Monitoring of Insecticide Resistance of *Culex pipiens* (Diptera: Culicidae) Colonies-Collected from California

M. A. I. Ahmed, A. Cornel, and B. Hammock

***Abstract*—Several populations of Culex pipiens sensu latu mosquitoes, a vector of mosquito-born flaviviruses such as west nile virus, have developed resistance to pyrethroids in the United States. To design and then institute resistance monitoring, mitigation, and management strategies, knowledge of the mechanisms of resistance will be required. Preliminary investigations conducted on Culex pipiens s.l. populations in California and Southern Africa strongly suggest that this resistance is mediated by enzymatic detoxification and/or target-site sensitivity. The aims are to determine permethrin resistance levels in first generation progeny of colonies-collected Culex spp. from various regions of California and evaluate the levels of detoxification enzyme [esterase and glutathione S-transferase (GST)] and improve diagnostic Knockdown resistance (kdr) in F1 progeny of Culex spp. mosquitoes with confirmed permethrin resistance. In this study, mosquitoes in the Culex pipiens complex were collected at various sites throughout California and tested for esterase, GST, and kdr activities. The esterase, GST, and kdr activities were compared to the corresponding activities found in a pyrethroid-sensitive laboratory strain Culex quinquefasciatus (CQ1). A correlation was found between elevated esterases activities and kdr assay indicating that further investigation should be done to figure out the potential role of enzyme detoxification and kdr assay in conceding resistance to pyrethroids class.**

***Index terms*—*Culex pipiens*, pyrethroids, knockdown resistance (*kdr*), pesticides resistance.**

I. Introduction

Insecticide application is considered the most important component in the global mosquito vector control effort [1]-[3]. Currently, Pyrethroid insecticides are the most widely used insecticides for controlling mosquitoes worldwide. However, mosquito-borne diseases are now once again becoming a problem, largely because of the insecticide resistance (one of the biggest problem related to health and the environment) that has developed in mosquito vectors [4]-[11]. Insecticide resistance is the development of an ability in some individuals of a given organism to tolerate doses of a toxicant which would prove lethal to a majority of individuals in a normal population of the same organism" [12]. Mechanisms of resistance in mosquitoes have attracted attention of many scientists, since they elucidate pathways of resistance development and help those designing novel strategies to prevent or, at least, minimize the spread and evolution of resistance. The resistance mechanisms have been identified in several strains of mosquitoes [13]-[16] and multi-mechanisms appear to be common phenomena in pyrethroid resistance of these insect pests, [16] which can interact to increase the levels of resistance. Furthermore, more than 100 mosquito species are known to have developed resistance to one or more insecticides [17], including 56 species of anopheline and 39 species of culicine mosquitoes [18]. The resistance mechanism of increased sequestration or detoxification has contributed to a decrease in the effective dose of insecticides available at the target site, while decreased target site sensitivity contributes to the ineffective binding of a given dose [19]-[21]. The three gene families, cytochrome P450 monooxygenases (cytochrome P450s), hydrolases, and GSTs, are mainly involved in the detoxification of insecticides, whereas, three proteins, including sodium channels, acetylcholinesterase, and GABA receptor, have been mainly implicated in decreased target site sensitivity in insect nervous system [20]. The other mechanism conferring pyrethroid resistance in mosquitoes is the target site insensitivity of sodium channels. The voltage-gated sodium channel is the primary target of pyrethroid insecticides [13] and its insensitivity has been associated with pyrethroid resistance in several kdr insect species, including mosquitoes [22]-[23]. A substitution of leucine to phenylalanine, termed the kdr mutation, in the S6 hydrophobic segment of domain II of the para-type sodium channel gene is consistently associated with knockdown resistance in several insect species, including mosquitoes. [22]-[25]. In our study, we evaluate the levels of detoxification enzyme (esterase and GST) and detect kdr in F1 progeny of Culex spp. mosquitoes with confirmed permethrin resistance.

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II. Material and Methods

A. Chemicals

Fluorescent substrate S-acetate for esterases assay and general substrate chlorodinitrobenzene (CDNB) for GST assay.

B. Mosquitoes

Ten field colonies (5-7 days old adults) obtained from different cities with different environmental community in California (Table I).

TABLE I: CITIES FROM CLAIFORNIA STATE WHICH THE COLONIES OBTAINED FROM

City	Community
Orange Cove	urban
Parlier	rural
Exeter	rural
Fresno	urban
Chico	urban
Kingsburg	urban
Clovis	urban
Santa Fe Springs	urban
La Quinta	urban

CQ1 colony was used as pyrethroid-susceptible colony that originated from Merced, California

C. Preparation of Mosquito Extracts

Approximately 20 of whole 5-7 days old adults were homogenized in 500 µl 0.1 M ice-cold sodium phosphate buffer (pH 7.6) manually in Ependorf tube. The homogenate was centrifuged at 10000 Xg for 15 min and the supernatant was filtered through glass wool for 15 min and centrifuged at 40 C at 10,000 Xg for 1 h. The resulting microsomes were suspended in 2 ml buffer. The supernatant and the microsomes were stored at 800 C until used.

D. Enzymes Activities

The activity of esterases for fluorescent esterase activity assays were measured in black 96-well at 30 C with a spectrafluor Plus Fluorometer (Tecan, Research Triangle, NC). Substrate was prepared in 10 mM ethanol. Reaction mixture contained 20 µl protein, 180 µl 20 mM Tris/HCl buffer (pH 8.0), and 1 µl substrate (total volume 201 µl). Three replicates were carried out for the substrate. Fluorescence was monitored with excitation at 330 nm and emission at 465 nm.

GST activity assay generated by adding 10 µl of mosquito homogenate, 10 µl of CDNB, and 10 ul glutathione in 270 µl 0.1 M sodium phosphate buffer (pH 6.5) (total volume 300ul). The reaction was measured my using Spectramax microplate spectrophotometer by measuring absorbance at 340 nm at 300 C.

E. Kdr Detection

Kdr detection was carried out by PCR elongation assay. PCR elongation assays were conducted and modified as described previously [26]. Briefly, The optimized PCR reaction mix for detecting the Leu-Phe substitution included 0.25 µL of the AGSWA primer (5'-GGCCACTGTAGTGATAGGAAATTTA-3') labeled with green fluorescence (5'-Hex modification), 0.12 µL of the AGRWA primer (5'-GGCCACTGTAGTGATAGGAAATTTT- 3') labeled with blue fluorescence (5'-Fam), 0.25 µL of the AGREV primer (5'-GCAAGGCTAAGAAAAGGTTAAGCA- 3'), 0.45 µL of dNTPs (10 mmol/µL), and 0.125 L of Taq (Ependorf) and 1 uL of DNA template in a 25-µL reaction with 10× buffer, 5× PCR enhancer (Ependorf), and 2.5 µL magnesium chloride (25 mmol/µL). PCR amplifications were done on MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and included an initial 2 minutes at 95°C, followed by 25 cycles of 1 minute at 95°C, 30 seconds at 63°C, 30 seconds at 72°C, and a final extension step at 72°C for 5 minutes. PCR products were diluted in H_2O 10–40 times before they were mixed with Genescan 400HD size standard (Applied Biosystems, Foster City, CA) and run on an ABI 3100 capillary sequencer (Applied Biosystems). Individuals were genotyped for *kdr* based on the presence and intensity of the allele specific green and blue peaks at size 110 bp.

Statistical analyses of changes in *kdr* frequency. The changes in the frequency of susceptible *SS*, resistant *RR*, and heterozygote *RS* individuals were analyzed using logistic regression analysis. The variable collection date was considered an ordinal variable [27].

III. RESULTS AND DISCUSSIONS

Esterase activities are presented in Fig. 1. Compared to CQ1 as susceptible colony, elevated activities of esterase toward *S*-acetate substrate were measured in adult stage of all field colonies and the highest significant value was in Fresno colony (2.49-fold compared to CQ1). Whereas, the rest of the colony didn't show any significant elevation. Interestingly, *S*-acetate considered a general fluorescent substrate that is specific mimics to pyrethroid type II, however, it shows that it was specific in detecting the elevated esterase activities associated in pyrethroid resistant problem in California.

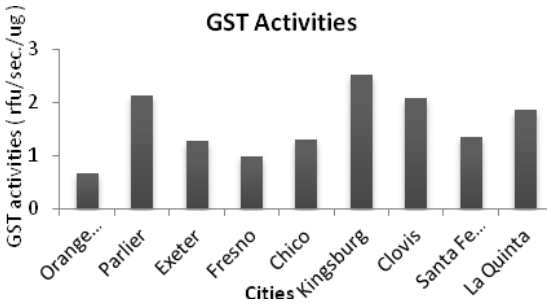

Fig. 1. Comparison of 5-7 days old adults GST activities as relative fluorescent units/second/µg protein (rfu/sec./µg) of *Culex pipiens* colonies with CDNB.

Furthermore, the elevation of GST activities was detected in the field colonies using the general substrate (CDNB) and the most significant values were in Parlier, Kingsburg, and Clovis and the values were 2.13-, 2.52-, and 2.07-fold respectively compared to CQ1 colony (Fig. 2).

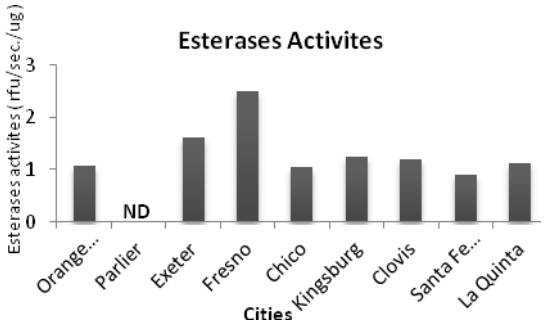

Fig. 2. Comparison of 5-7 days old adults GST activities as relative fluorescent units/second/µg protein (rfu/sec./µg) of *Culex pipiens* colonies with *S*-acetate.

The *kdr* allele was detected in all colonies. *Kdr* was found at particularly high frequencies (R-allele) in Fresno, Chico, La Quanta, and Exeter with percentage of 63.64%, 55%, 55%, and 54.62% respectively (Fig.3). Whereas, highest counted homozygote-resistant allele (RR-allele) was for Fresno, Chico, Exeter, and La Quanta with percentage of 40.91%, 40%, 33.85%, and 30% respectively. However, the highest percentage of heterozygous (RS-allele) was found in Kingsburg, Santa Fe Spring, Parlier, and Clovis (71.79%, 61.11%, 54.55%, and 54.46% respectively) (Fig. 4).

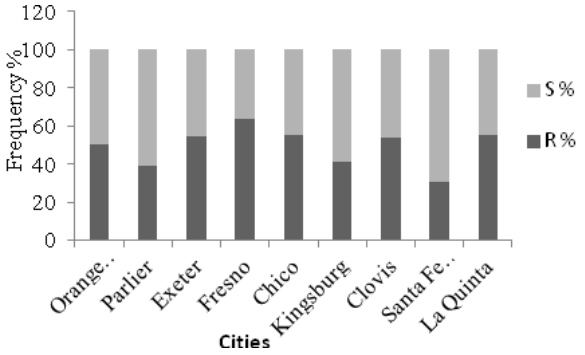

Fig. 3. Changes in the frequencies of R-allele and S-allele in different colonies of 5-7 days old adults *Culex pipiens*.

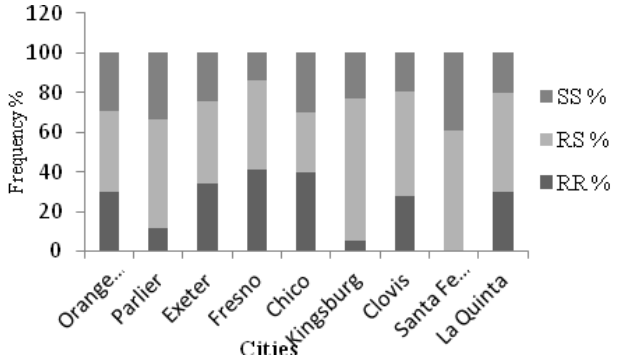

Fig. 4. Changes in the frequencies of homozygote susceptible (SS), heterozygote (RS), and homozygote resistant (RR) in different colonies of 5-7 adults *Culex pipiens*.

The results of the study showed that there is correlation between the elevated esterase activities and kdr resistance gene detection, especially in Fresno city. However, the intensive uses of pyrethroid class as common pesticides in California brought this kind in crucial issue of pyrethroid resistance. Furthermore, [8] demonstrated the first discovery of pyrethroid-resistance in field mosquitoes (Culex pipiens) in the USA. After that, many efforts have been done to create flexible tools for monitoring resistance. In our study, we optimize simple methods to detect the resistance using the enzymatic assays and kdr mutation methods. However, the kdr allele was found in the field colonies suggest that the frequency of resistant individuals in these populations could reach new levels in the near future. Further investigation should be done for monitoring resistance and developed more sensitive fluorescent substrates. Moreover, improving methods and using alternative ways for controlling pests rather than pyrethroids alone that could help to reduce the chance to develop persistent resistance to pyrethroids.

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