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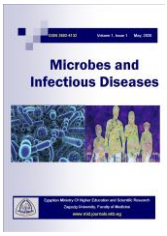

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Original article

Bio-larvicidal activity of *Bacillus thuringiensis* on the larvae of chikungunya virus vector

Abdirasak Sharif Ali Mude[1] Yahye Ahmed. Nageye[1] Kizito Eneye Bello[2]

1- *Faculty of Medicine and Health Sciences, SIMAD University, Mogadishu, Somalia. 252.*
2- *Department of Microbiology, Faculty of Natural Science, Kogi State (Prince Abubakar Audu) University, Anyigba. PMB 1008, Anyigba, Kogi State, Nigeria.*

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ABSTRACT

Background: Mosquitoes carry numerous diseases, such as malaria, chikungunya, yellow fever, dengue fever, and lymphatic filariasis. The control of these diseases is geared towards vector control. Still, using artificial chemical insecticides for mosquito control has been linked to the emergence of resistance and harmful impacts on both humans and the environment. **Methods**: Adult mosquitoes were collected, sorted, and identified using a standard identification key. Aedes spp were bred in an enclosed setting and fed with albino rat blood, sugar, and yeast. Third-stage instar larvae were used for larvicidal screening. *Bacillus thuringiensis* (Bt) was isolated and characterized following conventional microbiological and molecular methods. Bt bioassay was prepared into three concentrations (0.5mg/mL, 0.25mg/mL, 0.125mg/mL). **Results**: As the concentration of Bt increases, there is a significant decrease in the number of surviving larvae. There was a correlation between the concentration and duration of Bt exposure and the death of larvae. The higher the concentrations and more extended exposure periods, the higher the effectiveness of the Bt bioassay. A 0.5mg/mL concentration had the highest mortality rate and efficacy compared to 0.125mg/mL. **Conclusion**: The larvicidal assay revealed the effectiveness of Bt against Aedes larvae. The higher the concentration, the higher the mortality rate. These findings contribute to the ongoing efforts in integrated vector management, providing a sustainable and ecologically friendly alternative to synthetic insecticides for managing mosquito-borne diseases.

Introduction

The incidence of vector-borne diseases is still a significant healthcare challenge globally [1]. Mosquitoes are one of the most successful and adaptable insects in the world. Mosquitoes are agents of several diseases ranging from viral to protozoan diseases [2]. The associated morbidity of arboviral diseases in tropical and subtropical nations is still very high [3].

Several mosquito species are vectors for transmitting various diseases, such as malaria, chikungunya, zika, dengue, and yellow fever. They also cause environmental nuisance and human unrest due to their biting habits [4].

The persistent increase of diseases transmitted by vectors presents a significant obstacle to worldwide public health, requiring creative approaches for controlling vectors [5]. Chikungunya virus (CHIKV) is a notable disease that poses a

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* *Corresponding author:* Abdirasak Sharif Ali Mude
E-mail address: *arshamyare@gmail.com*

danger, leading to extensive outbreaks and generating severe symptoms in affected people [6]. The primary carrier of CHIKV is the Aedes mosquito, specifically *Aedes aegypti* and *Aedes albopictus* species [7]. These mosquitoes are most common in tropical and subtropical areas, increasing the likelihood of transmission [8].

Bacillus thuringiensis (Bt) is a viable contender for effective vector control techniques [9]. Bt, a widely distributed Gram-positive bacteria, has attracted interest due to its strong larvicidal action against many mosquito species, particularly those involved in the transmission of CHIKV [10]. This bacterium synthesizes a wide range of insecticidal proteins, referred to as δ-endotoxins or Cry toxins, that exhibit specific toxicity towards insect larvae while harmless to humans and other non-target animals [11].

Investigation into the larvicidal characteristics of Bt against CHIKV vector larvae has provided persuasive knowledge regarding its effectiveness and mode of operation. Multiple studies have extensively recorded the efficacy of Bt formulations in specifically targeting Aedes larvae at different phases of development, including early instars and pupae [12-14]. This disruption of their life cycle efficiently prevents the emergence of adult mosquitoes. The mechanism by which Bt toxins work includes larvae consuming them, which is then followed by their activation through proteolysis in the alkaline environment of the midgut [13]. This activation leads to the creation of pores in the epithelium of the gut, resulting in paralysis and eventual death of the gut [15].

Moreover, studies on the ecological consequences of Bt-based larvicides have highlighted their potential as eco-friendly substitutes for traditional chemical insecticides [16]. Contrary to wide-ranging pesticides, Bt formulations have a particular affinity for specific mosquito species, hence reducing the risk of harm to helpful insects and non-target creatures in aquatic ecosystems [17]. Moreover, the ability of Bt toxins to naturally break down in the environment addresses worries about any lingering environmental pollution, providing a sustainable method for controlling disease-carrying organisms [18].

Although Bt-based larvicides have favorable characteristics, there are still difficulties in maximizing their implementation and effectiveness in practical environments. Obstacles to wider deployment include mosquito resistance development, the formulation's stability, and the viability of operational procedures [19,20]. To tackle these issues effectively, it is crucial to foster interdisciplinary collaboration involving the fields of entomology, microbiology, biotechnology, and public health [21]. This collaboration will allow us to fully use the potential of Bt-based interventions in the fight against CHIKV and other diseases transmitted by vectors [22].

Thus far, conventional pesticides have been the primary means employed to battle mosquitoes [23]. Nevertheless, synthetic pesticides are known to induce health complications and contaminate the ecosystem [24]. In addition, certain artificial insect repellents can induce encephalopathy in youngsters [25]. Hence, there is a pressing demand for efficient and secure alternatives to traditional pesticides. Regarding natural pesticides, essential oils are employed to combat adult mosquitoes [26]. However, it is important to note that while these oils can deter mosquitoes, they cannot eliminate them. *Bacillus thuringiensis*, the most effective bioinsecticide, has been utilized for 30 years to control mosquito larvae and continues to be used presently [27]. This bacterium possesses numerous advantages compared to traditional pesticides. It exhibits specificity towards certain pest species, is environmentally friendly, and does not pose a threat to non-target organisms [11, 27]. Additionally, mosquitoes have not yet demonstrated considerable resistance to this bacterium in field conditions. Because of the increasing resistance to chemical insecticides and the lack of new alternative methods to control mosquitoes, the search for bio-control agents has given rise to several larvicidal studies [19- 21, 28, 29].

The present study, Bio-larvicidal activity of *Bacillus thuringiensis* on mosquito larvae, was carried out using *Bacillus thuringiensis* isolated from dead larvae of *Aedes aegypti.*

Material and methods

Mosquito identification method

Adult mosquitoes were collected using a CDC insect trap in the early morning and the late hours before dusk. Standard identification keys were used to identify and sort mosquitoes into their respective strata as described by Sharma et al. [30]. The retrieved Aedes mosquito species were bred under suitable conditions.

Mosquito collection and breeding

Female Aedes mosquitoes fed on albino rat blood were captured from the field sampling mosquito collections. The methodology for collecting indoor resting mosquitoes followed the guidelines outlined by Kim et al. [31]. The captured mosquitoes were transferred to the insectary and kept in cages, where they were raised and nourished with a 10% sugar solution [32]. Upon noticing the gravidity of the mosquitoes, egg cups were introduced into the cages. Filter sheets were positioned over the egg cups with 30ml of water to ensure the filter paper was constantly moist. Following the mosquito's gravid state, the eggs are gathered and placed into three containers measuring 45×20cm, each filled with unchlorinated distilled water [33]. Food was withheld from the containers until the first instar emerged. At this point, they were nourished with a yeast diet of 10% concentration [34]. Sieving was performed when the water became contaminated. The process was iterated until the larvae reached the third instar of development.

Isolation and identification of test organism (*Bacillus thuringiensis*)

Wild mosquito larvae were gathered from their native breeding grounds. The specimens were cleansed using sterile distilled water and then crushed by introducing 1 ml of sterile distilled water with a glass rod. The suspension was further diluted in a series of steps until reaching a dilution factor of 10-7. Specimens from different dilutions were cultured on a nutrient agar medium and placed in an incubator at 37 ºC ± 2ºC for 24 hours. The morphological features were observed after 24 hours. The isolates were identified using morphological characterization, standard biochemical procedures, and molecular characterization [35]. The processes of Gram staining and spore staining were executed. The conducted biochemical tests include the assessment of catalase, indole, coagulase, and amylase activities [36].

DNA was extracted from the isolated bacteria suspected to be Bacillus sp based on their previous morphological and biochemical tests using a Machery Negal DNA extraction kit with strict adherence to the manufacturer's instructions.

The PCR reactions utilized the following primers: REP1 5′-IIIICGICGICATCIGGC-3′, REP2 5′-ICGICTTATCIGGCCTAC-3′, ERIC1 5′-ATGTAAGCTCCTGGGGATTCAC-3’ and ERIC2 5′-AAGTAAGTGACTGGGGTGAGCG-3′ [37]. The amplification reactions were conducted in a 30µL thermoscientific PCR master mix reaction volume, utilizing 20 ng of genomic DNA. The amplifications were conducted using a thermocycler Mastercycler (Eppendorf, Hamburg, Germany) with the following thermocycler conditions: An initial denaturation at 94°C for 1 minute, followed by 30 cycles at 94°C for 60 seconds and an initial extension at 72°C for 2 minutes. The final extension was performed at 72°C for 10 minutes. The amplicons underwent gel electrophoresis using a 2.0% agarose gel stained with DNA fluorochrome stain and X0.5 TBE buffer at 80 volts and 400 watts for 60 minutes. The separated bands were observed using a UV transilluminator, and the gel images obtained were effectively saved.

The resulting amplicons were sequenced using Sanger's method, and the resulting sequences were blasted in the National Center for Biotechnology Institute. A phylogenetic tree was constructed using the neighbor-joining method.

***Bacillus thuringiensis* bioassay**

The isolated *Bacillus thuringiensis* was subjected to starvation by inoculating them into a cocktail containing 1% sugar and no carbon source for 48 hours at 37 ºC ± 2ºC in an orbital shaking incubator (IVigene, Germany). The resulting culture was centrifuged at 1000 rpm for 30 minutes and was then filtered and concentrated to powder using lactose-acetone precipitation as described by Yasutake et al. [38].

The *B. thuringiensis* isolate was diluted in 10 ml of sterile water, resulting in an average count of 109 colony-forming units (CFU)/ml. This diluted solution was then utilized for initial assessments of larvicidal activity against 3rd instar larvae. Three different concentrations were made via a two-fold dilution method according to the standard for larvicidal testing. The concentrations were 0.5mg/mL, 0.25 mg/mL, and 0.125 mg/mL [39].

Experimentation

One hundred eighty healthy third instar Aedes larvae were chosen and divided into three treatments, each consisting of 60 larvae. These treatments were then reproduced three times. The initial, subsequent, and third treatments were administered with 2 ml of 0.5mg/mL of the bioassay into a vial containing 8 mL of sterile distilled water and 60 larvae. They were repeated for 0.25 mg/mL and 0.125 mg/mL, respectively. At the same time,

the control group received no *Bacillus thuringiensis* treatment.

Mortality determination and relative mortality rate

Mortality was assessed by employing a glass rod to ascertain the viability of the larvae after one hour. The glass rod was immersed into the solution containing the larvae and positioned near the deceased larvae under suspicion [40]. If the larvae are alive, they would promptly exhibit either movement or bending, resulting in a wrinkle-like motion, thereby confirming their vitality. When the mortality rate of the control group is more than 10%, Abbot's formula is employed to adjust the mortality rate in the treatment group.

$$M=Mo-Mc/100-Mc\times100$$

Where Mo = Observed mortality

Mc = Control mortality

Data analysis

The data obtained from this study was inputted into SPSS version 21.0. The mortality of mosquito larvae was assessed using Analysis of Variance (ANOVA) to identify any significant differences across all variables. The tests were conducted using a confidence interval of 95% and a significance level 0.05.

Results

Three isolates suspected to belong to the genus Bacillus were recovered. They were oval to spherical colonies with milky to yellowish areal coloration, raised elevation, an entire edge, and an oval spore shape, with evidence of crystal formation. All the isolates tested positive for Gram's reaction, catalase, and coagulase test. The isolates had similar sugar fermentation ability. Details of the morphological characteristics are presented in **Table 1**.

The molecular characterization revealed that the tested isolates were approximately 300bp in molecular weight **(Figure 1)** and were of close ancestral lineage with *Bacillus thuringiensis* with a low divergence score, as presented in **Figure 2**. The molecular analysis reveals that the three isolates were *Bacillus thuringiensis*. Therefore, an isolate with code BT-1 was used for the larvicidal assay.

The efficacy of *Bacillus thuringiensis* (Bt) against Aedes larvae at different doses and exposure times is demonstrated **Figure 3**. **Table 2** presents the exact number of Aedes larvae alive after exposure to various concentrations of Bt over specific periods. As the concentration of Bt increases, there is a significant decrease in the number of surviving larvae. At higher concentrations and longer exposure times, complete larval mortality is observed. The efficacy of Bt therapy is further emphasized by the percentage of larval mortality presented in **Figure 3**. With greater concentrations and more extended periods of exposure, there is a noticeable and substantial rise in the death rate of larvae, with mortality approaching 100% in certain instances. This highlights the strong larvicidal efficacy of Bt against Aedes larvae. The data demonstrate a correlation between the concentration and duration of Bt exposure and the death of larvae. Higher concentrations and more extended exposure periods lead to increased effectiveness.

Table 1. Morphological and biochemical characteristics of Bacillus sp isolates.

Variables	Isolate code		
	BT-1	BT-2	BT-3
Morphological traits			
Shape	Rod	Rod	Rod
Edge	Entire	Entire	Entire
Elevation	Raised	Raised	Raised
Arial color	Cream-Yellow	Cream-Yellow	Cream-Yellow
Spore formation	+	+	+
Shape of spore	Oval	Oval	Oval
Biochemical traits			
Gram stain	+	+	+
Catalase	+	+	+
Coagulase	+	+	+
Citrate	+	+	+
Sugar fermentation			
Glucose	+	+	+
Maltose	+	+	+
Xylose	-	-	-
Arabinose	+	+	+
Sucrose	+	+	+
Suspected isolate	Bacillus spp	Bacillus spp	Bacillus spp

Key: +, Positive; -, Negative

Table 2. Number of larvae at different times and concentrations of *Bacillus thuringiensis*.

Concentration	**Number of Aedes larvae alive**				
	15mins	**30mins**	**60mins**	**12hrs**	**24hrs**
Negative (water)	**60**	**60**	**60**	**60**	**60**
0.5mg/mL	**20**	**8**	**4**	**0**	**0**
0.25mg/mL	**31**	**13**	**4**	**0**	**0**
0.125mg/mL	**47**	**15**	**0**	**0**	**0**

Figure 1. Agarose gel image showing the molecular weight of *Bacillus thuringiensis* (300bp).

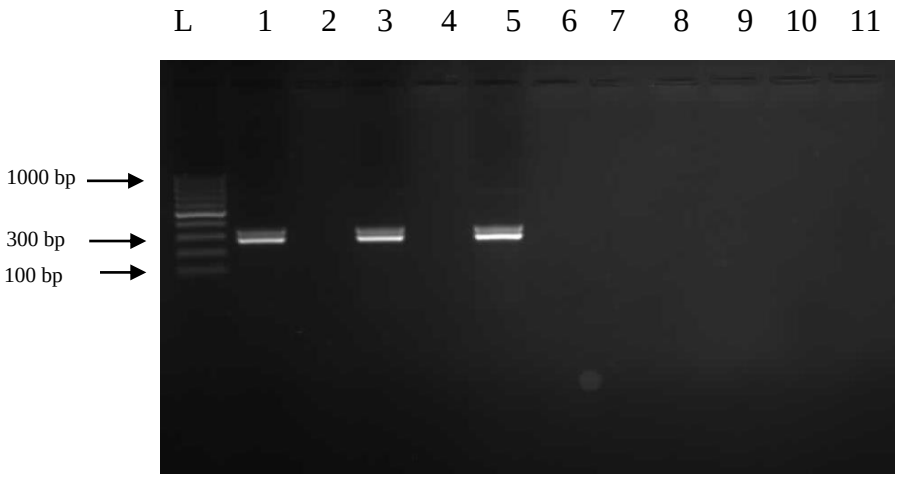


Figure 2. A neighbor-joining method phylogenetic tree showing the genomic relatedness of BT-1, BT-2, and BT3.

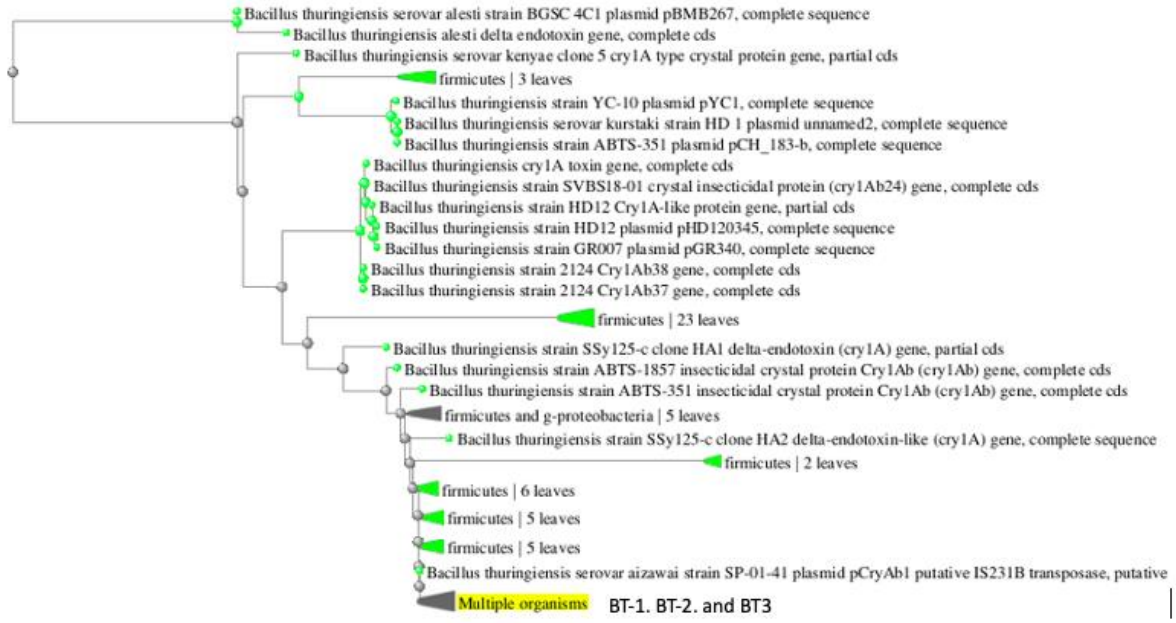
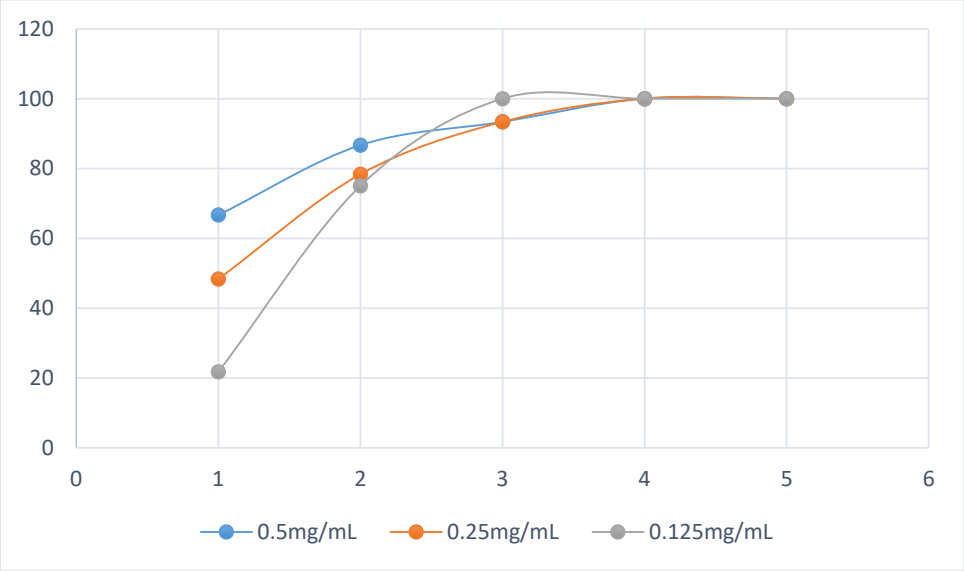


Figure 3. Percentage mortality of Aedes larvae on different concertation of *Bacillus thuringiensis* bioassay.



Discussion

The burden of mosquito-borne diseases is on the rise globally [41,42]. Efforts towards controlling and managing these diseases are targeted towards vector control schemes [43, 44]. The use of synthetic insecticides has both environmental and health implications [24, 45-47]. The emergence of novel concepts and approaches towards vector-borne disease control and prevention through integrated vector management has wholly modified the traditional narrative of vector control. This study focuses on the detailed analysis of the physical, chemical, and genetic properties of Bacillus isolates that are believed to be part of the Bacillus genus.

Additionally, it examines the effectiveness of *Bacillus thuringiensis* (Bt) in killing Aedes

larvae. The findings are crucial for comprehending the potential of these isolates as biological control agents against disease vectors such as Aedes mosquitoes. This study focuses on the bio-larvicidal activity of *Bacillus thuringiensis* on the larvae of the chikungunya vector. There was a high mortality rate of larvae after the tested bioassay intervention. The findings of this study are in alliance with the report of others [19 -21, 28].

The morphological and biochemical characteristics of the isolates align with the standard characteristics commonly seen in Bacillus species. The sugar fermentation assays demonstrated comparable metabolic activity among the isolates, providing additional evidence for their identification. The results are consistent with earlier reports describing Bacillus species' morphological and chemical characteristics [9, 27, 38].

The molecular study yielded additional information regarding the genetic similarity of the isolates, indicating a strong ancestral connection with *Bacillus thuringiensis* (Bt). Furthermore, the neighbor-joining phylogenetic tree had low divergence scores, suggesting a significant level of genomic similarity between the isolates and Bt reference strains. The findings of this study are in alliance with other reports [48, 49]. The molecular characterization confirms that the isolates are *Bacillus thuringiensis*, verifying their suitability for larvicidal uses. The larvicidal assay confirmed the effectiveness of *Bacillus thuringiensis* against Aedes larvae. The findings of this study demonstrate a correlation between the dosage and the reaction, where higher concentrations and longer exposure durations led to a higher rate of larval death. The probable reason for this bioassay potency could be attributed to the Bt crystal proteins that contain chitinase and Beta-lactamase enzymes that degrade the chitins and cell walls of insects and mosquito larvae. The findings of this study are in consonant with the report of others [21, 28, 29]. Significantly, total larval death occurred at elevated concentrations over 24 hours of exposure, underscoring the efficacy of Bt as a larvicide. The findings align with prior research that assessed the larvicidal efficacy of *Bacillus thuringiensis* against mosquito larvae [9, 15,27]. The effectiveness of Bt toxins as a biological control agent is highlighted by their ability to target the midgut epithelium of insect larvae, causing paralysis and death [27].

Conclusion

This study has provided significant findings about the potential of Bacillus isolates, specifically *Bacillus thuringiensis* (Bt), as biological agents for controlling Aedes larvae responsible for transmitting mosquito-borne diseases like chikungunya. The larvicidal assay revealed the effectiveness of Bt against Aedes larvae. The higher the concentration, the higher the mortality rate. These findings contribute to the ongoing efforts in integrated vector management, providing a sustainable and ecologically friendly alternative to synthetic insecticides for managing mosquito-borne diseases. The study affirms the promise of *Bacillus thuringiensis* as a promising instrument in combating mosquito-borne diseases, providing a safer and more focused method for controlling larvae.

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Conflicts of interest

The authors declare that they do not have any conflict of interest.

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