The potential role of urbanization in the resistance to organophosphate insecticide in *Culex pipiens pipiens* from Tunisia

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Abstract

Objective: To examine the effects of urbanization on the resistance status of field populations of *Culex pipiens pipiens* to organophosphate insecticide.

Methods: Bioassays and biochemical assays were conducted on Tunisian field populations of *Culex pipiens pipiens* collected in four various areas differing in the degree of urbanization. Late third and early fourth larvae were used for bioassays with chlorpyrifos and adults mosquitoes for biochemical assays including esterase and acetyl cholinesterase (AChE) activities.

Results: The distribution of resistance ratios in this study appears to be influenced by the degree of urbanization. The highest resistance was recorded in the population from most urbanized areas in Tunisia whereas the lowest resistance was found in relatively natural areas. Both metabolic and target site mechanisms were involved in the recorded resistance.

Conclusion: This is the first study in Tunisia showing evidence of the impact of urbanization on the resistance level in *Culex pipiens pipiens*. Proper management of the polluted breeding sites in the country and effective regulation of water bodies from commercial and domestic activities appear to be critical for managing insecticide resistance.

Keywords: *Culex pipiens pipiens*, urbanization, organophosphate resistance.

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Introduction

Culex pipiens mosquito has been strongly suspected as the most likely vector in the transmission of West Nile virus outbreaks that have affected Tunisia[1] in 1997, 2003, 2007, 2010, 2011 and 2012. Vector control by insecticides is the main tool to prevent these diseases and organophosphate insecticides are one of the most effective mosquito larvicides used in many places. Unfortunately, the massive use of insecticides during the malaria eradication program between 1967 and 1978 has led to the development of strong resistance worldwide in *Culex pipiens* from Tunisia[2]. This situation becomes a serious problem in Tunisia where very high resistance to the organophosphate chlorpyrifos (> 10,000-fold) was described in *Culex pipiens pipiens*[2]. The various mechanisms that enable insects to resist the action of insecticides can be different in different populations and be resumed into four distinct categories: metabolic resistance, target-site resistance, re-

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duce penetration and behavioral avoidance. 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[2,3,4]. Among factors likely to influence insecticide resistance in mosquitoes, urbanization has been strongly implicated but rarely studied in detail.

As *Culex* mosquitoes adapt to the polluted environment of the urban areas, transmission of pathogens may increase. The effect of this adaptation on the mosquitoes tolerance to insecticides used in vector control is unknown. A detailed knowledge of the biology of urban vectors, including the processes and mechanisms by which these vectors adapt to pollutants as well as to the many insecticides is needed to plan and implement urban vector control strategies.

Historically, urbanisation has always been closely linked to economic development, which leads to the increase of vector-borne diseases[5,6]. Many problems have emerged as a result of urbanization, including environmental pollution, crowding, and the destruction of natural ecology. Changes in environmental conditions as a result of urbanization may directly and/or indirectly affect the ecology of mosquitoes, e.g., larval habitat availability and suitability, development, and survivorship facilitating the invasion and establishment mosquito populations in proximity to their hosts and therefore leading to an uncontrolled use of insecticides[7]. Previous studies showed that an additional selective pressure favoring insecticides resistance in urban areas may be presented as results for such human's practices[8]. On the other hand, many anthropogenic pollutants in water bodies are always associated to urbanization and may puts indirect pressure of the resistance of mosquitoes to chemical insecticides. These urban pollutants are often not toxic to mosquitoes but may affect rapidly their resistance to different insecticides inducing mainly detoxification enzymes activities[9-12]. As a result, knowledge on resistance status of vectors against organophosphate and the mechanism involved as well as factors that influence the resistance have become important. In this context, it is important to note that most previous studies have been focused on resistance level and associated mechanisms. However, less research effort have been carried out to study the influence of urbanization on mosquito's resistance. This study was therefore carried out to assess resistance status of *Culex pipiens pipiens* to organophosphate insecticide in four various areas differing in the degree of urbanization and the possible mechanisms involved as well as environmental factors associated with its distribution. Our objective was to investigate factors facilitating the vectors adaptation to setting differing in the degree of urbanization in view to develop an integrated vector control strategy to successfully vector control in urban settings. Indeed, a top-down approach and methods, based on a limited or inadequate understanding of mosquito ecology, evolution, and urban social ecology, will fail.

Materials and methods

Mosquitoes

Four populations of *Culex pipiens pipiens* were collected in four various areas differing in the degree of urbanization (anthropogenic ie densely populated urban area, semi-anthropogenic ie moderately populated urban area, semi-natural ie rural area weakly populated and natural sites ie rural area without human population) (Figure 1). The characteristics of study areas including insecticides usage is given in Table 1. Data were collected according to both ministries of health and agriculture and during individual interviews with the collection sites residents. A susceptible strain named S-Lab[13] was used to calculate the resistance ratios of field populations. Two resistant strains named SA2 (A2-B2) and SA5 (A5-B5) were used as references in starch gel electrophoresis[14].

Figure 1: Geographic origin of Tunisian populations.

Bioassays

Different bioassays were performed on late third and early fourth larvae according to standard methods of Raymond et al[15], using ethanol solutions of organophosphate chlorpyrifos and carbamate propoxur under standard laboratory conditions (25 ± 1°C and 70 ± 5% RH) . Chlorpyrifos bioassays included 5 concentrations providing between 0 and 100% mortality and 5 replicates per concentration on sets of 20 late 3rd and early 4th instars in a total volume of 100 ml of water containing 1 ml of ethanol solution of the tested insecticide. It should be noted that we used a series of five beakers in the case of control larvae and we added only 1 ml of ethanol. A standard sub lethal doses of 0.08 mg/l for DEF (S,S,S-tributyl phosphorotrithioate), and 2.5 mg/l for Pb (piperonyl butoxide), 4 hours before the addition of the insecticide was added to all synergized treatments to estimate the role of detoxification enzymes in the recorded resistance. Dead larvae were counted 24 hours after treatment; larvae that did not move when touched with a thin needle were considered dead. The carbamate propoxur bioassays included just one dose (1mg/liter) and five replicates to detect the involvement of the common mechanism of resistance to both insecticides. This concentration kills all susceptible mosquitoes.

Biochemical assays

The identification of different esterases was performed using starch electrophoresis according to the methods of Pasteur et al[16]. Detected esterases were identified by comparing their electrophoretic mobility to that of known over-produced esterases.

AChE activity

The enzymatic assay was investigated according to the standard protocol of Bourguet et al[17] to measure the susceptibility of AChE1 to a propoxur and detect the presence of AChE1S and AChE1R.

Data analysis

Obtained data were analyzed using log probit program of Raymond et al[18] based on Finney[19] (1971). Values of LC_{50}, LC_{95}, confidence limits at 95% and slopes were computed. Resistance ratio at LC_{50} (RR50 = LC_{50} of field population/LC_{50} of sensitive strain) and synergism ratio at LC_{50} (SR50 = LC_{50} in absence of synergist/LC_{50} in presence of synergist) were calculated.

Results

Details on Log-dosage probit-mortality analysis are shown in Table 2. Resistance ratios ranged from 1.8 to 8929. The highest and the lowest resistance ratio was observed in sample 1 (anthropogenic site) and 4 (naturel site), respectively. The resistance ratio values of samples collected from semi-antrhpogenic and semi-natural were 163 and 75, respectively. The highest resistance was recorded in the population from most urbanized areas in Tunisia whereas the lowest resistance was found in relatively natural areas.

Table 1: Geographic origin of Tunisian populations, breeding site characteristics, and insecticide control.

Code	Locality	Breeding site	Date of collection	Mosquito control (used insecticides)	Agricultural pest control	Nature of breeding site
1	Ezzahra	Ditch	Nov 2005	Very frequent	None	Anthropogenic
2	Sidi thabet	Ditch	Aug 2004	Rare	Yes	Semi-anthropogenic
3	Sidi khalifa	Waste water pond	July 2004	None	None	Semi-natural
4	Bordj El Khadra	Water pond	March 2002	Occasional	None	Natural

Very frequent: one time by week in summer season
Rare: one time by 6 weeks in summer season
None: any insecticides application
Occasional: When alerted by the complaints about mosquito biting

As shown in Table 2, the use of synergists showed that detoxification enzymes were not involved in the recorded resistance of studied samples. However, five esterases of high activity were observed in studied field samples except for sample 4 using starch electrophoresis (Table 3). The esterase C1 encoded by the Est-1 locus and four esterases encoded by the Ester super locus: A1, A2-B2, A4-B4 (or A5-B5, which has the same electrophoretic mobility) and B12. The high level of chlorpyrifos resistance observed in resistant populations was correlated with propoxur resistance indicated an insensitive AChE 1. The frequencies of the resistant genotypes were 0.83, 0.85 and 0.44 for samples 1, 2 and 3, respectively. These findings may be due to the higher insecticide selection pressure in anthropogenic areas than the rest of the study sites.

Table 2: Chlorpyrifos resistance characteristics of Tunisian *Culex pipiens pipiens* in presence and absence of synergists DEF and Pb

Population	*Chlorpyrifos*			*Chlorpyrifos +DEF*					*Chlorpyrifos +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	0.56 (0.53-0.58)	9.0 ± 1.04	-	0.17 (0.14-0.20)	2.85 ± 0.26	-	1.4 (1.08-1.8)	-	0.45 (0.17-1.3)	1.16 ± 0.43	-	0.53 (0.35-0.79)	-
1-Ezzahra	4950 (3830-6850)	1.67 ± 0.19	8929 (6773-11773)	4740 (3690-6430)	1.43 ± 0.22	28199 (21728-36598)	1.04 (0.80-1.3)	0.31	6340 (2910-55400)	0.95 ± 0.33	14228 (8361-24209)	0.78 (0.50-1.2)	0.62
2-Sidi thabet	90 (49-166)	0.79 ± 0.08	163 (121-221)	846 (220-3230)	0.99 ± 0.23	5032 (2769-9145)	0.10 (0.06-0.17)	0.03	241 (87-531)	0.97 ± 0.17	481 (276-838)	0.42 (0.29-0.61)	0.33
3-Sidi khalifa	41 (20-84)	±0.84 0.15	75.0 (53.7-104)	203 (108-437)	0.69 ± 0.13	1210 (915-1600)	0.20 (0.14-0.28)	0.06	18 (4.1-86)	0.88 ± 0.24	42.3 (21.6-83.0)	2.2 (1.3-3.6)	1.7
4- Bordj El Khadra	1.0 (0.68-1.6)	±3.01 0.66	1.8 (1.08-3.3)	-	-	-	-	-	-	-	-	-	-

(a), 95% CI.
RR_{50}, resistance ratio at LC_{50} (RR_{50}=LC_{50} of the population considered /LC_{50} of Slab); SR_{50}, synergism ratio (LC_{50} observed in absence of synergist/LC_{50} 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).
[-]: The empty cells were due to the loss of some populations.

Table 3: Frequencies of insensitive acetylcholinesterase and over-produced esterases phenotypes in Tunisian populations of *Culex pipiens pipiens* sampled in 2005.

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-Ezzahra	36	0.03	0.06	0.36	0.06	0.08	-	0.03	0.33	0.11	0.17	0.08	0.75	0 (100)
2-Sidi thabet	34	-	0.09	0.44	-	0.03	-	0.06	0.38	0.06	0.15	0.59	0.26	0.46 (99)
3-Sidi khalifa	36	-	0.06	0.19	0.14	0.06	-	0.05	0.50	0.03	0.56	0.33	0.11	0.68 (99)
4- Bordj El Khadra	36	-	-	-	-	-	-	-	1	-	1	-	-	1 (100)

Phenotype [i] corresponds to genotypes $Ester^i$ / $Ester^0$ or $Ester^i$ / $Ester^i$, and phenotype [ij] correspond to genotype $Ester^i$ / $Ester^j$.
N represents the total number of mosquitoes analyzed for each sampling site.

Discussion

In Tunisia, *Culex pipiens pipiens* is an important member of *Culex pipiens* complex and act as an important vector for West Nile virus that recently affected the country[1]. For these reasons, it was necessary to address the insecticide resistance problem. Here, we had undertaken the most comprehensive research into insecticide resistance in *Culex pipiens pipiens* mosquitoes from four various areas differing in the degree of urbanization. It is important to note that the general characteristics of study areas showed that insecticide usage varied in different ecological settings (anthropogenic, semi-anthropogenic, semi-naturel and naturel sites).

The distribution of resistance ratios of Tunisian *Culex pipiens pipiens* in this study appears to be influenced by the degree of urbanization. Indeed, the highest resistance was recorded in the population from most urbanized areas in Tunisia whereas the lowest resistance was found in relatively natural areas. The characteristics of study areas showed that agricultural and domestic use of insecticides may be as the major cause of resistance in urban areas[8]. However, despite the absence of both public health and agricultural applications, mosquitoes collected from semi-naturel area were resistant and therefore cannot fully explain the cause of the recorded resistance. In this preliminary assessment, it is clear that urban populations are exposed to higher levels of anthropogenic pollutants exhibit stronger signals of selection. These observations must take a critical look at what needed to be done to manage the polluted breeding sites in the country and regularized water bodies from commercial and domestic activities. The impact of urban pollutants on insecticides resistance in mosquitoes has been confirmed in previous studies[10,11]. Contrary to agricultural pest control, the role of urban pollutants and uncontrolled use of insecticides for personnel protection were strongly involved although Essandoh et al[20] suggested the important impact of agricultural use of pesticides on organophosphates resistance. Other studies showed that *Anopheles* mosquitoes were found susceptible to organophosphates which were detected in large quantities in their breeding sites. These finding are in agreement with those of our study where mosquitoes collected from naturel site were found susceptible although the occasional use of insecticides in this area.

In addition to both public health and agricultural applications, several unknown chemicals or insecticides in polluted breeding sites can affect and select multiples resistance mechanisms in mosquitoes that can confer important resistance levels to new insecticides in the country. In this context, it is important to noted that both metabolic and target site mechanisms were identified to be involved in the recorded resistance of studied field populations. An insensitive acetyl cholinesterase (AChE1) and detoxification esterases were detected in resistant samples and these mechanisms were positively associated with chlorpyrifos resistance. These findings are consistent with previous investigations which related both mechanisms with resistance to organophosphates insecticides[2-4,21-23]. Thus, monitoring the resistance mechanisms may help the surveillance of organophosphate resistance in *Culex pipiens pipiens*.

An interesting result from this study was the detection of the impact of urbanization on insecticide resistance in *Culex pipiens pipiens*. Resistance differed widely between anthropogenic, semi-anthropogenic, semi-naturel and naturel sites. Data on the distribution of resistance in various studied areas will be of great importance to develop efficient mosquito control strategies.

Conclusion

This is the first study in Tunisia showing evidence of the impact of urbanization on the resistance level in *Culex pipiens pipiens*. Besides the public health and agricultural applications, anthropogenic pollutants may be an important cause of resistance in mosquitoes. Proper management of the polluted breeding sites in the country and effective regulation of water bodies from commercial and domestic activities appear to be critical for managing insecticide resistance. In this context, it is important to mention the necessity of alternative effective vector control methods including larval resource reduction and biological control, as well as new chemical insecticides.

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Conflict of interest

The authors declare that they have no conflict of interest.

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