Effect of *Bacillus thuringiensis var. israelensis* (H-14) on Culex, Aedes and Anopheles larvae (Cotonou; Benin)

Gbehou Nounagnon Achille [1,*], Houssou S. Christophe[2], Li Yilian [3]

1. School of Environmental Studies, China University of Geosciences, Hubei province, 388 lumo Road, 430074 Wuhan, P.R China, , 008615927014830
2. University of Benin, (Abomey-Calavi), Dean of the Faculty of Human sciences Box: 526- Abomey-Calavi- Benin
3. China University of Geosciences, Department of Environmental Sciences, 388 Lumo Road, Hongshan District, Wuhan City, Hubei province, 430074, P.R.China

*For correspondence: achillegbehou@yahoo.fr

Abstract: The use of insect-specific toxins from *Bacillus thuringiensis var. israelensis* is forming an increasingly important component of biological control strategies that are either being implemented or planned for use in mosquito control. In terms of morbidity and mortality caused by vector-borne diseases, mosquitoes are the most dangerous animals confronting mankind. They threaten more than 2 billion people and have substantially influenced the development of mankind, not only socio-economically but also politically. In this study, the use of *Bacillus thuringiensis var. israelensis* crystals for controlling insect's larvae was carried out at laboratory scale. Three species of insects were tested. The operational parameters for the most efficient use and monitoring of *Bacillus thuringiensis var. israelensis* toxins against insect's larvae in the laboratory were discussed. [Stem Cell. 2010;1(1):60-68] (ISSN 1545-4570).

Key words: Larvae, mosquitoes, *Bacillus thuringiensis var. israelensis*, operational parameters

1- Introduction

Amongst vector borne diseases, malaria occupies a predominant position since it is probably the leading cause of death in the world despite intense national and international efforts to control it (Pickett, 1990; Smyth, 1994). It is estimated that there are 300-500 million new cases every year, with 1.5 to 2.7 million deaths world wide particularly in Africa (WHO, 1992).

Malaria is a major health problem in Benin, where the entire population lives in areas with malaria transmission. Malaria is a leading cause of morbidity and mortality among children under five, accounting for 44 percent of outpatient visits and 40 percent of all hospitalizations.

This disease is the leading dominant disease and is one of the most important causes of mortality among infants and young children. Two main vectors are responsible of the disease transmission in Africa: Anopheles gambiae and Anopheles funestus.

To reduce the intensity of human contact - vector, WHO has recommended since 1996 the use of insecticide-treated nets and indoor spraying of insecticides at home residual where the context permits. Unfortunately today, the resistance of vectors to insecticides has been observed and constitutes a barrier to their use (VULULE et Al. 1994).

The first case of resistance to pyrethroids has been reported in Bouaké, Côte d'Ivoire because of the massive use of home Aerosol (Ellisa, N, Mouchet, 1993).

Later, the same remark was made in Kenya in the area where the nets impregnated with pyrethrum are used by the population.

In Benin in 1996, according to the work of Laleye and Akinotcho, a resistance of Anopheles gambiae ss to pyrethrum has been reported in Cotonou. This resistance was expressed by 20% mortality of Anopheline adults exposed to papers impregnated with pyrethrum.

Most of chemical larvicides create environmental problems because they are lethal to non-target species. To meet the challenges of vector resistance to chemical larvicides and environmental safety, we considered the use of biological insecticides Bacillus thuringiensis var. israelensis (Bti)

The biological agent has proved to be useful for control of mosquito species in a variety of breeding habitats (Mulla et al. 1984; Karch et al. 1992), and shown very high environmental safety. However, experimental evaluation of the agent specifically as control agent against malaria vectors is still limited.

The performance of the microbial agent may be affected by water quality parameters such as organic content, salinity, pH, and water temperature, all which vary by ecology and type of breeding habitat. These variables provide the basis for

evaluating the efficacy of this compound in a variety of ecological and epidemiological settings.

The larvicidal activity of the granular formulation of *Bacillus thuringiensis var. israelensis* was evaluated.

The main objective was to determine the optimal application rates and duration of activity for the biological larvicide.

2- Materials and methods

2-1 Literature review

2-1-1 Characterization of Bacillus thuringiensis israelensis

In 1975-76, a World Health Organization sponsored project in Israel examined mosquitoes for the presence of pathogens or parasites. During this survey, a new *Bt* strain was discovered with high toxicity to mosquito larvae (Goldberg and Margalit 1977) which was later identified and designated *Bt* var. *israelensis,* serotype H14 since raised to subspecies status as *B. thuringiensis israelensis*. This strain was significantly more toxic to mosquitoes than other known bacterial strains at that time. It was collected from mosquitoes in the Negev desert of Israel. While dipteran active *Bt*s were known, *Bti was* found to be relatively specific to Diptera and was quickly shown to be toxic to a range of mosquito and black fly species. Therefore, it was considered to have commercial potential as a control agent of nuisance Diptera around the world. Rapid development of *Bti* strains occurred in the early 1980 and several products were developed. The need for a more environmentally benign mosquito control agent and rising incidence of resistance to chemical pesticides provided a platform for rapid *Bti* development.

The species in the complex are only differentiated from one another by a few characters, most of which are located on plasmids. Therefore, characterization of *B. thuringiensis has* been problematic and several systems have been used. Phenotypic methods used include flagellar serotyping, description of crystal morphology, biochemical reactions and bioassays. Classification of subspecies or varieties based on serotyping using H-serovars resulted in identification of almost 60 varieties (Hansen *et al.* 1996). Serotype does not necessarily relate to the presence of δ-endotoxins, which determine host specificity, as flagellar genes are carried on the chromosome, while toxin genes are usually encoded on plasmids.

Characterization methods based on phenotypic characters are insufficient when used alone in studies on the environmental ecology and fate of *B. thuringiensis*, as these methods do not provide unambiguous identification. A number of DNA-based methods have been developed for characterization: specific primed polymerase chain reaction (PCR); Random amplified polymorphic DNA (RAPD), DNA: DNA colony hybridisation (Hansen *et al*. 1996) and rRNA-based probe (Akhurst *et al*. 1997). These methods can distinguish individual strains and isolates, allowing the tracking of the environmental fate of strains used for pest control.

Such methods can also be used to identify the presence/absence of specific endotoxin genes, which mean it is possible to establish whether a particular strain has lost or acquired specific δ-endotoxin genes in the environment.

2-1-2 Action

The products contain the spores and parasporal crystals of *Bti* H-14 serotype which must be ingested by the larval stage of the mosquito to cause mortality. Following ingestion, the parasporal crystals are solubilised in the alkaline larval midgut, followed by proteolytic activation of the soluble insecticidal crystal proteins. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell, which leads to death of the larva.

Bacillus thuringiensis var. israelensis treated mosquito larvae generally cease feeding within 1 hour, show reduced activity by two hours, extreme sluggishness by four hours and general paralysis by six hours after ingestion (Chilcott *et al*. 1990).

2-1-3 Occurrence

An understanding of the ecology of *Bti* in the environment is essential in assessment of its environmental risk. While originally recovered mainly from insects, improved isolation and identification techniques have indicated that *Bacillus thuringiensis may* be ubiquitous in soil. The lowest percentage recovery of *Bacillus thuringiensis from* soil reported was in the USA (60% of soils sampled) (Meadows 1993). In New Zealand, Chilcott and Wigley (1993) found that between 60-100% of soils sampled contained *Bt*, depending on source (urban, horticulture etc.). *Bacillus thuringiensis* is also indigenous in many other environments, being found in stored products, dust, on deciduous and coniferous plants and in aquatic environments. *Bacillus thuringiensis has* also been isolated from insect habitats such as rotting wood, wasp nests and stored products in many countries.

2-2 Area of study

This study was conducted in Cotonou, Benin's economic capital due to the high rate of malaria which is filled each year. The city was built on a

sandy beach to create a harbour close to the only waterway between the Gulf of Guinea and Lake Nokoué. The total area is 74 square kilometer. It is the largest city in Benin and situated between latitude 6.2°-6.3° N and longitude 2.2°-2.3° E. The relief is relatively flat and the elevation ranges between 0.3 and 6 meters.

The climate is equatorial, alternating with two rainy seasons (April - July and September - October, 800 to 1,200 mm of rain per year) and two dry seasons. From December to January, the city is affected by winds. The annual temperature varies between 18 and 35 °C. The population has multiplied rapidly from 3,300 inhabitants in 1921 to 383,000 in 1981 and 780,657 in 2006.

Cotonou always faces the most devastating floods during the rainy season in West Africa. These floods preceded by heavy rains in the catchment areas of Lake Nokoue, and consequent massive releases of water from the lake, inundated the city, and slums were the worst hit. Over half of Cotonou (Benin) suffers every year from several months of flooding, allowing mosquito larvae breeding and leading to an increase in malaria transmission.

2-3 Mosquitoes

Mosquitoes are vectors of many diseases around the world like malaria, dengue fever, yellow fever and many types of encephalitis.

In Cotonou and many provinces of Benin, insecticides are used to control mosquito populations, but larvicides like *Bacillus thuringiensis var. israelensis* are not used. Almost 100 % of the products used in the last 15 years to control mosquitoes and black flies are insecticides. Depending of the species, the adult female mosquito will lay eggs either on the water surface (egg rafts or single eggs) or on damp soil (single eggs). In favourable conditions, eggs will hatch and give birth to larvae. There are four different larval instars. The last aquatic stage is the pupa from which will emerge the adult. *Bacillus thuringiensis var. israelensis* is active only against the larvae and there is no toxic activity neither against the eggs or the pupae.

Mosquitoes can breed in many types of habitats. One can find them in roadside and irrigation ditches, pastures, woodland pools, tidal waters, salt marshes, polluted waters (with organic and/or inorganic matter), small containers, tires and tree holes. They are also present in your own backyard in abandoned pools, bird baths, roofs, clogged gutters, etc. Almost everywhere where stagnant waters are present, you get high probabilities to find mosquito larvae. Different formulations of *Bacillus thuringiensis var. israelensis* can be used to control larvae in these various breeding sites. The type of formulations and dosages must be adjusted to the types of sites encountered.

2-4 Larvae sampling

This research was a laboratory experiment which investigated on the control of malaria vectors with the use of a bacterial agent (*Bacillus thuringiensis var. israelensis*). The types of mosquito larvae which had shown a resistant to the pyrethroids were tested with the Bti in the process of evaluating its use in the control of malaria vector in Cotonou.

The mosquito larvae were collected from three areas (**Fifadji, Ladji** and **Placodji**). A high rate of resistant to the insecticides was found in those three areas (Akogbéto & al. 1999).

The larvae collected were brought to the laboratory and then sorted and classified according to their evolutionary stage (1 to 4). For the results accuracy, two larvae stage were chosen (L2 and L4).

Estimates of larval density were then made by calculating the number of larvae dipped in each container. Larval samples were preserved in 70% alcohol and identified later by standard larval taxonomy.

After that a test of sensitivity of larvae to the Bti was made. Every 30 minutes, the mortality rate of the larvae was recorded. A control experiment also was set up.

2-5 Characterization of larval habitats

- pH
- Average depth
- Volume
- Water temperature
- Light intensity

2-6 Materials

- Granular formulation of Bti, serotype H-14 (VectoBac G – 200 International toxic units per mg [ITU/mg])
- Anopheles gambiae larvae (L2 and L4)
- Culex larvae (L2 and L4)
- Aedes larvae (L2 and L4)
- A graduated ladle
- Graduated test-glass
- Glass containers
- Sucking pipettes
- Deionized water
- Pasteur pipette
- Glass beads

2-7 Experiment Procedures

The *Bacillus thuringiensis var. israelensis* solution was prepared following the procedure of de Barjac and Larget (1984), cited by Dulmage *et al.* (1990): 50 mg of primary powder was suspended in

10 ml of deionized water and homogenized on a mixing machine for 10 min at 500 strokes/min using a bead mill (20-ml penicillin flask with several 6-mm glass beads). This homogenate was further diluted with deionized water to form a stock solution of 10 mg/L. A mixing machine was used for resuspension. Subsequent dilutions were made directly in the 500-ml test dishes.

The larvae were tested against the above mentioned *Bti* preparations at concentration of 10 mg/L. Using a Pasteur pipette with the narrow tip removed, 25 individuals were transferred to each test dish containing 500 ml. A control experiment was always run at each step of the procedure. The tests were run at room temperature. Larval mortality was assessed 30 minutes to 6 hours after *Bacillus thuringiensis var. israelensis* application. Each test was carried out at least twice and the standard deviation was less than 3%.

3- Results and discussions

Table 1: Larvicidal activity of Bti on larvae (L2) of Anopheles gambiae

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	24	22	19	14	12	11	9	8	6	4	1	0
X	0	1	3	6	11	13	14	16	17	19	21	24	25
Mortality (%)	0	4	12	24	44	52	56	64	68	76	84	96	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2: Larvicidal activity of Bti on larvae (L4) of Anopheles gambiae

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	24	24	20	17	10	7	5	3	2	1	1	0
X	0	1	2	5	8	15	18	20	22	23	24	24	25
Mortality (%)	0	4	8	20	32	60	72	80	88	92	96	96	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3: Larvicidal activity of Bti on larvae (L2) of Culex

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	20	6	3	2	1	0	0	0	0	0	0	0
X	0	5	19	22	23	24	25	25	25	25	25	25	25
Mortality (%)	0	20	76	88	92	96	100	100	100	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4: Larvicidal activity of Bti on larvae (L4) of Culex

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	25	19	11	7	4	3	2	1	0	0	0	0
X	0	0	6	14	18	21	22	23	24	25	25	25	25
Mortality (%)	0	0	24	56	72	84	88	92	96	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: Larvicidal activity of Bti on larvae (L2) of Aedes

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L2	25	22	16	4	3	2	1	0	0	0	0	0	0
X	0	3	9	21	22	23	24	25	25	25	25	25	25
Mortality (%)	0	12	36	84	88	92	96	100	100	100	100	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6: Larvicidal activity of Bti on larvae (L4) of Aedes

Time(min)	0	30	60	90	120	150	180	210	240	270	300	330	360
L4	25	25	24	23	15	12	7	5	5	3	2	0	0
X	0	0	1	2	10	13	18	20	20	22	23	25	25
Mortality (%)	0	0	4	8	40	52	72	80	80	88	92	100	100
Control experiment													
X	25	25	25	25	25	25	25	25	24	25	25	25	25
Mortality (%)	0	0	0	0	0	0	0	0	0	0	0	0	0

The larvicide had caused significant mortality of larvae during the experiment. Reduction of larval population was pronounced at each experiment, except the control experiment. The mortality rate of 100% was observed at 360 minutes (graph 1 and 2) for both type of larvae (L2 and L4) for Anopheles species. The L2 larvae population for Anopheles species have lower mortality speed than L4. Both Anopheles larvae had virtually the same sensitivity to Bti.

The impact of *Bacillus thuringiensis var. israelensis* was also followed in the laboratory on two larval stages of Culex spicies (L2 and L4). The results showed the death rate of 100% after three hours and half of exposure time and five hours for L2 and L4 respectively. A death rate of 50% of the larvae was observed after fifteen and one hour and twenty minutes for L2 and L4 larvae respectively. All these variations of population death rate of tested larvae are perceptible on graph 3 and 4. We can then say that the L2 larvae have a high sensitivity compared to the L4 larvae. The same experimental protocol was used again on Aedes larvae. After three hours and a half, we observe a 100% mortality rate for larvae L2 and five hours for L4 larvae (graph 5 and 6). However, after a one hour period of time, the death rate of 50% was observed for the larvae L2 and after 2 hours, the same percentage was reached for L4 larvae. Contrary to what we observed on the level of Aedes and Culex larvae, *Bacillus thuringiensis var. israelensis* has a less intense activity on the larvae of *Anopheles gambiae*. The death rate of 100% was observed after six hours and a half for both of L2 and L4 larvae. The two types of *Anopheles gambiae* larvae have practically the same sensitivity to *Bacillus thuringiensis var. israelensis*. The effectiveness of *Bacillus thuringiensis var. israelensis* on the larvae L2 of Aedes and Culex is higher compared to that of Anopheles of the same stage. The results obtained show that the larvae of Culex and Aedes are more sensitive to the crystals of *Bacillus thuringiensis var. israelensis*. This remark would be caused by behavioral and physiological variations for species studied. The position of the larvae in water would be one of the causes for these variations. Indeed, whereas the larvae of Anopheles are parallel to the water surface, those of Culex and Aedes are immersed because of their respiratory siphon. These results are connected with those provided by Lacoursière et al., (2004). This degree of susceptibility is also related to the dispersion of the crystals of *Bacillus thuringiensis var. israelensis*. The results obtained show that the larvae of anopheles are less affected than the larvae of Culex and Aedes when they are exposed to the same quantity of crystals of *Bacillus thuringiensis var. israelensis*. For Aly et al., (1987), the larvae of Anopheles would show a higher death rate if the crystals of *Bacillus thuringiensis var. israelensis* are delivered under a floating formulation. Moreover, the food behavior would be the essential component for this observation. The larvae of Culex and Aedes feed on

themselves actively through the entire water column, and since the crystals forms a deposit slowly towards the bottom of the lodging; they have a facility to introduce these crystals more than the larvae of Anopheles. The larvae of Anopheles which practically nourish themselves at water surface will not have time to introduce a sufficient quantity of crystals before their sedimentation. The low level of mortality recorded on the level of the L4 larvae could be explained by the fact why they are nourished little at the beginning of the pupal stage (stage where the metamorphosis at the adult stage occurs). The nymphs are completely insensitive to the crystals of *Bacillus thuringiensis var. israelensis* because they do not need to be fed on.

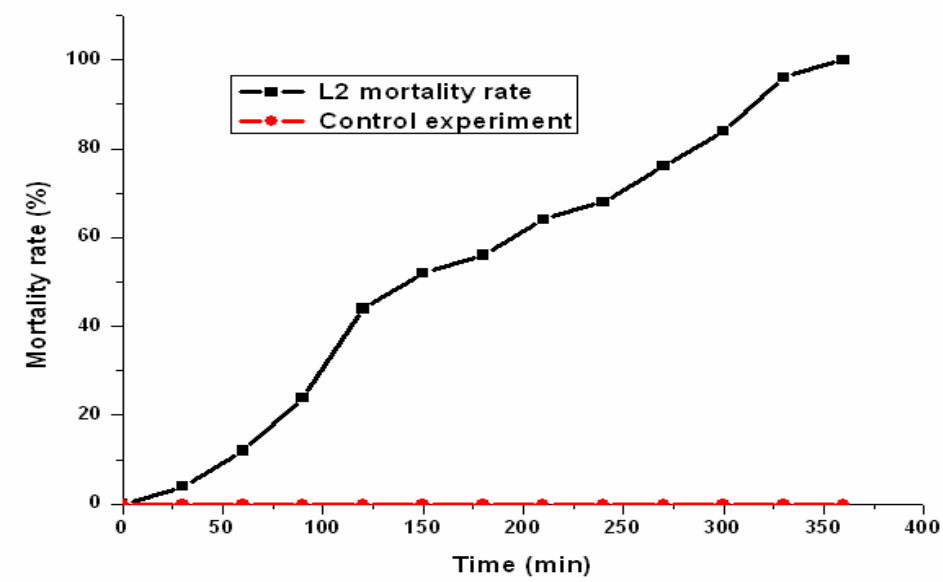

Figure1: Effect of *Bacillus thuringiensis var. israelensis* on Anopheles L2 larvae

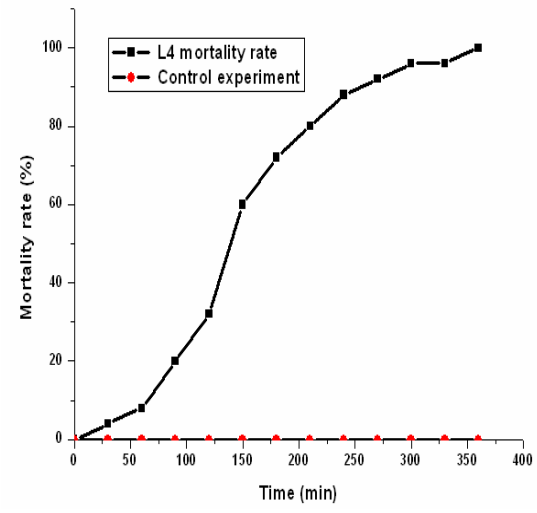

Figure2: Effect of *Bacillus thuringiensis var. israelensis* on Anopheles L4 larvae

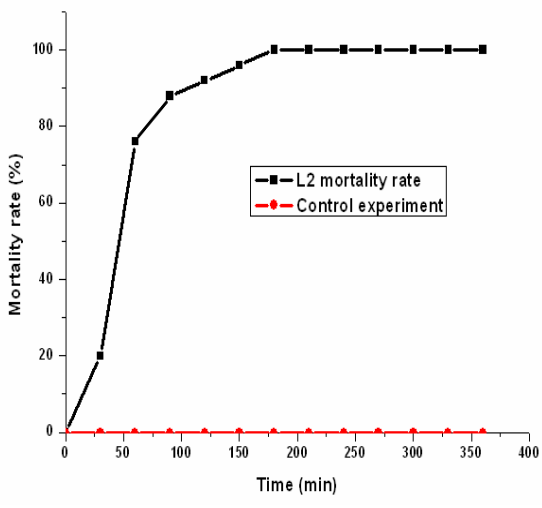

Figure3: Effect of *Bacillus thuringiensis var. israelensis* on culex L2 larvae

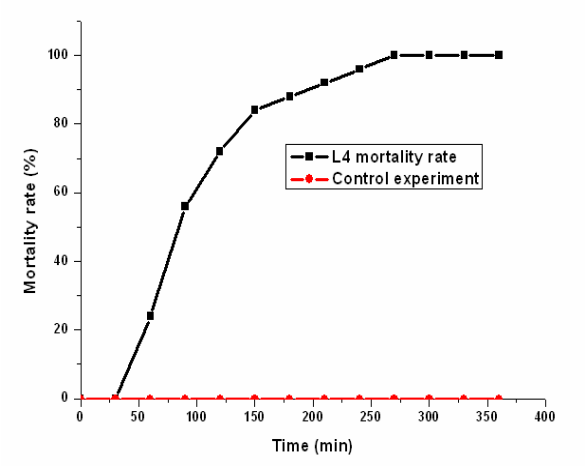

Figure4: Effect of *Bacillus thuringiensis var. israelensis* on culex L4 larvae

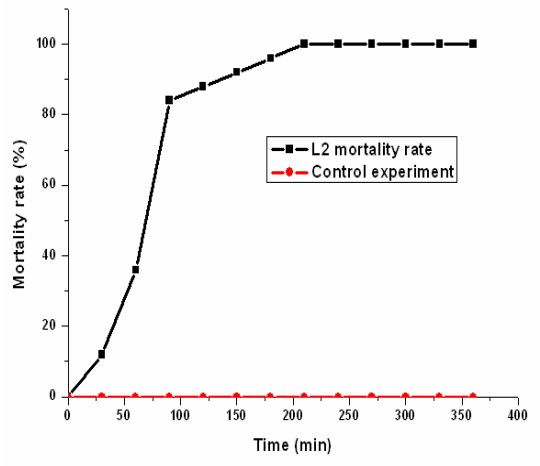

Figure5: Effect of *Bacillus thuringiensis var. israelensis* on Aedes L2 larvae

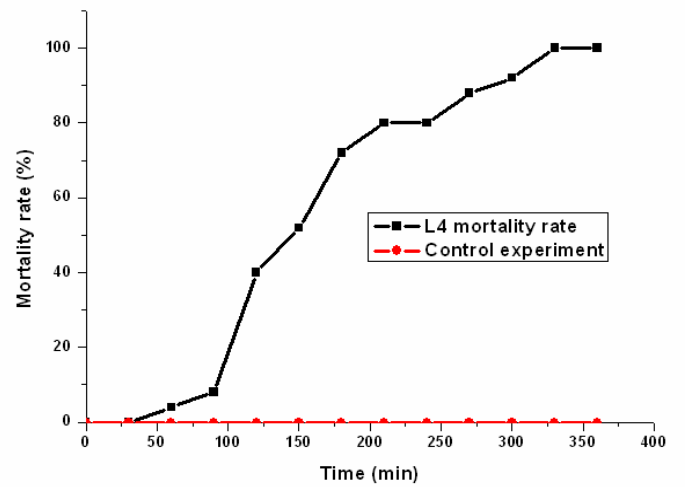

Figure6: Effect of *Bacillus thuringiensis var. israelensis* on Aedes L4 larvae

4- Conclusion

Since its commercial arrival in the early 1980's, *Bacillus thuringiensis var. israelensis* has been considered as an environmentally safe biopesticide for the control of mosquitoes and black flies. Compared to chemicals, the high degree of specificity, the low impact on non-target organisms and the short persistence have meant that *Bacillus thuringiensis var. israelensis* formulations are now useful in malaria vector control.

A promising future of this microbial control agent in mosquito control programs is ensured by its high efficacy, its specificity, its feasibility to be fermented on an industrial scale, its long shelf-life, its transportability, and finally and maybe the most important, there is actually no known field resistance documented until today.

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