# EFFICACY OF LIQUID BACTERIAL FORMULATION OF *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* 1593 MIXTURE IN CONTROLLING MOSQUITO LARVAE IN BANGKOK

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**Abstract.** Subsequent to flood disasters in Thailand subsided, vast areas become breeding sites for mosquitoes, and people complained about the huge number of mosquitoes. The National Science and Technology Development Agency and the Faculty of Science together with the Faculty of Tropical Medicine, Mahidol University launched a project to use locally produced bacterial larvicide for controlling mosquito larvae numbers. A liquid formulation of a 1:1 mixture of *Bacillus thuringiensis* subsp. *israelensis* ($10^8$ cfu/ml) and *B. sphaericus* ($10^4$ cfu/ml) was applied in spraying on water surface at 0.1 liter/$m^2$. Over an 18-week study period, a test area was sprayed three times, on week 0 (W0), W7 and W14. Mosquito larvae were monitored using a dipping method and adults using a Center for Disease Control light trap. By W14 bacterial larvicide application achieved >80% and 50% reduction of immature and adult mosquito numbers, respectively, demonstrating the effectiveness of this bacterial larvicide formulation.

**Keywords:** *Bacillus thuringiensis*, *Bacillus sphaericus*, bacterial mosquito larvicide formulation, mosquito control

## INTRODUCTION

Thailand is prone to floods due to its local topography and location in a tropical region affected by seasonal monsoon rains. The most severe flooding occurred at the end of July 2011, caused by tropical storm "Nock-ten" and resulting in flooding throughout the northern, northeastern and central Thailand (Wikipedia, 2018). In total, 65 of 77 provinces of Thailand were declared flood disaster zones, and over 20,000 square kilometers (2,000,000 hectares) of farmland were damaged. Flooding persisted in some areas until mid-January 2012, leading to the worst flooding in Thailand recent history and severe impairments to the country's economy, industrial sector and society (Wikipedia, 2018).

Natural disasters, such as flooding, can affect insect vectors breeding sites and transmission of vector-borne and water-related diseases. Subsequent to water subside, stagnant pools, which accumu-

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late garbage, are left behind, providing breeding grounds for polluted breeder mosquitoes, such as *Culex quinquefasciatus* and *Armigeres subalbatus*, causing serious problems in these areas (Rajavel, 1992). Emergency mosquito control involving fogging can reduce mosquito numbers for a few days; however, application of larvicides, such as synthetic chemical or bacterial agents to confined areas provides a more effective alternative control measure.

*Bacillus thuringiensis* var. *israelensis* (*Bti*) is a gram-positive, spore-forming entomopathogenic bacterium, first isolated in 1976 (Goldberg and Margalit, 1977). *Bti* is a bioagent with proven high efficacy against target organisms, primarily mosquitoes, such as *Aedes*, black fly larvae (Mittal, 2003; Lacey, 2007). The effectiveness of *Bti* in control of mosquitoes has been clearly demonstrated (Fry-O'Brien and Mulla, 1996). Specificity of crystalline protein toxins, which disrupt cell membrane of target organism digestive tract is well documented and the toxins exhibit no adverse effects on non-target invertebrates and vertebrates (Lacey and Mulla, 1990; Saik *et al*, 1990). This bioagent is safe even in drinking water (WHO, 1999). In addition, the multiple protein toxins involved in the killing mechanism decreases the potential development of resistance by target organism. *Bti* exhibits rapid killing activity; however, it had a short residual efficacy (Lee and Zairi, 2005; Lee and Zairi, 2006). Improvement in formulation has increased efficacy, *eg*, different formulations and concentrations of Mosquito DunksB Briquette formulation to improve persistence in controlling *Aedes*, particularly in fast-flowing or turbulent waters (Fansiri *et al*, 2006). Water-dispersible granule (WDG) *Bti* formulation, such as WDG *Bti* H-14 (VectoBac ABG 6511, 3000 ITU/mg), also shows longer residual activity (for at least 35 days) in direct field application to control *Aedes* larvae (Lee and Zairi, 2006).

On the other hand, larvicidal activity of *B. sphaericus* (*Bs*) is mainly due to crystal toxin Bin, composed of two polypeptides, BinA and BinB, acting synergistically through binding to a specific receptor present on apical midgut brush border membranes of target mosquito larvae (Rao *et al*, 1995; Charles *et al*, 2000). The resulting damage to the midgut cells kills mosquito larvae, but *Bs* is at high risk of selecting resistance in mosquito populations because its binary toxin binds to a single receptor type on midgut microvilli. A field population of *Cx. quinquefasciatus* can rapidly develop a high level of resistance to *Bs* when exposed to 35 rounds of spraying with *Bs* 1593M over a 2-year period (Rao *et al*, 1995).

*Cx. pipiens pipiens* (L.) in southern France develops high resistance (>10,000-fold) to *Bs* crystal toxin after >8 generations of laboratory selection (Nielsen-Leroux *et al*, 1997). There are at least two different mechanisms, which could confer high levels of *Cx. pipiens* complex resistance to *Bs* crystal toxin, namely, the mutation of the recessive gene with or without alteration of the toxin binding site in larval midgut receptors (Charles *et al*, 2000).

A key strategy for delaying resistance to insecticidal proteins is to use mixtures of toxins with different targets of the insect, especially mixtures that act synergistically. Wirth *et al* (2005) demonstrated selection pressure of *Cx. quinquefasciatus* for 20 generations using *Bs* alone results in >1,000-fold resistance, whereas a mixture of *Bs* and Cyt1A from *Bti* showed only low resistance. The strategy of using a mixture of *Bs* and *Bti* endotoxins

can delay development of *Bs* resistance of *Cx. quinquefasciatus* in the field (Mulla *et al*, 2003). After a 9-month period, mosquitoes treated with *Bs* strain 2362 alone show emergence of resistance by the ninth treatment and almost complete resistance by the 17th treatment. On the other hand, at another site treated with a mixture of *Bti* and *Bs* for nine treatments *Cx. quinquefasciatus* show no noticeable change in susceptibility to *Bs*.

The National Science and Technology Development Agency (NSTDA), Thailand launched a project using locally produced bacterial larvicide to control mosquito larvae populations in affected areas after a flood disaster. This preliminary project was to assess the efficacy of a bacteria larvicide combination formulation in controlling larvae and mosquitoes in a flooded area in Thailand. The data should assist in the development and improvement of locally produced bacterial larvicides and promote their use as eco-friendly bioagents.

## MATERIALS AND METHODS

### Study area

The test area was located at Phahonyothin 45 Community, Chatuchak District, Bangkok, consisting of an area of 10 Rai (16,000 $m^2$) with 400 houses built over stagnant, slow-flowing water. All waste water from the community is collected in a pond located in front of the community entrance before flowing into a drain. Control (non-treated) area was located at Vagas Mu 9 community, Pak Kret District, Nonthaburi Province, covering an area of 3.48 Rai (5,568 $m^2$) with 87 houses built over stagnant slow-flowing water.

### Bacterial larvicides

Equal volumes of *Bti* ($10^8$ cfu/ml) (TFI Green Biotech, Ratchaburi Province, Thailand) and *Bs* ($10^4$ cfu/ml) (Micro Innovate, Ratchaburi Province, Thailand) were mixed together prior to spraying. Bacterial larvicides were not tested for their potency. The solution was sprayed using a mist blower spraying machine at a rate of 0.1 l/$m^2$ over the body of water in the test community three times, at week (W)0, W7 and W14 of the 18-week study period. Control area was not sprayed.

### Collection of mosquito larvae and adults

Monitoring of mosquito larvae was carried out using a dipping method at two-week intervals after spraying in the treated area and at similar times in control area for 18 weeks.

Five and three dipping sites were selected around treated and control area, respectively (Table 1). Three dips at each site were taken and numbers of larva stage 1-2 (L1-2), stage 3-4 (L3-4) and pupae were counted.

Monitoring of adult mosquitoes was carried out using Center for Disease Control (CDC) light traps (BioQuip Products, Rancho Dominguez, CA) for one night (from 06:00 PM to 06:00 AM) before and after spraying at W0, W1, W2, W4, and W8. One trap was located near houses in a shaded area at the center and at the four corners of the community. Collected mosquitoes were counted and species identified according to Bram keys (Bram, 1967).

### Data analysis

Statistical analyses were performed using PASW Statistics for Windows Version 18.0 (IBM, Armonk, NY). Number of mosquito larvae are presented as mean of larvae per dip and percent reduction was calculated as follows (Mulla *et al*, 2003):

$$\% \text{ reduction} = 100 - \left(\frac{C1}{T1} \times \frac{T2}{C2}\right) \times 100$$

where C1 = number of larvae in non-treated area at time of spraying in treated

Table 1
Sampling sites in bacterial larvicide treated (Phahonyothin 45 Community, Chatuchak District, Bangkok) and non-treated (Vagas Mu 9 Community, Pak Kret District, Nonthaburi Province) areas.

| Site | Treated area | | Site | Non-treated area | |
|---|---|---|---|---|---|
| | Coordinate | | | Coordinate | |
| 1 | 13 51′ 24.38″N | 100 34′ 45.67″ E | 1 | 13 53′ 38.21″ N | 100 28′ 38.45″ E |
| 2 | 13 51′ 21.71″ N | 100 34′ 43.31″ E | 2 | 13 53′ 36.32″ N | 100 28′ 36.72″ E |
| 3 | 13 51′ 21.81″N | 100 34′ 42.65″ E | 3 | 13 53′ 36.02″ N | 100 28′ 37.30″ E |
| 4 | 13 51′ 22.90″ N | 100 34′ 42.54″ E | | | |
| 5 | 13 51′ 19.98 ″N | 100 34′ 41.66″ E | | | |

area; C2 = number of larvae in non-treated areas after spraying in treated area; T1 = number of larvae in treated areas before spraying; T2 = number of larvae in treated areas after spraying.

Comparisons between larvae numbers in non-treated and treated areas were carried out using non-parametric Mann-Whitney test, and between pre- and post-treatment at each sampling week were compared using Wilcoxon signed-rank test, with $p$-value <0.05 considered significant.

## RESULTS

**Effect of bacterial larvicide on mosquito larvae numbers**

Prior to treatmment, numbers of collected larvae per dip in L1-L2 and L3-L4 stages are not significantly different in non-treated and treated areas, only numbers of pupae stage are significantly different (Fig 1). The majority of collected larvae were L3-4 stages. Spraying of bacterial larvicide resulted in marked decrease in numbers per dip of mosquito larvae L1-L2 and L3-L4 and pupae stages at W1, and this was maintained after the second (W7) and third larvicide (W14) applications. However at W13, numbers of larvae stage L3-L4 and pupae were higher than the pre-spraying period. It is worth noting there was rain during the nights before collecting days at W6, W9, W11 and W18.

**Effect of bacterial larvicide on mosquito adult population**

Five CDC light traps were set only in the treated area and operated for one night (from 06:00 PM to 06:00 AM) on pre-spray day, and during W1, W2, W4, W6, and W8. Mosquito species ($n$ = 13) collected were *Armigeres subalbatus*, *Aedes aegypti*, *Ae. albopictus*, *Culex gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Cx. vishnui*, *Mansonia indiana, Ma. annulifera, Ma. annulata, Ma. uniformis, Mimomyia,* and *Uranotaenia*, the majority being polluted water breeders, with *Cx. gelidus* and *Cx. quinquefasciatus*, vectors of Japanese encephalitis and filariasis the most abundant (data not shown). However, the average number of mosquitoes per trap is not significantly different between pre- and post-spraying (Table 2).

## DISCUSSION

Stagnant pools and inadequate drainage of polluted water with a high organic

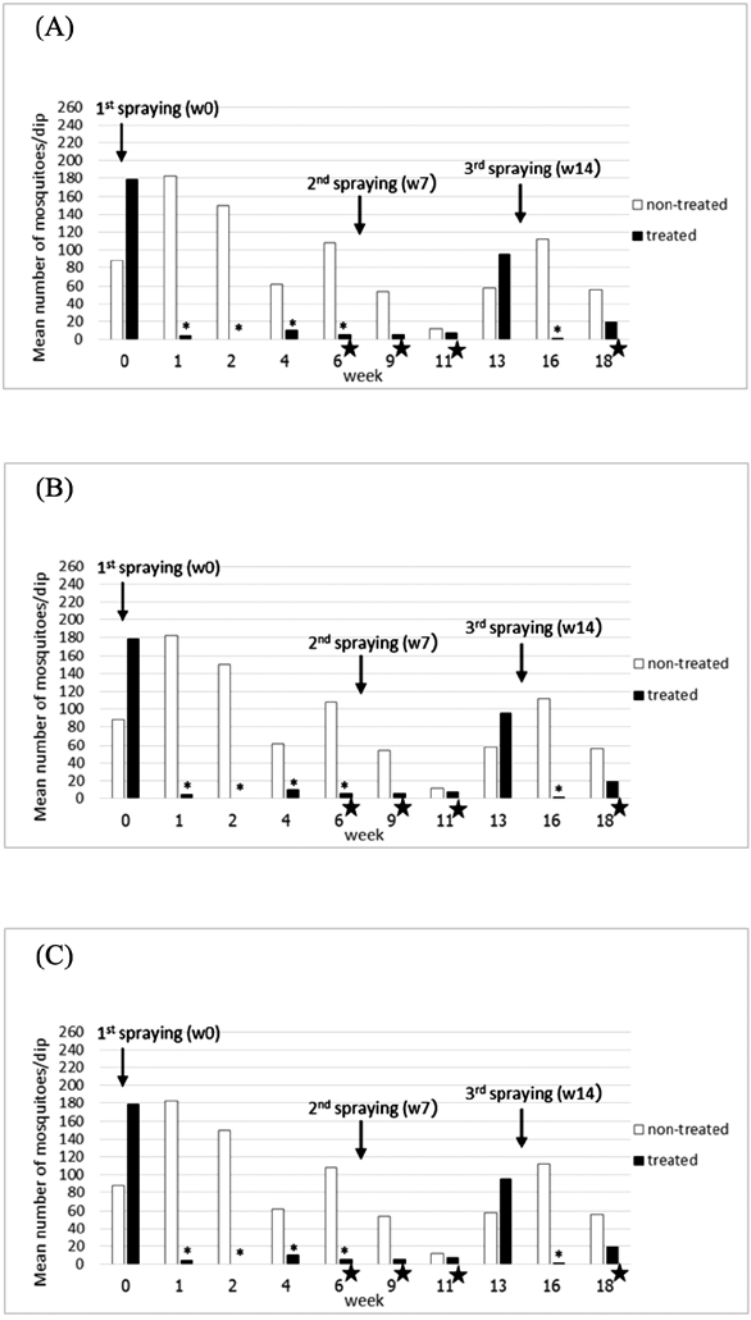


Fig 1-Numbers of mosquito larvae in L1-L2 (A), L3-L4 (B) and pupa (C) stages collected from bacterial larvicide treated (Phahonyothin 45 Community, Chatuchak District, Bangkok) and non-treated (Vagas Mu 9 Community, Pak Kret District, Nonthaburi Province) areas during a 18-week (W) period. Mosquito larvae/pupae were counted from three dipping samples. Asterisk (*) on bars indicate a significant difference at each specific sampling week . Significance at $p$-value <0.05, Mann-Whitney test. Star indicates rain during the night prior to collection day.

Table 2
Numbers of adult mosquitoes collected from five traps during 18-week period of bacterial larvicide treated area, Phahonyothin 45 Community, Chatuchak District, Bangkok.

| | Pre-spraying (D0) | 1st SPRAYING | W1 | W2 | W4 | W6[a] | W7 | W8[a] |
|---|---|---|---|---|---|---|---|---|
| Mean number of mosquitoes/trap | 124 | | 77 | 62.5 | 93.4 | 77.8 | 2nd SPRAYING | 63.2 |
| *p*-value[b] | | | 0.144 | 0.068 | 0.500 | 0.500 | | 0.345 |

[a]Rain at night before the collection day. [b]Significant at $p<0.05$, Wilcoxon signed-rank test between pre- and post-spraying. D, day; W, week.

content support the colonization of high numbers of immature mosquitoes that breed in such water conditions (UC Riverside, 2002). Controlling numbers of larvae in a confined and accessible habitat is more convenient and more effective than controlling flying adult mosquitoes.

Many commercial bacterial larvicide products show effective control of immature insects in agricultural fields (Deist *et al*, 2014). The effectiveness of *B. thuringiensis* H-14 in controlling mosquito propagation, primarily *Aedes* larvae, has been demonstrated (Fry-O'Brien and Mulla, 1996). The application of microbial agents, such as *Bti*, as a mosquito control tool that is highly effective and absent of potential toxin resistance, has been adopted in many countries (Becker, 1997; Regis *et al*, 2001; Flacio *et al*, 2015). Improvement in formulations of *Bti* and *Bs*, such as *Bti* WDGs, has increased effectiveness for mosquito larvae control in the field. In order to avoid or delay development of bacterial toxin resistance, many studies have demonstrated the advantage of using a combination of *Bti* and *Bs* (Wirth *et al*, 2004).

The present study demonstrates a 1:1 liquid mixture of *Bti* and *Bs* sprayed over a water-logged area within an urban community resulted in nearly 100% reduction after two weeks. However, the number of mosquito larvae gradually increased until the nest spraying, possibly due to a decrease in bacterial spores as a result of ingestion by larvae and/or because spores sank to the water bottom. It is worth pointing out raining just before larvae collection resulted in a spurious drop in larvae numbers, probably due to increase in water volume. Overall, there was more than 80% reduction in mosquito larva/pupa numbers over an 18-week with bacterial larvicide spraying every seven weeks.

On the other hand, the numbers of adult mosquitoes decreased over a period of two weeks, then rose again. This may be due to an influx of mosquitoes from outside the treated area. No dissection of collected mosquitoes was performed to assess the ratio of newly emerging mosquitoes and existing mosquitoes. Disappointingly, the number of collected mosquitoes is not significantly reduced

from bacterial larvicide spraying program. Nevertheless, residents expressed satisfaction with mosquito reduction for the two weeks after spraying.

In conclusion, the research demonstrates a combination of two bacterial larvicides produced in Thailand was highly effective in limiting mosquito larvae and pupae populations in a defined area of stagnant water located within the confine of an urban community. Use of bacterial larvicidal products should be encouraged by local administrations for mosquito control management. Furthermore, as these bacterial larvicides are produced within the country, their adoption in mosquito control programs is in line with the country's goal of self-reliance. However, requirement for large storage space, short shelf life and inconvenience of transportation of liquid bacterial larvicide formulation, highlight the need for further development to overcome these limitations.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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