Development of Resistance to Chlorpyrifos in *Culex pipiens* (Diptera: Culicidae) Population under Temephos Selection Pressures

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Abstract: Development of resistance to chlorpyrifos in *Culex pipiens* population from Tunisia under temephos selection pressures was investigated. The objective of this study was to follow the evolution of resistance responses in *Culex pipiens* larvae under laboratory conditions. The selection to temephos caused a significant increase of resistance to chlorpyrifos levels. Indeed, the LD50 resistance rate increased from 75.90 in the natural population to approximately 6620.66 in the 4th generation of selection. This resistance dropped sharply in the 5th generation (1686.35) to increase again in the last generation of selection reaching 3929.36. The reason for this unstable variation is unknown and effects related to uncontrolled environmental parameters and/or inter-strain genetic variability is probably involved. Our results showed the involvement of insensitive acetylcholinesterase (AChE), which is a common target for temephos and chlorpyrifos insecticides, in the recorded resistance. The potential to develop resistance to the chlorpyrifos is very clear in this species.

To cite this article
[Tabbabi, A., Lamari, A., & Ben Cheikh, H. (2017). Development of Resistance to Chlorpyrifos in *Culex pipiens* (Diptera: Culicidae) Population under Temephos Selection Pressures. *The Journal of Middle East and North Africa Sciences*, 3(5), 1-4]. (P-ISSN 2412- 9763) - (e-ISSN 2412-8937). www.jomenas.org**. 1**

Keywords: *Culex pipiens*, temephos, chlorpyrifos, selection pressure, resistance**.**

1. Introduction:

Fighting against harmful insects is a necessity for humans. Various means have been used for this purpose which has constantly evolved, to combat them, until the development of organic insecticides by the year 1930. These have been very effective at the beginning of their use. Unfortunately, insects quickly respond by developing physiological mechanisms of resistance to these insecticides.

The first cases of mosquito resistance to insecticides, including DDT, were reported in 1947 for *Culex pipiens* in Italy (Brown and Pal, 1971). This problem was retained to provide a clear framework for an in-depth analysis of the modalities of adaptive change. This allows better management of resistance phenomenon in populations of insects subjected to high insecticide selection pressures (vector control operation). In fact, understand, why, how these resistances appear and how they evolve in agronomic, medical and economic fields, is very important.

Subsequent work on the populations of *Culex pipiens* in Tunisia (Ben Cheikh et al., 1993; 1995; 1998; 1999; 2008; 2009; Daaboub et al., 2008; Tabbabi et al., 2016; 2017) showed that these populations have developed very high resistance rates to insecticides, especially to organophosphates. Indeed, the massive use of pesticides over many years has led to a very specific situation of resistance in Tunisia: A very strong resistance to chlorpyrifos (organophosphate), reaching record levels (10,000 times), was recorded so that it remained low, not exceeding about 10 times to temephos (Ben Cheikh, 1999). How can we explain this important rate of resistance to chlorpyrifos in Tunisia? Why it appeared to chlorpyrifos and not to temephos, which is also an organophosphate?

The objective of this study was to determine the rate of resistance development to chlorpyrifos in the presence of temephos selection pressure.

2. Materials and Methods:

2.1. Mosquitoes

Culex pipiens larvae, which have been maintained in the insectarium of Unit of Genetics, the University of Monastir for many years and have not been exposed to any insecticide and/or biological control agent was used as reference strain and designated as S-Lab. The mosquitoes were bred and reared in the insectarium. A population of *Culex pipiens* was collected from natural habitats (Boussalem, Northwestern Tunisia) in 2004. Mosquitoes were reared in standard insectary conditions with tap water (larvae) and net cages (adults). The population was selected with the organophosphorus insecticide temephos in the laboratory.

2.2. Chemical Insecticides

Three technical grade insecticides were used for selection and bioassay: the organophosphates temephos (9l%o; American Cyanamid, Princeton, NJ), chlorpyrifos (99.5%, Laboratoire Dr. Ehrenstorfer GmbH – Germany) and the carbamate propoxur (997o; Mobay).

2.3. Synergism Test and Esterase's Detection for Confirmation of the Defense Mechanism

This test was similar to the bioassay tests except that 0.5 ml of the maximum sub-lethal concentration of an esterase inhibitor DEF, S,S,S-tributyl phosphorotrithioate, (0.5 µg/ml) was added to each cup with 0.5 ml of insecticide and piperonyl butoxide (PB), an inhibitor of mixed function oxidases. Esterase phenotypes were established by starch electrophoresis (TME 7.4 buffer system) as described by Pasteur et al. (1981, 1988) using homogenates of thorax and abdomen.

2.4. Bioassay Test for Mosquito Larvae and Data Analysis

The larval stages were subjected to selection pressure against temephos at the concentrations that caused 50% and 75% mortality, using the WHO standard bioassay. The selection was applied to mosquitoes for 6 consecutive generations. Briefly, bioassays were carried out in triplicates, with five different temephos concentrations by serial dilutions. Controls without insecticide were done in five repetitions. Bioassays were carried out on twenty early fourth instar larvae. Larval mortality was recorded after 24 hours of exposure. Surviving larvae were reared and bred to subsequent generations.

Mortality data were analyzed by using the log-probit program of Raymond (1993), based on Finney (1971).

3. Results:

The linearity of the dose-mortality response is rejected ($p > 0.05$) only for Bou.nat.C and Bou.tem1.C (Table 1). In addition, their curves have very important inflections. Concerning their parallelism with that of S-Lab.C, it is rejected for all the strains studied (Table 1).

The selection to temephos caused a significant increase in resistance to chlorpyrifos levels. Indeed, the LD50 resistance rate increased from 75.90 in the natural population to approximately 6620.66 in the 4th generation of selection. But, this resistance dropped sharply to the 5th generation (1686.35) to increase again in the last generation of selection reaching 3929.36 (Table 1).

The results showed that the DEF caused, on the contrary, an antagonistic effect (SR <1). In addition, statistical analysis showed that the SR of the strain studied is less than the S-Lab SR (Table 2). The esterases and GSTs are therefore not at the origin of the resistance recorded in Bou.tem6.

The SR value of Bou.tem6.C.PB at LD50 is not significantly different from S-Lab.C.PB. This value is less than 1 (table 2). This implied that the equilibrium between activating oxidases and degradation oxidases is not modified compared to the sensitive strain.

Acetylcholinesterase (AChE) is a common target for temephos and chlorpyrifos. Except for Bou.nat, c*ulex pipiens* of Bou.tem6 showed resistance to Propoxur which indicates an acetylcholinesterase insensitive. Synergism tests (Table 2) and electrophoresis gel showed that resistance was not associated with monooxygenase and esterases or (GST).

Table 1: *Chlorpyrifos resistance evolution of Bou.tem larvae under selection pressures with temephos Bou: Boussalem; nat: natural population; tem: temephos; C: chlorpyrifos*

Name of population	LD_{50} (a)	LD_{95} (a)	Slope (b)	H (df)	RR_{50} (c)	RR_{95} (c)
S-Lab.C	0.00098 (0.00089-0.0010)	0.0029 (0.0024-0.0039)	3.42± (0.29)	1 (3)	-	-
Bou.nat.C	0.0748 (0.0420-0.1317)	0.4351 (0.1563-1.2672)	2.15± (0.42)	5.01 (3)	75.90 (48.46-118.87)	145.97 (61.48-346.55)
Bou.tem1.C	0.0910 (0.0521-0.1582)	1.4459 (0.5564-3.8334)	1.63± (0.19)	5.30 (6)	92.32 (64.97-131.20)	485.09 (248.21-948.02)
Bou.tem2.C	0.0976 (0.0665-0.1314)	3.4905 (1.8187-10.0702)	1.05± (0.13)	1 (2)	98.99 (79.94-122.58)	1170.98 (700.43-1957.64)
Bou.tem3.C	0.9046 (0.7265-1.3037)	15.2541 (5.5609-256.5709)	1.34± (0.32)	1 (2)	917.41 (743.53-1131.95)	5117.37 (2010.05-13028.30)
Bou.tem4.C	6.5286 (2.3964-155.9082)	530.4879 (43.5941-1920025.0000)	0.86± (0.23)	1 (2)	6620.66 (3692.43-11871.08)	177964.90 (40744.93-777311.00)
Bou.tem5.C	1.6629 (1.3708-2.0925)	11.1575 (7.4884-19.2014)	1.99± (0.16)	1 (4)	1686.35 (1332.61-2133.99)	3743.06 (2195.46-6381.59)
Bou.tem6.C	3.8747 (3.3426-4.5119)	24.5456 (18.3208-36.1044)	2.05± (0.16)	1 (3)	3929.36 (3212.09-4806.81)	8234.42 (5179.67-13090.73)

(a) In mg/liter, 95% CI in parentheses. (b) Standard errors in parentheses. H: Heterogeneity, (df): testing linearity of the probit mortality/log dose response. (c) RR, resistance ratio (LC50 of the population considered / LC50 of S-Lab); 95% CI in parentheses.

Table 2: *Responses of Bou.tem strains of Culex pipiens to chlorpyrifos with and without synergists*

Name of population	LD_{50} (a)	LD_{95} (a)	Slope (b)	H (df)	RR_{50} (c)	RR_{95} (c)	SR_{50} (d)	SR_{95} (d)
S-Lab.C	0.00098 (0.00089-0.001)	0.0029 (0.0024-0.0039)	3.42± (0.29)	1 (3)	-	-	-	-
Bou.tem6.C	3.8747 (3.3426-4.5119)	24.5456 (18.3208-36.1044)	2.05± (0.16)	1 (3)	3929.36 (3212.09-4806.81)	8234.42 (5179.67-13090.73)	-	-
S-Lab.C.DEF	0.00005 (0.00004-0.000055)	0.00001 (0.000013-0.000019)	3.31± (0.25)	1 (6)	-	-	19.13 (15.99-22.89)	18.44 (11.67-28.66)
Bou.tem6.C .DEF	5.1234 (4.3445-6.2644)	24.2844 (16.5575-44.2138)	2.43± (0.29)	1 (2)	99435.21 (80613.02-186184.78)	150300.60 (8618.78-262113.90)	0.75 (0.60-0.95)	1.01 (0.56-1.79)
S-Lab.C.PB	0.0045 (0.0040-0.0051)	0.0180 (0.0130-0.0315)	2.75± (0.39)	1 (1)	-	-	0.2159 (0.1744-0.2673)	0.1654 (0.0904-0.3024)
Bou.tem6.C .PB	5.0581 (4.1906-6.4375)	32.4867 (20.0555-71.6253)	2.03± (0.26)	1 (1)	1107.5610 (874.8061-1402.2440)	1802.9400 (879.0307-3697.9220)	0.76 (0.61-0.95)	0.75 (0.41-1.38)

(a) In mg/liter, 95% CI in parentheses.
(b) Standard errors in parentheses. H: Heterogeneity, (df): testing linearity of the probit mortality / log dose response.
(c) RR, resistance ratio (LC50 of the population considered / LC50 of S-Lab); 95% CI in parentheses.
(d) SR, synergism ratio (LC50 observed without synergist / LC50 observed with synergist); 95 CI in parentheses.

4. Discussion:

Our studies showed that the natural population of *Culex pipiens* harvested from the Boussalem region has high levels of resistance to chlorpyrifos (RR at LD50 is 75.90). However, the work of Ben Cheikh, (1999) showed a very strong resistance to chlorpyrifos, unique in the world by its magnitude.

Regular exposure to temephos leads to a continuous increase in resistance to this insecticide and to chlorpyrifos over the first 4 generations of selection. Then, an irregularity in the evolution of the resistance rates was recorded for the two insecticides (Tables 1): the fact that the two products belong to the same family of organophosphorus, can at least in part explain this. But why has there been a very high resistance to chlorpyrifos and not to temephos: selection insecticide?

How can this irregularity be explained in the evolution of resistance to temephos? Effects related to uncontrolled environmental parameters or inter-strain genetic variability is likely to be involved.

The synergistic effect of DEF and PB is not more important in the strain studied than in the reference strain. In addition, the curves of the dose-mortality response of Bou.tem6 obtained by the effect of the insecticide added with synergist are almost identical with those in which the insecticide is used alone. This demonstrates the non-involvement of esterases and/or GSTs in the detoxification of the insecticides used. The disappearance of inflections at the dose-mortality response curve for Chlorpyrifos of Bou.tem6C indicates the existence of phenotypes with identical characteristics of resistance to this insecticide.

Raymond et al. (1989) have shown that the association of detoxification with an insensitive target is additive. The absence of detoxification enzymes in the strains studied suggests the intervention of other factors in the detoxification of insecticides. The modifications that seem to be the most effective, that is to say that give the mosquitoes the strongest resistance, are those that affect the targets of insecticides and detoxification. Thus, this last mechanism (enzymatic conjugation) cannot be neglected, which could be responsible, at least in part, for the resistance recorded. Indeed, the action of the synergist employed in the toxicological tests (DEF) does not always result in the inhibition of esterases and GSTs. Similarly, some cytochrome P450 enzymes may be insensitive to the action of PB. How can we explain the appearance of a very high resistance to organophosphorus insecticides (especially chlorpyrifos) after a few generations of selections?

In the studied strains, the overproduced esterases are not involved in this resistance. The latter can be explained by the existence of insensitive acetylcholinesterase (AChE) (with the exception of the natural population which showed an increased sensitivity of AChE to propoxur) and by the presence of other factors (Pasteur et al., 1999): The very high resistance to chlorpyrifos (organophosphates) present in the region of Tunis is unique in the world by its magnitude. A subsequent study showed that all enzymes inhibited by DEF and PB synergists (esterases, oxidases) play a very weak role in this enormous resistance and the mechanism involved remains to be elucidated (Ben Cheikh, 1999; 2003).

Financial support

This study was supported by the Tunisian Ministry of High Education and Research in the frame of the Laboratory of Genetics, Faculty of Medicine of Monastir, Monastir University, 5019, Monastir, Tunisia.

Conflicts of Interest:

Authors declared no conflicts of interest.

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Received March 03, 2017; revised March 23, 2017; accepted April 01, 2017; published online May 01, 2017.