Research Article

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An evaluation of *Bacillus thuringiensis israelensis* (AM65-52) treatment for the control of *Aedes aegypti* using vehicle-mounted WALS® application in a densely populated urban area of Puerto Rico

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Abstract

BACKGROUND: With a shortage of effective options for control of *Aedes aegypti* in Puerto Rico due to widespread resistance to conventional mosquito adulticides, an alternative approach was investigated to reduce vector populations. In two areas (totaling 144 ha) of the municipality of Bayamón, Puerto Rico, *Bacillus thuringiensis israelensis* (*Bti*) AM65-52 WDG was applied at a rate of 500 g/ha using vehicle-mounted aqueous wide-area larvicide spray applications weekly for 4 weeks and then every other week for a further 16 weeks. Bioassay jars were placed in the field to monitor for deposition of *Bti* droplets in open spaces, and under vegetation and building coverage. Autocidal gravid ovitraps were placed throughout the field site to monitor the population of adult female *Ae. aegypti* in both treatment and control sites.

RESULTS: Larvicide spray was successfully deposited into jars in an array of open and covered locations, as confirmed by larval bioassays. After the fourth weekly spraying, differences in autocidal gravid ovitrap densities were observed between treatment and control sites resulting in 62% ($P = 0.0001$) and 28% ($P < 0.0001$) reductions in adult female *Ae. aegypti* numbers.

CONCLUSION: Repeated wide-area larvicide spray application of *Bti* AM65-52 WDG to residential areas in Puerto Rico effectively suppressed dengue vector populations. The success of this trial has led to expansion of the WALS® program to a larger area of Bayamón and other municipalities in Puerto Rico.

Supporting information may be found in the online version of this article.

Keywords: *Aedes aegypti*; WALS®; *Bacillus thuringiensis israelensis* AM65-52 WDG; dengue vector control; larviciding; container mosquito

1 INTRODUCTION

In recent years, Puerto Rico has suffered several major epidemics of mosquito-borne disease, notably dengue in 2010, chikungunya in 2014 and Zika in 2016, with 26 766, 28 327 and 40 000 suspected cases respectively.[1–3] In 2020, dengue circulation is present, threatening to expand to an outbreak for which 415 cases have been reported to 3 November.[4] Each of these diseases is vectored by the mosquito *Aedes aegypti* L. which is highly abundant in Puerto Rico.[5] Enjoying close proximity to humans, its larval habitats are typically man-made items such as tires, plant pots, garbage and any other water-holding

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containers.[6] Puerto Rico is one of the most densely populated islands in the Caribbean with over 3.7 million people, most living in urban areas surrounding the capital of San Juan, which has the highest population density at an estimate of 8262.3 people per square mile.[7]

In the wake of the Zika crisis of 2016, recognizing the need for organized vector control in the model of mosquito control districts commonly found in other parts of the USA, the Centers for Disease Control and Prevention (CDC) funded the Puerto Rico Science, Technology and Research Trust to set up an independent vector control unit (the Puerto Rico Vector Control Unit or PRVCU). The mission of the PRVCU is 'to protect the people of Puerto Rico from the *Ae. aegypti* mosquito, while educating and empowering citizens to dramatically and sustainably reduce mosquito populations across Puerto Rico.' The unit focuses on three main activities: vector surveillance, community education and mobilization, and vector control. As part of the unit's surveillance activities, over 1200 autocidal gravid ovitraps (AGOs) have been deployed in seven of the 78 municipalities that make up the island, namely San Juan, Carolina, Bayamón, Caguas, Dorado, Guaynabo, and Ponce. The community outreach team provides classes in schools and attends and organizes community events across the island. The Puerto Rico environment poses significant barriers to effective, affordable and sustainable vector control solutions. Mosquito resistance to a wide range of adulticidal pesticides has been reported,[8, 9] meaning that a typical adulticidal chemical approach to control of *Ae. aegypti* is not feasible. Additionally, the dense urban population, tight home security and presence of cryptic larval habitats mean that door-to-door patio inspections and community clean-up are neither feasible nor sustainable.

The inspiration for this study came as a result of the success of wide-area larvicide spraying in other locations.[10–14] Because Puerto Rico has a very different type of architecture from other parts of the USA reflecting more the Latin American style of small yards, properties located closely together, having flat roofs and high security often meaning access to back yards is limited, it was necessary to determine whether this type of larvicidal application was appropriate for control of *Ae. aegypti* in neighborhoods on the island. Other previous efforts by the PRVCU to control *Ae. aegypti* in Puerto Rico using the US style of patio inspections, source reduction and hand-applied larviciding proved to be too labor intensive and did not allow ground crews the access they required to reach yards often enough. Some yards were repetitively treated due to occupants being home when crews called, and their general support of the program, whereas other yards never received a visit because residents were not home or access was barred. Additionally, PRVCU ground crews observed a high number of abandoned houses in urban areas of Puerto Rico, reported by residents to be due to migration of the population to the continental USA. Thus, it was hoped that wide-area larvicide spraying, which is an application strategy trademarked by Valent Biosciences LLC. as WALS® would help to address the problems of accessibility and result in control of *Ae. aegypti* in urban Bayamón.

WALS® is a biorational larvicide application strategy that uses wind-distributed aqueous microdroplets of mosquito larvicide to target cryptic larval sources, such as containers, across complex landscapes. WALS® application of *Bti* AM65-52 WDG has been successful for dengue vector and disease suppression in Malaysia[10, 11] and in Key West, Florida.[12] WALS® was also used successfully in Miami, Florida[13] and Brownsville, Texas[14] during the Zika crisis of 2016. Therefore, in an effort to find a solution that can be applied to entire communities at speed and at scale, a trial was launched in Puerto Rico to test the effectiveness of applying *Bacillus thuringiensis israelensis* (*Bti*) AM65-52 WDG (VectoBac® WDG, Valent Biosciences LLC) using vehicle-mounted WALS® across multiple areas in the island's urban environments to control *Ae. aegypti*. The intention of this study was to evaluate whether control of *Ae. aegypti* could be achieved by repeated (weekly and biweekly) WALS® micro-droplet application of *Bti* AM65-52 pushed high into the air using vehicle-mounted air-blast spraying equipment, allowing the larvicide to be distributed by wind across neighborhoods and into a range of larval sources.

2 MATERIALS AND METHODS

2.1 Communities and field sites

Bayamón, a municipality of 208 116 people,[15] suffers repeated episodes of arboviral diseases, with 761 cases of dengue in 2010 and 3906 cases of Zika in 2016.[4] No official data are available for chikungunya, but the Bayamon Department of Health confirmed that cases occurred during the outbreak of 2014 (Santiago C, pers. commun.). Bayamón is a municipality in the San Juan metropolitan region to the west of San Juan and Guaynabo. To the north is Cataño where the island's main container depot for shipping is situated. Bayamón is one of the municipalities included in the PRVCU vector surveillance program and over 400 surveillance AGOs (SpringStar Inc.) were deployed across the urban and suburban areas of Bayamón at the time of this study, bringing in data on numbers of *Ae. aegypti* weekly.

Typical housing in Bayamón is of concrete construction, comprising one or two floors, louvered windows and an outside covered area or 'marquesina.' Houses are situated close to the road, sometimes with a small front yard and often a backyard with no access from the road. Security is important in urban Puerto Rico and many homes and back yards are inaccessible without entering through locked security gates (Figure S1). This high security is a barrier to conventional patio inspections and typical backpack applications carried out by mosquito control personnel.

Two sites in Bayamón were selected for the application of VectoBac® WDG by vehicle-mounted air-blast sprayer WALS® applications. Treatment site 1 was situated in northwest Bayamón, a 68 ha site incorporating the neighborhoods of Parque Valencia, Reparto Valencia, El Sopapo and El Frutal (18.3995168, −66.1885805). This site includes 12 of the PRVCU's surveillance traps (Figure 1). Treatment site 2 to the southeast of Bayamón is a 76 ha area including the neighborhoods of Santa Juanita and Rivera (18.354369, −66.157573), and includes 16 of the PRVCU's surveillance traps (Figure 2). The remaining area of the Bayamón surveillance network served as the aggregated, untreated control (378 traps).

Treatment sites were selected to be of a size that could be sprayed within 2 h (between 6 and 9 pm), using a single 380 L tank of suspended *Bti*. This was to allow for mixing and filling to be carried out at the Bayamón Department of Health pesticide storage facility and then be driven to the field site for application.

2.2 Application of the product

WALS® applications were carried out using an A1 Super Duty Mist Sprayer® (A1 Mist Sprayers) equipped with a Micronair® AU 5000 (Micron Group) atomizer equipped with short (7 cm) fan blades (EX6353) set at 55° and a 20-mesh screen. An automated mixing system was set up for suspension of the product prior to loading into the spray equipment and characterization of the equipment

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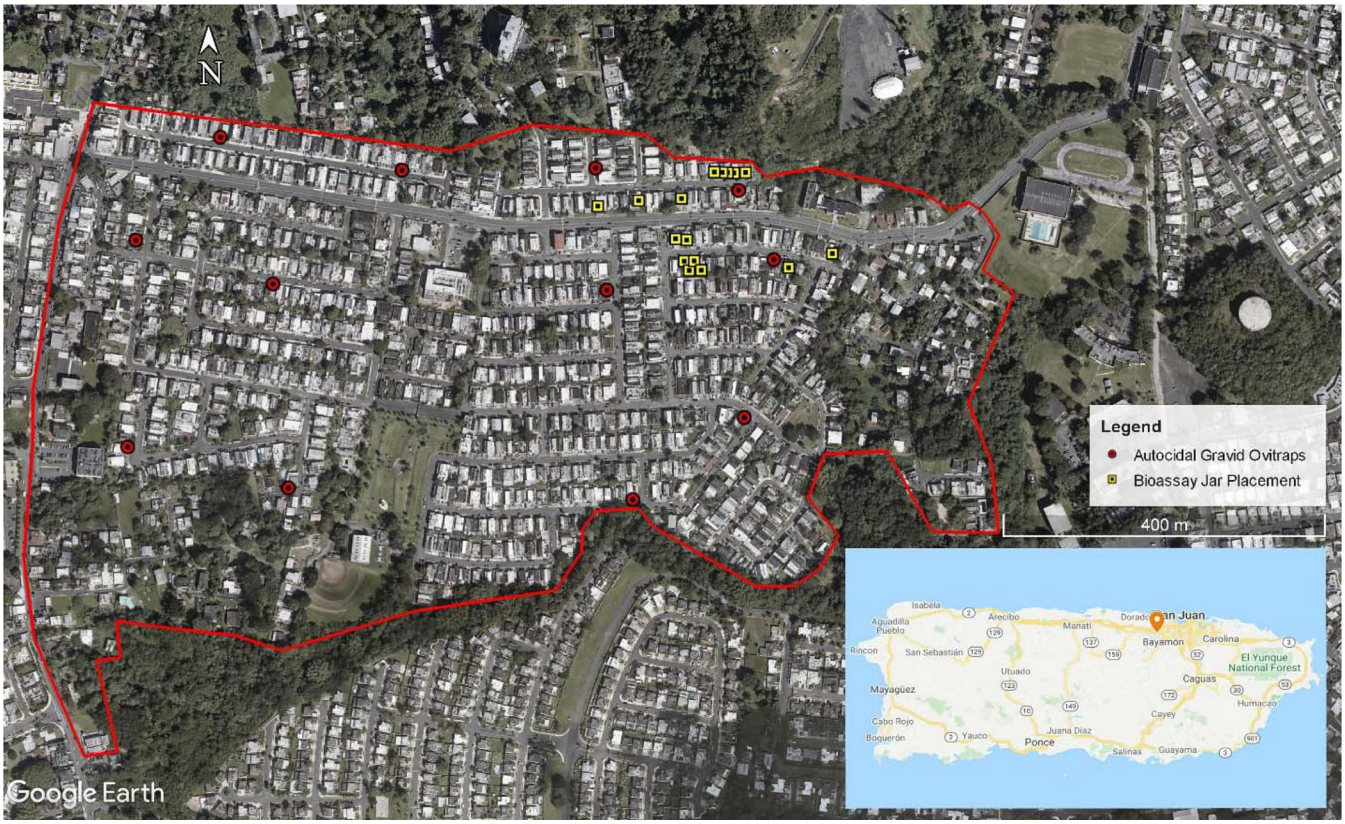

FIGURE 1. The spray block for treatment site 1 is depicted by the red outline. Surveillance traps (autocidal gravid ovitraps collected weekly) are represented by red circles. Houses where bioassay cups were placed prior to each spray mission are represented by yellow squares. Location of the field site in Puerto Rico can be seen embedded. Map Data: ©2020 Google.

carried out (details can be found in Supporting Information). The treatment schedule (based on previous work carried out by Valent Biosciences LLC [Lake L, pers. commun.]) was for each treatment area to be sprayed weekly for 4 weeks and then every other week for the following 16 weeks to a total of 12 treatments. Treatment began in site 1 on 14 November 2018 and finished on 27 March 2019. Treatment in site 2 began on 29 November 2018 and should have finished on 11 April 2019, however delayed delivery of the *Bti* product to the island meant the application did not take place as planned on 14 February 2019 so an additional application was carried out in site 2 on 25 April 2019 instead.

The target product application rate was 500 g/ha. This was achieved using a 12% VectoBac® WDG suspension applied using a flow rate of 8 L /min and a vehicle speed of 11 km/h. Application took place shortly after sunset once the ground temperature was cooler than the air temperature (measured by a digital anemometer with thermometer and a non-contact infrared thermometer; Holdpeak) to facilitate settling of droplets.

2.3 Bioassays

To confirm that spray droplets were reaching a range of sites in different locations around homes in the treatment blocks, bioassay jars (polystyrene straight-sided clear jar, 6.5 cm diameter, US Plastics Corp.) were deployed in a cluster of selected properties at the start of the spray route, in the northeast corner of treatment site 1 (Figure 1) and in a cluster of properties along the eastern edge of treatment site 2 (Figure 2) prior to treatment. Houses were selected based on finding residents at home and confirming their willingness to have staff enter their properties. On occasion, the residents of the selected properties were not home at the time of placing the bioassay jars, so properties could not be entered, in this case alternative properties close by were identified where a resident gave permission and permitted entry to the field team and these houses were used instead. During the study, 16 and 18 houses were used in sites 1 and 2 respectively, however for each treatment only ten houses at a time would be used. In each of the ten properties per treatment, technicians placed four empty, 175-ml plastic jars with the lid removed in different types of locations. The jars were placed so that one jar was entirely open to the sky, another was placed covered (without open sky, for example under a porch or inside a marquesina), two more jars were placed with slight vegetative cover and dense vegetative cover under plants and shrubs. Assessment of cover was subjective and based on the judgement of the technician placing the jars. An additional 12 jars were placed outside the treatment site in the neighborhood of Juan Sanchez (18.364498, −66.142748) during each application to act as negative controls. Thirty minutes after spraying all the jars were retrieved, lids screwed on and returned to the PRVCU laboratory and stored at −20 °C until bioassays were performed.

Larvae for use in the bioassays were collected as eggs from the Irlanda neighborhood of Bayamón (18.352517, −66.145748) using standard ovitraps[16] with seed germination paper as the substrate. Larvae were reared to third instar in the PRVCU insectary and fed on Tetramin™ fish food (Spectrum Brands Pet LLC).

When ready for processing, bioassay jars were removed from the freezer in batches that included all the control and treatment site jars from a single date and allowed to stabilize to room temperature. Bioassays were performed modelled on the WHO

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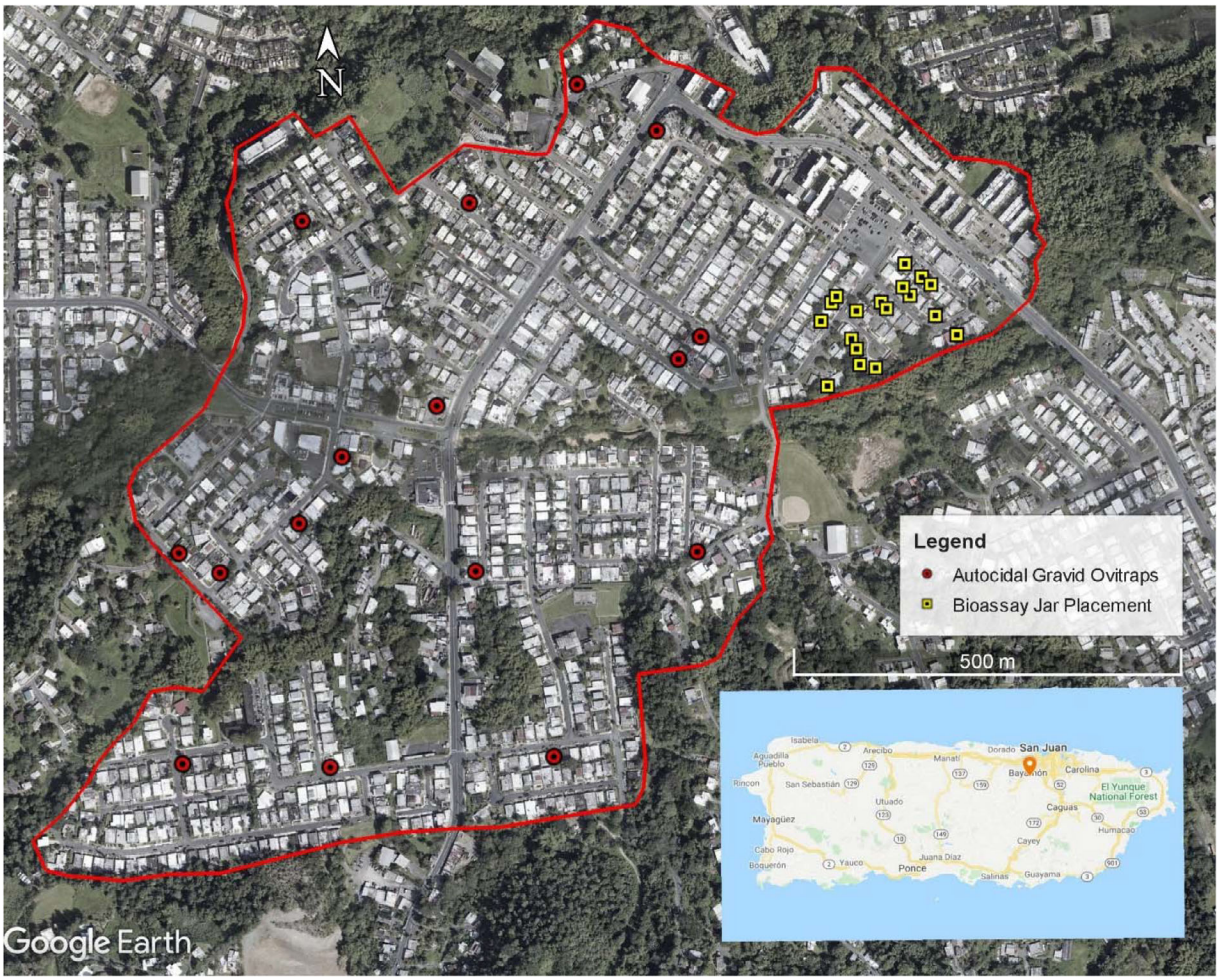

FIGURE 2. The spray block for treatment site 2 is depicted by the red outline. Surveillance traps (autocidal gravid ovitraps collected weekly) are represented by red circles. Houses where bioassay cups were placed prior to each spray mission are represented by yellow squares. Location of the field site in Puerto Rico can be seen embedded. Map Data: ©2020 Google.

guidelines.[17] Bioassay jars had 100 ml of distilled water added to them, the lid replaced, and the jar shaken vigorously to ensure all traces of *Bti* deposited on the inner walls of the jars was released into the water. Some 20 third-instar larvae were added to each jar and mortality was scored after 48 h.

2.4 Effect of treatment on adult *Ae. aegypti* populations

Adult populations were monitored through trapping in the treatment sites, as well as across the municipality of Bayamón to determine the success of the trial at reducing numbers of adult female *Ae. aegypti* in treatment sites compared with the control. AGOs were collected and reset weekly from May 2018. Traps were tracked using affixed QR codes that were scanned using a PRVCU custom-built tracking app. The capture chamber from each trap was removed, replaced and returned to the laboratory for identification of mosquito specimens to species and sex. The study was divided into three distinct phases: a pre-treatment phase, a treatment phase and a post-treatment phase. The pre-treatment phase ran from 31 May 2018 to 14 November and 29 November 2018 for treatment sites 1 and 2, respectively. The treatment phase for site 1 was from 14 November 2018 to 27 March 2019 and for site 2 was from 29 November 2018 to 25 April 2019. The post-treatment phase was from 27 March and 25 April 2019 to 19 August 2019 for sites 1 and 2, respectively.

2.5 Statistical analysis

Percent mortality was scored for each bioassay jar 48 h after the addition of larvae. Because of concerns regarding the subjective interpretation by technicians as to what constitutes 'slight' and 'full' coverage, analysis of bioassay results by coverage type could not be carried out. However, a *z*-test was carried out to determine whether mortality seen in 'covered' jars was significantly different from that in the control group using the Microsoft® Excel® (version 2008) data analysis tool.

Because placement of bioassay jars in positions that were open to the sky was not open to misinterpretation by technicians, mean mortality and standard error were calculated by application date (n = 239) and plotted to show percent mortality as vertical bars with standard error shown as error bars. (Figure 3). To detect if there was a difference in the mean mortality by application date, a one-way analysis of variance (ANOVA) was used both combining data across the sites and by each site separately. ANOVA was carried out using the Microsoft® Excel® (version 2008) data analysis tool.

A negative binomial regression was fit to the mean number of adult *Ae. aegypti* females calculated for each treatment site and compared with the mean of the remaining traps in Bayamón (n = 378) following Pruszynski *et al.*[12] Initial statistical analysis (mean, standard deviation, and standard error of *Ae. aegypti* female trap counts in each site) indicated that variances were

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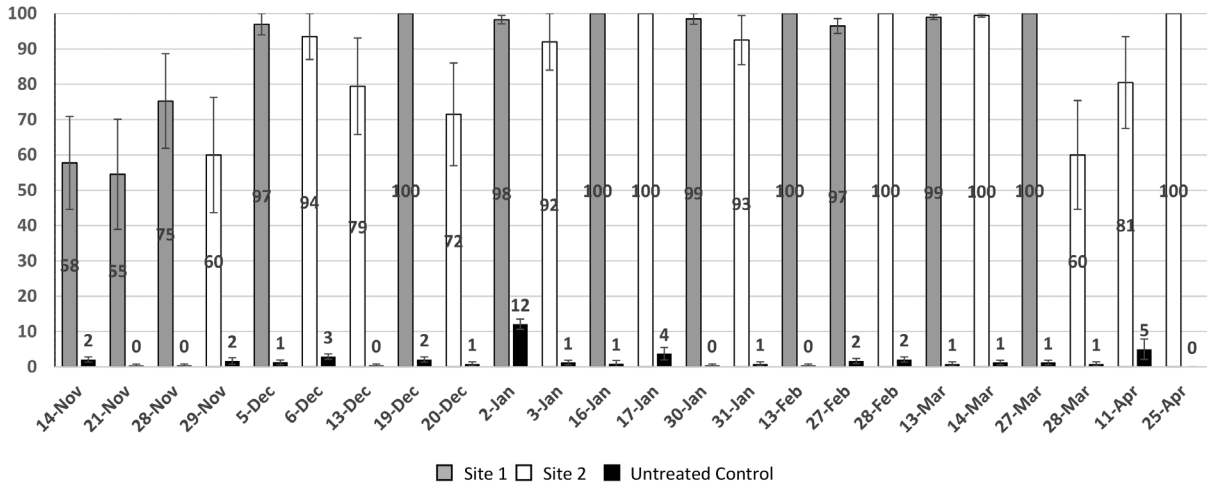

FIGURE 3. The overall mean percentage mortality and standard error for larvae after 48 h bioassays in cups placed in the open, exposed to WALS(R) using *Bti* AM65-52 sprayed on different dates (data collected 2 January have been corrected using Abbott's formula as a result of high control mortality).

almost twice as high as the mean, indicating overdispersion of trap data hence negative binomial regression was used for comparing the treatments.[18, 19] To visualize the data, locally weighted polynomial regression (LOESS model) curves[12, 18, 20] were plotted for the weekly collections of female *Ae. aegypti* from AGOs in the different sites, with each treatment site plotted in comparison to the control traps. Standard error was also plotted. Statistical analyses were conducted using the open-source software 'R' (version 3.6.1)[21] and graphs were plotted using the ggplot2 package (version 3.3.2).[22]

3 RESULTS

3.1 Bioassays

Bioassays indicated that vehicle-mounted WALS® application of *Bti* resulted in larvicide deposit in bioassay jars, regardless of coverage by roofs or various densities of vegetation; this was confirmed by larval mortality in bioassays for all coverage types and a significant difference between covered jars ($n = 714$) and control jars ($n = 287$) ($P < 0.0001$, $z = 59$).

Mean mortalities in bioassay jars exposed to the open sky varied for each application but were consistently > 55%, with eight applications resulting in 100% mortality in all jars exposed to the sky (Figure 3). ANOVA of all bioassays from open jars in relation to application date indicated a statistical difference ($F = 3.38$, $P = 1.5 \times 10^{-6}$). The same was found when these data were separated by site (site 1: $F = 5.75$, $P = 3.01 \times 10^{-7}$; site 2: $F = 2.31$, $P = 0.01$).

This is not unexpected due to the possibility of a range of climatic effects such as wind, temperature and humidity. Mean mortality in the control bioassay jars for each treatment date remained < 5% with the exception of the control jars collected on 2 January. On this occasion, mean mortality after the 48 h bioassay was 12% so treatment data was corrected using Abbot's Formula[23] per the WHO guidelines.[17]

3.2 Effect of treatment on adult *Ae. aegypti* populations

Negative binomial regression analysis (Figures 4 and 5) showed that there was no significant difference in the mean number of female *Ae. aegypti* between treatment and control sites prior to

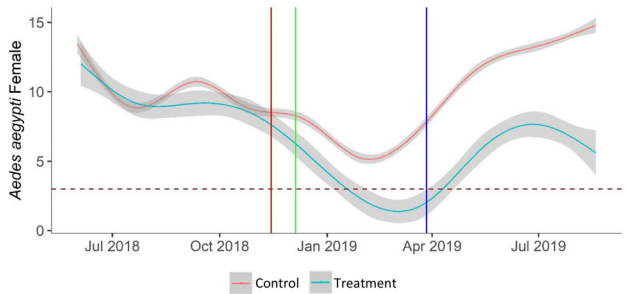

FIGURE 4. Locally weighted polynomial regression (LOESS model) of the mean number of female *Aedes aegypti* caught per week in autocidal gravid ovitraps in treatment site 1 compared with the control (the rest of Bayamón). Standard error is represented by the gray shaded areas and is greater in the treatment site than the control due to there being many more traps in the aggregated control site ($n = 378$) than in the treatment site ($n = 12$). Vertical lines represent the following: red, start of once per week treatments; green, reduction to once every 2 weeks treatments; blue, cessation of treatments. The horizontal broken red line represents three *Ae. aegypti* females.

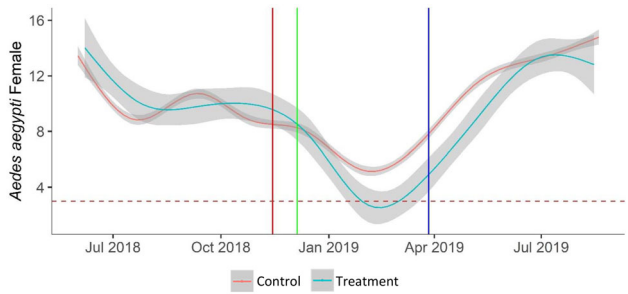

FIGURE 5. Locally weighted polynomial regression (LOESS model) of the mean number of female *Aedes aegypti* caught per week in autocidal gravid ovitraps in treatment site 2 compared with the control (the rest of Bayamón). Standard error is represented by the gray shaded areas and is greater in the treatment site than the control due to there being many more traps in the aggregated control site ($n = 378$) than in the treatment site ($n = 16$). Vertical lines represent the following: red, start of once per week treatments; green, reduction to once every 2 weeks treatments; blue, cessation of treatments. The horizontal broken red line represents three *Ae. aegypti* females.

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Table 1. Comparison of autocidal gravid ovitrapping data (adult *Aedes aegypti* females) between treated and untreated control in both field sites

Site	Phase	Estimate	Standard error	*Z*-value	*P*-value
Site 1	Pre-treatment	−0.007	0.095	−0.143	0.886
	Treatment	−0.618	0.065	−9.454	<0.0001
	Post-treatment	−0.741	0.056	−13.12	<0.0001
Site 2	Pre-treatment	−0.017	0.033	−0.513	0.608
	Treatment	−0.287	0.052	−5.547	<0.0001
	Post-treatment	−0.098	0.052	−1.885	0.059

initiation of wide-area larvicide spraying for either site (treatment site 1, $P = 0.886$ and treatment site 2, $P = 0.608$). During the period of treatment, a significant reduction in the mean number of female *Ae. aegypti* was observed in both sites. In treatment site 1 between 15 November 2018 and 28 March 2019 a reduction of 62% in the treatment site *versus* the control was observed ($P = 0.0001$). Although the effect was less marked in treatment site 2, between 29 November 2018 and 25 April 2019, female *Ae. aegypti* numbers dropped in the treatment site by 28% compared with the control ($P < 0.0001$). After treatment ceased, the difference in treatment site 1 remained significant ($P < 0.0001$) for over 4 months with a mean reduction between treatment and control of 74%. In treatment site 2, mean numbers of female *Ae. aegypti* recovered within 2 months and were comparable with numbers within the control site again ($P < 0.059$) (Table 1).

Previous work carried out in Puerto Rico using AGOs for surveillance, suggests that disease transmission is less likely if the number of *Ae. aegypti* females collected in traps each week is less than three.[24] Mosquito numbers were reduced below this threshold in both sites (Figures 4 and 5) during the current study. In site 1, numbers recovered above this level shortly after the cessation of spraying. In site 2, numbers reduced briefly during spraying but were beginning to recover again before the end of spraying.

4 DISCUSSION

Overall, the results from this study suggest WALS® as a possible tool for the control of *Ae. aegypti* in urban areas of Puerto Rico and confirm the ability to treat open, covered and cryptic breeding sites. During this study, WALS® treatment in two field sites in Bayamón resulted in significant reductions in numbers of adult *Ae. aegypti* with reductions of 62% ($P = 0.0001$) and 28% ($P < 0.0001$) in comparison with an untreated control. Additionally, the use of *Bti* provides an alternative to the problem of resistance to adulticides. *Ae. aegypti* from Puerto Rico have been reported to be resistant to a wide range of adulticidal active ingredients[8, 9] notably used by the local department of health. Resistance to *Bti* strain AM65-52 (the strain used in the VectoBac® product) has not been reported in the scientific literature and resistance to natural *Bti* strains is considered unlikely.[25]

Although this trial was successful in reducing numbers of female *Ae. aegypti* in treatment sites, there was some need for finetuning of the application method used during the first three applications (14, 21 and 28 November 2018) in site 1. Initially 500 g/ha of product was achieved by spraying a 24% VectoBac® WDG solution at 5.68 L/min using a vehicle speed of 16 km/h. After this first application, it was determined that maintaining a consistent vehicle speed of 16 km/h was not possible; the narrowness of roads combined with oncoming traffic caused the spray vehicle to have to regularly slow down to pass cars parked on the side of the street, and speed bumps also caused the vehicle to have to slow down. The application rate was therefore adjusted to use a 12% VectoBac® WDG suspension, at a flow rate of 5.68 L/min and a vehicle speed of 8 km/h. However, this approach also caused problems with the vehicle moving too slowly and backing up traffic, which resulted in running out of fuel for the spray equipment before the application was complete. A final adjustment was then made resulting in an application of 12% VectoBac® WDG suspension, applied at a flow rate of 8 L/min and a vehicle speed of 11 km/h; after this final adjustment on 28 November 2018, all applications were made using these parameters.

Bioassay mortality in the sprayed blocks was higher than in the untreated control indicating that applications were successful in delivering *Bti* to the jars placed on properties within the sprayed blocks. Bioassays also confirmed that it is possible to reach a variety of different larval habitats on residential properties whether open to the sky, under vegetative cover or covered such as inside a marquesina or under a porch. Regardless of the position of the simulated larval habitats, it was possible to achieve deposition of droplets of *Bti* that was sufficient to result in the death of larvae added to the jars in the lab.

During the study, it became apparent that jars could not be placed consistently enough to allow analysis by coverage type to take place. The fact that different technicians were employed in the placing of the jars each week meant that bioassay jars were unlikely placed in the exact same locations for each treatment. Decisions surrounding what might constitute the different coverage types proved to be subjective, for example the difference in definition between slight *versus* dense vegetative cover. Additionally, the definition of full cover also proved open to interpretation by technicians, with some considering this to mean covered from the sky and others thinking the bioassay jar needed to be closely covered. This resulted in covered jars being placed in a range of environments from inside empty marquesinas to directly underneath parked cars. A jar placed on the floor of a marquesina having more space around it could be assumed to more easily allow for droplets of *Bti* to drift in and reach the bioassay jar as the spray vehicle passes by, than that closely protected underneath a parked car. For future similar studies, a stricter definition of coverage and placing of bioassay jars would need to be provided.

Despite limitations, these data do provide evidence that aqueous *Bti* microdroplets broadcast from air-blast equipment can reach a wide variety of larval habitats across spray blocks. It also raises interesting questions around the dynamics of spray cloud movement, the existence of microclimates and thermal inversions in the urban environment that cannot be answered here.

During the trial, the mean mortality of larvae in bioassays varied widely by week of spray application ($P < 0.01$), suggesting spray deposition was being influenced by factors that could not be controlled for. Factors that could have contributed to differences in larval mortality related to application date may include meteorological conditions such as wind speed, wind direction or

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temperature. Of the two treatment sites, site 1 repeatedly had higher mortalities after 48 h bioassays than site 2, suggesting differences in the topography of the sites, the buildings and local weather patterns which may have affected spray cloud dynamics resulting in site 1 receiving greater deposition of *Bti* as a result. However, without further, greater analysis of external factors, determining the reason for the variation seen by application date is beyond the scope of this study.

Lower mortality was observed in the bioassays from jars that were placed on properties on the edges of the spray blocks compared with those closer to the middle (data not shown). This is not surprising because vehicle-mounted spray paths are limited to roadways and spray cloud distribution is largely dependent on wind (prevailing winds in Bayamón are typically from the southeast). Operationally, an attempt to address this has now been made by the PRVCU by adjusting their spray routes, multiple routes around the spray blocks have been defined and are driven on alternate treatment dates with the aim of getting better coverage and avoiding areas where spray deposition is negatively impacted by buildings or prevailing wind. Another risk to the edges of spray blocks relates to the immigration of adult mosquitoes from outside the treated area. In the case of an established ongoing program in which mosquito numbers are reduced within the treatment block, it may be possible to see higher numbers of adult mosquitoes in surveillance traps on the edge of the spray block as a result of immigration into the area from outside the treatment site.

On two occasions during the trial, supply chain issues meant that spraying did not take place as planned. On 21 November 2018 there was not enough newly purchased *Bti* to carry out the application in treatment site 1 so an alternative supply was sourced from the storage facility at the Bayamón Department of Health and the treatment was carried out using product that was 2 years old. Larval mortality in bioassays on this date was the lowest for the whole trial (55%; Figure 3), but the error bars suggest this is not out of line with the other treatments carried out in November while application was being optimized.

On 14 February 2019 supply chain issues once again meant that there was not enough *Bti* to carry out the application leading to it being cancelled. From the female *Ae. aegypti* abundance data (Figure 5) it is not possible to see any impact as a result of the cancelled application. Prior to and following this date the abundance of *Ae. aegypti* females in the treatment site appears to follow the same trend as in the untreated control with abundance in the treatment site being significantly less than in the control. This raises a question for future investigation regarding what the appropriate cadence is for application of *Bti* once control has been achieved. It appears that missing this application and spraying at a one-month interval did not adversely affect the level of control achieved.

Although it was possible to significantly suppress female *Ae. aegypti* populations in both treatment sites during the trial, consideration should be given to what is an acceptable level of control. Work by Barerra *et al.*[24] using AGOs for surveillance (as well as control) determined in the case of chikungunya that if numbers of *Ae. aegypti* females caught in traps were above three per trap per week then disease transmission was more likely. In the current study, numbers of females reduced to below this threshold within two months of spraying (six applications) in both sites. Although suppressed, mean numbers of female *Ae. aegypti* in both sites continued to follow the trend seen in the untreated control group. In site 1, numbers of females remained below three per trap per week until the end of spraying and recovered shortly after spraying ceased (Figure 4). In site 2, suppression below the threshold was not maintained until the end of spraying, with both the treated site and untreated control following an upward trend in mosquito numbers in March and April (Figure 5). Ideally, it would be hoped that application of *Bti* using this method would result in a crash in populations rather than just suppression. The fact it was possible to get below this threshold shows promise and perhaps through further optimization of application, suppression of numbers to below the desired three per trap per week thought to be required for prevention of local arboviral disease transmission in Puerto Rico could be achieved.

The amount of time it took the *Ae. aegypti* populations within the treatment sites to recover contrasts widely. In treatment site 1, the significant difference between treatment and control was maintained throughout the course of the summer and by the end of data collection on 19 August 2019, the mean number of female *Ae. aegypti* in the untreated control was more than double that of the treatment site, with the two groups having followed the same trend since cessation of spraying. Interestingly, in the post-treatment phase the reduction in female *Ae. aegypti* in treatment site 1 had increased to 74% from 62% during the treatment phase. By contrast, the female *Ae. aegypti* population in treatment site 2 recovered very quickly and no significant difference could be detected between treatment site and untreated control by the end of May once the normal mosquito season had begun. Why there was such a contrast in recovery times is unclear at this time. This raises questions for the operational use of WALS®, and how to optimize its impact. Such questions may include when seasonally is the best time to spray and how long a spray campaign may need to last, or whether once a certain threshold is reached it is possible to stop spraying until mosquito numbers recover. Most likely the best approach to using WALS® for operational control of *Ae. aegypti* would be to establish a level of control that is acceptable to the program, carry out applications until this is achieved and then use larval surveys or set up larval sentinel sites (that are visited very regularly) to determine when spraying should be carried out. Spray campaigns based on the application of adulticides are typically reactive, meaning that when a rise in adult mosquito numbers is observed in surveillance traps, spraying is initiated. Because *Bti* is a larvicide and there can be a lag phase of over a week between larvae hatching and the resultant increase in adult trap catches, it is advised to use a larval indicator to initiate application rather than the traditional adult indicators described.

This trial was carried out at the end of 2018 and into 2019 due to a need to plan around other activities at the PRVCU and in Bayamón municipality. In order to see the greatest reduction in mosquito numbers between the treatment and control sites it may have been preferable to have planned the trial so that it began just prior to the start of the mosquito season, typically April/May,[6] so that *Bti* was applied to containers and other larval habitats as rains began, attending to larvae hatching from any egg bank that may have built up during the drier period of the year. Instead, this trial was carried out during the dry period when mosquito numbers were already comparatively low, it was expected that it may have been hard to determine if there was any difference between treated areas and untreated controls. Regardless, a significant difference between both treatment sites and the untreated control was observed, which is very encouraging for this technique as a control tool for use in the Puerto Rican context. In early planning it was also

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hoped to detect an epidemiological impact as a result of reducing mosquito numbers with WALS®; however, due to logistical considerations and timing, this was not possible. The trial site was likely too small, spraying was not carried out during the typical dengue season and at that time there had not been a large outbreak for over 5 years. It is hoped that continued WALS® operations by the PRVCU in Bayamón and other parts of Puerto Rico in 2020, with the ongoing dengue epidemic may help to reveal any epidemiological impact as a result of spraying.

5 CONCLUSION

In recent years, WALS® has increasingly become a recognized method of control for container-breeding mosquitoes in a variety of geographical locations, using a range of equipment from aerial spraying[12] to ground treatment using backpacks.[11] In comparison with another study using vehicle-mounted equipment where the net level of control reported was a 29% reduction of *Ae. aegypti* in treatment sites,[14] the study in Bayamón showed some improvement of the technique, with 28% and 62% reductions in numbers of *Ae. aegypti* in treatment sites compared with the control. In summary, we are confident that a wide range of larval habitats can be reached using this technique, higher levels of control can be achieved through optimization of vehicle-mounted sprays, and that it is an appropriate method for control of *Ae. aegypti* in urban Puerto Rico. With widespread pyrethroid insecticide resistance across Latin America and beyond, there is an absence of other suitable alternatives to vector control and WALS® offers a substantial improvement to conventional door-to-door approaches for dengue vector control. Based on the results of this trial, the PRVCU have invested in additional spraying equipment and expanded the WALS® program to six areas of the municipality of Bayamón as well as two areas in Dorado, with further future expansion planned in other municipalities on the island. The PRVCU now use WALS® as a standard part of their vector control program.

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CONFLICT OF INTEREST

The authors state that no conflict of interests exists in the publishing of this manuscript.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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