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EMERGENCE OF RESISTANCE AND RESISTANCE MANAGEMENT IN FIELD POPULATIONS OF TROPICAL *CULEX QUINQUEFASCIATUS* TO THE MICROBIAL CONTROL AGENT *BACILLUS SPHAERICUS*

MIR S. MULLA,[1] USAVADEE THAVARA,[2] APIWAT TAWATSIN,[2] JAKKRAWARN CHOMPOSRI[2] AND TIANYUN SU[1]

ABSTRACT. In recent years, highly potent mosquitocidal strains of the microbial agent *Bacillus sphaericus* (*Bsph*) have been isolated and developed for the control of mosquito larvae around the world. Laboratory selection experiments with the most active strains and their use in large-scale operational mosquito control programs resulted in the emergence of resistance in larvae of the *Culex pipiens* complex. This generated great concern among vector control agencies around the world, who feared reduced efficacy of this highly active larvicidal agent. To address this issue, the current studies were started to find practical strategies for controlling resistant mosquitoes and more importantly to develop resistance management strategies that would prevent or delay development of resistance. We initiated field studies in 3 low-income communities in Nonthaburi Province, Thailand. In 1 of the communities, larvae of *Culex quinquefasciatus* that were highly resistant (>125,000-fold) to *Bsph* strain 2362 were successfully controlled with applications of *Bacillus thuringiensis* var. *israelensis* (*Bti*) alone or in combination with *Bsph.* To prevent or delay resistance to *Bsph,* 2 other sites were selected, 1 treated with *Bsph* 2362 alone and the other treated with a mixture of *Bsph* 2362 and *Bti.* Mosquitoes treated with *Bsph* 2362 alone showed some resistance by the 9th treatment and almost complete failure of control occurred by the 17th treatment. After 9 treatments with the mixture over a 9-month period at another site, no noticeable change in susceptibility to *Bsph* was detected. During this period, the site treated with *Bsph* alone required 19 treatments, whereas the site treated with mixtures took only 9 treatments because of slower resurgence of larvae at the site treated with the mixture than at the site treated with *Bsph* alone. This is the 1st field evidence for delay or prevention of resistance to microbial agents in larval *Cx. quinquefasciatus* by using mixtures of *Bti* and *Bsph.* Further studies on the use of mixtures for the management of field resistance are warranted.

KEY WORDS *Culex quinquefasciatus, Bacillus sphaericus,* resistance, resistance management, prevention

INTRODUCTION

In the past 25 years, several isolates and strains of 2 species of spore-forming bacteria have been found that produce parasporal proteins that show high toxicity against mosquito larvae (WHO 1985, de Barjac and Sutherland 1990). *Bacillus thuringiensis* var. *israelensis* de Barjac (*Bti*) was discovered in 1976 (Goldberg and Margalit 1977) and was developed for use in mosquito and black fly control programs (de Barjac 1990a, Mulla 1990). During this same period, research on isolating and developing mosquitocidal strains of *Bacillus sphaericus* (*Bsph*) Neide was intensified. Of the approximately 300 strains isolated, 17 were highly toxic to mosquito larvae (de Barjac and Sutherland 1990). Of these, strains 2297 from Sri Lanka (Wickremesinghe and Mendis 1980), 1593M from Indonesia (de Barjac 1990b, Singer 1990), 2362 from Nigeria (Weiser 1984), and C3-41 from China (Liu et al. 1989) were studied extensively and are now available commercially as mosquito larvicides. Strain 2362, which is the most commonly used material, has been used in Europe and elsewhere since 1989 and in the USA since 1996.

In 1990, we initiated studies on the potential development of resistance to *Bsph* 2362 in larvae of *Culex quinquefasciatus* Say in the laboratory. A moderate to high level of resistance was obtained on selection with this strain (Rodcharoen and Mulla 1994, Wirth et al. 2000, Zahiri et al. 2002). Moderate to high levels of resistance in the *Cx. pipiens* L. complex were reported in the field after 2–3 years of use of strains 2362 (Sinègre et al. 1994, Silva-Filha et al. 1995), 1593M (Adak et al. 1995, Rao et al. 1995), and C3-41 (Yuan et al. 2000). In the evaluation and development process, *Bsph* strains were tested once or twice in field trials for initial activity in several countries (Liu et al 1989; Zhang et al. 1989; Karch et al. 1992; Mulla et al. 1997, 1999; Ali et al. 2000). Once implemented in field control programs, little or no monitoring of susceptibility levels of mosquito species was conducted and resistance was only detected when gross failure in field control programs was noted.

During the course of several years of field evaluation of *Bsph* formulations against *Cx. quinquefasciatus* in polluted water in squatter communities in Thailand (Mulla et al. 1997, 1999, 2001), almost 100% initial and 35 days of residual control of larvae was achieved with the 1st few treatments of *Bsph* 2362. However, with further treatments, the level of control declined and longevity of control also progressively decreased (Mulla et al. 2001). Rapid failure of control occurred with the VectoLex water dispersible granules (WDG) formulation at Wat Pikul Community in Nonthaburi Province in 2000. Control of larvae was reduced after the 4th

[1] Department of Entomology, University of California, Riverside, CA 92521.
[2] NIH, Department of Medical Sciences, Ministry of Public Health, 87/7 Tiwanon Road, Nonthaburi 11000, Thailand.

treatment, and no control was achieved in the 5th treatment at the maximum label dosage of 200 mg/m^2 (Mulla et al. 2001). A high level of resistance in *Cx. quinquefasciatus* evidently had developed after 4–5 repeated applications of *Bsph* at this site. This rapid development of resistance caused concern in vector control programs.

To respond to these concerns of rapid development of resistance, the current studies were initiated to document the magnitude of resistance in *Cx. quinquefasciatus* at Wat Pikul and to develop practical strategies for the control of *Bsph*-resistant mosquitoes. Laboratory assays provided evidence for lack of cross resistance to *Bti* in *Bsph*-resistant mosquitoes (Rao et al. 1995; Rodcharoen and Mulla 1996; Zahiri et al. 2002; Su and Mulla, unpublished data) and this information led to the hypothesis that *Bsph*-resistant populations at Wat Pikul could be controlled with *Bti* alone or with a mixture of *Bti* and *Bsph. Bacillus sphaericus* produces higher mortality and longer residual activity than does *Bti* in polluted water. The belief was held that a mixture of *Bsph* and *Bti* could yield initial and persistent control. Therefore, field studies were initiated to ascertain control of *Bsph*-resistant mosquitoes by mixtures of *Bsph* and *Bti,* to investigate prevention of resistance by use of *Bsph–Bti* mixtures, and to study the emergence of resistance as a result of treatments with *Bsph* 2362 alone. Results are presented in figures.

MATERIALS AND METHODS

Control of Bsph*-resistant mosquitoes at Wat Pikul:* To control *Bsph*-resistant *Cx. quinquefasciatus,* treatments with *Bti* or *Bsph* 2362 alone or in a mixture were initiated. Wat Pikul (Bang Yai District, Nonthaburi Province) is a low-income community with high-density housing and a dense human population (Mulla et al. 2001). In some dwelling units, as many as 9 families live together, in some cases with 1 family per room. As in other low-income communities in this area, wastewater and solid organic and inorganic wastes are thrown outside. These accumulate under structures built above ground on posts and pylons. These conditions (solid waste mixed with polluted water) create ideal habitats for the propagation of mosquitoes, especially *Cx. quinquefasciatus.* The breeding area for mosquitoes at this location was about 4,000 m^2, a relatively large mosquito-producing area of this type. After detecting resistance to *Bsph* in this area in December 2000, the total area was subjected to treatments of WDG formulations of *Bti* or *Bsph* 2362 alone or in various mixtures from January to late October 2001.

Another community, Soi Jumpa (Pak-Kret District, Nonthaburi Province), was used as an untreated control. Mosquitoes at this site were not resistant to *Bsph,* because a treatment with 100 mg/m^2 of the WDG formulation yielded almost 100% initial control. Detailed descriptions of this site were published by Mulla et al. (1999, 2001). This community is similar in size to Wat Pikul and has similar potential for supporting polluted-water mosquitoes. However, monitoring of this site was terminated at the time of the 10th treatment at Wat Pikul on May 23, 2001. The trends of immature mosquitoes were rather stable during the 1st 4 months of the experiment and we saw no point in further following these populations as a control. We have noted that mosquitoes in these types of developmental sites resurge quickly after cessation of treatment and reach pretreatment populations; a comparison of posttreatment counts versus pretreatment counts provides an accurate estimation of control.

Emergence of resistance to Bsph *and prevention of resistance with mixtures:* These studies were carried out in 2 communities in the Nonthaburi Province, Thailand. The purpose of these studies was to determine if repeated treatments of a small breeding source with *Bsph* 2362 alone would result in the development of resistance, as was observed at Wat Pikul. At the same time, we examined whether mixtures of *Bti* and *Bsph* could prevent or delay emergence of resistance to *Bsph.* The 1st community, Wat Lahan, Bang Bua Thong District, was subjected to repeated treatments of *Bsph* WDG formulation at 100 mg/m^2, a highly effective dosage for susceptible larvae.. This site encompassed a total mosquito-producing area of 400 m^2, an area much smaller than that of Wat Pikul and Soi Jumpa. This area also had some inaccessible breeding sources of mosquitoes that could not be treated. The studies in Wat Lahan community started on May 17, 2001, and terminated on March 15, 2002, after the last (19th) treatment at 200 mg/m^2 failed to provide satisfactory control of larvae.

In previous years, Soi Jumpa community was treated intermittently with WDG formulations of *Bsph* 2362, receiving 7 treatments in 1997, 3 treatments in 1998, and 4 treatments in 1999 (Mulla et al. 1999, 2001). In each period, the last treatment was found to be highly effective, pointing to the absence of resistance in this population. Since October 1999, this site remained untreated. To test the susceptibility of larvae at this site, it was treated in early May 2001 at 100 mg/m^2 of *Bsph* WDG, which yielded almost 100% initial control. Because of the lack of apparent resistance to *Bsph,* this site was selected for the mixture trials.

Sampling: Mosquito larval populations were sampled by 400-ml-capacity dippers. Larvae were sorted into 1st, 2nd, 3rd, and 4th instars and pupae and counted. The sites were sampled before treatment and at various intervals after treatments. When the larval and pupal counts resurged to within 60–80% of the pretreatment levels, the sites were treated again.

Larval sampling was biased toward areas where mosquito larvae and pupae were dense because we wished to judge effectiveness of control in areas of dense aggregations of larvae and pupae. The number of dips taken varied depending on the size of the treated area. In Wat Pikul, Wat Lahan, and Soi Jumpa, 20, 10, and 20 dip samples were taken each time, respectively.

Materials and application: Commercial products of *Bti* (VectoBac WDG) and *Bsph* 2362 (VectoLex WDG) formulations were used in these experiments. Both products were provided by Valent BioSciences Corporation (Libertyville, IL). The dosages given in mg/m^2 are for the formulations used. The product containers were kept at room temperature until used. The required amounts of the WDG formulation was mixed with water in a plastic bucket, transferred to a 7-liter stainless steel spray tank, pressurized, and sprayed onto the water surface of the habitat. The aqueous spray was applied through a cone jet nozzle that projected the spray jet up to 4–5 m from the sprayer. Most, if not all, of the habitat was covered by the application.

In Wat Pikul, the treatable area was 4,000 m^2 through the 9th treatment, but drained to 2,000 m^2 after this time. Wat Lahan had only 400 m^2 of mosquito-breeding area. In Soi Jumpa, the control area for the Wat Pikul test, and subsequently an area treated with the mixture to examine prevention of resistance, had a breeding source of 3,000 m^2. At Wat Pikul, the large area was sprayed with 7 tanks (7 liters each), whereas the reduced area was sprayed with 5 tanks. Wat Lahan was sprayed only with one 7-liter tank, whereas Soi Jumpa was sprayed with 5 tanks (7 liters each) each time.

Population reduction: Population reduction in percent was calculated by comparing counts of 3rd and 4th instars and pupae to the pretreatment counts of the same stages. Pretreatment counts used in the calculations were those just before each treatment at a given site. Although *Bti* and *Bsph* do not kill pupae, the pupal numbers quickly declined because of larval kill by the treatments and, therefore, they were included in calculations of percent reduction. Comparison of posttreatment with pretreatment populations in the treated site provide consistent and reliable levels of control.

RESULTS AND DISCUSSION

Control of resistant mosquitoes at Wat Pikul

Culex quinquefasciatus, the dominant mosquito, was treated 5 times at this site with *Bsph* 2362 WDG formulation in 2000. After the 4th and 5th treatments, larval control was not satisfactory (Mulla et al. 2001). The magnitude of resistance in this population was further quantified by laboratory bioassays, which showed more than 125,000-fold resistance compared to susceptible mosquitoes from Soi Sirichai, Nonthaburi Province, Thailand, to *Bsph* 2362 technical powder (Su and Mulla, unpublished data). To control this highly resistant mosquito population, treatments were initiated mostly with *Bti–Bsph* 2362 mixtures starting in January 2001 and continued them until late October 2001. In total, 18 treatments with single agents or mixtures of the 2 agents were made (Fig. 1).

To further confirm the resistance at this site, the 1st treatment of VectoLex WDG was made on January 22, 2001, at 200 mg/m^2, a highly efficacious dosage against susceptible mosquitoes in polluted water. This rate of application gave no control of 3rd and 4th instars and pupae 48 h after treatment. The larval density increased after this treatment (Fig. 1A). In our studies at this site in 2000, before the mosquitoes developed resistance to *Bsph* 2362, we obtained almost 100% control of larvae with the 1st treatment at 200 mg/m^2 of VectoLex WDG for over 35 days (Mulla et al. 2001). After documenting lack of control at a high dosage of VectoLex, the effectiveness of an equal dosage of VectoBac WDG was tested against this *Bsph* 2362–resistant population. This treatment gave almost complete control of larvae, lasting, as expected, for 14 days. After this treatment, the efficacy of lower dosages of VectoBac WDG was determined by applying this formulation at 50, 25, and 20 mg/m^2 in the next 3 treatments. The 50 mg/m^2 treatment gave 100% control of larvae 2 days after treatment, with the control dropping to 78% 8 days after treatment; the population resurged to the pretreatment level 13 days after treatment. The treatments at 25 and 20 mg/m^2 yielded 98 and 91% initial control of larvae, which dropped to 40 and 47% 7 days after treatment, respectively (Fig. 1A). Two more applications of *Bti* alone (13th and 14th treatments) were made at the 20 mg/m^2 dosage of VectoBac WDG. These treatments gave 99–100% control of larvae 2 days after treatment. The level of control dropped to 60 and 33% in the 13th and 14th treatments 7 days after treatment, respectively. The 17th treatment in this series was made with VectoBac WDG at 200 mg/m^2. This treatment, as for the 2nd treatment at 200 mg/m^2, yielded 100% control initially, which declined to 92% in 7 days and to 64% 15 days after treatment (Fig. 1B). These treatments with *Bti* showed that the *Bsph*-resistant mosquitoes at Wat Pikul are completely susceptible to *Bti.* This was confirmed by laboratory studies (Su and Mulla, unpublished data). The 7 treatments (2nd through 5th, 13th, 14th, and 17th) made with VectoBac WDG also provided evidence that *Bti* treatments have no residual activity (although this increased slightly at higher dosages) beyond 7 days and that repeat treatments would be necessary every 7–10 days at practical dosages. This study also indicated that the optimum dosages for the control of this *Bsph*-resistant polluted-water mosquito are between 20 and 50 mg/m^2 for initial and short-term control.

After 4 treatments (2nd through 5th) with *Bti* against *Bsph*-resistant mosquitoes, various mixtures of *Bti* and *Bsph* 2362 were used for treatment. The 6th and 7th treatments were made with mixtures of

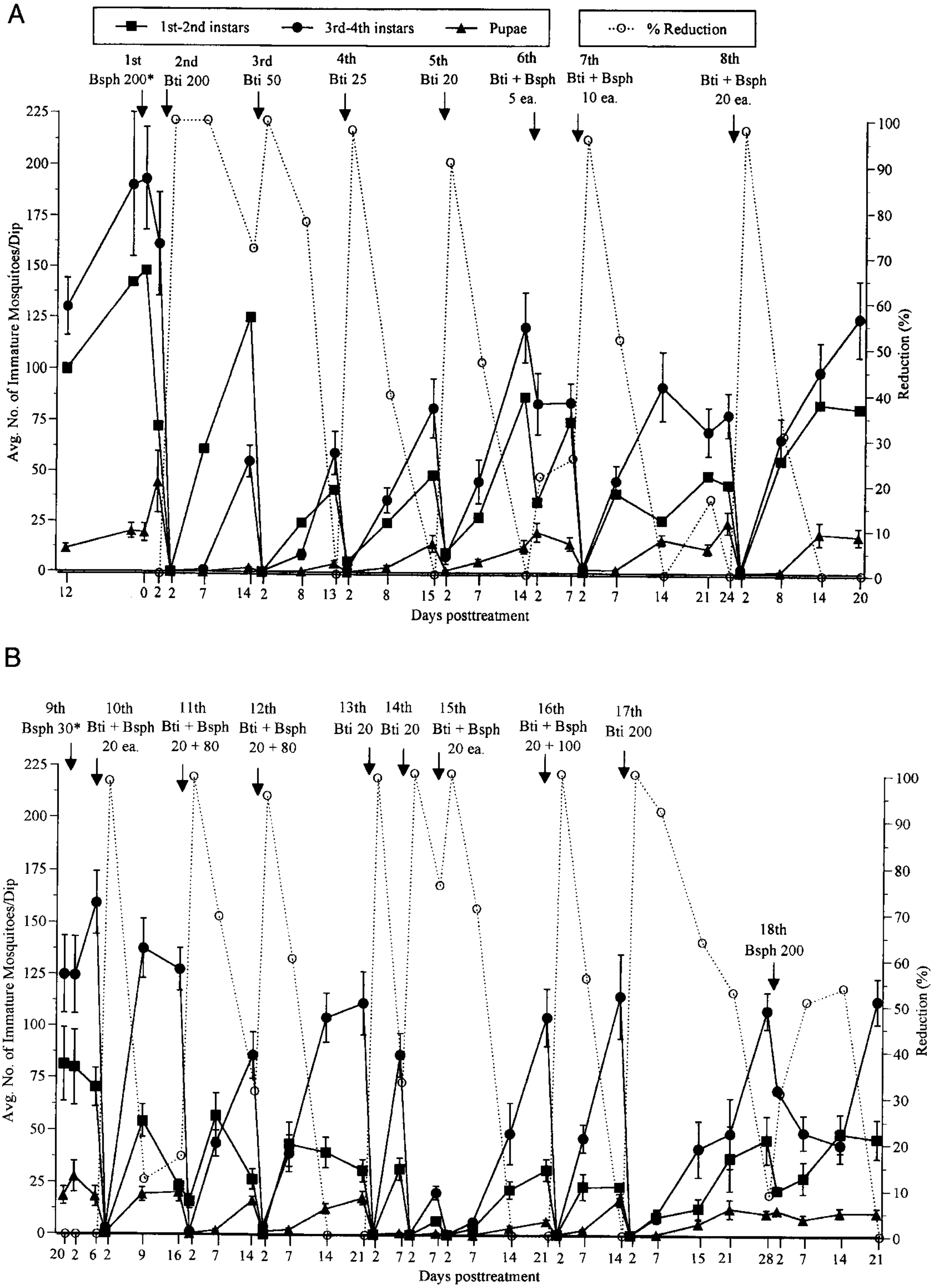

Fig. 1. Control of *Culex quinquefasciatus* resistant to *Bacillus sphaericus* (*Bsph*) 2362 (resistance ratio 125,000-fold) with (A) 1st through 8th treatments (January to April 2001) or (B) 9th through 18th treatments (May to October 2001) of *Bacillus thuringiensis* var. *israelensis* (*Bti*) or *Bsph* alone or *Bti–Bsph* mixtures at Wat Pikul, Bang Yai District, Nonthaburi Province, Thailand. Dosage is given as mg/m².

VectoBac WDG and VectoLex WDG at 5 mg/m^2 of each (total 10 mg/m^2) and 10 mg/m^2 of each (total 20 mg/m^2), respectively. The treatment with 5 mg/m^2 of each formulation yielded little or no control of larvae, but treatment with 10 mg/m^2 of each yielded 96% control of larvae 2 days and 52% control 7 days after treatment. The populations increased beyond the pretreatment level 14 days after the 7th treatment. For the 8th treatment, the dosages were increased to 20 mg/m^2 each (total 40 mg/m^2). This treatment yielded similar control as the 7th treatment, 98% at 2 days after treatment and 31% 1 week later (Fig. 1A).

After 7 treatments with *Bti* alone or with a *Bti–Bsph* mixture, a single treatment with VectoLex WDG at 30 mg/m^2 was administered to determine if a shift in level of *Bsph* resistance occurred. This treatment yielded no control of the larvae, hence showing no decline in *Bsph* resistance (Fig. 1B). After noting these results, 20 mg/m^2 of each agent was applied in the 10th treatment. This treatment yielded 98% control initially and only 12% control 9 days after treatment (Fig. 1B). The mixture treatments thus far offered no residual activity against *Bsph*-resistant mosquitoes, and the results were similar to those with *Bti* alone.

To determine if a higher ratio of *Bsph* in the mixture would generate residual activity beyond that of *Bti* alone, a mixture of *Bti–Bsph* at 20 and 80 mg/m^2, respectively, was applied in the 11th and 12th treatments. In both treatments, initial control was high (95–99%), but control 7 days after treatment was 69 and 60% in the 11th and 12th treatments, respectively. No residual control was noted in either treatment at 14 days after treatment. These 2 experiments indicated that residual activity against *Bsph*-resistant populations could not be increased substantially by increasing the ratio or dosage of *Bsph* in the mixture. As evidenced in the 13th and 14th treatments with *Bti* WDG alone at 20 mg/m^2, the initial control and longevity were essentially the same as in the mixture with *Bsph* at 80 mg/m^2 and *Bti* at 20 mg/m^2. Addition of *Bsph* to *Bti*, even at 4 times the dosage of *Bti*, did not increase residual activity. Use of *Bti* and *Bsph* at 20 mg/m^2 each (15th treatment) yielded 100% initial control, similar to that with *Bti* alone at 20 mg/m^2. Addition of *Bsph* to *Bti* did not improve the extent or longevity of control. In 1 further treatment (16th), where the quantity of *Bsph* in the mixture was increased further (*Bti* 20 mg/m^2, *Bsph* 100 mg/m^2), the level of control was similar to that with *Bti* alone (20 mg/m^2) or with the mixture of *Bti–Bsph* at 10 mg/m^2 each. For the 17th treatment, *Bti* was again applied at 200 mg/m^2 to test for residual activity. This treatment produced 100% initial control, but the level of control was 92 and 64% at 7 and 15 days after treatment, respectively. No significant level of persistence was noted beyond 7 days (Fig. 1B).

For the final treatment (18th) against *Bsph*-resistant mosquitoes at Wat Pikul, the efficacy of the high label dosage of *Bsph* WDG at 200 mg/m^2 was tested. Little or no initial control (31%) was achieved with this treatment (Fig. 1B). This clearly indicated that the *Bsph* resistance in this population was stable, with no marked shift noted after 15 treatments of *Bti* or *Bti–Bsph* mixtures, showing that once a high level of resistance was established, it could not be reversed with 15 treatments of *Bti* or of *Bti–Bsph* mixtures and that use of *Bsph* 2362 against *Bsph*-resistant *Cx. quinquefasciatus* would not be practical. Increasing the ratio of *Bsph* in mixture did not result in persistence against resistant larvae. Thus, development of strategies to prevent or delay development of resistance to mosquitocidal *Bsph* strains is needed. From the foregoing, *Bsph*-resistant *Cx. quinquefasciatus* in the tropics clearly can be controlled easily by the use of *Bti* alone at relatively low rates, with the drawback being that *Bti* would have to be applied at least weekly, which would increase treatment costs.

Development of resistance in sites treated only with *Bsph*

To provide further information on the speed of development of resistance as found in Wat Pikul in previous studies (Mulla et al. 2001), a small polluted water source of mosquitoes (400 m^2) at Wat Lahan was subjected to repeated treatments with VectoLex WDG alone at 100 mg/m^2. In total, 19 treatments were made (Fig. 2) between May 2001 and March 2002. The larval threshold for repeat treatments was set low at 30 larvae/dip.

The 1st 6 treatments yielded approximately 99% control for 7 days and 79% control 14 days after treatment (Fig. 2). However, the 7th treatment yielded a low level of control on day 2 (85%) and day 7 (18%). The 8th treatment yielded excellent initial control (96%) on day 2 and 76% control on day 8. During the following treatments (9th through 16th), extent of control declined substantially both on day 2 and day 7 after treatment. Likewise, substantial residual activity was not noted in any of these 8 treatments (9th through 16th). The 17th and 18th treatments exhibited complete failure of control, because initial control was only 8% for the 17th treatment. After the 18th treatment, the initial population density increased 30% and then doubled over the pretreatment level 14 days after treatment. For the next treatment (19th), the dosage of VectoLex WDG was increased from 100 mg/m^2 to 200 mg/m^2. At this relatively high dosage, only 60% control on day 2, 55% control on day 9, and 29% control on day 15 was achieved. The conclusion was made that larval *Cx. quinquefasciatus* at Wat Lahan community had developed resistance to *Bsph* 2362, and therefore further treatments were stopped.

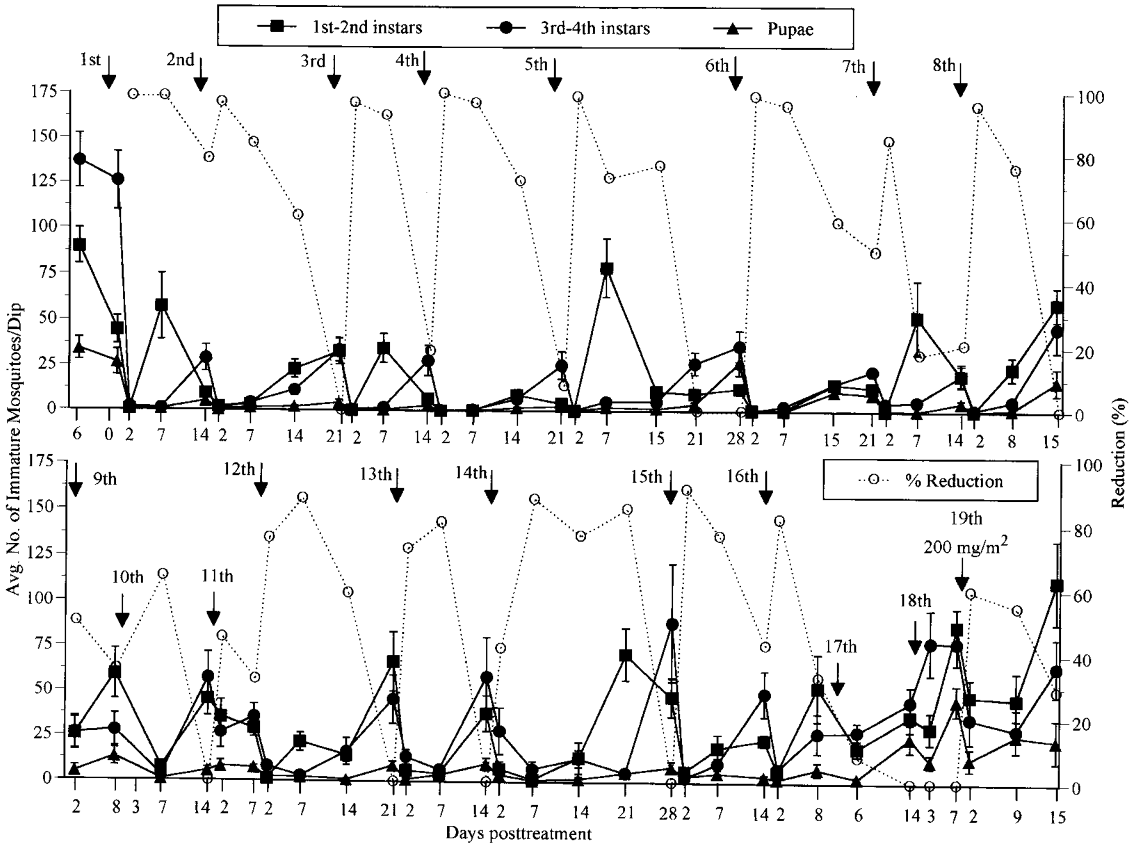

Fig. 2. Emergence of resistance in larval *Culex quinquefasciatus* treated 19 times (May 2001 through March 2002) with *Bacillus sphaericus* 2362 water dispersible granules formulation at 100 mg/m² at Wat Lahan Community, Bang Bua Thong District, Nonthaburi Province, Thailand.

Preventive measures against resistance to *Bsph*

As shown in the previous section, once *Bsph* resistance emerges, use of *Bsph* after treatments with *Bti* or a mixture of *Bti* and *Bsph* is impractical. Establishment of proactive antiresistance measures is more desirable before the widespread use of *Bsph* or other chemical and microbial agents. Based on the previous study and our laboratory studies (Zahiri and Mulla, unpublished data), a mixture of VectoBac and VectoLex WDG at 50 mg/m² each (total 100 mg/m²) was tested in the low-income community of Soi Jumpa, where the total breeding habitat was treated repeatedly.

In Soi Jumpa community, 9 treatments with a mixture of *Bti–Bsph* were made from May 23, 2001, to January 2002 (Fig. 3). The 1st 6 treatments provided 98–100% control 2 days after treatment, 98–99% control 7 days after treatment, and up to 86% control 14 days after treatment, indicating residual activity for up to 2–3 weeks after treatment. Thereafter, moderate levels of control were achieved 21–28 days after treatment (Fig. 3). The 7th and 8th treatments produced 96–99% control on days 2 and 7 after treatment; however, the level of control was quite low on day 14 after treatment (54 and 46%, respectively). However, the following treatment (9th) yielded excellent control on day 2 (99%) and day 7 (91%) after treatment; this declined to 84% but was still good on day 14 after treatment.

Although the field treatments at Wat Lahan (*Bsph* alone) and Soi Jumpa (with mixture) covered the same period, 19 treatments were made at the former site compared to 9 at the latter site. The reason for fewer treatments at Soi Jumpa was that resurgence of larvae here was slower than at Wat Lahan.

From these studies, mixtures of *Bti–Bsph* (1:1) used against *Bsph*-susceptible larvae did not cause any noticeable shift in the susceptibility of larval *Cx. quinquefasciatus* at Soi Jumpa. All treatments were equally effective, with good residual activity up to 14 or 21 days after treatment. This is in contrast to the *Bsph*-resistant populations at Wat Pikul, where mixtures readily controlled the resistant larvae but provided no residual control. One other aspect that was observed was that over the 8-month period of our study at Soi Jumpa, the immature

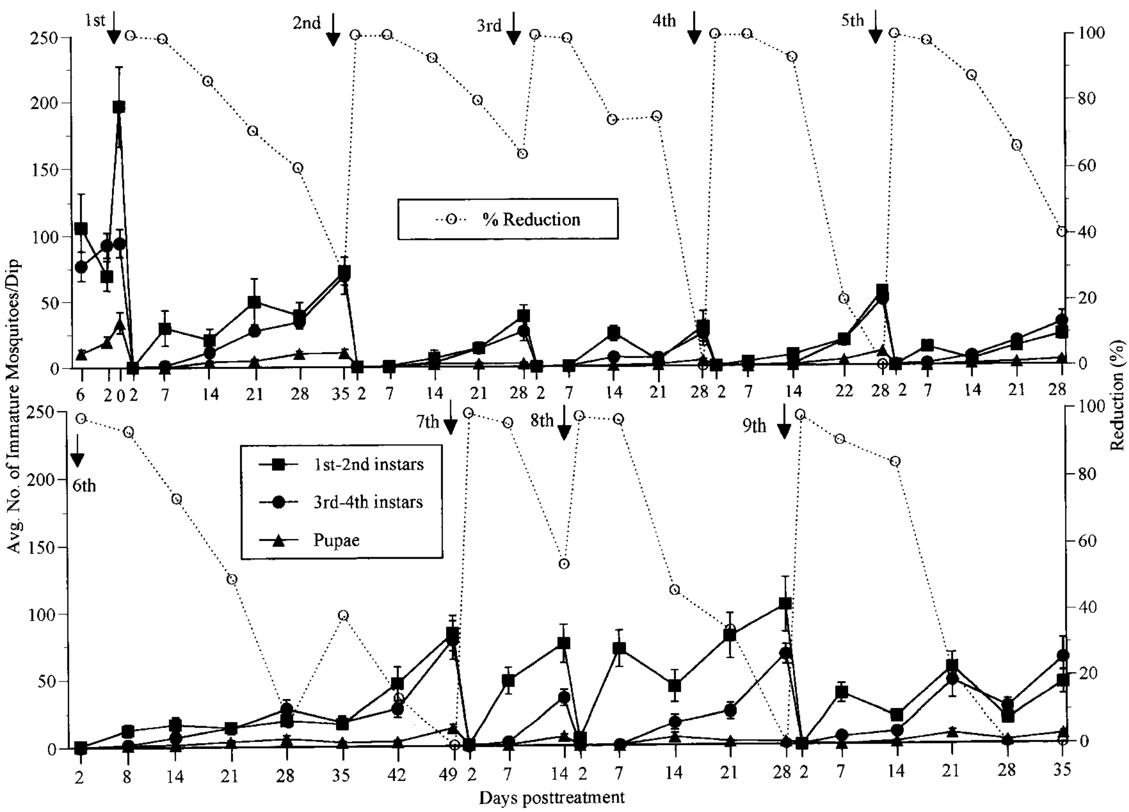

Fig. 3. Prevention of resistance development in *Culex quinquefasciatus* treated 9 times (May 2001 through January 2002) with mixture of water dispersible granules formulation of *Bacillus sphaericus–Bacillus thuringiensis* var. *israelensis* (1:1) at 100 mg/m² total in Soi Jumpa Community, Pak Kret District, Nonthaburi Province, Thailand.

populations subjected to treatments with *Bti–Bsph* mixtures occurred at lower numbers, and never recovered to the level prior to the 1st treatment. On the basis of these few treatments, the conclusion was made that development of resistance will be materially delayed or possibly prevented by administering a mixture of *Bti–Bsph* to susceptible larval populations. However, additional field tests and possibly more treatments with mixtures are warranted to document prevention of resistance. At the present time, adequate information is not available on the optimum ratios of *Bti–Bsph* that will result in desired level of initial and persistent control of susceptible larvae.

CONCLUSIONS

In previous and present studies, evidence was provided that tropical *Cx. quinquefasciatus* can develop resistance rapidly to *Bsph* 2362. Emergence of a high level of resistance (>125,000-fold) at Wat Pikul (Su and Mulla, unpublished data) was noted after 5 treatments. This study documents the re-emergence of resistance at Wat Lahan after 17 treatments. Possible reasons that more treatments at Wat Lahan than at Wat Pikul were needed for development of resistance to *Bsph* are that only 400 m² of possible mosquito-breeding source was treated at Wat Lahan and that sources that could not be treated were nearby. Factors such as refugia, size of area treated, and migration of susceptible genes into the target area can influence the speed, nature, and magnitude of emergence of resistance in mosquitoes. Note that resistance to *Bsph* is a feature of *Cx. pipiens* complex and no reports of development of resistance to *Bsph* in other species of mosquitoes have surfaced to date.

As a result of these studies, the conclusion is made that *Bsph*-resistant mosquitoes can be controlled with *Bti* alone or mixtures of *Bti* and *Bsph*. The conclusion also is made that use of a mixture of *Bti* and *Bsph* against susceptible *Cx. quinquefasciatus* can delay or prevent emergence of resistance, although additional studies are needed. The inclusion of *Bsph* in the mixture is justified, because it provides longer residual control of susceptible, but not resistant, mosquito larvae. Prolonging the efficacy of control for 1 or 2 more weeks has advantages in operational vector control programs in terms of costly surveillance, monitoring, and application of treatments.

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