Comparative efficacy of *Bacillus thuringiensis israelensis* crystal proteins in free and Montmorillonite bound state as a larvicide in the ovitraps for *Culex quinquefasciatus* Say.

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ABSTRACT

The usage of Insecticidal Crystal Proteins (ICPs) from *Bacillus thuringiensis israelensis* incorporated in an ovitrap can make the ovitraps leathal to the mosquito larvae thus reducing the mosquito population. Montmorillonite is a clay mineral which is known to be strong adsorbent for organic molecules such as proteins. The effect of adsorption of ICPs to montmorillonite on the larval mortality and the oviposition response of *Culex quinquefasciatus* and the persistence of protein in the ovitrap as a larvicide for a period of 21 days are analysed. The LC_{50} value (24 hrs) for the larvicidal activity of the free and montmorillonite bound insecticidal proteins from *Bacillus thuringiensis israelensis* against third instar larvae of *Culex quinquefasciatus* were 0.0578 µg/ml and 0.0388 µg/ml, respectively. The ovitraps which served as controls consisted of 30% grass infusion which is an attractant. The test ovitraps contained a single lethal dose of free ICPs and Montmorillonite bound ICPs from *Bacillus thuringiensis israelensis* along with grass infusion. No significant difference were found in the number of egg rafts laid in the ovitraps for both the treatments. The larvicidal activity of the bacterium is significantly enhanced in ovitraps that contained montmorillonite bound ICPs in comparison with the free ICPs from *Bacillus thuringiensis israelensis* (t = 4.7491).

Key words: *Culex quinquefasciatus*, Insecticidal Crystal Proteins (ICPs), *Bacillus thuringiensis israelensis* Montmorillonite , Ovitrap

INTRODUCTION

Ovitraps are used as a tool to monitor, detect and control the container breeding mosquito populations. They give an appropriate gauge of the adult population in an area and act as an early warning signal to pre-empt any impending disease outbreak (Ai-leen and song, 2000). Ovitrap surveys could be considered as a sensitive and an efficient technique for detecting and monitoring economical and environment friendly (Al-leen and song, 2000 and Chan *et al.*, 1977). Sudden increase in the mosquito population can be detected with ovitrap surveys. This will reduce insecticide consumption and will be cost effective (Sivagnaname *et al.*, 1994).

The use of an insecticide treated oviposition strip incorporated in an ovitrap was suggested by Zeichner and Perich (1999) in order to make the ovitrap lethal to both larvae and the adult. The use of biological larvicide *Bacillus thuringiensis israelensis* which is highly effective against the mosquito larvae would reduce the possibility of resistance because of its highly specific mode of action. (Gill *et al.*, 1992).

Few studies have been performed previously for the usage of *B. thuringiensis israelensis* in the ovipositional response studies in mosquitoes. Evaluation of the oviposition response of *Aedes aegypti* (Santos *et al.*, 2003), *Aedes albopictus* (Stoops, 2005) to ovitraps loaded with *B. thuringiensis israelensis* as a larvicide were performed. The ovipositional response of *Culex quinquefasciatus* to aqueous suspensions of *B. thuringiensis israelensis* and *B. spaericus* were studied (Zahiri and Mulla, 2005). The toxins from *B. thuringiensis* are rapidly adsorbed and tightly bound to clay minerals such as montmorillonite and kaolinite, as well as on the clay size fraction separated from the soil (Venkateswerulu and Stotzky, 1992; Tapp *et al.*, 1994). Of the various clay minerals studied, montmorillonite is known to be strong adsorbent for organic molecules, such as proteins (Rigou *et al.*, 2006). Moreover, the structure of these proteins did not appear to have been modified as a result of their binding with clays. (Tapp *et al.*, 1994). The adsorption and the desorbability of the Cry1AaBt insecticidal protein were measured on montmorillonite (Helesa *et al.*, 2009).

In the present study, the efficacy of *B. thuringiensis israelensis* in free and montmorillonite bound states as larvicides in the ovitraps which serve as oviposition sites for gravid *C. quinquefasciatus* mosquitoes are analyzed under field conditions. Persistence of *B. thuringiensis israelensis* in free and montmorillonite bound states are compared over 21 days, with the objective of enabling the ovitrap to be used safely for a more prolonged period.

MATERIALS AND METHODS

Collection and maintenance of mosquito population

The egg rafts of *C. quinquefasciatus* mosquito were collected by placing the ovitrap consisted of tap water and 30% grass infusion (Santos *et al*., 2003). The larvae were maintained in trays at 28 ±2°C and 75% humidity in the laboratory. They were fed with yeast pellets and allowed to grow until pupation. The metamorphosed adult mosquitoes emerged from the pupae were grown in screened cages provided with cotton soaked in glucose and honey. A healthy immobilized pigeon was placed in the cage on alternative nights for 2 hours as a blood meal. The female mosquitoes were allowed to lay eggs on a small bowl of water placed inside the cage. Thus, the mosquito population is maintained in the laboratory and the third instar larvae were selected for the bioassay.

The microbial toxin preparation

Bacillus thuringiensis israelensis lyophilized culture (MTTC- 869) was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The potato extract media (Poopathi *et al*., 2002) was used for the toxin preparation. Total amount of protein in the spores were estimated spectrophotometrically (Spectronic-20) following Lowry method (Lowry *et al*., 1951)

Adsorption of the Insecticidal Crystal Protein by Montmorillonite

The Montmorillonite adsorption study was followed according to the procedure of Lee *et al*. (2003) with minor modifications. Serial concentrations of 0 to 500 ìg of protein were added with 100ìg montmorillonite in 0.2M phosphate buffer (pH-6.0) to a total volume of 1ml. The clay toxin complexes were rotated at 50 rpm on a motorized wheel at 28 ºC .The mixtures were centrifuged at 26,300 Xg and equilibrium supernatant was collected. The loosely bound proteins were desorbed by two washes with distilled water. The concentration of protein in the supernatant was discovered by Lowry (1951) method. The amount of toxin bound to the clay was calculated by subtracting the amount of toxin recovered in the equilibrium supernatant in all washes and from the amount of toxins added to montmorillonite and the optimum adsorption was found out.

Bioassay

The grid bioassay proposed by Shah and Shethna (1994) was followed in this study. The bioassay, in general, consisted of testing various concentrations of the toxin, suspended in 150 ml of distilled water against 12 larvae and recording the mortalities at the end of 24 hours at room temperature (28ºC to 32ºC). The bioassay was carried out in trays designed with grids for this purpose. The dimension of the trays is 10 cm X 10 cm X 2 cm. The larvae were picked up with a help of a mesh, drained and placed in the compartments of assay tray containing the aqueous toxin suspension. The larvae were checked for mortality at the end of 24 hours. The LC_{50} values were calculated by Probit analysis. (Finney, 1971).

The ovitrap experimental design

Ovitraps were adapted from the model proposed by Santos *et al*. (2003).The ovitraps consisited of 2000 ml round earthen pots, 12 cms wide and 10 cms tall, with 30% grass infusion. The grass stock infusion was prepared by adding 30g of grass to two litres of tapwater and stagnated for 7 days. The oviposition substrate was 10 cm piece of white filter paper, as proposed by Stoops (2005).

A single lethal dose of free and montmorillonite bound insecticidal crystal proteins were used to find out whether they influence the oviposition behaviour. The concentration was fixed as 0.14 ìg for free proteins and 0.12 ìg for the montmorillonite bound proteins. An ovitrap without any larvicide was maintained as a control. The ovitraps were placed 30 cms apart in a row. To test the efficacy of Bacillus *thuringiensis israelensis* applications in free and montmorillonite bound conditions over time, filter papers were collected after 72 hours period after the placement of ovitraps in the field. New filter papers were replaced in the ovitraps .The collected filter papers were brought to the laboratory and the eggs were counted with the aid of a dissection microscope. At the end of 21 days, the ovitraps were brought to the laboratory and the live larvae, pupae and the pupal exuviae were counted in all the ovitraps.

Study area

The study was carried out in the Bethanakshi nagar residential area, towards the south of V.V.V. College for Women, Virudhunagar, Tamil Nadu, India. The ovitraps were placed in the verandahs or open space in the houses which are surrounded by bushes and gutters.

Oviposition Positivity Index (0PI)

The results of oviposition are expressed as mean number of egg rafts and oviposition activity index was calculated according to Kramer *et al.* (1979), where the activity index = (NT – NS) / (NT + NS); NT denoted the number of egg rafts laid in the test ovitraps and NS, the number of egg rafts laid in the control ovitraps. The Oviposition Positivity index values lie within the range from + 1 to -1. The Values of Zero indicates no difference between the treatments, but it indicates there has been no avoidance of oviposition in the test containers. The index is only a course measurement of whether or not the females are influenced by a particular treatment (Stoops, 2005). Only the end point (actual number of eggs laid) is measured, ignoring all potential pre-egg laying behaviors (Kramer *et al.*, 1979).

Statistical analysis

The numbers of egg rafts in each treatment were counted and the results were statistically analyzed using ANOVA to find out the variation between them. Students "t" test of significance was used to find the efficacy of *B. thuringiensis israelensis* to control the *C. quinquefasciatus* larvae in the ovitraps at the end of the test period.

RESULTS AND DISCUSSION

The LC_{50} value (24 hrs) for the larvicidal activity of the free and montmorillonite bound insecticidal proteins from *Bacillus thuringiensis israelensis (Bti)* against third instar larvae of *C. quinquefasciatus* at various concentrations were 0.0578 µg/ ml and 0.0388µg/ ml respectively. The montmorillonite bound protein has low LC_{50} value showing its high efficacy. The higher mortality of larvae in the montmorillonite bound proteins could have reflected greater ingestion of bound toxins than that of free toxins.

In the oviposition response studies, female mosquitoes did not oviposit in ovitraps that contained *Bti* protein in free and montmorillonite bound states along with grass infusion. More eggs were laid in B*ti* treated ovitraps than in controls .The mosquitoes laid egg rafts on the filter papers in one or more of the ovitraps in all the five replicates. Eggs were found on the filter papers in both the treated and untreated ovitraps. The mean number of egg rafts per ovitrap and minimum and maximum number of eggs per replicate during the sampling periods are given in Table 1. A total of 241 egg rafts were collected in all the ovitraps during the study period. The control ovitrap received 77 egg rafts. The ovitraps that contained free protein received 80 egg rafts and the ovitraps that contained montmorillonite bound proteins received 84 egg rafts. No significant difference were found in the number of egg rafts laid in the ovitraps for both the treatments, when compared with the control (F = 0.0025, 0.0157) and within themselves (F = 0.0045).

The oviposition behavior of mosquitoes is governed by several factors such as pheromone, water chemical composition, presence of pathogens and predators etc., (Bentley and Day, 1989). Santos *et al.* (2003) reported that the presence of *Bti* appears not to influence the choice of ovitrap as an oviposition site, when combined with grass infusion. Their results suggest that the larvicide does not influence the number of eggs laid and that this effect may depend on the infusion concentration. The oviposition activity index was determined on every third day from the day of introduction of the ovitraps to the field until 21 days and the results were tabulated (Table 2).

Out of 7 observations, the ovitrap that contained free protein produced 3 positive values, two negative values and two values were zero. The ovitraps contained montmorillonite bound proteins produced 4 positive values and three values were zero. These results reveal that the Oviposition activity of the *C. quinquefasciatus* were at random in all the ovitraps. The presence of *Bt* proteins neither attracted nor repelled the gravid female *Culex quinquefasciatus* mosquitoes to lay their eggs. It is possible that female mosquitoes are not able to detect the larvicide when used with grass infusion, which is an attractant.

The mean number of alive III and IV instar *C. quinquefasciatus* larvae was 14.4 ± 1.74.On treatment with *Bti* protein in a free state , the mean number of III and IV instar larvae of *C. quinquefasciatus* were reduced to 8.4 ± 1.50. On treatment with *Bti* protein in montmorillonite bound state, the mean number of III and IV instar larvae of *C. quinquefasciatus* were further reduced to 3.8 ± 1.17 (t = 4.7491).

In the present study, 41.66% of the *C. quinquefasciatus* larvae were dead in the ovitraps that contained free protein from *Bti* and grass infusion on comparison with the control at the end of the study period of 21 days. Santos *et al.* (2003) have shown that 100% of the larvae of *Aedes aegypti* were eliminated for 15 days in the ovitraps containing *Bti* with 10% or 30% grass infusions. This permits the ovitraps to be used in the field for longer than 7 to 10 days, the normal post embryonic development time for *C. quinquefasciatus* mosquitoes, removing the real risk of transferring an ovitrap to a breeding site.

The larvicidal activity of the bacterium is enhanced for more period as it was observed that 73.61% of the larvae

Table 1. The mean number of egg rafts (± SD) collected per ovitrap and minimum and maximum number of eggs per replicate during the sampling period and Oviposition Positivity Index (OPI) between the number of egg rafts.

Day	Control (Grass infusion) (A)	Grass infusion +Free protein(B)	Grass infusion +Montmorillonite bound protein(C)	OPI(A) and (B)	OPI(A) and (C)
3	0.4 ± 0.49(0 – 1)	0.4 ± 0.49(0 – 1)	0.3 ± 0.49(0 – 1)	0	0.2
6	0.8 ± 0.96(0 – 2)	0.9 ± 0.83(0 – 1)	1.2 ± 0.98(0 – 2)	0.05	0.2
9	1.8 ± 0.75(2 – 3)	1.6 ± 1.04(0 – 3)	1.8 ± 0.98(0 – 3)	- 0.08	0
12	2.6 ± 0.49(2 – 3)	2.2 ± 0.96(1 – 4)	3 ± 0.62(2 – 4)	- 0.04	0.07
15	2.6 ± 1.36(1 – 4)	3.4 ± 1.50(1 – 5)	2.6 ± 0.48(2 – 3)	0.8	0
18	3.6 ± 0.75(2 – 4)	3.8 ± 0.97(2 – 5)	4 ± 0.636(3 – 5)	0.02	0.05
21	3.4 ± 0.49(3 – 4)	3.4 ± 0.49(3 – 4)	3.4 ± 0.49(3 – 4)	0	0

were dead in the ovitraps that contained montmorillonite bound protein from *Bti* and grass infusion in comparison with the control, at the end of the study period of 21 days. Hydrophobic interactions with soil organo-mineral surfaces play an important role in both the adsorption and subsequent changes in conformation of the protein (Hellasa *et al.*, 2009). The increased time for which an ovitrap can remain in the field could greatly reduce the operational cost of the control programmes. The number of eggs collected may also increase. The presence of rotting organic material in the suspension does not reduce its efficacy over this period. The resultant metabolites from the bacterial growth with in the ovitrap may also a contributing factor for increasing oviposition and ovitrap attractiveness. If the traps serve as a permanent oviposition site, female mosquitoes will tend not to disperse in search of other breeding grounds. In the absence of ovitraps, the eggs would be deposited in the unmonitored breeding sites in the vicinity (Santos *et al.*, 2003) . The use of these traps are effective in dry season in order to collect the eggs. If these eggs are deposited in other sites, they could enter a period of dormancy even up to a year (Silva and Silva, 1999). The results of the present study show that when the insecticidal crystal proteins from *Bacillus thuringiensis israelensis* is bound with montmorillonite, the protein shows enhanced larvicidal activity, over a period of time. The binding of proteins with montmorillonite involves primarily physical forces such as hydrogen bonds, Vander Waal's interactions, co-ordination bonds and water bridging. (Theng, 1979; Rutter and Vincent, 1980) . On the basis of x-ray diffraction and electron microscopic analysis, the protein appears to enter clay inter layers.

Some of the mechanisms by which proteins bound on montmorillonite resist utilization or inactivation by microbes and remains active for long period were postulated by Koskella and Stotzky (1997). Additional studies are warranted to elucidate the molecular mechanisms binding of *Bti* protein on montmorillonite, its ability to withstand degradation and precise mechanism of delivery and mode of action on the larval gut.

The useful ness of *Bti* has been shown in mosquito control programmes for a long time. Larval control is a long term investment that minimizes human annoyance while maintaining healthy ecosystems (Swadner., 1992). By using the combination of these larvicidal and ovitrap methods, it would be possible to reduce the mosquito population. These techniques are more selective and have only few negative effects on humans, wild life and environment.

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