Physiological Studies on the *Aedes eagypti* larvae Culicidae, Diptera

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Abstract: The total and dry body weight treated larvae of *Aedes aegypti* was significantly decreased at different time intervals under investigation (6, 12, 24, and 48 hrs) post-treatment with *LC30* of *Bt* subsp. *Kurstaki* HD-1. The body water content was decreased significantly at 6 hrs post-treatment, while it was highly significant at the three other treatments. Also hemolymph volume was significantly decreased. A significant increase in hemolymph density was observed only at 12 hrs post treatment. The main of the total hemocyte count in the treated larvae at all inspected time was highly significant increased, also there was a marked variation in the hemocytes percentage of untreated and treated larvae of *Aedes eagypti* at time intervals.
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1. Introduction

Aedes eagypti is one of the most serious and abundant medical insect pest in many countries in the world. *Aedes eagypti* Microbial pest control agents are considered as one of the new methods for the control of such insect pest, which have low impact on the peoples associated with the pest in environment. The production and application of Bt has been developed quickly . *Bt* toxins contain crystal proteins. Researchers have been working to develop environmentally responsible method for pest control based on microbiological control strategies. *Bacillus-thuringiensis (B.t)* is a gram-positive bacterium that can produce parasporal crystal protein during sporulation. These crystal proteins often show insecticidal activity against particular insect species and thus, *B.t.* is widely used in microbiological-based insect control. To date, the Cry genes used in transgenic plants have primarily been CryIA-type, CryZA and CryIF**; Schmidt *et al.,* 2009; Tabashnik *et al* .,2009; Grahan *et al*.,2010**). The increasing potential for resistance to Cry toxins to develop in insect pests has been demonstrated in previous laboratory research (**Tabashnik *et al.,* 1994; *et al.,*; Wu *et al.,* 2009**) and tolerance to the Cry toxins by insect pests has been observed in the field (**Li *et al*.,2004; Li *et al*., 2007**).To delay further insect resistance, it is important to discover and evaluate new Cry proteins with different mechanisms of action from those currently applied to pest control.Cry1Aa, CryIAbl, CryAc, Cry2A and Cry 2B. These *Bt* toxins are effective against dipterous pests. A generally accepted mode of action for Cry toxins describes the sequential steps of protoxin, activation, specific binding and cell toxicity). Both the required activation and more importantly binding steps confer remarkable pest specificity to Cry proteins (**Piott and Ellar 2007**). Ingested insecticidal crystal proteins are activated to a toxic form by proteinases from the digestive insect gut fluids. This paper describes the toxicity of *Bt* subsp. *Kurstaki* HD-1.against a serious medical disease vector. Thus the present investigation is preformed to encourage the using of *Bt* subsp. *Kurstaki* HD-1 as alternative method to chemical insecticide. This work is planned to investigate the following ; the effect of LC_{30} of the tested bacterium on some physiological parameters such as body water content, total and dry body weight as well as hemolymph volume, hemolymph density and also to determine its effect on the total and differential hemocyte counts of 2nd instar larvae of *Aedes eagypti*.

2. Materials and Methods

Test insects:-

Sources of colony:-

Adults susceptible strain of *A.aegypti* used in present study were obtained from a well established colony originated from Biology Department Faculty of Science for girls, King Abdul Aziz University.

Rearing Technique:-

Egg masses were used to maintain a colony in the laboratory under constant conditions of temperature and humidity (27 ± 2°C and 60 ±5% R.H). Each egg mass was placed in a clean Petridish (10cm diameter), previously washed with 10% formalin solution to avoid any contamination according to a constant technique .

2. Source of the bacterial pathogen.

Bt strain YBT – 226 was identified in *Aedesa egypti* screen and is the property of E.I Dupont de Nemours, *Bt* subsp. *Kurstaki* HD-1 was obtained from H.D. Burges, Institute for Horticultural Research , Little Hampton , UK . The conditions for

growth and sporulation on CCY medium were as described for *B. megaterium* KM . Purification of protein inclusions were purified from spore /crystal mixtures by centrifugation through discontinuous sucrose gradients (Thomas **and Ellar, 1983**). Protein yield was determined by the method of **Lowry *et al.* (1951)**.

3. Experimental larvae:

All the following investigated tests were accomplished on newly moulted 2nd instar larvae of *Aedesa egypti* treated with sublethal concentration (LC_{30}) of the bio-insecticide *Bt* subsp. *Kurstaki* HD-1 the tested larvae were obtained from the stock colony maintained in the laboratory of Biology Department-Faculty of Science for Girls, King Abdul Aziz University. Just after moulting and starved for about 12hrs then allowed to feed on treated larval media with LC_{30} of the bacterial pathogen. All the experiment were carried out under lab conditions (25 ± 2°C and 60 ± 5% R.H).

4. Physiological studies

4.1 Determination of total body weight and body water contents of the 2nd instar larvae.

Body water content was determined by the method of (Shapiro (1967). Measurements were adopted on ten larvae per each time interval (6, 12, 24, and 48hrs) after treatment. The body water content was calculated as the difference between fresh (total) body weight and body weight after drying for one day at 80°C in an oven.

4.2 Estimation of some physical parameters of larval hemolymph.

a. Hemolymph density

The density of hemolymph was determined for normal and treated larvae after the different time intervals, it was expressed as mg/ µ l. Ten measurements were used for each time interval.

b. Hemolymph volumes

The blood density was determined as described above. The blood weight was determined as the difference between filter paper weighted before and after 10 larvae was squeezed on this filter paper. The following equation was adopted to evaluate the blood volume:

The blood volume is expresses as µl/ Larva.

$$\text{Blood volume} = \frac{\text{Blood weight}}{\text{Blood denisty}}$$

4.3 Cellular immune response

4.3.1 Differential hemolymph counts (DHC_S)

Differential hemolymph counts were determined according to the technique of **Lim and Li (1981)**. The smears were examined under oil immersion (× 1000) and 100 cells from random fields were differentiated on each slide to determine the percentage of each type. Cell – shape, diameter, nuclear, cytoplasmic ratio and cytoplasmic inclusions were used for classification of the hemocytes using the classification a scheme of Li et al., (2007) types calculated by the formula:

$$\% = \frac{\text{Number of each hemocyte type}}{\text{Total number of hemocytes counted}} \times 100$$

$$\text{Number of WBCs\mm3} = \frac{x}{64} \times \frac{1}{\text{Vol. of each small square}} \times \frac{1}{\text{Dillution}}$$

$$\text{volume of each small square} = \frac{1}{4} \times \frac{1}{4} \times \frac{1}{10} = \frac{1}{160}$$

$$= \frac{x}{64} \times 160 \times 20 \text{WBCs\mm3}$$

determine 30% and 50% mortalities and slope value of the tested material.

3. Results

1. Effect of *Bt* subsp. *Kurstaki* HD-1. at LC_{30} on the total body, dry body weights and on the body water content of 2nd larval instar.

The total body weight of the treated larvae was significantly decreased at different time intervals under investigation (6, 12, 24, and 48 hrs) post-treatment. The same trends were also observed in the case of measuring the dry body weight, (Table 1). It is clear from presented results that the body water content was decreased significantly ($P< 0.05$) at 6 hrs post treatment, were as highly significant decrease was recorded at the other three time intervals post-treatment. The decrease in the total body weight (fresh weight) after larval treatment with microbial agent appeared to be mainly due to the decrease in the dry body weight and secondary due to the decrease in the body water content.

Table (1): Body water content of 2nd instar larvae of A.aegypti determined at different time intervals post-Treatment with LC_{30} of *Bt* subsp. *Kurstaki* HD-1Corresponding author

Hours post treatment	Body water content (mg)	
	Control mean± S.E.	Treated mean± S.E.
6	7.11 ± 0.241	6.07 ± 0.217 *
12	12.88 ± 0.555	9.32 ± 0.374**
24	18.68 ± 0.853	4.59 ± 0.401**
48	29.02 ± 1.431	9.17 ± 0.651**

n 10 larvae per test .
* Significant ($P< 0.05$) .
** Highly significant ($P< 0.01$) .

2. Effect of microbial agent (*Bt* subsp. *Kurstaki* HD-1) at LC_{30} on the hemolymph volume and density of the treated larvae:

Results in table (2) indicate that the hemolymph volume was significantly decreased at 12, 24 and 48 hrs post – treatment. These values were 2.23 ± 0.41, 1.56 ± 1.94 and 4.68 ± 0.245 µL as compared to 2.71± 0.111, 7.38 ± 0.331 and 13.44 ± 0.197 µL, respectively. A significant increase in the hemolymph density was observed only at 12 hrs post treatment. On the other hand, there is no significant differences in the values of hemolymph density in the untreated larvae as well as in treated larvae at 6, 24, 48 hrs post- treatments (Table 3).

3. Effect of *Bt* subsp. *Kurstaki* HD-1 at concentration of LC_{30} on the total hemocyte counts (THC_s) and Differential hemocytes (DHC_s) of 2nd larval instar of *A. aegypti*.

a. Total hemocyte (THC_s)

The blood cells, or hemocytes of insects form part of defense mechanism against insecticides, bacteria and other foreign bodies, they are mesodermal in origin and analogous to the leucocytes of the vertebrates. In the present study, the blood cells of the 2nd instar larvae of ***A. aegypti*** were classified into five types:- prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids . The mean THC_s at different time of treated and untreated 2nd instar larvae intervals was obtained in Table (4), it is clear from the present results that the mean of THC_s (cells / mm^3) in the treated larvae at all inspected times (6, 12, 24, and 48 hrs) was highly significant increased for example, the mean of THC_5 during the period was 33690 ± 395, 38000 ± 495, 24010 ± 403 and 20210 ± 409 (cells / mm^3) respectively. The corresponding figures of the untreated larvae during these periods were 25685 ± 245, 26335 ±211, 16040 ± 284 and 18855 ± 230 cells/ mm^3 respectively.

b. Differential hemocytes (DHC_s)

Results in Table (5) indicated that the differentiated hemocytes were greatly affected as result of post–treatment with B.t. When the 2nd instar larvae treated with tested material at $LC_{30,}$ the prohemocytes became smaller than intreated larvae. The injury causes other effects on the cytoplasm, break down of the cell wall; clumped with each other and extruding of their cytoplasm were observed.

Some morphological changes were observed in those of treated larvae. There are numerous shapes such as podocytes, amaobocytes and spindle shape. Some forms of plasmatocytes containing no distinguishing inclusion bodies in their cytoplasm which separated from the nucleus and contained large vacuoles**.**

Table (2): Hemolymph volume (µL/ larva) of 2nd instar of *A. Aegypti* determined at different time intervals post-treatment with LC_{30} of *B.t*

Hours post - treatment	molymph volume (µL /larva)	
	Control mean± S.E.	Treated mean± S.E.
6	2.14 ± 0.159	1,79 ± 0.145
12	2.71 ± 0.111	2.32 ± 0.141*
24	7.38 ± 0.331	1.56 ± 0.194 **
48	13.44 ± 0.197	4.68 ± 0.245 **

n 3 replicates per test .
* Significant ($P< 0.05$)
** Highly significant ($P< 0.01$) .

Table (3): Hemolymph density (mg / µL) of 2nd instar of *A. aegypti* determined at different time intervals post- treatment with LC_{30} of *B.t*

Hours post - treatment	Hemolymph density (mg/µL)	
	Control mean± S.E.	Treated mean± S.E.
6	0.86 ± 0.01	0.89 ± 0.008
12	0.87 ± 0.007	0.89 ± 0.014*
24	0.88 ± 0.007	0.88 ± 0.090
48	0.88 ± 0.005	0.88 ± 0.007

n 3 replicates per test .
* Significant ($P< 0.05$) .

Table (4): Total hemocyte counts (THC_s) (cells/ mm^3) of 2nd instar of *A. aegypti* determined at different time Intervals post -treatment with LC_{30} of *B.t*.

Hours post - treatment	Hemocyte counts (THC_s) (cells/ mm^3)	
	Control mean± S.E.	Treated mean± S.E.
6	25685 ± 245	33690 ± 395 **
12	26335 ± 211	38000 ± 495 **
24	16040 ± 284	24010 ± 403 **
48	18855 ± 230	20210 ± 409 **

n 10 replicates per test .
* Significant ($P< 0.05$)

Table(5): Differential hemocyte counts (DHCs) of 2nd instar of *A. aegypti* determined at different time intervals Post-treatment with LC_{30} of *B.t.*

ours post treatment	Percentage of hemocyte type (Mean ± SE)									
	Prohemocytes		Plasmatocytes		Granulocytes		Spherulocytes		Oenocytoids	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
6	10.50 ±0.05	7.70 ±0.21**	41.40±0.33	48.70±0.43**	24.70±0.35	18.30±0.20**	19.30±0.23	23.40±0.32**	4.10 ±0.13	1.90±0.11**
12	4.90 ±0.14	2.20±0.09**	42.40±0.16	49.80±0.45**	29.70±0.35	18.50±0.42**	20.20±0.31	27.20±0.62**	2.80±0.09	2.30±0.12*
24	1.60±0.08	3.64±0.15**	55.20±0.12	56.46±0.29**	24.50±0.29	20.60±0.27**	15.80±0.32	16.50±0.43	2.90 ±0.18	2.80±0.15
48	1.01±0.03	1.43±0.09**	52.89±0.31	58.70±0.65**	28.60±0.29	19.10±0.49**	15.40±0.24	18.70±0.57**	2.10±0.07	2.07±0.20

* Significant ($P< 0.05$) . ** Highly significant ($P< 0.01$) .

The effect of tested material on spherulocytes cells was small, they are often have an irregular shape the membrane is broken the nucleus moved to become laterally located.While, the injuries on granulocytes as a side effect of treatment were degeneration in cell wall with vacuolized cytoplasm, some cells were divided into two cells and other are aggregated together. Extrusion of nucleus from the cytoplasm is also observed in infected cells.Cytopathological changes were clearly observed on oenocytoids, indicated that these cells become irregular in shape with extruded cytoplasm and divided nucleus. Few cells appeared small with two nuclei and vacuoles in their cytoplasm.

4. Discussion

The larvicidal activity of *Bt* is due to toxins found in parasporal inclusions that are produced at time of sporulation (delta- exotoxins), they comprise a diverse group of proteinaceous toxins comparison of the amino acid sequences of the individual toxing resulted in the development of a new nomenclature and enabling correlations to be made between the sequence, larvicidal activity and evolutionary origin of toxin (**Crickmore, 2000**). Toxins with highest larvicidal activity (Cry4A, Cry4B, CryllA and Cyt 1A) are found in *Bti*. In addition to the 4 toxins listed above (**Perez, *et al.*, 2005**) also include CryloAa and Cyt2Ba. However, (Paul**, *et al.*, 2005**) recently reported high levels of resistance to *Bti* in isolated field population. So, the mechanism responsible for resistance Lead to develop modified methods for protein purification (**Tabashnik *et al.*, 2009**).

The total body weight, dry body weight and body water content of the treated larvae were highly significant decrease at different time intervals as compared to control (untreated larvae), and this decrease appeared mainly due to the decrease in the dry body mass, as reflected from the increase in water content percent, wet body weight and also due to the decrease in the body water content. This enables us to say feeding of *A.aegypti* larvae on diet treated with LC_{30} *B.t.i.* decreased the larval dry body weight. results' who found that in the Indian meal moth *lodia interpunctella* larvae, the B.T. induced gradual decrease in the fresh, dry body weights and body water content at three time intervals (6, 12, 24, and 48hrs). **Hegazi *et al.*(1998)** came to the same conclusion on the greater wax moth, *Galleria mellonella* larvae after injection with *Bacillus circus.*

The hemolymph volume was significantly decreased at 12, 24, and 48 hrs post treatment. The estimated significant decrease of the blood volume in the larvae may be attributed to water lose from blood and tissues as a result of bacterial infection. The present results are in accordance with that demonstrated by **Hegazi *et al.*, 1999; Crickmore, 2000 and Guo *et al.*, 2011.** The hemolymph density of untreated larvae showed non-significant difference in this parameter was observed at 12hrs post treatment, and this increase may be due to the increase in the total hemocyte counts and the increase of blood volume as well as the increase of bacterial metabolites. These results are in conformity with Guo[l] **e*t al.*, 2011** on *Plodia interpunctella* larvae with staphylococcus (**Hegazi *et al.*, 1998**) on *Galleria mellonella* larvae injected with *B. circus* (**Schmidt *et al.*, 2009, Saengwiman *et al.*, 20011 and Ritchie *et al.*, 2011**).

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