Development of a *Culex kdr* Assay for the Detection of Pyrethroid Resistance

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Introduction

Many species of mosquitoes within the *Culex* genus are vectors for pathogens such as West Nile virus (WNV), St. Louis Encephalitis (SLEV) and filariasis (Farajollahi et al. 2011). Chemical controls, among other measures, are used to mitigate the spread of vector borne diseases, but may result in pesticide resistance. A single nucleotide polymorphism (SNP) in the knockdown resistance (*kdr*) locus of the *voltage gated sodium channel* (*vgsc*) gene of *Culex* mosquitoes confers knockdown resistance to pyrethroids. The most common mutation conferring pyrethroid resistance among *Culex* species is the L1014F mutation. PCR-based assays that detect these SNPs in *Culex* species are currently available only for *Culex pipiens* and *Culex quinquefasciatus* (Chen et al. 2010). Under the threat of widespread resistance, we sought to develop a quantitative reverse transcriptase (qRT)-PCR assay that detects the most common *kdr* mutation in *Culex* species that leads to pyrethroid resistance. Our original goal was to develop this assay for use in *Culex tarsalis*. However, after comparing the cDNA sequences of other *Culex* vectors, we discovered that the qRT-PCR method created a more conserved template compared to its quantitative PCR counterparts, allowing the assay to perform for multiple *Culex* species.

Methods

We designed primer and probe sequences (Integrated DNA Technologies, Coralville, Iowa) using Primer3Plus software based on the *Cx. tarsalis vgsc* complementary DNA sequences (Table 1). Mosquitoes were collected from various trap sites within Alameda County and their RNA was isolated using the MagMAX – 96 Viral RNA Isolation Kit (ThermoFisher Scientific) and the *kdr* single nucleotide polymorphism evaluated using qRT-PCR. Briefly, each RT-PCR reaction featured a volume of 25 microliters consisting of 6.25 microliters Taqman™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Waltham, MA), 2.25 microliters (0.9mM) of RT*kdr*_Fwd and RT*kdr*_Rev primers, 0.6 microliters (0.25mM) of RT*kdr*_WT and RT*kdr*_Mutant probes, 1 microliter of RNA template and 12.05 microliters of nuclease free water. Cycling conditions were based on TaqMan Fast Virus 1-Step Master Mix's Fast Cycling Mode and are as follows: 50°C for 5 minutes, 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The *Culex* RT-PCR *kdr* assay was validated through sanger sequencing of the PCR products.

Results and Discussion

We ascertained that a substantial increase in FAM or HEX fluorescence indicated homozygous wildtype or mutant genotype, respectively. An increase in both FAM and HEX in relatively equal fluorescence indicated a heterozygous genotype (Fig. 1). Using sequencing as a reference, we determined the accuracy of the *Culex* RT*kdr* assay to be 99% (not shown). We tested 1,383 *Culex* specimens collected from Alameda County with the *Culex* RT*kdr* assay and found 362 (26%) were resistant, 285 (21%) were heterozygous, and 736 (53%) were susceptible. The resistant allele frequency was 0.57 among *Cx. pipiens*, 0.15 among *Cx. tarsalis* and 0.00 among *Culex erythrothorax*. *Culex pipiens* complex mosquitoes are notorious for their resistance. Prior studies also found high resistant allelic frequencies among *Cx. pipiens* mosquitoes (Yoshimizu et al. 2020, McAbee et al. 2003, Ahmed et al. 2012). Additional, we discovered that *Cx. pipiens* and *Cx. tarsalis*

Table 1.—Primer and probe sequences used in the *Culex* RT*kdr* assay.

Name	Sequence (5′ -> 3′)
Primers	
RTSeq_Fwd	ATCTGACGTTTGTGCTCTGC
RT*kdr*_Fwd	CCTGCATTCCGTTCTTCTTG
RT*kdr*_Rev	GCGATCTTGTTCGTTTCGTT
Probes	
RT*kdr*_WT	FAM-GGTTAAGTA/ZEN/ CGACTAAGTTTCCTATCACTAC-3IABkFQ
RT*kdr*_Mutant	HEX-GGTTAAGTA/ZEN/ CGACAAAGTTTCCTATCACTAC-3IABkFQ

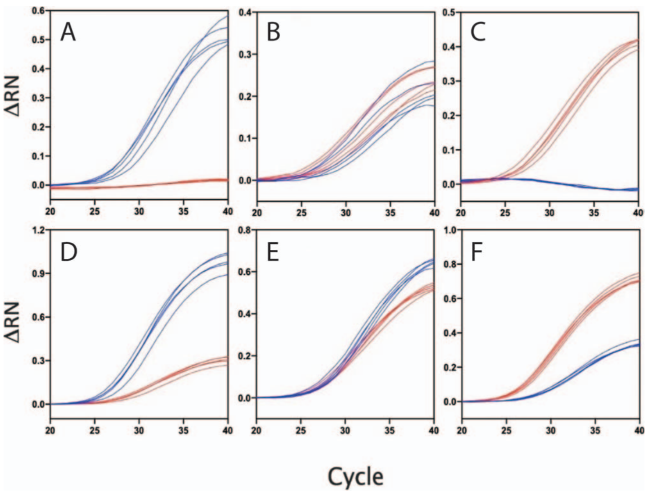

Figure 1.—Amplification plots (ΔRN vs Cycle Number) for *Culex pipiens* (A-C) and *Culex tarsalis* (D-F) with 5 representative specimens selected for each genotype. The blue and red lines represent amplification of the wildtype and mutant probes, respectively. (A) *Culex pipiens* homozygous wildtype; (B) *Culex pipiens* heterozygous; (C) *Culex pipiens* homozygous mutant; (D) *Culex tarsalis* homozygous wildtype; (E) *Culex tarsalis* heterozygous; (F) *Culex tarsalis* homozygous mutant.

mosquitoes from the inland region of Alameda County were more resistant than their coastal counterparts, with resistant allelic frequencies of 0.54 and 0.21, respectively (Fig. 2). The *Cx. erythrothorax* mosquitoes were collected from constructed marsh habitats within the coastal region where they may be exposed to all manner of surface runoff that contain pyrethroid insecticides. That none of the *Cx. erythrothorax* contained an allele that is associated with pyrethroid resistance suggests that if the runoff contained pyrethroids, it had been degraded or diluted to the point of being functionally inactive. According to the California Pesticide Information Portal (Calpip) database, pyrethroids applied in the County are mainly for agriculture and commercial pest control. Runoff due to rainfall is likely transporting pyrethroid residues to mosquito larval habitats, possibly contributing to resistance in the inland region of the County (Tang et al. 2018).

Conclusions

Despite Alameda County Mosquito Abatement District applying less than 10 ounces of adulticides between 2010 and 2019, pyrethroid resistance remains prevalent in Alameda County. Commercial use of pesticides for both structural and agricultural control of pests may be contributing to the higher proportion of resistance we observed inland. Additionally, species behavior and habitat preferences may contribute to insecticide resistance. The *Culex* RT*kdr* assay not only satiates the need for a *Cx.*

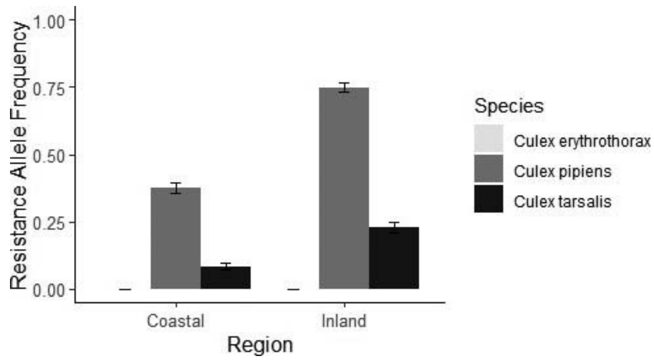

Figure 2.—Resistant allele frequency (F_R) by species and region. Light Grey, dark grey and black bars represent F_R for *Culex erythrothorax* (no resistance detected), *Culex pipiens* and *Culex tarsalis*, respectively.

tarsalis PCR pyrethroid detection assay, but also allows for testing multiple *Culex* species on one PCR run.

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References Cited

Ahmed, M. A. I., A. Cornel, and B. Hammock. 2012. Monitoring of insecticide resistance of *Culex pipiens* (Diptera: Culicidae) colonies-collected from California. Int. J. Environ. Sci. Dev. 3: 346–349.

(CalPip) California Department of Pesticide Regulation. 2020. California Pesticide Information Portal Application. https://calpip.cdpr.ca.gov/main.cfm. Accessed 10 March 2020.

Chen, L., Zhong, D., Zhang, D., Shi, L., Zhou, G., Gong, M., Zhou, H., Sun, Y., Ma, L., He, J., Hong, S., Zhou, D., Xiong, C., Chen, C., Zou, P., Zhu, C., and Yan, G. 2010. Molecular ecology of pyrethroid knockdown resistance in *Culex pipiens pallens* mosquitoes. PLoS ONE, *5*(7): e11681.

Farajollahi, A., Fonseca, D. M., Kramer, L. D., and Kilpatrick, A. M. 2011. Bird biting mosquitoes and human disease: A review of the role of *Culex pipiens* complex mosquitoes. Infect. Genet. Evol. 11: 1577–1585.

McAbee, R. D., Kang, K.-D., Stanich, M. A., Christiansen, J. A., Wheelock, C. E., Inman, A. D., Hammock, B. D., and Cornel, A. J. 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var *molestus* from Marin County, California. Pest. Manag. Sci. 60: 359–368.

Tang, W., Wang, D., Wang, J., Wu, Z., Li, L., Huang, M., Xu, S., and Yan, D. 2018. Pyrethroid pesticide residues in the global environment: An overview. Chemosphere 191: 990–1007.

Yoshimizu, M. H., Padgett, K., & Kramer, V. 2020. Surveillance of a *kdr* resistance mutation in *Culex pipiens* (Diptera: Culicidae) and *Culex quinquefasciatus* in California. *J. Med. Entomol*. 57: 645–648.