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RESIDUAL ACTIVITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* AGAINST MOSQUITO LARVAE

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ABSTRACT

Residual effect of *Bacillus thuringiensis* var. *israelensis* (Bti) Technical powder, WDG (water Dispersible Granules) and a *Bacillus sphaericus* (Bsph) was evaluated against laboratory-reared Anopheles *stephensi* and field collected *Culex quinquefasciatus* late third instars from Lahore, Pakistan. In general residual activity of both strains in seven different concentrations (100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm) for laboratory reared and in semi-field bioassays varied in both species of mosquito larvae. *Bti* TP residual activity varies from 51 days to 24 hours against laboratory reared *A. stephensi* larvae using maximum concentrations 100 ppm to minimum concentration 0.0001 ppm (fresh stock replacement every 24 hours). Whereas, *Bsph* TP indicated maximum residual effect for 18 days and minimum for 48 hours against the same species. Percent mortality was significantly higher (P=0.000) for *A. stephensi* against 100 ppm as compared to *Bsph*. However, the same effect (P=0.054) was observed for field collected *Culex quinquefasciatus* against 100 ppm *Bsph.* In conclusion field collected *Culex quinquefasciatus* larvae were more susceptible and have prolonged residual effect as compared to laboratory reared *A. stephensi* against *Bsph* while *Bti* have effect vice versa. Residual effect for field evaluation of *Bti* WDG under low treatment (0.2 mg/litre) against *Culex quinquefasciatus* lasted 14 days indicating more efficient for field bioassays as compared to laboratory.

Key words: *Anopheles stephensi*, Residual activity, *Bacillus thuringiensis*, *Bacillus sphaericus*, *Culex quinquefasciatus*.

INTRODUCTION

Malaria is the most important parasitic disease of human worldwide even today. An estimated 300 million people (of which 1 to 3 million die each year) are infected with one or more species of the parasites. In fact, almost 40% of the world's population lives in parts of world where malaria is endemic (WHO Report, 1997). In Pakistan three main groups of mosquitoes belonging to the genera, *Culex*, *Anopheles*, and *Aedes* predominates. Lahore is one of the districts in the Punjab that have large population of above three genera which cause different diseases (*Filariasis, Malaria and Dengue*) in human beings.

In Pakistan, 24 species of genus *Anopheles* have been reported (Amerasinghe *et al*., 2002). Among these species *Anopheles culicifacies* is a confirmed primary vector of malaria in rural areas (Pervez and Shah, 1989). Vectoral capacity of *Anopheles stephensi* has only been confirmed by laboratory investigations from different parts of the country especially peri-urban areas of Karachi and the Indian subcontinent (Rehman and Mutalib, 1967).

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 (Aldemir 2009; Rydzaniez *et al.,* 2009; 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. These two biological control insecticides have become control agents of choice due to environmental safety and specificity to nematocerandiptera (including mosquitoes) almost throughout the world (Mulla *et al.,* 1999; Fillinger *et al*., 2003).

Bti have shown highest level of biological activity with different levels of toxicity to mosquito species i.e.; *Culex* and *Aedes* are highly susceptible while *Anopheles* is less susceptible (Anonymous, 1985; Balaraman *et al*., 1993; Charles *et al*., 1996). However, even within one genus, some species are more susceptible than others (Chui *et al*., 1995).

Biological control with *Bti* and *Bsph* larvicides proved highly effective yet selective in action (Charles and Nielsen, 2000) and therefore, environmentally safe to non-target organisms as well as for human exposure

(WHO 1997). These *Bacillus* products are cost effective, can be produced locally and highly acceptable in the community (Becker, 1992).

The current study was designed with specific objective to evaluate the bio- efficacy and residual activity of *Bti a*nd *Bsph as* biological larvicides against late 3rd instars of laboratory reared *Anopheles stephensi* and wild caught *Culex quinquefasciatus*. In addition field evaluation of *Culex quinquefasciatus* was also conducted against *Bti* in a semi-field condition. This study is the first report of its kind against malarial vector and *Culex* species of mosquitoes from Pakistan.

MATERIALS AND METHODS

***Bacillus* formulations:** Test Strains of *Bacillus thuringiensis* var. *israelensis* (*Bti* VectoBac® 5000 ITU/mg) Technical powder (TP), *WDG* (water Dispersible Granules) 3000 ITU/mg and a *Bacillus sphaericus* (*Bsph*) 2363 VectoLex® Technical powder (TP) 1380 ITU/mg by Valent Bioscience Crop; IL, USA was used to evaluate bio-efficacy and residual activity against laboratory reared *Anopheles stephensi* and wild collected *Culex quniquefasciatus* larvae.

Animals: Late 3rd instars (L3) of *Anopheles stephensi* and *Culex quinquefasciatus* were used for laboratory and field experiments. *Anopheles stephensi* (strain from Malaria Center Lahore) were reared in Govt. College University, Lahore (GC University) insectory while *Culex quinquefasciatus* were periodically collected from Nasir Garden (in front of GCU) Lahore. Breeding habitat of wild collected *culex* larvae for laboratory and semi-field assays was a semi-permanent small artificial pond with grassy banks and without any shade. There was low-level detritus in the bottom of pond due to the litter present around the pond.

Mosquitoe rearing and maintenance: *Anopheles stephensi* were reared in GCU insectary under standardized condition at 27ºC ±3ºC and 80% ±3% relative humidity and a photoperiod of 16:8 (L: D) hours with minor modifications Jahan and Hurd (1997). Larvae for both colony and experiment were maintained in batches of 200 each in l200 m1 of deionized water in steel trays (35 cm, 30cm, 5cm) was fed with two drops of 10% sugar solution daily for the first 3 days then finely course fish food (available from local market). Measured amount of food was applied to the surface of water and allowed to spread evenly upto pupae formation (9-12 days post hatching). Adults emerging within 24 hours period were maintained in cages (30 cm^3) and fed on 10% glucose solution and water. Nulliparous females of the same age (4-6 days post emergence) were fed periodically on restricted albino mice to maintain the colony.

Experimental protocol

Evaluation of residual effect in laboratory: For laboratory bioassays test were performed with seven different concentrations (100, 10, 1, 0.1, 0.01, 0.001, 0.0001 ppm) of each *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* technical powder in distilled water. Each concentration was replicated three times and three untreated cups were used as control. For larval bioassays 25 Larvae of late 3rd instar of each species viz. *Anopheles stephensi* and wild caught *Culex quinquefasciatus* were placed in plastic cups (7.8 cm diameter) consisting 150 ml of distilled water.

Mortality in each concentration was recorded after every 24 hours. Mortality was counted by separating dead larvae from live with the help of camel hair brush. Larvae were replaced with fresh (late 3rd instar) stock every 24 hours until the dose effect could be monitored with respect to mortality. No food material was added during whole experiment. Water was daily added to compensate the water loss by evaporation. Larval mortality in treated cups was corrected for any larval mortality in corresponding controls and percentage reduction in each group was calculated using the following

Percentage reduction (%RD) = NC-NT/ NCx100

Where

NC = No. of larvae in control

NT = No. of larvae in treatment

Evaluation of residual effect in field: Open field experiments were conducted with *Bti* WDG formulation from August 15 to August 28, 2006 (rainy season) in GC University lawn at Lahore. During experiment air temperature ranged from 17°C to 38°C (mean = 30.8°C) and relative humidity from 45% to 84% (mean = 65%). In field evaluation treatment concentration was calculated on the basis of standard water depth 7cm and fixed surface area 412.61 cm^2 (Schnetter *et al*., 1981) of round plastic tubs used for experiment. Four tubs were placed in open field covering an area 10 m^2 with distance 20 cm apart in Govt. College University lawn. In the lawn the habitat was dense green with direct sunlight similar to that of Nasir Garden Lahore (original stock place) in front of GCU Lahore (as described in section 3.3). Each tub was with two and half liters capacity, placed under shade made with hard board to protect treatments from dilution by Moon Soon rain falls of season, other insects and debris from surroundings. A plastic string was encircled around the legs of stools to avoid excess of wild cats and other animal.

To introduce larvae in each tub, plastic cups (surface area 12.5 cm^2, depth 4 cm) with deep lids were designed. Side walls of each cup was removed at three places and replaced with nylon mesh. This design allows free movement of water containing *Bti* between tub and cups at all levels. Three designed cups were inverted in each tub containing 25 larvae in each cup, with upper porous surface for respiration of testing larvae. Three tubs were placed each containing 0.2 mg / lit *Bti* WDG (low concentration was selected on the bases of laboratory results (LC_{95} *Bti*) with three replicates in each tub. Similarly untreated fourth tub with three replicates was placed as control.

In each cup 25 *Culex quinquefasciatus* late 3rd instars (larvae from original stock) were introduced and replaced with fresh stock after every 24 hours. No food material was added during whole experiment. Water was daily added to compensate water lost by evaporation.

Larval mortality was evaluated by picking the cups carefully so that all the dead and live larvae along with some water come to deep bottom lid of the cup. Live and dead larvae were counted directly by separating them with the help of camel hair brush. Mosquito larval morality in treated cups was corrected for any larval mortality in corresponding controls and percentage reduction in each group was calculated using the same formula mentioned in laboratory evaluation.

Data analysis: Data from all replicates were pooled and analyzed using computer software SPSS for Probit–regression analysis to estimate the dosage response of exposed larvae after Finney (1971). LC_{95} (lethal concentration at 95% death) was determined for the first 24 hours exposure in each group. LT_{100} (lethal time for 100 % mortality) and LT_{50} (lethal time for 50% mortality) was determined by basic statistic. Mean percent mortality in each group was compared for significance by one-way analysis of variance (ANOVA) using Minitab software. (SPSS, Inc., version 14).

RESULTS AND DISCUSSION

Evaluation of residual effects in laboratory: Residual activity along with LT_{100} (Lethal Time for 100% mortality) and LT_{50} (Lethal Time for 50% mortality) of *Bacillus thuringiensis israelensis* VectoBac® TP 5000 ITU/mg and *Bacillus sphaericus* VectoLex.® TP 1380 ITU/mg was evaluated against laboratory reared *Anopheles stephensi* and wild collected *Culex quinquefasciatus* larvae in laboratory. In general 100 ppm *Bti* treatment indicated maximum residual activity against *Anopheles stephensi* i.e. 51 days whereas 0.0001 ppm was minimum effective dose with residual effect 24 hours (Table 1). Residual effect with 10, 1, 0.1, 0.01 and 0.001 ppm was 31, 20, 13, 6, and 2 days respectively 24 hours exposure post-treatment (Table: 1). LT_{100} of *Anopheles stephensi* larvae was observed upto 19, 15 and 6 days against 100, 10 and 1ppm *Bti* treatments respectively. LT_{50} was 44, 25, 15 and 8 days against 100, 10, 1 and 0.1 ppm respectively while before 24 hours against low doses 0.01, 0.001 and 0.0001 ppm. (Fig 1).

Residual activity of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm *Bti* treatments against field collected *Culex quinquefasciatus* larvae was 47, 29, 17, and 11, 7, 5 and 3 days respectively. Maximum residual activity was 47 days against 100 ppm while minimum effect was 3 days against 0.0001 ppm 24 hours post-treatment. LT_{100} was 17 days, 24 hours and less than 24 hours against 100, 10 and 1 ppm respectively whereas LT_{50} was 42, 24, 9, 5, 2, 2 and 1 day/ days against 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm respectively (Table 2; Fig 2). *Anopheles stephensi* had maximum residual activity with 100 ppm *Bsph* treatment *i.e.* 18 days whereas 0.0001 ppm indicated minimum residual effect *i.e.* 48 hours. Residual effect with 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm was 15, 13 days, 10, 7, and 5 days by 24 hours post exposure (Table 3; Fig 3). LT_{100} of *Anopheles stephensi* was observed upto 4, 2 days and 24 hours against 100, 10, and 1ppm respectively whereas LT_{50} was 12, 11, 8 and 5 days against 100, 10, and 1ppm *Bsph* treatment. LT_{50} against 0.01, 0.001, and 0.0001 ppm was before 24 hours. Residual activity of *Bsph* treatments 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm was 24, 17, 15, 13, 12, 10 and 6 days respectively against wild caught *Culex quinquefasciatus* after 24 hour exposure. Maximum residual effect was 24 days against 100 ppm while minimum residual activity was 6 days against 0.0001 ppm. LT_{100} was 12, 5, 6, 4, and 2 days against 100, 10, 1, 0.1 and 0.01 ppm respectively whereas LT_{50} was 17, 9, 10, 8, 8, 7 days (1week) and 1day against 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001ppm respectively (Table 4; Fig 4).

A comparisons made between both mosquito species indicated a prolonged residual effect of *Bti*100 ppm against laboratory reared *Anopheles stephensi i.e.* 51 days as compared to wild caught *Culex quinquefasciatus i.e.* 47 days. However there is no significant difference between two species for all concentrations ($P = 0.781$). In addition percent mortality is significantly higher in *Anopheles stephensi* (Mean = 79.92±3.62) against 100 ppm *Bti* as compared to 100 ppm *Bsph* (Mean = 68.44±4.66) (Table 5; Fig 5).

Comparison made for *Bsph* 100 pmm treatment indicated more prolonged residual effect for *Culex quinquefasciatus i.e.* 24 days as compared to *Anopheles stephensi i.e.* 18 days. ANOVA for all concentrations regarding *Bsph* also indicated no significant difference ($P = 0.218$) between the two species. However, percent mortality is significantly higher ($P = 0.054$) in *Culex quinquefasciatus* (Mean = 71.08±7.41) as compared to *Anopheles stephensi* (Mean = 68.44±4.66) against 100 ppm *Bsph* (Table 5; Fig 5).

A comparison of percent mortality of lower dose *i.e.* 0.001 ppm *Bti* indicated significantly higher mortality ($P = 0.054$) for *Culex quinquefasciatus* (Mean = 39.20±2.47) as compared to *Anopheles stephensi* (Mean = 20.00±0.30). Whereas, comparison of percent mortality against lower doses of *Bsph i.e.* 0.01, 0.001, and 0.0001 ppm also indicated significantly higher mortality (P = 0.000, 0.000, 0.008, respectively) in *Culex quinquefasciatus* (Means = 62.66±5.02, 62.80±2.14, 36.00±2.10 respectively) as compared to *Anopheles stephensi* (Means = 18.02±4.17, 16.00±2.00, 6.00±2.00 respectively). These results indicated that *Anopheles stephensi* is more resistant as compared to *Culex quinquefasciatus* against *Bsph* lower doses.

Probit-Regression analysis indicated that LC_{95} is 0.5519 and 0.6254 for *Bti* against *Anopheles stephensi* and *Culex quinquefasciatus* respectively. In addition LC_{95} was 0.5519 and 0.4352 for *Bsph* against late 3^{rd} instar (larvae) of same species. These results also indicated that wild caught *Culex quinquefasciatus* larvae are more susceptible as compared to laboratory reared *Anopheles stephensi* against *Bsph*, while *Anopheles stephensi* larvae were more susceptible against *Bti* as compared to field collected *Culex quinquefasciatus* larvae.

Evaluation of residual effect in field Field evaluation of *Bti* WDG was conducted during August 2006 when temperature ranged 17 to 38^{o}C and relative humidity ranged from 47 to 84 %. Low treatment 0.2mg/ lit. of *Bti* WDG was used as experimental dose against wild collected *Culex quinquefasciatus* larvae. Residual activity of this formulation prolonged for 14 days. LT_{100} was 2 days (48 hours) and LT_{50} was 10 days post-treatment.

The rapid increase in mosquito resistance to various chemical insecticides and the growing public concern over environmental pollution has resulted in the development of alternatives for mosquito control, such as use of the biological agent *Bacillus thuringiensis* var *israelensis* (*Bti*) and *Bacillus sphaericus* (Zahiri *et al.*, 2004; Araujo *et al.*, 2007). Microbial larvicides safety to the environment and their efficacy against a variety of mosquito species have been demonstrated by several authors, both in laboratory and field conditions however, their use in vector control program is less studied (Fillinger *et al*., 2003; Prabakaran and Hoti 2008).

Data on field and laboratory evaluation of residual activity *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* against mosquitoes in Pakistan is not available. However, the effectiveness of these strains in the study is comparable to that in several other studies reported on mosquito larvae in different countries of the world, (Pantuwatana *et al*., 1989; Mittal *et al*., 2001; Lima *et al*., 2005). The difference in potencies and differential levels of susceptibility of various instars of a mosquito species of various test formulations should be considered while making their activity comparison.

Current study of a semi-field assay using plastic tubs and special designed cups provided more accuracy in evaluating the persistence and residual effects of these larvicides in natural conditions.

In current study wild caught *Culex quinquefasciatus* late 3^{rd} instar larvae were found comparatively more susceptible against *Bsph* where LC_{95} 0.4352 ppm was the lowest effective concentration as compared to 0.5519 ppm for laboratory reared *Anopheles stephensi* after 24 hours exposure. These results on Bio-efficacy are consistent with the previous studies in different countries (Charles and Nielsen, 2000) that *Culex quinquefasciatus* is more susceptible against *Bsph* as compared to *Bti* irrespective to the potency about 4X higher than *Bsph.* Various *Bti* and *Bsph* treatments had different residual activity against different species of mosquito and general ecology of the area. Our data with 100 ppm had maximum residual activity 51 days and 47 days against late 3^{rd} instar laboratory reared *Anopheles stephensi* and wild caught *Culex quinquefasciatus* respectively under laboratory conditions while 14 days with 0.2 mg/lit *Bti* WDG against *Culex quinquefasciatus* under field conditions comparable with several studies (Skovmand and Sanogo, 1999; Hameed and Jahan, 2007; Poopathi, 2012) where residual activity of commercial and experimental *Bti* formulations against mosquitoes generally does not exceed four weeks.

All these studies indicated LT_{100} and residual activity was dose dependent. Higher doses of *Bti* and *Bsph* enhanced the residual effects against various species of mosquitoes (Sabrina, *et al.,* 2011). In current study LT_{100} of *Bti* with minimum (0.0001 ppm) and maximum treatment (100 ppm) was less than 24 hours-18days and less than 24 hours-17 days against *Anopheles stephensi* and *Culex quinquefasciatus* respectively. LT_{100} of *Bsph* with same minimum and maximum treatments range less than 24 hours-4 days and less than 24 hours-12 days against *Anopheles stephensi* and *Culex quinquefasciatus* respectively. These results are comparable with previous studies indicating *Bsph* has long residual effect against *Culex quinquefasciatus* as compared to *Anopheles stephensi*.

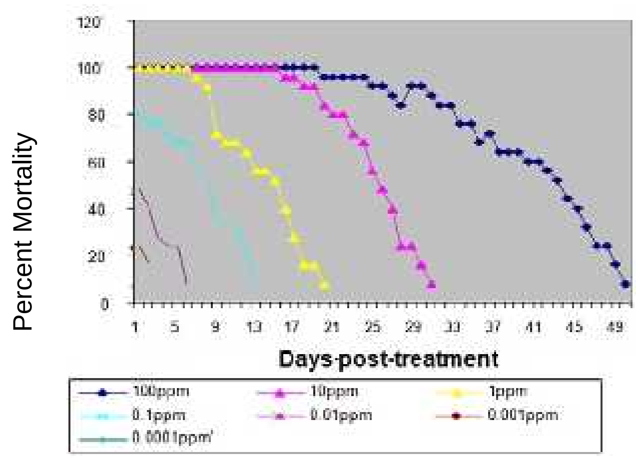

Figure 1. Residual activity of *Bacillus thuringiensis israelensis* against laboratory reared *Anopheles stephensi* larvae

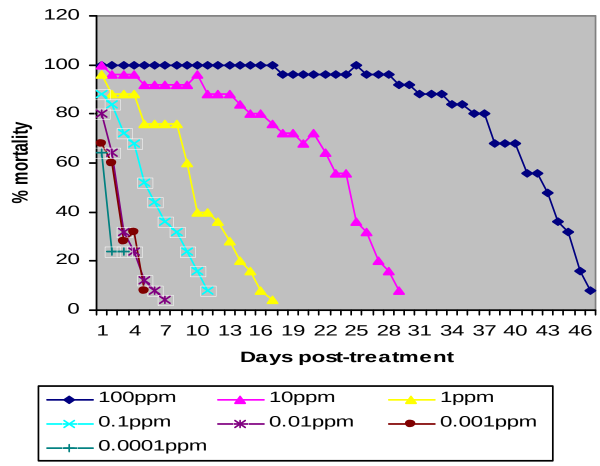

Figure 2. Residual activity of *Bacillus thuringiensis israelensis* against field collected *Culex quinquefasciatus* larvae in laboratory

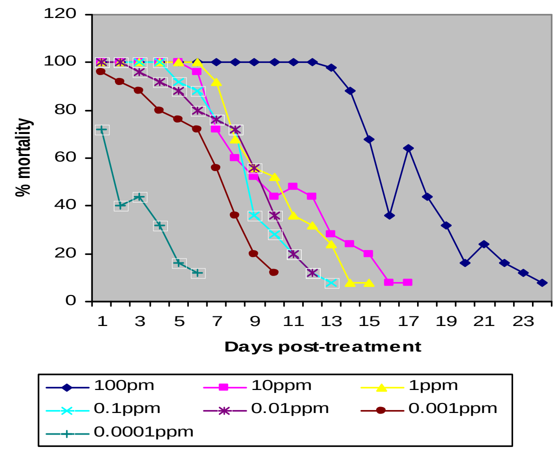

Figure 4. Residual activity of *Bacillus sphaericus* against field collected *Culex quinquefasciatus* larvae in laboratory

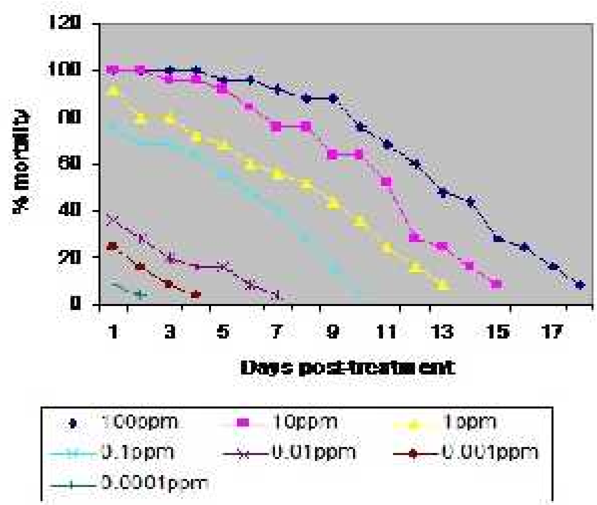

Figure 3. Residual activity of *Bacillus sphaericus* against laboratory reared *Anopheles stephensi* larvae

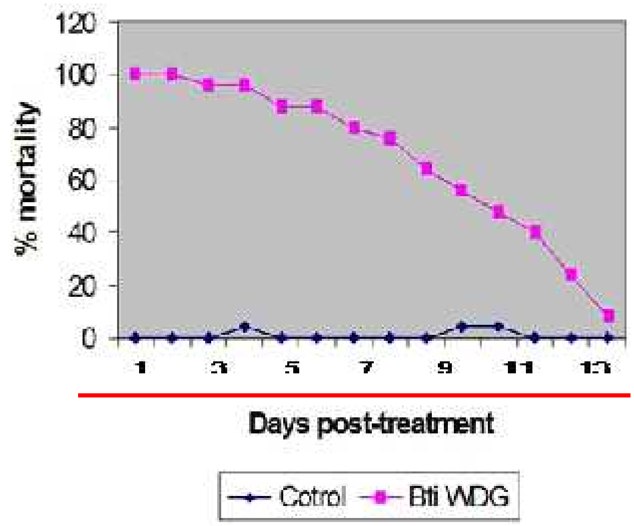

Figure 5. Residual activity of *Bti* WDG against field collected *Culex quinquefasciatus* larvae in a semi-field assay.

Table 1. Residual activity (maximum no. of days) of *Bacillus thuringiensis israelensis* and percent mortality of *Anopheles stephensi* larvae / week

Doses	Max. days of Residual activity	% mortality in No. of weeks							
		1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week
100ppm	51	100	100	98.86	92	84.57	64.57	38.5	3.43
10ppm	31	100	100	91.43	55.43	6.86	---	---	---
1ppm	20	99.43	68	22.86	---	---	---	---	---
0.1ppm	13	70.86	25.71	---	---	---	---	---	---
0.01ppm	06	24.57	---	---	---	---	---	---	---
0.001ppm	02	5.71	---	---	---	---	---	---	---
0.0001ppm	01	1.14	---	---	---	---	---	---	---

In a semi-field assay *Bti* WDG (0.2 mg/lit) indicated less residual activity (14 days) against *Culex quinquefasciatus* as compared to *Bti* TP in laboratory. These results are consistent with the previous studies indicating higher doses are needed to control wild mosquitoes as compared to laboratory conditions (Becker and Rettich, 1994). Moreover, in current study 3X lower doses than laboratory conditions (LC_{95}) were applied in the field to find the optimum effective dosage to control *Culex quinquefasciatus* in Pakistan.

Current evaluation of LT_{100} and residual activity of *Bti* and *Bsph* against laboratory reared *Anopheles stephensi* and wild caught *Culex quinquefasciatus* may be presumed as the first reference from laboratory and wild habitats under local conditions. However, extensive study is needed with different *Bti* and *Bsph* formulations in natural conditions.

Table 2. Residual activity (maximum no. of days) of *Bacillus thuringiensis israelensis* and Percent mortality of *Culex quinquefasciatus* larvae / week.

Concentrations In ppm	Max. days of Residual activity	% mortality in No. of weeks		
		1st week	2nd week	3rd week
100	18	97.71	67.43	10.86
10	15	92	42.28	1.14
1	13	72.57	25.71	---
0.1	10	60	6.86	---
0.01	07	18.28	---	---
0.001	05	11.43	---	---
0.0001	02	1.17	---	---

Table 3. Residual activity (maximum no. of days) of *Bacillus sphaericus* and Percent mortality / week of *Anopheles stephensi* larvae

Concentrations in ppm	Max. days of Residual activity	Percent mortality in no. of weeks						
		1st Week	2nd week	3rd week	4th week	5th week	6th week	7th week
100	47	100	100	97.71	96.57	88	68	20
10	29	94.86	90.28	76	47.71	3.43	---	---
1	17	84	42.86	4	---	---	---	---
0.1	11	63.43	11.43	---	---	---	---	---
0.01	7	32	---	---	---	---	---	---
0.001	5	28	---	---	---	---	---	---
0.0001	3	16	---	---	---	---	---	---

Table 4. Residual activity (maximum no. of days) of *Bacillus sphaericus* and Percent mortality / week of *Culex quinquefasciatus* larvae

Concentrations in ppm.	*Bti* Mean Percent mortality ± SEM		*Bsph* Mean Percent mortality ± SEM	
	An. stephensi	*C. quinquefasciatus*	*An. stephensi*	*C. quinquefasciatus*
100	79.92±3.62	84.00±3.41	68.44±4.66	71.08±7.41
10	79.20±5.39	72.42±4.9	65.06±3.33	69.05±6.64
1	64.84±3.30	53.88±4.68	52.92±5.25	65.06±4.37
0.1	49.60±1.7	47.63±4.67	46.80±5.23	64.00±3.47
0.01	24.80±5.12	32.00 ±4.07	18.02±0.15	62.66±5.20
0.001	20.00±0.30	39.20±2.47	16.00±1.03	62.80±2.14
0.0001	---	37.30±1.63	6.00±2.00	36.00±2.10

Table 5. Mean Percent mortalities for Laboratory-reared *Anopheles stephensi* and and wild caught *Culex quinquefasciatus* larvae treated against various concentrations of *Bti* and *Bsph*

Concentrations In ppm	Max. days of Residual activity	% mortality in No. of weeks			
		1st Week	2nd week	3rd week	4th Week
100	24	100	98	44.57	5.14
10	17	95.43	42.86	5.14	---
1	15	98.86	39.43	1.14	---
0.1	13	93.71	25.14	---	---
0.01	12	90.86	20.00	---	---
0.001	10	80	9.71	---	---
0.0001	06	30.86	----	---	---

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