

Potential for sublethal insecticide exposure to impact vector competence of *Aedes albopictus* (Diptera: Culicidae) for dengue and Zika viruses

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Abstract: Chikungunya, dengue, and Zika viruses (CHIKV, family *Togaviridae*, genus *Alphavirus*; DENV and ZIKV, family *Flaviviridae*, genus *Flavivirus*) are arboviruses that cause human epidemics. Due to the lack of vaccines for many mosquito-borne diseases, there is a need for mosquito control. In the US and other regions, residual barrier insecticide sprays are applied to foliage where female mosquitoes rest and/or sugar feed between blood meals. Residual sprays are an important control method for anthropogenic day-active sylvan mosquitoes such as *Aedes albopictus* (vector of CHIKV, DENV, and ZIKV) that are difficult to control using ultralow-volume sprays applied only at dusk or dawn when these mosquitoes are not active. In this exploratory study, we analyzed the extent to which ingestion of a sublethal dose of the active ingredient bifenthrin affected vector competence (i.e., infection, dissemination, and transmission) of *Ae. albopictus* for DENV and ZIKV. Two incubation periods (IPs; 7 and 14 d) were tested at 28°C for insecticide-fed and sugar-fed mosquitoes. We show that mosquitoes that were fed bifenthrin (0.128 µg/mL) mixed with sucrose solution exhibited significantly lower DENV infection rates and body titers after a 14-d IP. During the 7-d IP, one mosquito (sugar group) transmitted ZIKV. During the 14-d IP, 100% of mosquitoes showed body and leg ZIKV infections, and one mosquito (sugar+bifenthrin group) transmitted ZIKV. This is a preliminary communication, and more studies will be required to further investigate these findings. We expect the findings of this small-scale study to spur larger-scale investigations of the impacts of insecticides on mechanisms regulating vector competence, and exposure to other active ingredients, and aid in development of new insecticides.

Keywords: mosquito, insecticide exposure, arbovirus

Introduction

Chikungunya, dengue, and Zika viruses (CHIKV, family *Togaviridae*, genus *Alphavirus*; DENV and ZIKV, family *Flaviviridae*, genus *Flavivirus*) cause human epidemics across the world. In recent years, DENV has become more important than malaria in morbidity and economic impact, and several recent ZIKV outbreaks have affected humans, with particular concern for development of microcephaly in infants.¹⁻⁴ The vectors of both arboviruses are *Aedes aegypti* L. and *Aedes albopictus* Skuse.⁵ It has been shown that CHIKV has increased its global spread, in part, due to the high degree of vector competence in *Ae. albopictus*.^{6,7}

Residual insecticide barrier sprays are increasingly used for mosquito control in conjunction with, or instead of, ultralow-volume applications with no residual effects. The effects of residual sprays can persist on foliage for up to 21 d, but degrade over

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time, depending on environmental conditions.⁸ Mechanisms for insecticide resistance (e.g., overproduction of detoxification enzymes) can lower energy reserves and reduce immune functions that may impact vector competence and fitness.^{9–11} Mosquito populations may develop resistance due to exposure to sublethal doses of insecticides; therefore, more information is needed to understand how insecticides may influence vector competence. While both *Ae. aegypti* and *Ae. albopictus* oviposit in natural and artificial water-holding containers within human dwellings, *Ae. albopictus* is found in sylvan environments, thereby making evaluation of barrier insecticides that are applied to foliage of adult resting sites important. One US study showed dichlorodiphenyltrichloroethane (DDT)-resistant populations of *Ae. albopictus*.¹² A separate study carried out by us showed possible resistance and/or resistance in *Ae. albopictus*, *Culex pipiens*, and *Culex quinquefasciatus* from several US regions exposed to bifenthrin, deltamethrin, etofenprox, malathion, permethrin, and phenothrin.¹³

Ae. aegypti adults were exposed to malathion-impregnated papers prior to exposure to a DENV-infected blood meal.¹⁴ The same study found no difference in infection rates (whole body) between treatment and control groups at 10 d postinfection. When insecticide-resistant *Anopheles gambiae* infected with *Plasmodium falciparum* were exposed to deltamethrin-treated bednets, they showed reduced parasite loads, compared to mosquitoes exposed to untreated bednets.¹⁵ Others have shown that insecticide-resistant mosquitoes infected with *P. falciparum* are more susceptible to DDT than uninfected mosquitoes.¹⁰ Nutritional and larvicide (*Bacillus thuringiensis israelensis* [*B.t.i.*]) stress reduced occurrence of *P. falciparum* in *An. gambiae*, but did not decrease parasite concentration.¹⁶ The exposure of *Ae. aegypti* larvae to sublethal concentrations of *B.t.i.* did not impact adult survivorship or DENV infection or dissemination rates; however, adults from *B.t.i.*-fed larvae laid more eggs, compared to the control.¹⁷ The same study showed that *B.t.i.*-fed mosquitoes developed faster than the control mosquitoes. There is clearly a link between insecticide exposure, mosquito infection, and potentially, mosquito fitness that should be explored.

There are limited published vector competence studies for *Ae. albopictus* and ZIKV, especially for US populations. There is no vaccine for ZIKV, and although there is a vaccine for DENV, it is not yet at sufficient production capacity and does not protect all age groups in the population.^{18,19} Thus, protection from mosquito bites and targeted vector control are of utmost importance. The anthropogenic nature of the DENV and ZIKV transmission cycle, along with international travel and vector range expansion, necessitates that

health agencies prepare for outbreaks. We investigate the extent to which mosquito–insecticide interactions impact vector competence for *Ae. albopictus* infected with DENV and ZIKV, thereby providing practical information to improve control of a primary vector.

Materials and methods

Insecticide detection in stock

A standard solution of bifenthrin was prepared by dissolving 0.01 g of technical-grade active ingredient (AI; Sigma-Aldrich, St. Louis, MO, USA) in acetone (40 mL) and used to prepare the calibration curve for quantification. The concentration of the prepared AI (bifenthrin) stock was confirmed in order to verify the bifenthrin dose being fed to mosquitoes by analyzing three to four replicate samples (1 μ L) of the stock solution via capillary gas chromatography with flame ionization detector (GC-FID; Agilent GC 6850 instrument; Agilent Technologies, Santa Clara, CA, USA). The GC conditions used were adapted from our previous study.⁸ The linearity of the detector response was checked before conducting analysis by using the calibration curves.

Mosquitoes and insecticide treatment

We used an *Ae. albopictus* (F₂₂) colony originating from Louisiana. We tested the insecticide resistance/susceptibility status of this population in accordance with the Centers for Disease Control (CDC) bottle bioassay protocol.²⁰ Our findings showed 100% mortality in this *Ae. albopictus* colony after exposure to a diagnostic dose of 12.6 μ g bifenthrin/bottle at a diagnostic time of 30 min.

To improve feeding rate and increase sample size, mosquitoes were deprived of sucrose and fed only water for 48 h prior to the experiments. To test the effects of insecticide ingestion on vector competence, mosquitoes in the insecticide group were fed a solution containing 0.128 μ g/mL bifenthrin mixed with a 20% sucrose solution for a feeding period of 4 h. Mosquitoes were observed feeding on the sucrose solution in both treatment and control groups. The concentration of bifenthrin was verified by GC-FID using our previously established methods.⁸ Mosquitoes in the control group were fed a 20% sucrose solution containing no insecticide for 4 h. After this period, the sucrose solution was removed from both groups and replaced with water until infectious blood meals were offered to mosquitoes 48 h later.

Vector competence evaluation

Vector competence assays were conducted using our previously established methods.²¹ Briefly, the mosquitoes were

assayed for DENV and ZIKV during 7- and 14-d IPs. A Southeast Asian DENV-2 isolate (16803) and a Puerto Rican ZIKV isolate (PRVABC59) were used for infecting mosquitoes. Viral cultures were propagated for use in blood meals according to standard procedures used in our laboratory.²² Freshly propagated DENV and ZIKV were mixed individually with defibrinated bovine blood (Hemostat, Dixon, CA, USA) prior to mosquito feeding. Mosquitoes were blood fed in 1-L cardboard cages with mesh screening and held at 28°C with 80% humidity in a 14:10 light:dark cycle before being transferred to incubators at 28°C for the duration of the experiments. Samples of the blood meal were stored at -80°C until further processing to determine viral titer. Mosquitoes (8- to 9-d old) were fed for 45 min on pledgets soaked with warmed (35°C) DENV- or ZIKV-infected blood at a dose of 5.4 log₁₀ plaque-forming unit equivalents/mL. Mosquitoes were anesthetized with cold, and each fully engorged blood-fed female was transferred to a 1-L cage (according to treatment) for the duration of the experiment.

At the end of 7- and 14-d IP, 5–10 live mosquitoes from each group were anesthetized with cold, and legs and wings were removed following standard methods used in our laboratory.²³ Legs were transferred to sample tubes containing 0.5 mL of RNAlater and two 5-mm glass beads and coded for each mosquito. Mosquitoes were allowed to expectorate saliva into capillary tubes filled with a 1:1 mixture of fetal bovine serum and 20% sucrose for 30 min using our methods as previously described.²³ Following the salivation period, bodies and saliva samples were transferred to separate tubes categorized by mosquito code containing 0.5 mL of RNAlater and two 5-mm glass beads, and all were stored at -80°C until further processing.

Virus assay

Samples were homogenized and centrifuged as described previously.²² Nucleic acids were extracted using the QIAmp viral RNA kit (Qiagen, Valencia, CA, USA). The amount of viral RNA in each sample was determined using quantitative real-time TaqMan reverse transcriptase polymerase chain reaction (qRT-PCR) with the LightCycler[®] 480 Instrument (Roche, Mannheim, Germany) and the Superscript III One-Step qRT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) programmed as follows: 48°C for 30 min, 95°C for 2 min, and 45 cycles at 95°C for 15 s, 60°C for 30 s, and finally, 40°C for 30 s. The following primers/probes were used: ZIKV 1163c: 5'-CCACTAACGTTCTTTTGCAGACAT-3', ZIKV 1087: 5'-CCGCTGCCCAACACAAG-3', ZIKV 1108: 5'-/56-FAM/AGCCTACCT/ZEN/TGACAAGCAGTCAGACACTCAA/

3IABkfq/-3'; DENV 237: 5'-CATGGCCCTKGTGGCG-3', DENV 305: 5'-CCCCATCTYTTTCAGTATCCCTG-3', DENV 254: 5'-/56-FAM/TCCTTCGTTTCCTAACAATC/C/3BHQ_2/-3' (IDTDNA, Coralville, IA, USA). Standard curves were based on plaque assays used to determine titer. The infection rate was the percentage of all blood-fed mosquitoes that had infected bodies. The dissemination rate was the percentage of blood-fed mosquitoes that had infected legs. The transmission rate was the percentage of blood-fed mosquitoes that had infected saliva.

Statistical analysis

The chi-square test was used to detect significant differences in rates of infection, dissemination, and transmission between groups ($P < 0.05$). Nonparametric analysis of variance was used to evaluate differences in titers of body and leg tissues and saliva between IPs and treatments. If significant differences were observed, then a Duncan test was used to determine differences in the means.

Results

Effects of insecticide ingestion on vector competence

During the 7-d IP, we observed 80%–90% DENV infection rate and 10%–30% DENV dissemination rate in both experimental groups (Table 1). Mosquitoes that were fed bifenthrin (0.128 µg/mL) exhibited statistically equivalent DENV infection rates ($\chi^2 = 5.49$, $df = 1$, $P = 0.057$) and significantly lower body titers ($F = 6.83$, $df = 1$, $P = 0.024$) after a 14-d IP, as compared to mosquitoes fed only sugar. No DENV transmission was observed during the 7- or 14-d IP.

During both the 7- and 14-d IP, 100% of mosquitoes showed ZIKV body infections. Mosquitoes in the bifenthrin group showed 100% dissemination rates during both the 7- and 14-d IP; however, mosquitoes fed only sugar prior to the ZIKV-infected blood meal showed 40% and 100% dissemination during the 7- and 14-d IP, respectively. Two mosquitoes transmitted ZIKV (sugar group – 7-d IP; sugar+bifenthrin group – 14-d IP; Table 1).

Discussion

Research aimed at elucidating the effects of insecticide–mosquito interactions has largely been focused on vector control and development of resistance. Little attention has been given to the impacts of insecticides on vector competence. Insecticide pressure on mosquito populations is a continuing threat as mosquito control is the primary method of protecting humans from mosquito-borne diseases.

Table 1 Mean titers (\log_{10} plaque-forming unit equivalents [PFUeq] DENV/mL or ZIKV/mL) \pm standard error of rate of infection, dissemination, and transmission for *Aedes albopictus* stratified by pre-blood-feeding meal, virus type, and incubation period

Pre-blood-feeding meal	Number tested	Number infected (%) ¹	Number disseminated (%) ¹	Number transmitted (%) ¹	Body titer ²	Leg titer ²	Saliva titer ²
7 d postinfection							
DENV							
Sugar	10	9 (90) ^a	3 (30) ^a	0	4.3 \pm 0.4 ^a	2.5 \pm 0.1 ^a	–
Sugar+bifenthrin	10	8 (80) ^a	1 (10) ^a	0	4.3 \pm 0.3 ^a	2.5 ^a	–
ZIKV							
Sugar	10	10 (100) ^a	4 (40) ^b	1 (10)	5.1 \pm 0.3 ^a	0.1 \pm 0.0 ^a	1.4
Sugar+bifenthrin	10	10 (100) ^a	10 (100) ^a	0	4.2 \pm 0.5 ^a	0.4 \pm 0.2 ^a	–
14 d postinfection							
DENV							
Sugar	10	9 (90) ^a	2 (20) ^a	0	5.3 \pm 0.2 ^a	3.7 \pm 0.3 ^a	–
Sugar+bifenthrin	10	4 (40) ^b	1 (10) ^a	0	3.5 \pm 1.1 ^b	2.3 ^a	–
ZIKV							
Sugar	10	10 (100) ^a	10 (100) ^a	0	4.9 \pm 0.4 ^a	2.1 \pm 0.6 ^a	–
Sugar+bifenthrin	5	5 (100) ^a	5 (100) ^a	1 (20)	5.4 \pm 0.2 ^a	3.3 \pm 0.2 ^a	3.2

Notes: Analyses were conducted separately for each virus. ¹Same letter in the same column for each virus indicates no significant difference between treatments by chi-square test. ²Same letter in the same column indicates no significant difference between treatments by means comparison.

Ae. albopictus is one of the primary vectors of DENV and CHIKV, is a competent vector in the field²⁴ and laboratory,^{6,7,21,25,26} and feeds on the blood of humans and other animals.²⁷ A study in Gabon (Central Africa) showed that 137 and 45 *Ae. albopictus* pools were positive for ZIKV and DENV, respectively, although transmission was not tested in these cases.²⁸ Another study showed transmission (capillary tube assay) of ZIKV by several *Aedes* spp., including *Ae. aegypti*.²⁹ The same study did not evaluate ZIKV vector competence in *Ae. albopictus*.

We have shown infection, dissemination, and transmission of DENV²⁶ and ZIKV (current study) for *Ae. albopictus*. After a 14-d IP, *Ae. albopictus* that ingested bifenthrin showed increased ZIKV transmission rate and decreased DENV infection rate. More information is needed to elucidate these differences between viruses and insecticide groups as the current exploratory study had small sample sizes. Additional experiments are planned to explore these differences at a larger scale. Sublethal doses of insecticides obtained (by contact and/or ingestion) from foliage utilized in barrier sprays may impact gut bacteria, the mosquito immune response, or affect other unknown factors that may impact subsequent vector competence.

We have previously shown that, after field application of bifenthrin, leaf surfaces contain 0–25.6 μ g/mL,⁸ and the current study exposed *Ae. albopictus* to 0.128 μ g/mL of bifenthrin. Future studies should assay a variety of bifenthrin doses in this range (0–25.6 μ g/mL) from sublethal to lethal concentrations. Exposure of mosquitoes to bottles (as in the CDC bottle bioassay) coated with active ingredients

of insecticide would be another alternative to insecticide ingestion, and we are planning to investigate this method of insecticide exposure. Our findings that ZIKV is transmitted at 7 d (one mosquito in the sugar-only group) and at 14 d (one mosquito in the sugar+bifenthrin group) should be interpreted with caution due to our sample sizes of 5–10 mosquitoes in each treatment group used in this exploratory experiment. Larger-scale studies with increased sample size should be planned to further characterize the vector competence of different populations of *Ae. albopictus*. This is a preliminary communication, and more studies are needed to investigate this aspect of vector competence.

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A poster of this paper was presented at the East Carolina University World Environmental Health Day in September 2016, and the abstract with interim findings can be viewed at: <http://thescholarship.ecu.edu/handle/10342/5963>.

Disclosure

The authors report no conflicts of interest in this work.

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