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Genetic Variability among Susceptible, Resistant and Hybrid *Culex pipens* (Diptera: Culicidae) Strain to *Bacillus sphaericus*

[1]*Wassim, N.M.*, [1]*Soliman, B.A.*, [2]*Hassanain, M.A.*,
[3]*Lotfy, N.M.*, [4]*Kadria N. Abdel Megeed and* [2]*Khalil, S.M.*

[1]Department of Biological and Geological Sciences, Faculty of Education, Suez Canal University, Egypt
[2]Department of Zoonotic Diseases, National Resarch Center, Dokki, Giza, Egypt
[3]Department of Entomology, Faculty of Science, Ain Shams Univeresty, Cairo, Egypt
[4]Department of Parasitology and Animal Diseases, National Resarch Center, Dokki, Giza, Egypt

Abstract: Investigations on mechanism of resistance of *Cx. pipiens* strains to *B. sphaericus* strain 2362 were performed in the present study including molecular characterization. The tested strains were: resistant (F_{28}), susceptible (F_{28}) and progeny of reciprocal crosses between susceptible and resistant strains. The molecular characterization focused on the amplification of a fragment of the D3 28S rDNA gene using PCR by specific primers. To detect the point of mutations, single strand confirmation polymorphism (SSCP) analysis was used. The analysis of SSCP bands revealed that high rate of polymorphisms were recorded in both resistant and susceptible females followed by resistant males. Low rates of polymorphisms were recorded in the progeny individuals. It was concluded that molecular techniques were used as powerful tool for the differentiation between susceptible,resistant and hybrid *Cx.pipiens* strains.

Key words: Molecular techniques · rDNA · Mosquitoes · *Culex pipens* · Resistance · *Bacillus sphaericus*

INTRODUCTION

Mosquitoes transmit serious human diseases, such as malaria, yellow fever, West Nile fever and dengue. Resistance to all the major classes of chemical insecticides has now been recorded in several mosquito species, threatening to undermine the mosquito vector control programmes [1]. To obviate resistance and the risks for human health and the environment caused by broad-spectrum conventional pesticides, new strategies for insect control have been developed based on the environmentally friendly biopesticides derived from the bacteria *Bacillus sphaericus* (*B. sphaericus*) and *B. thuringiensis*. Highly toxic strains of *B. sphaericus* Neide have been proven to effectively control mosquito-borne diseases of the genera *Culex* (*Cx.*) and *Anopheles* [2-4]. The main strains of *B. sphaericus* used in commercially available products for vector control are 2362 and 1593. It has been shown that continuous exposure to *B. sphaericus*, both in the field and in the laboratory, may lead to selection for resistance [5-8]. Studies have shown that the resistance mechanism in the majority of cases studied is related to the absence of the binary toxin receptor in *Cx. pipiens* (Cpm1) and *Cx.quinquefasciatus* (Cqm1) in the intestinal epithelium of larvae, making it impossible for the Bin toxin to recognize and act on cells [9 -12].The activity of the *B. sphaericus* binary toxin on *Cx. quinquefasciatus* larvae depends on its specific binding to the Cqm1 receptor, a midgut membrane-bound α glucosidase. Evaluation of Cqm1 expression in individual larvae through α glucosidase assays corroborated the allelic frequency revealed by PCR. The $cqm1_{REC}$ allele was present at a detectable frequency in nontreated populations, while the higher frequency in samples from the treated area is, perhaps, correlated with the exposure to *B. sphaericus* [13].

The ribosomal DNA cistron, one of the multigene families frequently distributed in genome in arrays of tandem repeats, is the preferred candidate region for PCR-based species-diagnostic assays because of useful feature of concerted evolution acting on the rDNA array that maintains sequence homogeneity in a species. The rDNA genes in eukaryotes occur in hundreds or thousands copies arranged in tandem arrays; therefore, small amounts of insect bodies are sufficient for the PCR assay [14].

Corresponding Author: Kadria N. Abdel Megeed, Department of Parasitology and Animal Diseases, National Resarch Center, Dokki, Giza, Egypt.

Wassim [15] studied the molecular alteration of the susceptible and resistant *Cx. pipiens* strains (F28) to *B. sphaericus* as homozygous and their progeny of reciprocal crosses as heterozygous based on the genetic variations of the third domain of 28S rDNA.

SSCP is a sensitive electrophoretic technique for detecting nucleotide polymorphisms [16]. SSCP analysis has been used to identify numerous polymorphic loci for the construction of linkage maps of the *Ae. aegypti* genome [17, 18] and holds promise as a tool for extensive and efficient genotyping of individual mosquitoes.

The present study aimed to characterize the molecular differences among the susceptible *Cx. pipiens* mosquito strains (F_{28}), resistant and the progeny resulted from reciprocal cross mating between resistant and susceptible strains by SSCP analysis.

MATERIALS AND METHODS

Mosquitoes: The *Cx. pipiens* colonies used in the study were; a resistant colony at F_{28} (RR>38.70) selected with *Bacillus sphaericus* strain 2362 and a susceptible one (RR>29.04). Both resistant and its susceptible counterparts were derived from the same original colony established from a large number of egg rafts deposited by adult females of field collected 4th larval instars. The resistant and susceptible colonies were obtained after laboratory selection pressure F_{28} [19]. All cross-experiments were done between resistant and susceptible strains from the same breeding area (Qalubiya Gov.). Resistant (R) and susceptible (S) pupae from each colony were kept separately till adult emergence. For reciprocal crosses a 156 virgin individuals were used to form 78 pairs of S♀ X R♂ and a S♂ X R♀. The offspring of the former pairs (F1a) and that of the latter (F1b) were reared to adults under controlled conditions of temperature (27± 2°C), relative humidity (70-80%) and a 16 h photoperiod in walk-in insectaries.

Extraction and Amplification of DNA: DNA was extracted from adult mosquitoes by two methods; Proteinase K digestion followed by organic solvents (Phenol: Chloroform: Isoamyl alcohol) extraction and by using Wizard Genomic DNA Purification Kit[20].A 400 bp region of the D3 ribosomal 28S rDNA gene was amplified using conserved primers Sequences according to Koekemoer [21] are as follows:

D3 A sense : 5` GACCCGTCTTGAAACACGGA 3`
D3 B antisense: 5` TCGGAAGGAACCAGCTACTA 3`.

DNA was amplified as described by Stothard [22] 50 ng of genomic DNA (3–5 µl) was used as PCR template. The reaction mixture (50 µl) contained the following: 50 pmol of each forward and reverse primers, 100 mM Tris-HCl, pH 8.8, 2.5 mM $MgCl_2$, 0.2 mM dNTP (Pharmacia, Sweden), 2.5 U *Bio-Taq* (Bioline, UK) and was overlaid with a single drop of mineral oil to prevent evaporation during thermal cycling.

PCR conditions were an initial 4 cycles (denaturing at 95°C for 30 sec, annealing at 45°C for 30 sec and extension at 72°C for45 sec) followed by 41 cycles (95°C for30 sec, 50°C for 30 sec and 72°C for 45 sec). Amplification product was separated on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and photographed using gel documentation system.

Single Strand Conformational Polymorphism Analysis: Amplification products were subject to SSCP analysis using a protocol detailed by Hiss [23]. PCR products were heated to 95°C for 2 min in equal volume of loading buffer (800 µl formamide, 100 µl 1% bromophenol blue, 100 µl 1% xylene cyanol, 2 ml 0.5 M EDTA, 1 ml 10 M NaOH) and then immediately cooled in an ice-bath for 10 min. The entire mixture was loaded onto a 6% T, 3.5% C polyacrylamide gel [Hoefer SE260 apparatus with 1.5 m spacers (Pharmacia, Sweden)], in TBE (1.34 M Tris-HCl, pH 8.8, 749 mM boric acid, 25.5 mM EDTA). This apparatus and gel were previously equilibrated to 4°C inside a laboratory refrigerator. Samples were separated by electrophoresis at 70 V for 7 h at 4°C, after which, the gels were stained with silver according to Hodgkinson [24].

RESULTS

According to the theory of SSCP technique, DNA polymorphism is detected when the electrophoretic mobilities of DNA bands are reproducibly different (SSCP pattern appears, defined as a constant combination of number and position of single stranded DNA). Particularly to obtain a high rate of mutation detection, the amplicons were subjected to SSCP analysis. The number of bands and their position in the gel showed very clearly the occurrence of DNA sequence variation. Sometimes additional bands could be observed, resulted from the incomplete denaturation of DNA. This however, did not affect the identification of the SSCP patterns. Within the tested specimens, several different SSCP patterns were observed.

Table 1: Molecular weight analysis of SSCP bands of *Cx. pipiens* females and males

Marker	S female	R female	F_{1a} female	F_{1b} female	S male	R male	F_{1a} male
1500	1470	1500	1500	1500	1470	1500	1500
1000							
900						900	
800	865	891	857	832	824		883
700	775	785	760	770	760	756	756
600							
500	561	556					
	500	500	556	556	535	515	
400	471	465	457	438	435	430	
	402	402	400	400	400	402	
300	321	333		314		373	
						318	329
200	278	276	278	276	274	274	274
100							

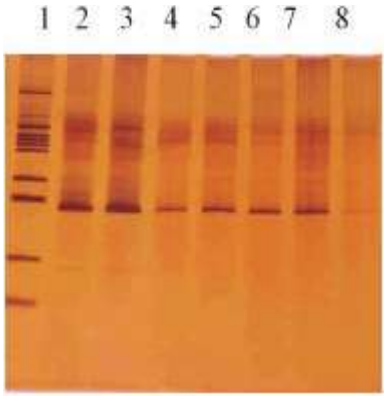

Fig. 1: Single strand conformation polymorphism profiles of 28S gene of resistant, susceptible and hybrid *Cx. pipiens* females and males

Lane 1 Molecular weight marker (Bioron, 100bp)
Lane 2 Susceptible female
Lane 3 Resistant female
Lane 4 Hybrid female (Susceptible female x Resistant male)
Lane 5 Hybrid female (Resistant female x Susceptible male)
Lane 6 Susceptible male
Lane 7 Resistant male
Lane 8 Hybrid male (Susceptible female x Resistant male)

The upper set of bands was the renatured single-strand DNA (RSS), where the lower bands represent denatured single-strand DNA (DSS). Renatured double-strand DNA is intermediate in mobility between the RSS and DSS, but it is seldom visible on a SSCP gel. The diagnostic bands for each species are shown in fig. 1 to show actual specimens of all tested individuals. In order to calculate the relative mobility (rm) of the single strand molecules, a double stranded DNA Ladder (Bioron, 100bp+1.5kb DNA Ladder) was used. The analysis was done by using the Lab image computer based program. The data analysis of SSCP of susceptible, resistant and hybrid *Cx pipiens* mosquito samples showed wide variation in the electrophoretic mobility of DSS bands as shown in table 1. The electrophoretic mobility of DSS bands differed among samples referring to their homozygosity or heterozygosity. At 400 bp the mobility rates of hybrid females and S male were the same while R, S females and R male have the same mobility rate at 402 bp.

DISCUSSION

SSCP analysis of rDNA provides a sensitive and specific method of species identification that is not dependent on having extensive information on interspecific and intraspecific sequence variation. Furthermore, SSCP analysis is sufficiently sensitive to detect single point mutations in rDNA genes. The present results demonstrated that the mobility of RSS and DSS of D3 28S rDNA gene during SSCP analysis were distinct among resistant, susceptible and hybrid *Cx. pipiens* mosquito strains. The D3 28S rDNA gene was previously amplified from resistant, susceptible and hybrid *Cx. pipiens* mosquito strains by Wassim [15]. The length of D3 in 28S rDNA was relatively longer in susceptible strain and decreased gradually from resistant to hybrid. Similar pattern was noted between males (399-345 bp) and females (375-343 bp).

The obtained results agreed with these of Hiss [22]. They found that the mobility of RSS bands was correlated

with the number of substitutions. Such the mobility of the RSS only weakly reflects the numbers and types of substitutions. The mobility of the DSS was independent of sequence length suggesting that its mobility is not a simple function of size and that intra-strand interactions may govern the mobility of this molecule. In the present study, polymorphisms among the resistant male and female in the D3 flanking region of 28S gene rDNA were identified. Banding patterns differed among resistant, susceptible and hybrid progeny. The number of bands was 9 in resistant strains and decreased to 7 in susceptible male. The hybrid progeny had fewer bands less than resistant or susceptible strains. The DSS banding patterns were not stable among all samples may be due to the sequence variation which may be resulted from point of mutations of selection pressure from *B. sphaericus* on *Cx. pipiens* mosquito samples. As a result of point of mutations, polymorphisms could be detected clearly. SSCP can distinguish between homozygous (resistant or susceptible) and heterozygous (progeny of reciprocal crosses) individuals. These results agreed also with Hiss [22]. They stated that there are many technological advantages of SSCP over existing PCR-based techniques for molecular taxonomy.

In SSCP, the PCR primers are either "universal" or at least specific to a particular arthropod group. This provides an advantage over species-specific primer groups because SSCP avoids the labor and expense associated with gathering complete interspecific and intraspecific sequence variation. In addition, some of the cost of primer construction is avoided. The technique is superior to RAPD-PCR or AP-PCR techniques because contamination with non-target DNA from bacterial or protozoan sources is minimal with SSCP. The obtained results of SSCP technique proved to be useful in designing species-specific primers by identifying nuclear and mitochondrial regions that exhibit low intraspecific variability while showing distinct interspecific patterns. Given the speed, sensitivity, low cost and ease of performing SSCP analysis, the technique is likely to find many applications in other fields of insect genetics. SSCP analysis of nuclear genes has been shown to identify heterozygous genotypes at an amplified locus making the technique useful for population genetics [22, 25]. On the other hand, Koekemor [21] used SSCP to distinguish 4 members of the *An. funestus* group collected from 10 different localities in Africa using conserved primers to amplify the D3 domain in the 28S gene. Ribosomal DNA genes have different numerous sites among closely related species. Intraspecific variation was detected in *An. funestus* specimens from east and West Africa. The authers did not expect any variation between males and females because rDNA is sex-linked.

It was concluded that use of rDNA is a good molecular tool to differentiate the susceptible, resistant and their progeny of *Cx. pipiens* treated *with B. sphaericus*.

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