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ULTRALOW VOLUME APPLICATION OF *BACILLUS THURINGIENSIS* SSP. *ISRAELENSIS* FOR THE CONTROL OF MOSQUITOES

H. L. LEE, E. R. GREGORIO, JR., M. S. KHADRI AND P. SELEENA

Division of Medical Entomology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

ABSTRACT. Evaluation of the effectiveness of *Bacillus thuringiensis* ssp. *israelensis* (*B.t.i.*) against mosquito larvae dispersed by ultralow volume (ULV) spraying was conducted in simulated field trials. Effectiveness was measured using 3 different indicators: larval mortality, colony-forming unit enumeration, and droplet analysis. *B.t.i.* was dispersed with a ULV generator using 2 different flow rates: 0.3 and 0.5 liter/min on 2 different days. Based on the results of this study, it can be concluded that an output of 0.3 liter/min is effective for controlling *Aedes aegypti,* although a dosage of 0.5 liter/min can be used when high residual activity is desired. For *Culex quinquefasciatus* control, both dosages were effective but with low residual activity. For *Anopheles maculatus* control, only a discharge rate of 0.5 liter/min was effective with low residual activity. *B.t.i.* application at both dosages penetrated tires well, indicating that *B.t.i.* ULV application is an effective method for controlling container-inhabiting mosquitoes. Good coverage of target area and penetration were attributed to satisfactory droplet profiles.

INTRODUCTION

Ultralow volume (ULV) application of insecticides is now an established method of vector control (Armstrong 1970; Mount et al. 1968, 1970). In Malaysia and other Southeast Asian countries, ULV application of malathion is commonly used in the control of dengue vectors (Lam and Tham 1988) and is now standard in suppressing *Aedes* vectors, especially during vector-borne disease outbreaks. Since ULV-applied malathion is not known to exhibit larvicidal efficacy, there is a need for a larvicidal agent capable of being dispersed through ULV spraying. Also, new control agents should be environmentally friendly and have minimal environmental impact. Today, two mosquito control agents having these characteristics are the microbial control agents *Bacillus thuringiensis* ssp. *israelensis* (*B.t.i.*) and *Bacillus sphaericus.* These agents are highly and specifically effective against larvae of mosquitoes and blackflies. Often, conventional methods—knapsack sprayers—are used to disperse the bacteria to target areas (World Health Organization 1982). However, the limitations of these methods—coverage and application speed—have reduced their effectiveness. Here, we report the use of ground ULV techniques for the dispersal of *B.t.i.* for mosquito control.

MATERIALS AND METHODS

Study site: An open field measuring about 150 × 100 m was selected as the study site.

ULV machine used: A vehicle-mounted IGEBA® ULV aerosol generator, model #U15M (IGEBA Geraetebau Gmbh, Germany) was used to disperse *B.t.i.* and was operated at an air pressure of 4.5 psi during the ULV operation. The insecticide was dispersed at 2 discharge rates, 0.3 and 0.5 liter/min (= 1.48 and 2.47 liter/ha, respectively), at a vehicle speed of 8–10 kph for 1 min covering a distance of about 45.7 m with its 0.8 × 2-mm nozzle elevated to 45° pointing toward the test samples. Prior to the tests the flow rates were calibrated by the supplier using distilled water.

Biological insecticide used: Vectobac® (Abbott Laboratories No. Chicago, IL), an aqueous suspension of *B.t.i.* (1,200 ITU/mg) against *Aedes aegypti* (Linn.) was used. Its LC_{50} value against laboratory-bred *Ae. aegypti* (L3/L4 instar) was 0.0027 mg/liter (Lee and Seleena, unpublished data).

Mosquitoes tested: Larvae of 3 species of laboratory-bred mosquitoes were used in the study: *Ae. aegypti, Anopheles maculatus* Theobald, and *Culex quinquefasciatus* Say.

Bioassay: Fifteen late 3rd/early 4th instar (L3/L4) larvae of each species were placed in a cup with 50 ml distilled water. Tires with 500 ml distilled water but without larvae were also used. Cups and tires were arranged side by side in a straight line so the 1st cup and tire were 3 m away from the ULV generator with the rest of the cups and tires spaced at 3-m intervals to a distance of 30 m.

Evaluation: To evaluate the effectiveness of *B.t.i.* applied by ULV, 3 different indicators were measured:

1. Larval mortality: The most important factor in evaluating the effectiveness of *B.t.i.* is larval mortality 24 h posttreatment. Larval mortality was scored by placing 15 L3/L4 laboratory-bred larvae of *Ae. aegypti, An. maculatus,* and *Cx. quinquefasciatus* in cups containing 50 ml of distilled water. These cups were placed at 3-m intervals, 3 m from the ULV generator out to 30 m in a straight line. One hour after treatment, the cups containing the larvae were removed from the test sites and brought back to the laboratory. Larval mortality was scored 24 h post-

ULV application. All larvae were subsequently removed from the cups. The cups with the test water were left at room temperature (28–32°C) with relative humidity of about 85% for 14 days. Untreated control cups (2 cups/species) were brought to the test site but kept away from the ULV spray. To determine whether *B.t.i.* toxins persist and exert a residual effect, larval mortality after 7 and 14 days was scored. This was done by adding 10 fresh L3/L4 instar larvae into the cups, and after an overnight exposure the number of dead larvae was counted.

2. *B.t.i.* colony-forming unit (CFU) enumeration: Another measurement employed in the evaluation was the CFU enumeration per ml, used to determine the residual activity caused by *B.t.i.* spores. This was accomplished by collecting water samples from the test cups and tires and putting them into sterile containers at intervals of 1 hour, 7 days, and 14 days post-ULV. Samples were then plated onto *B. thuringiensis* selective media (NYPC). This culture medium contains nutrient agar (23 g/liter), yeast (0.5 g/liter), $MnCl_2$ (6 mg/liter), $CaCl_2$ (80 mg/liter), and $MgCl_2$ (70 mg/liter). Polymyxin B sulphate (0.1 g/liter) and chloramphenicol (1 mg/liter) were incorporated into this medium (Yousten et al. 1982). This medium was inoculated with 0.3 ml of each water sample, and the CFU/ml was enumerated after a 24-h incubation at 32°C. Corresponding serial dilutions were done for samples whose CFUs were more than 300/plate. Only those plates with a CFU count of between 30–300/plate were counted and 2 replicates were done for each sample. The number of CFUs/ml in water samples enumerated 1 h post-ULV was indicative of the coverage of ULV spraying, and CFU counts for 7 and 14 days post-ULV water samples showed the persistence of *B.t.i.* in the test samples.
3. Droplet analysis: The 3rd factor was droplet analysis. Distribution and size of sprayed particles were monitored with MgO-coated slides. Prepared by burning a 25-cm strip of magnesium ribbon on the underside of a clean microscope slide to produce a uniform coat of MgO, these slides were placed horizontally with the coated surface up to receive the droplets amid the cups from a 3–30-m distance from the generator. After 1 h post-ULV, slides were collected and brought back to the laboratory. Using a calibrated micrometer, the diameter of each droplet was measured. Data were analyzed using the ULV droplet analysis program of Sofield and Kent (1984) to obtain the volume median diameter (VMD) and number median diameter (NMD) values. To determine the uniformity of the droplet size, the ratio of VMD to NMD was obtained. For uniform distribution the ratio should be near 1.

The trials were conducted on 2 different days, testing 2 different discharge rates. Two preliminary spraying trials were conducted to test-run the machines.

Trial 1—June 2, 1995: B.t.i. was dispersed downwind at 0.3 liter/min toward the test samples. Water samples taken from tires were transferred into cups and brought back to the laboratory where 15 fresh L3/L4 instar larvae of *Ae. aegypti* were added. Larval mortality 24 h post-ULV in the test samples from the tires was also scored. To determine the persistence of *B.t.i.* after field application, 7- and 14-day water samples were collected and CFU/ml was enumerated.

Trial 2—June 26, 1995: B.t.i. was dispersed 0.5 liter/min. Cups, MgO-coated slides, and tires were arranged as in the 1st trial, but, because of prevailing winds, the spray vehicle drove in the opposite direction from the previous trial. After 1 hour, larvae and water samples taken from the tires were brought to the laboratory. Larval mortality was scored 24 hours, 7 days, and 14 days post-ULV. CFUs/ml were enumerated and slides were analyzed.

RESULTS

Trial 1 (*B.t.i.* dispersed at 0.3 liter/min) results are presented in Table 1. *B.t.i.* sprayed at 0.3 liter/min achieved a 70–100% mortality for *Ae. aegypti* and *Cx. quinquefasciatus* at all distances 24 h post-spray, with a relatively high CFU count per ml. Few droplets were observed on MgO-coated slides. The VMD and NMD were 43.53 and 11.39 μm, respectively, with a ratio of 3.82. Low residual effect after 7 days and no residual activity after 14 days were observed for both *Aedes* and *Culex* larvae. In comparison, the CFU count was high in the 30-min water samples and low in both the 7- and 14-day water samples.

For *An. maculatus* low larval mortality (0–55%) at all distances was noted after 24 h although there were considerably higher CFU counts observed in all the 30-min water samples. Absence of CFU, and thus complete lack of residual activity, was also observed in 7- and 14-day post-ULV samples.

Table 1 shows *Ae. aegypti* were highly susceptible to the sprayed *B.t.i.* in tires as indicated by high mortality after 24 h, indicating that *B.t.i.* dispersed at 0.3 liter/min had good penetration of tires, a larval habitat for this mosquito. Mortality observed in the tires 6 m and 9 m away from the generator was only 40 and 30%, respectively, while no mortality was observed in one tire 24 m away. However, minimal mortality was noted in the 7- and 14-day post-ULV samples. There were high CFU counts from the 30-min samples and low counts in the 7- and 14-day post-ULV samples.

For the 2nd trial *B.t.i.* was dispersed at 0.5 liter/min. More droplets were observed on the MgO-coated slides. The VMD and NMD were 57.56 and

Table 1. Mean mosquito larval mortality (%) 24 hours, 7 days, and 14 days posttreatment using a discharge rate of 0.3 liter/min.

Distance from ULV generator (m)	*Ae. aegypti*			*Cx. quinquefasciatus*			*An. maculatus*			Tires		
	24 h	7 days	14 days	24 h	7 days	14 days	24 h	7 days	14 days	24 h	7 days	14 days
3	100	100	50	100	0	0	30	35	5	100	70	10
6	100	100	50	100	0	0	35	15	0	40	30	0
9	100	100	50	100	0	0	30	0	0	30	0	0
12	90	50	0	100	0	0	55	0	0	100	0	0
15	80	50	0	90	0	0	45	0	0	90	0	0
18	70	50	0	90	0	0	50	0	0	90	0	0
21	100	50	0	90	0	0	45	0	0	90	50	0
24	100	50	0	90	0	0	0	0	0	0	0	0
27	10	10	0	90	0	0	50	0	0	100	0	0
30	70	45	0	70	0	0	0	0	0	100	0	0

24.97 μm, respectively, with a ratio of 2.3. Results are presented in Table 2.

High larval mortality after 24 h at all distances was observed for both *Ae. aegypti* and *Cx. quinquefasciatus*. CFU counts for the 30-min samples were high. High residual activity against *Ae. aegypti* was observed in the 7-day water samples and persisted until 14 days. However, no residual effect was observed for *Cx. quinquefasciatus* in the 7- and 14-day water samples.

Table 2 also shows a high mortality for *An. maculatus* 24 h post-spraying, although low residual activity had been observed in the 7- and 14-day water samples. There was high larval mortality in almost all the 24-h post-ULV tire samples at all distances with the exception of tires 12–15 m away from the ULV generator, which only showed 30–60% mortality. Low mortality was observed in the 7- and 14-day water samples. In all trials, absence of mortality was observed in all species of the control (untreated) mosquito larvae.

DISCUSSION

In the 1st trial the discharge rate used to disperse *B.t.i.* was 0.3 liter/min. Few droplets were observed on MgO-coated slides, and the ratio of VMD to NMD was 3.82, indicating droplet size was not uniform, i.e., there were more bigger droplets than smaller droplets. Nevertheless, *B.t.i.* sprayed at a rate of 0.3 liter/min was able to reach a distance of 30 m from the ULV generator. High mortality at all distances was observed for both *Ae. aegypti* and *Cx. quinquefasciatus* 24 h post-fogging. CFU enumeration was in the range of 31,000–878,333 CFUs/ml for *Aedes,* while it was 163,333–993,333 CFUs/ml for *Culex.* But little mortality was achieved in the 7- and 14-day water samples for *Aedes,* with CFU counts ranging from 1,750–871,667 CFUs/ml. Mortality was not observed in the 7- and 14-day post-ULV evaluations for *Culex*; the CFU count ranged from 33–15,333 CFUs/ml. This signifies that *B.t.i.* dispersed at 0.3 liter/min

Table 2. Mean mosquito larval mortality (%) 24 hours, 7 days, and 14 days posttreatment using a discharge rate of 0.5 liter/min.

Distance from ULV generator (m)	*Ae. aegypti*			*Cx. quinquefasciatus*			*An. maculatus*			Tires		
	24 h	7 days	14 days	24 h	7 days	14 days	24 h	7 days	14 days	24 h	7 days	14 days
3	60	0	0	90	0	0	60	0	0	100	0	0
6	100	100	75	100	0	0	100	0	0	100	80	0
9	100	100	100	100	0	0	100	0	0	100	100	0
12	100	100	70	100	20	0	100	0	0	60	0	0
15	100	50	50	100	15	25	100	0	40	30	80	100
18	100	100	85	100	0	0	100	0	0	100	0	0
21	100	80	100	95	0	0	100	0	0	100	40	0
24	100	80	90	100	0	0	70	0	0	100	100	0
27	100	75	50	100	0	0	100	0	0	100	40	0
30	100	95	20	100	0	0	35	10	0	100	0	0

was effective for immediate, short-term control of *Aedes* and *Culex,* but because the flow rate used was too low, there was a lesser amount of toxin/spores deposited in the test samples. Thus, little residual activity was observed 7 and 14 days post-spraying.

Low mortality (less than 50–70%) had been scored after 24 h for *An. maculatus* despite the high CFU count per ml (103,333–676,667 CFUs/ml). The decrease in mortality observed for *Anopheles* can be attributed to the surface-feeding behavior of this mosquito. Bacterial toxins tend to sink to the bottom of the cups after 3 h and before the larvae are able to feed on them (Lee and Cheong 1985). Absence of CFUs was noted in the 7- and 14-day water samples and no residual activity was observed.

Results of the trial conformed with the findings of Seleena et al. (1995), in which the discharge rate was 1.6 liters/min. They found that *B.t.i.* sprayed at this flow rate caused high mortality for *Ae. aegypti, Cx. quinquefasciatus,* and *An. maculatus.* Because the discharge rate was much higher than 0.3 and 0.5 liter/min, very high residual activity was observed in the 7-day post-ULV samples. However, this study did not test for persistence 14 days post-ULV.

To determine the ability of the sprayed *B.t.i.* to penetrate tires, 24-h larval mortality in the water samples taken from the tires was also scored. *B.t.i.* sprayed at 0.3 liter/min penetrated tires, as indicated by the high *Ae. aegypti* larval mortality. Low mortality was observed in tires 6–9 m away from the generator. The low mortality can be explained because the tires used here had a much narrower rim opening which restricted spray penetration. A similar observation was made for the 2nd trial using 0.5 liter/min. The CFU count was in the range of 16,667–116,667 CFUs/ml. Very low residual activity was seen, as indicated by low mortality 7 and 14 days post-ULV with a CFU count ranging from 87 to 9,300 CFUs/ml. Only *Aedes* was tested for this purpose since this species has a predilection for inhabiting these kinds of containers. The ability of *B.t.i.* ULV to penetrate tires likewise agreed with earlier studies made in the Dominican Republic (Tidwell et al. 1994). They noted high mortality (95.1–100%) in all water-holding drums and tanks both inside and outside houses. The flow rates used were 788, 1,577, and 2,586 ml/ha. This study was made as an emergency control of dengue, and therefore only *Ae. aegypti* was used.

A higher flow rate of 0.5 liter/min was used in the 2nd trial. During this trial, more droplets were observed on the MgO-coated slides with an overall VMD and NMD of 57.56 and 24.97 μm, respectively. Here the ratio was closer to 1, indicating that the droplets were more uniform than in the 1st trial. Since more droplets could be sampled on the slides, it became possible to analyze the droplet profiles and ratio of VMD to NMD at all distances (Fig. 1).

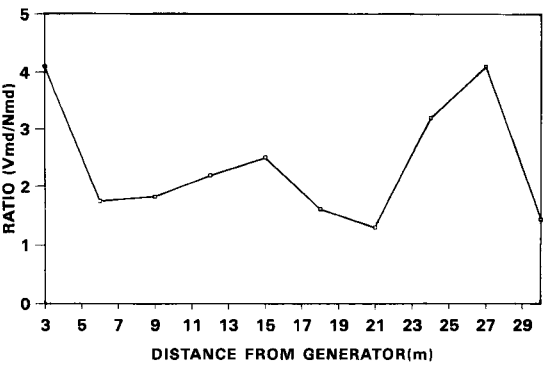

Fig. 1. Droplet profile of ULV fogging at a discharge rate of 0.5 liter/min.

The ratio ranged from 1.3 to 4.1, indicating that the 0.5-liter/min rate produced larger droplets (VMD) than the 0.3-liter/min rate. *B.t.i.* sprayed at 0.5 liter/min covered a distance of up to 30 m from the generator. This result shows that a flow rate of 0.5 liter/min increases the coverage in dispersing *B.t.i.* by ULV. This result also agreed with the suggestion of Seleena et al. (1995) that a flow rate lower than 1.6 liters/min could cover a distance of more than 21 m from the generator.

B.t.i. dispersed at the 0.5 liter/min rate gave high mortality (60–100%) at all distances after 24 h both for *Aedes* and *Culex* with a CFU count ranging from 80,000 to 796,667 CFUs/ml. High residual activity was observed for *Aedes* in the 7- and 14-day water samples, indicating that this discharge rate is effective in controlling *Ae. aegypti* with good residual activity even after 14 days. The CFU counts were between 1,110–9,967 CFU/ml. Low mortality and CFU count (1,750–15,000 CFUs/ml) were observed for *Culex* after 7 and 14 days, indicating that *Culex* was not as susceptible to comparatively low dosages as were *Ae aegypti* and *An. maculatus.*

Anopheles maculatus was fairly susceptible at this dosage, as indicated by a high mortality observed after 24 hours. This rate resulted from a higher dosage of bacterial toxin/spores settling on test samples as indicated by both CFU enumeration, which showed a count of between 113,333–747,762 CFUs/ml, and increased number of droplets seen on the MgO-coated slides. However, there was no residual activity observed for *Anopheles,* showing the toxin is only effective within 24 h on these larvae. Once the toxins settled on the bottom, there was a lower probability they would be ingested by these surface-feeding larvae.

For the tires, there was also good penetration of *B.t.i.,* as indicated by high mortality of *Ae. aegypti* larvae at all distances with the exception of tires placed 12–15 m away from the ULV generator (mortality of 30–60%). The CFU counts in the 30-min samples ranged from 100,000–766,667 CFUs/ml. The reason for lower mortality was prob-

ably the much narrower rim opening of these 2 tires compared to the openings of other standard tires used in this evaluation. Some residual activity was noted 7 and 14 days post-fogging, with the CFU counts ranging from 220 to 9,500 CFUs/ml. Based on this study, it can be concluded that *B.t.i.* dispersed by ULV spraying at a rate of 0.3 liter/min was effective in controlling *Ae. aegypti* and *Cx. quinquefasciatus.* However, at this dosage no residual activity was observed. When high residual activity is desired, we recommend that the discharge rate be increased to 0.5 liter/min. For control of *An. maculatus,* a rate of 0.5 liter/min appeared to be effective, but low residual effect was observed.

Analysis showed droplet profiles obtained by spraying at both flow rates were satisfactory when good penetration of tires is desired. Large droplets (50–100 μm) are desired in *B.t.i.* ULV spraying as this will ensure rapid settling of the spores/toxin complex into water containers and minimize loss of bacteria due to drift. However, large droplets produced as a result of high flow rate tend to travel shorter distances and are not cost-effective. Hence, the flow rates of the generator should be regulated to effect an optimal output based on the types of larvae to be controlled.

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