The Pattern of Knockdown Resistance Mutations Correlated to the Annual Average Temperature in Field Populations of *Aedes Albopictus* (Diptera: Culicidae) in China

Hanming Chen

Naval Medical University

Qiuming Zhou

Naval Medical University

Haowei Dong

Naval Medical University

Hao Yuan

Naval Medical University

Jie Bai

Naval Medical University

Jingpeng Gao

Naval Medical University

Feng Tao

Naval Medical University

Hui Ma

Chinese PLA General Hospital

Xiangyu Li

Naval Medical University

Heng Peng (✉ pengheng0923@126.com)

Naval Medical University

Yajun Ma

Naval Medical University

Research

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Abstract

Background:

Aedes albopictus is the main vector of dengue fever in China, distributed from north to south in China. Insecticides are an important method to control the mosquitoes, especially in the outbreak of dengue fever, but insecticide resistance raises the risk of failure to control vector-borne diseases. Knockdown resistance (*kdr*) caused by point mutations in the *VGSC* gene is a key mechanism that confers resistance to pyrethroids. To explore the characteristics and possible evolution trend of *kdr* mutation in *Ae. albopictus*, we analyzed the *kdr* mutations of field populations in China in this study.

Methods:

A total of 1 549 *Ae. albopictus* were collected from 18 sites in China from 2017 to 2019, as well as 50 individuals from three sites in the 1990s. A fragment of approximately 350 bp from part of S6 segment in the *VGSC* gene domain III was amplified and sequenced. The haplotypes of *VGSC* gene were recorded and the parsimony network was constructed using TCS 1.21. The data of annual average temperatures (AAT) of collection sites was acquired from national database. The correlation between AAT of the collection site and the *kdr* mutation rate was analyzed by Pearson Correlation using SPSS 21.0.

Results:

The overall frequency of mutant allele F1534 is 45.62%. Nine mutant alleles were detected at codon 1534 in fifteen field populations, namely TCC/TCG (S) (38.86%), TTG/CTG/CTC/TTA (L) (3.71%), TGC (C) (2.68%), CGC (R) (0.27%) and TGG (W) (0.10%). Only one mutant allele ACC (T) was found at codon 1532 with frequency of 6.39% in ten field populations. Moreover, multiple mutations at I1532 and F1534 in a sample appeared in five populations. The 1534 mutation rate was significantly positive related to AAT (Coefficient=0.624, $p=0.0056$), while the 1532 mutation rate was significantly negative related to AAT (Coefficient=-0.645, $p=0.0038$). Thirteen haplotypes were inferred, in which six mutant haplotypes were formed by one step, and the other six haplotypes were formed by one more mutations. In the samples from 1990s, no mutant allele was detected at codon 1532 of *VGSC* gene. However, F1534S/TCC was found in HNHK94 with an unexpected frequency of 100%.

Conclusions

Kdr mutations are widespread in the field populations of *Ae. albopictus* in China. Two novel mutant alleles F1534W/TGG and F1534R/CGC were the detected. The 1534 *kdr* mutation appeared in the population of *Ae. albopictus* no later than 1990s. F1534 mutation rate is positive correlated to AAT, while I1532 mutation rate is negative correlated to AAT. Insecticide using should be carefully managed to slow down the spread of high-resistance *Ae. albopictus* populations.

1. Background

Dengue fever is one of the most widespread mosquito-borne diseases around the world. It was estimated that there are 390 million dengue virus infections worldwide each year (1). Dengue fever has also been an important public health emergency in China. In 2019, 22 599 dengue fever cases were reported in China, and the distribution area reached an unprecedented level (2, 3). Due to the scarcity of therapeutic drug and effective vaccine, the vector controlling is still the main measure to prevent dengue fever(4).

Aedes aegypti and *Ae. albopictus* are the main vectors of dengue fever (5). Although *Ae. aegypti* is more susceptible to dengue virus, *Ae. albopictus* is considered the main vector of dengue fever in China due to its wide distribution range (6). *Ae. albopictus* is distributed from Liaoning Province in the north to Hainan Province in the south in China, covering tropical, subtropical and temperate climate (6). Insecticide is an essential method to control the *Ae. albopictus* vector, especially during outbreaks of mosquito-borne diseases.

With the advantages of high efficiency and low toxicity, pyrethroid insecticides have been widely used to control mosquito in China since 1980s (7). Although integrated control are emphasized in the general *Ae. albopictus* control, pyrethroid insecticides were sprayed heavily during the outbreak of dengue fever to control the epidemic quickly(8, 9). Long-term and mass application of insecticides has led to a serious problem — insecticide resistance. High level and multiple pyrethroid insecticides resistance have been observed in many *Ae. albopictus* field populations in China (10–12).

The target of pyrethroids is voltage-gated sodium channel (*VGSC*), which interferes with electronic signals in the nervous system, leading to paralysis and death, an effect known as knockdown (13). The target insensitivity was a critical mechanism of the pyrethroids resistance developing, which was caused by mutations in the *VGSC*; so-called knockdown resistance mutation (*kdr* mutation) (14, 15).

The *kdr* mutation in *Ae. albopictus* was detected firstly in Singapore in 2009 collected samples by Kasai et al (16). After that, more types of *kdr* mutation were reported in many *Ae. albopictus* field populations (17), including V1016G in Italy and Vietnam (18), F1534C in Singapore, Brazil, India, Italy, Vietnam and Greece (16, 19, 20), F1534L in America (19), F1534S in America, Italy and Vietnam (18, 19) and I1532T in Italy (19). In China, we reported F1534S and F1534L mutant alleles firstly in *Ae. albopictus* from Haikou, Hainan (21, 22). Soon after, The F1534S/L/C, I1532T mutations were reported in Shanghai, Jiangsu, Yunnan, Beijing, Shandong and Guangdong (19, 23–30). V1016G and D1736Y mutations were also detected in certain populations (27, 28).

More and more *kdr* mutations have been discovered, some of which have been confirmed to be closely related to insecticide resistance (12, 31, 32). However, how these mutations appear, accumulate and spread in field populations remain a puzzling and attractive issue. Moreover, the evolution trend of *kdr* mutation maybe the key which would determine the strategy of pesticide application in the future.

In this study, we collected eighteen field populations from North to South China, as well as three populations in the 1990s. The types and frequencies of *kdr* mutations were surveyed and analysis in order to explore the characteristics and possible evolution trend of *kdr* mutation.

2. Methods

2.1 Sample collection and species identification

Eighteen field populations of *Ae. albopictus* were collected from in 13 provinces (municipalities) in China from 2017 to 2019 (Fig. 1). Larvae and pupae were scooped from breeding sites and brought back to laboratory, which were reared to adults under standard conditions at $26 \pm 1^{\circ}\text{C}$ and $65 \pm 5\%$ relative humidity with a photoperiod of 12-h light: 12-h dark. Adult mosquitoes were collected using sucking aspirators, BG-trap (Biogents, Germany) or light trap (Houji Dianzi, China) in outdoor environment near human. To explore the historical changes of *kdr* mutations in *Ae. albopictus*, three field populations in the 1990s were also collected, including HNHK94 (Haikou, 1994), GDGZ95 (Guangzhou, 1995) and SCCD97 (Chengdu, 1997). The collection information was summarized in Table 1. Species of *Aedes* adult mosquitoes were identified by morphology characteristics (33) and confirmed by molecular markers (34).

Table 1
Sampling information of *Aedes albopictus* field populations in China

Collection sites		Code	Latitude/longitude coordinates	Sampling environment	Date	Collecting method/stages
Beijing	Fengtai	BJFT	39°51' N/116°16' E	Residential area, Urban	July 2018	Sucking tube/adult
Guangdong	Guangzhou	GDGZ95 ^{&}	23°07' N/113°15' E	Outdoor area of office building, Urban	June 1995	Scooping/larvae, pupa
		GDGZ			November 2019	
	Shantou	GDST	23°21' N/116°40' E	Park, Suburban	May 2018	Scooping/larvae
	Shenzhen	SZSK	22°29' N/113°54' E	Flower nursery, Urban	June 2018	Scooping/larvae, pupa
Guangxi	Nanning	GXNN	22°47' N/108°19' E	Kindergarten, Urban	May 2020	Scooping/larvae
Hainan	Haikou	HNHK94 ^{&}	20°02' N/110°11' E	Residential area, Suburban, Island	June 1994	Scooping/larvae, pupa
		HNHK*			June-October 2017	
	Yongxing Island	HNSS	16°49' N/112°20' E	Tropic Island	April 2019	BG-trap/adult
	Sanya	HNSY	18°15' N/109°30' E	Village, Island	June 2019	Scooping/larvae, pupa
Jiangsu	Nanjing	JSNJ	32°03' N/118°47' E	Park, Urban	October 2017	Scooping/larvae
Sichuan	Chengdu	SCCD97 ^{&}	30°34' N/104°03' E	Park, Suburban	June 1997	Scooping/larvae, pupa
Shandong	Jinan	SDJNan	36°39' N/117°06' E	Park, Urban	August 2017	Scooping/larvae, pupa
	Jining	SDJNing	35°24' N/116°34' E	Village	July 2018	Scooping/larvae, pupa
Shanghai (SH)	Baoshan	SHBS*	31°24' N/121°29' E	Residential area, Suburban	August 2017	Scooping/larvae
	Yangpu Park	SHGQ	31°19' N/121°32' E	Park, Urban	September-October 2017	Scooping/larvae
	Yangpu	SHYP*	31°15' N/121°31' E	Outdoor area of office building, Urban	June-October 2017	BG-trap/adult
Shanxi	Yangquan	SX	37°51' N/113°34' E	Residential area, Urban	June-October 2017	Scooping/larvae
	Yuncheng		35°01' N/111°0' E			
Shaanxi	Xian	SXXA	34°20' N/108°56' E	Residential area, Urban	September-October 2017	Light trap/adult
					June-October 2018	Light trap/adult
Yunnan	Jinghong	YNJH	22°02' N/100°47' E	Outdoor, Urban	October 2016	Scooping/larvae, pupa
					September 2017	Sucking tube/adult
Zhejiang	Hangzhou	ZJHZ*	30°14' N/120°12' E	Residential area, Urban; Surrounding area of restaurant, Suburban	September 2017	Scooping/larvae; Sucking tube/adult
*These population's information has been published in our previous work [12, 30]. ^{&} The field populations collected in 1990s.						

Table 2
The allele frequency at codon 1532 and 1534 of *VGSC* gene in each *Aedes albopictus* population from China.

Sample code	Sample size		The allele frequency at codon 1532 and 1534 of VSGC gene in each <i>Aedes albopictus</i> population from China.											
			Codon 1532		Codon 1534									
			Wildtype	Mutant	Wildtype	Mutant								
			ATC/I	ACC/T	TTC/F	TTG/L	CTG/L	CTC/L	TTA/L	TCC/S	TCG/S	TGC/C	CGC/R	TGG/W
BJFT	58	<i>N</i>	73	43	115	0	0	0	0	1	0	0	0	0
		<i>P</i> (%)	62.93	37.07	99.14	0	0	0	0	0	0.86	0	0	0
GDGZ	42	<i>N</i>	84	0	21	1	0	0	0	60	0	2	0	0
		<i>P</i> (%)	100.00	0	25.00	1.19	0	0	0	0	71.43	0	2.38	0
GDST	60	<i>N</i>	120	0	120	0	0	0	0	0	0	0	0	0
		<i>P</i> (%)	100.00	0	100.00	0	0	0	0	0	0	0	0	0
SZSK	60	<i>N</i>	120	0	37	26	4	10	0	9	2	21	8	3
		<i>P</i> (%)	100.00	0	30.83	21.67	3.33	8.33	0	7.50	1.67	17.50	6.67	2.50
GXNN	54	<i>N</i>	108	0	16	1	0	1	0	47	0	43	0	0
		<i>P</i> (%)	100.00	0	14.81	0.93	0	0.93	0	43.52	0	39.81	0	0
HNHK	139	<i>N</i>	278	0	95	0	0	0	0	172	0	11	0	0
		<i>P</i> (%)	100.00	0	34.17	0	0	0	0	61.87	0	3.96	0	0
HNSS	36	<i>N</i>	72	0	26	0	0	0	0	46	0	0	0	0
		<i>P</i> (%)	100.00	0	36.11	0	0	0	0	63.89	0	0	0	0
HNSY	91	<i>N</i>	182	0	119	42	0	1	0	18	0	2	0	0
		<i>P</i> (%)	100.00	0	65.38	23.08	0	0.55	0	9.89	0	1.10	0	0
JSNJ	47	<i>N</i>	87	7	90	0	0	0	0	4	0	0	0	0
		<i>P</i> (%)	92.55	7.45	95.74	0	0	0	0	4.26	0	0	0	0
SDJNan	108	<i>N</i>	216	0	190	0	0	0	23	3	0	0	0	0
		<i>P</i> (%)	100.00	0	87.96	0	0	0	10.65	1.39	0	0	0	0
SDJNing	44	<i>N</i>	81	7	88	0	0	0	0	0	0	0	0	0
		<i>P</i> (%)	92.05	7.95	100.00	0	0	0	0	0	0	0	0	0
SHBS	138	<i>N</i>	253	23	214	0	0	0	0	62	0	0	0	0
		<i>P</i> (%)	91.67	8.33	77.54	0	0	0	0	22.46	0	0	0	0
SHGQ	126	<i>N</i>	207	45	159	0	0	0	0	93	0	0	0	0
		<i>P</i> (%)	82.14	17.86	63.10	0	0	0	0	36.90	0	0	0	0
SHYP	98	<i>N</i>	161	35	102	2	0	0	0	91	1	0	0	0
		<i>P</i> (%)	82.14	17.86	52.04	1.02	0	0	0	46.43	0.51	0	0	0
SX	62	<i>N</i>	115	9	123	0	0	0	0	1	0	0	0	0
		<i>P</i> (%)	92.74	7.26	99.19	0	0	0	0	0.81	0	0	0	0
SXXA	25	<i>N</i>	32	18	50	0	0	0	0	0	0	0	0	0

N: number of the allele; *P*: frequency of the allele.

		<i>P</i> (%)	64.00	36.00	100.00	0	0	0	0	0	0	0	0	0
YNJH	77	<i>N</i>	144	10	72	4	0	0	0	73	1	4	0	0
		<i>P</i> (%)	93.51	6.49	46.75	2.60	0	0	0	47.40	0.65	2.60	0	0
ZJHZ	284	<i>N</i>	567	1	48	0	0	0	0	520	0	0	0	0
		<i>P</i> (%)	99.82	0.18	8.45	0	0	0	0	91.55	0	0	0	0
Total	1549	<i>N</i>	2900	198	1685	76	4	12	23	1200	4	83	8	3
		<i>P</i> (%)	93.61	6.39	54.39	2.45	0.13	0.39	0.74	38.73	0.13	2.68	0.26	0.10
<i>N</i> : number of the allele; <i>P</i> : frequency of the allele.														

2.2 Annual average temperature of collection sites

The annual average temperature (AAT) of collection sites were search and recorded from China National Meteorological Science Data Center (<http://data.cma.cn>) (Table S1).

2.3 DNA extraction

Genomic DNA was extracted from adult mosquitoes using DNAzol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. In brief, a single mosquito was put in a 1.5mL eppendorf tube, and powdered efficiently by firstly freezing in liquid nitrogen. Then 100μL DNAzol were added to lysis, and the supernatant was moved to another eppendorf tube. After adding 50μL ethanol, the mixed solution was stored at -20°C overnight. The DNA in the solution was adsorbed through a nucleic acid adsorption column. After washing with 70% ethanol, the DNA was eluted from the column with ddH₂O. The concentration of the DNA was quantified using OD₂₈₀ absorption.

2.4 PCR and kdr alleles detection

A fragment of approximately 350 bp of S6 segment in the *VGSC* gene domain III was amplified using PCR kit (Aidlab, China) and sequenced. The primers are aegSCF7 (5'-AGG TAT CCG AAC GTT GCT GT-3') and aegSCR8 (5'-TAG CTT TCA GCG GCT TCT TC-3') (12, 16). PCR reaction was carried out in a Verity 96 well 157 Thermal Cycler (Applied Biosystems, USA). The cycling parameter included an initial step of denaturation at 94°C for 2 min, followed by 35 cycles of amplification at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 8 min. After electrophoresis, PCR products were purified and sequenced from both directions. Sequences were aligned and analyzed by DNASTAR Lasergene 12.0 software (35).

2.5 Parsimony network the haplotypes of VGSC gene

The haplotypes of *VGSC* gene were recorded and the networks was constructed based on statistical parsimony using TCS 1.21 software (36).

2.6 Statistical analyses

The allele frequency of *kdr* mutations was calculated as:

$$R = \frac{k}{n \times 2} \times 100\%$$

“R” means the allele frequency. “k” represents the number of alleles, and “n” represents the sample size.

The mutation frequency was defined as the frequency of wildtype-mutant heterozygotes and mutant-mutant homozygotes, which was calculated as:

$$M = \frac{a + b}{n} \times 100\%$$

“M” means the mutation frequency. “a” represents the number of wildtype-mutant samples, and “b” represents the number of mutant-mutant samples. “n” is the sample size.

The correlation between the AAT of the collection site and the *kdr* mutation rate was analyzed by Pearson Correlation using SPSS 21.0. The difference of the mutation rate between groups was calculated by one-way ANOVA using SPSS 21.0.

3 Results

3.1 Mosquito samples

A total of 1 549 *Ae. albopictus* adults were collected from 18 sites in China from 2017 to 2019 (Table 2). The *kdr* information of 5 populations (SDJNan, SHBS, SHYP, ZJHZ, HNHK) has been reported in our previous work (12, 30).

3.2 F1534 mutation pattern

3.2.1 F1534 mutation rate at codon

The wildtype codon at codon 1534 is TTC (F). The overall frequency of mutant allele F1534 is 45.62%, which is the site with the most mutation frequency and types in *VGSC* of *Ae. albopictus*. A total of nine mutant alleles were detected, namely TCC/TCG (S) (38.86%), TTG/CTG/CTC/TTA (L) (3.71%), TGC (C) (2.68%), CGC (R) (0.27%) and TGG (W) (0.10%). The mutant allele TCC has the highest frequency (38.74%), whereas the lowest frequency allele is TGG (0.10%) (Fig. 1, Table 2). A total of 15 genotypes were detected (Table S2), including the wildtype genotype F/F, four wildtype-mutant heterozygotes F/L, F/S, F/C and F/R, and ten mutant genotypes L/L, C/C, S/S, C/R, L/C, L/R, C/W, S/R, L/S and S/C. The frequencies of wildtype genotype, wildtype-mutant heterozygote and mutant genotype were 40.61%, 27.57% and 31.82%, respectively (Table S2). In other words, 59.39% of *Ae. albopictus* adults have mutations at 1534 codon of the *VGSC* gene.

3.2.2 F1534 mutation pattern in the field populations

In the eighteen field populations, fifteen populations have F1534 mutations (Table 2). The highest frequency was in ZJHZ (91.55%), followed by GXNN (85.19%), GDGZ (75.00%), SZSK (69.17%), HNHK (65.83%), HNSS (63.89%) and YNJH (53.25%), and eight populations with frequencies below 50%. Three populations have no mutant in 1534 including GDST, SXXA and SDJNing. (Table 2).

In terms of the mutant allele types, the SZSK population carried the most types with eight mutant alleles, followed by GXNN, YNJH and HNSY with 4 mutant alleles, GDGZ and SHYP with 3 mutant alleles, HNHK and SDJNan with 2 mutant alleles. For the other seven populations, only one mutant allele as F1534S/TCC was detected (Table 2).

1534TCC/S is the most common mutant allele, which was detected in all of the 15 populations. A total of four mutant alleles were found in only one population, namely CTG/L, CGC/R, TGG/W and TTA/L. Among them, CTG/L (3.33%), CGC/R (6.67%) and TGG/W (2.50%) were only found in SZSK, and TTA/L (10.65%) was identified in the SDJNan.

3.3 I1532 mutation pattern

3.3.1 I1532 mutation rate at codon

Except for the wildtype alleles ATC (I), only one mutant allele ACC (T) was found at codon 1532 (Table 2). Compare to 1534 codon, the 1532 codon have much lower mutation frequency with 6.39%. Three genotypes were found, as wildtype genotype I/I (88.57%), wildtype-mutant heterozygote I/T (10.07%) and mutant genotype T/T (1.36%) (Table S3).

3.3.2 F1532 mutation pattern in the field populations

The mutant allele I1532T was detected in ten field populations, as YNJH, BJFT, SDJNing, SHBS, SHYP, SHGQ, ZJHZ, SXXA, JSNJ and SX. The highest frequency was 37.07% in the BJFT population, followed by 36.00% in the SXXA population (Table 2, Fig. 2). The mutant homozygous 1532T/T was found in six populations, namely BJFT (13.79%), SXXA (12.00%), SHYP (5.10%), SHGQ (2.38%), SDJNing (2.27%) and SHBS (0.72%) (Table S3).

3.4 Multiple mutations at codon 1532 and 1534

Multiple mutations at I1532 and F1534 appeared in five populations as YNJH, SHBS, SHYP, SHGQ and ZJHZ (Fig S3). The highest frequency of co-occurrence of the mutations was 21.43% in SHYP population, followed by 15.08% (SHGQ). Three types of combined mutations were present, as I/T + F/S, I/T + S/S, T/T + F/L. However, no individuals with mutant homozygotes at both codons were found (Table 3).

Table 3
Frequency of co-occurrence of I1532T + F1534S genotypes of *VGSC* gene in the *Aedes albopictus* populations from China.

Loci		Population				
I1532	F1534	SHBS	SHGQ	SHYP	YNJH	ZJHZ
I/T	F/S	5 (3.62%)	19 (15.08%)	19 (19.39%)	6 (7.79%)	0
I/T	S/S	0	0	0	0	1 (0.35%)
T/T	F/L	0	0	2 (2.04%)	0	0
Note: The number in brackets is the percentage of co-occurrence of I1532T + F1534S genotype.						

3.5 Geographic distribution of *kdr* mutants of *Ae. albopictus* in China

Interestingly, the pattern of *kdr* mutations in *Ae. albopictus* seems to be related to geographical factors. F1534S/L/C/R/W mutant alleles were mainly found from southern and central China (Fig. 2A), while I1532T was mainly found in populations from northern and central China (Fig. 2B). If the field populations were divided to the southern group and the northern group by 32°N (Fig. 2), there is a significant difference in the 1534 mutation rate ($p = 0.0003$) and the 1532 mutation rate ($p = 0.0443$) between the two groups.

To further explore the phenomenon, we collected the AAT of the collection sites. The 1534 mutation rate was significantly positive related to AAT (Coefficient = 0.624, $p = 0.0056$), while the 1532 mutation rate was significantly negative related to AAT (Coefficient=-0.645, $p = 0.0038$).

It is worth noting that the ZJHZ population was collected during the 2019 Hangzhou dengue fever epidemic. At that time, a large number of insecticides had been used, so the collected mosquitoes were likely to be a screened resistant population. The collection site of the GDST population is very close to the sea. It was learned that almost no pesticides was used in the local area through oral interviews. If the ZJHZ and GDST population were removed from the correlation analysis, the 1534 mutation rate showed more significantly positive related to AAT (Coefficient = 0.843, $p < 0.0001$), and the 1532 mutation rate was also significantly negative related to AAT (Coefficient=-0.657, $p = 0.0057$).

3.6 Genealogical relationships among the haplotypes of VGSC gene

Based on the alleles in codons 1532 and 1534, thirteen haplotypes were inferred, which has been submitted to the NCBI (<https://www.ncbi.nlm.nih.gov/>) (Table 4). The 95% parsimony network showed a parallel evolution pathway. The ancestral wildtype haplotype (H01_IF) weight was only 0.13. Six mutant haplotypes were formed by one step mutation from ancestral haplotype, as H02_IL, H04_IL, H05_IL, H06_IS, H08_IC and H11_TF. The other six haplotypes were formed by one more mutation (Fig. 3). This result supports the view that *kdr* mutations of *Ae. albopictus* have multiple origins.

Table 4
Haplotype of *VGSC* gene according mutant alleles of codons 1532 and 1534 in *Aedes albopictus* populations from China.

Haplotype	Allele		GenBank Submission Number
	Codon 1532	Codon 1532	
H01_IF	I/ATC	F/TTC	MT559317
H02_IL	I/ATC	L/TTG	MT559318
H03_IL	I/ATC	L/CTG	MT559319
H04_IL	I/ATC	L/CTC	MT559320
H05_IL	I/ATC	L/TTA	MT559321
H06_IS	I/ATC	S/TCC	MT559322
H07_IS	I/ATC	S/TCG	MT559323
H08_IC	I/ATC	C/TGC	MT559324
H09_IR	I/ATC	R/CGC	MT559325
H10_IW	I/ATC	W/TGG	MT559326
H11_TF	T/ACC	F/TTC	MT559327
H12_TL	T/ACC	L/TTG	MT559328
H13_TS	T/ACC	S/TCC	MT559329

3.7 Historic change of *kdr* mutants in *Ae. albopictus* in China

A total of 50 individuals from three sites in the 1990s were collected to explore the historic change of the *kdr* mutation, including HNHK94 (Haikou, 1994, $n = 12$), GDGZ95 (Guangzhou, 1995, $n = 18$) and SCCD97 (Chengdu, 1997, $n = 20$). No mutant allele was detected at codon 1532 of *VGSC* gene. However, F1534S/TCC was found in HNHK94 with an unexpected frequency of 100%. HNHK94 samples were collected from Haikou in 1994, when 15 years earlier than the firstly reported *kdr* mutant populations by Kasai et al (16). Although the number of samples available is limited, the result clearly showed that the F1534S *kdr* mutation of *Ae. albopictus* appeared no later than 1994.

3.8 Synonymous mutations

In addition to above non-synonymous mutations, we also found 5 synonymous mutations at codon 1516 CCG (P), 1528 TTC (F), 1539 ACC (T), 1540 CTC (L) and 1541 AAC (N) in the fragment. Only one mutant allele was detected at codons 1516 (CCA), 1528 (TTT) and 1541 (AAT), respectively, while two mutant alleles were found at codons 1539 (ACG, ACT) and 1540 (CTT, CTG). The frequencies of the mutation alleles were 20.80% (1516), 8.85% (1528), 3.78% (1539), 50.96% (1540) and 51.52% (1541) (Table S4). However, we did not find any correlation between these synonymous mutations and non-synonymous mutations at codon 1534 and 1532. The role of these synonymous mutations remains to be explored.

Discussion

Insecticide resistance has become an important threat to the control of diseases transmitted by *Ae. albopictus*. In this study, we collected the field populations of *Ae. albopictus* in China, detected and analyzed the *kdr* mutation to explore the characteristics and possible evolution trend of the *kdr* mutations. The sample collection sites covered the main distribution range of *Ae. albopictus* in China from 16°N to 40°N in this study (6). The climate includes tropical, subtropical and temperate climates.

The results showed that types of *kdr* mutations have appeared widely in the field populations of *Ae. albopictus* in China. Certain population (SZSK) showed a high mutation rate of 1534 with complex mutation types. Moreover, two novel mutant alleles F1534W/TGG and F1534R/CGC were detected in SZSK population. The AAT of this group collection point is relatively high (23.0°C), where mosquitoes reproduce all year round. Moreover, developed trade and

logistics, high population mobility, and frequently used pesticides in Shenzhen are all factors that promote insecticide resistance in mosquito populations (37). If the use of pesticides cannot be controlled, the high frequency and multiple types of 1534 mutations appeared in SZSK population may be the trend of *kdr* mutations in the field populations of *Ae. albopictus* in the future.

An extremely high mutation rate of 1534 was detected in the HNHK94 population collected in Haikou City in 1994, indicating that some populations of *Ae. albopictus* had high insecticide resistance decades ago. The result pushed the discovery time of *kdr* mutation in *Ae. albopictus* samples forward by 15 years (16). The phenomenon may be related to the large-scale use of pesticides in Hainan Province after three dengue fever outbreaks in the last century, as 1979 ~ 1982, 1985 ~ 1988, 1991(38). The ZJHZ population was also collected after the occurrence of dengue fever in Hangzhou (11), which similarly showed a very high percentage of 1534 mutation rates, suggesting that the large-scale use of pesticides is a “closely related” factor for the 1534 mutation rate of the *Ae. albopictus* population. Therefore, a more cautious, precise and strict insecticide resistance management (IRM) is urgently to be established (39).

In northern populations where the average temperature was low, the *kdr* mutation rate of *Ae. albopictus* was generally low, and the I1532 mutation rate was relatively high. However, in the southern populations with higher average temperature, the *kdr* mutation rate was higher, and the F1534 mutation rate was relatively high. Research on the correlation between gene mutations and insecticide resistance showed that F1534S and F1534C were significantly related to insecticide resistance (12, 21), while the correlation between I1523T and insecticides was still unclear (12, 32). The results of this study clearly show that the southern population is more resistant to pesticides than the northern population. The temperature in the south is high, and mosquitoes have more generations, which can be regarded as fast-evolving populations, while the northern populations can be regarded as slow-evolving populations. The results in this study suggested that the highly resistant population may be screened out in a relatively short period of time in the fast-evolving populations. Due to the long cold winter in the north, pesticides are often used seasonally, which may slow down the development of pesticide resistance. This suggests that seasonal use of pesticides may be a strategy to slow down the development of pesticide resistance. However, how to use insecticides seasonally in a certain area and effectively control the risk of vector-borne disease transmission requires further research. This is very important for the prevention and control of mosquito-borne diseases in the context of global climate change (40).

Although some meaningful results were obtained, there are still some limitations in this study. First, there are only a few historical samples available, which make it impossible to systematically analyze the historical changes of *kdr* mutations. In addition, accurate information on the use of insecticides cannot be collected, which affected the interpretation of the results. Moreover, this study focused on the most common *kdr* mutations in *Ae. albopictus*, leaving some types of *kdr* mutations with low mutation rate (18). Finally, although the synonymous mutations and intron length polymorphisms have been documented in the populations of *Ae. albopictus* and *Ae. aegypti* (19, 28, 41), the significance of the synonymous mutations found in this study is not clear, and further research is needed.

Conclusions

From this study, we can found the *kdr* mutations are widespread in the field populations of *Ae. albopictus* in China. Two novel mutant alleles F1534W/TGG and F1534R/CGC were the detected for the first time. The 1534 *kdr* mutation appeared in the population of *Ae. albopictus* no later than 1994. HNHK94 was the earliest known *Ae. albopictus* field population with *kdr* mutation. F1534 mutation rate is positive correlated to AAT, while I1532 mutation rate is negative correlated to AAT. Insecticide using should be carefully managed to slow down the spread of high-resistance *Ae. albopictus* populations.

Abbreviations

VGSC
voltage-gated sodium channel;
kdr
knockdown resistance;
WHO
World Health Organization;
AAT
Annual Average temperature;
CDC
Center for Disease Control and Prevention;
IRM
insecticide resistance management.

Declarations

Competing interests and consent to participate

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request, and sequences are available in GenBank.

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Authors' contributions

All authors contributed to the collection of mosquitoes. YM and HP designed the study. MC, HD, HY, JB, JG, HM and HP did the experiment and performed data analysis. QZ, FT, HP and YM wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

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References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. *Nature*. 2013;496(7446):504–7.
2. Liu Q. [Dengue fever in China: new epidemical trend, challenges and strategies for prevention and control.]. *Chinese Journal of Vector Biology Control*. 2020;31(1):1–6.
3. Du JW, Pan XH. [Prevalent status and features of dengue fever in China]. *Chinese Journal of Epidemiology*. 2010;31(12):1429–33.
4. Wellekens K, Betraains A, De Munter P, Peetermans W. Dengue: current state one year before WHO 2010–2020 goals. *Acta clinica Belgica*. 2020:1–9.
5. Lambrechts L, Scott TW, Gubler DJ. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl Trop Dis*. 2010;4(5):e646.
6. Yang S, Liu Q. [Trend in global distribution and spread of *Aedes albopictus*]. *Chinese Journal of Vector Biology Control*. 2013;24(1):1–4.
7. Meng F, Wang Y, Feng L, Liu Q. [Review on dengue prevention and control and integrated mosquito management in China]. *Chinese Journal of Vector Biology Control*. 2015;26(1):4–10.
8. Lin H, Wang X, Li Z, Li K, Lin C, Yang H, et al. Epidemiological characteristics of dengue in mainland China from 1990 to 2019: A descriptive analysis. *Medicine*. 2020;99(36):e21982.
9. Wang Y, Jiang Z. [Development and application of public health pesticides in China, 2013–2016.]. *Chinese Journal of Vector Biology Control*. 2016;27(1):421–5.
10. Li Y, Zhou G, Zhong D, Wang X, Hemming-Schroeder E, David RE, et al. Widespread multiple insecticide resistance in the major dengue vector *Aedes albopictus* in Hainan Province, China. *Pest management science*. 2020.
11. Hou J, Liu Q, Wang J, Wu Y, Li T, Gong Z. Insecticide Resistance of *Aedes albopictus* in Zhejiang Province, China. *Bioscience trends*. 2020;14(4):248–54.
12. Gao JP, Chen HM, Shi H, Peng H, Ma YJ. Correlation between adult pyrethroid resistance and knockdown resistance (kdr) mutations in *Aedes albopictus* (Diptera: Culicidae) field populations in China. *Infectious diseases of poverty*. 2018;7.
13. Narahashi T. Neuronal ion channels as the target sites of insecticides. *Pharmacology toxicology*. 1996;79(1):1–14.
14. Silva JJ, Scott JG. Conservation of the voltage-sensitive sodium channel protein within the Insecta. *Insect molecular biology*. 2020;29(1):9–18.
15. Scott JG. Life and Death at the Voltage-Sensitive Sodium Channel: Evolution in Response to Insecticide Use. *Ann Rev Entomol*. 2019;64:243–57.
16. Kasai S, Ng LC, Lam-Phua SG, Tang CS, Itokawa K, Komagata O, et al. First detection of a putative knockdown resistance gene in major mosquito vector, *Aedes albopictus*. *Jpn J Infect Dis*. 2011;64(3):217–21.
17. Auteri M, La Russa F, Blanda V, Torina A. Insecticide Resistance Associated with kdr Mutations in *Aedes albopictus*: An Update on Worldwide Evidences. *BioMed research international*. 2018;2018:3098575.
18. Kasai S, Caputo B, Tsunoda T, Cuong TC, Maekawa Y, Lam-Phua SG, et al. First detection of a Vssc allele V1016G conferring a high level of insecticide resistance in *Aedes albopictus* collected from Europe (Italy) and Asia (Vietnam), 2016: a new emerging threat to controlling arboviral diseases. *Eurosurveillance*. 2019;24(5):48–59.

19. Xu J, Bonizzoni M, Zhong D, Zhou G, Cai S, Li Y, et al. Multi-country Survey Revealed Prevalent and Novel F1534S Mutation in Voltage-Gated Sodium Channel (VGSC) Gene in *Aedes albopictus*. PLoS Negl Trop Dis. 2016;10(5):e0004696.
20. Aguirre-Obando OA, Martins AJ, Navarro-Silva MA. First report of the Phe1534Cys kdr mutation in natural populations of *Aedes albopictus* from Brazil. Parasites & vectors. 2017;10.
21. Chen H, Li K, Wang X, Yang X, Lin Y, Cai F, et al. First identification of *kdr* allele F1534S in VGSC gene and its association with resistance to pyrethroid insecticides in *Aedes albopictus* populations from Haikou City, Hainan Island, China. Infectious diseases of poverty. 2016;5:31.
22. Wang X, Chen H, Yang X, Lin Y, Cai F, Zhong W, et al. [Resistance to pyrethroid insecticides and analysis of knockdown resistance (*kdr*) gene mutations in *Aedes albopictus* from Haikou City]. Academic Journal of Second Military Medical University. 2015;36(8):832–8.
23. Zhu CY, Zhao CC, Wang YG, Ma DL, Song XP, Wang J, et al. Establishment of an innovative and sustainable PCR technique for 1534 locus mutation of the knockdown resistance (*kdr*) gene in the dengue vector *Aedes albopictus*. Parasites & vectors. 2019;12(1):603.
24. Li Y, Xu J, Zhong D, Zhang H, Yang W, Zhou G, et al. Evidence for multiple-insecticide resistance in urban *Aedes albopictus* populations in southern China. Parasites vectors. 2018;11(1):4.
25. Zhao C, Zhu C, Kai W, Liu G, Liu Q, Lin L, et al. [Genotypes of knockdown resistance gene and their distribution in *Aedes albopictus* in Haikou, China, in 2018.]. Chinese Journal of Vector Biology Control. 2019;30(1):7–11.
26. Lan X, Xu J, Jiang J. [An analysis of voltage-gated sodium channel gene mutation in *Aedes albopictus* resistant populations against pyrethroid insecticides in Ruili, Yunnan Province, China.]. Chinese Journal of Vector Biology Control. 2019;30(2):158–62.
27. Zhu C, Zhao C, Lun X, Zhu J, Li H, Jiang J, et al. [Distribution of knockdown resistance genotypes in *Aedes albopictus* in Jinghong, Yunnan Province, China, 2018–2019.]. Chinese Journal of Vector Biology Control. 2020;31(1):7–11.
28. Zhou XJ, Yang C, Liu N, Li M, Tong Y, Zeng XP, et al. Knockdown resistance (*kdr*) mutations within seventeen field populations of *Aedes albopictus* from Beijing China: first report of a novel V1016G mutation and evolutionary origins of *kdr* haplotypes. Parasites & vectors. 2019;12.
29. Liu HM, Liu LH, Cheng P, Yang LL, Chen JH, Lu Y, et al. Bionomics and insecticide resistance of *Aedes albopictus* in Shandong, a high latitude and high-risk dengue transmission area in China. Parasites & vectors. 2020;13(1).
30. Chen H, Gao J, Jiang J, Peng H, Ma Y. [Detection of the I1532 and F1534 *kdr* mutations and a novel mutant allele I1532T in VGSC gene in the field populations of *Aedes albopictus* from China.]. Chinese Journal of Vector Biology Control. 2018;29(2):120–5.
31. Gao J, Chen H, Ma Y. Establishment of diagnostic doses of three pyrethroid insecticides for detecting resistance in *Aedes albopictus* (Diptera: Culicidae) in China. Acta Entomologica Sinica. 2018;61(1):18–24.
32. Yan R, Zhou Q, Xu Z, Zhu G, Dong K, Zhorov BS, et al. Three sodium channel mutations from *Aedes albopictus* confer resistance to Type I, but not Type II pyrethroids. Insect Biochem Mol Biol. 2020;123:103411.
33. Lu B, [Fauna Sinica I. Diptera, Culicidae I]. Vol. 9. Beijing: Science Press; 1997.
34. Manni M, Gomulski LM, Aketarawong N, Tait G, Scolari F, Somboon P, et al. Molecular markers for analyses of intraspecific genetic diversity in the Asian Tiger mosquito, *Aedes albopictus*. Parasites & vectors. 2015;8.
35. Burland TG. DNASTAR's Lasergene sequence analysis software. Methods in molecular biology. 2000;132:71–91.
36. Clement M, Posada D, Crandall KA. TCS: a computer program to estimate gene genealogies. Molecular ecology. 2000;9(10):1657–9.
37. Huang YW, Peng SQ, Lian CL. [Survey on Mosquito at Shenzhen Shekou Port]. Chinese Journal of Frontier Health Quarantine. 2010;2:112–4.
38. Wang CZ, Chen WZ. [Overview of Dengue Fever Prevalence in Hainan Province.]. Hainan Medical Journal. 1992;3(3):1–4.
39. Dusfour I, Vontas J, David JP, Weetman D, Fonseca DM, Corbel V, et al. Management of insecticide resistance in the major *Aedes* vectors of arboviruses: Advances and challenges. PLoS Negl Trop Dis. 2019;13(10):e0007615.
40. Tidman R, Abela-Ridder B, de Castaneda RR. The impact of climate change on neglected tropical diseases: a systematic review. Trans R Soc Trop Med Hyg. 2021;115(2):147–68.
41. Chung HH, Cheng IC, Chen YC, Lin C, Tomita T, Teng HJ. Voltage-gated sodium channel intron polymorphism and four mutations comprise six haplotypes in an *Aedes aegypti* population in Taiwan. PLoS Negl Trop Dis. 2019;13(3):e0007291.

Figures

Figure 1

A schematic map of collection sites for *Aedes albopictus* in China and the composition of the codon 1534 alleles of VGSC gene in the samples. The red balloons indicate the sampling sites (see Table 1 for details). The codon 1534 alleles composition in each site was displayed by a pie chart with green as wildtype F1534, yellow as mutant allele F1534S and blue as mutant allele F1534L/C/R/W (see Table 2 for details). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

The histogram of allele frequency at codon 1532 (A) and 1534 (B) of VGSC gene in *Aedes albopictus* populations. SH was the pool of three populations (SHBS, SHYP, SHGQ). For information details, see Table 1 and Table 2. The populations on the left of the dotted line were from central and northern China, and the populations on the right side were from southern China. Horizontal axis indicates the populations while vertical axis denotes the allele frequency. The populations with asterisk was co-occurrence of mutations both codons 1532 and 1534.

Figure 3

Genealogical relationships among haplotypes of kdr mutations of VGSC gene estimated by TCS. The circle indicates one of haplotypes and the number in each circle represents the haplotype type. The yellow square indicates the wildtype haplotype. Every unit branch represents one mutation.

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