. albopictus* females, were constructed by combining mosquitoes according to their insecticide exposure status and phenotypic outcome (alive=resistant; mortality =susceptible; total mosquitoes=resistant+susceptible). Genomic DNA was extracted from each pool using the TIANamp Genomic DNA Kit (Tiangen Biotech) following the manufacturer's protocol, which included tissue lysis with proteinase K, column-based DNA purification, and elution in TE buffer.

Targeted polymerase chain reaction (PCR) amplification and Sanger sequencing were performed for the *VGSC* gene regions known to harbor *kdr* mutations: Domain Ⅱ (encompassing the V1016G locus) and Domain Ⅲ (encompassing the I1532T and F1534S loci) (Table 1). The primer sets included:

Domain Ⅱ (V1016 region): Primers aegSCF20/aegSCR21[22].

Domain Ⅲ (I1532/F1534 regions): Primers aegSCF7/aegSCR7[31].

The PCR was initiated with an initial denaturation step at 95 ℃ for 5 min. This was followed by 10 cycles of denaturation at 94 ℃ for 30 s, annealing at 63 ℃ for 30 s, and extension at 72 ℃ for 30 s. Subsequently, 30 cycles were performed under the following conditions: denaturation at 95 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 30 s. A final extension step was conducted at 72 ℃ for 10 min to complete the reaction. Amplicons were verified by 1% agarose gel electrophoresis and sequenced by Sangon Biotech using the additional sequencing primers (aegSCR22 and aegSCR8).

2.6. Statistical analysis

The Pudong New Area encompasses approximately 1 210 km^2, comprising 36 streets (towns), and exhibits considerable variability in pesticide application. Consequently, standardized areas of 3.36 km^2 and 33.6 km^2 were employed for classification purposes.

The criteria for determining resistance in *Ae. albopictus* larvae are as follows[30]:

The resistance ratio (RR) is calculated as the LC_{50} value of the wild population divided by the LC_{50} value of the sensitive strain. A resistance ratio of 0≤RR<3 indicates sensitivity; 3≤RR<10 denotes low resistance; 10≤RR<40 signifies moderate resistance; and RR≥40 represents high resistance.

The LC_{50} value was utilized as the parameter for assessing pesticide toxicity against *Ae. albopictus*. Data processing and analysis were performed using Excel 2019 and SPSS 22.0 software to derive LC_{50} values and their 95% confidence limits (mg/L), toxicity regression equations, and resistance ratios for *Ae. albopictus* larvae in response to three pesticides. The municipal CDC provided the LC_{50} of the sensitive strain. The significance level was set at α=0.05.

The criteria for determining resistance in adult *Ae. albopictus* are as follows:

$$\text{Mortality rate (\%)} = \frac{\text{number of dead mosquitoes}}{\text{total number of test mosquitoes}} \times 100\%$$

A mortality rate of ≥98% stands for sensitivity, 80%≤mortality rate<98% indicates suspected resistance, and mortality rate<80% denotes a resistant population.

Table 1. Primer sequences and target regions.

Target region	Primer name	Sequence (5'-3')	Type	Reference
Domain Ⅱ	aegSCF20	GACAATGTGGATCGCTTCCC	Forward	[21]
	aegSCR21	GCAATCTGGCTTGTTAACTTG	Reverse	
	aegSCR22	TTCACGAACTTGAGCGCGTTG	Sequencing	
Domain Ⅲ	aegSCF7	GAGAACTCGCCGATGAACTT	Forward	[21,22]
	aegSCR7	GACGACGAAATCGAACAGGT	Reverse	
	aegSCR8	TAGCTTTCAGCGGCTTCTTC	Sequencing	

3. Results

3.1. Study on the use of insecticides in the research area

The insecticide usage survey in Pudong New Area (2023-2024) identified eight common public health insecticides, including four compound formulations, categorized into organophosphates, pyrethroids, and carbamates. In adult mosquito control, pyrethroid-based insecticides predominated, with Permethrin (161.5 g/L) combined with *S*-bioallethrin (7.1 g/L) and Tetramethrin (4.5%) combined with Permethrin (5%) being the most commonly utilized formulations for spatial spraying in residential areas (standardized area >33.6 km^2). Residual spraying primarily employed β-Cypermethrin (3%) combined with Propoxur (2%) and Lambda-cyhalothrin (2.5%), demonstrating extensive application in residential zones, farmers' markets, and outdoor environments. For larval control, organophosphates such as Temephos (1%) and Fenthion (5%) were exclusively utilized for treatment of breeding sites, exhibiting widespread coverage across all settings, as shown in Table 2.

Temephos, Fenthion, β-Cypermethrin, and Propoxur were selected for larval experiments. β-Cypermethrin, Lambda-cyhalothrin, Permethrin, and Propoxur were selected for adult mosquito experiments. For larval experiments, Temephos and Fenthion were chosen as they are the organophosphates exclusively and widely used for larval control in breeding sites. β-Cypermethrin, Lambda-cyhalothrin, and Permethrin (pyrethroids) were selected for adult experiment as they predominate in adult mosquito control strategy—Permethrin in spatial spraying, and β-Cypermethrin/Lambda-cyhalothrin in residual spraying. Propoxur was included as it is commonly used in combination with the above mentioned adult-targeted formulations, with extensive use in residential and other areas.

3.2. Resistance testing results of Ae. albopictus larvae

Field collections yielded 1 249 *Ae. albopictus* eggs and 4 732 adult mosquitoes that were reared to establish laboratory colonies (Supplementary file 2). From these F1 generation populations, a total of 2 610 third- to early fourth-instar larvae were utilized for resistance testing. The bioassay results revealed that *Ae. albopictus* larvae exhibited low resistance levels to the insecticides Fenthion, Temephos, and β-Cypermethrin, with resistance ratios of 5.845, 4.628, and 3.197 (3≤RR<10), respectively. In contrast, the larvae displayed a high sensitivity to the insecticide Propoxur, with a resistance ratio of 0.325 (0≤RR<3), as shown in Table 3 and Supplementary file 3.

Table 2. Revised pesticide usage in Pudong New Area, Shanghai, 2023-2024.

Mosquito stage	Ingredients	Insecticidal category	Application type	Residential area	Government office	Farmers' market	Outdoor environment	Vector control
Adult	Permethrin (161.5 g/L), *S*-bioallethrin (7.1 g/L)	PY	Spatial spraying	+++	+	+	++	++
Adult	Tetramethrin (4.5%), Permethrin (5%)	PY	Spatial spraying	+++	+		++	++
Adult	Permethrin (102.6 g/L), *S*-bioallethrin (1.4 g/L)	PY	Spatial spraying		+			
Adult	β-Cypermethrin(3%), Propoxur (2%)	PY, C	Residual spraying	+++	+	+	++	++
Adult	Lambda-cyhalothrin (2.5%)	PY	Residual spraying	+++	+	+	++	++
Adult	*Cis*-Cypermethrin (5%)	PY	Residual spraying		+			
Larvae	Temephos(1%)	OP	Breeding site treatment	+++	+	+	++	++
Larvae	Fenthion (5%)	OP	Breeding site treatment	+++	+	+	++	

OP: Organophosphorus; PY: Pyrethroids; C: Carbamates; -: No recorded use; +: Standardized area of use < 3.36 km^2; ++: 3.36 km^2 ≤ Standardized area of use ≤ 33.6 km^2; +++: Standardized area of use > 33.6 km^2.

Table 3. Sensitivity of *Ae. albopictus* larvae in Pudong New Area to four insecticides in 2024.

Insecticides	Regression equation (y=a+bx)	LC_{50} and 95% confidence limits (mg/L)	Sensitive strain LC_{50} (mg/L)	Resistance ratio (RR)	Resistance level
Temephos	y=5.919+2.673x	0.006 (0.002-0.004)	0.132×10^{-2}	4.63	Low resistance
Fenthion	y=6.381+3.444x	0.014 (0.012-0.016)	0.024×10^{-1}	5.85	Low resistance
β-Cypermethrin	y=4.110+1.617x	0.003 (0.002-0.004)	0.009×10^{-1}	3.20	Low resistance
Propoxur	y=1.151+1.236x	0.117 (0.087-0.158)	0.36	0.33	Sensitive

3.3. Resistance assessment and phenotypic screening results of *Ae. albopictus*

In the controlled laboratory setting, a total of 836 female *Ae. albopictus* mosquitoes (3-5 days post-emergence) were used to assess resistance to four insecticides within three main categories. The adult *Ae. albopictus* showed potential resistance to three insecticides: β-Cypermethrin, Lambda-cyhalothrin, and Permethrin, with 24-hour mortality rates of 92.67%, 96.79%, and 86.58%, respectively. In contrast, they demonstrated susceptibility to Propoxur, achieving a 24-hour mortality rate of 100%. These differences were statistically significant when compared to the control group, as illustrated in Figure 2. Upon assessing resistance to β-Cypermethrinn, 153 sensitive and 11 resistant phenotypes of *Ae. albopictus* were identified. Similarly, testing for resistance to Lambda-cyhalothrin revealed 182 sensitive and 6 resistant phenotypes. Resistance testing with Permethrin resulted in the identification of 155 sensitive and 24 resistant phenotypes, as depicted in Figure 2 and Supplementary file 4.

3.4. Genotypes and frequency distribution of VGSC gene mutation sites in *Ae. albopictus*

Wild-type homozygotes (I/I at I1532, F/F at F1534, V/V at V1016) were predominant in susceptible pools, while heterozygous mutations were enriched in resistant pools. F1534S was the most prevalent mutation, detected in 75% of resistant pools. V1016G and I1532T exhibited weaker individual associations with resistance but played critical roles in combination with F1534S. Notably, the co-occurrence of F1534S+V1016G was exclusively observed in lambda-cyhalothrin-resistant pools, indicating a synergistic effect. Similarly, I1532T+F1534S co-occurred in permethrin-resistant pools, strongly linked to resistance, as shown in Figure 3.

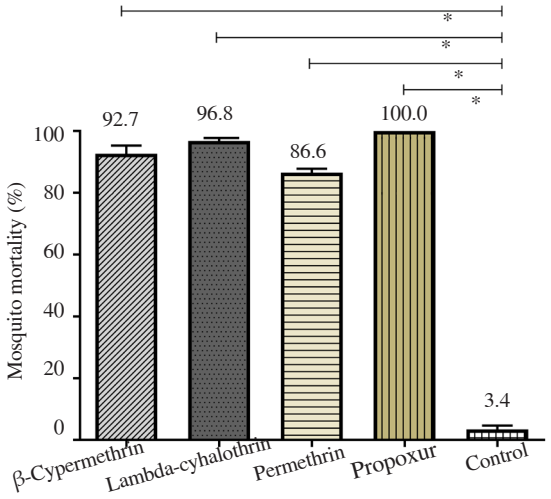

Figure 2. The mortality rates of *Aedes albopictus* after being exposed to diagnostic doses of four insecticides on insecticide-impregnated paper.

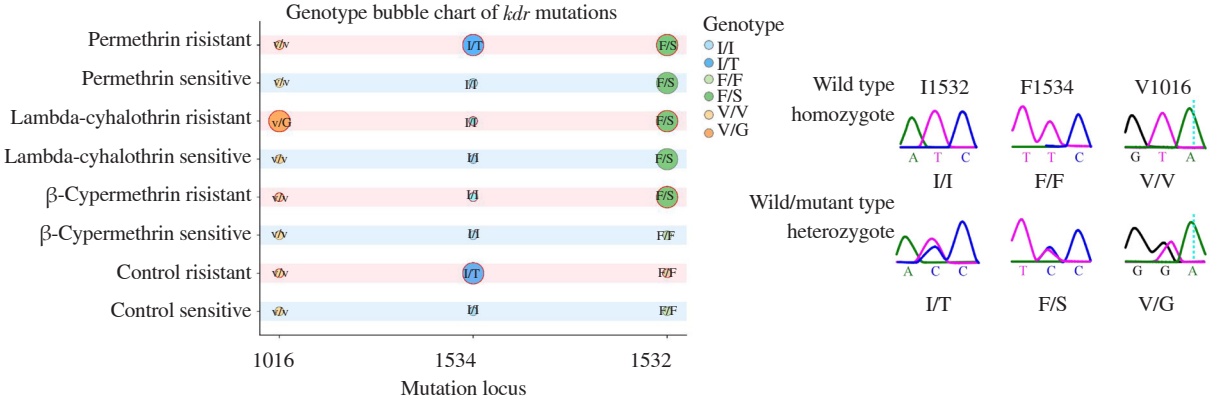

Figure 3. Genotype distribution and sequencing results of *kdr* mutations in *Aedes albopictus* populations from Pudong New Area.
A. Genotype bubble chart of kdr mutations. B. A sequencing chromatogram of *kdr* gene mutations in *Aedes albopictus* populations exposed to three insecticides in Pudong New Area. Note: Bubble size is for distinguishing mutated genotypes. Larger bubbles are for non-mutated ones, not indicating percentage.

4. Discussion

The full susceptibility of *Ae. albopictus* larvae to Propoxur and adults to Propoxur underscores their utility in targeted vector control. Propoxur could be prioritized for larval source reduction in Pudong, while Propoxur may serve as a rotational adulticide to mitigate pyrethroid resistance. However, integrated strategies combining environmental management and judicious insecticide use are critical

to sustain efficacy—especially given the widespread application of pyrethroids in the region, where Propoxur's susceptibility provides a vital alternative for vector control programs. Incorporating Propoxur into rotational regimes will reduce selection pressure on pyrethroids and delay further resistance development.

Notably, this study represents the first systematic linkage between on-site insecticide usage surveys and resistance monitoring in the Pudong New Area. We conducted a comprehensive investigation of public health insecticide use across five towns, covering residential areas, government institutions, and farmers'markets. Using a standardized formula to quantify insecticide application intensity and type distribution across regions, we directly correlated field usage data with laboratory resistance assay results. This analysis confirmed the association between high-frequency pyrethroid application and the pyrethroid resistance phenotype in *Ae. albopictus*, establishing a complete "usage-resistance" evidence chain that strengthens causal inferences about resistance drivers—an advance that addresses the historical disconnect between real-world insecticide use and resistance data.

In the Pudong New Area, adult *Ae. albopictus* exhibited resistance to three classes of pyrethroid insecticides. Monitoring data showed that adult mortality rates following exposure to β-Cypermethrin (96.81%), Lambda-cyhalothrin (93.29%), and Permethrin (86.59%) were significantly higher than the 2020 national averages (77.97%, 87.30%, and 79.31%, respectively) reported by the Chinese CDC across 13 provinces[32]. Given inconsistencies in diagnostic dosages in recent years, there is an urgent need to accurately assess resistance trends in adult *Ae. albopictus* in Pudong—underscoring the value of our study's first-established genotype-phenotype joint monitoring system for *Ae. albopictus* resistance in the region. Beyond traditional phenotypic assays, we innovatively integrated *VGSC* gene *kdr* mutation detection, targeting three critical loci (V1016, I1532, F1534). Through pooled sample sequencing and statistical analysis, we for the first time revealed species-specific associations between pyrethroid resistance and both the F1534S single mutation and the double-mutation haplotypes "F1534S+V1016G" and "I1532T+F1534S"—filling a longstanding gap in understanding the molecular mechanisms of *Ae. albopictus* resistance in Pudong.

Kdr mutations in the *VGSC* gene are a key indicator of mosquito resistance to pyrethroid insecticides[33]. Our pooled sequencing analysis identified three *kdr* mutations (V1016G, I1532T, and F1534S) in *Ae. albopictus* populations from Pudong New Area, with distinct association patterns with resistance phenotypes. F1534S was the most prevalent mutation, detected in 75% of resistant pools, and showed a significant association with overall pyrethroid resistance. This aligns with observations from multiple Chinese regions, where F1534S is recognized as a primary marker of pyrethroid resistance[34-36]. Notably, F1534S alone was sufficient to confer resistance to β-Cypermethrin, consistent with Chen *et al.*'s findings that this mutation independently contributes to reduced susceptibility to type Ⅱ pyrethroids[24]. V1016G and I1532T exhibited weaker individual associations with resistance but played critical roles in combination with F1534S. The co-occurrence of V1016G and F1534S was exclusively observed in Lambda-cyhalothrin-resistant pools, suggesting a synergistic effect. This is consistent with Kasai *et al.*'s report that V1016G enhances resistance when combined with F1534 mutations, particularly against Lambda-cyhalothrin[20].

Similarly, I1532T + F1534S co-occurred in Permethrin-resistant pools, corroborating Liu *et al.*'s observation that these two mutations collectively strengthen resistance to type Ⅰ pyrethroids[37]. The absence of homozygous mutants in all pools suggests that resistance is in an incipient stage, potentially due to recent selection pressure from intensive pyrethroid use in residential areas. Geographical variations in mutation frequencies were observed: V1016G frequency in Pudong (25% in resistant pools) was higher than in Beijing (2.50%) but lower than in Yunnan (25.32%), likely reflecting differences in local insecticide application histories[35,38]. These findings suggest a potential synergistic interaction between residues (V1016 and F1534, or I1532 and F1534), which may synergistically alter the conformational structure of the insecticide target site. For proactive resistance management, these mutations should not be assessed in isolation but rather tracked as high-risk haplotypes, as their co-occurrence signifies a potent resistance mechanism against a major insecticide class. Critically, these mutations and their combinations serve as specific molecular markers for pyrethroid resistance in *Ae. albopictus* from Pudong. Regular monitoring of their frequency dynamics enables the development of an early warning system that is more sensitive than traditional phenotypic assays, thereby establishing the foundation for a"molecular early-warning and precision-guided intervention"strategy for smart vector control.

As the first systematic investigation of *kdr* mutations in *Ae. albopictus* in Pudong, our primary goal was to establish a baseline profile of insecticide resistance genotypes across the region. Given the lack of prior data, a pooled analysis provides a critical snapshot of the overall prevalence and distribution of resistance mutations, which will inform future spatially targeted interventions. This approach aligns with similar foundational studies in other regions[22,39].

This study has several limitations. Sample sizes at individual sites were insufficient for robust statistical comparisons, and our standardized resistance monitoring protocol prioritizes rapid assessment of regional trends over microgeographic variability—consistent with methodologies used in other CDC-led surveillance programs and necessitating our pooled analysis approach[31].

Future research will explore insecticide resistance patterns across the ecological gradients of Pudong New Area, analyzing resistance

levels and mutation frequencies across urban, suburban, and rural landscapes and among larval habitats, including artificial containers, natural water bodies, and green spaces. Additionally, we will design longitudinal surveillance to track spatial-temporal dynamics of resistance, akin to the multi-year frameworks in Li *et al.*, to monitor how resistance patterns change over time in different habitats and inform adaptive control strategies[40]. Beyond these upstream field-based analyses of polymorphism across locations, downstream molecular studies will incorporate haplotype network analysis and phylogenetic analysis of *VGSC* gene variants, aiming to unravel the evolutionary pathways of resistance mutations. These future studies will build upon the current baseline data and provide a more nuanced understanding of *Ae. albopictus* resistance in Pudong, ultimately improving vector control and chikungunya prevention efforts.

Conflict of interest statement

All authors declare no competing financial interests.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Authors´ contributions

CL and YTY, were responsible for conceiving the study, participating in its design and coordination, and drafting the manuscript. JL, QL, HHL and LF contributed to the study by participating in the design and conducting statistical analyses. HXL and HZL also played a role in the conception of the study, design, coordination, and manuscript drafting. All authors read and approved the final manuscript.

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Supplementary file 1. Survey on the use of vector control agents in Pudong New Area, Shanghai (2023-2024)

1. Questionnaire Instructions.

Hello! This questionnaire is designed to investigate the use of insecticides by the Public Health Pest Control Operator (PCO) in Pudong New Area. The data is only used for scientific research analysis and kept strictly confidential. Please fill in according to your actual work situation. Thank you for your support!

2. Basic Information

2.1 Unit Name: _____

2. 2 Belonging Street Town: _____

3. Insecticide usage records (each township fills in 6 survey questionnaires, each containing at least 1 type of insecticide)

No.	Mosquito Stage	Ingredients	Insecticidal Category	Application Type	Residential Area	Government Office	Farmers' Market	Outdoor Environment	Vector Control
1	Adult	Chlorpyrifos(9%), β −Cypermethrin (3%)	OP, PY	Spatial spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
2	Adult	β −Cypermethrin (3%), Propoxur (2%)	PY, C	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
3	Adult	Tetramethrin (4.5%), Permethrin (5%)	PY	Spatial spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
4	Adult	Permethrin (102.6 g Ł$^{-1}$), S-bioallethrin (1.4 g Ł$^{-1}$)	PY	Spatial spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
5	Adult	Permethrin (161.5 g Ł$^{-1}$), S-bioallethrin (7.1 g Ł$^{-1}$)	PY	Spatial spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++

6	Adult	Cis-cypermethrin (5%)	PY	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
7	Adult	Cypermethrin (10%)	PY	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
8	Adult	Lambda-cyhalothrin (10%)	PY	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
9	Adult	Lambda-cyhalothrin (2.5%)	PY	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
10	Adult	Methylparathion (30%)	OP	Residual spraying	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
11	Larva	Temephos (1%)	OP	Breeding site treatment	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
12	Larva	Fenthion (5%)	OP	Breeding site treatment	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++
13		Others				○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++	○− ○+ ○++ ○+++

Instruction for filling: Standardized area symbol: According to the formula $M = (\sum_{dosage}) / (6 \times \text{recommended dosages})$:

-: no usage record; +: standardized usage area <3.36 km^2; ++: 3.36 km^2 ≤ standardized usage area ≤ 33.6 km^2; +++: standardized usage area > 33.6 km^2.

4. Survey questionnaire results

No.	Unit Name	Street Town	Mosquito Stage	Ingredients	Insecticidal Category	Application Type	Residential Area	Government Office	Farmers' Market	Outdoor Environment	Vector Control
1	Unit 1	Street Town A	Larva	Temephos (1%)	OP	Breeding site treatment	++	++	+	++	+
2	Unit 1	Street Town A	Larva	Fenthion (5%)	OP	Breeding site treatment	+++	+	+	++	−
3	Unit 2	Street Town B	Adult	β −Cypermethrin (3%), Propoxur (2%)	PY, C	Residual spraying	+++	+	+	++	++
4	Unit 2	Street Town B	Adult	Permethrin (161.5 $g \cdot L^{-1}$), S-	PY	Spatial spraying	+++	+	+	++	++

				bioallethrin (7.1 g·L^{-1})							
5	Unit 3	Street Town C	Adult	Tetramethrin (4.5%), Permethrin (5%)	PY	Spatial spraying	+++	+	−	++	++
6	Unit 3	Street Town C	Larva	Temephos (1%)	OP	Breeding site treatment	+++	+	++	+++	++
7	Unit 4	Street Town D	Adult	Permethrin (102.6 g·L^{-1}), S-bioallethrin (1.4 g·L^{-1})	PY	Spatial spraying	–	+	−	–	–
8	Unit 4	Street Town D	Adult	Cis-cypermethrin (5%)	PY	Residual spraying	–	++	−	–	–
9	Unit 5	Street Town E	Larva	Temephos (1%)	OP	Breeding site treatment	+++	+	++	++	++
10	Unit 5	Street Town E	Adult	Tetramethrin (4.5%), Permethrin (5%)	PY	Spatial spraying	+++	+	−	++	++
11	Unit 1	Street Town A	Adult	Cyhalothrin (3%), Carbofuran (2%)	PY, C	Residual spraying	++	+	+	++	++
12	Unit 1	Street Town A	Adult	Lambda-cyhalothrin (2.5%)	PY	Residual spraying	+++	+	+	++	++
13	Unit 2	Street Town B	Larva	Fenthion (5%)	OP	Breeding site treatment	++	+	+	++	–
14	Unit 2	Street Town B	Adult	Permethrin (102.6 g·L^{-1}), S-bioallethrin (1.4 g·L^{-1})	PY	Spatial spraying	–	+	−	–	–
15	Unit 3	Street Town C	Larva	Temephos (1%)	OP	Breeding site treatment	++	+	+	++	++
16	Unit 3	Street Town C	Adult	Cis-cypermethrin (5%)	PY	Residual spraying	–	+	−	–	–
17	Unit 4	Street Town D	Adult	Tetramethrin (4.5%), Permethrin (5%)	PY	Spatial spraying	+++	+	−	++	++

18	Unit 4	Street Town D	Larva	Fenthion (5%)	OP	Breeding site treatment	++	+	+	++	−
19	Unit 5	Street Town E	Adult	β −Cypermethrin (3%), Propoxur (2%)	PY, C	Residual spraying	+++	++	+	++	++
20	Unit 5	Street Town E	Adult	Permethrin (161.5 $g \cdot L^{-1}$), S-bioallethrin (7.1 $g \cdot L^{-1}$)	PY	Spatial spraying	+++	+	+	++	++
21	Unit 1	Street Town A	Adult	Cis-cypermethrin(5%)	PY	Residual spraying	–	+	–	–	–
22	Unit 1	Street Town A	Larva	Temephos (1%)	OP	Breeding site treatment	+++	++	+	+++	++
23	Unit 2	Street Town B	Larva	Fenthion (5%)	OP	Breeding site treatment	++	+	++	++	−
24	Unit 2	Street Town B	Adult	Lambda-cyhalothrin (2.5%)	PY	Residual spraying	+++	+	+	++	++
25	Unit 3	Street Town C	Adult	Permethrin (102.6 $g \cdot L^{-1}$), S-bioallethrin (1.4 $g \cdot L^{-1}$)	PY	Spatial spraying	–	+	–	–	–
26	Unit 3	Street Town C	Larva	Temephos (1%)	OP	Breeding site treatment	++	+	+	+++	++
27	Unit 4	Street Town D	Adult	Propoxur (4.5%), Permethrin (5%)	PY	Spatial spraying	+++	+	−	++	++
28	Unit 4	Street Town D	Larva	Fenthion (5%)	OP	Breeding site treatment	+++	+	++	++	−
29	Unit 5	Street Town E	Adult	β −Cypermethrin (3%), Propoxur (2%)	PY, C	Spatial spraying	+++	+	+	++	++

Supplementary file 2. Mosquito collection of *Aedes albopictus* in Pudong New Area, 2024.

Location	Habitat	Latitude(N)	Longitude(E)	Altitude(m)	Adult mosquitoes	Eggs
Huinan	Residential	31°05′24.69″	121°76′77.94″	3.1	644	143
Huinan	Park	31°22′11.40″	121°54′40.90″	3.3	548	158
Weifang	Residential	31°22′32.65″	121°51′91.13″	3.2	796	270
Hangtou	Residential	31°04′29.19″	121°57′87.73″	3.1	1242	66
Puxing	Residential	31°27′36.44″	121°59′20.82″	3.15	354	89
Puxing	Park	31°22′08.81″	121°53′19.91″	3.2	480	93
Zhangjiang	House	31°17′80.28″	121°63′03.15″	3.14	668	430
Total					4732	1249

Supplementary file 3. *Aedes albopictus* larvae pesticide resistance test results in Pudong New Area.

Insecticide Name	Final Concentration (mg/L)	Mortality (No.) Trial 1	Total (No.) Trial 1	Mortality (No.) Trial 2	Total (No.) Trial 2	Mortality (No.) Trial 3	Total (No.) Trial 3	Total Mortality	Total Tested
Control	0	0	30	0	30	0	30	0	90
Temephos	0.025	30	30	29	30	27	30	86	90
	0.01	25	30	18	30	17	30	60	90
	0.0075	18	30	16	30	18	30	52	90
	0.005	16	30	12	30	14	30	42	90
	0.00375	8	30	12	30	10	30	30	90
	0.0025	3	30	4	30	4	30	11	90
	0.00125	1	30	0	30	1	30	2	90
Fenthion	0.03	28	30	30	30	24	30	82	90
	0.025	30	30	27	30	21	30	78	90
	0.02	20	30	15	30	14	30	49	90
	0.015	18	30	14	30	13	30	45	90

	0.01	15	30	10	30	11	30	36	90
	0.0075	6	30	4	30	8	30	18	90
	0.005	1	30	2	30	0	30	3	90
	0.02	30	30	30	30	30	30	90	90
	0.015	27	30	26	30	24	30	77	90
	0.01	20	30	21	30	23	30	64	90
β -Cypermethrin	0.005	17	30	18	30	19	30	54	90
	0.001	9	30	11	30	13	30	33	90
	0.0005	3	30	2	30	3	30	8	90
	0.00025	0	30	1	30	0	30	1	90
	1	30	30	30	30	29	30	89	90
	0.5	24	30	18	30	18	30	60	90
Propoxur	0.25	19	30	17	30	16	30	52	90
	0.1	17	30	14	30	14	30	45	90
	0.05	9	30	11	30	11	30	31	90

	0.01	5	30	3	30	4	30	12	90
	0.005	0	30	1	30	1	30	2	90
Total	-	-	-	-	-	-	-	1212	2610

Supplementary file 4. ***Aedes albopictus*** **adult mosquitoes' pesticide resistance test results in Pudong New Area**

Pesticide name	**Mortality (No.) trial 1**	**Total mosquitoes (No.) trial 1**	**Mortality (No.) trial 2**	**Total mosquitoes (No.) trial 2**	**Mortality (No.) trial 3**	**Total mosquitoes (No.) trial 3**	**Mortality (No.) trial 4**	**Total mosquitoes (No.) trial 4**	**Mortality (No.) trial 5**	**Total mosquitoes (No.) trial 5**	**Mortality (No.) trial 6**	**Total mosquitoes (No.) trial 6**	**Total mortality**	**Total mosquitoes**	**Mortality rate (%)**	**Resistance level**
Control	1	18	0	19	1	31	2	41	-	-	-	-	4	109	3.67	-
β-Cypermethrin	34	38	13	16	17	18	28	29	33	35	28	28	153	164	93.29	Possible resistance
Lambda-cyhalothrin	32	35	31	32	30	30	38	38	26	27	25	26	182	188	96.81	Possible resistance
Permethrin	24	27	20	24	20	22	25	29	20	24	46	53	155	179	86.59	Possible resistance
Propoxur	32	32	23	23	36	36	39	39	28	28	38	38	196	196	100.00	Sensitive
Total	123	150	87	114	104	137	132	176	107	114	137	145	690	836	82.54	