EFFICACY OF A *BACILLUS THURINGIENSIS ISRAELENSIS* TABLET FORMULATION, VECTOBAC DT®, FOR CONTROL OF DENGUE MOSQUITO VECTORS IN POTABLE WATER CONTAINERS

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Abstract. VectoBac DT®, a tablet formulation of *Bacillus thuringiensis israelensis (Bti)* was evaluated for the potential control of dengue vectors in various types of potable water containers. On introduction to containers, the tablet sinks to the bottom and the *Bti* toxins are found concentrated at the sides and the base, while the treated water column is free of *Bti* toxins within 24 hours after tablet introduction. In a simulated study, earthen, HDPE and plastic containers were kept covered and laboratory-bred larvae were introduced to determine the control by the tablet. The efficacy and persistence of the tablet, with a control of >90%, was significantly longer in earthen containers in comparison to the HDPE and plastic containers. Efficacy and persistence were observed in earthen containers for a minimum period of 5.5 months (166 days) both without water replenishment and with weekly, 50% water volume, replenishment, and for a maximum period of 2.2 months (66 days) with daily, 50% water volume, replenishment. In plastic and HDPE containers, the tablet activity had a persistence of 2.1 months (63 days) without water replenishment and 1.8 months (54 days) with weekly water replenishment. The efficacy and persistence of the VectoBac DT® was significantly longer in the earthen containers, with or without regularly treated water exchange, due to the *Bti* toxins being embedded in the porous earthen container surfaces, which protects them from rapid degradation. Lesser toxin amounts are removed from the water column during water exchange. The efficacy of VectoBac DT® was also evaluated for the control of natural infestation of *Aedes* larvae which were resistant to temephos at the WHO diagnostic dosage of 0.012 mg/l. The tablet significantly reduced the pupal density by 8 fold in earthen containers for 67 days and 5 fold in HDPE containers for 55 days in comparison to untreated containers ($p<0.05$). However, the tablet was effective for a shorter period of 25 days post-tablet-introduction due to fungal infestation in the treated plastic containers. There is a need to determine the capacity of the VectoBac DT® to reduce the dengue vector population to a threshold which will prevent dengue outbreaks in dengue endemic areas.

INTRODUCTION

Large ceramic, clay or cement containers, as well as plastic, HDPE and metal drums, used for storing water for household use, are a common feature in and around the houses in Southeast Asia (SEA). They represent one of the most important larval habitats for the dengue vector, *Aedes aegypti* (L.) in this region (Scanlon, 1967). Temephos employed in artificial containers as 1% sand core granules at a dosage of 1.0 mg a.i./l has been the mainstay of *Ae. aegypti* larval vector control for 30 years in this region.

The prevalence of widespread temephos resistance in larval populations has been recorded in detail in the Americas and the following alternatives are implemented to replace temephos: environmental sanitation; alternative insecticides; insect growth regulators; and *Bacillus thuringiensis israelensis* (*Bti*) (Rawlins, 2002). In spite of reports of larval resistance to temephos in the Americas, it is continuously being introduced to artificial containers during the peak dengue season in Asian countries. The only 2 published reports from this region on the

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development of temephos resistance in field populations of *Ae.albopictus* (Skuse) and *Ae.aegypti* mosquitoes, collected in Kuala Lumpur, Malaysia, were by Lee *et al* (1998) and Nazni (unpublished data, 2003) at resistance ratios of 0.07-4.5.

Since 2001, the alternative larvicide, *Bti*, has been used effectively in all municipalities in Brazil to control *Ae. aegypti* which showed resistance to temephos (Ponce *et al,* 2002; Lima *et al*, 2003). *Bti* has yet to be evaluated in larviciding programs in SEA to determine its capacity to suppress *Aedes* populations to a threshold that will prevent dengue outbreaks. As source reduction and larviciding with temephos and *Mesocyclops* in potable water containers have not prevented the annual dengue outbreaks in SEA countries (Center for Dengue Vector Research, 2002), there is a need to evaluate *Bti* to control dengue vectors in these containers.

This study reports the evaluation of VectoBac DT®, a commercial tablet formulation of *Bti* by Valent BioSciences Corporation, to control dengue mosquito vectors, *Ae. aegypti* and *Ae. albopictus*, in potable water containers. The tablet designed for use in the containers: weighs 0.34 g/tablet, contains 2,250 ITU/mg against *Ae. aegypti,* is irradiated, and is formulated to be used in direct application in the water at a dosage of 1 tablet per 50 liter water.

The efficacy of VectoBac DT®, in varied potable water container types was evaluated at the Institute for Medical Research, Malaysia from November 2001 to June 2003. This paper reports three research findings: 1) the distribution of the VectoBac DT® particles in the treated containers, 2) the efficacy and persistence of VectoBac DT® against laboratory-reared *Aedes* larvae in varying container types, and 3) the efficacy and persistence of VectoBac DT® for the control of natural infestation of *Aedes* mosquitoes.

MATERIALS AND METHODS

Test containers

Earthen, HDPE and plastic containers were used in all our studies. All the containers held 50 l tap water. For each study, 6 to 8 containers were used as treated containers and 2 as untreated controls. Before initiating each test all the containers were washed with tap water and were tested for the presence of any larvicidal contaminant by introducing 30 *Ae. aegypti* (L3) larvae. The larvae were observed until complete pupation.

Test larvae

Laboratory-bred *Ae. aegypti* and *Ae. albopictus* larvae, from the insectarium of the Entomology Division, Institute for Medical Research were used in some parts of this study. The L1 larvae were fed with dried liver powder and the L2 to L4 larvae were fed on pieces of fresh beef liver before the larvae were introduced into the potable water containers. In the containers the larvae were not fed.

Study 1. Distribution of VectoBac DT® particles in treated water containers.

A tablet was added to each of 3 earthen, HDPE and plastic containers holding 50 l tap water. At one day post tablet introduction, 200 ml water samples were removed from just below the water surface, mid water column and 10 cm above the container base. Twenty laboratory-bred *Ae. aegypti* larvae (L3) were introduced into each removed sample in paper cups and the mortality was observed for 24 hours and 48 hours after exposure.

In addition to removing the treated water samples, 50 laboratory bred *Ae. aegypti* larvae (L3) were introduced into the tablet treated containers and mortality was observed for 24 hours and 48 hours post-exposure.

Study 2. Efficacy of VectoBac DT® against *Aedes albopictus* larval instars

Fifty *Ae. albopictus* larvae (L1) were introduced into each treated earthen, HDPE and plastic container. The larval feeding behavior and the larval mortality were observed for 48 hours post-exposure. The study was repeated with L2/L3 and L4 larvae.

Study 3. Efficacy and persistence of VectoBac DT® in different potable water container types to control *Ae.albopictus* larvae

Different container types have been shown to support significantly varying *Aedes* larval and pupal densities (Seleena, unpublished data). For

this study, the larval numbers were made consistent by introducing 50 laboratory-bred *Ae. albopictus* larvae into each container.

One tablet was introduced into each of the containers holding 50 l tap water. The covered containers were placed outdoors in a shaded area. At weekly intervals, 50 *Ae. albopictus* larvae (L2) were introduced into the containers. Larval mortality was recorded 24 hours and 48 hours after exposure. Any surviving larvae were left remaining in the containers until mortality or pupation. The study for each container was terminated when one larva pupated.

All dead larvae floating on the water surfaces were removed to prevent the surviving larvae and the fresh larvae that were introduced in the following weeks from acquiring *Bti* toxins by ingesting the dead larvae.

The tablet efficacy in the containers was evaluated under the following conditions: 1) Treated water was not replenished, but evaporated water was replaced with tap water weekly; 2) 25 l treated water was replenished with 25 l fresh tap water weekly, and 3) 25 l treated water was replenished with 25 l fresh tap water, daily. Studies (2) and (3) were conducted to simulate natural conditions and water was removed using a measuring jug. The untreated containers were subjected to the same conditions as the treated containers.

Study 4: Determining the reason(s) for VectoBac DT® efficacy and persistence in earthen containers

In study 3, it was observed that VectoBac DT® had the longest efficacy in the earthen containers, compared to the plastic and HDPE containers. The treated water in all container types had a temperature range of 27º-30ºC and a pH of 6.6-6.10; the recorded temperature and pH range were not significant contributing factors to the rapid loss of tablet efficacy in the plastic and HDPE containers. In study 3, when all the containers were washed with tap water and were tested for the presence of remnant *Bti* toxins from the previous study by introducing 30 *Ae. aegypti* (L3) larvae and observing the larvae till pupation, it was observed that one wash for the HDPE and plastic containers was sufficient for the containers to be free from all larvicidal components. The earthen container surfaces required vigorous scrubbing and at least 3 washes before the containers were free from *Bti* toxins. This indicated that the *Bti* toxins were embedded in the porous surfaces of the earthen containers, thus protecting them from rapid degradation. Study 4 was undertaken to determine whether the *Bti* toxins were embedded in the porous surfaces of the earthen containers.

Eight new earthen containers, not previously used for any study, were treated with VectoBac tablets, and an additional two were used as untreated controls. The covered containers were placed outdoors in a shaded location. On weekly intervals for 4 consecutive weeks, 50 *Ae. albopictus* larvae (L2) were introduced into the containers. The larval mortality was recorded 24 hours after exposure. All dead larvae floating on the surface were removed and evaporated water was replaced during the 4 weeks.

On the 28th day post tablet introduction, all of the containers were drained by removing the plugs from the bases. All tablet particles were removed from the bases by washing with 2 l tap water per container. Fresh plugs were used to seal the holes and the containers were refilled with 50 l tap water. At weekly intervals, 50 *Ae. albopictus* larvae (L2) were introduced into the containers. The larval mortality was recorded 24 hours and 48 hours after exposure. Any surviving larvae were left in the containers until mortality or pupation. The study for each container was terminated upon first observation of pupation. All dead larvae floating on the surfaces were removed.

Study 5.0. Efficacy and persistence of VectoBac DT® in potable water containers for control of natural infestation of *Aedes* mosquitoes

Study 5 was conducted from August 2002 to June 2003 under harsh conditions, where the containers were not covered, placed outdoors under a building eave, exposed to sunlight, and within the vicinity of a densely populated *Ae. albopictus* site. The landing rate of *Ae. albopictus* at this site was observed to be 25.5±3.5 mosquitoes/man/hr.

This study was conducted with 2 preliminary studies to determine the time taken for natural *Aedes* mosquitoes to infest an untreated container, and to determine the effect of VectoBac DT® treated water on the natural oviposition of *Aedes* mosquitoes and on their hatching of eggs.

Study 5.1. Time taken for natural infestation of *Aedes* mosquitoes in the varied container types. For each container type, 6 to 10 containers were filled with 50 l tap water and placed in the test site. The containers were monitored daily to detect the presence of *Aedes* larvae. As soon as the containers were placed in the test site, *Aedes* mosquitoes hovered over the water surfaces and rested on the sides of all container types. *Aedes* larvae were found in all the plastic and earthen container types within 2 days of introduction, and pupae were observed within 10-14 days. In the HDPE containers larvae and pupae were not observed until 30 days after introduction. The study of the HDPE containers was repeated with 50 l tap water per container and with increased volumes of tap water (75, 100, 150 and 200 l per container). The larvae and pupae populations were observed only after 30 days. The reason for late infestation in the HDPE containers is not known.

As there was immediate infestation of *Aedes* mosquitoes in the earthen and plastic containers, it was decided that VectoBac DT® would be introduced into the containers on filling with water. For all HDPE containers the tablet would be introduced when larval presence was observed in all the HDPE containers.

Study 5.2. Effect of VectoBac DT® treated water on oviposition by *Aedes* mosquitoes and on the hatching of eggs. This study was undertaken to determine whether the oviposition behavior of *Aedes* female mosquitoes was influenced by VectoBac DT® treated water. Six containers of each type were treated with tablets and four were used as untreated controls. A day after treatment, 100 ml water samples were removed from the water surface of each container and were placed in ovitraps with paddles. The ovitraps were placed in a cage holding 200 blood-fed *Ae. albopictus* (F4) adults for 2 days. The number of eggs in each ovitrap was counted. The eggs were then placed on filter paper, dried for 4 days and then set for hatching in their respective water samples. The larvae hatched within 24 hours in all the containers and were fed with liver powder. The larvae were counted during the L3/L4 stage. The ratio of the number of larvae hatched per number of eggs deposited in each water sample was also determined.

The statistical significance of oviposition and larval hatching in the tablet-treated and non-treated water samples was analysed using the Student's *t*-test. There was no significant difference between the number of eggs oviposited in the treated waters (508.67±82.23) and in the untreated waters (354.75±38.24) ($p>0.05$). There was also no significant difference between the number of larvae hatched in the treated waters (313.17± 136.10) and in the untreated waters (178.0± 58.38) ($p>0.05$). There was no significant difference between the ratio of the number of larvae hatched versus the number of eggs oviposited in either the treated waters (60.54±7.73) or the untreated waters (51.52±11.80) ($p>0.05$).

As there was no significant difference in oviposition and larval hatching between the VectoBac DT® treated and untreated water, it was concluded that *Aedes* mosquitoes were not inhibited from oviposition by the tablet-treated water, and the tablet-treated water had no adverse effects on the development of eggs.

Study 5.3. Efficacy and persistence of VectoBac DT® in potable water containers to control the natural infestation of *Aedes* mosquitoes. Earthen, plastic and HDPE containers were filled with 50 l tap water. With reference to the results obtained for Study 5.1, the VectoBac DT® was introduced into the earthen and plastic containers as soon as the containers were filled with 50 l tap water. For the HDPE containers, the tablet was introduced when *Aedes* infestation was observed in each of the containers, about 33 days after filling the containers with tap water.

The *Aedes* immature population was recorded after regular observations by the same person, with the aid of a flashlight. Dipping, the conventional method of sampling for immatures in water receptacles, was not used in this study as in previous studies at the Institute for Medi-

cal Research, Malaysia. Lee (unpublished data) and Tun-Lin *et al* (1994) had indicated that immature counts from dipping did not correlate with the population in the receptacles. Visual observation and counting of all immatures in the container conducted by the same person, with the aid of a flashlight, gave a consistent and realistic count of the population in the container.

Larval and pupal numbers in each container were noted, with estimated larval counts and actual pupal counts. All pupae were removed from the containers and allowed to emerge in the laboratory. The emerged adults were identified. On the last day of the study, the contents of each container were passed through a sieve, the larvae and pupae were collected and counted. Evaporated water was regularly replaced. It was essential to replace the evaporated water to have maximum colonization in the containers, as most of the *Aedes* eggs were found adhering to the inner walls of the containers above the water surface.

RESULTS

Study 1. Distribution of VectoBac DT® particles in treated water containers

Larval mortality was not observed in the treated water samples that were removed from below the water surface, mid water column, or 10 cm above the container base. Complete mortality was obtained in the larval populations introduced into the treated containers. The results indicate that the tablet particles were not present in the treated water column, but were concentrated along the sides and the base of the containers.

Study 2. Efficacy of VectoBac DT® against *Aedes albopictus* larval instars

The L1 larvae upon introduction into the potable containers remained at the water surfaces and did not graze the container surfaces. The L1 larvae survived beyond 48 hours after exposure; on moulting into L2 larvae, they grazed the sides of the container, ingested the *Bti* toxins and complete mortality was observed.

The L2-L4 larvae on introduction into the containers actively grazed the sides and the base of the container and larval mortality was observed within 24 hours of exposure. This result reconfirmed the results of Study 1, that the tablet particles are concentrated along the sides and the base of the container, when the larvae come in contact with them they are killed.

Study 3.0. Efficacy and persistence of VectoBac DT® in potable water container types for the control of *Ae.albopictus* larvae

Study 3.1. Treated water was not replenished, but evaporated water was replaced with fresh tap water weekly

Earthen containers (Table 1 and Fig 1a). Six earthen containers were treated with tablets, and two were used as untreated controls. During the study period of 166 days, there was consistent water loss from evaporation. The volume of water loss varied widely among the containers, from 46 l to 160 l per container. The evaporated water was regularly replaced.

Complete larval mortality was achieved within 48 hours of exposure for 46 days (6.6 wk) post tablet introduction. The larval mortality (%) then averaged 94.6±1.10 up to 166 days (23.7 wk) post tablet introduction. There was no pupation in 5 containers for 166 days, except in one container (No.J8), which had 12% pupation and emergence on the 151st day (21.6 wk) post tablet introduction. The study was terminated on the 166th day. The VectoBac DT® efficacy for >90% control of *Ae. albopictus* larvae in earthen containers extended beyond 23 weeks.

HDPE and plastic containers (Table 1 and Fig 1a). For each container type, seven were treated with a tablet each, and two were used as untreated containers. During the study period of 81 days, there was very minimal water loss from evaporation of just 2 l per container, and this was replaced. Complete larval mortality was observed within 48 hours after exposure, and for 21 days (3 wk) post tablet introduction. After this, the larval mortalities (%) were 97.7±1.4 and 96.4±0.83 for the plastic and HDPE containers, respectively, up to 63 days (9 wk) post tablet introduction. The study was terminated upon pupation in all the containers on the 81st day (11.6 wk), with 84.8±4.03 and 83.7±5.7 (%) larval mortality in the plastic and HDPE containers, respectively.

All the treated plastic containers were coated with black filamentous fungus from the 20th day post tablet introduction.

The earthen containers had the longest significantly tablet efficacy for control of >90% of *Ae. albopictus* larvae, a minimum period of 23 weeks, in comparison with the plastic and HDPE containers, which had a maximum period of 9 weeks. Large volumes of water loss due to evaporation in the earthen containers did not have any detrimental effect on *Bti* toxins.

Study 3.2. Twenty-five liter l treated water was replaced with 25 l fresh tap water weekly in the earthen containers (Table 1 and Fig 1b). Seven earthen containers were each treated with a tablet, and two were used as untreated controls. Complete larval mortality was achieved within 48 hours of exposure and for 74 days (10.6 wk) post tablet introduction, with a total of 10 replenishments. After this, the larval mortality (%) was 96.8±0.82 until the 166th day (23.7 wk) post tablet introduction, with a total of 22 replenishments. There was no pupation in the 6 containers for 166 days, except in one container (No.J4) which had 2% pupation and emergence on the 162nd day (23.1 wk) post tablet introduction.

The study was terminated on the 166th day. The VectoBac DT® capacity to control >90% of *Ae. albopictus* larvae in the earthen containers, with weekly replenishments of 50% of the total treated water volume, extended beyond 23 weeks.

HDPE and plastic containers (Table 1 and Fig 1b). For each container type, seven were treated with a tablet each, and two were used as untreated controls. Complete larval mortality was achieved within 48 hours of exposure and for 22 days post tablet introduction with a total of 3 replenishments. After this, the larval mortalities (%) were 97.7±1.4 and 98.3±1.1 for plastic and HDPE containers, respectively, until the 54th day (7.7 wk) post tablet introduction. The study was terminated upon pupation in all containers on the 66th day (9.4 wk) with a total of 9 replenishments. All treated plastic containers were coated with a black filamentous fungus from the 20th day post tablet introduction.

The efficacy and persistence of VectoBac DT®, with weekly replenishments of 50% of the total treated water volume, was at least 3 times longer in the earthen containers than in the plastic and HDPE containers.

Study 3.3. Twenty-five liter l treated water was replenished with 25 l fresh tap water daily

Earthen containers (Table 1 and Fig 1c). Seven earthen containers were treated with a tablet each, and two were used as untreated controls. Water was replenished daily, except on public holidays and weekends. Complete larval mortality was achieved within 48 hours of expo-

Table 1
Efficacy and persistence of VectoBac DT® for the control of *Ae.albopictus* larvae in varied potable water container types.

Container type	Percent larval mortality (Mean ± SE) in relation to time (days post tablet introduction)		
	Treated water not replenished	25 l treated water replenished weekly	25 l treated water replenished daily
Earthen	100±0 for 46 days 94.6±1.1 for 166 days	100±0 for 74 days 96.8±0.82 for 166 days	100±0 for 38 days 99.7±0.3 for 66 days
Plastic	100±0 for 21 days 97.7±1.4 for 63 days	100±0 for 22 days 97.7±1.4 for 54 days	NA
HDPE	100±0 for 21 days 96.4±0.83 for 63 days	100±0 for 22 days 98.3±1.1 for 54 days	NA

NA : not available

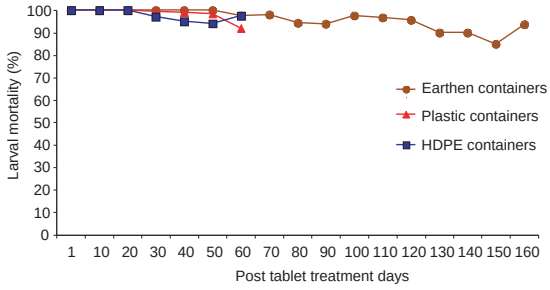

Fig 1a–treated water not replenished.

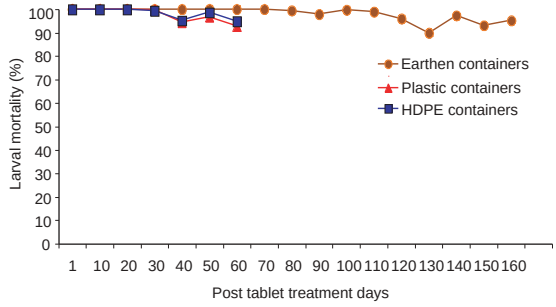

Fig 1b–50% treated water replenished weekly.

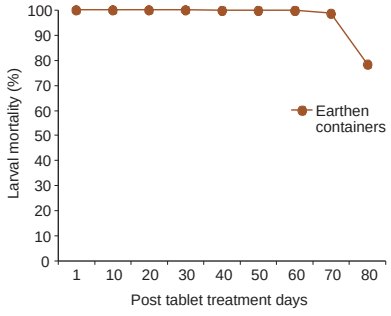

Fig 1c–50 % treated water replenished daily

Fig 1–Efficacy and persistence of VectoBac DT, for the control of *Ae.albopictus* larvae in varied potable water container types.

sure and for 38 days (5.4 wk) post tablet introduction, with a total of 29 replenishments. The larval mortality (%) averaged 99.68±0.25 until the 66th day (9.4 wk) post tablet introduction, with a total of 49 replenishments and pupation in two of the containers. There was pupation in all seven treated containers on the 82nd day (11.7 wk) post tablet introduction, after 53 replenishments.

HDPE and plastic containers. (This study was not conducted for HDPE and plastic containers).

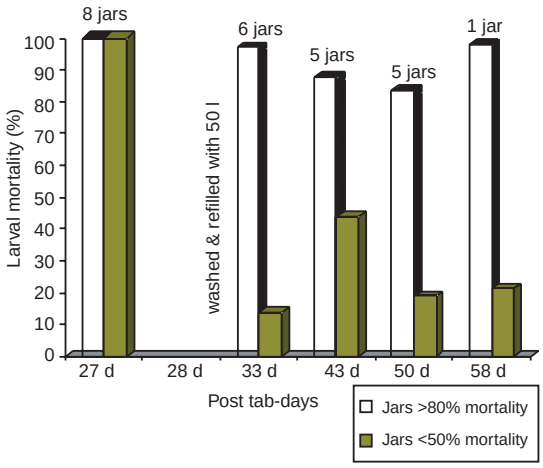

Fig 2–Efficacy and persistence of VectoBac DT®, in earthen containers before and after replenishing all the treated water.

Earthen containers had the longest tablet efficacy for the control of *Ae. albopictus* larvae in both the daily and weekly treated water replenishments. The first pupation was observed in one of the earthen containers (14.3%) after a total volume of 800 l was displaced in 32 replenishments. Pupation was observed in other treated earthen containers after 1,325 l of water was replaced in 53 replenishments. The plastic and HDPE containers could only support a pupa free environment for a change of 175 l/container.

The reasons for the significantly longer tablet efficacy in the earthen containers were subsequently established in Study 4.

Study 4. To determine the reason(s) for the long VectoBac DT® efficacy and persistence in the earthen containers (Fig 2)

Complete larval mortality was achieved in all eight treated containers for 4 weeks post tablet introduction, before the treated waters were replaced with fresh tap water. After replenishing the entire treated water volume with 50 l fresh tap water on the 28th day post tablet introduction, 6 containers (J10, J11, J12, J13, J18, J19) had 97±1.7 larval mortality (%) and 2 containers (J14, J15) had 14±4.0 larval mortality (%) on the 33rd day post tablet introduction. Larval mortality reduced with time and pupation was observed in one of the containers (J13) on the 43rd day post tablet introduction. Seventeen days after

replenishing (50th day post tablet introduction) five containers had 83.3±4.3 larval mortality (%) and two containers had 19.0±15.0 larval mortality (%). On the 58th day post tablet introduction, there was pupation in all seven containers with 21.4±7.1 larval mortality (%). One container (J15) had 98% larval mortality and no pupation. Container - J15 was the only container that had increasing mortality with time: 18% larval mortality 48 hours after exposure, when the larvae were introduced immediately after replenishing with 50 l of treated water, on the 33rd day post tablet introduction, the larval mortality surged to 86% on the 43rd day post tablet introduction, and then to 100% on the 50th day post tablet introduction.

The treated water containing VectoBac DT® particles was drained from the containers on the 28th day post tablet introduction. The replenished water in 5 of the 8 treated containers gave significant larval mortality for 17 days after replenishing. This indicated that VectoBac DT® particles were embedded in the container surfaces and they were not drained out with the treated water. One container had increasing mortality with time, indicating that the amount of toxin embedded in the container surface and the rate of toxin released into the water column differed between the containers, depending on the porosity of the container surfaces.

The same containers used for this study were later utilized in a field study (Study 5) to determine the tablet efficacy for control of the infestation of wild *Aedes* larvae in the earthen containers. The five containers which had ≥80% larval mortality in the replenished water for 17 days in Study 4 supported a significantly lower *Ae.albopictus* larval and pupal density during the study period of 67 days in Study 5, in comparison to the containers with ≤50% larval mortality in the replenished water.

Study 5. Efficacy and persistence of VectoBac DT® in potable water containers for the control of natural infestation of *Aedes* mosquitoes

The efficacy and persistence of VectoBac DT® for the control of natural *Aedes* infestation in potable water containers is shown in Tables 2 and 3. Table 2 shows the infestation of immatures and the presence of black dead larvae in treated containers with relation to time (post introduction days). The blackened dead larvae indicated the presence of viable *Bti* toxins in the containers. Table 3 shows the mean larval and pupal numbers in the treated and untreated containers for the study period.

General observation

With reference to untreated containers, earthen containers harbored significantly higher *Aedes* larval and pupal densities than the HDPE and plastic containers (Table 3) ($p<0.05$). It is not known whether the container material or the container color influenced the gravid female mosquitoes to oviposit more in the earthen containers or they oviposited equally in all the container types, but *Aedes* eggs had better adhesion to the porous surfaces of the earthen containers and better survival into the larval stage in the earthen containers than in the HDPE and plastic containers.

A surge in larval density was always observed a day after evaporated water was replaced in all the containers, submerging the *Aedes* eggs that were adhering to the inner walls of the containers above the water surface.

In the VectoBac DT® treated containers, it was observed, as confirmed in Study 2, that L1 *Aedes* larvae remained on the water surface, did not graze the container surfaces and survived to the L2 stage. Reduction in larval numbers due to larval mortality was always observed 1- 2 days after L1 larvae infested the treated containers.

Blackened dead larvae (L3/L4), indicating the presence of viable *Bti* toxins, were found floating on the water surface. The intense darkness in the dead larvae decreased with time due to natural degradation of *Bti* toxins and lesser toxin amounts in the treated water.

An increase in larval mortality (L3/L4) was usually observed a day after evaporated water was replaced in all treated containers. This was due to wild *Aedes* larvae (L3/L4) exhibiting territorial behavior within the containers, remaining in their respective positions near the sides of the container, but not coming into contact with the *Bti* toxins. Thus, when the water column was disturbed during replacement of evaporated

Table 2
Efficacy and persistence of VectoBac DT® for the control of natural infestation of *Aedes* mosquitoes in potable water containers. Duration of infestation of *Aedes* immatures (post treatment days) and the observance of black dead larvae in treated containers.

Container	Study period	Larval infestation	Black dead larvae[a] in treated containers	Pupal infestation (percent positive containers)
1. Earthen				
Treated	67 days	7th day	61 days	24th day (25)
				46th day (100)
Untreated		2nd day	-	11th day (100)
2. Plastic				
Treated	42 days	16th day	31 days	25th day (14.3)
				41st day (100)
Untreated		4th day	-	14th (50)
				17th (100)
3. HDPE [b]				
Treated	55 days	19th day	40 days	25th day (20)
				41st day (100)
Untreated		1st day	-	1st day (100)

[a]No. of days for which black, dead larvae were consistently found on the water surface in treated containers.
[b]HDPE containers were treated with a tablet 33 days after setting up for *Aedes* oviposition at the test site. Thus, the untreated containers had immature infestations from day 1 of this study.

Table 3
Efficacy and persistence of VectoBac DT® for control of natural infestation of *Aedes* mosquitoes in potable water containers. Mean larval and pupal numbers ± SE for study period.

Container	Study period	Mean larval No. ± SE	Mean pupal No. ± SE
1. Earthen			
Treated	67 days	16.9 ± 2.01	1.13±0.24
Untreated		101.2±4.51	9.51±0.74
2. Plastic			
Treated	42 days	16.5±4.1	0.19±0.08
Untreated		47.6±1.6	0.43±0.13
3. HDPE			
Treated	55 days	5.4±2.4	0.43±0.14
Untreated		64.9±4.3	2.07±0.32

water, causing an agitation of the tablet particles and a disturbance in the larval position, it induced the larval contact with the tablet particles.

All pupae removed from the containers emerged successfully and were identified: 92.22% were *Ae. albopictus* and the remainder were *Ae.aegypti*.

Fungal infestation was observed in the treated plastic and earthen containers from the 3rd week of the study period. A more dense fungal growth was observed in the plastic containers than in the earthen containers. There was no fungal infestation in the untreated containers.

Earthen containers (Tables 2 and 3). Eight containers were treated with a tablet each and two

were used as untreated controls. The tablets were introduced into containers holding 50 l tap water (day 1). Observations were made for *Aedes* immature infestations until the 67th day. The study was terminated on the 67th day due to green algae bloom in both the untreated containers, making it impossible to observe and count the *Aedes* population. Algae bloom was not observed in the treated containers.

In the untreated containers, larval infestation was observed from the 2nd day, at an average of 50±0 larvae (L1/L2) per container; and pupae on the 11th day, at an average of 4.5±0.5 pupae per container. Throughout the study period, an infestation of *Aedes* larvae (L1-L4) and pupae was present in the untreated containers. The final larval and pupal counts on the 67th day were 264.5±20.5 and 18.5±1.5 per container, respectively.

In the treated containers, larval infestation was observed from the 7th day in two containers (0.63±0.42 larvae per container) and pupae from the 24th day in two containers at 0.38±0.3 per container. The larval and pupal densities increased to a maximum of 62.3±19.3 and 6.75±1.54 per container, respectively, on the 67th day. In comparison to the untreated containers the larval and pupal densities were 4 fold and 2.5 fold less in the treated containers, respectively, on the 67th day.

For 61 days, black dead larvae were observed floating on the treated water surface, indicating the presence of sufficient viable *Bti* toxins in the containers. Pupal infestation was observed in all eight treated containers on the 46th day.

For the study period of 67 days, the mean larvae and pupae densities in the treated containers were significantly less than in the untreated containers ($p<0.05$) (Table 3).

The larval numbers varied significantly between the eight treated containers: the five containers which had ≥80% larval mortality in the replenished water for 17 days (Study 4), supported a larval density of 0 - 30 larvae per container during the study period of 67 days, and on the 67th day when the *Aedes* population was counted the five containers had significantly lower larval (L4) and pupal densities of 14±10.7 and 4.2±2.4 per container, respectively. Treated containers with ≤50% larval mortality in replenished water (Study 4), supported a larval density of ≥50 larvae per container during the study period of 67 days, and for the final count the containers had a significantly higher larval (L4) and pupal densities of 54±10.1 and 11±2.5 per container, respectively.

Plastic containers (Tables 2 and 3). Seven containers were treated with a tablet each and two were used as untreated controls. The tablets were introduced into the containers holding 50 l tap water (day 1). Observations were made for *Aedes* immature infestations until the study was terminated on the 42nd day.

In the untreated containers, larval infestation was observed from the 4th day, and pupae on the 14th day. Throughout the study period, an infestation of *Aedes* larvae (L1-L4) and pupae was present in the untreated containers.

In the treated containers, larval infestation was observed from the 16th day in four containers and pupae from the 25th day in one container. The larval numbers uniformly increased in all the containers with time and there was no significant difference in the larval density among the treated containers ($p>0.05$).

Black dead larvae were observed floating on the treated water surfaces for 31 days. Pupae were observed in all the containers on the 41st day.

For the study period of 42 days, the mean larval density in the treated containers was significantly less than in the untreated plastic containers ($p<0.05$), but there was no significant difference in the mean pupal densities between treated and untreated containers ($p>0.05$) (Table 3).

All treated container surfaces were coated with a black filamentous fungus from the 20th day.

HDPE containers (Tables 2 and 3). Five containers were treated with a tablet each and two were used as untreated controls. The containers were observed for 33 days after they were set for oviposition in the test site, and treated when all the containers were infested with *Aedes* immatures.

Observations were made for *Aedes* larvae and pupae until the study was terminated on the 55th day.

Throughout the study period, *Aedes* larvae (L1-L4) and pupae were present in the untreated containers. The larval and pupal counts on the 55th day were 57.0±24.0 and 3.0±2.0 per container, respectively.

In the treated containers, larvae were observed from the 19th day in one container and pupae from the 25th day in one container. The larval and pupal numbers increased to a maximum of 22.4±3.4 and 2.6±1.2 per container, respectively on the 55th day.

Black dead larvae were observed floating on the treated water surfaces for 40 days. Pupae were observed in all containers on the 41st day.

For the study period of 55 days, the mean larvae and pupae densities in the treated containers were significantly less than in the untreated containers ($p<0.05$) (Table 3).

DISCUSSION

The potential for *Bacillus thuringiensis israelensis* (*Bti*) to control dengue vectors in potable waters has been evaluated in countries, with widespread temephos resistance in the *Ae.aegypti* larval population. Becker *et al* (1991), Lerdthusnee *et al* (1996) and Santos *et al* (2001) evaluated *Bti* tablets under simulated conditions, whereby batches of laboratory bred larvae were introduced periodically into treated containers over a control period of 1 to 3 months without water replenishment. Other *Bti* formulations: VectoBac, 12AS, an aqueous suspension formulation, and VectoBac WDG, a water dispersible granule formulation, have been evaluated for *Ae. aegypti* control in portable containers. Ponce *et al* (2002) dispensed VectoBac 12AS treated water into metal drums and larval indices were reduced to zero during the 2 wk observation period. This study was suspended when residents complained of a fine dusty film on the water surface. Another study was conducted with VectoBac WDG: 0.5 g was introduced into 50 l tap water in earthen containers and complete larval control was achieved during 1 month observation period, but this study was terminated because of a dusty film on the treated water surface, cloudiness in the water column, and with time there was a fishy odor in the treated containers (Seleena, unpublished data). Therefore, appropriate *Bti* formulations that do not have undesirable effects on the domestic water source, are easily applied, and provide durable periods of protection against *Aedes* infestation are needed to be accepted by the community.

In this study, VectoBac DT® packaged in individual blister packs, has proven to be an appropriate candidate to be used in portable containers. It was easy to measure and to introduce into the container, it sank to the bottom on introduction, the treated water remained clear, and there was no unpleasant odor throughout the study period of 6 months. VectoBac DT® sank to the bottom of the container, did not disintegrate unless it was agitated, but fizzles, releasing *Bti* toxin into the container. The released *Bti* toxins were not present in the water column 24 hours post tablet introduction, but were concentrated along the sides and at the base of the treated container. Thus, the treated domestic water used by the consumers contained little or no *Bti* toxin.

The efficacy of VectoBac DT® was significantly longer in the earthen containers than in the HDPE and plastic containers, with or without regular treated water exchange. Becker *et al* (1991) reported a similar observation, with lower *Bti* tablet activity in the plastic containers than in the earthen containers. This was due to *Bti* toxins embedded in the porous earthen container surface which protects it from rapid degradation; lower toxin amounts were removed from the water column during water exchange. The long residual action of VectoBac DT® in earthen containers is similar to the long residual action of OMS-786® (Abate) emulsion concentrate (EC) in earthen containers (Bang and Tenn, 1969). It was reported that OMS-786® sprayed on to the inner walls of earthenware containers in Bangkok gave 3 months control against natural infestation with *Ae.aegypti* larvae, even under constant water exchange. Since OMS-786®

has an affinity to adhere to the internal surfaces of the earthen container, the EC formulation was superior to the temephos sand granule formulation in protecting earthen containers from natural infestation by *Aedes* larvae, and the number of weeks of protection was not influenced as much by constant water exchange with the EC formulation as with the sand granule treatment.

The length of effective and persistent residual action of VectoBac DT® in earthen containers is due to the ability of the inner wall of the earthen containers to hold the tablet particles. This affinity did not appear to be present in the plastic and HDPE containers.

The efficacy of VectoBac DT® was also studied in a field trial conducted under harsh conditions. The uncovered containers were placed outdoors under a building eave, exposed to sunlight and placed within the vicinity of a densely populated *Ae.albopictus* site. *Aedes* mosquitoes were not repelled from ovipositing by the VectoBac DT® treated water, and the treated water did not have any adverse effect on the development of eggs. Thus, the significantly lower immature density in all the tablet treated waters was due to VectoBac DT® treatment, not due to any other obvious reason.

In the field study, the efficacy of VectoBac DT® cannot be determined by counting and comparing larval numbers between the treated and untreated containers, because L1/L2 larvae remained on the water surface and were not in contact with the tablet particles, which were concentrated at the sides and base of the container. Thus, mortality was not observed in the L1/L2 stage. Larval mortality was observed in the L3/L4 population, which grazed the container surfaces, and black dead larvae (L3/L4) were observed floating on the water surface. Some field larvae (L3/L4) were observed to exhibit territorial behavior, grazing in their specific position in the container. These larvae were observed to move away from their position when they were disturbed, when the water column was disturbed during the replacement of evaporated water. An increase in larval mortality (L3/L4) was usually observed a day after evaporated water was replaced in all the treated containers. In view of the above information, the efficacy of VectoBac DT® was evaluated better by counting and comparing the pupal numbers between treated and untreated containers.

VectoBac DT® significantly reduced the pupal density in the treated earthen and HDPE containers throughout the study periods of 55 and 67 days, respectively ($p<0.05$). In the plastic containers, the tablet significantly reduced the pupal density for the first 25 days post treatment (0.03±0.03 pupae per treated container; 0.39±0.10 pupae per untreated container) ($p< 0.05$). Beyond 25 days there was no significant difference in the pupal density between the treated and untreated plastic containers ($p > 0.05$). In fact, the mean pupal numbers were higher in the treated containers (0.52±0.18) than in the untreated plastic containers (0.39±0.18). The reason for the poor tablet efficacy in the plastic containers was due to a black filamentous fungus which was observed coating the inner surface of all the treated plastic containers, from the 20th day post treatment.

In the fungal infested containers, the *Aedes* larvae grazed and fed on the fungus, pupae were more active and larger in size, and the emerged *Aedes* adults were also larger in size in comparison to the *Aedes* immatures and adults in the untreated containers, which were devoid of the fungal infestation. The fungal material from the containers were cultured on Sabouraud dextrose agar with chloramphenicol (SDA-Cl) and the plates were incubated at room temperature (28ºC) for 48 hours. A greyish white spreading cottony mold grew on the agar surface, and the agar reverse was black. The mold did not sporulate and further identification was not carried out. The fungus toxicity against laboratory bred *Ae. aegypti* and *Ae.albopictus* larvae was tested. The larvae fed on the fungus, survived and successfully pupated. The fungal material in the treated plastic containers could be an environmental contaminant, and their growth in the treated containers could have been supported by the formulated materials in the VectoBac DT®. A denser fungal growth in the treated plastic containers could be due to the container type providing better adhesion for the attachment of the filamentous fungi.

The fungus could have reduced the tablet

efficacy by coating the tablet particles that were adhering to the container surface, thus preventing the larvae from acquiring the *Bti* toxin; or the fungus could have degraded the *Bti* toxin in the container, or the fungus could have neutralized the *Bti* toxin in the larval mid gut. The fungus was found not to be toxic to the larvae. It may have provided the required nutrients for better larval survival and development into the pupal stage, thus explaining the higher pupal numbers in the treated containers than in the untreated containers after fungal infestation was observed in the treated containers.

In the field study, a higher efficacy of VectoBac DT® was obtained in the earthen containers than in the HDPE containers to control the natural infestation of *Aedes* mosquitoes. Black dead larvae, which indicated the presence of viable *Bti* toxins, were observed on the water surface for 61 days post treatment in the earthen containers, but for only 40 days post treatment in the HDPE containers (Table 3). The pupal density was an average of 8 fold less in the earthen containers, but 5 fold less in the HDPE containers in comparison to untreated containers. The longer tablet efficacy and persistence in the earthen containers was due to *Bti* toxins being embedded in the porous earthen container surface, which protected it from rapid degradation.

In the field study, susceptibility to temephos was determined for the wild *Ae. albopictus* larval population, which were collected from the untreated containers. A bioassay was conducted with 96% TG temephos, at the WHO diagnostic dosage of 0.012 mg/l. The bioassay protocol was as described in the WHO (1996). The susceptibility status of the wild larvae was compared to lab-bred *Ae. albopictus* (F25) larvae from the insectarium of the Entomology Division, IMR. The wild larvae collected from the untreated potable containers were resistant to temephos at the WHO diagnostic dosage of 0.012 mg/l, with a resistance ratio of 12.15, but the temephos resistant wild population was susceptible to VectoBac DT®.

Bti has proven to be an effective larvicide in public health programs because of its environmental friendliness. It is exceedingly low in mammalian toxicity, highly specific to its targets with little or no effect on non-target insects and other invertebrates breeding in the same habitats, and does not have any documented cases of resistance. Thus, tablets of *Bti* have been recommended to be used in drinking water to control dengue vectors (WHO, 1999). VectoBac DT®, an easy to use *Bti* formulation in potable containers, has proven its effectiveness in reducing *Aedes* immature populations in varied container types. Nevertheless, there is a further need to determine the efficacy of VectoBac DT® to reduce the dengue vector population to a threshold that will prevent dengue outbreaks in dengue endemic areas.

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