Biological Control of the Dengue Transmitted Mosquitoes *Aedes aegypti* Using *Bacillus thuringiensis*

A. M. Mohammad

Biology Department, University College of Al-Darb, Jazan University, Jazan, 45142, Saudi Arabia

* Corresponding author E-mail: ayousuf@jazanu.edu.sa (A. M. Mohammad)

ABSTRACT

Dengue fever (DF) is a viral infection caused by the dengue virus. The virus is spread by the *Aedes aegypti* mosquito and is found in several Saudi Arabian cities, including Jazan. Chemical insecticides used in pest control programs can be harmful to human health and the environment. Therefore, there is a need for safe and effective alternatives to eliminate these pests. This study investigated the use of the bacteria Bacillus *thuringiensis* as a natural pest control method against the larval stages of *Aedes aegypti* mosquitoes. A laboratory experiment investigated the effectiveness of two Bacillus thuringiensis var. israelensis formulations (powder and liquid) against both young and adult *Aedes aegypti* mosquitoes. The study aimed to determine the lethal concentration (concentration causing 50% mortality) of B.t. *israelensis* against 4th instar larvae was 8.31x105 colony forming units (CFU) per milliliter for the liquid formulation and 6.72x105 CFU per gram for the wettable powder. The bioassay data further revealed that the wettable powder had a greater impact on pupation percentage and adult emergence compared to the liquid formulation. The liquid formulation of *Bacillus thuringiensis* was more effective at killing adult *Aedes aegypti* mosquitoes than the wettable powder formulation. The liquid formulation caused a higher mortality rate among adult mosquitoes, ranging from 49.33% to 64.23%, compared to the wettable powder formulation.

Keywords: Biological Control: *Aedes aegypti: Bacillus thuringiensis*.

INTRODUCTION

Flies and mosquitoes pose significant risks to public health by serving as carriers for various human diseases. Among these disease vectors, Culex and Aedes species, particularly *Aedes aegypti*, play a significant role in transmitting diseases like dengue fever (DF). The mosquito *Aedes aegypti* is the most common mosquito that spreads viral diseases around the world. This includes several cities in Saudi Arabia, like the Jazan area (Alhaeli et al., 2016). The climate of this region, characterized by high humidity, provides suitable breeding conditions for mosquitoes. Additionally, the presence of water storage containers serves as natural breeding sites for mosquitoes (Elisa et al., 2014). However, Using chemical insecticides to kill pests can be harmful to people and the environment, and many insects have become resistant to these chemicals. So, we need to find other ways to get rid of pests that are safe and effective. *Bacillus thuringiensis subsp israelensis* (Bti) is a bacteria that was first found in 1976. It kills mosquito larvae by making crystal proteins that paralyze their digestive system. This leads to a fatal infection called septicemia (Angelo et al., 2010; Boisvert, 2007). Researchers have also found that a fungus called Clonostachys spp can kill *Aedes aegypti* mosquitoes. Using biological control agents to kill insect pests that can spread diseases to humans is important for public health. It is a safer alternative to using chemical pesticides, which can harm people and the environment (Huang et al., 2017; Couret et al., 2020).

MATERIALS AND METHOD

Breading of Mosquitoes:

Mosquitoes undergo a four-stage metamorphosis, starting as eggs, then transitioning to larvae, pupae, and finally reaching adulthood. The eggs hatch, and the larvae go through four growth stages. Eventually, the fully developed larvae transform into pupae. Once mature, the pupae undergo further development and emerge as adults. The entire life cycle, from egg to maturity, typically spans 6 to 14 days (Wada, 1989).

Researchers employed a 7 cm diameter glass jar to observe mosquito reproduction. Ten adult mosquito pairs were confined together for 24 hours and provided a solid diet of wheat germ, sugar, and yeast (Concalves et al., 2013). Once the females laid eggs, these were collected and placed in running water. The eggs were then transferred to glass jars with filter paper and sustained on an artificial diet. This same diet was provided to the hatched larvae until they pupated and emerged as adult mosquitoes.

*Address correspondence to this author at the Department of Biology, University College of Al-Darb, Jazan University, Jazan, 45142, Saudi Arabia. *E-mail: ayousuf@jazanu.edu.sa

***Bacillus thuringiensis* Strains**:

Researchers obtained strains of the bacteria *Bacillus thuringiensis israelensis* from the Agricultural College at Ain Shams University in Cairo, Egypt. To increase the number of bacteria, they grew them in a liquid nutrient medium called LB medium. Afterwards, the bacteria were stored on a solid nutrient medium called slant agar medium.

Production of Delta-Endotoxin:

The researchers prepared the inoculum by adding a small amount of bacteria from a slant agar culture to 10 ml of broth medium in a shake flask using a loop. This initiated the growth of the bacteria in LB medium. The mixture was then placed in a rotary shaker set at 28°C and 200 rpm for a duration of 8 hours, allowing the bacteria to grow and multiply. After this initial incubation, 3% (v/v) of the culture was transferred into a larger volume of 500 ml of fresh LB medium. The culture was once again placed on the rotary shaker, following the same conditions as before, and left to incubate for 2 days. At the end of the incubation period, the culture was subjected to centrifugation to collect the sporulated culture. After harvesting, the cake-like biomass was used in the preparation of the formulation (Mehrabi et al., 2015).

Preparation of ɓ- Toxin Formulation:

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

A liquid formulation was prepared using a concentrated suspension of Bti spore-crystal complex. This suspension was created by mixing the complex with various additives like detergents, emulsifiers, UV protectants, and dispersants. These additives prevented the bacteria from settling rapidly. The final bacterial concentration in the suspension was adjusted to 3 x 10^7 colony-forming units (CFU) per milliliter (ml). Prior to incorporating the suspension into the diet, 10 ml of it was homogenized with other ingredients using a glass apparatus (Ejiofor & Okafor, 1991).

Fly ash served as the carrier material for a water-dispersible powder formulation. The mixture was dried at 45°C and then pulverized into a fine powder. This powder was sieved to achieve a particle size of less than 30 micrometers. After adjusting the moisture content to 5%, the powder was stored. The final product was a fine, gray powder that readily dispersed in water. The bacterial concentration in this powder formulation was also adjusted to 3 x 10^7 CFU per gram (Lopez et al., 2010).

Bioassay:

The laboratory experiments employed both early 4th instar larvae and adult *Aedes aegypti* mosquitoes for bioassay testing.

Against Immature Stages:

Ten fourth-instar mosquito larvae (*Aedes aegypti*) were raised in plastic containers and given an artificial food source. The larvae were kept at a constant temperature of 28°C. Five different concentrations of the Bti formulation were mixed into the food, with three replicates for each concentration. Mortality was monitored and documented every 24 hours. The experiment was conducted in a completely randomized manner. Probit analysis was used to determine the 50% lethal concentration (LC50), slope, and confidence intervals. A control group was also included, in which no Bti was added to the food.

Against Adults:

Ten adult mosquitoes were starved for 12 hours and then placed on plastic plates with a diameter of 14 cm. Mosquito larvae were provided with artificial food containing varying concentrations of a bacterial formulation. The experiments were conducted in a randomized manner, with three replicates for each treatment. A control group was included, in which larvae were fed food without the bacterial formulation. Mortality was recorded daily for seven days and corrected using Abbott's formula 1925. Additionally, the percentage of larvae that pupated and emerged as adults was calculated.

Statistical Analysis:

The recorded mortality data for both immature and mature stages underwent mortality analysis of variance (ANOVA). Statistical analysis was performed using Tukey's test to compare the mean results, with a significance threshold of 0.05. Mortality in the control group (ranging from 5% to 20%) was adjusted using Abbott's formula. The corrected mortality data were then analyzed using a mortality-concentration regression model to determine the LC50 and LC90 values. This analysis was conducted using specialized statistical software.

RESULTS

Bacterial Culture and Toxin Production:

Acillus thuringiensis colonies exhibit distinct characteristics on LB agar medium after 24 hours of incubation. Their appearance can be described as large, cream-colored, and expansive. To induce sporulation, these colonies are subsequently incubated in a shaking incubator over a 5-day period. Following the completion of sporulation, a centrifugation is used to isolate crystal toxin, spores, and cellular debris from the culture broth. This centrifugation is carried out at a speed of 12,000 revolutions per minute and sustained for 10 minutes.

Larval Bioassay:

The results of the bioassay indicated that the susceptibility of mosquito larvae to *Bacillus thuringiensis israelensis* increases with higher concentrations of spore crystals. The findings also showed that the powder formulation was more effective against 4th instar larvae of *Aedes aegypti* compared to spore-crystal liquid suspensions, as shown in Table 1. The 50% lethal concentration (LC50) for the suspensions was more than 20% higher than that of the powder formulation in the larval bioassay.

When tested against a target organism, the liquid formulation demonstrated an LC50 value of 8.31 x 105 CFU/l and an LC90 value of 8.93 x 108 CFU/l. In comparison, the powder formulation exhibited an LC50 value of 6.72 x 105 CFU/l and an LC90 value of 6.58 x 108 CFU/l. Statistical analysis confirmed a substantial difference ($P<0.001$) between the two formulations in terms of their LC50 and LC90 values. Notably, the powder formulation displayed enhanced efficacy, necessitating a lower concentration to achieve the same level of control compared to the liquid formulation.

Table 2 presents data on the impact of varying concentrations of B.t.i. on the pupation rate, malformation of pupae, and emergence of adults. Notably, a clear pattern emerged: as the concentration of B.t.i. increased, the proportion of insects that successfully pupated declined significantly. The wettable powder formulation resulted in a 36% reduction in pupation percentage, with a malformed pupae percentage of 9.6%. The adult emergence percentage was only 23.2%, with 17.3% of the adults being malformed. In contrast, the liquid formulation resulted in a pupation percentage of 49%, a malformed pupae percentage of 6.3%, an adult emergence percentage of 22.6%, and a malformed adults percentage of 9.8%.

Adult Bioassay:

The efficacy of the formulations was evaluated in bioassays targeting the adult stage, and the mortality values were recorded, as shown in Table 3. The wettable powder formulation exhibited the highest activity against *Aedes aegypti*, with a mortality value of 64.23% (LC50=9.73 x 105), and 95% Confidence Limits of 1.321 and 3.564. The liquid formulation, on the other hand, showed significantly different mortality values compared to the wettable powder formulation ($P<0.01$). The mortality value for the liquid formulation was 49.33% (LC50=12.88 x 105), with 95% Confidence Limits of 0.797 and 1.966.

DISCUSSION

Mosquitoes are known vectors of diseases such as dengue fever, prompting the search for effective and eco-friendly control methods (Priest, 1992). Chemical pesticides have faced challenges due to insect resistance and environmental concerns. *Bacillus thuringiensis israelensis* has emerged as a promising alternative to chemical insecticides. This gram-positive bacterium produces toxic crystal proteins during sporulation. These crystal toxins specifically target insect pests and are considered environmentally safe (Roh et al., 2007).

Bioassay studies have shown that B.t. toxin is more effective against first instar larvae than fourth instar larvae, and pupae are not affected by the bacterium or its toxin (Mulla et al., 1990).

Numerous studies have demonstrated the susceptibility of mosquito larvae and adults to B.t. israelensis toxins. Cossentine et al. (2016), Shishir et al. (2015), and Saravanan et al. (2017) tested various water-dispersible powder formulations, including a new isolate (LFB-Fiocruz), against *Aedes aegypti* larvae. The LC50 values for the tested formulations were found to be low, indicating high efficacy. Other research by Gad & Al-Dakhil (2018) and Zaki et al. (2020) investigated the biological effects of B.t. on dipteran insects, including pupation percentage, malformation, and adult emergence. Their findings support the effectiveness of B.t. as a mosquito control agent.

CONCLUSION

Bacillus thuringiensis is a promising biological control agent for combating the mosquito pest *Aedes aegypti*. This study investigated the efficacy of two B.t.

formulations, a water-dispersible powder and a liquid formulation, against different life stages of the insect. The results showed that both formulations exhibited varying mortality rates, with larvae being more susceptible than mature stages. The water-dispersible powder formulation was found to be more effective in reducing pupation and adult emergence compared to the liquid formulation.

REFERENCES

Abbott, W.S. 1925: A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology,* 18:265-267.

Alhaeli, Alaa, Bahkali, S., Anna Ali, El-Metwally, A. 2016: The epidemiology of Dengue fever in Saudi Arabia. *Journal of Infection and public health,* 9(2): 117-124.

Angelo, E.A., Vilas, G.T., Castro, R.J. 2010: *Bacillus thuringiensis* general characteristics and fermentation. *Semina: Ciencias Agranas*, 31(4), 945-958.

Biosvert, M. 2007: Utilization of *Bacillus thuringiensis* var. israelensis, *B.t.* based-formulations for the biological control of mosquitoes in Canada. 6th Pacific Rim Conference on the biotechnology of *Bacillus thuringiensis* and its environmental impact. *Victoria BC, Canada,* Oct. 30 –Nov. 3, 2005. Pp. 87-93.

Convalves, R.S., Nava, D.E., Valgas, R.A. 2013: Biology and fertility life table of Agmapis pelleranoi, in larvae of *Arasterapha fraterculus*. *Annals of the Entomological Society of America,* 106: 791-798.

Cossentine, J., Robertson, M., Xy, D. 2016: Biological activity of *Bacillus thuringiensis* in *Drosophila suzkii*. *Journal of. Economic Entomology,* 109: 1071-1078.

Couret, J., Notarangelo, M., Veera, S., LeClaire-Conway, N., Ginsberg, H.S., LeBrun, R.L. 2020: Biological control of *Aedes* mosquito larvae with carnivorous aquatic plant, *Utricularia macrorhiza.* Parasites Vectors, 13,208. 08 https://doi.org/10.1186/s13071-020-04084-4.

Elisa, M.Z., Gamil, M.A., Eifan, S.A., Al-Sum, B.A. (2014). Prevalence of Dengue Fever in Jizan Area, Saudi Arabia. *Journal of Pure* and *Applied Microbiology*, 8(1), p. 225-231.

Ejiofor, A., Okafor, N. 1991: Formulation of a flowable liquid concentrate of *Bacillus thuringiensis* serotype H-14 spores and crystals as mosquito larvicide. *Journal of Applied Bacteriology,* 71 (3): 202-206.

Huang, Y.S., Higgs, S., Dana Vanlandingham, L. 2017: Biological Control Strategies for Mosquito Vectors of Arboviruses. Insects 2017, 8, 21; doi: 10.3390/insects8010021

Gad, A., Al-Dakhil, A. 2018: Efficacy of *Bacillus thuringiensis israeleinsis* and for plant extracts on the mortality and development of Culex quinquefasciatus Say. *Egyptian journal of biological pest control,* 28 (62), 1-5.

Lopez, J., Arantes, O., Cenci, M. 2010: Evaluation of a new formulation of *Bacillus thuringiensis israelensis. Brazilian Journal* of *Biology*,70 (4), 1109-1113.

Mehrabi, M., Zoghimofrad, L., Mazinani, M. 2015: A study of the effect of *Bacillus thuringiensis* serotype H14 (subspecies israelensis) delta endotoxin on Musca larvae. *Turkish Journal* of *Medical Sciences*, 45: 794-799.

Mulla, M.S., Darwazeh, H.A., Zgomba, M. 1990: Effect of some environmental factors on the efficacy of *Bacillus sphaericus* 2362 and *Bacillus thuringiensis* (H-14) against mosquitoes. *Bulletin of the Society of Vector Ecology,* Vol.15, pp.166–175.

Priest, F.G. 1992: A review. Biological control of mosquitoes and other biting flies by *Bacillus sphaericus* and *Bacillus thuringiensis*. *Journal of Applied Bacteriology,* Vol.72, pp.357-369.

Rodrigues, J., Luiz, F.N., Martinez, R.J.M., Montalva, C., Humber, R.A., Luz, C. 2022: *Clonostachys* spp., natural mosquito antagonists, and their prospects for biological control of *Aedes aegypti.* Parasitology research, 121, 2979-2984.

Roh, J.Y., Choi, J.Y., Li, M.S., Jim, B.R., Je, Y.H. 2007: *Bacillus thuringiensis* as a specific, safe and effective tool for insect pest control. *Journal of Microbiology and Biotechnology,* 17: 547-559.

Saravanan, Tamilsevan, Arulsamy Mary Manonmani, Purushothaman Jambulingam Indian. 2017: Fly ash-based water dispersible powder formulation of *Bacillus thuringiensis var. israelensis*: Development & laboratory evaluation against mosquito immatures. *Journal of Medical Research*, 146, December 2017, pp 714-721 DOI: 10.4103/ijmr.IJMR_651_15.

Scott, A., Luke, R., Benjamin, S. 2010: *Bacillus thuringiensis* var *israelensis* provides residual control of *Ades aegypti* in small containers. *American Journal of Tropical Medicine and Hygiene,* 82(6), 1053-1059.

Shishir, M.A., Akter, A., Bodiuzzaman, M., Hossain, A., Hoq, M.M. 2015: Novel toxicity of *Bacillus thuringiensis* strains against the melon fruit fly, *Bactrocera cucurbitae*. *Biocontrol Science*, 20: 115-123.

Wada, Y., Takajai, M., Tsuda, Y. 1989: Distribution of Mosquitoes on a Hill of Nagasaki City, with Emphasis to the Distance from Human Dwellings. *Tropical Medicine*, 33 (3), 55-60.

Zaki, A.Z., Nazri, C., Alhothily, I. 2020: Efficacy of *Bacillus thuringiensis* treatment on Ades

population using a different application. *Tropical Medicine and Infectious Disease*, 5, 67.

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.

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.

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

المكافحة البيولوجية لبعوض حمى الضنك ،الزاعجة المصرية ،باستخدام *Bacillus thuringiensis*

عبير محمود محمد

قسم الأحياء, الكلية الجامعية بالدرب, جامعة جازان, المملكة العربية السعودية.

* البريد الإليكتروني للباحث الرئيسي: ayousuf@jazanu.edu.sa

الملخص العربي

حمى الضنك (DF) هو مرض فيروسي يسببه فيروس حمى الضنك الذي ينتقل عن طريق بعوضة الزاعجة المصرية *Ades aegypti* ويستوطن في بعض مدن المملكة العربية السعودية مثل منطقة جازان. وبسبب سمية المبيدات الحشرية الكيميائية المستخدمة في برامج مكافحة الآفات، لذلك دعت الحاجة إلى استخدام طرق بديلة فعالة وآمنة للقضاء على الآفات. لذلك هدفت الدراسة الحالية إلى استخدام بكتيريا *Bacillus thuringiensis israelensis* كمبيد حيوي ضد الأطوار اليرقية لحشرة *Ades aegypti*. تم استخدام السلالة البكتيرية في مستحضرين مختلفين (مسحوق قابل للانتشار في الماء ومستحضر سائل) وتم فحص سميتها ضد المراحل غير الناضجة والبالغة من *Ades aegypti* في المختبر. أشارت النتائج إلى أن التركيز القاتل 50 % لبكتيريا *B.t.israelensis* ضد العمر الرابع، كان $8.31 x 10^5$ CFU/ ml في التركيبة السائلة و $6.72 x 10^5$ CFU/ ml للمسحوق القابل للبلل. كما أظهرت بيانات الاختبار الحيوي أيضًا أن نسبة التشرنق وظهور البالغين تأثرت أكثر عند إخضاع اليرقات للتغذية بالمسحوق قابل للبلل مقارنة بالتركيبة السائلة. حيث تراوحت قيم الوفيات للبالغين بين 49.33 و64.23% عند استخدام التركيبة السائلة والمسحوق القابل للبلل على التوالي.

الكلمات الاسترشادية: الزاعجة المصرية، المكافحة الحيوية، *Bacillus thuringiensis*