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Original Article

Validation of VectoBac WDG for aerial larviciding of *Aedes* against Zika introduction in East Central Florida

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ABSTRACT

To block the local transmission of Zika virus (ZIKV) in Volusia County, its primary vector mosquitoes *Aedes aegypti* and *Ae. albopictus*, at larval stage, were controlled by spraying very-fine to fine (VF/F) droplets of VectoBac WDG (*Bacillus thuringiensis israelensis*) at the rate of 0.56 kg/ha in four areas using a helicopter low-volume (LV) system. The spray effects were verified using droplet-collecting cards and cups set in the fields. Cup bioassay on *Ae. aegypti* larvae was conducted to estimate the mortalities. The droplet sizes and densities ranged between 80-364 µm and 16-29 droplets / cm^2, respectively. The overall mortality reached 93 ± 15% ($\tilde{x}$ ± SD) at 24 hours and 95 ± 13% at 48 hours, and no significant mortality difference was detected among the coverage treatments, between the yard and forest settings, and among the spray areas. The results indicate expected larval control effects. The effectiveness influenced by weather, humidity and forest were discussed, and the relevant improvements were recommended.

KEYWORDS: *Aedes*, mosquito larvae, area-wide control, helicopter LV spray, *Bacillus thuringiensis israelensis*, biological control.

INTRODUCTION

Aedes aegypti (L.) and *Aedes albopictus* (Skuse) are peridomestic mosquito species and are the known vectors transmitting Zika (ZIKV, family *Flaviviridae*, genus *Flavivirus*), dengue (DENV, family *Flaviviridae*, genus *Flavivirus*) and chikungunya (CHIKV, genus *Alphavirus*, family Togaviridae) viruses to people throughout the world [1, 2]. *Aedes aegypti* and *Ae. albopictus* are both present in Florida, and the state often leads the US in travel-related human cases of each arbovirus [3, 4]. In addition, autochthonous transmission of each virus has occurred in the state [5].

In Volusia County of East Central Florida (Figure 1), both *Ae. aegypti* and *Ae. albopictus* adults have been captured on traps year round, and winter oviposition has been observed in *Ae. albopictus* (Unpublished data). During 2015-2016, there were 4,593 travel-associated ZIKV cases and 216 locally acquired Zika cases in the contiguous US with 98% of these autochthonous ZIKV cases occurring in Florida. While local transmission was absent in Volusia County in 2016, twelve travel-related ZIKV cases were identified [6]. A potential autochthonous ZIKV case, ultimately determined to be a false positive, provided the impetus for the Volusia County Mosquito Control (VCMC) to initiate aerial larviciding directed at peridomestic mosquito production sites.

Larval control and larviciding are fundamental in integrated mosquito management (IMM) [7]. Among several active ingredients used to control mosquito larvae, *Bacillus thuringiensis israelensis* (*Bti*) bacteria has been used extensively and aerially as a biological control agent since US registration in 1983 [8-14]. VectoBac® *Bti* formulations (Strain AM65-52, Valent BioSciences LLC., Libertyville, IL) have previously demonstrated efficacy against larvae of *Aedes*, *Anopheles* and *Ochlerotatus* [15-24].

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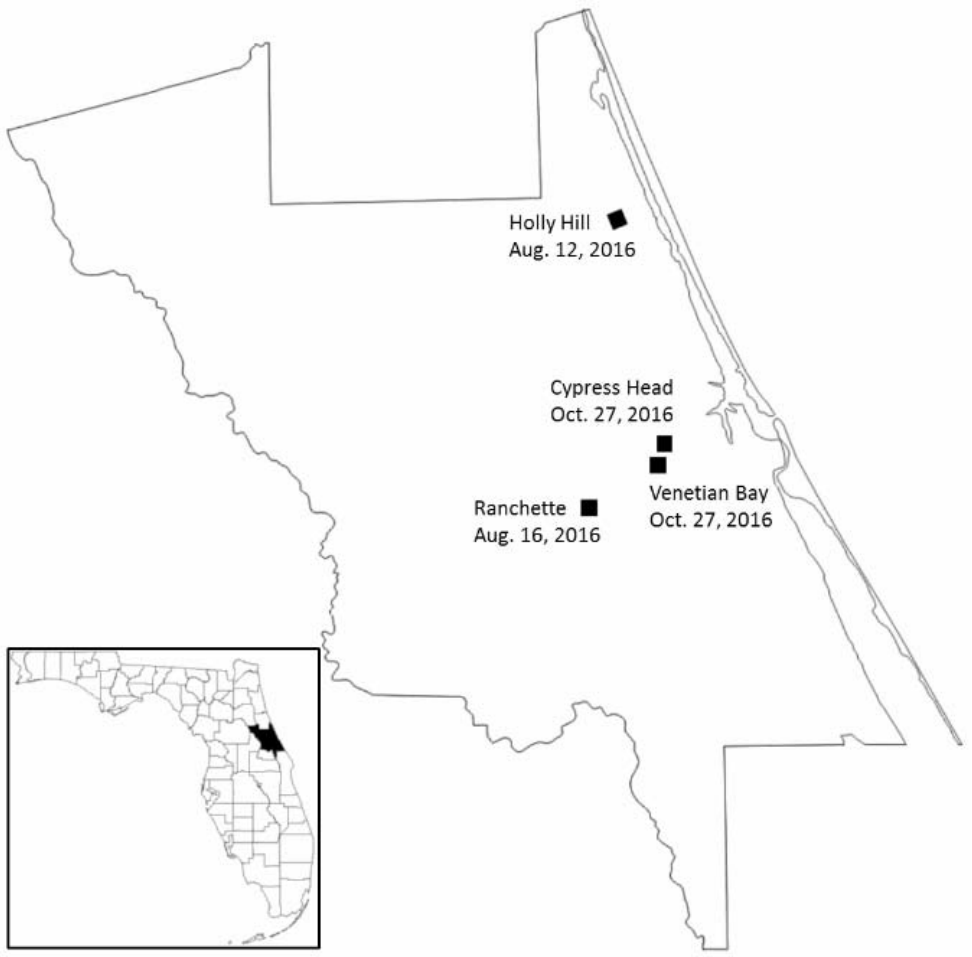

Figure 1. Map of the spray areas in Volusia County, Florida. Each area, marked with the spray date, is 1.61 x 1.61 km. The inset map shows the location of Volusia in Florida.

Among the available *Bti* formulations, VectoBac® WDG (Water Dispersible Granule) was developed for both ground and aerial applications including the low-volume (LV) spray with very-fine to fine (VF/F) droplet spectrum [25, 26]. Following an outbreak of dengue in 2012, the Florida Keys Mosquito Control District worked in collaboration with Valent BioSciences LLC and Helicopter Applicators, Inc. (HAI) to explore the potential for control of *Ae*. *aegypti* in Key West, FL. VectoBac® WDG was aerially applied *via* a Bell 206B helicopter using six Micronair AU5000 atomizers. In addition to varied application rates, the droplet size, drop distribution and deposit under different vegetative coverage were evaluated in Key West [27]. With subsequent suppression on mosquito adults, this methodology has been incorporated into the routine IMM program in the Keys. In an emergence response to invasive *Aedes aegypti* in the California desert in 2016, VectoBac® WDG was also sprayed using a Hiller 12E helicopter with two Micronair AU 6935 electric rotary atomizers and two AU7000 propeller-driven atomizers. During the spray operation, open cups were placed under various vegetation coverage to collect the sprayed drops. Later in a lab, water was added and *Ae. aegypti* larvae were also transferred into the cups. The larval mortalities reached 72-99% in these cups [28].

The successful integration of VectoBac® WDG, applied aerially to mitigate peridomestic mosquito species production in southern Florida coupled with recent disease trends – highlighted by the rapid expansion of CHIKV and ZIKV into Florida, with local transmission taking place – led VCMC to

attempt adaptation of both the active ingredient and aerial spray system for use in the aerial larviciding program in Volusia, and to verify the efficacy of area-wide control of container mosquito production.

MATERIALS AND METHODS

To evaluate spray efficacy in different landscape settings found in Volusia, four residential areas where *Ae. aegypti* and *Ae. albopictus* production had been identified were selected, each with the size of 1.61 x 1.61 km (1 mi^2) (Figure 1, Figure 2, Table 1). The first aerial application at Holly Hill was conducted in the early morning hours before sunrise. The other applications, at Ranchette, Cypress Head and Venetian Bay, were made in evening after sunset when VCMC aerial control operations typically take place. HAI (Gettysburg, PA), was contracted to conduct the sprays. HAI utilized a Bell 206L helicopter equipped with the AG-NAV Guia and Platinum guidance systems (AGNAV, Barrie, ON, Canada) and the Simplex 7900 tank/pump spray system (Simplex, Porland, OR) with six Micronair AU5000 atomisers (Micron, Bromyard, UK) and a 40° blade pitch/angle. The spray system delivered a 112 µm volume median diameter (VMD) as estimated in a wind tunnel [27].

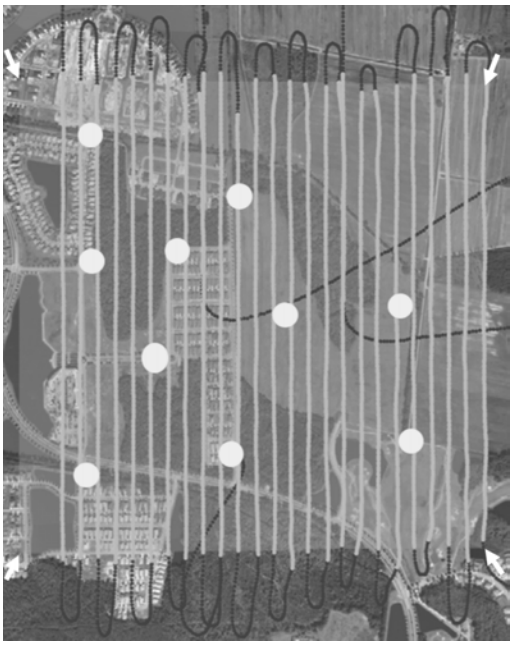

Figure 2. The flight courses with spray on (grey vertically-parallel lines) and off (black dotted lines) in Venetian Bay. The shaded square (4 arrows pointing to its corners) is the assigned spray area (1.61 x 1.61 km) and the white circles indicate the ten sites where the cups were set up.

Flights took place at 61 m (200 ft) above ground level (AGL) and 129 km/hr (80 mph) speed; the application's swath width was 61 meters (200 ft). Weather conditions specified by the manufacturer were met for each evaluation area, with wind ≤ 19 km/hr (12 mph) and relative humidity (RH) > 65% [26, 27]. VectoBac® WDG (*Bacillus thuringiensis israelensis* Strain AM65-52, Valent BioSciences LLC, Libertyville, IL) was first suspended in water and sprayed at the rate of 4.68 liter/ha (1.89 liter/acre), corresponding to the dry rate of 0.56 kg/ha (0.5 lb/acre) [26, 27]. To characterize spray droplets, ten water-sensitive cards (52 x 76 mm in size, Syngenta, Switzerland) were set out at open sites in Ranchette (Figure 3). Given the high humid situation, the cards were

Table 1. Spray areas, situations and validation methods used.

Location	Landscape	Spray time	Weather and humidity (RH)	Validation methods
Holly Hill	Old residential blocks, with few trees	Earlier morning of Aug. 12, 2016	Clear, ~ 95%	Cups and water-sensitive cards*
Ranchette	Under-developed forest with fewer houses	Evening of Aug. 16, 2016	Right after a shower, ~ 100%	Cups and water-sensitive cards**
Cypress Head	Combination of newer houses and forests	Evening of Oct. 27, 2016	Clear, ~ 75%	Cups only
Venetian Bay	Combination of newer houses and forests	Evening of Oct. 27, 2016	Clear, ~ 75%	Cups only

*Unsuccessful due to ≥ 1 hour exposure in higher humidity; **~30 min exposure.

Figure 3. Sprayed droplets on a Syngenta water-sensitive card (52x76 mm) scanned using the DropVision.

collected in about 30 minutes after the spray. The droplets on the cards were measured using a stage micrometer (Ward's Sci. Rochester, NY) under a Motic® AB310 microscope with a 10x reticle eyepiece (Carlsbad, CA). The droplet sizes were calculated according to size and the corresponding spread factors [29]. The droplet densities were estimated using two methods: the Syngenta aid (it is a 1-cm^2 square window provided with the purchase of the water sensitive cards. On each card, three counts along a diagonal were made and averaged) and the DropVision AG Ver. 2.7.2. (Leading Edge Associates, Fletcher, NC). The Valent BioSciences protocol [30] was followed to collect spray droplets using small cups and to conduct bioassays using mosquito larvae. Due to lower cost and local availability, 162 ml disposable cups with snap-on lips (5.5 oz, Diamond™, Jarden Cooperation, Fishers, IN) were purchased and used for droplet collection in this study.

In each spray area, a total of forty cups were used as a proxy for residential containers and water bearing debris during each helicopter spray application; ten cup sites were selected, each with four cups under four coverage treatments, respectively (Figure 2). The coverage treatments were defined as: E (completely Exposed to the sky), S (Sparse vegetative cover, ~ 30 % cover), D (Dense vegetative cover, ~70 %) and C (Covered, 100% cover and sky completely obstructed). At the cup sites where no full coverage naturally occurred, a bucket cover was set up 0.5 m above a cup. The cups were set out immediately before the aerial spray commenced; all cups were capped and collected one hour after spray.

Cups were stored in a refrigerator and shipped within a week *via* a cooler with ice packs to the lab of Benzon Research Inc. (Carlisle, PA) for bioassay. For the cup bioassay, 100 ml of non-chlorinated water was added to each cup, ensuring that the cup's internal wall was rinsed into the cup's contents using a disposable pipette, and 20 larvae of 3rd to early 4th instar *Ae. aegypti* were then transferred into each cup. As a control, ten un-sprayed cups were handled and processed in the same way in the bioassay for each spray area. Larval death in each cup was evaluated or monitored at two, four, six, 24 and 48 hours after introduction. The mortality was corrected and calculated [31, 32].

Due to the similar landscape settings and the same weather condition in Cypress Head and Venetian Bay (Table 1), the bioassay data from the two areas were pooled and categorized into two groups, namely yard and forest, to compare the spray effects between the two landscape settings with and without forest coverage at the cup sites.

To evaluate the difference in mortality among the four cup treatments or between yard and forest settings, one-way ANOVA and Tukey pairwise comparisons with 95% simultaneous confidence intervals in the Minitab Version 14.20 were used to analyze the data [33].

RESULTS

The estimated droplet sizes and densities on the water-sensitive cards from the Ranchette application are listed in Table 2. The droplet sizes ranged between 80-364 µm, with an average of 142 µm. The droplet densities were between 16-26 droplets / cm^2 (21 on average) and 16-29 droplets / cm^2 (20 on average), estimated by the Syngenta droplet counting aid and the DropVision AG, respectively. The two methods delivered comparable results.

Bioassays of the four coverage treatments, collected from the four spray areas, and the larval mortalities across five time intervals are shown in Figure 4 and Table 3. The overall mortality (across all coverage treatments and areas) reached 93 ± 15% ($\tilde{x}$ ± SD) at 24 hours and 95 ± 13% at 48 hours. For each coverage treatment, mortalities increased over time while the corresponding standard deviations had a trend of reduction (Table 3). No significant

Table 2. Sprayed droplet characterization on Syngenta water-sensitive card.

	Stain size (µm)	Droplet size (µm)	Droplet density (per cm^2)
Range	140-800	80-364	16-26*, 16-29**
Average	265	142	21*, 20**

*Estimated by the Syngenta droplet counting aid; **Estimated by the DropVision AG.

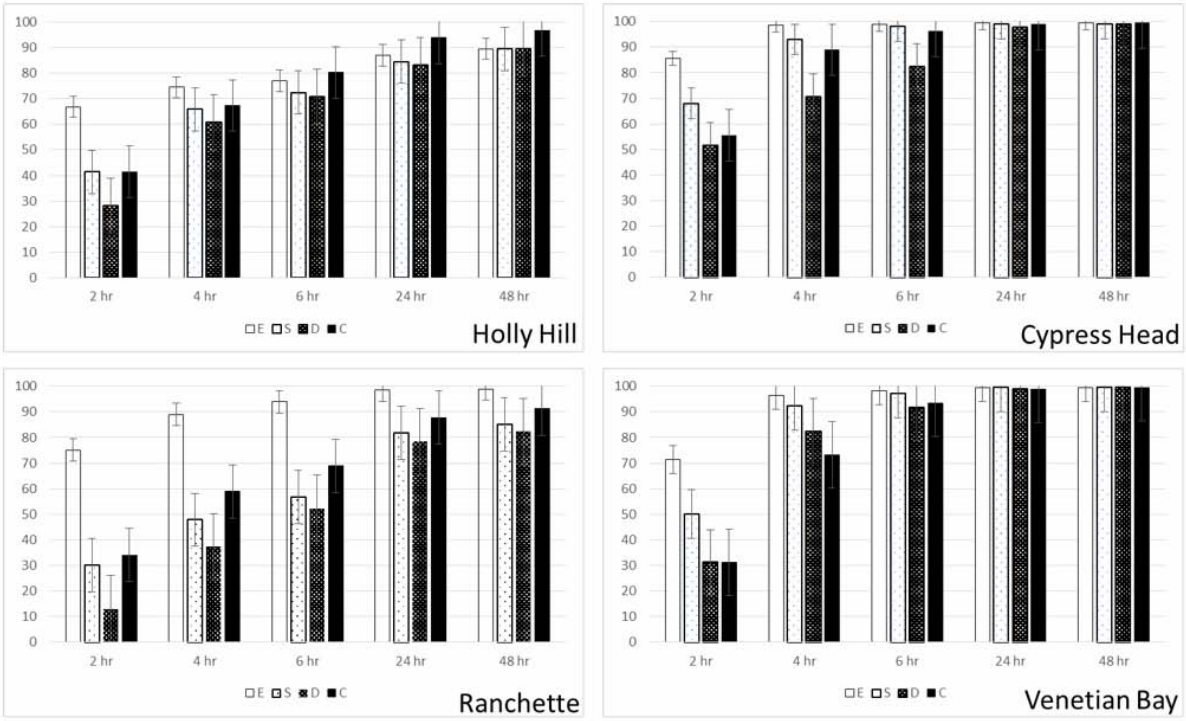

Figure 4. Larval mortalities for the cup coverage treatments from the four spray areas at different time intervals. The treatments are E (Exposed to the sky), S (Sparse vegetative cover), D (Dense vegetative cover) and C (Covered, obstructed from the sky). The standard error bars are on the data columns.

difference in the mortalities at the same time interval was detected between the coverage treatments, between the spray areas, or between the morning spray in Holly Hill and the evening sprays in the other areas ($P > 0.05$).

In Cypress Head and Venetian Bay (Figure 5), the average mortalities for all coverage treatments at 24 hours were higher than 98% in the forests and higher than 99% in the yards, and the mortalities for the same treatments at various time intervals were not significantly different between the two kinds of landscape, namely forest and yard ($P > 0.05$). The comparisons demonstrated the effective and similar droplet penetration in both the yard and forest settings.

DISCUSSION

Good control effects by the aerially sprayed VectoBac® WDG is supported by the high average mortalities in the cup bioassay. For all coverage treatments across the spray areas, most of the average mortalities at 48 hours (14 among 16 sprayed-cup groups) reached to 90-100%. The higher average mortalities and no significant difference in the mortalities among the coverage treatments at 24 and 48 hours show effective coverage of various small containers by the sprayed droplets. The similar mortalities in the 4 coverage treatments (E, S, D and C) between the yard and forest landscapes in Cypress Head and

Table 3. Average mortalities (%, $\tilde{x}$ ± SD) of the cup coverage treatments across the four spray areas.

Cup treatments	2 hours	4 hours	6 hours	24 hours	48 hours
Exposed to the sky (E)	75 ± 30	90 ± 24	92 ± 20	96 ± 11	97 ± 9
Sparse vegetative cover (S)	48 ± 37	74 ± 34	81 ± 30	91 ± 18	93 ± 14
Dense vegetative cover (D)	31 ± 30	62 ± 34	74 ± 30	90 ± 19	92 ± 17
Covered (C)	41 ± 34	73 ± 30	85 ± 24	95 ± 10	97 ± 8

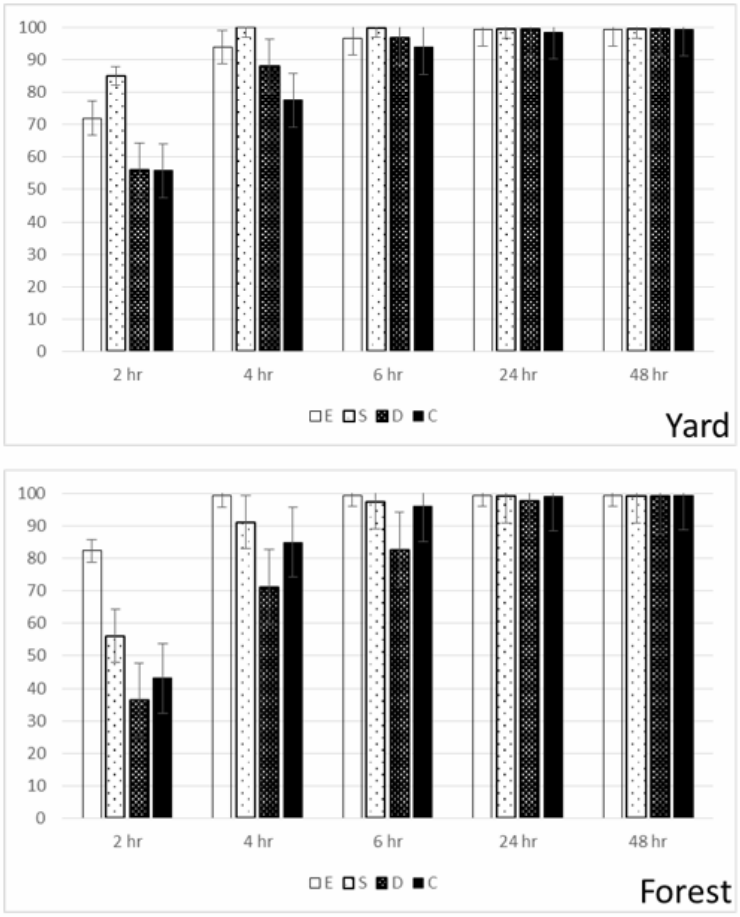

Figure 5. Larval mortalities for the cup coverage treatments at different time intervals in the yard and forest settings of Cypress Head and Venetian Bay. The treatments are E (Exposed to the sky), S (Sparse vegetative cover), D (Dense vegetative cover) and C (Covered, obstructed from the sky). The standard error bars are on the data columns.

Venetian Bay demonstrate effective droplet penetration in the forest conditions.

Large variations in the mortality data were observed in all spray areas (Figure 4), especially Ranchette, sprayed right after a rain and with more woods. Although the more open cups (E and S in Table 3) started with higher larval mortalities, the mortality differences among the coverage treatments were not significant across time. The wider ranges of the mortalities of coverage treatments in every area were the main reason contributing to the non-significances statistically revealed in this study. For the future aerial spray of VectoBac® WDG, more attention should be given to the areas with

heavy forests in order to reach more uniformed spray effects.

More knowledge of LV fine drop aerial spray of different larvicides is needed to predict their effects on populations of mosquito larvae since the aerial larviciding is less applied [34, 35] and most publications were about the aerial applications of adulticides, organophosphate larvicides or lack of considerations of landscape factors on control effect [15, 36-40]. In Volusia, weather conditions and various densities of trees or forests in the residential communities are concerned to affect spray effectiveness.

A wide variety of climatological conditions, including but not limited to wind, precipitation and fog, variously influence spray droplets and drift [27, 34]. During a helicopter spray, local wind is one of the factors observed to affect the droplet landing in the cups, as revealed by the wide variations of larval mortalities in the cup bioassay. At some sites, strong gusts were noted and lower mortalities in the cups open to the sky were observed later in the bioassay. In the foggy condition right after a rain in summer, bigger stains on the Syngenta water-sensitive cards were found, probably formed by merging the sprayed droplets and water dews in the air. These bigger-size droplets could reduce the penetration effect of helicopter spray. On the other hand, low humidity causes evaporation, especially for droplets smaller than 50 μm to fly away from the target area [27].

Over the years, there are three bio-validation methods developed for aerially sprayed *Bacillus thuringiensis* agents against mosquito larvae. Small field habitats with mosquito larvae were checked and compared before and after an aerial spray to evaluate control effect [20]. Small cages or containers infested with mosquito larvae were set out in fields to verify aerial spray effect [15, 41]. Valent BioSciences placed small empty containers in fields to collect sprayed drops, and conducted a larval bioassay in lab later [30].

The validation protocol developed by Valent BioSciences [30] and the modifications used in this study are suitable for field use. The small plastic cups used in this study are easy to handle and require less field work. This validation method can be applied in residential, commercial and forest areas, and in emergency response against mosquito-borne diseases. The cups with VectoBac® WDG collected from fields and sealed with lids can be kept in room temperature or in a refrigerator for a couple of weeks, providing more flexible time for shipping and bioassay later (DeChant and Royals, personal communication).

However, there are several aspects that need to be discussed and improved. First, if there are more than one ground crew to set up the cups and multiple areas to spray in one night, it is better to have all crews start in the same area to save time for helicopter spray. Secondly, it suggests that the cup sites should be marked with small flags, reflective strips, LED lights or glow sticks for easy post-spray collection in dark. Thirdly, the plastic cups with screw-on caps described in the original protocol are water-tight and can hold small amount of water accidently collected in field for shipping to a later bioassay. The disposable cups used in this study are locally available and of lower cost. Due to their light weight, a heavier anchor may be necessary in windy or uneven ground conditions.

The water-sensitive cards used in this study have a specially coated, yellow surface which will become dark blue by aqueous droplets landing on it, and have been developed for field use to quickly evaluate sprayed drops (Figure 3) [29]. In both this study and Mickle and DeChant [27], Micronair AU5000 rotary atomizers were used, and the droplet sizes on the cards estimated in this study are consistent with the drop size range in the previous report. The average droplet densities estimated by the Syngenta droplet counting aid card and the DropVision AG are very close to each other (21 and 20 droplets per cm^2), and fall into the range of 20-30 droplets per cm^2 for insecticide spray as suggested by Syngenta [29]. The drop densities estimated in this study tend to be higher than those in Mickle and DeChant [27]. No further analyses have been conducted since the water-sensitive cards were not placed along a flight course in this study. Due to the high humidity in Florida summer, the water-sensitive cards will be stained dark blue by the water in the air if left uncovered for about one hour, making them undistinguishable for droplet edge. Such failure happened in the spray in Holly Hill. To avoid the failure, the cards were exposed to the

sky right before the helicopter spray in Ranchette. The exposure time of the droplet-collecting cards can be further reduced to 15 min after spray if necessary [23].

Aerial spray of mosquitocides was conducted at various time of day: earlier morning, day time or evening [27, 35, 42]. For mosquito adult control, a spray time may need to be balanced between mosquito activity, mosquitocide characteristic and the annoying effects by aerial spray to the local residents. However, for larval control, the main concerns are to minimize disturbance of residents from aerial spray and photoabilation on the *Bit* toxins [43]. In this study (Table 1), the first helicopter spray was launched in an earlier morning. Close to its end at sunrise, more outdoor residential activities and traffic were observed in the community. Therefore, evening sprays were conducted in other areas later. No difference in the cup bioassay was revealed between the morning spray in Holly Hill and the evening sprays in the other areas.

In this study, the average spray cost was about USD 14,750 per acre, excluding the labor, materials and biossay for validation. If sprayed by the VCMC Helicopter Program, the cost may be reduced by a couple hundred dollars as predicted.

The lab colony of *Ae. aegypti* maintained in Benzon Research was used in the cup bioassay of this study. In such future validation, a locally collected field population of vector mosquito larvae is recommended, or at least, a co-relation in the mortalities by the same larvicide between the lab colony and field population from a target region should be established in order to reveal more accurate field effect of the larvicide.

CONCLUSION

Vector mosquito control is one of the most effective and widely used approaches to fight Zika introduction and spread. Due to the high efficacy and eco-friendly feature of *Bti*, it has being promoted in area-wide management of Zika vector larvae although most aerial applications are of synthetic adulticides. As the research has demonstrated, *Bti* sprayed from a helicopter low-volume system can be used to effectively control vector mosquito larvae in residential communities with various landscape conditions during an emergence response to Zika. To implement the larviciding efficacy, the range of drop sizes should be validated before an operation and monitored during a spray for better drop penetration though various coverage and drop landing into larval aquatic habitats within a target area. Furthermore, local weather conditions including rain, humidity, wind speed and direction should also be considered to minimize their negative influences on drop size and flying pathway.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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