AEDES ALBOPICTUS CONTROL WITH SPRAY APPLICATION OF *BACILLUS THURINGIENSIS ISRAELENSIS*, STRAIN AM 65-52

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Abstract. A *Bacillus thuringiensis israelensis* (Bti) formulation, VectoBac®WG (strain AM 65-52), was evaluated for mosquito control in a training area with dense vegetation. Bti was spray applied to target *Aedes albopictus* larval habitats of 130 ha once every 2 weeks using a motorized back pack mist blower, Stihl ® SR420, and a vehicle mounted ultra low volume generator (ULV), IGEBA® U40. Ovitrap index (OI) and larval density (LD) were used to measure the efficacy of larviciding. In the Bti treated area the OI and LD significantly decreased with time ($p < 0.05$); OI decreased from 84.3 ± 1.7 to 27.5 ± 2.5 (%) and LD decreased from 27.9 ± 1.5 to 3.2 ± 1.8 larvae per ovitrap by 3 months from the start of treatment. During the same period of time there was no significant reduction in OI and LD at the untreated site which was under a conventional mosquito control program. This large scale study indicates larvicidal spraying with Bti of natural breeding sites, was able to reduce *Ae. albopictus* adult density. This significant reduction was not achieved with conventional manual application methods.

Key words: *Aedes albopictus* control, vegetation, larviciding, spray application of Bti

INTRODUCTION

Dengue is endemic in Singapore with year-round transmission. In the past two decades, a surge in cases was reported in 1992, 1998, and 2004. Since 2005, this affluent country has experienced epidemic levels of dengue cases for 3 consecutive years with several fatalities. Some weeks during these 3 years had more than the "epidemic threshold" of 378 cases per week. In 2005 there were 14,209 confirmed cases with 25 deaths, 2006 had 2,844 cases, with 10 deaths and 2007 had 8,826 cases and 20 deaths (Ministry of Health Singapore, 2007). *Aedes aegypti* (L.) and *Aedes albopictus* Skuse are the two recognized dengue vectors in this island-state.

The Singapore National Environment Agency (NEA) and members of an interagency taskforce have intensified daily inspections of possible larval habitats in homes and outdoor areas, such as drains, construction sites, parks and vacant lands. Source reduction and larval habitat treatment with temephos sand granules and antimalarial oil are the primary measures used to combat the mosquito vectors. Adulticiding is used in the event of dengue outbreaks within the vicinity via outdoor thermal fogging and indoor ULV application with organophosphates.

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In a comprehensive blanket entomological survey conducted from 2003 to 2004 in various training areas used by the Singapore Armed Forces (SAF), *Mansonia* sp Blanchard and *Ae. albopictus* were the principal human biting mosquito species, with small numbers of *Culex gelidus* Theobald and *Culex vishnui* Theobald. Among the *Mansonia* sp, *Mansonia indiana* Edwards was the most commonly collected species.

In view of the high incidence of dengue in Singapore, there is a need to adopt an effective mosquito management strategy that can provide good control of dengue vectors within the SAF training areas and camps without compromising the natural ecosystem within these grounds. SAF training grounds are by and large undeveloped and contain a mixture of uninhabited primary and secondary forest. Such training grounds are off limits to the general public and are protected. These grounds provide ideal larval habitats for *Ae. albopictus* in the form of natural containers, such as leaf litter, stagnant pools of clear water with leaf litter and vegetation with leaf axils and tree holes that can hold small volumes of water. Conventional mosquito control techniques used include treating larval habitats with anti-malarial oil and temephos sand granules on a weekly basis and fogging adulticides on an ad hoc basis when the adult mosquito density is determined to be high.

The SAF mosquito control program focuses on source reduction and a weekly mosquito control program administered by commercial pest control companies. Source reduction has limited effectiveness in these training grounds since the larval habitats of *Ae. albopictus* consist largely of numerous natural breeding sites. Furthermore, the natural larval habitats of *Ae. albopictus*, which are generally microhabitats within a forest ecosystem, are not treated by conventional methods of treating water bodies using anti-malarial oil by hand compression sprayers and temephos sand granules applied by hand. Limitations in the present mosquito control program and the extensive area of the microecosystem have contributed to the successful colonization of the training grounds by a high density of *Ae. albopictus* mosquitoes.

Many adulticides and larvicides used for mosquito control are not target specific and hence eliminate a wide spectrum of beneficial insects in the forest ecosystem of the training sites.

A 6-month study managed by the SAF was undertaken to investigate the efficacy of *Bacillus thuringiensis israelensis* (Bti), an established biological larvicide, to tackle the above mentioned problems. Bti was discovered in 1976 and has seen widespread use for the control of mosquito and black fly larvae (Lacey, 2007). A water-dispersible granule Bti formulation (strain AM 65-52) formulation, VectoBac® WG of Valent BioSciences Corporation, USA, was used in this study. This formulation of 3,000 ITU/mg against *Ae. aegypti* has been successfully evaluated by the WHO Pesticide Evaluation Scheme (WHOPES) for product efficacy and safety and the product is manufactured in accordance with the WHO specification 770/WG (WHO 2004, 2007). WHOPES has found this formulation to have high efficacy against larvae of many mosquito species in a variety of habitats, not harmful to non-target organisms and free of chemical and microbial contaminants.

This paper reports the impact of Bti larvicidal spraying against *Ae. albopictus* adult mosquito density in a forested training ground.

Fig 1–Study sites, TS 1 and TS 2 separated by a 6 lane expressway (Kranji Expressway). Source of map: Singapore Land Authority

MATERIALS AND METHODS

Study site

Two study sites were selected from 5 training plots. Both had similar characteristics (Fig 1). The sites were located in western Singapore and in this paper they are designated as training sites TS1 and TS 2. The sites were separated by a 6 lane road, the Kranji Expressway, with a minimum distance of 0.2 km between the 2 study sites. A baseline survey was conducted from 2004 to 2005 in order to determine the predominant human biting species and to map the target larval habitats in each site. In both sites *Ae. albopictus* was the predominant human biting species.

TS 1 has a total land area of 900 ha. The target larval habitats were concentrated in an area of 130 ha which was treated with Bti spray application. Larval habitats consisted of natural breeding sources, which included tree holes, leaf axils, clumps of bamboo plants, and forest ground covered with layers of curled dried leaves that remained moist at all times, holding in water after rain. There are at least 15 different ponds with stagnant clear water and leaf litter at the base of the ponds. There are also numerous earth drains that aid in the drainage within this site. However, due to the design of these earth drains there are numerous "dead spots" holding water within the drainage system.

TS 2 (128 ha), used as the untreated control (UTC) site, is entirely covered with tall vegetation and little undergrowth. The forest grounds are covered with dried curled leaves, just as in TS 1. There is a concrete lined drain running through the area but due to a structural defect it also allows pockets of stagnant water to form in the drain. During the period of study, conventional mosquito control strategies were used, which were comprised of weekly oiling in all possible ground larval habitats and monthly treatment of permanent water bodies with temephos sand granules. Thermal fogging was also conducted when adult mosquito densities were high.

Bti treatment

All target *Ae. albopictus* larval habitats mentioned above in the description of study site TS 1 were treated with Bti via spray application, which provided complete uniform coverage. Motorized back pack mist blowers, Stihl® SR420 and a vehicle mounted ultra low volume generator (ULV), IGEBA® U40 were used for spray application. Areas inaccessible to the vehicle mounted sprayer, were treated using the back pack mist blowers. The discharge rates, and the swaths of the spray equipment were pre-calibrated. A pre-treatment exercise was conducted to determine the required amount of water to disperse the Bti into all larval habitats and the size of the discharged droplets, volume median diameter (VMD), were also determined using Magnesium Oxide slides.

Six cycles of Bti treatments with VectoBac® WG (Lot No 125 - 097 PG), Valent Biosciences, Chicago, IL) commenced in the 1st week of October 2005, with the last treatment on 25 December 2005. The treatments were conducted once every 2 weeks, at a dosage of 500 g/ha. Bti was suspended onsite into the required amount of water. For the back pack mist blowers 4 kg was suspended in 120 liters of water using moderate agitation. Each back pack unit holding 13 liters of the mixture was used to apply the mixture to target habitats. The flow control dial was set at orifice No. 2 providing an average discharge rate of 500 ml/minute. During the scheduled treatment week, spraying was carried out by 3 applicators between 7:00 AM - 4:00 PM covering a total area of 70 ha. For the vehicle mounted ULV generator, 5.6 kg was suspended in 75 liters of water and the suspension was delivered through 4 adjustable nozzles which were set at -5º, 0º, 5º and 15º. Set at a discharge rate of 1 liter/minute, and a vehicle speed of approximately 5 km/h, the spray droplets of VMD ≤ 50 μm were delivered into target larval habitats over wide areas to an optimum swath of 15 m in dense vegetation, covering a total area of 60 ha. The insecticide tank was topped up after a run of 1 hour, because a minimum volume of 10 liters of mixture in the tank was required to produce a uniform spray from the ULV generator when the vehicle was passing over uneven ground. The ULV spray application was conducted during the cool hours in the late evening or early morning, 7:00 PM - 5:00 AM. The cool air allowed the settling of the sprayed Bti droplets into the target larval habitats.

Ovitrap surveillance

The efficacy of Bti treatment was evaluated by measuring the adult mosquito population. The larval habitat in the SAF study sites was vast, and it was too tedious to search and measure the larvae/pupae density among the dense vegetation. The *Ae. albopictus* adult populations were thus monitored with ovitrap surveillance. Ovitraps are the most sensitive tools

Table 1
Mean ovitraps positive with *Ae. albopictus* larvae (%) ± SE from training sites, TS 1 and TS 2 during the study period, July-December 2005.

Training site	Pre-treatment period	Treatment period		
	July - September 2005	October 2005	November 2005	December 2005
TS 1 Bti treated	84.3 ± 1.7 [a]	77.2 ± 2.22 [a] (14.2)	44.4 ± 5.9 [b] (47.9)	27.5 ± 2.5 [b] (66.0)
TS 2 Untreated control site	93.6 ± 3.5 [a1,c]	100 [a1,c]	94.6 ± 5.4 [b1,c]	89.8 ± 3.3 [b1,c]

Figures in parentheses represent percent reduction as calculated by Mulla's (1971) formula.
[a, b, c] Significant analysis determined between pre-treatment and treatment periods within a site; [a1, b1] Significant analysis determined between sites during the same period. Similar alphabets denote no significant difference at $p = 0.05$; [a1 and b1] are significantly different from the corresponding OI values [a and b] over time.

to detect the presence of gravid female *Aedes* sp mosquitoes in a habitat where immature mosquitoes are difficult to locate (Jakob and Bevier, 1969; Lee, 1992).

The ovitraps used are described by Lee (1992): 300 ml plastic containers (7.8 cm x 6.5 cm x 9.0 cm) with the exterior coated with a layer of black paint. An oviposition paddle (10 cm x 2.5 cm x 0.3 cm) made from dark brown colored hard board was placed in each ovitrap. The containers were filled with tap water to a level of 5.5 cm in each container. In between surveys, the ovitrap containers and paddles were washed thoroughly with hot water to destroy any remaining unhatched eggs.

Forty ovitraps were placed in TS 1 and 30 ovitraps in TS 2. The selection of ovitrap sites was predetermined through adult mosquito landing rates during the day. The selected ovitrap sites were determined to have a high *Ae. albopictus* adult mosquito density with a landing rate of at least 90 mosquitoes/man/hour. Efforts were also made to ensure that all the ovitrap locations were well distributed in each study site to ensure that adequate coverage was achieved during the study. All ovitraps were placed on the ground within the vegetation at the same locations throughout the study period. Ovitraps were placed once every 2 weeks from July to December 2005 with 6 pretreatment surveillance events (July- September), followed by 6 concurrent ovitrap surveillance events during Bti treatments (October - December).

Ovitraps were collected 5 days after placement and brought to the SAF camp. The contents of each ovitrap, including the paddle, were transferred to a plastic container. Sufficient water was then added to ensure the paddle was fully submerged. Larval counts commenced on the third day after collection, only mosquito larvae from the second instar or larger were selected for counting and identification. Larval counting was subsequently carried out on alternate days until Day 7 post-collection, thus providing sufficient time for eggs to hatch.

Data analysis

The total number of ovitraps with *Ae. albopictus* larvae (positive ovitrap) and total number of *Ae. albopictus* larvae found in all the collected ovitraps at each surveillance were recorded. The ovitrap index

Table 2
Mean larval density per ovitrap ± SE from training sites, TS 1 and TS 2 during the study period, July - December 2005.

Training site	Pre-treatment period	Treatment period		
	July - September 2005	October 2005	November 2005	December 2005
TS 1 Bti treated	27.9 ± 1.5 [a]	17.8 ± 2.1 [b] (59.3)	9.7 ± 1.8 [b] (53.2)	3.2 ± 1.8 [b] (80.0)
TS 2 Untreated control site	30.4 ± 7.9 [a0, c]	47.7 ± 21.4 [b0,c]	22.5 ± 1.8 [b1,c]	17.1 ± 1.6 [b1,c]

Figures in parentheses represent percent reduction as calculated by Mulla's (1971) formula.
[a, b, c] Significant analysis determined between pre-treatment and treatment periods within a site; [a0, b0, b1] Significant analysis determined between sites during the same period. Similar alphabets denote no significant difference at $p = 0.05$; [a0 and b0] are not significantly different from the corresponding LD values [a and b] over time at $p = 0.05$; [b1] is significantly different from the corresponding LD values [b] over time at $p = 0.05$.

and larval density were determined as follows: the Ovitrap Index (OI) was the number of positive ovitraps/total number of collected ovitraps x 100; and the Larval Density (LD) was the total number of larvae/total number of collected ovitraps. All data were statistically analyzed using the *t*-test at $p = 0.05$.

The percent reduction in the post-treatment ovitrap index and larval density were calculated according to Mulla (1971) as follows:

%Reduction = 100 - (C1/T1 x T2/C2) x 100

Where C1 is the pretreatment number in the control site (TS 2), C2 is the post-treatment number in the control site (TS 2), T1 is the pre-treatment number in the Bti treated site (TS 1), and T2 is the post-treatment number in the Bti treated site (TS 1).

RESULTS

Pre-treatment ovitrap surveillance took place from 18 July to 26 September 2005 ($n = 6$) and indicated a stable highly dense population of *Ae. albopictus* mosquitoes at both study sites (Fig 2), with a significantly higher mosquito population in TS 2 ($p < 0.05$). The mean ovitrap index (OI) for TS 1 was 84.3 ± 1.7% and for TS 2 was 93.6 ± 3.5% (Table 1). In TS 1, the larval density (LD) over the 3 month pre-treatment surveillance period remained stable (Fig 3) with an average of 27.9 ± 1.5 larvae per ovitrap (Table 2). The LD in TS 2 varied from 14 to 60 larvae per ovitrap (Fig 3), giving an average of 30.4 ± 7.9 (Table 2). Overall, the pre-treatment LD did not differ significantly between the sites ($p > 0.05$).

In TS 1, during the 3 months of Bti treatment from October to December 2005 a highly significant reduction in the *Ae. albopictus* adult population was measured by the OI was observed with time with a strong correlation ($R^2 = 0.81$), but the population in TS 2 remained stable during those months ($R^2 = 0.016$) (Fig 2). A similar pattern was also observed for larval density in the ovitraps (Fig 3). In the first month of Bti treatment reductions were observed in the OI and LD in comparison to the pre-treatment period (Tables 1 and 2). During the same period, TS 2, which was under the conventional mosquito control program, had an increase in *Ae. albopictus*

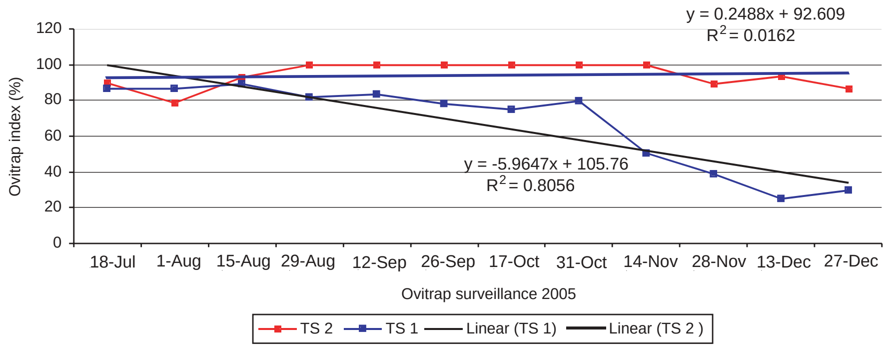

Fig 2–Average ovitrap index (%) for each surveillance week in study sites TS 1 (Bti treated site) and TS 2 (untreated control site) with linear regression lines.

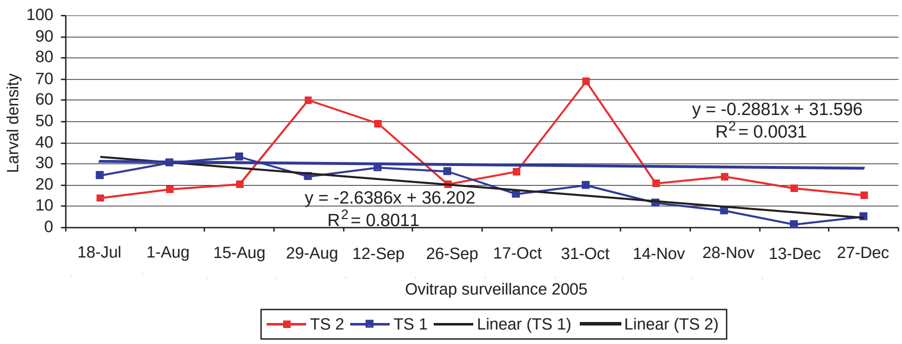

Fig 3–Average larval density for each surveillance week in study sites TS 1 (Bti treated site) and TS 2 (untreated control site) with linear regression lines.

adult density, and it remained consistently high throughout the month with an OI of 100 % (Fig 2). The LD also peaked at 69.08 larvae per ovitrap (Fig 3).

During the next 2 months of Bti treatment, a highly significant reduction in the *Ae. albopictus* population was observed in TS1 ($p < 0.05$). During the second month of treatment (November 2005) the OI reduction was 47.9%, followed by a 66.0% reduction in December 2005. There were 53.2% and 80.0% reductions in the larval density in the Bti treated site during the second and third months, respectively.

In the control site (TS 2), the OI remained at 87 - 100% positive from October to December 2005, indicating no significant change in the number of adult gravid female mosquitoes at that site (Table 1). There was also no significant reduction in the LD ($p > 0.05$) (Table 2). Seasonal monsoon rains occur in late November/early December, which at times caused the water to overflow in the ovitrap containers which were kept on site for 5 days. This could have contributed to the reduction in the LD but not to the ovitrap index. However, the LD at the control site was 5.4 fold higher than at the Bti treated site ($p < 0.05$).

DISCUSSION

Of the 2 mosquito vectors, *Ae. aegypti* is the more likely transmitter of dengue viruses and *Ae. albopictus* ranks second (Knudsen, 1995). *Ae. albopictus* is considered to have a lower vectorial capacity because of its lack of host specificity. However, in some situations, *Ae. albopictus* has been the sole vector for dengue and chikungunya (CHIK) epidemics in Hawaii, Reunion Island and Italy, confirming its vectorial capacity. Epidemic sites have had high densities of *Ae. albopictus* mosquitoes (Effler *et al*, 2005; Reiter *et al*, 2006; Renault *et al*, 2007), utilizing larval habitats in dense vegetation located within these endemic sites (Effler *et al*, 2005; ECDC/WHO, 2007).

Natural occurrence of vertical transmission of arboviruses in the field among *Ae. albopictus* mosquitoes has been reported, suggesting the viruses are maintained in nature by transovarial transmission. Dengue 2 virus was isolated from adult males and females emerging from field-collected larvae in Kerala, South India (Thenmozhi *et al*, 2007). CHIK virus was also isolated from field collected *Ae. albopictus* larvae in Reunion Island (Delatte *et al*, 2008). *Ae. albopictus* has been recognized for its ability to cause epidemics through its high vectorial capacity and possibly through vertical transmission, thus there is a need for an efficient vector control program to prevent or contain arbovirus transmission.

In the absence of a specific treatment or medicine for dengue or CHIK infected persons and with no available effective vaccine for public use, mosquito vector control plays the primary role in prevention and management of these diseases. The current dengue vector control program as outlined by the WHO under vector management is targeted for the control of *Ae. aegypti* and its container habitats (WHO, 2009). Recommended strategies include environmental management, chemical control in larviciding and adulticiding, individual and household protection, and biological control with fish and copepods. WHO guidelines state larvicides may be impractical to apply in hard-to-reach natural sites, such as leaf axils and tree holes, the common habitats of *Ae. albopictus*. Delatte *et al* (2008) stated that in sites where epidemics are caused primarily by *Ae. albopictus*, larval control measures must be implemented in natural habitats that are close to human activity sites. Larval control of *Ae. albopictus* is easier since it has a longer larval stage (Knudsen, 1995) and shorter longevity in the adult stage in comparison to *Ae. aegypti* (Nazni WA and Choh FH, unpublished data).

During the CHIK epidemic in Reunion Island, *Bacillus thuringiensis israelensis* (Bti), was used for larval control (Renault *et al*, 2007). In this program, temephos was replaced with Bti to reduce hazards to non-target orgainsms and possible damage to the environment.

Bti can be sprayed using conventional ground adulticide insecticide sprayers without any need for modifications. In simulated field studies back pack misters, vehicle mounted cold sprayers and thermal foggers were able to disperse the required doses of Bti, killing all *Aedes* spp larvae in their habitats within 24 hours post-treatment and also providing a residual efficacy for a minimum of 14 days post-treatment (Seleena *et al*, 1996, 1998, 1999, 2001). Field bio-efficacy studies of dengue endemic sites in Malaysia and

Singapore have shown that spraying Bti in larval habitats significantly reduced both *Ae. aegypti* and *Ae. albopictus* adult populations at the time of Bti treatment. Larval habitats in these field sites were mapped and treated once every 2 weeks with back pack misters targeting the concrete drainage systems, roof gutters and vegetation. The adult mosquito populations remained at low levels (< 30% ovitrap index) and did not require any control interventions for 4 weeks post-treatment (Lee *et al*, 2008; Rama Chandramogan, unpublished data).

In this 6 month pilot study with the Singapore Armed Forces, motorized back pack sprayers and vehicle mounted ULV generators dispersed Bti droplets at an average VMD of 60-100 μm and 20-40 μm, respectively, into vast areas of *Ae. albopictus* larval habitats in dense vegetation. Motorized spray application with wide treatment swaths of an average 8 m for back packs and 15 m for vehicle mounted ULV sprayers enabled a blanket coverage of the target area and caused the micro-droplets to penetrate into "micro" habitats: leaf axils, tree holes and bamboo stumps. In this pilot study, Bti spray applications conducted once every 2 weeks significantly reduced adult mosquito numbers and the reduction was sequential with time. Such reductions were not observed in the untreated site which was under the conventional mosquito control program. Continuous Bti application was required because prior to this study the "micro" larval habitats were not treated under the conventional mosquito control program. *Ae. albopictus* eggs in their dried form are abundant in such habitats, hatching when water is present. With suppression of adult mosquito numbers re-treatment with Bti can be lengthened to beyond 2 weeks and applied at lower doses than that used initially of 500 g/ha. The ovitrap index can be used to determine the re-treatment intervals.

During the last 3 decades *Ae. albopictus* has spread widely throughout Asia and has become established globally (Knudsen, 1995; Haddad *et al*, 2007). In a recent reviewed article on the global spread of *Ae. albopictus*, it was noted this species on Reunion Island strongly prefers human hosts and this makes it a dangerous vector (Enserink, 2008). In the same article entomologists were doubtful that the present mosquito control program, which emphasizes source reduction and aerosol spraying of adulticides, can stop the global spread of *Ae. albopictus* and prevent future epidemics. The reported dengue and CHIK outbreaks caused by large populations of *Ae. albopictus* were in sites with dense vegetation that provided natural larval habitats for this vector. Vegetation cannot be removed as a part of the source reduction measure. There is a need to explore new and innovative approaches, such as the novel method of using insecticides in this situation (Reiter *et al*, 2006). The significant reduction in adult mosquito density in this pilot study at the SAF site has shown wide application of Bti into vegetation to treat all natural breeding sites is an innovative approach that can be easily adapted to all communities to successfully suppress the *Ae. albopictus* adult population.

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