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AMPLIFIED ESTERASES B1 AND A2-B2 IN FIELD POPULATIONS OF *CULEX PIPIENS* FROM CHINA

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ABSTRACT. The main organophosphate (OP) resistance mechanism in the *Culex pipiens* complex is increased activity of esterases A and B. Fourth-stage larvae from 2 field populations of *C. pipiens* from Gaomi and Kunming, China, were compared for tolerance to parathion, dichlorovos (OP), and bassa (carbamate) insecticides. Both populations were resistant to OPs but not to bassa. Starch gel electrophoresis indicated that elevated esterase activity was correlated with OP resistance. High frequencies of amplified esterase genes B1 and A2-B2 (0.85 and 0.50) were discovered in Gaomi and Kunming, respectively. However, only low levels of gene amplification were detected.

KEY WORDS Mosquitoes, insecticide resistance, esterases, organophosphates

INTRODUCTION

The wide use of organic insecticides to control pests has resulted in the evolution of resistance in many insect pest species, including mosquitoes, the primary vectors of many diseases (Georghiou and Mellom 1983). Organophosphate (OP) resistance in *Culex pipiens* is achieved by the overproduction of nonspecific carboxylesterases that detoxify the insecticide by sequestration (Cuany et al. 1993). Two closely linked loci (Est-3 and Est-2) code for esterases A and B, respectively. When OP resistance develops, it often is associated with changes in both the activity of these detoxification enzymes (Curtis and Pasteur 1981, Maruyama et al. 1985) and the frequencies of advantageous alleles (Yebakima et al. 1995). In *Cx. pipiens,* 10 high-activity esterases have been observed in natural populations (Georghiou et al. 1980, Pasteur et al. 1981, Wirth et al. 1990, Poirier et al. 1992, Rivet et al. 1993, Xu et al. 1994). The increased activity is due to the overproduction of esterases, resulting from gene amplification (Mouches et al. 1986, 1990; Raymond et al. 1991; Poirie et al. 1992) or regulation of A1 gene expression (Rooker et al. 1996).

Overproduced esterase B1 has been found in Europe, North America, and China (Georghiou et al. 1980, Qiao and Raymond 1995). All of the amplified esterase B1 genes have the same DNA haplotype, indicating that the B1 amplification has a unique origin (Qiao and Raymond 1995). Neutral polymorphisms flanking the esterase B structural gene in susceptible mosquitoes, and the presence of the same amplified haplotype in populations from distant geographic areas (Raymond et al. 1991, Qiao and Raymond 1995) have been taken as evidence that all B1/B2 alleles are identical by descent. A unique amplification event has been proposed to have occurred before extensive migration of esterase B haplotypes. Electrophoretically identical esterases from different countries correspond to amplification of identical haplotypes (Qiao and Raymond 1995). This is true with the exception of the esterases A4-B4 (VIM) and A5-B5 (Cyprus), which have the same electrophoretic mobility but do not share homologous restriction sites in regions flanking the esterase B gene (Poirie et al. 1992). A 2nd mechanism involves overproduction of A2-B2 esterases and is expanding worldwide (Raymond et al. 1991, Yebakima et al. 1995). Restriction maps of A2-B2 alleles in 12 strains sampled from 4 continents were extremely similar (Callaghan et al. 1998), supporting the hypothesis that the A2-B2 allele amplification has spread worldwide by migration (Raymond et al. 1991).

The use of OP insecticides began in 1953 in China and has resulted in OP resistance in natural populations of *Cx. pipiens.* Amplified B1 was observed in southern China in 1992 (Xu et al. 1994). The main resistance mechanism is also increased esterase A and B activity (Xu et al. 1994, Qiao and Raymond 1995). The present study was undertaken to further investigate the distribution and amplification of amplified A and B genes in the *Cx. pipiens* complex from China.

MATERIALS AND METHODS

Two populations, named KM and GM, of the *Cx. pipiens* complex were collected as larvae and pupae, laboratory reared to adults, and stored at −80°C awaiting analysis (Fig. 1). Population KM was collected from a pool in the suburb of Kunming, Yunnan Province, in south-central China in May 1998. Population GM was collected from a ditch in Gaomi, Shandong Province, in northeast coastal China in August 1998.

The TEM-R strain, a *Cx. p. quinquefasciatus* Say strain (Georghiou et al. 1980), is homozygous for overproduced esterase B1 that has resulted in a 250-fold amplification of the B1 gene (Mouches et al. 1990). The SELAX strain (Wirth et al. 1990) is

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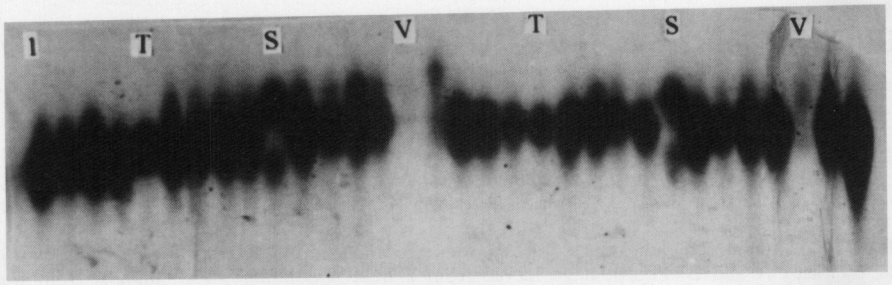

Fig. 1. Study localities for *Culex pipiens* complex in China.

homozygous for A2-B2 amplification. The VIM strain (Poirier et al. 1992) is homozygous for an A4-B4 amplification. Beijing-s is a susceptible *Cx. p. pallens* reference strain that originated from a suburb of Beijing 30 years ago and has been reared in the laboratory without exposure to any known insecticides.

Technical grades of parathion, dichlorovos, and bassa (Insecticide Factory of Qingdao, China) were dissolved in alcohol and used in larval bioassays. Tap water (99 ml) was placed in plastic cups and 20 4th-stage larvae were added. Each cup received 1 ml of insecticide at an appropriate concentration. Larval survival was recorded after 24 h. Each test was repeated 3 times and results were analyzed with PROBIT software (Raymond et al. 1993b).

Presence of highly active esterases was tested in single adult mosquitoes using starch gel electrophoresis with the TME buffer system (Pasteur et al. 1988). Each gel contained mosquitoes from TEM-R, SELAX, and VIM. At least 81 individuals were analyzed from each breeding site. The DNA was extracted from single adult mosquitoes (Qiao and Raymond 1995) and digested with *Eco*RI in a total volume of 20 µl. Digested DNA was loaded onto 0.8% agarose gels and the fragments were separated by electrophoresis and transferred onto nylon membranes by Southern blotting (Sambrook et al. 1989). The filters were prehybridized and hybridized at 65°C with ^{32}P-labeled B1 (Mouches et al. 1990) and washed at high stringency at 65°C. After autoradiography, filters were stripped of radioactive signal and reprobed with ^{32}P-labeled A2 polymerase chain reaction product (Guillemaud et al. 1996). The TEM-R and SELAX strains were used as positive controls.

RESULTS

Resistance of larvae

Every population was bioassayed with 2 OP insecticides and 1 carbamate. Analysis of larval bioassay results (Table 1) showed that the OP resistance levels of GM and KM were higher than in the susceptible strain, Beijing-s. The OP resistance was about 3.7 times higher in GM than in KM. The GM population showed almost the same resistance level to parathion and dichlorovos. No significant difference was found in bassa sensitivity between the field populations and susceptible strains. This is consistent with the fact that carbamates are seldom used in China.

Esterase activity

Starch gel electrophoresis revealed that the activity and electrophoretic mobility of esterases were different between GM and KM. Esterase A2-B2 was absent from GM. Esterase B1 was present in 80 of 81 individuals in GM. Only 1 GM mosquito had an esterase B with low activity and higher mobility.

Table 1. Parameters of the probit lines of each population of *Culex pipiens* complex, with parathion, dichlorovos, and bassa.[1]

Insecticide	Population	LC_{50} (µg/liter)	LC_{95} (µg/liter)	95% confidence interval	Slope	SE	df	χ^2	RR
Parathion	Beijing-s	0.00034	0.00234	0.00172–0.00358	1.96	0.19	4	7.91	1.0
	KM	0.00265	0.00917	0.00764–0.01204	3.05	0.37	4	0.40	7.8
	GM	0.01025	0.1685	0.01510–0.02024	7.61	1.07	3	1.08	30.1
Dichlorovos	Beijing-s	0.02227	0.04181	0.03702–0.04932	6.01	0.56	2	0.57	1.0
	KM	0.19263	0.86481	0.58207–2.04939	2.56	0.56	3	0.18	8.6
	GM	0.68502	1.21024	1.03135–1.81837	6.66	1.53	2	3.29	30.8
Bassa	Beijing-s	0.15734	0.42839	0.12812–1.61638	3.78	1.11	3	12.96	1.0
	KM	0.16445	0.45554	0.34307–0.79421	3.72	0.64	5	1.36	1.05
	GM	0.28469	0.52108	0.45943–0.63971	6.27	0.83	4	1.99	1.8

[1] LC_{50}, median lethal concentration; LC_{95}, 95% lethal concentration; RR, resistance ratio.

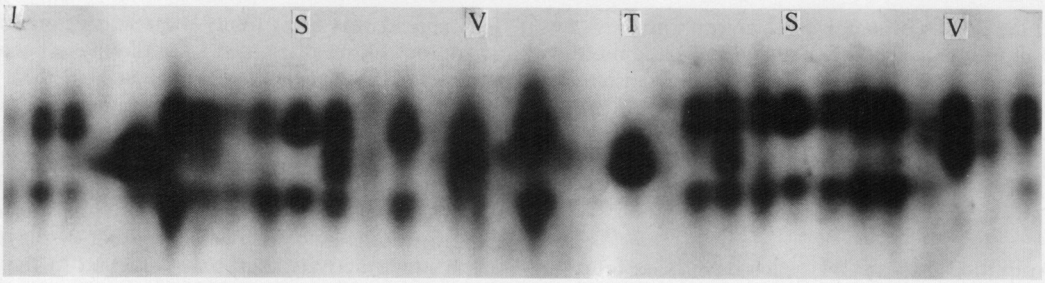

Fig. 2. Starch gel electrophoresis of esterases from the KM field sample. T is strain TEM-R (diluted 4-fold) showing esterase B1, S is strain SELAX (diluted 4-fold) showing esterase A2-B2, V is strain VIM (not diluted) showing esterase A4-B4.

Mosquitoes from KM (127 individuals) were analyzed by starch gel electrophoresis (Fig. 2). The electrophoretic esterase patterns in KM were more complex than in GM. Sixty-six KM mosquitoes (57%) displayed increased esterase A2-B2 with a variable activity. Twenty-eight mosquitoes (22%) did not possess active enzymes at any esterase locus (Fig. 2, lanes 14, 19, 21), indicating that either null alleles existed or its esterase activity was so low that it could not be detected by starch gel electrophoresis. Twenty-eight mosquitoes showed low esterase A and B activity, and their mobilities were different from that of A2-B2 (Fig. 2, lanes 12, 29, 31). In 5 mosquitoes (4%), electromorphs had the same mobility as esterase B1 (Fig. 2, lanes 11, 23), as well as A2 and B2, but none of these mosquitoes displayed B1 activity alone. The A4-B4 (and A5-B5) esterases were absent from KM and GM.

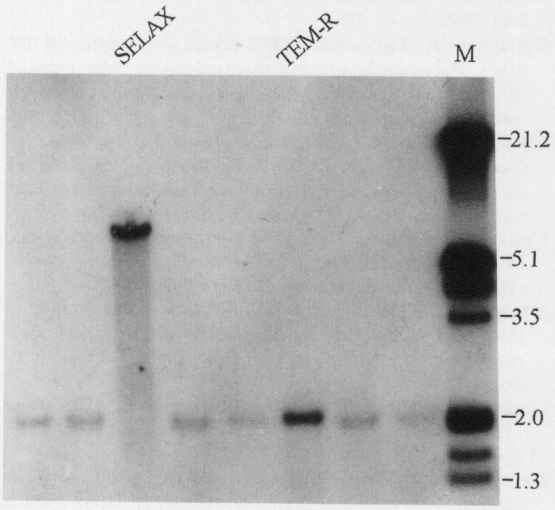

Fig. 3. Restriction fragment length polymorphism analysis on DNA from single mosquitoes from the GM field sample probed with B1 cDNA. M = size marker (kilobases). The DNA from TEM-R and SELAX strains was loaded as controls.

DNA polymorphism on single individuals

Esterase A and B genotypes were scored in mosquitoes from Gaomi (n = 33) and Kunming (n = 20). Twenty-eight Gaomi mosquitoes displayed the same B1 genotype at 2.1 kilobases but the intensity of this band varied among individuals. No signal was detected with the A2 probe, indicating the absence of amplified esterase A in this population. These observations are consistent with the results of starch gel electrophoresis, although the B1 allele frequency detected at the DNA level was somewhat lower than that measured at the protein level.

In contrast, A2 and B2 alleles were detected in the KM population. Because of the known association of A2-B2 in the *Cx. pipiens* complex, only restriction fragment length polymorphisms of esterase A genes are shown in KM (Fig. 4). The frequency of A2 in KM was 50%, which was not as high as that of B1 in GM, and the intensity of A2 in KM was not as strong as that of A2 in SELAX. No mosquitoes had the B1 allele. This did not agree

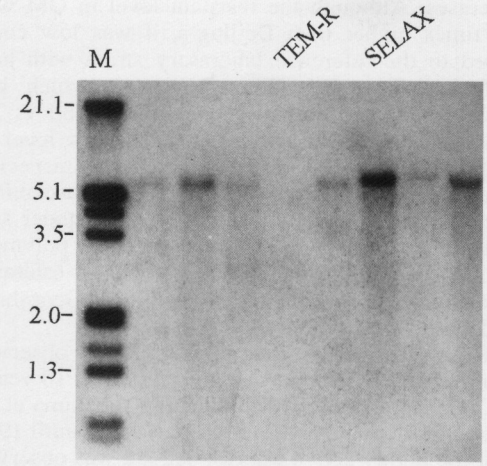

Fig. 4. Restriction fragment length polymorphism on single mosquitoes from the KM field sample probed with A2 cDNA. M = size marker (kilobases). The DNA from TEM-R and SELAX strains was loaded as control.

with the results of starch gel electrophoresis. A few KM individuals possibly showed the same mobility of esterase as B1 in TEM-R or that the sample size was too small to include those individuals with a B1 allele. No signal was detected in 10 individuals with the A2 cDNA probe.

DISCUSSION

Amplified esterase B1 genes have been found in *Cx. pipiens* from many areas around the world, such as North America, the Caribbean region, South America, and some provinces in China (Georghiou et al. 1980, Bisset et al. 1990, Raymond et al. 1991, Xu et al. 1994). Organophosphates have been used for more than 40 years in China. Gaomi is only 700 km from Beijing and is the main cotton planting area in northern China where OPs were used against agricultural pests until 1980 when, because of resistance, they were replaced by pyrethroids mixed with OPs (Han et al. 1996). The frequency of amplified B1 in GM is very high (85%). A high frequency of B1, albeit with low amplification levels (Fig. 3), was also detected in GM. The 1st observation of B1 esterase from *Cx. pipiens* in Beijing was demonstrated in 1995 (Qiao and Raymond 1995). Subsequently, B1 seems to have spread to the southeast. *Culex pipiens* with B1 esterase was detected in Yuncheng in Shandong province, where OP treatments against *Cx. pipiens* are intensive. It seems likely that one or a few resistant mosquitoes containing the B1 allele migrated into Gaomi and under OP selection, the frequency of B1 increased gradually, and increased further under pyrethroid + OP mixtures. *Culex p. quinquefasciatus* highly resistant to lambda-cyhalthrin and deltamethrin (pyrethroids) also had high cross-resistance to malathion (Bisset et al. 1997, 1998). Both types of resistance probably resulted from amplified esterases. Although the resistant level in GM was 30 times higher than Beijing-s, it was low compared to the reference laboratory strain with amplified esterase B1 (TEM-R strain), which has 1,230-fold temephos resistance (Raymond et al. 1993a). This can be explained by the low level of amplification of B1 in GM (Fig. 3). Nonspecific esterases are highly polymorphic in susceptible populations (Raymond et al. 1996), but under significant insecticide selection pressure, polymorphism can be largely reduced when an esterase-based resistance mechanism is involved (Georghiou and Pasteur 1978).

Highly active esterase A2-B2 was 1st observed in mosquitoes from Tanzania. In less than 10 years, A2-B2 had spread to 4 continents (Yebakima et al. 1995, Guillemand et al. 1996). It was not until 1992 that the highly active esterase A2-B2 was observed in southern China (Xu et al. 1994). It is very likely that the resistant mosquitoes in KM migrated from the nearby Chengdu Sichuan Province and the frequency of A2-B2 increased under OP selection. This spread was most likely the result of an active migration. Flight rates of 0.1 km/day have been reported for this species (Schreiber et al. 1988). Esterase A2-B2 has a broad geographic distribution and strains with this genotypes have a high relative fitness (Qiao et al. 1998). Esterase A2-B2 confers a lower resistance than is provided by B1 (Raymond et al. 1987). The low level of resistance in KM was associated with esterases that displayed a much lower activity than that observed in the reference strain, indicating that overproduced esterases and gene amplification were still at a low level.

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