

Multiple resistance mechanisms associated with low pirimifos-methyl resistance in *Culex pipiens pipiens* in three populations of Tunisia

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Received 18 January 2018; received in revised form 14 February 2018; accepted 8 August 2018

Abstract. The aim of this study was to evaluate the resistance status of *Culex pipiens pipiens* to pirimifos-methyl insecticide. Three field populations of mosquitoes were collected from Tunisia and analyzed in laboratory. The samples studied showed low level of resistance not exceeding 5-folds. The low resistance recorded is particularly interesting, because it leaves a range of tools useable by vector control services. Both metabolic and target-site resistance mechanisms were identified. Different esterases of high activity including A2-B2, A4-B4 (and/or A5-B5) and B12 were observed in studied field samples using starch electrophoresis although opposite results were found using synergists tests on samples # 1 and 3. The polymorphism of AChE1 (Acetylcholinesterase) was analyzed and three phenotypes were detected: susceptible (ACHE1S, phenotype [SS]), resistant (ACHE1R, phenotype [RR]), and heterozygous (phenotype [RS]) of ACHE1. The resistance of *Culex pipiens pipiens* to pirimifos-methyl remains low although the occurrences of multiple resistance mechanisms are able to confer high resistance levels to organophosphate insecticides. Therefore, continuous monitoring of resistance is fundamental for rational use of insecticides and mosquito control programs.

INTRODUCTION

Mosquitoes are considered as one of the main groups of arthropods causing nuisance and public health problems. In Tunisia, *Culex pipiens pipiens* is an important member of *Culex pipiens* complex and acts as an important vector for West Nile virus that recently affected the country (Tabbabi & Bekhti, 2017). This species is known as the main vector of many arboviral diseases and even *Plasmodium relictum* that causes bird malaria worldwide (Horsfall, 1955; Service, 2003; Mullen, 2009). Their ecologic plasticity allows them to colonize all continents of the world (Savage *et al.*, 2007; Mullen, 2009; Strickman & Fonseca, 2012) and particularly different parts of Tunisia

where chemical insecticides including organophosphates have been used by both public health and agricultural departments (Ben Cheikh *et al.*, 1998; 2008; Daaboub *et al.*, 2008). Previous studies of Tunisian *Culex pipiens* reported different level of resistance because of the continuous exposure to several insecticides causing selection pressure and apparition of resistance mainly in waste water habitats (Tabbabi *et al.*, 2017; Daaboub *et al.*, 2017).

With few rare reports on the susceptibility of *Culex pipiens*, the majorities of studies have shown their high level of resistance to different chemical insecticides (Davidson, 1964; Mukhopadhyay *et al.*, 1993; Ben Cheikh *et al.*, 1998; Bisset *et al.*, 1999; Martinez-Torres

et al., 1999; Ben Cheikh *et al.*, 2008; Tantely *et al.*, 2010; Toma *et al.*, 2011; Jones *et al.*, 2012; Pocquet *et al.*, 2013; Daaboub *et al.*, 2017). The current study aimed to determine the tolerance status of pirimiphos-methyl in Tunisian *Culex pipiens pipiens*. We also investigated the effect of synergists, the S,S,S-tributyl-phosphorotrithioate (DEF) and the piperonyl butoxide (Pb), on the resistance to the studied insecticide. The cross-resistance between pirimiphos-methyl and propoxur, and the polymorphism of over-produced esterases and AChE 1 were also investigated.

MATERIALS AND METHODS

Mosquitoes

Three field-populations of *Culex pipiens pipiens* were collected along three districts from northern and southern Tunisia between July 2003 and October 2005 to evaluate their resistance status to pirimiphos-methyl insecticide. The population densities were the main criteria for choosing sampling sites. The location of study areas are given in Fig. 1. It should be noted that general characteristics of study areas including insecticides usage were collected according to the ministry of health and during individual interviews with the residents in the collection sites. Data showed the irregularly use of organophosphates and pyrethroids insecticides by both public health and agricultural departments. Bioassays results of studied field-populations were compared to those of S-Lab which is a susceptible strain without any known resistance genes isolated from a Californian population (Georghiou *et al.*, 1966). It has been maintained for 50 years without exposure to insecticides under laboratory conditions and has been used as reference strain in comparison with our field populations. We note that larval rearing was done for part of each sample to ensure to finalize all necessary tests. Collected larvae were transported to the laboratory and directly transferred to distilled water with rabbit croquette which served as food. Adults were fed with sugar and pigeon blood.

Larval Bioassays

The organophosphate pirimiphos-methyl was used to evaluate the resistance/susceptibility status of studied samples. Late 3rd and early 4th instars were used in bioassays. Groups of 20 larvae were assayed in 99 ml of distilled water and 1 ml of insecticide solution at the required concentration. We used five replicates of 20 larvae per concentration and 5-9 concentrations providing between 0 and 100% mortalities for each bioassay as described by Raymond *et al.* (1986). The effect on pirimiphos-methyl resistance of 2 synergists, the DEF (98% [AI], Chem Service, England), and the Pb (94% [AI], Laboratory Dr Ehrenstorfer, Germany), was evaluate to estimate the role of detoxi-



Figure 1. Geographic origin of Tunisian populations.

fication enzymes. Propoxur bioassays included one dose (1mg/liter) and five replicates. This concentration kills all susceptible mosquitoes.

Biochemical Assays

Overproduced Esterases: Different esterases were revealed using starch electrophoresis according to the methods of Pasteur *et al.* (1988). Electrophoretic patterns of field mosquitoes were compared with reference strains with known esterases: SA2 for A2-B2 esterases, and SA5 for A5-B5 esterases (Berticat *et al.*, 2002).

Acetylcholinesterase (AChE): AChE1 polymorphism was analyzed according to the method described by Bourguet *et al.* (1996) comparing AChE1 activity of homogenates of adult heads in the absence or presence of propoxur.

Data Analysis

Mortality data were analyzed using log dose-probit mortality software developed by Raymond *et al.* (1993) based on Finney (1971). This program tests the linearity of a dose-mortality response, computes different lethal doses (LCs) and their confidence interval (CI) at the chosen probability (here P=95%). Susceptible strain was used to calculate the Resistance ratio at LC₅₀ which is LC₅₀ of field population/LC₅₀ of sensitive strain and synergism ratio at LC₅₀ which is LC₅₀ in absence of synergist/LC₅₀ in presence of synergist.

RESULTS

Pirimiphos-methyl resistance characteristics of Tunisian *Culex pipiens pipiens* are shown in Table 1. It is important to note that the effectiveness of the studied insecticide appeared completely after 24 hours of exposure. The resistance ratio was low and varied from 2.2 in sample # 1 to 5.15 in sample # 3. The last population can be considered slightly as resistant sample according to the resistance scale of Sinègre *et al.* (1976). The resistance ratio value of sample # 2 was 3.02. Enzyme inhibition

assays were performed using two synergists (DEF and Pb) acting as inhibitor and enzyme levels were compared with and without inhibitors. It should be note that synergists were used for the sensitive strain to do comparisons. The synergism study showed that resistance to pirimiphos-methyl was not affected (RSR<1, Table 1) by both synergists, indicating that detoxification enzymes were not involved in the recorded resistance of studied samples.

Biochemical tests revealed the presence of both target site and metabolic detoxification as main mechanisms of resistance. Different esterases including A2-B2, A4-B4 and or A5-B5 and B12 were detected in studied samples using starch electrophoresis (Table 2) although opposite results were found using synergists tests on samples # 1 and 3 (Table 1). This should be due to the insensitivities of some detoxification enzymes toward the used synergists. The frequencies of detected esterases were positively correlated to the level of resistance to pirimiphos-methyl. Indeed, the sample # 1 and 3 having the lowest and the highest resistance ratio showed 0.09 and 0.34 of total esterases, respectively. The sample # 2 having the medium level of resistance showed a medium frequency of esterases (0.20). The percentage of 34% of esterase in the most resistant population (sample # 3) could suggest a selection for detoxification enzymes as a biochemical mechanism for pirimiphos-methyl resistance.

The susceptibility to 1mg/l of propoxur insecticide was used to detect altered acetylcholinesterase 1 (AChE1). The cross-resistance between the organophosphate pirimiphos-methyl and the carbamate propoxur showed the involvement of the target site (AChE1) in the recorded resistance (Table 2). Three phenotypes were identified: susceptible (ACHE1S, phenotype [SS]), resistant (ACHE1R, phenotype [RR]), and heterozygous (phenotype [RS]) of ACHE1 (Table 2). The two last phenotypes expressed a resistant character with the dominance of the heterozygote's ones [RS] in all studied samples # 1, 2 and 3 (0.64, 0.52,

Table 1. Pirimifos-methyl resistance characteristics of Tunisian *Culex pipiens pipiens*

Population	Pirimifos-methyl			Pirimifos-methyl +DEF				Pirimifos-methyl +Pb					
	LC ₅₀ in µg/l (a)	Slope ± SE	RR ₅₀ (a)	LC ₅₀ in µg/l (a)	Slope ± SE	RR ₅₀ (a)	SR ₅₀ (a)	RSR	LC ₅₀ in µg/l (a)	Slope ± SE	RR ₅₀ (a)	SR ₅₀ (a)	RSR
Slab	2.9 (2.5-3.4)	2.34 ± 0.18	-	0.30 (0.16-0.56)	1.70 ± 0.42	-	9.79 (6.16-15.5)	-	0.40 (0.31-0.55)	1.47 ± 0.18	-	7.24 (5.73-9.14)	-
1-Ousja	6.4 (4-10.3)	1.28 ± 0.21	2.2 (1.63-2.99)	10 (7.3-13)	2.06 ± 0.28	34.1 (20.1-57.7)	0.63 (0.42-0.94)	0.06	14 (9.9-20)	1.99 ± 0.36	35.4 (25.0-50.2)	0.45 (0.29-0.69)	0.06
2-Mjedz El Beb	8.8 (7.7-10)	2.23 ± 0.21	3.02 (2.47-3.70)	-	-	-	-	-	-	-	-	-	-
3-Ibeniana	15 (12.2-18.9)	1.99 ± 0.22	5.15 (4.05-6.56)	4.6 (3.7-5.6)	2.17 ± 0.26	15.5 (9.73-24.8)	3.25 (2.50-4.22)	0.33	-	-	-	-	-

(a), 95% CI;

RR₅₀, resistance ratio at LC₅₀ (RR₅₀=LC₅₀ of the population considered/LC₅₀ of Slab); SR₅₀, synergism ratio (LC₅₀ observed in absence of synergist/LC₅₀ observed in presence of synergist). RR and SR considered significant (P<0.05) if their 95%CI did not include the value 1.

RSR, relative synergism ratio (RR for insecticide alone / RR for insecticide plus synergist).

Note: the empty cells was due to the loss of some populations.

Table 2. Frequencies of insensitive acetylcholinesterase and over-produced esterases phenotypes in Tunisian populations of *Culex pipiens pipiens*

Population	N	Ester Locus				Est-1 Locus			ace-1 Locus		Propoxur mortality at 1 mg/liter (sample size)			
		[1]	[2]	[4]	[12]	[24]	[212]	[412]	[0]	[C1]		[SS]	[RS]	[RR]
1-Ousja	42	-	0.02	0.07	-	-	-	-	0.91	-	0.36	0.64	-	0.26 (100)
2-Mjedz El Beb	40	-	0.07	0.13	-	-	-	-	0.80	-	0.43	0.52	0.05	0.38 (99)
3-Ibeniana	36	-	0.03	0.25	0.03	-	-	0.03	0.66	-	0.45	0.33	0.22	0.40 (103)

Phenotype [i] corresponds to genotypes *Ester^r / Ester^r / Ester^r / Ester^r*, and phenotype [ij] correspond to genotype *Ester^r / Ester^r*.

N represents the total number of mosquitoes analyzed for each sampling site.

0.33, respectively). According to our finding, it seems that AChE 1 conferred only low resistance to pirimiphos methyl. Indeed, the sample # 1 and 3 having the lowest and the highest resistance ratio showed 0.64 and 0.55 of total AChE1, respectively.

DISCUSSION

Low levels of resistance to pirimiphos-methyl insecticide were recorded in Tunisian *Culex pipiens pipiens* and these results could be explained by the low insecticide selection pressure in the country and also by gene flow among districts. Previous studies (Schaefer & Wilder, 1970) related the high rate of resistance to an operational failure of public health service. We can conclude that the present status of resistance to pirimiphos-methyl constitutes a good opportunity to vector control services to better rationalize the use of available insecticides. Therefore, the use of this insecticide in alternation may be of great importance for efficiency results.

It is important to note that the used insecticide (pirimiphos-methyl) has different mode of action including increasing rate of detoxification and target site changes which make it effective (Chang *et al.*, 1991). Its use by world departments of public health including Tunisia is due to the limited number of available insecticides used in resistance management strategies (Chavasse & Yap, 1997). This insecticide is recommended against both larval and adult mosquitoes including *Culex pipiens* (Gallo & Lawrejk, 1991; Khazraji *et al.*, 1984). Previous studies showed its efficiency against *Culex pipiens* resistant to many chemical insecticides including pyrethroid and carbamates without any detection of cross-resistance (Bisset *et al.*, 1991) hence its necessity as alternative insecticide.

Our findings were consistent with those found in Tunisia by Tabbabi *et al.* (2017) and Daaboub *et al.* (2017) who reported different level of resistance to organophosphate

insecticides including pirimiphos-methyl. The present results may be explained by the limited use of chemical compounds by both public health and agricultural departments in the studied areas. However, Ben Cheikh *et al.* (1998) reported very high rates of organophosphate resistance in this species and explained their finding by the massive and continuous mosquitoes control using chemical insecticides, which leads to the apparition of cross-resistance to other organophosphate insecticides. It is known that intensive, continuous and uncontrolled use of insecticides against mosquitoes can probably create multiple mechanisms which make the employment of insecticides inefficient. It is the case of our finding where different mechanisms were involved in the recorded resistance.

The frequencies of detected esterases were positively correlated to the level of resistance to pirimiphos-methyl. Indeed, low frequencies of esterases activity was associated with low level of resistance to the used insecticide not exceeding 5-folds and agree with previous studies that reported a positive correlation between a low elevation of esterase activity and a low levels of resistance to a variety of organophosphate insecticides (Villani *et al.*, 1983; Breeden *et al.*, 1984; Brown, 1986; Bisset *et al.*, 1990).

The dominance of the heterozygote's phenotypes [RS] in all studied samples should be due to the fitness cost caused by natural selection and always associated to the presence of single resistant allele. In this context, we note that fitness cost related to the insensitive AChE 1 has been confirmed in field populations and laboratory strains of *Culex pipiens pipiens* (Raymond *et al.*, 1985; Chevillon *et al.*, 1995). According to our finding, it seems that AChE 1 conferred only low resistance to pirimiphos methyl. In this context, modified AChEs that confer less resistance to organophosphate insecticide have been reported in *Culex* mosquitoes (Raymond *et al.*, 1986). In contrast, the target site (AChE 1) has been considered as the main mechanisms of resistance to organophosphate insecticide

in many insects including mosquitoes (Ben Cheikh *et al.*, 1998, 2008; Berticat *et al.*, 2002).

CONCLUSIONS

In conclusion, the resistance of *Culex pipiens pipiens* to pirimiphos-methyl remains low despite the occurrence of multiple resistance mechanisms. Therefore, the low resistance recorded to organophosphates insecticides is particularly interesting, because it leaves a range of tools useable by vector control services. The massive and intensive use of chemical insecticides will probably increase the frequency of both detoxification enzymes and target site changes. Next step of this research will focus on the molecular investigation.

Acknowledgements. This work was kindly supported by the Ministry of Higher Education and Scientific Research of Tunisia by funds allocated to the Research Unit (G n tique 02/UR/08-03) and by DHMPE of the Minister of Public Health of Tunisia. We are very grateful to S. Ouanes, for technical assistance, A. Ben Haj Ayed and I. Mkada for helping in bioassays, S. Saïdi, Tunisian hygienist technicians for helping in mosquito collection, and M. Nedhif and M. Rebhi for their kind interest and help.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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