Exploration of New Mutations for Resistance of Houseflies to Organophosphate Insecticides in Housefly Populations in Guizhou Province, China

Rong Mou
Characteristic Laboratory of Modern Pathogen Biology,Basci Medical Science College,Guizhou Medical University
Xi Yang
Department of Basic Medical Science,Qiannan Medical college for Nationalitiies
Qiuguo Liang
Department of Basic Medical Science,Qiannan Medical college for Nationalities
Jinzhi Cheng
Characteristic Laboratory of Modern Pathogen Biology, Basic Medical Science College,Guizhou Medical University
Xiaohui Mou
Characteristic Laboratory of Modern Pathogen Biology, Basic Medical Science College,Guizhou Medical University
Zhengling Shang
Research Center of Basic Medical Science College, Guizhou Medical University
Gang Wang
Zhejiang International Travel Healthcare Center
weilong tan
Huadong Research Institute for Medicine and Biotechnics
Jiahong Wu (jiahongw@gmc.edu.cn)

Research

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Abstract

Background The campaign to establish the national sanitary city has been launched across major places in Guizhou Province, which leads to the extensive use of insecticide to eradicate the disease-carrier *Musca domestica* found everywhere while keeping the cleanliness of environment.

Methods: In order to perceive the resistance of houseflies to the commonly used organophosphate insecticides in 7 housefly populations belonging to Guizhou province (China), the susceptibility bioassays, detection of resistance-associated mutations, and the carboxylesterase activity assay were conducted.

Results: The bioassays exhibited 142.16~303.54-fold to dichlorvos (DDVP) and 122.13~363.98-fold to temephos. The molecular analysis unveiled mutant ACE gene at loci of 260, 342 and 407 in all populations, which led to high frequencies at 27.4~73.8% of 260L, 59.1~76.7% of 342A, 23.7~40.9% of 342V, and 83.4~100.0% of 407Y, with inclusion of 8 genotypes and 10 mutant combinations. Further analysis of mutations showed a linkage disequilibrium of L/V+A+Y at locus 260 & 342, indicating a significant association with the DDVP resistance. The Hardy-Weinberg equilibrium test demonstrated that the observed ACE heterozygosity mostly exceeded 0.5 and deviated from the equilibrium. In the fixation index, an insignificant differentiation was noted among the 7 housefly populations.

Conclusions: However, further research should concentrate on the use of insecticides to avoid the abuse of insecticides, and to regularly monitor the resistance of houseflies using novel methods.

Background

Musca domestica L. (Diptera: Muscidae), commonly called the house fly, is a major domestic, medical and veterinary pest that causes irritation, spoils food and acts as a vector for many pathogenic organisms[1, 2]. Houseflies can spread a deadly strain of Escherichia coli and transmit life threatening antibiotic-resistant bacteria, which constitute an ever increasing threat in hospitals and other healthcare facilities [1].

The housefly containment measures emphasize a comprehensive regulation of breeding places, including biological, physical and chemical pest control, in which the chemical control predominates with its fast and effective insect lethality. For instance, the pesticide used in China amounted to 1.8 million tons and the insecticide used in the United States reached 31.75 million kg in urban settings[3, 4]. However, the long-term extensive use of insecticide makes the selection pressure works and forces housefly to activate various mechanisms for adaptation and resistance to insecticide. A study concentrated on 48 Chinese cities demonstrated that the houseflies showed a strong resistance to several commonly used insecticides, such as dichlorvos (DDVP), temephos, etc.[5], Moreover, the resistance of houseflies to deltamethrin and DDVP was reported [5]. This indicated that the resistance to the organophosphate (OP) insecticides, including DDVP and temephos, may cause a serious concern to control local houseflies.

It has been previously revealed that the resistance of houseflies to OP is attributable to insensitive acetylcholinesterase (AChE) and enhances carboxylesterase activity. The AChE enzyme is a serine hydrolase vital for regulating the neurotransmitter acetylcholine in mammals, birds, and insects[6, 7]. However, the modification or mutation of ACE gene encoding AChE may change the structure of enzyme, thereby reducing or eliminating the binding affinity of insecticide with the target-site [8]. It was suggested that V260L, G342A/V, and F407Y mutations of ACE gene in a particular active site are responsible for the resistance to changing the current of the catalytic triad, as well as restricting binding to insecticides[9, 10]. Carboxylesterase is widely found in insects, and changes in carboxylesterase activity were proven to be associated with resistance to OP insecticide in some species of houseflies, mosquito, aphids, blowflies, and western flower thrips where the resistant strains of flies showed a tendency to suppress the level of the carboxylesterase activity. The main reason is that structural mutation and overexpression of carboxylesterase inhibit hydrolyzation of methyl butyrate and naphthyl acetate, triggering increase of OP[11, 12]. An evidence suggested that multiple carboxylesterase genes were co-upregulated in resistant houseflies [12, 13].

Regrettably, since 1999, only a research conducted by Lin & Zhu (1999) concentrated on housefly resistance in Guizhou province (China). Subsequently, in the campaign of establishing the national sanitary city that was spread gradually across mainland China [14], Guizhou province played an active role using a series of insecticides for reducing the number of houseflies, which could be an indicator to determine whether that city was qualified to be a national sanitary city or not. Thereafter, the resistance of houseflies to propoxur and DDVP in Anshun, Guiyang and Xingy, major cities of Guizhou, was reported to be terrible [15–17]. In the present study, an attempt was made to understand the current status of the resistance of houseflies to OP in other places across Guizhou province, and to predict the future trend of resistance.

Methods

Collection and rearing of houseflies

Adult *Musca domestica* houseflies were collected from urban or suburban areas distributed in 7 different places across Guizhou province, as shown in Fig. 1. In the current study, there were around 100 houseflies with the sweep net mainly in waste transfer stations and refuse dump of the farmer markets or old residential buildings, and those were mixed to represent a local population. All of the collected houseflies were routinely reared with the mixture of milk powder: granulated sugar (1:1) plus an appropriate amount of water at a constant indoor temperature of 25 ± 1 ℃, with humidity of 70 ± 10% under a 12 h light/12 h dark cycle [11]. Houseflies' eggs were laid in wheat bran (100 g) containing milk powder (5 g), granulated sugar (5 g), and water (130 mL) and hatched to pupate in some dry surface of the feed during within 7 days [18]. The World Health Organization (WHO) susceptibility bioassay, as well as chemical and molecular tests were conducted using the first-generation of collected houseflies that aged 3 ~ 5 days old with a similar body

weight of 18–22 mg. The insecticide-susceptible housefly population was transferred to the laboratory of the Chinese Center for Disease Control and Prevention (CDC), without exposure to any insecticide for decades and treated as the control group [19].

Bioassays

The resistance of houseflies to DDVP in 5 populations and to temephos in 4 populations were examined. The LD_{50} (μg/housefly) of houseflies to DDVP and temephos ranged from 0.56865–1.21415 and 13.8005-41.12605, respectively. Two OPs were characterized by an extremely high resistance, which reached 122.13 ~ 363.95-fold in temephos and 142.16 ~ 303.54-fold in DDVP, respectively (Table 1).

Table 1
Toxicity of insecticides to the field-collected houseflies

Insecticide	Population	Generation	N	LD_{50} & 95% CI (μg/housefly)	Slope	RR
DDVP	AS	F1	450	0.7231 (0.6468 ~ 0.8071)	2.56	180.78
	GY	F1	450	1.21415 (0.90335 ~ 1.6856)	2.621	303.54
	HS	F1	450	0.56865 (0.52115 ~ 0.69685)	3.538	142.16
	KL	F1	450	0.72205 (0.6538 ~ 0.79625)	2.58	180.51
	ZY	F1	450	0.63315 (0.55825 ~ 0.721)	3.712	158.29
	Susceptible	-	-	0.004 (0.003 ~ 0.005)	1.052	1
Temephos	AS	F1	450	24.01385 (11.1251 ~ 35.161)	2.16	212.51
	GY	F1	450	17.26095 (11.1251 ~ 26.95385)	2.356	152.75
	HS	F1	450	13.8005 (11.64275 ~ 17.2522)	1.858	122.13
	KL	F1	450	41.12605 (33.2248 ~ 54.005)	1.326	363.95
	Susceptible	-	-	0.113 (0.054 ~ 0.178)	0.894	1

Extraction Of Genomic Dna

A whole adult housefly was fully homogenized in a 300 μL extraction buffer (100 mM Tris-HCl (pH, 8.8), 50 mM NaCl, 10 mM EDTA, with 1% SDS) in a 1.5 mL Eppendorf tube, and then, proteinase K (50 μg) was added to each sample. The homogenates were incubated in a water bath at 56 ℃ overnight. On the next-day, solution of chloroform (300 μL) and isoamyl alcohol (24:1) was added to the tube for separation of the protein from DNA. After that, it was turned upside down for several times and centrifuged at 10000 rpm/min for 10 min at 4 ℃. The supernatant with aqueous phase containing DNA was sucked up to a new tube, added with sodium acetate (3 M) of 0.1-fold volume and absolute ethanol of 2-fold volume supernatant, and frozen to precipitate for longer than 2 h. Afterwards, the supernatant was discarded after centrifugation at 12000 rpm/min for 5 min at 4 ℃. Moreover, 70% ethanol (1 mL) was used to wash the DNA for once or twice after centrifugation at 12000 rpm/min, 4℃, for 10 min. Finally, the supernatant in the tube was discarded and the DNA remained on the tube bottom or the wall, in form of mixed with ddH_2O (15 μL). DNA (10–15 μg) was extracted from a housefly and more than 30 DNA samples were isolated from each field-collected population as described previously [20, 21].

Amplification And Sequencing Of Ace Gene

The ACE gene fragment was amplified in a 25 μL reaction containing Premix Taq™ (12.5μL), with Taq (0.625 U), dNTP Mixture (0.4 mM), Mg^{2+} (3 mM) and Pigment Marker (RR901A; TAKARA Bio Inc., Shiga, Japan), ddH_2O (8.5 μL), DNA template (2 μL), 10 μM forward primer S90MdAce (1 μL) (5'-CATCTAAAACCGATCAGGACCATTTAATAC-3'), and 10 μM reverse primer AS89MdAce (1 μL) (5'-TCATCTTTAACATTTCCAATCAGAATATCG-3') (Baskurt et al. 2011). The reactions were completed in a SimpliAmp™ Thermal Cycler (A24811; Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: at 94 ℃ for 3 min, followed by 40 cycles of PCR (at 95 ℃ for 30 s, at 55 ℃ for 30 s, and at 72 ℃ for 90 s), and a final extension at 72 ℃ for 10 min [22]. PCR products (5 μL) were identified with electrophoresis of 1% agarose gel and imaged by an ultraviolet (UV) transilluminator (3500R; Tanon Science & Technology Co., Ltd., Shanghai, China). The samples showing a band of ~ 800 bp fragment was bi-directionally sequenced by Majorbio Biotech Co., Ltd. (Shanghai, China).

Assay Of Carboxylesterase Activity

Preparation and quantification of crude enzyme: A decapitated housefly (age, 4 days old; body weight, 18–22 mg) was homogenized with 500 μL cold phosphate-buffered saline (PBS; 0.1 M, pH 7.5, containing 0.1% Triton-x100) in a pre-cold Eppendorf tube (1.5 mL). Subsequently, the homogenate was centrifuged at 10000 g for 15 min, and the supernatant was stored at 4 ℃. A total of 150 houseflies were chosen from the field-collected population of AS, GY, and HS. The enzyme was quantified using the Thermo Scientific Pierce BCA Protein Assay Kit [23].

Standard curve of α-naphthol: 600 µL of α-naphthol solution (1, 0.8, 0.5, 0.25, 0.125, and 0.0625 mM) and 100 µL of Fast Blue BB salt solution (3 mM containing 5% SDS, Shanghai Yuanye Biotech Co., Ltd., Shanghai, China) were added into each well of a 96-well enzyme-linked immunosorbent assay (ELISA) plate. The mixtures exhibited a different level of yellow-brown color and were measured with the Bio-tech Epoch spectrophotometer at wavelength of 570 nm after 5 min. The average absorbance of the diluting solutions minus that of the PBS control was calculated to plot the curve. An equation of y = ax + b was formulated (x = 1, 0.8, 0.5, 0.25, 0.125, and 0.0625 mM). Then, the following assay was conducted to calculate the unknown concentration of the produced α-naphthol using the standard curve [24, 25].

Measurement of carboxylesterase activity: Carboxylesterase catalyzes α-naphthylacetate and produces α-naphthol, and can be detected by the reaction product of α-naphthol. The mixture with 20 µL of crude enzyme and 200 µL of α-naphthylacetate (0.3 mM Shanghai Yuanye Biotech Co., Ltd., Shanghai, China) was incubated for 15 min at room temperature. Subsequently, the Fast Blue BB salt solution (50 µL) was added for 5 min, and the absorption was recorded at 570 nm with the assistance of the Bio-tech Epoch spectrophotometer. Three duplicates were required for each housefly and PBS control. According to the quantified enzyme (µg/µL) and the standard curve of α-naphthol (mM), the carboxylesterase activity was converted to α-naphthyl acetate (µmol α-naphthol/min/mg protein) [24, 25].

Statistical Analysis

The LD_{50} of tested houseflies was calculated by conducting probit analysis of concentration-mortality data using SPSS 17.0 software (IBM, Armonk, NY, USA). The resistance ratio (RR) was obtained from the LD_{50} of different insecticides dividing the respective LD_{50} of the susceptible housefly. The Pearson's correlation coefficient between the mutation frequency of each housefly population and the respective insecticide resistance was analyzed by GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). The mutation frequency was examined by Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium with Arlequin 3.5, while the fixation index (F_{ST}) was applied to analyze the population differentiation by the Analysis of Molecular Variance (AMOVA) [34]. The analysis of carboxylesterase activity was carried out using GraphPad Prism 6.0 software.

Results

Bioassays

The resistance of houseflies to DDVP in 5 populations and to temephos in 4 populations were examined. The LD_{50} (µg/housefly) of houseflies to DDVP and temephos ranged from 0.56865-1.21415 and 13.8005-41.12605, respectively. Two OPs were characterized by an extremely high resistance, which reached 122.13~363.95-fold in temephos and 142.16~303.54-fold in DDVP, respectively (Table 1).

Ace Mutation And Aa Substitution

Eight genotypes were detected at loci of 260, 342, and 407 in all field-collected populations, as shown in Fig. 2. New mutations of the ACE gene were at loci of 260, 342, and 407 in all populations. The frequency of heterozygous substitution L/V at locus 260 was 53.3%, whole houseflies were completely found at locus 342 substitution of A/V or A/A, and Y/Y substitution occurred at locus 407 in houseflies that occupied more than 80% (Table 2). KL had the highest substitution in 260L, and GY had in 342A. With respect to 407Y, CS and LPS even reached 100% substitution, as shown in Table 2. Besides, 10 combinations of ACE were observed among all detected samples, where ZY involved 7 combinations and CS involved only 2 (Fig. 3). The combination of L/V + A/V + Y topped each field-collected population, followed by L + A + Y and L/V + A + Y. According to the Pearson's correlation coefficient, combination of 342A/V substitution (r = 0.844/-0.835, $P < 0.05$) and L/V + A + Y substitution (r = 0.970, $P = 0.003$) exhibited a strong correlation with LD_{50} of DDVP (Table 3).

Table 2
Genotype and allele frequency of ACE gene at loci of 260, 342, and 402

Population	N	Locus and Genotype Frequency %								Locus and Allele Frequency %				Simultaneous Mutation Frequency of the Three Loci %
		260			342		407			260	342		407	
		L/V	L/L	V/V	A/V	A/A	F/Y	Y/Y	F/F	L	A	V	Y	
AS	31	77.4	12.9	9.7	71.0	29.0	0	90.3	9.7	51.6	64.5	35.5	90.3	46.6
CS	53	54.7	45.3	0	54.7	45.3	0	100.0	0	27.4	72.7	27.3	100.0	27.4
GY	30	77.7	23.3	0	46.7	53.3	6.7	93.3	0	72.7	76.7	23.7	96.7	70.3
HS	33	81.8	18.2	0	81.8	18.2	15.2	81.8	3	59.1	59.1	40.9	89.4	52.8
KL	30	53.3	46.7	0	56.7	43.3	3.3	96.7	0	73.8	71.7	28.3	98.4	72.6
LPS	30	93.3	6.7	0	70.0	30.0	0	100.0	0	53.4	65.0	35.0	100.0	53.4
ZY	30	66.7	13.3	20	66.7	33.3	6.7	80.0	13.3	46.7	66.7	33.3	83.4	38.9

Table 3
Pearson correlation between DDVP LD50 and mutant Ace frequency

Mutation Frequency	DDVP LD_{50}	*P*
	Pearson Correlation Coefficient	
260L	0.590	0.147
342A	0.844	0.036*
342V	-0.835	0.039*
407Y	0.577	0.154
Simultaneous mutation of the three loci	0.596	0.145
L/V + A/V + Y	-0.787	0.057
L + A + Y	0.322	0.298
L/V + A + Y	0.970	0.003*
*$P < 0.05$		

The observed and the projected heterozygosity of the 10 combinations among 7 populations were validated by analysis of variance (ANOVA) using Arelequin 3.5 software. CS and LPS were detected without any heterozygosity, as presented in Table 4. Each population exhibited a linkage disequilibrium with AS, HS, ZY at loci of 260 & 342 and 260 & 342, and with GY and HS at loci of 342 & 407 (Table 5). AMOVA results unveiled a F_{ST} of 0.02819 ($P < 0.05$), suggesting that the differences in ACE genotypes in each population were statistically significant (Table 6). The F_{ST} between ZY and CS reached the maximum (0.08246, $P < 0.05$), followed by ZY and KL (0.07023, $P < 0.05$), while the F_{ST} between other populations mainly ranged from 0 to 0.05 ($P < 0.05$), as shown in Table 7.

Table 4
Hardy-Weinberg using a Markov chain Monte Carlo test on loci of 260, 342 and 407

Locus	Population	N	Observed heterozygosity	Expected heterozygosity	*P*	SD
260	AS	31	0.77419	0.50767	0.00406*	0.00006
	CS	53	0.54717	0.40126	0.00551*	0.00007
	GY	30	0.76667	0.48079	0.00113*	0.00003
	HS	33	0.81818	0.49091	0.00007*	0.00001
	KL	30	0.53333	0.39774	0.074840	0.00027
	LPS	30	0.93333	0.50621	0.00000*	0.00000
	ZY	30	0.66667	0.50621	0.138340	0.00033
342	AS	31	0.70968	0.46536	0.00387*	0.00006
	CS	53	0.54717	0.40126	0.00566*	0.00007
	GY	30	0.46667	0.36384	0.295310	0.00045
	HS	33	0.81818	0.49091	0.00005*	0.00001
	KL	30	0.56667	0.41299	0.065330	0.00026
	LPS	30	0.70000	0.46271	0.00459*	0.00007
	ZY	30	0.66667	0.45198	0.01093*	0.00010
407	AS	31	0.00000	0.17768	0.00009*	0.00001
	CS	53	-	-	-	-
	GY	30	0.06667	0.06554	1.000000	0.00000
	HS	33	0.15152	0.19254	0.297450	0.00046
	KL	30	0.03333	0.03333	1.000000	0.00000
	LPS	30	-	-	-	-
	ZY	30	0.06667	0.28249	0.00043*	0.00002
*$P < 0.05$						

Table 5
Linkage disequilibrium using a Markov chain Monte Carlo test on loci of 260, 342, and 407

Population	260 & 342		260 & 407		342 & 407	
	χ^2	*P*	χ^2	P	χ^2	P
AS	31.9688	0	7.0857	0.008416*	3.6536	0.078119
CS	15.0356	0	-	-	-	-
GY	11.3514	0	1.2861	0.143663	6.7980	0
HS	31.6331	0	5.4211	0.032970*	0.0199	0.033663*
KL	8.6258	0	0.3698	0.263069	2.5723	0.285842
LPS	28.2692	0	-	-	-	-
ZY	22.6339	0	6.4821	0.000297	2.9400	0.135842
*$P < 0.05$						

Table 6
AMOVA of Ace genotypes in houseflies

Source of Variance	df	Sum of Squares	Variance of Components	Percentage of Variation	*F*	*P*
Among Populations	6	7.572	0.01435	2.82	F_{ST}: 0.02819	0
Among Individuals within Populations	230	68.829	-0.019531	-38.38	F_{IS}: -0.39491	1
Within Individuals	237	163.500	0.68987	135.56	F_{IT}: -0.35559	1

Table 7
FST of ACE gene among houseflies

F_{ST} *P* Value	AS	CS	GY	HS	KL	LPS	ZY
AS	-						
CS	0.04934 0.00901*	-					
GY	0.01146 0.16216	0.00449 0.19820	-				
HS	-0.00829 0.56757	0.03706 0.01802*	0.01886 0.10811	-			
KL	0.04060 0.06306	-0.01269 0.97297	0.00201 0.28829	0.02670 0.05405	-		
LPS	-0.00733 0.46847	0.03563 0.02703*	0.00635 0.26126	0.00086 0.28829	0.03164 0.06306	-	
ZY	-0.00997 0.70270	0.08246 0.01802*	0.02921 0.06306	0.00467 0.20721	0.07023 0.00901*	0.01259 0.19820	-
*P < 0.05							

Carboxylesterase Activity

The increased metabolic detoxcification is another significant resistant mechanism, in which carboxylesterase has indicated a close association with OP resistance. In a previous research, a variety of carboxylesterases were detected to be associated with OP metabolism and resistance [32], which is attributed to changes or overexpression of the carboxylesterase. For instance, a recent duplication event was noted in L. cuprina that was resulted in the duplication of the chromosomal region containing αE7, with two copies of this gene and others from the α-esterase cluster being carried on one chromosome [33]. In the current study, we attempted to assess carboxylesterase activity of houseflies in three places in Guizhou province. AS housefly population was considered to have a high carboxylesterase activity. However, measurement of enzymatic activity demonstrated that the HS housefly catalyzed more substrate (Fig. 4). Thus, it can be concluded that structural changes in carboxylesterase contributed to a reduced affinity to the substrate. Therefore, further research needs to be conducted to validate this hypothesis. Recently, Feng and Liu (2018) suggested that up-regulation of carboxylesterase genes is a major component of insecticide resistant mechanisms in insects, and concluded that multiple carboxylesterase genes are co-upregulated in resistant houseflies, providing further evidence for their involvement in the detoxification of insecticides and development of insecticide resistance.

Discussion

China is faced with a widespread resistance of houseflies to the commonly used insecticides, with even an extremely high resistance to certain chemical products (e.g., propoxur). In recent years, several researches have assessed the resistance of houseflies to insecticides in some cities across mainland China (e.g., Anshun, Guiyang, and Xingyi), which is consistent with a higher resistance to OP than that of pyrethroid[15]. Compared with previously obtained results, DDVP resistance was found more serious than pyrethroid in the present research. To our knowledge, pyrethroid is a less harmful insecticide to human and environment, while DDVP is being slathered in several places and resistance of houseflies to DDVP has gradually increased. A housefly obtained from the field-collection of Guizhou exhibited an extremely high resistance to DDVP with a LD_{50} of 0.56865 ~ 1.21415 μg/housefly, in

which the RR of GY reached 303.54, and a relatively high resistance to temephos was noted with a LD_{50} of 13.8005 ~ 41.12605 μg/housefly; besides, the RR of KL reached 363.95, and that of AS stood in the second place (212.51). The results of bioassays unveiled that the housefly resistance in different populations varied, and prompted a specific control program for houseflies according to local conditions. Although houseflies in Hangzhou, Wuhan, and Zhangjiagang cities (China) showed a resistance to DDVP and pyrethroid to some extent between 2014 and 2016, the average resistance to DDVP in Guizhou province was significantly higher than that in other provinces[26–28]. This resistant was found to be associated with duration of pest control in China[29]. For temephos, it is more frequently used as mosquito larvicide, and few reports are available, highlighting the resistance of houseflies to temephos [30]. In the present research, the resistance of houseflies to temephos seemed hardly optimistic in 4 detected places of Guizhou, especially KL for the RR that reached 363.95-fold. Therefore, continuous application of DDVP and temephos might be harmful to control houseflies in Guizhou province, while the application of insecticide could be well coordinated and a regular supervision was enforced.

The Relationship Between Ace Mutation And Resistance To Op

The ACE mutation was associated with resistance to OP, including V260L, G342A/V, F407Y, *etc.* [6, 7, 11]. ACE gene fragment of 7 housefly populations was amplified and sequenced, and 8 genotypes were identified (Table 2). All of the detected populations were mutated at loci of 206, 342, and 407, with a mutation frequency of 27.4 ~ 73.8% at locus 260, an entire mutation at locus 342 (100%), where 342A mutation led to a distribution of 59.1 ~ 76.7%. In GY, the frequency of L/V + A + Y and the DDVP (LD_{50}) reached the maximum. In KL, the frequency of L/V + A/V + Y and the temephos (LD_{50}) was the highest. Besides, the Pearson's correlation coefficient confirmed that L/V + A + Y and DDVP showed an increasing trend simultaneously (R^2 = 0.970, P = 0.003), indicating that L/V + A + Y was associated with OP resistance. Different from Qiu et al.'s research conducted in Guangdong, Shanghai, Shandong, Beijing, and Jilin regions, which are located in the south, east, and north China, V/V at locus 260 and F/F at locus 407 were detected in Guizhou province that is located in the southwest China. However, G/A at locus 342 was not detected in the current research. On the basis of AMOVA results, there was no significant difference between each pair of populations, except for CS and KL for F_{ST} of 0.05 ~ 0.15 (P < 0.05). Therefore, it can be concluded that in a housefly, ACE gene is commonly observed in Guizhou province, which could explain why the majority of housefly populations were resistant to OP.

HWE test revealed a disequilibrium that the observed heterozygosity was more than expectation in AS, CS, GY, HS, and LPS at locus 260, in AS, CS, LPS and ZY at locus 342, and in ZY at 407, while an observed disequilibrium indicated that the heterozygosity was less than expectation in AS at 407. Due to excessive heterozygosity, the disequilibrium occurred, suggesting that all the populations survived at the highest heterozygosity. When it could not reach the genetic equilibrium, the relative gene from the population was mutated for a survival benefit [31]. In order to avoid the possible fixed mutant population, we may adjust the use of various insecticides before the resistance of houseflies to OP become steady. For balance-keeping resistant population, it may exhibit a stable inheritance of resistance, demonstrating loss of the best opportunity to take containment measures. In this survey, KL showed the equilibrium at the three loci and the resistance ratio reached 363.95, while it needs to be reduced in the future control program of houseflies in KL. The LD test disclosed a linkage of 260 and 342 in all detected populations, a linkage of 260 and 407 in AS, HS and ZY, and a linkage of 342 and 407 in GY and HS. It was previously uncovered that 342V and multiple mutations could lead to increased resistance[9]. Thus, the correlation among the three loci increased the existence of L/V + A + Y, leading to a threatening resistance to OP.

Carboxylesterase Activity

The increased metabolic detoxcification is another significant resistant mechanism, in which carboxylesterase has indicated a close association with OP resistance. In a previous research, a variety of carboxylesterases were detected to be associated with OP metabolism and resistance [**32**], which is attributed to changes or overexpression of the carboxylesterase. For instance, a recent duplication event was noted in L. cuprina that was resulted in the duplication of the chromosomal region containing αE7, with two copies of this gene and others from the α-esterase cluster being carried on one chromosome [**33**]. In the current study, we attempted to assess carboxylesterase activity of houseflies in three places in Guizhou province. AS housefly population was considered to have a high carboxylesterase activity. However, measurement of enzymatic activity demonstrated that the HS housefly catalyzed more substrate (Fig. 4). Thus, it can be concluded that structural changes in carboxylesterase contributed to a reduced affinity to the substrate. Therefore, further research needs to be conducted to validate this hypothesis. Recently, Feng and Liu (2018) suggested that up-regulation of carboxylesterase genes is a major component of insecticide resistant mechanisms in insects, and concluded that multiple carboxylesterase genes are co-upregulated in resistant houseflies, providing further evidence for their involvement in the detoxification of insecticides and development of insecticide resistance.

Conclusion

In summary, the different housefly populations in Guizhou province caused a terrible resistance to the OP insecticide, and bioassays exhibited the large mutant ACE with stable inheritance of partial loci to the offspring was widespread and kept the Hardy-Weinburg equilibrium, and the carboxylesterase activity assays presented a possible strong OP hydrolysis. However, further research should concentrate on the use of insecticides to avoid the abuse of insecticides, and to regularly monitor the resistance of houseflies using various methods.

Declarations

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Ethics approval

Pre-permission (from April 2018 to December 2019) was granted for the housefly observation, adult housefly collection, and field studies in Guizhou province for this study, and the study was financially supported by the Science and Technology Planning Project of Guiyang city (Grant No. [2012103]28). All the experimental studies on houseflies were confirmed by the Institutional Animal Care and Use Committee of the Guizhou Medical University (China).

Authors' contributions

RM, XY and QG performed field investigation and samples collection. JZ, XH and ZL carried out all laboratory work and subsequent analysis of data, and wrote the manuscript document. GW, WL and JH are the primary investigator of this study and a major contributor in writing the manuscript.

Consent for publication

All the authors agreed to publish our findings in the journal of **Parasites & Vectors**.

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Competing interests

The authors declare that they have no competing interests.

Author details

1 Characteristic Laboratory of Modern Pathogen Biology, Basic Medical Science College, Guizhou Medical University, Guiyang 550025, China.

2 Department of Basic Medical Science, Qiannan Medical College for Nationalities, Duyun 558000, China.

3 Research Center of Basic Medical Science College, Guizhou Medical University, Guiyang 550025, China.

4 Zhejiang International Travel Healthcare Center, Hangzhou Customs District, Hangzhou, China.

5 Huadong Research Institute for Medicine and Biotechnics, 293 Zhongshan East Rd, Nanjing, 210002, P. R. China.

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Figures

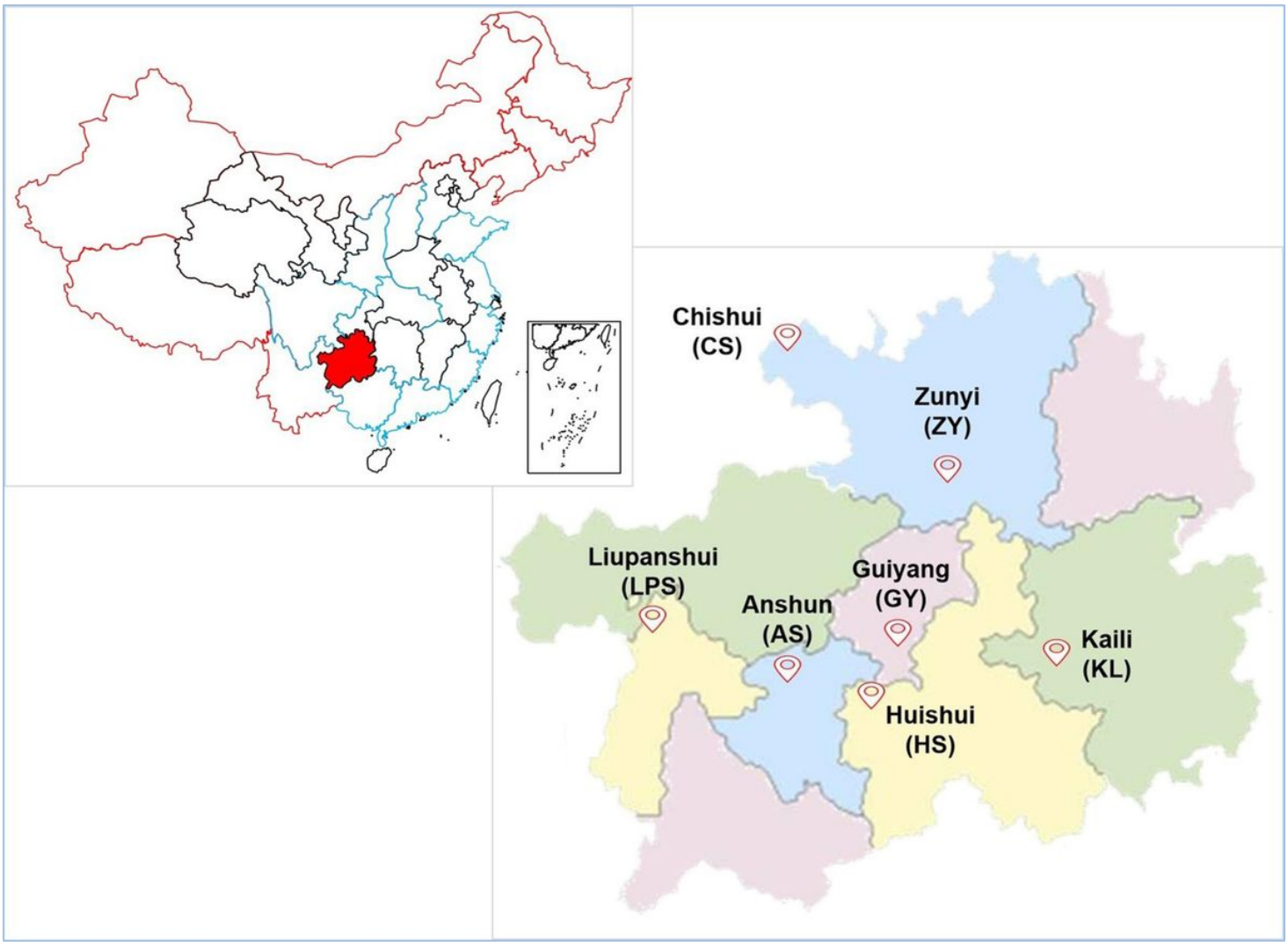

Figure 1

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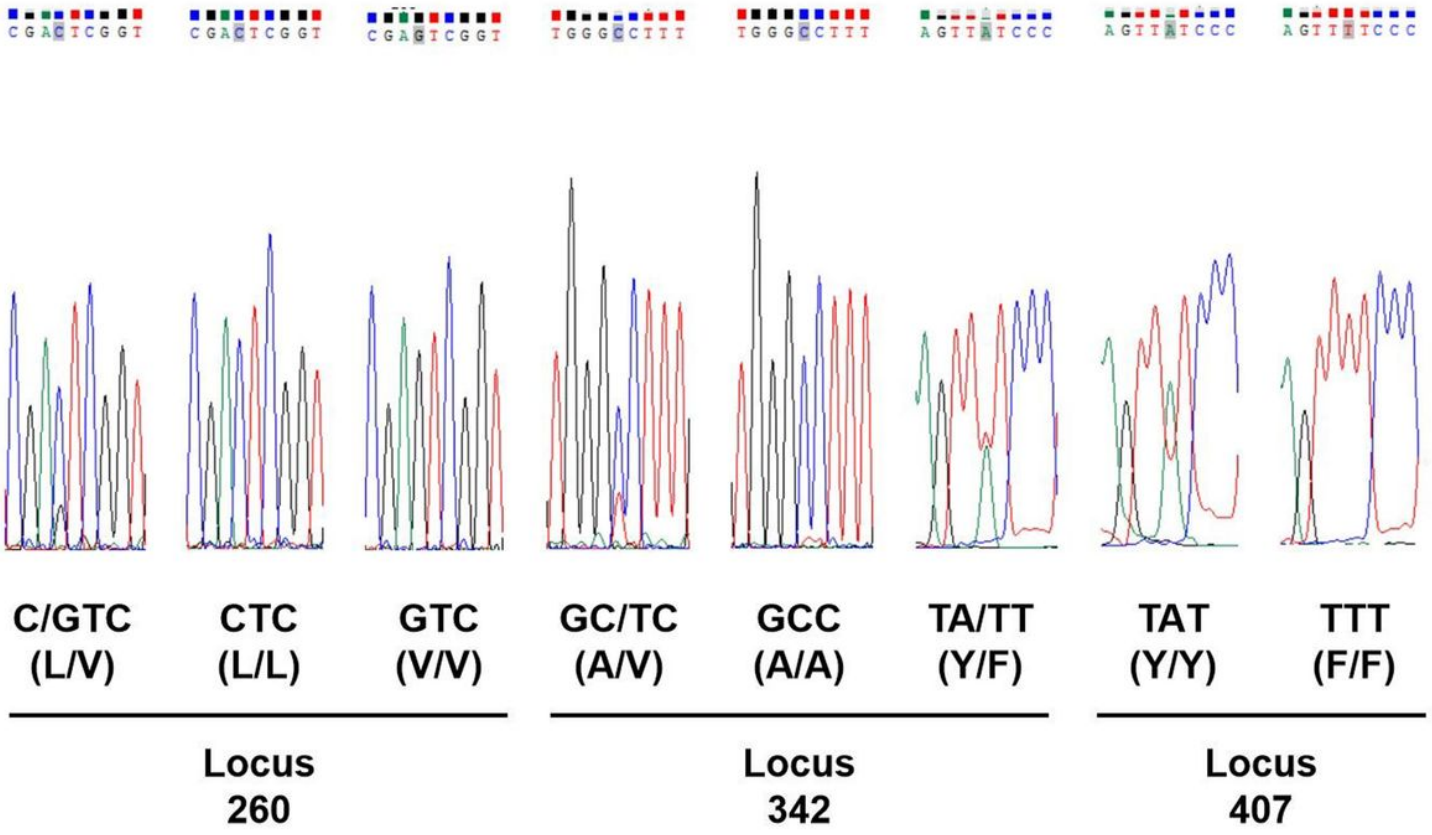

Figure 2

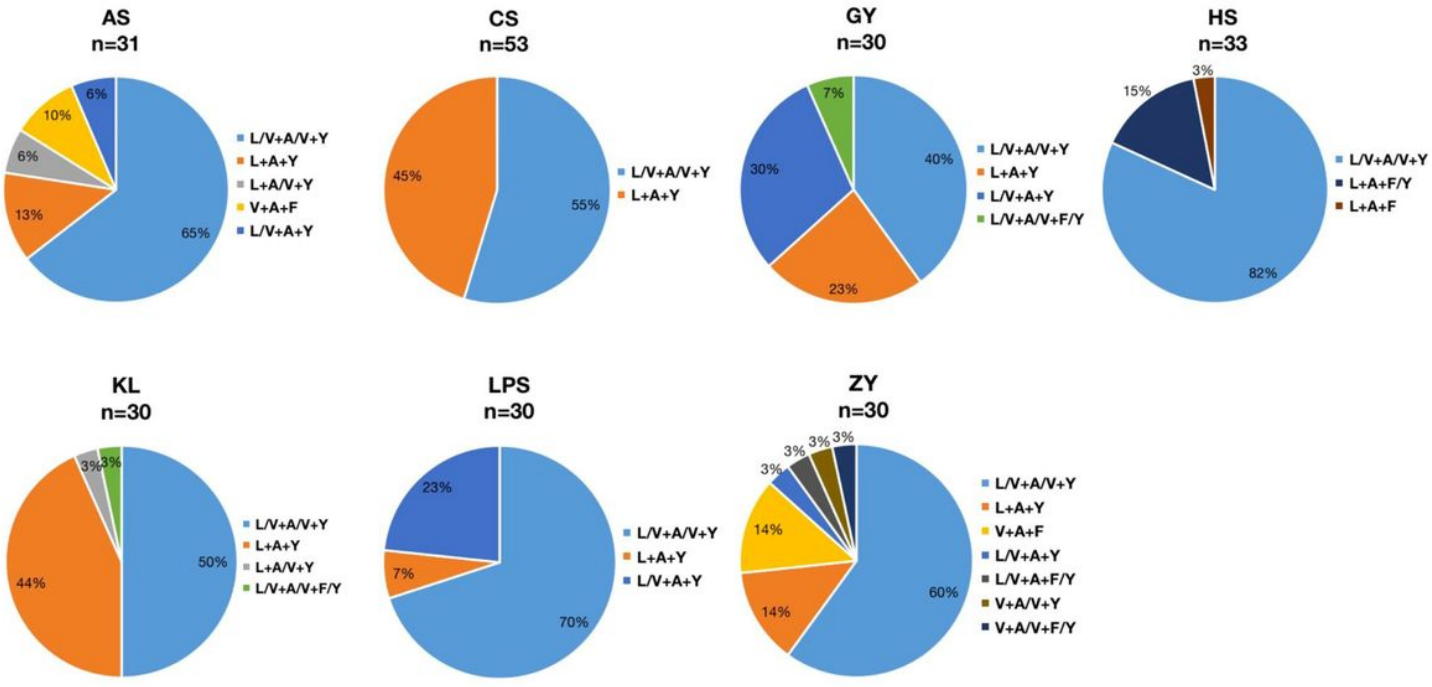

Figure 3

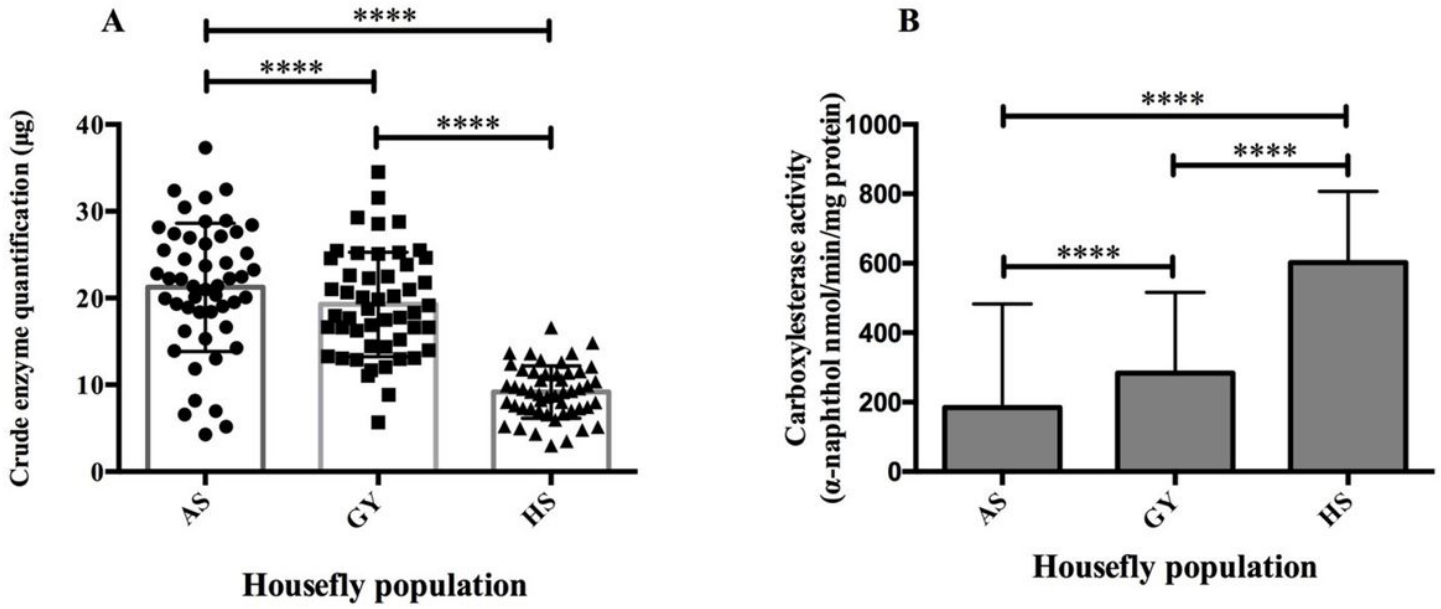

Figure 4

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