Assiut Vet. Med. J. Vol. 39 No. 77, April 1998

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STUDIES ON THE BIOLOGICAL CONTROL AND ECOLOGY OF MOSQUITOES IN ASWAN GOVERNORATE 1- EVALUATION OF BACILLUS SPHAERICUS STRAIN ABG 6232 AS BIOLOGICAL LARVICIDE

(With 4 Tables and 6 Figures)

By

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(Received at 8/1/1998)

دراسات على المكافحة الحيوية وبيئة "البعوض بمحافظة أسوان"
١ – تقييم كفاءة المبيد البكتيري باسيلس أسفيركس (أ ب ج ٦٢٣٢) ضد بعض يرقات البعوض الكيوليسيني

عبدالعال عبدالمجيد عبدالعال ، نور الدين فرغلي حمد ، صفوت أحمد عكاشه ، عصام عبدالسلام عبد الحليم شعلان

أثبت المبيد البكتيري باسيلس أسفيركس (أ ب ج ٦٢٣٢) فعالية أكبر تجاه يرقات كيولكس أنتيناتس عن يرقات كيولكس بيبينز موليستس وكان العمر الثاني ليرقات كيولكس بيبينز موليستس أكثر تأثراً من العمر الرابع . كما أثبت أيضاً المبيد ذاته تأثيراً طفيفاً على النسبة المئوية للموت في يرقات كيولكس بيبينز موليستس خلال الساعات الأولى من تعرضها له إلا أنه بعد ١٢ ساعة من التعرض له يتلاشى هذا التأثير خاصة عند أستخدام تركيز عال منه . أثبت الفحص الهيستولوجي ليرقات كيولكس بيبينز موليستس المعالجة بالمبيد البكتيري باسيلس أسفيركس (أ ب ج ٦٢٣٢) أن خلايا المعى المتوسط والغشاء المبطن لها من الداخل هما أماكن التأثير الباثولوجي لهذا المبيد.

SUMMARY

Laboratory experiments with *Bacillus sphaericus* strain ABG 6232 against *Culex pipiens molestus* and *culex antennatus* larvae proved that this larvicide was highly toxic to *Culex antennatus* larvae compared to *Culex pipiens*

molestus larvae and the 2nd instar larvae of the latter species were more susceptible than the 4th instar larvae. Treating *Culex pipiens molestus* larvae with *Bacillus sphaericus* strain ABG 6232 for different periods ranging from 1 hr to 48 hr indicated that, the bacterial suspension has a slight effect on the larval mortality percentage during the first hours but after 12 hours it has no significant effect on the larval mortality percentage especially when the larvae were exposed to high bacterial concentrations. The histopathological examination of treated fourth instar larvae of *Culex pipiens molestus* with *Bacillus sphaericus* strain ABG 6232 revealed that the mid-gut epithelial cells and the peritrophic membrane were the sites of action of this pathogen.

Key words: *Biological control, Bacillus sphaericus strain ABG 6232, susceptibility, Histopathology.*

INTRODUCTION

The present paper is the first of a series of papers concerned with studies on the biological control and ecology of mosquitoes in Aswan governorate.

Because of the fact that health and environmental considerations have limited the use of most insecticides for insect control, attention has been directed towards biocontrol agents especially bacterial pathogens. Among the most promising bacterial pathogens for mosquito control is *Bacillus sphaericus.* Various studies have indicated the susceptibility of different mosquito species to this biological larvicide (Davidson et al., 1975). Spores of *Bacillus sphaericus* kill their host by means of a toxin, recycling can occur in the dead host with net increase in the number of spores and this toxin affects the larval mid-gut within 30-60 minutes, but 8 hours are required for all mortality to be expressed (Davidson, 1984).

Many authors carried out experimental and field studies on *Bacillus sphaericus* as a mosquito larvicide, this is clear in the works of the following authors:

Davidson (1979) observed the fate of *Bacillus sphaericus* (strain SSII-I) cells ingested by *Culex pipiens quinquefasciatus* larvae and the cytological events preceding death of the host by using electron microscopy. He found that *Bacillus sphaericus* cells were digested rapidly in the anterior and central mid-gut. The outer cell wall layer and cytoplasmic ground substance disappeared sone after ingestion. Cytolysosomes became

prominent in the mid-gut cells as these cells become gradulally separated from one another.

El-Sayed and Lotfy (1990) evaluated the efficiency of *Bacillus sphaericus* 1593-4 against third instar larvae of *Culiseta longiareolata* and investigated its latent effect on produced adults. The tested larvae were found susceptible to this biologicl agent. Larvae survived from bacterial treatment were reared in the laboratory until adult stage. The resulting adults were examined for sex ratio as well as for egg production and hatchability. The results indicated the domination of males over females (2:1) in the group treated with a concentration of 0.05 mg/L.; while the sex ratio of the check and the group treated with a lower concentration 0.01 mg/L. was almost 1:1. On the other hand, the number of eggs laid by females developed from treated larvae as well as their hatchability were greatly reduced. No effect, however, was extended to subsequent generations. Histopathological effects on the ovaries revealed that susceptible ovaries contained normal and abnorml oocytes. The oocytes were found in a state of deterioration or being reabsorbed.

Raus-Neto et al. (1994) compared the efficacy of chemical and biological larvicides for the control of mosquitoes in Rio Grande du Saul State, Brazile. In the laboratory, bioassays using vectobac 12AS, Teknar 3000 (both containing *Bacillus thuringiensis* subsp. *israelensis)* and ABG 6262 *(Bacillus sphaericus* 2362) against *Culex quinquefasciatus* indicated that these larvicides are highly effective in both liquid and powder forms.

Mercer et al. (1995) tested thirteen strains among 3 species of entomopathogenic bacteria against 3 medically important mosquito species in French Polynesia. Six of seven strains of *Bacillus sphaericus.*

Rodcharoen and Mulla (1994) subjected larvae of laboratory and field collected colonies of *Culex quinquefasciatus* Say to selection pressure with preparation of *Bacillus sphaericus* Neide strain 2362 in the laboratory. Young fourth instars of every generation of both colonies were treated with the *Bacillus sphaericus* 2362 preparation at a concentration that yielded 80% mortality (Lc_{80}).

MATERIALS and METHODS

Bio-assay Experiments:

Test insects:

Larvae of 2 different mosquito species were tested during the present study. These mosquitoes were:

Culex pipiens molestus: A laboratory colony was raised in the insectary. Field collected larvae were transported to laboratory and were kept in white enamel bowls containing tap water till pupation. Pupating larvae were gathered daily in a small plastic cups (7 cm in diameter) filled with tap water and were subsequently introduced into the adult cage (40x40x45 cm) until the emergence of adults. Females were fed on blood meals through the introduction of a domestic pigeon into the adult cage, during feeding, cage was covered with black cloth to achieve a more rapid feeding. A sponge soaked with 10% sugar solution was used for feeding males, from time to time the sponge was replaced by a new one to avoid fungal contamination.

Both males and females were left for about 4 days to permit mating. Plastic cups filled with tap water were placed inside the cage for oviposition.

The laid egg rafts were removed daily from cage and left till hatching. The newly hatching larvae were transferred to breeding bowls (40 cm diameter and 20 cm depth) filled with tap water. A mixture of dried biscuit and Brewer's yeast in the ratio of 2:1 respectively were fround, sieved and used as larval food.

Pupae were collected daily by means of a small medicinal dropper and transferred to plastic cups filled with tap water and introduced inside adult breeding cage.

Culex antennatus: Trials to rear this mosquito in the laboratory did not succeed, so, field collected larvae were transported to laboratory and were kept in white enamel bowls containing tap water and homogenous larvae were selected to be used in the susceptibility tests.

Bacteria: The biological larvicide *Bacillus sphaericus* strain ABG 6232 was used in the present study and was kindly obtained as a wettable powder formulation from Dr. Adel Kmal El-Sayed, Departmen of Entomology, Faculty of Science, Ain Shams University.

Susceptibility tests: A fresh stock was prepared from the bacterial powder by adding 1 gm powder to 100 ml distilled water in a 250-ml flask and shaking vigorously by hand for 5 minutes.

Serial 1/10 the dilutions were made from the stock suspension to reach the requested concentration for each bioassay treatment. Dilutions were freshly prepared and shacked in order to assure homogenous distribution of the bacterial materials before each bioassay.

For testing the larval susceptibility levels to bacterial larvicide, plastic cups (150 ml capacity), each containing 100 ml of distilled water were used.

One ml of the tested larvicide preparation at the desired serial concentration was infiltrated under the water surface with apipette. A group of 10 2nd or 4th instar larvae were placed in each cup. All treatments, including the controls, were replicated 5 times. Throughout the tests, larvae were feed daily on ground Brewer's yeast and biscuit (1:2).

Mortality was recorded at 24 and 48 hours, and regression lines were drawn on log-probit paper. Mortality of the larvae was assured when no larval response was observed due to needle stimuli and when larvae were unable to retun to the surface after being forced to the cup bottom. When control larval mortality exceeded 5%, mortalities were corrected according to Abbott's formula (1925). Tests with 10% mortality in the controls were discarded.

Susceptibility tests were carried out to investigate the following:

1. Susceptibility of 2nd and 4th larval instars of *Culex pipiens molestus* to *Bacillus sphaericus* strain ABG 6232.
2. Susceptibility of 4th instar larvae of *Culex antennatus* to *Bacillus sphaericus* strain ABG 6232.

Effect of exposure period: Third instar larvae of *Culex pipiens molestus* were exposed to the bacterial suspension of *Bacillus sphaericus* strain ABG 6232 at different periods of time (1,3,6,12,24,48 hr) in order to evaluate the influence of the exposure period on larval mortality. In this experiment 40 larvae were bioassayed as previously described using three concentrations of the bacterium (1, 0.5 and 0.05 mg/L) at each exposure period. The tested larvae were exposed to the bacterial suspension for each period of time and were then transferred to distilled water after being washed with water. Mortality reading was taken 48 hr post-treatments including the exposure period.

Histopathological Studies:

The objectives of this study were to throw the light on the cellular and tissue response to the action of the bacterial toxins during the infected mosquito larvae in order to trace the route of infection as well as to explain the mode of action of *Bacillus sphaericus.*

Treated larvae were fixed in aqueous Bouin's fixative for 24 hours, followed by washing in 70% alcohol, transferred to 90% alcohol followed by absolute alcohol for 4 hours.

The sections were then passed into a descending series of alcohol and immersed in haematoxyline stain for 5 minutes. They were differentiated into acid alcohol solution (ethanol 70% + few drops of IN HCL) for few seconds,

washed in distilled water and then immersed in eosin counter stain for 30 seconds.

RESULTS

Susceptibility testes:

Susceptibility levels of culicini larvae of *Culex pipiens molestus* and *Culex antennatus* following treatment with different concentrations of *Bacillus sphaericus* strain ABG 6232 are shown in Tables (1 & 3) and illustrated by Fig. (1, 2, & 3).

Susceptibility of *Culex pipiens molestus* larvae:

Data in Table (1) indicated the following: Second and fourth instar larvae of *Culex pipiens molestus* appeared susceptible to the tested larvicide, and this susceptibility was positively correlaed with the larvicidal concentrations.

The susceptibility of tested larvae was significantly increased with the exposure time.

A dose of 0.1 mg/L resulted in 100% mortality among second instar larvae only, after 48 hr exposure to the larvicide.

Second instar larvae were more susceptible to the tested larvicide than fourth instar larvae.

The Lc_{50s} for second and fourth instar larvae were 0.0015 and 0.0056 mg/L after 24 hr exposure; 0.0003 and 0.0015 mg/L after 48 hr exposure to the larvicidal concentrations. Thus, the second instar larvae were 3.7 times more susceptible than the fourth instar larvae when mortality readings are taken after 24 hr, but they were 5 times more susceptible after 48 hr.

Susceptibility of *Culex antennatus* larvae:

Data in Table (3) indicated the following: Fourth instar larvae of *Culex antennatus* appeared highly susceptible to the tested larvicide, and the susceptibility was positively correlated with the larvicidal concentrations.

High mortality percentage of tested larvae occurred after 24 hr exposure, and slight increase in mortality percentage occurred after 48 hr exposure to the larvicidal concentrations.

Doses of 0.1 mg/L and 0.01 mg/L resulted in 100% mortality among larvae after 24 hr exposure to the larvicide.

The Lc_{50s} for tested larvae were 0.009 mg/L and 0.00054 mg/L after 24 hr and 48 hr exposure to the larvicidal concentrations respectively.

Comparison between susceptibility of ***Culex pipiens molestus*** and ***Culex antennatus larvae.***

Data in Table (4) indicated the following. Fourth instar larvae of *Culex antennatus* appeared highly susceptible to the tested larvicide compared to *Culex pipiens molestus* larvae.

The susceptibility of tested *Culex pipiens molestus* larvae was increased with the exposure period more than that of *Culex antennatus* larvae.

Doses of 0.1 mg/L and 0.01 mg/L resulted in 100% mortality among larvae of *Culex antennatus*. Whereas no 100% mortality resulted among larvae of *Culex pipiens molestus*.

The larvae of *Culex antennatus* were 6.2 time more susceptible than those of *Culex pipiens molestus* when mortality readings were taken after 24 hr, but they were 2.8 times more susceptible after 48 hr. This is due to the slight increase in mortality percentage occurred after 48 hr exposure to the larvicide among *Culex antennatus* larvae.

Effect of exposure period:

The effect of different exposure periods to different concentrations of *Bacillus sphaericus* on the mortality percentage of third instar larvae of *Culex pipiens molestus* were studied. Results are tabulated in Table (3) and graphically illustrated in Fig. (4).

Data in Table (2) indicated the following. At high bacterial concentrations (1 & 0.5 mg/L) larval mortality percentage increased with the increase in the exposure period.

At relatively low bacterial concentration (0.05 mg/L), larval mortality percentage increased with the increase in the exposure period from one hour up to six hours. It was decreased at the 12 hr exposure period and increased again when the exposure period up to 48 hr.

Mortality percentage at concentrations 1 and 0.5 mg/L was the same under all exposure periods.

Twelve hours exposure period was found enough to cause 100% mortality at concentrations 1 and 0.5 mg/L. In this case, exposure periods up to 24 hr and 48 hr gave the same mortality percentage as did 12 hr exposure period at the two concentrations.

Histopathological studies:

The histopathological examination of treated fourth instar larvae of *Culex pipiens molestus* with *Bacillus sphaericus* strain ABG 6232 indicated that the mig-gut epithelium was the first site of infection. The alimentary canal of the infected larvae was filled with vegetative cells of *Bacillus sphaericus* particularly the part of the mid-gut, which exhibited extensive

cellular damage, destorted nuclei and abundant multiplication of the pathogen (Fig. 6).

The mid-gut epithelial lining cells became enlarged, vacuolated, separated from each other and the cell contents were discharged into the lumen. Cells causing hypertrophy and basement membrane was deprived of the epithelial cells. The fore-gut was unaffected. The presence of vegetative cells of *Bacillus sphaericus* in the haemolymph at late stages of infection indicated septicemia rather than toxima. The muscles were also affected (Fig. 6).

The pathological action on the epithelial mid-gut cells first appeared as an enlargement of the nucleus followed by the darkening of the cytoplasm in comparison with the normal larvae (Fig. 5). The cell size and the cytoplasm became vacuolated then the cell wall towards the gut lumen protrudes to form the characteristic balloon shaped cell and possessed ruptured peritrophic membrane. At this stage the cellular linear layer is partly detached from the basement membrane. Owing to the increase of cell size, the cell membrane became ruptured, liberating the cytoplasm or part of it to be discharged in the gut lumen. Goblet cells were aslo found to respond to the toxic action of ingested bacteria where the cytoplasm change the to black colour.

On higher magnification (Fig. 6) in advanced treated larvae, most cells and peritrophic membrane appeared hypertrophied, sloughed and completely damaged. The nuclei seem to be enalrged, evacuted at late stage of infection and it could be seen in close contact with each other.

DISCUSSION

In the present work the susceptibility of *Culex pipiens molestus* and *Culex antennatus* larvae to the larvicide *Bacillus sphaericus* strain ABG 6232 and also the effect of exposure period on mortality percentage were studied.

Susceptibility tests:

Results of laboratory bioassay tests proved that *Culex pipiens molestus* and *Culex antennatus* larvae were susceptible to the different concentrations of our tested larvicide *Bacillus sphaericus* strain ABG 6232. The susceptibility is dose dependent and is proportional to the exposure time (Salama, 1988). This may be due to the fact that *Bacillus sphaericus* is either a slow acting pathogen or that it multiplies in the larval mid-gut resulting in

higher mortality rates (Gharib et al., 1989), or due to the action of the bacterial larvicide depending on the number of spores digested by the larvae (El-Sayed and Lotfy, 1990).

The Lc_{50s} values of *Culex pipiens molestus* larvae indicated that the second instar larvae being 3.7 - 5 times more susceptible than fourth instar larvae. These results indicate a possible correlation between larval instar and susceptibility. The higher susceptibility observed among 2nd instar larvae may be attributed to their lower resistance to the toxic action of the bacterium. Other authors have also found that, in general, younger instar larvae are more susceptible to *Bacillus sphaericus* than the older larvae (Wraight et al., 1981a and 1981b; Mulla et al., 1984; Daiwan, 1988 and Gharib et al., 1989).

In case of field collected *Culex antennatus* larvae, the larvae appeared highly susceptible to our bacterium and the high mortality percentage of larvae occurred after 24 hr exposure and a slight increase in mortality percentage occurred after 48 hr of exposure to the larvicidal concentrtions.

Comparison of larvicidal activity of *Bacillus sphaericus* strain ABG 6232 against the present culicine larvae indicated that this pathogen was highly toxic to larvae of *Culex antennatus* compared to *Culex pipiens molestus* larvae. This result was based on Lc_{50s} values obtained for fourth instar larvae especially after 24 hr. Therefore, we can conclude that, the change in the trend of toxicity is not only due to the larval instars tested but also due to the mosquito species used.

Nevertheless, the results of this investigation emphasize the fact that *Bacillus sphaericus* strain ABG 6232 has considerable potential as a microbial control agent of *Culex pipiens molestus* and *Culex antennatus* larvae.

Effect of exposure period:

The obtained results of exposing *Culex pipiens molestus* larvae to *Bacillus sphaericus* strain ABG 6232 for different periods ranging from 1 hr to 48 hr, indicated that the period of larval exposure to the bacerial suspensions has a slight effect to some extent on larval mortality percentage during the first 6 hr but after 12 hr the bacterial suspensions have no significant effect on larval mortality percentage particularly when larvae were exposed to relatively high concentrations (1 & 0.5 mg/L). This result is due to the bacterium ingested by each larva seen propagates inside the gut and

hence induces toxicity leading to larval mortality, and as a result of that, the amount of bacteria taken by larvae during the first hour is responsible for the induction of larval mortality through its multiplication inside the gut (Daiwan, 1988).

The decrease of larval mortality percentage after 12 & 24 hr (0.05 mg/L) in comparison to that after 6 hr can be explained as aresult of the used larvae were late third instar larvae or the number of bacterial spores taken by larvae were few and so its multiplication inside the larval gut was decreased and leading to this decrease of larval mortality percentage.

Histopathological studies:

The aim of the present investigation is to clarify the histopathological effects of the bacterial control agents against *Culex pipiens molestus* larvae.

The pathogenesis of *Bacillus sphaericus* strain ABG 6232 as has been revealed during the histopathological study, indicated that the mid-gut cells and the peritrophic membrane were the sites of action of this pathogen. The epithelial cells of the gut are swelled and deteriorated with abnormal nucleus. Similar results were described (Kellen et al., 1965; Davidson et al., 1975; Daiwan, 1988 and Salama, 1988).

The bacterial larvicides of *Bacillus sphaericus* strain ABG 6232 is an effective larvicide against *Culex pipiens molestus* larvae, whereas Mohamed and Abdel-Aal, 1995, found that *Bacillus thuringiensis* Var. *israelensis* was an extremely effective larvicide on local strains of *Culex poicilipes and Culex pipiens molestus* respectively.

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Table (1): Susceptibility levels of second and fourth instar larvae of *Culex pipiens molestus* to *Bacillus sphaericus* strain ABG 6232 (temp. 26^{0}C)

Conc.	Mortality %			
	2nd instar		4th instar	
	24 hr	48 hr	24 hr	48 hr
0.1 mg/L	98	100	88	98
0.01 mg/L	88	92	36	64
0.005 mg/L	66	82	30	42
0.001 mg/L	28	60	32	38
0.0005 mg/L	30	58	14	26
0.0001 mg/L	8	12	4	8
check	0	2	0	2

Table (2): Mortality of third instar larvae of *Culex pipiens molestus* after exposure for different periods to different concentrations of *Bacillus sphaericus* strain ABG 6232 (temp.26C^0).

Bacillus sphaericus concentrations mg/L	Mortality % after 48 hr including the following exposure periods					
	1 hr	3 hr	6 hr	12 hr	24 hr	48hr
1 mg/L	77.5	92.5	97.5	100	100	100
0.5 mg/L	77.5	92.5	97.5	100	100	100
0.05 mg/L	62.5	82.5	85	82.5	80	97.5
check	1	1	5	2	1	1

Table (3): Susceptibility levels of field collected fourth instar larvae of *Culex antennatus* to *Bacillus sphaericus* strain ABG 6232 (temp. 26°C)

Conc.	Mortality %	
	4th instar	
	24 hr	48 hr
0.1 mg/L	100	100
0.01 mg/L	100	100
0.005 mg/L	82	84
0.001 mg/L	48	52
0.0005 mg/L	36	40
0.0001 mg/L	12	18
check	1	3

Table (4): Comparison between susceptibility levels of fourth instar larvae of *Culex pipiens molestus* and *Culex antennatus* to *Bacillus sphaericus* strain ABG 6232 (temp. 26°C)

Conc.	Mortality %			
	Culex pipiens molestus		*Culex antennatus*	
	24 hr	48 hr	24 hr	48 hr
0.1 mg/L	88	98	100	100
0.01 mg/L	36	64	100	100
0.005 mg/L	30	42	82	84
0.001 mg/L	32	38	48	52
0.0005 mg/L	14	26	36	40
0.0001 mg/L	4	8	12	18
check	0	2	1	3

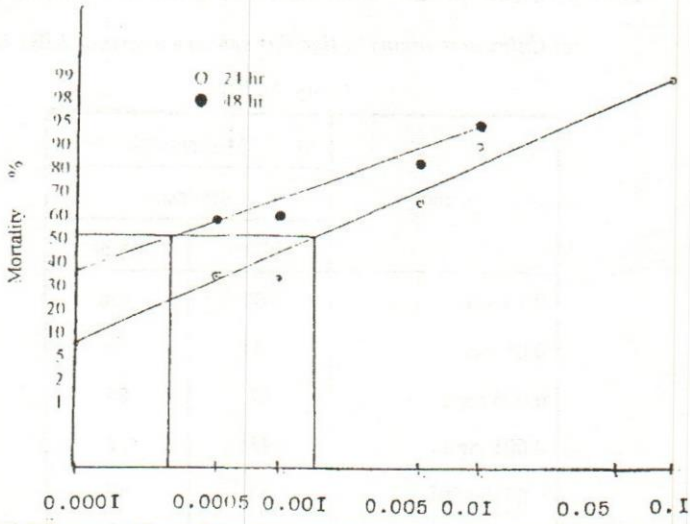

Figure (1) Regression lines of mortality among second instar larvae of *Culex pipiens molestus* after 24 hr and 48 hr of treatment with different concentrations of *Bacillus sphaericus* strain ABG 6232.

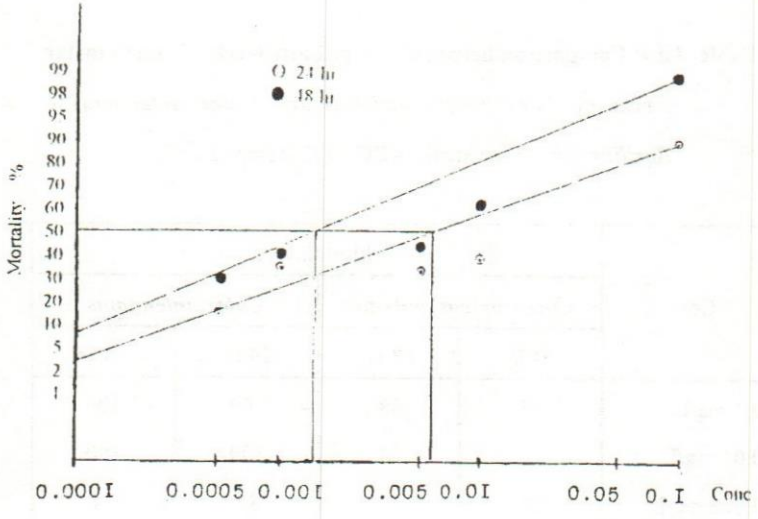

Figure (2) Regression lines of mortality among fourth instar larvae of *Culex pipiens molestus* after 24 hr and 48 hr of treatment with different concentrations of *Bacillus sphaericus* strain ABG 6232.

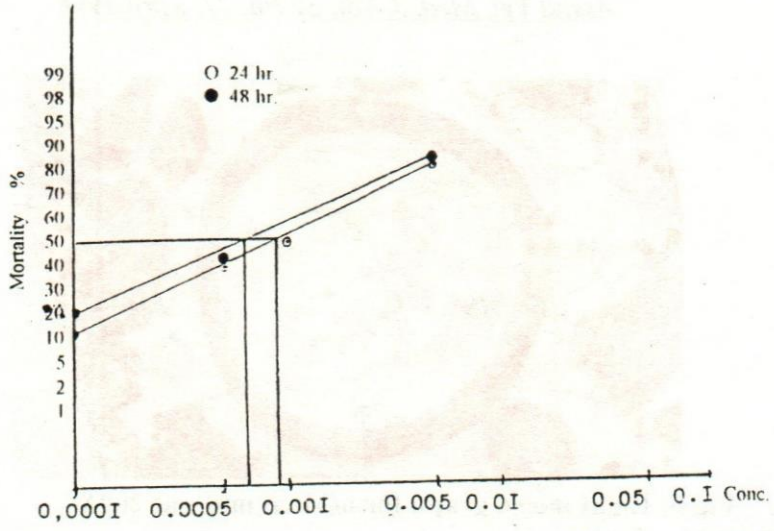

Figure (3) Regression lines of mortality among fourth instar larvae of *Culex antennatus* after 24 hr and 48 hr of treatment with different concentrations of *Bacillus sphaericus* strain ABG 6232.

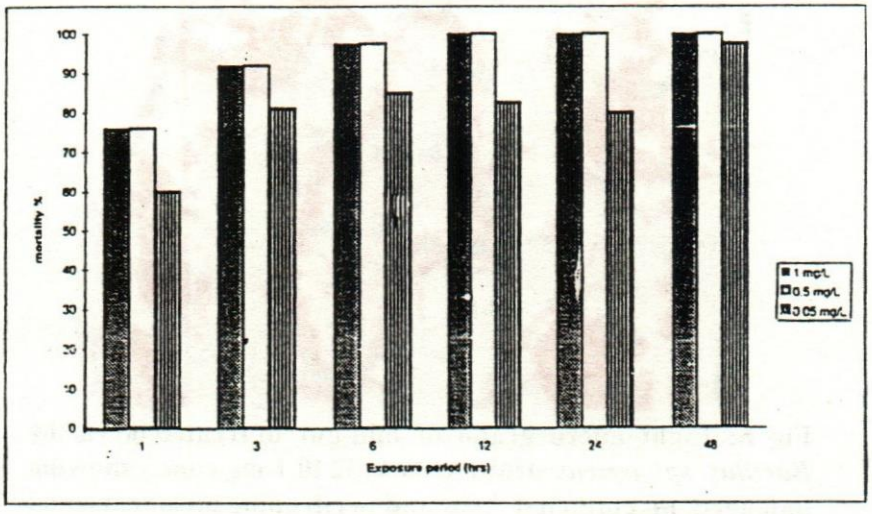

Figure (4) Effect of larval exposure period to *Bacillus sphaericus* strain ABG 6232 on the mortality percentage of *Culex pipiens molestus* third instar larvae

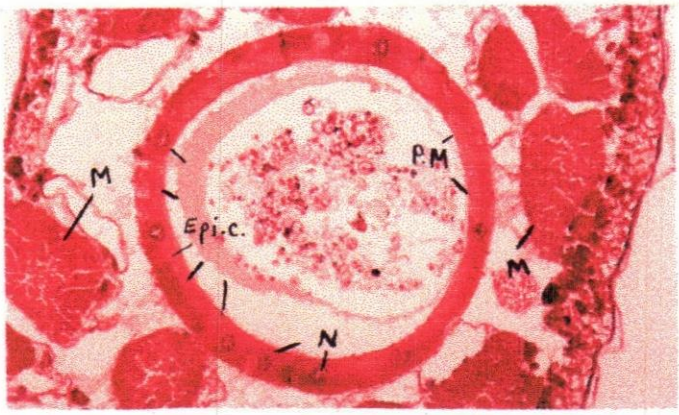

Fig 5: Light micro graph for normal mid-gut 200X.
P.M.= Peritrophic membrane
Epi.C= Epithelial cells N= Nucleus M= Muscles

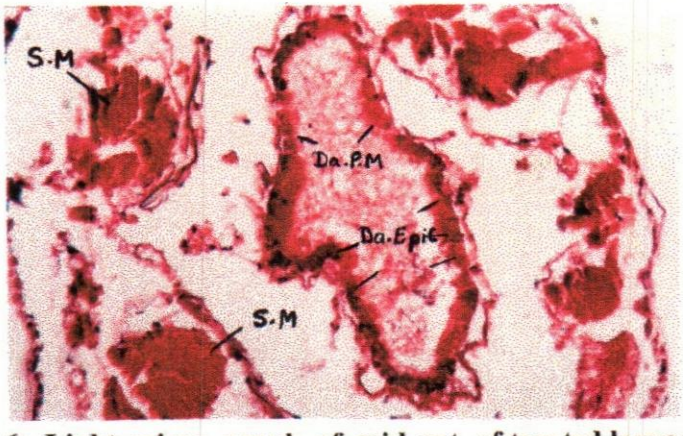

Fig 6: Light micro graph of mid-gut of treated larvae by *Bacillus sphaericus* strain ABG 6232 (0.1 mg conc.) showing damaged of epithelial cells and peritrophic membrane and separation of muscle fibrillae. 200X.
Da. P.M.= Damaged of peritrophic membrane
Da. Epi.C= Damaged of epithelial cells
S.M= separated muscles.