CROSS-RESISTANCE TO *BACILLUS SPHAERICUS* STRAINS IN *CULEX QUINQUEFASCIATUS* RESISTANT TO *B. SPHAERICUS* 1593M

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Abstract. *Bacillus sphaericus* 1593M resistant larvae of *Culex quinquefasciatus* were reared in the laboratory since 1995. Resistance in the larvae was monitored by subjecting selection pressure using *B. sphaericus* 1593M at every generation. Bioassays were conducted with different strains of *B. sphaericus* (*Bs* 2297, *Bs* 2362 and *Bs* IAB 59) and confirmed cross-resistance in the present study. The level ranged between 27.3 to 18.2 fold in comparison with susceptible larvae. But *Bacillus thuringiensis* var *israelensis* strains (*Bti* PG14 and *Bti* 426) did not show any cross-resistance in the larvae and it emphasized a need to study the mode of action of *B. sphaericus* toxin that induces cross-resistance in the larval strain.

INTRODUCTION

Crystal toxin from different serotypes of microbial larvicides like *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis* var *israelensis* (*Bti*) exhibit a high larvicidal activity against mosquito larvae. The binary toxin (42 and 51 kDa proteins) of *Bs* and the multiple toxin (27, 65, 128 and 135 kDa proteins) of *Bti* are the most important toxins that interact and produce complex effects to kill the larvae (Wu and Chang, 1985; Federici *et al,* 1990; Broadwell *et al,* 1990; Poncet *et al,* 1995). The mode of action of these bacterial toxins is different from that of synthetic insecticides. The sequence involves (i) ingestion of spore toxins (ii) toxin dissolution in the midgut (iii) activation of protoxin by protease into active toxins (42 and 51 kDa of *B. sphaericus* into 39 and 43 kDa proteins) (iv) binding of the active toxin with specific binding receptors in the midgut brush border membrane (MBBM) (v) internalization and excretion of the toxin and cell lysis (Broadwell and Baumann, 1987; Davidson, 1988; Baumann *et al,* 1991; Porter *et al,* 1993). Therefore, the high efficacy of *B. sphaericus* and *B. thuringiensis* var *israelensis* strains is unique.

Recent reports point out development of high level resistance to the binary toxins of *B. sphaericus* and low or no resistance to the multiple toxins of *B. thuringiensis* var *israelensis* (Georghiou *et al,* 1983, 1992; Goldman *et al,* 1986; Rodcharoen and Mulla, 1994; Rao *et al,* 1995; Silva and Regis, 1997; Wirth and Georghiou, 1997). Development of cross-resistance by a Californian strain of *Cx. quinquefasciatus* to *B. sphaericus* strains has also been recently reported (Rodcharoen and Mulla, 1996). We found recently that *B. sphaericus* 1593M resistant strain of *Cx. quinquefasciatus* larvae displayed a low tolerance to *B. thuringiensis* var *israelensis* H14 (IPS-82) strain (Poopathi, unpublished results). We have also observed on management of microbial resistance in mosquito larvae that *B. sphaericus* in combination with a neem based biopesticide (Neemtox®, 0.03% azadirachtin) acts synergistically and inflicts higher mortality of the larvae of *Cx. quinquefasciatus* resistant to *B. sphaericus* 1593M (Poopathi *et al,* 1997). In the present study, we have evaluated cross-resistance to different strains of *B. sphaericus* in *Cx. quinquefasciatus* larvae resistant to *B. sphaericus* 1593M spore toxin.

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MATERIALS AND METHODS

Background

A *Culex quinquefasciatus* control trial was carried out in an area of 8 km^2 in Gandhinagar (Kochi, Kerala, South India) with a formulation of *B. sphaericus* 1593M based Biocide-S (produced by Center for Biotechnology, Anna University, Chennai) over a period of two years. Good control of breeding was achieved during the first year of control and in the next year, satisfactory control was not obtained despite good coverage of biolarvicide spraying (Mani, 1992). It was therefore suspected that the poor results could be due to the development of resistance in the field. Samples of larvae collected from the treated area were transported to the laboratory, confirmed a high level resistance and colonized (Rao *et al*, 1995). The resistant strain in the laboratory was maintained by subjecting to moderate selection pressure with *B. sphaericus* at each generation and maintained as field - collected selected line (Gandhinagar resistant strain, GR). This strain was subjected selection pressure continuously for the last five years in the laboratory. Besides *B. sphaericus* susceptible larvae collected from Madurai urban area (KK Nagar, S Madurai) where no biocide was sprayed earlier was also reared in the laboratory as Madurai susceptible strain (MS).

Mosquito colonies

Larvae of *Cx. quinquefasciatus* strains (GR and MS) were reared in the laboratory at ambient laboratory temperature (29-31°C) in enamel trays providing yeast and dog biscuit in the ratio of 40 : 60 in water as nutrient source. The pupae were allowed to emerge in cages and the adults were sexed. Females were provided with blood meal from live chicken and males with raisin and 5 to 10% glucose solution through cotton pads. The adults were allowed to oviposit in water in enamel cups kept inside emergence cages. The freshly emerged larvae from egg rafts of both strains were individually cultured for next generation. The Gandhinagar resistant (GR) strain of *Cx. quinquefasciatus* was subjected to selection pressure by *B. sphaericus* 1593M during each generation. The early third instar were treated at a concentration to yield 50 % mortality (LC_{50}) in 48 hours and the surviving larvae were reared to the next generation. This type of selective breeding was continued for the maintenance of resistance to *B. sphaericus*.

Bioassays

Three *B. sphaericus* strains (*Bs* 2297, *Bs* 2362 and *Bs* IAB 59) other than the selection pressure subjected strain (*Bs* 1593M) and two *B. thuringiensis* var *israelensis* strains (*Bti* PG14 and *Bti* 426) were cultured in appropriate growth medium and formulated (see Poopathi, 1995). Bioassay tests were conducted in disposable paper cups (200 ml capacity). To 150ml of water, appropriate volume of *Bs* or *Bti* sample was added to obtain the desired concentration of the toxin in the medium as recommended by WHO (1981, 1985). Twenty-five freshly moulted third and fourth instar GR larvae (for *Bs* and *Bti* toxin respectively) belonging to 34th and 35th generation of selection for resistance to *B. sphaericus* 1593M were exposed to the test media and mortality was observed for 24 hours in the larvae exposed to *Bti* and for 48 hours in those exposed to *Bs*. Bioassays were repeated at the selected concentrations for five times and duplicates were maintained for each concentration. Larvae exposed to water served as controls. Considering the mortality, the critical lethal concentrations (LC_{50}, LC_{90} and LC_{95}) for *Bs* and *Bti* toxin were calculated by using software package 'ASSAY' (provided by Dr CF Curtis, London School of Tropical Medicine and Hygiene, UK). Resistance ratios (RR) at the LC_{50}, LC_{90} and LC_{95} levels were calculated by the method of Robertson and Preisler (1992).

RESULTS AND DISCUSSION

Table 1 provides data on cross-resistance to *B. sphaericus* of *Cx. quinquefasciatus* larvae selected for resistance to *B. sphaericus* 1593M toxin. All three *B. sphaericus* strains mentioned in the present study (*Bs* 2297, *Bs* 2362 and *Bs* IAB 59) have induced significant cross-resistance to *B. sphaericus* 1593M resistant larvae. These bacterial strains have indicated a cross-resistance range at LC_{50}, LC_{90} and LC_{95} levels from 9.6 to 27.3, 4.7 to 23.0 and from 3.9 to 24.4 fold, respectively. The *B. sphaericus* 2297 had the highest cross-resistance at LC_{50} level by 27.3 fold. Followed by this, *B. sphaericus* 2362 occupied highest cross-resistance at LC_{90} and LC_{95} levels by registering 23.0 and 24.4 fold, whereas the third strain of *B. sphaericus* IAB 59 occupied a lower cross-resistance from LC_{50} to LC_{95} levels, registering 9.6 to 3.9 fold. The results signal caution to find alternate measures to overcome cross-resistance in mosquito control operations. This observation is identical to a report on cross-resistance to *B. sphaericus* of the Californian strain of *Cx. quinquefasciatus* (Rodcharoen and Mulla, 1997). As indicated earlier in the result, the *Bti* formulations (*Bti* PG14 and *Bti* 426) did not show any cross-resistance or tolerance to *B. sphaericus* 1593M strain in resistant larvae. This cross-resistance level was negligible, ranging from 0.8 to 1.9 fold only at LC_{50} to LC_{95} levels. This variation may be attributed due to biological differences between the strains of mosquitos tested or experimental errors as suggested by Rodcharoen and Mulla, (1996) in their study. It is worthwhile to point out here that so far no report is available about cross-resistance by *B. thuringiensis* var *israelensis* strains in *B. sphaericus* resistant mosquito larvae. However, a lowest level of (2 to 3

Table 1

Cross-resistance to *Bacillus sphaericus* strains in *Culex quinquefasciatus* selected for resistance to *B. sphaericus* 1593M.

Strains	Intercept	Slope±SE	LC_{50} (mg/l)	LC_{90} (mg/l)	LC_{95} (mg/l)	X^2 (df)	RR (at LC_{50})[f]	RR (at LC_{90})[f]	RR (at LC_{95})[f]
B. sphaericus 1593M	6.86[a]	1.64±0.4	0.073 (0.122-0.044)[c]	0.442 (1.157-0.169)[d]	0.736 (2.396-0.226)[e]	19.65 (4)			
	13.99[b]	4.25±1.8	0.0076 (0.014-0.004)	0.015 (0.042-0.005)	0.0187 (0.053-0.006)	16.78 (2)	9.6 (8.7-11)	29.5 (27.5-33.8)	39.4 (45.2-37.7)
B. sphaerjcus 2297	6.631	1.64±0.5	0.101 (0.252-0.04)	0.612 (1.726-0.065)	1.02 (2.29-0.068)	44.18 (4)			
	8.430	1.402±0.3	0.0037 (0.0086-0.0016)	0.031 (0.148-0.006)	0.056 (0.399-0.007)	38.01 (4)	27.3 (29.3-2.5)	19.7 (38.7-10.8)	18.2 (38.3-9.7)
B. sphaericus 2362	6.11	1.66±0.7	0.213 (0.309-0.075)	1.267 (1.961-0.073)	2.099 (3.169-0.063)	30.81 (3)			
	8.65	1.89±0.3	0.0116 (0.013- 0.01)	0.055 (0.072-0.043)	0.086 (0.119-0.05)	6.85 (4)	18.4 (23.1-7.5)	23.0 (27.2-1.7)	24.4 (26.6-1.26)
B. sphaericus IAB 59	6.808	3.9±1.8	0.344 (0.804-0.147)	0.733 (3.962-0.136)	0.909 (6.714-0.123)	24.48 (2)			
	7.908	2.01±0.4	0.036 (0.065-0.019)	0.156 (0.414-0.058)	0.236 (0.769-0.072)	35.24 (4)	9.6 (12.4-7.7)	4.7 (9.6-2.3)	3.9 (8.7-1.7)
B. thuringiensis var *israelensis* PG14	9.65	1.66±0.5	0.0016 (0.004-0.0007)	0.0095 (0.061-0.002)	0.016 (0.157-0.002)	30.74 (3)			
	12.34	2.77±05	0.002 (0.0025-0.0019)	0.0065 (0.018-0.005)	0.0088 (0.04-0.0067)	5.15 (3)	0.8 (1.6-0.4)	1.5 (3.4-0.3)	1.8 (3.9-0.23)
B. thuringiensis var *isaelensis* 426	9.5	2.37±0.6	0.013 (0.019-0.008)	0.044 (0.088-0.022)	0.063 (0.145-0.027)	12.22 (3)			
	10.39	2.56±07	0.0078 (0.015-0.004)	0.025 (0.094-0.0065)	0.034 (0.17-0.0069)	9.68 (2)	1.7 (2.0-1.2)	1.8 (2.5-0.4)	1.9 (2.9-0.5)

[a] Gandhinagar resistant strain (GR); [b] Madurai susceptible strain (MS)

[c, d, e] 95% Fiducial limits of upper and lower at LC_{50} , LC_{90} and LC_{95} l evels

[f] Resistance ratio = Experimental values (GR) ÷ Control values (MS)

fold) tolerance was noticed by above authors. Similarly, a low level resistance (2 and 11 fold) was observed in *B. thuringiensis* var *israelensis* strains against *Aedes aegypti* and *Cx. quinquefasciatus* larvae (Georghiou *et al,* 1983; Goldman *et al,* 1986). Interestingly in the present study, the original strain that was used for selection pressure (*B. sphaericus* 1593M) did not show any marked resistance in GR strain in the test generation studied (34th and 35th generation). The resistance ratio observed was 9.6 fold at LC_{50}, 29.5 fold at LC_{90} and 39.4 fold at LC_{95} levels. However, it was reported earlier that the very same GR strain after exposure to selection by *B. sphaericus* 1593M had developed a high level resistance (2,556 and 853.7 fold at LC_{50} and LC_{90}) in the 7th generation (Rao *et al,* 1995). In this context it is worthwhile to point out here that *B. sphaericus* resistance in *Cx. quinquefasciatus* is encoded by a single major recessive gene on linkage group I at 22.1 recombination units from the sex locus. Thus we assume *B. sphaericus* resistance differed from highest level to lowest level (Nielsen-LeRoux *et al,* 1997). Hence, in the present study it is assumed that resistant variation from 7th generation to 34th and 35th generation may be due to random seggregation of recessive genes that cause resistance in the larval population by subjecting them to selection pressure for the last five years.

Bacterial toxins, after being activated, become internalized in the midgut epithelium of the host through the toxin binding receptors in the midgut brush border and cause perforations in the gut (Davidson, 1988; Baumann *et al,* 1991; Nielsen-LeRoux and Charles, 1992; Porter *et al,* 1993). In resistant larvae, loss of toxin receptors in the midgut brush border membrane (MBBM) confers resistance to the toxin (Nielsen-LeRoux *et al,* 1995). For instance, the binary toxin of *B. sphaericus* 1593 failed to bind to the midgut brush border membrane of *Cx. quinquefasciatus* (due to loss of functional receptors) which was highly resistant to *B. sphaericus* 2362. Rodcharoen and Mulla (1996) have also suggested that cross-resistance to *Bs* 1593 and *Bs* 2297 in *Bs* 2362 resistant larvae might be due to partial alteration or reduction in toxin receptor sites and binding affinities. In the present study, we point out that the cross-resistance to *Bs* strains (*Bs* 2297, *Bs* 2362 and *Bs* IAB 59) in *Cx. quinquefasciatus* larvae which are resistant to *Bs* 1593M strain is due to these factors. Further studies on mode of action of bacterial toxin through *in vitro* binding assays in MBBM of *B. sphaericus* resistant and susceptible *Cx. quinquefasciatus* larvae will be studied shortly.

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