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Toxins of *Bacillus thuringiensis var. Israelensis* for Control of Malaria Vector *Anopheles stephensi* under Laboratory and Semi Field Conditions

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Abstract

Two commercial preparations of *Bacillus thuringiensis var. israelensis* VectoBac WDG and Mousticide WP were evaluated and compared for larval control of *Anopheles stephensi* a malaria vector in Pakistan under laboratory and semi field conditions. *An. stephensi* larvae were susceptible to VectoBac WDG and Mousticide WP but susceptibility was higher for VectoBac WDG. In semi field experiment, VectoBac WDG @ 0.4 mg/L (1X LC_{100}) and1.6 mg/L (4X LC_{100}) remained more effective against *An. stephensi* L_3 larvae up to day 7 whereas larval mortality dropped below 50% at day 14 of post VectoBac WDG application. Mousticide WP @ 1.22 mg/L (1X LC_{100}) produced 79.33% mean mortality of *An. stephensi* L_3 at 1 day post treatment. The larval mortality dropped to 30% at day 14 of post treatment with Mousticide WP. No significant difference was seen in larval mortality between two dose rates of VectoBac WDG and Mousticide WP. © 2014 Friends Science Publishers

Keywords: *An. stephensi; Bacillus thuringiensis var. israelensis;* Bio larvicides

Introduction

Malaria is a mosquito-borne protozoan parasitic disease, which badly affects almost half a billion people living in 109 countries in Africa, Asia and Latin America (WHO, 2010). Despite of known gross under-reporting of malaria in Pakistan, the estimated burden of malaria is 1.5 million cases per year (Murtaza *et al.,* 2009). 140 million people in the country are at risk of malaria and 18% of the total population is living in high risk situations (WHO, 2009). Malaria is predominantly more prevalent in the rural areas (Mukhtar, 2004).

Among 22 *Anopheles* species, two sub-species and one variety reported from Pakistan, only two species, *Anopheles culicifacies* and *Anopheles stephensi* are known vectors of malaria. *An. culicifacies* is a confirmed primary vector of malaria in rural areas (Hick and Majid, 1937; Pervez and Shah, 1989; Herrel *et al.,* 2004). *An. stephensi* has been generally considered to be a secondary vector of malaria (Rehman and Muttalib, 1967). However, recent evidences from rural areas of Punjab, suggest that it may be playing an important role in transmission of malaria in rural areas as well (Kakar *et al.,* 2010).

Vector control with residual indoor spraying and insecticide treated bed nets are effective approaches to combat malaria. Due to rise and spread of resistance to residual insecticides in mosquito vectors (Hemingway *et al.,* 2002; WHO, 2004) the reliance on adulticides as sole tool may be inappropriate for control of mosquitoes in many situations. The treatment of mosquito breeding places with microbiolarvicides is good option to reduce the vector burdens thus, resulting a decrease in malaria transmission (Killeen *et al.,* 2002; Majambere *et al.,* 2007).

Bacillus thuringiensis var. Israelensis (*Bti*) a gram positive entomopathogen bacteria has rapid larvicidal activity against black flies and mosquitoes and due to its environmental safety and specificity to nematoceran Diptera especially mosquitoes (Aly *et al.,* 1987; Fillinger *et al.,* 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.

Resistance in vectors of malaria against different classes of insecticides has been reported from Pakistan (Rathor *et al.,* 1985). But studies on complementary/ alternative method like *B. thuringiensis var. Israelensis* (*Bti*) or other biological control agents against vectors of malaria in the country are rather limited (Jahan and Hussain, 2011).

Present study was designed to determine and compare the efficacy and residual effect of two commercial preparations of microbial larvicide *B. thuringiensis var. Israelensis* (*Bti*) against larvae of malaria vector *An. stephensi*.

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Materials and Methods

Larvicidings

Two commercial preparations of bio larvicides with trade names viz.,

1) VectoBac WDG: water dispersible granules of *B. thuringiensis var. Israelensis* strain AM65-52, 3000 ITU/mg from (Valent biosciences, USA).

2) Mousticide WP: a wettable powder of *B. thuringiensis var. Israelensis* strain H14 1410 ITU/mg with 20% TNOF-Yeast (Ento Genex, Malaysia) were used.

Mosquito Larvae Source

Bio assays were conducted with laboratory reared larvae originally derived from wild-caught *An. stephensi* mosquitoes.

For rearing the colony of wild *An. stephensi*, hand collection of indoor resting *An. stephensi* mosquitoes was carried out by using suction tube and torch-light. Mosquitoes were held in $30cm^3$ plastic cages and were provided cotton wool pads soaked in 20% sucrose. Mosquitoes were reared at 27±3°C, 70±10% relative humidity and a 16L: 8D photo-period Female were fed on restricted albino mice for 30minutes two times per week. After 72 h of blood feeding engorged females were set for egg laying in cages having container filled halfway with non-chlorinated drinking water and lined with paper towel for oviposition. Eggs on paper strips were removed, dried for one day and then stored in a zip lock bag with wet paper towel to maintain high humidity. Eggs were placed in plastic cups for hatching into first instar larvae. A pinch of fish food was added to cups and held overnight until larvae were large enough to sort in trays. These larvae were transferred to disposable plastic trays (35 cm × 30 cm × 5 cm) filled with 1200 mL of distilled water. Larvae were reared at a fixed density of 200 larvae per tray to reduce variation in adult size at emergence. Larvae were fed with 2 drops of 10% sugar solution for 3 days and then a pinch of liver powder was spread on the surface of water twice daily. On 11 to 15 days post hatching, pupae were collected in plastic cups containing distilled water and placed in emergence cages. Adult emerging within 24 h were provided with 10% glucose solution. This rearing and maintenance regime was adopted throughout the study period.

Laboratory Bio Assays

Bio assays were conducted following the guide line of World Health Organization for testing larval susceptibility to larvicides with some modification as described by Brown *et al.* (2000).

Laboratory bio assays were carried in 120 mL disposable plastic cups each holding 100 mL of tap water (non-chlorinated). To each cup 3rd instar larvae of *An. stephensi* (25 No.) were introduced and left for acclimatization for 2-3 h. Laboratory bio assays were conducted with 5 to 6 concentrations from 0.01-2 ppm (Becker and Bozhao, 1989). First 1% stock suspensions of VectoBac WDG and Mousticide were made and calculated amounts of larvicide suspensions were transferred to test cups to achieve the required concentrations. Test and control cups were kept at an ambient temperature (26-30°C), and humidity 80% for 24 h. After 24 h exposure death or the lack of reaction to gentle prodding with a glass pipette were observed and observations were recorded. No food was added to the test and control bioassay cups. Each concentration was replicated thrice and three untreated cups were used as control. During whole experiment if larval mortality in control container exceeded 10% the test was discarded and repeated. Each formulation was tested on three different occasions. The stock suspensions and their dilutions were freshly prepared on each occasion.

Determination of Residual Effect of *Bti* larvicides under Semi Field Conditions

Trials regarding evaluation of residual effect of two microbial insecticides viz. VectoBac® WDG and Mousticide were conducted from July to October, 2011. Fifteen plastic buckets (each with a diameter of 30 cm) were filled with 10 L of water collected from irrigation channel. All the 15 buckets were evenly distributed in five groups (A-E) and placed outdoors in sunlight at least 48 h prior to the experiment. Twelve buckets in groups A, B, C and D were treated with the test products and three buckets (in group E) with no treatment served as controls. VectoBac® WDG was applied @ 1X LC_{100} to buckets in group A and VectoBac® WDG @ 4X LC_{100} to buckets in group B. Mousticide was applied @ 1X LC_{100} to buckets in group C and @ 4X LC_{100} to buckets in group D. Each test product was applied once to each test bucket at the start of experiment. Thirty, third-instar larvae of *An. stephensi* in a cage (locally developed which allowed free movement of water inside) were introduced into each bucket on day1 day 7, day 14, day 21, day 28 post treatment or until the efficacy was less than 50%. First evaluations of larvae were carried out after 24 h of introduction into containers. Larval mortality was recorded daily, up to 48 h at each introduction period. The experiment was repeated on three different times.

Statistical Analysis

To estimate the dosage response of exposed larvae Probit – regression analysis was used: LC_{50}, $LC_{95,}$ at 95% confidence limits of each lethal level and slope values were determined. To determine possible effect of time periods on the efficacy of two larvicides under semi field conditions, mean larval mortalities were compared using analysis of variance

(ANOVA) and multiple comparisons were done using LSD technique. All analyses were carried out using Microsoft Excel 2007 and statistical software, SPSS version 16 (SPSS Inc, Chicago, IL, USA).

Results

Susceptibility of *An. stephensi* Larvae to Commercial Preparation of *Bacillus thuringiensis var. Israelensis* (*Bti*) Larvicides

Probit analysis of mortality data of 3rd instar *An. stephensi* (Table 1) revealed that LC_{50} and LC_{95} values of the *Bti* VectoBac WDG (strain AM65-52 3000 I1TU/mg) after 24 hours exposure against *An. stephensi* (field strain) amounted to 0.046 mg/L and 0.149 mg/L, respectively. At 24 h post-treatment of *Bti* Mousticide WP, the LC_{50} and LC_{95} values for *An. stephensi* were 0.203 mg/L and 0.564 mg/L respectively. In terms of potency, less amounts of *Bti* VectoBac WDG compared to *Bti* Mousticide WP were required to cause 50% and 95% mortalities in populations of 3rd instar *An. stephensi* at 24 h exposure periods.

Residual effect of VectoBac WDG and Mousticide WP

In semi field experiment, VectoBac WDG @ 0.4 mg/L (1X LC_{100}) performed effectively against *An. stephensi* L_3 larvae up to day 7 with 98.66±0.84 larval mortality whereas at day 35, the lowest larval mortality was 14.17±0.70. The larval mortality dropped below 50% at day 14 of post VectoBac WDG @ 0.4 mg/L (1X LC_{100}) treatment (Table 2). The mean mortality±SE of *An. stephensi* L_3 larvae at day 1,7,14,28 and 35 of post VectoBac WDG @ 1.6 mg/L (4X LC_{100}) treatment are shown in (Table 2). Post Hoc test (LSD) showed that mean mortality±SE between two treatment dosages of VectoBac WDG 0.4 mg/L (1X LC_{100}) and 1.6 mg/L (4X LC_{100}) differed insignificantly at day 1,7,14,28 and 35 of post treatments (P>0.05) (Table 3).

Mousticide WP @ 1.22 mg/L (1X LC_{100}) produced mean mortality±SE of *An. stephensi* L_3 at 1 day post treatment was 79.33 ±0.67, whereas at day 28 post treatment the larval mortality was 07.83±0.40. The larval mortality dropped below 50% at day 14 of post treatment with Mousticide WP @ 1.22 mg/L (1X LC_{100}) (Table 2). The mean mortality±SE of *An. stephensi* L_3 larvae at day 1,7,14,28 and 35 of Mousticide WP @ 4.88 mg/L (4X LC_{100}) post treatment are shown in (Table 2). Post Hoc test (LSD) showed that mean mortality±SE between two treatment dosages of Mousticide WP 1.22 mg/L (1X LC_{100}) and 4.88 mg/L (4X LC_{100}) differed insignificantly at day 1,7,14,28 and 35 of post treatments (P>0.05) (Table 3).

Discussion

Bacillus thuringiensis var. Israelensis (*Bti*) has been recognized as an efficient biolarvicide against many malaria vector species (Lacey and Lacey, 1990; Becker and Margalit, 1993; Das and Amalraj, 1997; Fillinger and Lindsay, 2006; Mwangangi *et al.*, 2011). In Pakistan *Bti* mosquito larvicides are not available locally. So published studies on laboratory and field trials of *Bti* against vectors of malaria and other mosquito species in Pakistan are quite limited (Rathor *et al.*, 1985).

In this study, larvae of *An. stephensi* were found almost highly susceptible to *Bti* formulations VectoBac WDG and Mousticide WP 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.

The level of larvicidal and hence the mortality encountered was different for VectoBac WDG and Mousticide WP. The doses of VectoBac WDG caused 50% and 95% mortality of 3rd instars of *An. stephensi* in this study were consistent with LC_{50}, LC_{95} of *Bti* formulations reported by Fillinger *et al.* (2003); Majambere *et al.* (2007) against 3rd instars of African *Anopheles* vectors of malaria. However, the doses of Mousticide TP caused 50% and 95% mortality of 3rd instars of *An. stephensi* was higher than the doses recorded for VectoBac WDG. It could be attributed to potency of Mousticide TP which contained less toxic units of *Bti* than VectoBac WDG. However the efficacy of a *Bti* may also be affected by physical nature, release mechanism of active ingredient and settling rate of the formulation (Lacoursiere and Charpentier, 1988). For VectoBac WDG formulation the crystals of *Bti* form deposits slowly towards the bottoms of the lodging than Mousticide TP formulation and provide sufficient quantity of *Bti* crystals to *Anopheles* larvae before their sedimentation. Larvae of *Anopheles* would show higher death rate if crystals of *Bti* are delivered under a floating formulation (Aly *et al.*, 1987).

Laboratory trials show a residual effect of *Bti* for 100 days (Ignoffo *et al.*, 1981) but adequate formulations that display long term persistence in the field are not yet available (Kramer, 1990; Skovmand and Sanogo, 1999). In this study VectoBac WDG caused 99% mortality of *An. stephensi* larvae up to 7day of post application and Mousticide WP showed maximum mortality 80% by 1day post application. The findings of this study are more or less similar to the studies conducted in other parts of the world. Barbazan *et al.* (1998); Fillinger and Lindsay (2006); Kahindi *et al.* (2008); Mwangangi *et al.* (2011) reported complete reduction of *Anopheles* larvae by 6 day post treatment of *Bti* in open field trials in East African region.

A number of environmental factors such as direct exposure to increased sunlight and high atmospheric temperature influence *Bti* effectiveness in the field (Ignoffo

Table 1: Probit analysis of *Bti* VectoBac WDG (strain AM65-52 3000 1TU/mg) and *Bti* Mousticide WP (strain H_{14} 1410 IU) against 3rd instar larvae of *An. stephensi* for 24 h exposure time

Bti formulations	LC_{50}, 95%CL	LC_{95}, 95%CL	Slop±SE	χ^2
VectoBac WDG, (AM65-52 3000 1TU/mg)	0.046; (0.038-0.054)	0.149; (0.112-0.243)	3.213±.465	1.638
Mousticide, WP; (H14,1200 ITU/mg + TMOF)	0.203; (0.170-0.242)	0.564; (0.439-0.822)	3.710±.464	3.786

LC lethal concentration CL confidence limits SE standard error

Table 2: Residual effect of VectoBac WDG and Mousticide WP at two treatment dosages under semi field conditions

Days Post Treatment	Mean Mortality %age of *An. stephensi* ±SE				
	Control	VectoBac WDG, 0.4 mg/L	VectoBac WDG, 1.6 mg/L	Mousticide, WP, 1.22 mg/L	Mousticide, WP, 4.88 mg/L
1	2.00±0.89	99.23±0.66	100.00±0.00	79.33±0.66	80.00±1.03
7	0.66±0.66	98.66±0.84	99.33±0.66	61.33±2.23	62.66±2.45
14	0.33±0.14	47.33±0.66	46.66±0.84	30.00±1.36	30.33±1.30
21	1.33±0.84	40.00±1.03	40.33±1.08	21.33±0.84	22.16±0.98
28	2.78±1.33	20.00±0.63	20.50±0.92	7.83±0.40	7.83±0.40
35	1.83±0.43	14.16±0.70	14.33±0.84	0.50±0.34	0.50±0.34

Table 3: Pair Wise Comparison of Treatments

Days post treatment	Larvicide	Control	VectoBac 0.4 mg/L	VectoBac 1.6 mg/L	Mousticide 1.22 mg/L	Mousticide 4.88 mg/L
1	VectoBac 0.4mg/L	0.000*	-	0.531	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.531	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.000*	0.000*	0.000*	-	0.531
	Mousticide 4.88mg/L	0.000*	0.000*	0.000*	0.531	-
7	VectoBac 0.4mg/L	0.000*	-	0.769	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.769	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.000*	0.000*	0.000*	-	0.558
	Mousticide 4.88mg/L	0.000*	0.000*	0.000*	0.558	-
14	VectoBac 0.4mg/L	0.000*	-	0.655	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.655	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.000*	0.000*	0.000*	-	0.808
	Mousticide 4.88mg/L	0.000*	0.000*	0.000*	0.808	-
21	VectoBac 0.4mg/L	0.000*	-	0.808	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.808	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.000*	0.000*	0.000*	-	0.556
	Mousticide 4.88mg/L	0.000*	0.000*	0.000*	0.556	-
28	VectoBac 0.4mg/L	0.000*	-	0.655	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.655	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.000*	0.000*	0.000*	-	1.000
	Mousticide 4.88mg/L	0.000*	0.000*	0.000*	1.000	-
35	VectoBac 0.4mg/L	0.000*	-	0.884	0.000*	0.000*
	VectoBac 1.6mg/L	0.000*	0.884	-	0.000*	0.000*
	Mousticide 1.22mg/L	0.469	0.000*	0.000*	-	1.000
	Mousticide 4.88mg/L	0.469	0.000*	0.000*	1.000	-

LSD at significance level 0.05

et al., 1981; Kramer, 1990; Becker *et al.*, 1992). The climate conditions of the study area may have contributed to lower persistence because containers were placed outdoors. Intensity of sunlight as well as the water temperature was high during the study period it is most probably that the combined effect of higher intensity of sunlight and high temperature reduced the potency of *Bti* formulations. Becker and Margalit (1993) found that sunlight reduced the effectiveness of *Bti* approximately four fold against *Cx. pipiens* in sunlit sites than shaded sites. Similarly to other authors, in this study higher concentrations of *Bti* formulations failed to enhance the residual activity (Karch *et al.*, 1991; Gelernter and Schwab, 1993; Kroeger *et al.*, 1995; Fillinger *et al.*, 2003).

In conclusion, it is evident that the *Bti* offers a good potential for larval control of local strain of *An. stephensi* under laboratory conditions. Under semi field conditions, the floating preparation of *Bti* VectoBac® WDG remained toxic to larvae of *An. stephensi* for longer duration than wettable powder preparation Mousticide.

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