Efficacy of *Bacillus thuringiensis* serovar *israelensis* against *Bacillus sphaericus* resistant and susceptible larvae of *Culex quinquefasciatus* Say

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ABSTRACT: A potential biological larvicides like *Bacillus sphaericus* (*Bs*) has been advocated in mosquito control operations. However, due to rapid development of resistance of mosquitoes to *Bs* toxin alternate mosquito control measures are needed. A strain of *Culex quinquefasciatus* Say was found highly resistant (resistance ratio (RR) = 6223 folds) in the field (Gandhinagar, Kochi, Kerala) during the year 1995 and was reared in the laboratory by subjecting to moderate selection pressure using *Bs* 2362 strain to maintain resistance. A susceptible mosquito colony of *C. quinquefasciatus* was also reared simultaneously. Bioassays were conducted with lyophilized bacterial culture of spore / crystal toxin of *Bs* 2362 with the inbred generations obtained from these colonies. We found that a high level of resistance to *Bs* (2362) toxin (RR at LC_{50} = 249.2 and at LC_{90} = 225.5 folds) in this resistant larvae. We also observed that the resistant larvae exhibited cross-resistance to *Bs* B42 (Serotype H5a5b) toxin (RR at LC_{50} and LC_{90} = 687.5, 473.4 folds, respectively). However, we did not come across any significant difference in the susceptibility level between *Bs* resistant and susceptible larval strains, when they were treated with another bacterial strain of *Bacillus thuringiensis* serovar *israelensis* (*Bti*) (VeetoBac®). This observation suggest that *Bti* based biopesticide can be used for the management of *Bs* resistant mosquito control operations.

KEY WORDS: *Bacillus sphaericus, Bacillus thuringiensis* serovar *israelensis, Culex uinquefasciatus,* resistance management

Mosquito borne diseases form a major part of communicable diseases in human society and several control strategies have been adopted to manage the same. Biological mosquito larvicides like *Bacillus sphaericus* Neide *(Bs)* and *Bacillus thuringiensis* serovar *israelensis* de Barjac *(Bti)* are promising and superior to conventional insecticides (Skovmand and Bauduin, 1997; Su and Mulla, 1999). The toxic activity is specific to mosquito larvae, and the activity is directly attributed to the parasporal crystal toxins that are produced during sporulation (Payne and Davidson, 1984; Baumann *et al.*, 1985). The binary toxin (42 and 51 kDa proteins) from *Bs* and the multiple toxin (27, 65, 128 and 135 kDa proteins) from *Bti* are the most important toxins that interact and produces a complex effect on the mosquito larvae (Broadwell *et al.*, 1990; Poncet *et al.*, 1995). When the crystal toxin from *Bs* is ingested by the susceptible larvae it undergoes solubilization and proteolytic cleavage in the alkaline environment in the midgut and the activated toxins interact with the receptors found in the brush border membrane (MBBM) midgut epithelium, leading to death of the larvae (Broadwell and Baumann, 1987; Nicolas *et al.*, 1990; Poopathi, 1999). Though,

the mode of action of *Bs* to MBBM of mosquitoes were well established (Nielsen Le-Roux and Charles, 1992; Charles, *et al.*, 1997) the mode of action of *Bti* in mosquito is not clearly understood (Ravoahangimalala and Charles, 1995).

The development of resistance in *Culex* species to *Bs* has impeded the success of mosquito control programme (Rao *et al.*, 1995; Poopathi *et al.*, 1999 a,b). Hence, identification of an alternate method to manage *Bs* resistant *C. quinquefasciatus*, is urgently needed. Therefore, we undertook this study to evaluate the relative efficacy of *Bti* against *Bs* resistant and susceptible larvae of *C. quinquefasciatus*.

MATERIALS AND METHODS

Mosquito colonies

Culex quinquefasciatus 3rd instar larvae (susceptible) were used from a colony maintained for more than five years in the laboratory of Centre for Research in Medical Entomology (ICMR), Madurai and named as Madurai susceptible strain, (MS).

A field trial was carried out in an 8 km² area in Gandhinagar (Kochi, Kerala) by spraying *Bs* 1593M (Biocide-S) during 1991 to 1993 and successful mosquito control operation was made by this centre. However, after resumption of regular spraying, satisfactory control was never obtained and it was suspected that the poor results could be due to the development of resistance in the field. The field collected larvae were transported to Madurai, tested for resistance and colonized and named as Gandhinagar resistant strain, (GR) (Rao *et al.*, 1995; Poopathi *et al.*, 1999 b, c). The GR strain has been subjected to moderate selection pressure (thousand early third instar larvae from GR strain were subjected at a concentration of 330 mg / l in 3 litre capacity bowl to determine the mortality of larvae at moderate level. The mortality ranged from 30 to 40 per cent. The surviving larvae from this experiment were pooled, rinsed in de-ionized water and reared to the next generation. Early third instar larvae of this

generation were again subjected to different doses to determine the lethal concentration level and the survivors were reared to the next generation for more than five years in the laboratory.

Both resistant (GR) and susceptible (MS) colonies were reared in the laboratory at ambient laboratory temperature (29-31° C) in enamel trays by providing yeast and dog biscuit at the ratio of 40: 60 in water as the nutrient source. Pupae were allowed to emerge in cages and the adults were sexed (male and female 4: 1 ratio). Females were provided with blood meal from live chicken and males with glucose solution (2-5%) through cotton pads and water soaked raisin. Adults were allowed to oviposit in water in enamel cups kept inside the rearing cages. Freshly hatched larvae from egg rafts of two larval strains (GR, MS) were cultured separately.

Bioassays

Lyophilized bacterial culture of *Bs* 2362 (SPH-88) (titre: 1500 International toxic units/ mg *Bs* toxin) received from Institute Pasteur, Paris, France, *Bs* H42 (H5a5b) received from Vector Control Research Centre (ICMR), Pondicherry and *Bti* H14 (VectoBac®) (titre: 1700 International toxic units / mg *Bti* toxin) received from Hoechest Schering AgrEvo Ltd, Mumbai were used in this study. Titration and preparation of stock solution from these bacterial strains and bioassays were made as described in WHO protocol (Anonymous, 1981,1985). In the present study, 6 grams of *Bs* and 25 milligrams of *Bti* toxins were homogenized in appropriate volume of de-ionized water as stock solutions. The aliquots of appropriate dilutions (for MS and GR strains) ranging from 8.5 gm to 0.02 mg / l. and from 0.36 mg to 0.011 mg / l were used from *Bs* (*Bs* 2362, B42) and *Bti* (*Bti* H14) toxins, respectively. These ranges of toxin were necessary to determine the susceptibility levels from 1.0 to 100 per cent by placing six to seven concentrations. Bioassays were conducted in disposable polythene cups (200 ml capacity). Test medium was prepared by adding appropriate volume of *Bs* or *Bti* toxin in 150 ml of water and twenty freshly moulted third instar GR and MS

strains were introduced individually in each test concentration. Larval food was given for *Bs* treated larvae, but it was not given for *Bti*- treated larvae as recommended by WHO. Because, the toxic component of crystals of *Bti* during mode of action was more serious (within 24 hours) than the *Bs* toxin. The test concentrations were replicated twice in each experiment. The experiments were repeated three times on different days. The experiment larvae were held at a room temperature (31°C) and larval mortality was assessed 24 and 48 hours after *Bti* and *Bs* treatment. At each test concentration, three trials were made and each trial consisted of two replicates. The larval mortality was scored after 24 and 48 hours for *Bti* and *Bs* respectively. If the mortality in control larvae was between 5 - 20 per cent, Abott's formula (Abott, 1925) was used to correct the mortality with experimental larvae as given below:

$$\text{Corrected control mortality} = \frac{\text{Percent test mortality} - \text{Percent control mortality}}{100 - \text{Percent control mortality}} \times 100$$

Moribund larvae if any, were counted as dead. The software package 'ASSAY' (courtesy of Dr. C.F. Curtis, London School of Tropical Medicine and Hygiene, U.K) were used for dosage mortality regression analysis. Resistance ratio (RR) at LC_{50} and LC_{90} levels were calculated by Robertson and Preisler (1992) :

$$\text{Resistance ratio} = \frac{LC_{50}/LC_{90}/\text{from } Bs\text{-resistant strain}}{LC_{50}/LC_{90}/\text{from } Bs\text{-susceptible strain}}$$

RESULTS AND DISCUSSION

In the present study, the toxic activity of *Bti* against *Bs* resistant and susceptible larvae of *C. quinquefasciatus* were studied. Table 1 represents the Probit regression analysis on resistance ratio (RR) between resistant (GR) and susceptible (MS) strains by exposing the larval strains with *Bs* or *Bti* toxin individually. As shown in the Table, in *Bs* 2362 (SPH-88) treated

larvae, the LC_{50} and LC_{90} values in MS strain were 7.15 and 30.01 mg / l, respectively. Whereas, the LC_{50} and LC_{90} values in GR strain were found to be 1782.04 and 6765.97mg / l, respectively. The resistance ratio at LC_{50} and LC_{90} levels were 249.2 and 225.5 folds, respectively. Thus, the results indicated clearly that resistance was found to be high in *C. quinquefasciatus* larvae, when subjected to selection pressure with *Bs* - toxin. Interestingly, the resistant strain GR was previously collected in the field (Gandhinagar, Kochi, Kerala) where, resistance at a high level (>6000 folds) was reported (Rao *et al.*, 1995; Poopathi *et al.*, 1999 a,b,c,d). The variations in resistance ratio were seen among lethal concentrations and it is expected that there may be variations in RR, since the mortality of larvae between the test-concentrations were high in the GR strain than the MS strain. However, statistically, no significant difference in resistance ratio was observed in all lethal concentrations (LC_{50} and LC_{90}).

Table 1 also represents a similar Probit regression analysis on resistance ratio between GR and MS strains by exposing the larval strain with *Bs* B42 (H5a5b) toxin. Here also, the LC_{50} and LC_{90} levels in MS strain were 0.24 and 1.23 mg / l, respectively and also it was found to be very low. Whereas, these lethal concentrations in GR strain were found to be moderately high in the levels of 165.04 and 582.34 mg / l, respectively. The RR at LC_{50} and LC_{90} were 687.7 and 473.4 folds, respectively. There is a strong correlation between the bacterial toxin and cross-resistance in *C. quinquefasciatus*. Rodcharoen and Mulla (1996) have reported cross-resistance to some *Bs* strains in Californian strain of *C. quinquefasciatus*. We have reported recently that the Indian strain of *C. quinquefasciatus* developed cross-resistance to *Bs* 2397, *Bs* 2362 and *Bs* IAB59 (Poopathi *et al.*, 1999 b). Bioassay results with *Bti* H14 (VectoBac®) against *Bs* susceptible and resistance larvae were also mentioned in Table 1, where the LC_{50} and LC_{90} levels in *Bs*-susceptible strain were 0.12 and 0.425 mg / l, respectively. Similarly, in *Bs*- resistance

Table 1. Toxicity of *B. sphaericus* 2362 (SPH-88)[a] and *B. thuringiensis* serovar *israelensis* H14[b] strains against
C. quinquefasciatus larvae

Bacterial strain	Mosquito strains	Intercept	Slope ± SE	LC_{50} 24 / 48h (mg / l) (95% FL)	LC_{90} 24 / 48h (mg / l) (95% FL)	χ^2 (df)	RR (at LC_{50})	RR (at LC_{50})
Bacillus sphaericus 2362 (SPH-88)	MS[c]	GR[d]	3.24 ±0.29	7.151 (8.209 - 6.229)[e]	30.01 (38.86-23.18)[e]	3.09 (4)	- -	- -
	GR[d]	2.19	2.21 ±0.29	1782.04 (20297.7-1564.6)	6765.97 (8528.9-5367.4)	4.66 (4)	249.2 (325.8- 190.6)	225.5 (367.9- 138.1)
Bacillus sphaericus B42 (H5a5b)	MS	6.12	1.8 ±0.29	0.24 (0.28-0.20)	1.23 (1.64-0.93)	5.1 (4)	- -	- -
	GR	0.19	2.34 ±0.38	165.04 (188.61-144.42)	582.34 (728.27-465.65)	272(3)	687.7 (943.05- 515.8)	473.4 (783.1- 283.8)
Bacillus thuringiensis serovar *israelensis* H14 VectoBac®	MS	7.12	2.27 ±0.35	0.116 (0.132-0.101)	0.425 (0.549-0.329)	0.60 (3)	- -	- -
	GR	7.16	2.38 ±0.37	0.123 (0.14-0.11)	0.427 (0.547-0.33)	1.15 (3)	1.06 (1.39- 0.83)	1.005 (1.66- 0.61)

[a] *Bacillus sphaericus* 2362 (SPH-88) = 1500 International toxic units / mg *Bs* toxin

[b] *Bacillus thuringiensis* serovar *israelensis* H14 = 1700 International toxic units / mg *Bti* toxin

Mosquito strains: MS[c] = Madurai susceptible strain; GR[d] = Gandhinagar resistant strain

[e] 95% f iducial limits of upper and lower at different lethal concentration levels

strain, the susceptibility levels in these lethal concentrations were 0.123 and 0.427 mg / 1, respectively. So, we did not come across any significant difference in the susceptibility levels between *Bs*-resistant and susceptible larvae (RR=1.005 to 1.06 folds), indicating that *Bti* toxin plays a major role to produce more mortality in *Bs* - resistant larvae. It is obvious that *Bti* contain multiple-toxin, so they interacts and produces a complex effect against mosquito larvae. This suggestion is in agreement with the findings of our earlier studies (Poopathi *et al.*, 1997, 1999c), which showed that neem based biopesticide helped to manage microbial resistance in mosquitoes. Results elsewhere have demonstrated (Regev *et al.*, 1996) similar observation in other insect also. The mechanism behind the management of resistance is not known. We suspect that these toxin may rejuvenate the *Bs* receptors in the midgut cells of *Bs* resistance larvae and render them more susceptible to the bacterial toxin or may directly involved for breaking major biochemical mechanism in the gut of mosquito to produce more mortality. It has been suggested that mixtures of functionally diverse toxins might be more effective than single toxin and might also delay evolution of resistance in target insects (Van Rie *et al.*, 1990; Tabashnik *et al.*, 1991). This suggestion strengthen our findings in the present study that *Bti* based biopesticide can also be used for the management of *Bs* resistant mosquito control operations.

ACKNOWLEDGEMENTS

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