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RESEARCH ARTICLE

INFLUENCE OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* ON MOSQUITOCIDAL, LONGEVITY AND FECUNDITY OF MALARIAL VECTOR, *ANOPHELES STEPHENSI* LISTON (INSECTA: DIPTERA: CULICIDAE)

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ABSTRACT

Mosquitoes are vectors of many human diseases and cause environmental nuisances. Due to their large geographical distribution and abundance, *Anopheles stephensi* represent the most important mosquito species in India. The management of these disease vectors using conventional pesticides has failed because of the high reproductive ability, development of insecticide resistance of mosquito species and environmental pollution. These reasons are leading to a search for novel molecules. As so the *Bacillus thuringiensis* var. *israelensis* (*Bti*) provide effective alternatives to broad spectrum larvicides in many situations with little or no environmental impact. Taking into account environmental benefits including safety for humans and other non-target organisms, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in aquatic ecosystems, their advantages are numerous. Bioassay was conducted to test the larval, pupal, and adult toxicity, longevity and mosquito fecundity. The mosquitocidal activity of *Bti* was tested at different concentrations, ranging between 10 to 40 ppm, against the different larval stages (1st instar to 4th instar) of *Anopheles stephensi*. Bioassays were done on newly moulted larvae under laboratory conditions. The technical material showed a high level of activity with mortality recorded for treated and following stages and happened after incomplete development. For the same treated series a significant decrease was also recorded in the longevity of the adult. In other experiments the compound was applied against the fourth instars larvae and its effects was investigated on fecundity of female emerged from larval treated series. The results showed that *Bti* reduced significantly the laying egg number, egg hatchability and the percentage of fecundity.

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INTRODUCTION

Mosquito control is a major public health concern, as mosquitoes transmit many severe human diseases such as malaria, filariasis, dengue, yellow fever, West Nile virus and the chikungunya virus. Malaria (Italian mala aria, bad air) is a protozoan (genus Plasmodium) infection transmitted by mosquitoes of the genus Anopheles. The four species of Plasmodium that infect humans appear to have evolved from a common ancestor during the early Tertiary period, some 60 million years ago (Garnham, 1963).

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Malaria is the world's most important parasitic infection, even with years of continual efforts, malaria is still one of the major causes of morbidity and mortality affecting third-world countries and still a threat to over 2 billion people, representing approximately 40% of the world's population in about 100 countries. Best estimates currently describe the annual global burden of malaria as 300-500 million caws and 1-2 million deaths (Na-Bangchang and Congpuong, 2007). These diseases represent a major health threat and economic burden in disease-endemic countries, and are currently in expansion due to increased worldwide exchanges, urbanization, and global warming. The only effective way of reducing the incidence of these diseases is to control the vector mosquitoes, mainly by application of insecticides to their breeding places. Mosquitoes have primarily been controlled with different kinds of chemical pesticides. A large number of these chemicals had shown adverse effects on the environment including their long-

term persistence, impact on non-target organisms in the aquatic food chain, biomagnifications and emergence of resistance (Chandre *et al*., 1999) in vector mosquitoes. Microbial larvicides (*Bacillus thuringiensis* var *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bsph*) are processed bacterial compounds which are registered for mosquito larvae control agents in outdoor areas. There are different commercial formulations of these two larvicides, ranging from emulsifiable liquids to granular pellets, available in different countries of the world (Pontes *et al.,* 2010). These biological insecticides have had a decimating effect on the use of chemical pesticides against mosquitoes during past 2-3 decades. *Bti* is highly pathogenic against *Culicidae* (mosquitoes) and *Simuliidae* (blackflies), and has some virulence against certain others Diptera, especially Chironomidae (midges). Mosquito have four distinct stages in their life cycle: egg, larva, pupa and adult. Depending on the specie a female lays between 30 and 300 eggs at a time on the surface of the water, singly (*Anopheles*), in floating rafts (*Culex*) or just above the water line or on wet mud (*Aedes*). Once hatched the larvae grow in four different stages (instars).

The first instar measures 1.5 mm in length, the fourth instar about 8-10 mm. The fully grown larvae then changes into a comma shaped pupa. When mature, the pupal skin splits at one end and a fully developed adult emerges. The entire period from egg to adult takes about 7-13 days under good conditions (Wada, 1989). First instar is more suceptible to *Bti* than fourth instar (Mulla *et al.* 1990). Pupa does not feed and therefore is not affected by *Bti*. For almost all species tested, increasing age of the larvae resulted in reduced susceptibility in mosquito (Chen et al. 1984: Mulla *et al.* 1985). *Bti* was found to be specific toxic to larvae of 109 mosquito species. *Bacillus thuringiensis* subsp. *israelensis* (Bti), serotype H14, is a subspecies of the diversified *Bacillus thuringiensis* species, an entomopathogenic bacterium able to survive in the environment as a spore and producing insecticidal toxins within an inclusion body during the process of sporulation. The different subspecies are characterized by different flagellar H antigens (serotypes). However, the specificity to a given group of insects is a consequence of the particular set of proteins a strain is producing and there is thus no strict correlation between serotypes, the toxins they produce during sporulation as insecticidal crystal inclusions and the host range.

Like other Bt toxins the mode of action of the Bti toxins is closely related to specific structure-function relationships. One particular feature of *Bti* is that its insecticidal activity relies on the combination of three distinct groups of toxins with respect to structure-function and thus specific mode of action, i.e. Cry4Aa+Cry4Ba, Cry11A and Cyt1Aa. This also shows at the level of the inclusion body which is a composite entity comprising three different crystal component, one for each the three groups mentioned above. Indeed, each group folds and accumulates separately into a specific sub-inclusion body of different shape assembled into a spherical parasporal body and held together by a lamellar envelope (Federici *et al*., 2003).
The crystal is ingested by larva, protoxins are solubilized in the alkaline midgut and activated to toxins that bind to specific membrane receptors, oligomerize and form a pore allowing the bacteria to proliferate in the host larva. Following binding, these proteins insert in the membrane to form a pore and more precisely an ionic channel triggering osmotic imbalance, cell death and ultimately insect death. However, important and not yet fully resolved steps are involved in this membrane insertion and permeation process. A first intermediate step seems to be oligomerization into a prepore structure with the probable involvement of membrane receptors. However, activated toxins are hydrosoluble intermediate forms which must undergo a conformational change to expose hydrophobic domains and insert stably into the membrane in order to form a transmembrane ionic channel. The two Cry4 toxins undergo this process in a way similar to that of the Cry1 or Cry3 proteins.

In general, *Bti* formulations were found more effective against larvae of *Aedes* and *Culex* species than *Anopheles* spp and among the two anopheline species tested in the laboratory, *An. stephensi* was more susceptible than *An. culicifacies* to different *Bti* formulations (Mittal, 2003). The larvicidal potentials of *Bti* have been recognized since 1977 (Goldberg and Margalit, 1977) and *Bti* has become mosquito control agents of choice almost throughout the world. Since 1977, biological control of mosquito was canied out by biolarvicides and till 2002 none of the biological control agents were reported to be mosquito pupicidal i.e. the ability to kill the pupal stages of mosquitoes. The first bacterium known to exhibit mosquito pupicidal activity is a gram-negative bacterium *Pseudomonasfluorescens.* The metabolites were toxic to larvae and pupae of mosquitoes (Prabakaran *et al*., 2003). This study aimed to determine the effect of *Bacillus thuringiensis* var. *Israelensis* as a mosquito larvicidal, pupicidal, adulticidal, biological and reproductive activity of *Anopheles stephensi* Liston.

MATERIALS AND METHODS

Preparation of *Bacillus thuringiensis* subsp. *Israelensis*

B.thuringiensis subsp. *israelensis* was obtained from Tuticorin Alkali Chemicals and Fertilizers Limited, Chennai, India. *B. thuringiensis*, 630 ITU/mg (a.i.) 5% w/w; total proteins (including the active ingredient 5% (*w/w*), 10% (*w/w*); fermentation solids, 10% (*w/w*); inert ingredient, 48% (*w/w*); non-ionic surfactant, 0.2 (*w/w*); food grade preservative, 0.3%; UV protectant, 0.1%; and water, 71.4% were used. Total 100% (*w/w*) was active specifically against mosquito larvae. The required quantity of *B. thuringiensis* was thoroughly mixed with distilled water and prepares various concentrations, ranging from 50 to 450, ppm respectively.

Mosquito culture

Mosquito larvae/eggs of *Anopheles stephensi* have been collected in an around Ooty. The mosquito colonies were maintained at 27 $\pm$ 2 ^{0}C, 75-85% relative humidity index a 14:10 light/dark photo period cycle (Murugan and Jeyabalan, 1999).

Larvicidal and Pupicidal assays

Larvae tested for the present study was obtained from our laboratory culture. Freshly hatched/moulted larvae were used

for the bioassay tests. The required quantity of different concentrations of *B.thuringiensis* subsp. *israelensis* were mixed thoroughly with 200 ml of rearing water in 500ml plastic troughs. One hundred early fourth instars mosquito larvae were released into each trough. Larvae food consisted of 1g of finely ground dog biscuits per day per trough. Dried coconut midribs were place over water as the substratum for pupation. The plastic trough containing 200 ml of rearing water with methanol served as the control. Dead larvae and pupae was removed and counted at 24 h intervals. Observations on larval and pupal mortality were recorded. The experiment was replicate five times. Percentage mortality observed in the control was subtracted from that observed in the treatments (Abbot, 1925). The day from moulting of the larvae to pupation and to adulthood was noted. Fecundity was assessed by counting the number of eggs laid during the life span by control and experimental mosquitoes. The larvae and pupal duration of treated and control individuals were compared and developmental rates were determined. The emergence day and mortality days of adults were recorded and the computed means gave mean longevity in days.

Adulticidal assay

Anopheles stephensi fresh adults were exposing to filter paper treated with different concentrations of *B.thuringiensis* subsp. *israelensis*. The paper was keep inside the beaker. Muslin cloth covering the beaker was also treated. Control insects was expose only to distilled water with methanol treated paper and muslin cloth. Mortality count was taken after 24h (Sharma *et al*., 1992).

Ovipositional Assay

Different concentrations of *B.thuringiensis* subsp. *israelensis* from a stock solution were mixed thoroughly with 200 ml of rearing food in 250 mil glass jars to obtain the concentration desired for the tests with *Anopheles stephensi*. The gravid females were give a choice between treated and control jars. During the tests, the groups of females were kept separate for 48h in cages measuring 25x 25x30cm. After the eggs were counted the oviposition activity index (OAI) was calculated using the formula:

$OAI = (Nc–Nt)/ (Nc+Nt) X 100$
Where Nc is the number of eggs in the control
Nt is the number of eggs in the treatment

Ovicidal assay

Anopheles stephensi eggs were released in water. The test extracts were added in desired quantities and hatching were observed for one week. The eggs were then exposed to deoxygenated water and the numbers of hatching eggs were recorded. Percentage hatching was compared with the control in which only distilled water with methanol was used (Sharma *et al*., 1992).

Statistical analysis

All data was subject to analysis of variance and the treatment mean was separated by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS

The larval and pupal mortality increased with increasing concentration of *Bti* (Table 1).

Table 1. Toxicity of *B.thuringiensis* subsp. *israelensis* on *A. stephensi*

Concentration (ppm)	Larval Mortality (%)	Pupal mortality (%)	Adult emergence (%)
Control	00[e]	00[e]	100[a]
10ppm	15[d]	10[d]	42[b]
20ppm	26[c]	21[c]	16[c]
30ppm	72[b]	40[b]	12[cd]
40ppm	98[a]	74[a]	8[d]

Within a column means followed by the same letters are not significantly different at 5% level by DMRT

The *Bti* was effective against both the larvae and pupae of *Anopheles stephensi*, yielding 98% and 74% mortality respectively at 40ppm. The efficacy of the *Bti* was readily demonstrated by a high initial mortality in larval population of the late instars. *Bti* was effective against early instars of *Anopheles stephensi* with a reduction of 96%-100% of the larval population within 24 hours. All the larvae reared in control successfully emerged. On the other hand exposure of the *A. stephensi* larva to sublethal doses of *Bti* inhibited the adult emergence. The adult emergence of *A. stephensii* after the treatment with *Bti* was only 8 percent at 40ppm. The larval and pupal duration of *Anopheles stephensi* treated with Bti were significantly extended compared to controls (Table 2).

Table 2. Biological parameters of *A. stephensi* after the treatment of *B.thuringiensis* subsp. *Israelensis*

Concentration (%)	Total Larval duaration (days)	Pupal duration (days)	Adult longevity (days)	Fecundity (no of eggs)
Control	8.0[d]	3.00[d]	70[a]	250[a]
10ppm	10.6[d]	6.1[d]	48[b]	102[b]
20ppm	14.6[c]	8.2[c]	35[c]	78[c]
30ppm	20.8[b]	10.4[b]	26[d]	52[d]
40ppm	22.4[a]	12.8[a]	16[e]	28[e]

Within a column means followed by the same letters are not significantly different at 5% level by DMRT

These results indicated that the treated larvae grew at a slower rate than the control larvae. The adult duration of *Anopheles stephensi* from the *Bti* treatment was significantly less than the control. It suggests that *Bti* had prolonged chronic effect. The total number of the eggs also significantly reduced after the treatment of *Bti*. Most larvae treated with *Bti* was arrested at the larval development stage, whereas most control larvae continued with the normal development to the 4th instar by eighth days. Treatment with *Bti* to the mosquito larvae significantly extended their total developmental duration. The duration from first instar to adult emergence for control was 11 days. Treatment of larvae with *Bti* (40ppm) extended the total developmental duration to 35.2 days. The ovipositional deterrency effect was significantly higher after the treatment of *Bti* to the *Anopheles stephensi* (Table 3). The data revealed a gradual decrease in egg hatchability due to treatment with *Bt*i (Table 3). The egg hatchability of *Anopheles stephensi* in control was 100 percent which markedly reduced (6 percent) after the treatment with *Bti* at 40ppm.

Table 3. Biological effect of *B.thuringiensis* subsp. *israelensis* on *A. stephensi*

Concentration (%)	Oviposional Deterrency (%)	Egg hatchability (%)
Control	00 [e]	100 [a]
10ppm	26 [d]	65 [b]
20ppm	55 [c]	53 [c]
30ppm	78 [b]	18 [d]
40ppm	98 [a]	6 [e]

Within a column means followed by the same letters are not significantly different at 5% level by DMRT

DISCUSSION

Bacillus thuringiensis, a gram positive bacteria produces a proteinaceous parasporal crystalline inclusion during sporulation. Upon ingestion by mosquitoes, the crystalline inclusion is solubilised in the midgut, releasing **δ-**endotoxins proteins (prototoxins). The protoxins are activated by midgut protease, and the activated toxins interact with larval midgut epithelium causing a disruption in membrane integrity, ultimately leading to insect death. *Bacillus thuringiensis var. Israelensis* (*Bti*) has been recognized as an efficient biolarvicide against many malaria vector species (Fillinger and Lindsay, 2006; Mwangangi *et al.,* 2011). In this study, larvae of *An. stephensi* were found almost highly susceptible to *Bti* formulations under laboratory conditions. Parallel to present study are findings of Jahan and Hussain (2011) who reported that 0.11 ppm of technical powder of *Bti* (VectoBac TP) containing 5000 ITU/mg caused 95% mortality in 3rd stage larvae of *An. stephensi* after 24 hours exposure. Rathor *et al.* (1985) in Pakistan using an aqueous suspension of *Bti* (ABG-6145) containing 587 ITU *Bti*/mg had reported complete mortality of the larvae of *An. stephensi* at 1 ppm dosage. Larvae of mosquitoes feed on small particulate mater in their breeding container upon ingestion, the crystal protein dissolves at the hight pH of the insect gut, proteolytic action releases toxix fragments the epithelial cells of the gut swell and lyses and death rapidly ensures.

At the molecular level, δ the model for toxicity proposed by Knowles and Ellar (1987) has a large experimental support. Processed toxins binds to a specific receptor (probably a glycoprotein) of the plasma membrances of susceptible cells in the midgut epithelium. Interestingly pathological changes in larvae following toxin ingestion, largely involve the midgut cells. Large vacuoles or cytolysosomes appear in the posterior midgut cells accompanying swelling of the gut. Eventually these cells separate from one another and slouch from the basement membrane (Charles and Nicolas, 1986). In cultural cells, there was very rapid swelling of the mitochondria christic and endoplasmic reticulum within 5 min of treatment (Davidson and Sweeny, 1933). The midgut environment also plays a crucial role in specificity, as shown with the activation of *Bti* subsp *Aizawai* (*Bta*) strain K1. When activated with lepidopteran *Pieris brassicae* midgut extract, the toxin kills both *P. brassicae* and dipteran *Aedes aegypti* lavae, when activated by *A. aegypti* midgut extract the isolate is toxic only to this mosquito larva (Haider and Ellar, 1989). The mosquito *A. stephensi* is susceptible to *Bti* formulations, with mortality ranging from 10 to 40ppm for third instar larvae. These results were similar to mosquitoes (Nayar *et al*. 1999) and other chironomids (Charbonneau *et al.,* 1994), with the susceptibility of older instar larvae to *Bti* declining as their age increases. Larval density can significantly affect the toxicity of *Bti* on mosquito larvae and chironomids (Nayar *et al*. 1999). Although the primary action of *Bt* toxins is in the midgut epithelium of sensitive insects, the investigators have only recently demonstrated that the toxin binds specifically to brush border membrane vesicles (BBMV) prepared from the midgut. The BBMV consists primarily of the apical brush border membrane of the midgut columnar cells. The BBMV studies show a positive correlation between the biological activity of the cry toxins and their ability to bind to BBMV of susceptible larvae (Hofman *et al*., 1988; Van Rie *et al*., 1989). The Cry toxins require a specific plasma membrance receptor on the midgut epithelial cells. The Cry toxins of mosquitocides Bt appear to act in a manner similar to that observed with other membrane disrupting toxins, some of which aggregate on cell membranes (Perker *et al*., 1989).

Ultra-structural effects have been reported in cultured cells of *A. stephensi* within a few minutes of treatment with soluble and activated *Bt* toxins. These alterations consisted mainly of swelling of mitochondrial cristae and endoplasmic reticulum followed by enlargement of vacuoles, and condensation of the mitochondrial matrix (Davidson and Titus, 1987). The transmission of diseases by the Culicidae remains an alarming phenomenon. *Bacillus thuringiensis* var. *israelensis* has been used in large scale due to its specificity for Culicidae. Our results show a toxic effect of *Bti* against the 4th instar larvae of *A. stephensi.* The *Bti* which specifically affects the Culicidae, contains spores and parasporal crystals of the serotype of *Bti* H – 14, that must be ingested by the larvae of the mosquito to cause mortality. After ingestion, the parasporal crystals are solubilized in the larval alkaline midgut, followed by proteolytic activation of proteins into soluble crystals. The toxin binds to a receptor cells of the midgut wall to form pores in cell, leading to larval death (Bauer, 1995). The results reported in this study have demonstrated that *Bti* effectively inhibited *A. stephensi* reproduction due to both a decrease in the maturation of oocytes and increased mortality of mature embryos.

The fecundity of insects is strongly influenced by treatments with *Bti* (Murugan *et al*., 2002). *Bti* applied retarded larval growth of the *A.stephensi*. These results are in agreement with Murray *et a*l. (1993) and Wierenga *et al*. (1996) indicating that larvae of the collorato potato beetle fed on potato foliage treated with *Bt* or on transgenic potatoes expressing a *Bt* gene (Cry IIIA) exhibited reduced weight gain and delayed development compared with those fed on regular potatoes. While larvae reoccurred within a relatively short time, virtually no pupae were able to develop of *Bti* applications. Within the weekly intervention intervals late instar larvae were not capable of developing into pupae and imagines. In the present study, *Bti* treatment reduced the larval duration, non the pupal and introverted the adult emergence. Those treated larvae escaped from mortality showed reduced longevity. The adult which emerged from treated larvae were morphologically normal but showed a great reduction in fecundity. the same results were mentioned when the *Bacillus sphaericus* was tested against malaria vector *Anopheles stephensi* (Kumar *et al*., 2013). Many reports show changes in fecundity after

treatment with *B. thuringiensis* (Murugan *et al*., 2002; 2003). Combined treatment of *Bacillus thuringiensis* with neem and pongamia showed an adult mortality and reduction in fecundity in *Culex quinquefasciatus* after the treatment with *B. sphaericus* (Makowski, 1993). From the present study we conclude that *Bti.* Vectobac G proved good larvicidal agent against *Cx pipiens and Cs. longiareolata* larvae in laboratory and also reduced the longevity of different developmental stages, egg productions and fecundity. In conclusion, it is evident that the *Bti* offers a good potential for larval control of local strain of *An. stephensi* under laboratory conditions.

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