

## Characterization of resistance to pirimiphos-methyl (an organophosphate insecticide) in *Culex pipiens pipiens* (Diptera: Culicidae) from Northern and Southern Tunisia

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**Abstract.** Despite the public health importance of *Culex pipiens pipiens*, their resistance to pirimiphos-methyl insecticides has not been explored enough. Late third and early fourth larvae of *Culex pipiens pipiens* were collected from three localities between 2003 and 2005 in Northern and Southern Tunisia. All bioassays were carried out using pirimiphos-methyl and propoxur insecticides. Populations of *Culex pipiens pipiens* were susceptible, moderate and resistant to pirimiphos-methyl insecticide. Resistance to this compound ranged from 2.62 in sample # 2 to 19.9 in sample # 1. The moderate resistance (5.25) was recorded in sample # 3. Synergist's tests showed that the resistance to pirimiphos-methyl was not affected by detoxification enzymes. However, biochemical assays showed the involvement of both metabolic (esterases) and target site (insensitive acetylcholinesterase) resistance mechanisms. The highest frequencies of the resistant phenotypes ([RS] and [RR]) (>0.74) were detected in the most resistant samples (#1). Four esterases enzymes including C1 encoded by the *Est-1* locus and three esterases encoded by the *Ester* super locus: A2-B2, A4-B4 (or A5-B5, which has the same electrophoretic mobility) and B12 were detected. The highest (0.61) and the lowest (0.22) frequencies of these esterases were recorded in samples # 1 (Sidi Hcine) and # 2 (El Fahs) which recorded the highest and the lowest level of resistance, respectively. Monitoring of insecticide resistance should be evaluated regularly for management of vector control.

### INTRODUCTION

*Culex pipiens* complex is considered as the most common mosquito in urban and suburban areas within North Africa including Tunisia (Tabbabi & Bekhti, 2017). This mosquito is a major nuisance and vector of West Nile virus (Tabbabi & Bekhti, 2017). It is known that vector control using chemical insecticides is an important element of strategies used to protect human populations and remains the most widely used approach. However, the effects of chemical insecticides on mosquito vector populations are usually transitory because vectors can rapidly develop resistance

against them (Hemingway & Ranson, 2000). Consequently, high levels of resistance to these insecticides have been documented in many field populations of *Culex pipiens* (Wirth & Georghiou, 1996; Bisset *et al.*, 1997; Ben Cheikh *et al.*, 1998; Liu *et al.*, 2004). In Tunisia, resistance to organophosphate chlorpyrifos was highly variable, reaching the highest level (> 10,000-fold) recorded worldwide (Ben Cheikh *et al.*, 1998). In this context, it should be noted that cross and multiple resistance to different insecticides have been frequently reported (Scott, 1995, Wirth & Georghiou, 1996, Rodriguez *et al.*, 2002).

Despite the public health importance of *Culex pipiens pipiens*, its ability to colonize a large range of larval habitats due to its adaptability and the extensive use of insecticides in public health sector as well as agriculture and urban pests, their resistance status to pirimiphos-methyl insecticide has not been explored enough. It should be noted that different insecticides including organophosphates are using in both agriculture and health purposes and the development of resistance in some vectors is possible. Indeed, large variation in susceptibility to various insecticides was observed in many species of mosquitoes (Hemingway *et al.*, 1997; Fonseca *et al.*, 2009; Faraj *et al.*, 2010; Perera *et al.*, 2008; Chanhin *et al.*, 2015). Two main mechanisms including increasing rate of detoxification and target site changes are known to be associated with insecticides resistance in mosquitoes. In the case of organophosphates, the insensitive acetylcholinesterases (AChE1) and enzyme system including esterases, oxidases (CYP450), and glutathione S-transferases (GSTs) have been frequently reported (Ben Cheikh *et al.*, 1998; Labbé *et al.*, 2007; Weill *et al.*, 2003, 2004).

The aim of this study was to evaluate the resistance and the susceptibility to pirimiphos-methyl insecticide in *Culex pipiens pipiens* from Northern and Southern Tunisia. The cross-resistance between pirimiphos-methyl /propoxur, and the polymorphism of over-produced esterases and AChE 1 were also evaluated. The periodic evaluation of used insecticides is necessary to monitor resistance status of mosquito's vectors.

## METHODS AND MATERIALS

**1. Mosquito strains:** Late third and early fourth larvae of *Culex pipiens pipiens* were collected from three localities in Northern and Southern Tunisia and were taken to the laboratory. The location of study areas are given in Fig. 1. The population densities were the main criteria for choosing sampling to ensure running of bioassays. A

susceptible strain (S-Lab) was used as susceptible reference strain. S-Lab is a susceptible strain without any known resistance genes isolated from a Californian population (Georghiou *et al.*, 1966). It has been maintained in laboratory and used as reference to do different comparison with field populations. All field collected population was identified morphologically using the key of Brunhes *et al.* (1999).

## 2. Chemicals insecticides and synergists:

Two chemical insecticides and two synergists were used for different bioassays: the organophosphate pirimiphos-methyl (99.5% [AI]) brought from laboratory Dr Ehrenstorfer, Germany, the carbamate propoxur (99.9% [AI], Bayer AG, Leverkusen,



Figure 1. Geographic origin of Tunisian populations.

Germany), the DEF (98% [AI], Chem Service, England), and the Pb (94% [AI], Laboratory Dr Ehrenstorfer, Germany). The synergists were used to estimate the role of detoxification enzymes in the recorded resistance.

**3. Resistance tests:** All bioassays were performed on late third and early fourth larvae of *Culex pipiens pipiens* in order to avoid misleading results related to the fragility of young and old instars. Bioassays were realized according to standard method of Raymond *et al.* (1986) using pirimiphos-methyl and propoxur insecticides. Bioassays were performed on field populations and/or F1 and F2 laboratory generations in order to finalize all necessary tests. Five replicates of 20 larvae included 5-9 concentrations providing between 0 and 100% mortality were used in a total volume of 100 ml of water containing 1 ml of ethanol solution of the tested insecticide. Mortalities were recorded after 24 hours. The Mazzarri and Georghiou (1995) criteria were followed to classify the resistance level of each population tested as follows: low ( $RR < 5$ ), moderate ( $5 \leq RR \leq 10$ ) or high ( $RR > 10$ ). S,S,S, tributylphosphorotrithioate (DEF) and piperonyl butoxide (Pb) synergistic effects were investigated by exposing larvae to a standard sub lethal doses of 0.08 mg/l for DEF, and 2.5 mg/l for Pb, 4h before the addition of the insecticide. Propoxur bioassays included one dose (1mg/l) and five replicates. This concentration kills all susceptible mosquitoes.

**4. Over-produced esterases:** We characterized Esterases activity using homogenates of adult thorax and abdomen in the presence of  $\alpha$ - and  $\beta$ -naphthyl acetate. Protein were separated using starch-gel electrophoresis (TME 7,4 buffer system) as described by Pasteur *et al.* (1988). The identification of detected enzymes was performed using electrophoresis mobility of known over-produced esterases.

**5. Insensitive AChE1:** According to the method described by Bourguet *et al.* (1996), AChE1 polymorphism was analyzed comparing AChE1 activity of homogenates of

adult heads in the absence or presence of propoxur. Individuals expressing only the susceptible (ACHE1S, phenotype [SS]), only the resistant (ACHE1R, phenotype [RR]), or both types (phenotype [RS]) of ACHE1 were discriminated using this enzyme bioassay.

**6. Data analysis:** The obtained results were analyzed by using the log probit program of Raymond (1993), based on Finney (1971) to obtain  $LC_{50}$ ,  $LC_{95}$  and regression line. Values of  $LC_{50}$ ,  $LC_{95}$ , confidence limits at 95% and slopes were computed. Susceptible strain was used to calculate the Resistance ratio at  $LC_{50}$  which is  $LC_{50}$  of field population/ $LC_{50}$  of sensitive strain and synergism ratio at  $LC_{50}$  which is  $LC_{50}$  in absence of synergist/ $LC_{50}$  in presence of synergist.

## RESULTS

**1. Insecticides resistance:** As shown in Table 1, populations of *Culex pipiens pipiens* were susceptible, moderate and resistant to pirimiphos-methyl insecticide. Resistance to this compound ranged from 2.62 in sample # 2 to 19.9 in sample # 1. The sample # 3 which located in southern Tunisia recorded a resistance ratio of 5.25. The homogeneity of regression slopes was observed (Table 1) and indicated intra-strain phenotypic homogeneity. In fact, the linearity of the dose-mortality response ( $p < 0.05$ ) was accepted for all studied samples including reference strain.

**2. Synergism tests:** DEF and Pb synergists did not suppress resistance to pirimiphos-methyl in all studied samples indicated that esterases (and/or GST) and CYP450 were not involved in the recorded resistance ( $0.28 < SR < 0.68$ ).

**3. Cross-resistance Pirimiphos-methyl/Propoxur:** Cross-resistance to pirimiphos-methyl organophosphate and propoxur carbamate were detected and let us suggests the involvement of their common target-site: AChE-1. The mortality due to propoxur was significantly correlated

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

Population	Pirimifos-methyl				Pirimifos-methyl +DEF				Pirimifos-methyl +Pb				
	LC <sub>50</sub> in µg/l (a)	Slope ± SE	RR <sub>50</sub> (a)	LC <sub>50</sub> in µg/l (a)	Slope ± SE	RR <sub>50</sub> (a)	SR <sub>50</sub> (a)	RSR	LC <sub>50</sub> in µg/l (a)	Slope ± SE	RR <sub>50</sub> (a)	SR <sub>50</sub> (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-Sidi Hcine	58,2 (21-159)	2,55 ± 0,72*	19,9 (9,44 - 42,2)	12 (9,9-15)	1,74 ± 0,17*	41,8 (26,2-66,6)	4,67 (2,20-9,89)	0,47	28 (24-32)	2,64 ± 0,23	69,9 (54,7-89,2)	2,07 (1,02-4,16)	0,28
2-El Fahs	7,6 (5,6-10)	1,77 ± 0,24	2,62 (1,98 - 3,46)	-	-	-	-	-	-	-	-	-	-
3-Dgach	15 (4,4-54)	2,33 ± 0,69	5,25 (2,37-11,6)	3,1 (2,7-3,7)	2,13 ± 0,20	7,71 (5,78-10,2)	4,79 (2,38-9,65)	0,68	-	-	-	-	-

(a), 95% CI. \* The log dose-probit mortality response is parallel to that of S-Lab;

RR<sub>50</sub>, resistance ratio at LC<sub>50</sub> (RR<sub>50</sub>=LC<sub>50</sub> of the population considered/LC<sub>50</sub> of Slab); SR<sub>50</sub>, synergism ratio (LC<sub>50</sub> observed in absence of synergist/LC<sub>50</sub> 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).

with the  $LC_{50}$  of the used insecticide (Spearman rank correlation,  $(r) = -0.62$  and  $-0.78$ , respectively,  $(P < 0.01)$ ). Indeed, the population # 1 showing the highest resistance to pirimiphos-methyl recorded the highest resistance to propoxur insecticide.

**4. Insensitive AChE1:** The polymorphism of AChE1 was analyzed in the three collected populations which showed an AChE1 insensitive to propoxur inhibition. The highest frequencies of the resistant phenotypes ([RS] and [RR]) ( $>0.74$ ) were detected in the most resistant samples (#1). The frequencies of [RR] phenotype were low in all studied samples. The [RS] phenotype frequencies ranged from 0.17 to 0.66, with an excess in the highest resistant population (#1). The highest frequencies of [SS] phenotype were recorded in samples # 2 (0.75) which showing the highest susceptibility to the used insecticides.

**5. Overproduced esterases:** Four esterases enzymes including C1 encoded by the *Est-1* locus and three esterases encoded by the *Ester* super locus: A2-B2, A4-B4 (or A5-B5, which has the same electrophoretic mobility) and B12 were detected. One or several esterases were detected in all the studied samples. The highest (0.61) and the lowest (0.22) frequencies of these esterases were recorded in samples #1 and 2 which recorded the highest and the lowest level of resistance, respectively.

## DISCUSSION

In the present study, the resistance was low, moderate and high to the used insecticide and additional investigations are required on the resistance status of this species. Our findings are in agreement with previous studies showing the same level of resistance to this insecticide (Tabbabi *et al.*, 2017). This low level of resistance to pirimiphos-methyl may be due to the absence of agricultural pest control in studied sites despite application of mosquitoes control using organophosphates insecticides, the

interruption of spraying for 3 months a year during the cold months and/or to the migration of the susceptible mosquitoes from the untreated populations in Tunisia. On the other hand, resistance of Tunisian *Culex pipiens pipiens* were highly variable and reached the highest level ( $>10,000$ -folds) recorded worldwide to chlorpyrifos which belonging to organophosphate insecticides (Ben Cheikh *et al.*, 1998). Authors explained these results by chlorpyrifos/DDT cross-resistance. In fact, the DDT was used as the main insecticide in the framework of the National Program for the Eradication of Malaria during the 60s and 70s against malaria vectors. It should be noted that several factors including the environmental conditions and the control programs implemented, which may vary in type and frequency of insecticide use may affect the resistance of mosquitoes to chemical insecticides. Therefore, it is not relevant to compare obtained results to bioassay results of mosquitoes that were exposed to different combinations and/or frequencies of insecticidal applications.

The general characteristics of study areas including insecticides usage were collected according to the ministry of health and showed the use of both organophosphates and pyrethroids insecticides in mosquitoes control. The moderate resistance detected in studied strains could be explained by cross-resistance to other insecticides that have common mechanisms resistance. The cross resistance between organophosphates, pyrethroids and organochlorates insecticides was detected and was associated with the monooxygenases, esterases and GST activity (Corbel *et al.*, 2013) considered as the most common mechanisms. Our results showed that oxidases, esterases and/or GST were not involved in the recorded resistance. We must remember that some detoxification enzymes may be insensitive to the action of synergists which explain the detection of different esterases using starch electrophoresis. In this context, it should be noted that resistance due to reduced penetration of insecticides was reported in previous studies but it was always governed by

metabolic resistance and mutation of target sites. In fact, Raymond *et al.* (1993) showed that resistances due to last mechanisms are additives.

Monitoring resistance in mosquito vectors and the characterizing the mechanism of resistance have the same importance. The cross-resistance detected between organophosphates and propoxur carbamate lets us suggest the involvement of their common target-site: AChE-1. Similar results were found in different insects and detected several mutations in the gene encoding for an acetylcholinesterase (Fournier, 2005) which result in reduced sensitivity to inhibition of the enzyme by these insecticides (Weill *et al.*, 2003; Alout *et al.*, 2009). The resistance allele, *ace-1<sup>R</sup>* is present worldwide and causes organophosphates resistance in several mosquito species (Labbé *et al.*, 2007). The G119S mutation (i.e. glycine to serine substitution at position 119) responsible for carbamate and organophosphates resistance has been detected and reported in vectors mosquitoes (Weill *et al.*, 2004; Djogbenou *et al.*, 2008). It is important to note that three different AChE 1 phenotypes were observed: *ace-1<sup>R</sup>* and *ace-1<sup>S</sup>* alleles, and duplicated haplotype which showed the higher frequency in the highest resistant sample. The overall fitness advantage of the duplicated haplotype may result from a lower fitness cost (Labbé *et al.*, 2007).

Overall, in the present study, it was found that *Culex pipiens pipiens* was moderately resistant to pirimiphos-methyl insecticide for all collected populations. The problem of resistance is very serious in Tunisia. Currently, the ministry of health has no alternative insecticide for effective vector control or for insecticide resistance management (Daaboub *et al.*, 2008) that's why an approach focused on the rational use of insecticides (rotational and/or mosaic strategy) can help in preventing the development of resistance in mosquitoes vectors as tried in a field test in Mexico to ameliorate multi-insecticide resistant *Anopheles albimanus* (Rodriguez *et al.*, 2006). Improving ecofriendly methods of vector control based on biocontrol and

biolarvicides would be of great importance (Ghosh & Dash, 2007; Tiwari *et al.*, 2011).

## CONCLUSIONS

We cannot overlook the insecticides as the most practical in controlling mosquito vector. The early detection of the status of resistance by monitoring of insecticide resistance at regular intervals is necessary to conceive some protocols to apply the foresaid strategy. The low and moderate resistance in *Culex pipiens pipiens* to carbamates and organophosphates presents greater opportunity for managing resistance in Tunisia.

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## Conflict of interest statement

The authors declare that they have no conflict of interest.

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