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Larvicidal efficacy of various formulations of *Bacillus sphaericus* against the resistant strain of *Culex quinquefasciatus* (Diptera: Culicidae) from southern India

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Abstract. Use of *Bacillus sphaericus* Neide (*Bs*) as potential biolarvicide in developing countries is limited due to development of resistance by target mosquitoes. Efforts are taken to look for appropriate formulations or combination of *Bs* to prevent or delay resistance problem. Here, we report the efficacy of a formulated *Bs* product to kill *Bs* resistant *Culex quinquefasciatus* Say larvae. The laboratory reared resistance colony was maintained by subjecting selection pressure with *Bs* (2362) toxin. Bioassays were conducted with lyophilized, standard formulated and *Bs* formulated by us (all belong to strain 2362, serotype H5a5b) against *Bs* resistant and susceptible colonies. The *Bs* resistant larvae showed a high level of resistance against lyophilized toxin with resistance ratio (RR) of 8375.2, 1055.6 and 11422.3 folds at LC_{50}, LC_{90} and LC_{95} levels, respectively, when compared with *Bs* susceptible larvae. With formulation of standard powder, the RR between *Bs* resistant and susceptible larvae were 1.01, 1.13 and 1.19 folds only at LC_{50}, LC_{90} and LC_{95} levels, respectively. This observation was comparable with our formulation prepared by a ground mixture of lyophilized *Bs* and a placebo (plaster of Paris). It is evident from our study, that the placebo present in our *Bs* 2362 formulation was responsible for increasing the efficacy of *Bs* lyophilized toxin against resistant larvae. The putative mechanism behind this toxicity phenomenon remains to be investigated to evolve new mosquito control strategies. A cross resistance to indigenous strain of *Bs* B42 (H5a5b) against *Bs* resistant larvae was also reported in this study.

INTRODUCTION

Mosquito-borne diseases form a major component of communicable diseases, malaria, filariasis, dengue and Japanese encephalitis in India and in other developing countries. Several strategies have been adopted to control these diseases but perils of epidemics still loom large in most states in the countries. Vector control as an in-built component of the nation-wide disease control strategy has been the main plank so far; wherein synthetic insecticides have been effectively used during past several decades to control varied dipteran pests. However, the use of chemical insecticides has been greatly impeded due to development of physiological resistance in the vectors, environmental pollution resulting in bio-amplification of food chain contamination and harmful effects on beneficial non-target animals. Therefore, the need for more effective and environment-friendly control agents became urgent and biological agents seemed to be the most promising ones. The discovery of entomopathogenic bacterium like *Bacillus sphaericus* Neide (*Bs*) which is highly toxic to dipteran larvae, has opened up the possibility of their use as alternative biolarvicides in mosquito control

programmes, the world over (Kalfon *et al.*, 1983). This bacterium has some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organisms, including human beings. Also, it is innocuous to the environment (Das & Amalraj, 1997). The main larvicidal activity of *Bs* is due to the parasporal crystal proteins, binary toxins (Bin toxins), which are ingested by the insect larvae (Poopathi *et al.*, 2002). The crystal protein is composed of two polypeptides with molecular weights of about 51 kDa and 42 kDa proteins (Charles *et al.*, 1997). The crystal proteins are ingested by larvae and after solubilization and proteolytic cleavage, the activated toxin interacts with specific receptors on the apical membrane of midgut epithelial cells, leading to death of susceptible larvae (Poopathi *et al.*, 2002).

Though the high efficacy and specificity of *Bs* toxins are useful in controlling mosquitoes, the recent appearance of resistance in *Culex* mosquitoes has inhibited its large scale use in developing countries. (Rao *et al.*, 1995; Rodcharoen & Mulla, 1996; Poopathi *et al.*, 1999a,b). In view of these facts, therefore, identification of more suitable formulations of *Bs* toxin to prevent or delay resistance problem is necessary in mosquito control programmes. We have studied the larvicidal activity of *Bs* 2362 which was formulated by us, containing lyophilized *Bs* toxin + placebo, on *Bs* resistant and susceptible larvae. We observed that there was mortality of *Bs* resistant larvae similar to that of *Bs* susceptible larvae. This suggested, that, similar suitable formulations would be useful in the management of *Bs* resistance. Hence, we have undertaken a study to evaluate the efficacy of our formulation of *Bs* 2362 (serotype 5Ha5b) against larvae of *Culex quinquefasciatus* Say.

MATERIALS AND METHODS

Test Larvae

Third instar larvae of *Cx. quinquefasciatus* susceptible to *Bs* were used from a colony maintained for more than seven years at the Centre for Research in Medical Entomology, Madurai, India and this strain was named as, Madurai susceptible strain (MS). *Bs* 2362 resistant *Cx.quinquefasciatus* mosquitoes collected from the field (Gandhinagar, Kochi, South India), where resistance has been reported by this centre (Rao *et al.*, 1995; Poopathi *et al.*, 1999b,c) were also used. This resistant mosquito colony named as, Gandhinagar resistant strain, GR has been submitted to continuous selection pressure at every generation for more than seven years in the laboratory. Thousand late third instar larvae were treated at a concentration of 1g *Bs* 2362 lyophilized powder per litre in 3 litre capacity bowl to determine the mortality of larvae after 48 h exposure period. The surviving larvae from these experiments were pooled, rinsed in distilled water and reared to next generation. A total of three consecutive generations (F_{27} to F_{29}) were studied for each mosquito strain.

Both *Bs* resistant (GR) and susceptible (MS) mosquito colonies were reared in the laboratory at ambient laboratory temperature (29-31°C) as per the method described earlier (Poopathi & Tyagi, 2002).

Bacterial strains:

Lyophilized *Bacillus sphaericus* (*Bs*) 2362 SPH-88 (serotype: H5a5b): This bacterial strain already available in the laboratory was used for the present study. This strain was earlier received from Bacteries et Champignons Entomopathogenes, Institute Pasteur, Paris, France (courtesy: Dr. Jean-Francois Charles and Dr. Christina Nielsen-LeRoux). We have been culturing the bacteria in NYSM medium (Nutrient Yeast extract Salt Medium containing: glucose, 5 gm; peptone 5g; NaCl, 5 gm; beef extract, 3 gm; yeast extract, 0.5 gm; mineral solutions ($MgCl_2$ $CaCl_2$, $MnCl_2$) 10ml in 1 litre of double distilled water at pH 7.5). The culture was allowed to grow under constant agitation 9120rev/min) under room temperature (30°C) in an orbital shaker. As soon as the cultures were fully sporulated, the spore/crystal toxin complex was recovered by centrifugation (10,000g/30 min/4°C) using super speed centrifuge (Kendro, USA) and the spore/crystal free supernatants were

discarded. The spore/crystal mixtures were thoroughly washed three times each with 0.1 M NaCl and sterile double distilled water. Finally, the *Bs*-spore/crystal complex were washed with protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF, 1mM, Sigma), re-suspended in sterile water, freeze dried and the lyophilized powder was stored at – 20ºC until further use, for analysis and toxicity bioassays. Since, the present bacterial strain (*Bs* 2362) is the WHO standard powder, its potency (International Toxic Unit, ITU) has already been assigned (1500 ITU/mg). Nevertheless, the potency of cultured samples (ITU/mg) of bacteria from our laboratory has been verified at regular intervals by following the formula: LC_{50} (standard *Bs*-SPH88) X 1500 / LC_{50} (laboratory cultured *Bs*-SPH88). The activity of this lyophilized sample is appreciable for about one year under normal laboratory (30ºC) and two to three years under controlled temperatures (-20ºC).

Formulated *B.sphaericus* (*Bs*) Sphericide (serotype: H5a5b): This is a powdered formulated product based on highly potent strain of *Bs* (serotype H5a5b), supplied by Bio-Tech International Ltd, New Delhi (courtesy: Dr. Vivek Singhal). Its potency was already calculated about 1500 ITU/mg. The formulated product was stored under controlled temperature (-20ºC) until further use.

Bacillus sphaericus lyophilized powder + placebo: The lyophilized sample of *Bs* (serotype: H5a5b) already cultured in the laboratory, as mentioned above, was mixed with a placebo (plaster of Paris) at the ratio of 1:5. This formulated product of ours was bioassayed simultaneously along with above samples against *Bs*-resistant and susceptible strain of *Cx. quinquefasciatus*.

Bioassay and data analyses:

Titration and preparation of stock solution from these bacterial samples and bioassays were made as described in Anon (1985). In the present study, 1800 mg of *Bs* sample was homogenized in 600 mL de-ionized water and this was used as the stock solution. Aliquots of appropriate dilutions ranging from 2.22 to 0.001 mg / litre and from 1000 to 0.003 mg / litre were used for MS and GR strains respectively, to determine the susceptibility levels at 48 h. Bioassays were conducted in wax-coated, disposable polyethylene cups (200 ml). Test medium was prepared by adding appropriate volume from the stock solution into 150 ml of water. Twenty freshly moulted third instar MS and GR larval strains were introduced separately in each of the test concentrations. Following exposure for 48 h at room temperature with food provided, the larval mortality was recorded. The lethal concentration values (LC_{50} and LC_{90}) were estimated by probit regression analysis, using a software package 'ASSAY' (courtesy: Dr. C.F. Curtis, London School of Tropical Medicine and Hygiene, UK). The test concentrations were replicated in each experiment and were repeated three times. Moribund larvae, if any, were counted as dead. Larvae exposed to water served as control. Control larval mortality was scored after 48 h and corrected by adopting Abbott's (1925) formula as described by Poopathi & Tyagi (2002). Resistance ratios (RR) at lethal concentrations (LC_{50}, LC_{90}, and LC_{95}) were calculated as described earlier by Robertson & Preisler (1992).

RESULTS AND DISCUSSION

In the present study, the efficacy of *Bs* toxin in three different forms were evaluated for their larvicidal activity against *Bs* resistant and susceptible *Cx. quinquefasciatus* larvae. The results obtained from the experiments are presented in Table 1 as probit regression analysis on resistance ratio (RR) as described by Robertson & Preisler (1992). As shown in the table, when larvae were treated with lyophilized *Bs* 2362, the LC_{50}, LC_{90} and LC_{95} values in *Bs* susceptible (MS) larvae were 0.087, 0.26 and 0.35 mg/L , respectively, whereas, the LC values for *Bs* resistant (GR) larvae were found to be very high at the levels of 728.6, 2,744.7 and 3,997.8 mg /L, respectively. The RR between GR and MS larval strains were 8,375.2, 10,556.6 and 11,422.3 folds, respectively. Thus, the results clearly indicate that resistance was found to be very high in *Cx. quinquefasciatus* larvae,

when subjected to selection pressure with *Bs* toxin. This resistant strain was formerly collected from the field (Gandhinagar, Kochi, South India) where, resistance at a high level was reported at 6,223 and 31,325 folds at LC_{50}, and LC_{90} levels, respectively. (Rao *et al.*, 1995). Variations in the resistance ratios were seen among lethal concentration levels and it is expected that there may be variations in RR, since, the percentage mortality of larvae between test concentrations were high in GR strain than in MS strain (data not shown). However, it is evident from Table 1, the RR at different lethal concentration levels have shown no significant variation (P>0.05), since, fiducial limits were overlapping.

The table also represents a similar probit regression analysis on resistance ratio between GR and MS strains by exposing the larval strain with a standard formulated powder (*Bs* 2362, H5a5b). Here, the LC_{50}, LC_{90}, and LC_{95} values in *Bs* - susceptible (MS) strain were 0.088, 0.24 and 0.31 mg/L respectively. Statistically, no significant difference on the toxicity level was found between lyophilized and formulated sample when exposed to *Bs* susceptible larvae. But, interestingly, this observation was in contrast with *Bs* resistant larvae when subjected to formulated sample. In this case, the toxicity levels were only 0.093, 0.27 and 0.37 mg/L at LC_{50}, LC_{90} and LC_{95} levels, respectively. So, we did not find any resistance in the larvae since the RR was close to 1. These negligible RR may be due to biological variations or experimental errors and not a real development of resistance as described earlier by Rodcharoen & Mulla (1996) in their study.

Bioassays were also carried out to test the efficacy of *Bs* lyophilized sample with plaster of Paris mixed in 1:5 ratio against MS and GR strains to compare the results with the standard formulations. The LC_{50}, LC_{90}, and LC_{95} values for MS strain were 0.71, 4.67 and 7.97 mg/L respectively and this did not differ significantly (P>0.05) from GR strain which ranged from 0.70 to 9.12 mg/L in all lethal concentration levels. The RR between GR and MS strains were 1.01, 1.11 and 1.14 folds only at LC_{50}, LC_{90}, and LC_{95} levels, respectively. This observation is similar as seen in standard formulation treated against GR and MS strains.

In another study, we did find cross-resistance to indigenous isolate of *Bs* B42 (H5a5b) strain against *Cx. quinquefasciatus* resistant to *Bs* 2,362 with RR at LC_{50}, LC_{90} and LC_{95} were 593.8, 540.4 and 525.3 folds, respectively (Table 1).

Studies elsewhere have reported (Rodcharoen & Mulla, 1994, 1996; Rao *et al.*, 1995) a high level of resistance to *Bs* toxin in *Culex* mosquitoes. Poopathi *et al.* (1999a,b) have also reported cross-resistance to different strains of *Bs* and *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) in *Cx. quinquefasciatus*. In the present study, we examined the toxic effect of formulated product of ours (*Bs* 2362) in comparison with lyophilized *Bs***.** We found that resistance was very high in larvae exposed to lyophilized *Bs***.** However, no resistance was found in these resistant larvae when exposed to formulated toxin of *Bs* 2362. Experiments were repeated several times and we obtained similar results.

The highest larvicidal activity on *Bs* resistant larvae may be contributed by the ingredients present in the formulation of *Bs* 2362, which may be responsible for killing *Bs* resistant larvae. This phenomenon was not seen with these larvae when exposed to unformulated *Bs* toxin. We also did experiments with placebo alone as control agent against these larval strains (GR and MS), but we did not find any mortality of larvae. However, comparative larvicidal effect of *Bs* lyophilized, formulated and formulated product of our own preparation on *Bs* resistant larvae has shown that the larvicidal activity may have been enhanced due to ingredients present in the formulations. In addition, there is no significant difference in larvicidal activity of these two (lyophilized and formulated sample) on *Bs* susceptible larvae, except, our own formulation and this could be due to variation in the formulation ratios. But the relative toxicity level between GR and MS strain was comparable. It is known that selection of the most potent biopesticide formulation is based on the suitable

combination ratios from active and inert ingredients (Yap, 1990). Pesticides are rarely used in their pure or technical form. Usually, the technical grade material (active ingredients) is mixed with various non-insecticidal ingredients to create a pesticide formulation. These inert ingredients serve a variety of functions. They may be combined with active ingredient to enhance stability, reduce toxicity, improve efficacy or facilitate handling of product (Yap, 1990). In the present study, *Bs* formulations have exerted highest larvicidal activity against *Bs* resistant larvae in combination with placebo. This observation strongly suggests that the standard formulation (like placebo) may increase the efficacy of *Bs* toxin for killing mosquitoes.

The mode of action of *Bs* toxin has been elaborately studied and found that the bacterial toxins after being activated, get internalized in the gut epithelium of the host through the toxin binding receptors in the midgut brush border membrane (MBBM) and cause perforations in the gut of *Bs* susceptible larvae (Davidson, 1988; Baumann *et al.*, 1991; Nielsen-LeRoux & Charles, 1992; Porter *et al.*, 1993; Charles *et al.*, 1996; Poopathi *et al.*, 2002). The ultrastructural studies did not show any differences in the *Bs* resistant and susceptible larvae before and after exposure to *Bs* toxin (Poopathi *et al.*, 2000). So, the putative mechanism behind the larvicidal activity of standard and placebo formulated product on *Bs* resistant larvae remain to be explored. However, results from our study strongly suggests that suitable formulations with *Bs* toxin can be used in mosquito control operations even in areas where there were reports of *Bs* resistance in *Cx. quinquefasciatus*.

The indigenous strain of *Bs* B42 (H5a5b) has shown cross-resistance in *Bs* 2362 resistant larvae of *Cx. quinquefasciatus* in this study. Earlier, we found similar observations on cross-resistance to three different bacterial strains (*Bs* 2397, *Bs* 2362, *Bs* IAB59) against *Bs* 1593M resistant strain of *Cx. quinquefasciatus* (Poopathi, *et al.*, 1999a). It is our assumption that cross-resistance to *Bs*-strain is continued to be seen in *Cx. quinquefasciatus* larvae to *Bs* toxin. In this connection, it is worthwhile to mention here that the toxicity levels in mosquitoes were diverse among different bacterial strains, serotype and even with in the same serotype (de Barjac *et al.*, 1988; Thiery & de Barjac, 1989; Thiery *et al.*, 1992; Nicolas *et al.*, 1992). As a result, cross resistance to bacterial strain is unavoidable, provided suitable formulations are developed in mosquito control operations.

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