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Biological Control of *Aedes aegypti* Mosquitoes Using *Bacillus thuringiensis*

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ABSTRACT

Dengue fever (DF) is a human arbovirus disease transmitted by the female mosquito of the genus Aedes, mainly *Aedes aegypti*, which is the most serious mosquito-borne viral disease worldwide and endemic in certain cities of Saudi Arabia, such as Jazan area. And because of the toxicity of the chemical- insecticides used in pest control programs, therefore, there is a need to use effective and safe alternative methods to eliminate pests. Therefore, the current study aimed to use the bacterium *Bacillus thuringiensis* as a bioinsecticide against the larval stages of *Ades aegypti*.

Two formulations of *Bacillus thuringiensis* var. *israelensis* (water-dispersible powder and Liquid formulation), were examined for their toxicity against immature and adult stages of *Ades aegypti* in the laboratory. Results indicated that the 50 % lethal concentration (colony forming unit, CFU) of *B.t.israelensis* against 4th instars, was 8.31x10^{5} CFU mL^{-1} in liquid formulation and 6.72 x10^{5} CFU gm^{-1} for wettable powder. Bioassay data also showed that pupation percent and adult emergence were affected more by subjecting the larvae to wettable powder than liquid formulation. The mortality values for the adults ranged between 49.33 & 64.23 % when using liquid formulation and wettable powder, respectively.

INTRODUCTION

Flies and mosquitoes are public health threats by transmitting various human diseases. Culex and Aedes species are insect vectors of several diseases such as malaria and dengue fever (DF). DF is a human arbovirus disease transmitted by the female mosquito of the genus Aedes, mainly *Aedes aegypti*, which is the most serious mosquito-borne viral disease worldwide and endemic in certain cities of Saudi Arabia, such as Jazan area, (Alhaeli *et al.*, 2016). And because of the climate of this area enhances the breeding of mosquitoes, especially during rainfall and high humidity. Another factor is the water storage in containers that served as natural habitats for mosquitoes, (Elisa *et al.*, 2014). Chemical- insecticides used in pest control programs, are toxic for both humans and the environment, also, many insect species gained resistance to the insecticides. Therefore, there is a need to use effective and safe alternative methods to eliminate pests. According to (Boisvert, 2007), *Bacillus thuringiensis* subsp *israelensis* was isolated for the first time in 1976 and found to be toxic to mosquito larvae. *B.t.t.* has entomopathogenic effects because of the crystal proteins produced during the sporulation stage. The crystal proteins paralyze the microvilli of the insect's digestive tract which leads to death by septicemia (Angelo *et al.*, 2010). The application of bio-control agents against insects that carry human pathogens is considered

one of the public health importance and alternatives to the usage of chemical pesticides, which harm both, man and the environment.

MATERIALS AND METHODS

Breading of Mosquitoes:

The life cycle of a mosquito has four stages: egg, larva, pupa, and adult. The female lays between 30 and 300 eggs at a time on the surface of the water line. Once hatched the larvae grow in four different instars. After then, the full-grown larvae change into a pupa. When mature, the pupae developed into adults. The period of the lifecycle, from egg to mature stage, takes about 6-14 days under good conditions (Wada, 1989).

Ten adult couples of mosquitoes were placed in a glass jar with a diameter of 7 cm and held for 24 h. Insects were fed on a solid diet composed of refined sugar, wheat germ, and brewer's yeast. (Concalves *et al.,* 2013). After oviposition eggs were collected in running water and transferred to glass jars, on filter paper, containing an artificial diet. The larvae were raised also on the artificial diet until pupation occurred and adults emerged.

***Bacillus thuringiensis* Strains**:

B. thuringiensis israelensis were obtained from CairoMercin, Agric. College, Ain Shams University. Inoculants were enriched in liquid LB medium, then preserved on slant agar medium.

Production of Delta-Endotoxin:

The inoculum was produced using LB medium in shake flasks, by inoculating 10 ml of broth medium with one loop from slant agar and incubating at 28°C on a rotary shaker (200 rpm) for 8 h. Then inoculating 3 % (v/v) from the culture into 500 ml of LB medium in a 2 L Erlenmeyer flask and incubating on a rotary shaker as done first, for 2 days. Sporulated culture was harvested by centrifugation. The bacterial biomass in the form of a cake was harvested and used for the preparation of the formulations, **(**Mehrabi *et al.,* 2015).

Preparation of ƃ- Toxin Formulation:

In this study, 2 *B.t.* formulation was prepared:

1- Liquid formulation as concentrated suspension, was produced by mixing *Bti* spore-crystal complex with detergent, emulsifier, UV protectant, and dispersal agents for slow sedimentation. The final concentration of *B.t.i*. in suspension formulation was adjusted to contain 3 x 10^7CFU/ml. Then before adding to the diet, 10 ml of formulation suspension was mixed well with ingredients using a glass homogenizer. (Ejiofor & Okafor, 1991).

2- Water dispersible powder formulation was made using fly ash as a carrier material. The formulation was dried at 40°C, ground to a fine powder, sieved to a size of ≤ 30 μ, and stored after ensuring the final moisture content was 5 percent. The end product of this formulation was a fine-sized gray powder that dispersed easily when mixed with water, containing 3 x 10^7CFU/gm. (Lopez *et al.,* 2010).

Bioassay:

The bioassay experiments were conducted in the laboratory, using early fourth instar larvae and adult stages of *A. aegypti*.

Against Immature Stages:

Ten 4^{th}–instar larvae of *A. aegypti* were reared in plastic jars and fed on an artificial diet, then incubated at 28^{o}C. *B.t.* formulation was incorporated into the diet; in five concentrations, with 3 repetitions of each treatment. Mortality was examined every 24 hrs. The experimental design was completely randomized. The 50 % lethal concentration, slope, and confidence limits were calculated by probit analysis. The control treatment was done without adding *B.t.* mixture to the formulation.

Against Adults:

Ten adults were reared without food for 12 hrs then incubated in plastic containers and placed on acrylic plates (12 cm diameter), and fed with nutrient solutions containing bacterial formulation. All treatments were designed completely randomized with 3 reps. In the negative control, insects were only fed food without bacterial formulation. Dead and live insects were determined daily for 7 days, and the mortality rate was corrected using (Abbott's formula, 1925). Also, pupation % and adult emergence % were calculated.

Statistical Analysis:

Data analysis of recorded mortality (immature & mature stages) was assumed in the mortality analysis of variance (ANOVA) that was applied to the data, and average results were compared using Tukey's test (p=0.05). Mortality in the control treatment (5-20%) was corrected according to Abbott's formula. Corrected mortality was exposed to mortality-concentration regression analysis to calculate 50 and 90 percent lethal concentration (LC_{50} and LC_{90}) values as well as their 95 percent fiducial limits (95% FL) using log-probit analysis software (SPSS Statistics ver. 21, IBM Corporation, NY, USA).

RESULTS

Bacterial Culture and Toxin Production:

Colonies of the *Bacillus thuringiensis* appear as large, cream-colored, and wide on LB agar medium, after 24 h of incubation, then incubated within a shaker incubator for 5 days to ensure reaching the sporulation phase. After ensuring that the sporulation had been completed (using staining), the crystal toxin, spores, and cellular remnants were separated from the culture medium by centrifugation (12000 rpm- 10 min).

Larval Bioassay:

Results indicated that the susceptibility of mosquitoes' larvae to *B.t. israelensis* increased, in a positive relationship with spore crystal concentrations. Results also demonstrated that powder formulation was more effective than spore-crystal liquid suspensions against the 4th instars of *A. aegypti*, Table (1). The 50% LC for suspensions was over 20% times greater than that for powder formulation in larval bioassay.

Table 1. Efficacy of *B.t.i.* formulations against 4th instars of A. *aegypti*.

Formulation	LC_{50} values* CFU gm^{-1}	Fiducial limits CFU gm^{-1}		LC_{90} values CFU gm^{-1}	Fiducial limits CFU gm^{-1}		Slope ± SE**	Larval Mortality (%)
		Lower limit	Upper limit		Lower limit	Upper limit		
Liquid formulation	$8.31x10^5$	$1.3x10^5$	$3.4x10^6$	$8.93\ x10^8$	$7.81\ x10^7$	$2.34\ x10^{11}$	0.632±0.109	70
Wettable Powder	$6.72\ x10^5$	$78.1\ x10^4$	$2.09\ x10^6$	$6.58\ x10^8$	$4.91\ x10^7$	$9.82\ x10^{10}$	0.768±0.181	80

* The concentration causing 50% mortality after 24 h. of exposure.

******Slope of the concentration-inhibition regression line **±** standard error.

As for liquid formulation, the LC_{50} value was 8.31×10^5 cfu/l and the $LC_{90} = 8.93\ x10^8$ cfu/l. Meanwhile, they were 6.72×10^5 cfu/l and $6.58\ x10^8$ cfu/l for powder formulation, respectively. Data analysis showed that the LC_{50} and LC_{90} values between the two formulations were significantly different at $P<0.001$. The activity of the wettable powder formulation was significantly different and required less concentration when comparing its LC values with that of the liquid formulation.

The effect of *B.t.t* formulations on pupation percent, malformed pupae, and adult emergence, was recorded as shown in Table (2). The pupation rate was reduced significantly as the bacterial concentration increased (CFU). As represented in table 2, the reduction in

pupation percent, using a wettable powder formulation, was 36%. The malformed pupae % was 9.6. The adult emergence percentage was only 23.2 %, with 17.3 % malformed adults. As for the liquid formulation, results indicated that the percentage of pupation, malformed pupae, adult emergence, and malformed adults were 49, 6.3, 22.6, and 9.8%, respectively.

Table 2. Biological aspects of A. *aegypti* larvae exposed to *B.t.* formulations.

Formulation	Pupation %	Malformed pupae %	Adult emergence %	Malformed adults %
Wettable Powder	64±0.43^{b}	9.6	23.2±0.11^{b}	17.3
Control	91±0.33^{a}	0.0	96±0.43^{a}	00
Liquid formulation	49±0.43^{c}	6.3	22.6±0.23^{c}	9.80
Control	93±0.33^{a}	0.0	97±0.23^{a}	00

Means within column followed by letter are not significant different (P≥0.05) Duncan's multiple range test.

Adult Bioassay:

Regarding the adult stage, the formulation products were evaluated in bioassays, and the mortality values were recorded. The efficacy of the two formulations is given in Table (3). Wettable powder formulation tested against *A. aegypti* was found to be the most active, with a mortality value of 64.23 %, (LC_{50}=9.73 x10^{5}), 95% Confidence Limits, 1.321 &3.564. As for the liquid formulation, bioassay tests indicated that the activity of the formulation was significantly different when comparing its mortality values with that of the wettable powder formulation (P<0.01). The mortality value was 49.33 %, (LC_{50}=12.88 x10^{5}), 95% Confidence Limits, 0.797 &1.966.

Table 3. Insecticidal activity of the spore-crystal formulation of *Bt* against adults stage of A. *aegypti*.

formulation	Mortality (%)	50% lethal concentration	95% Confidence Limits		Slope ± SE
			Lower limit	Upper limit	
Liquid formulation(cfu/ml)	49.33	12.88 x10^{5}	0.797	1.966	± 0.31^{bc}
Wettable Powder (cfu/gm)	64.23	9.73 x10^{5}	1.321	3.564	± 0.56^{c}

Means followed by the same letter in columns are not different from each other by the Tukey's test at 5% significance

DISCUSSION

From a medical point of view, mosquitoes are among the most important insects due to their ability to transmit human diseases such as malaria and dengue (Priest, 1992). And because of the problem that appeared with applying chemical pesticides, such as insect resistance to pesticides and environmental pollution, the urgent need for new agents and strategies to control these diseases' vectors, was appeared. *B. thuringiensis israelensis* was found to be highly pathogenic against mosquitoes. It is a gram-positive, facultative anaerobic bacterium that produces crystal proteins during sporulation that are highly toxic, specific to insect pests, and safe for the environment. These crystal toxins are the optimum alternative to chemical insecticides. (Roh *et al.,* 2007). Bioassay studies represented that, the first instars are more susceptible to *Bt toxin* than the fourth instars, also pupae do not affect by the bacteria or its toxin. (Mulla *et al.,* 1990).

In the same direction as the current study, larval and adult susceptibility to *B.t. israelensis* toxins has been demonstrated by (Cossentine *et al*., 2016; Shishir *et al*., 2015. Also, Saravanan *et al*., 2017), who tested different water dispersible powder formulations against *A. aegypti* larvae. They tested a new isolate of *Bti*, called LFB-Fiocruz. The LC_{50}

values of the tested formulation against *Aedes aegypti* larvae were 0.0417mg/L .Some biological properties such as pupation percent, malformation, and adult emergence of dipteran insects after being exposed to *B.t.*, were studied by (Gad & Al-Dakhil, 2018 & Zaki *et al.*, 2020). Their results agreed with the results of the current research results.

Comparable to results cited by (Zaki, *et al.*, 2020 & Lopez *et al*., 2010, and Scott *et al*., 2010), who stated, in similar results of this study, that, *Bacillus thuringiensis* is an effective biological insecticide for killing mosquito larvae. However, choosing the suitable application method for larviciding is critical in increasing its effectiveness.

The results of the current study recommended that the *B.t.t.* formulation treatment is recommended for use as a bio-insecticide against larvae and adult stages of *Aedes aegypti* which have a high sensitivity when treated with bacterial preparations.

Conclusions

This study demonstrates the importance of using *Bacillus thuringiensis* as an environmentally safe biological control agent, in the elimination of the insect pest *Aedes aegypti*. Both types, water-dispersible powder, and liquid formulation represented different mortality rates among the tested insect stages. Larvae exhibited greater susceptibility to both formulations than mature stages. Meanwhile, both stages were more susceptible to wettable powder than to liquid formulation. Also, the reduction of pupal formation and adult emergence was greater in the case of the wettable powder formulation.

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