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Biocontrol of Mosquito Larvae using *Bacillus thuringiensis* Isolated from the Soil

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ABSTRACT

A study on the efficacy of *Bacillus thuringiensis* on larvae of mosquitoes aimed at introducing the potential of *Bacillus thuringiensis* isolated from the soil as a natural control agent of mosquitoes by comparing two species; *Aedes albopictus* and *Culex pipiens* was undertaken. A total of 200 (one hundred each) mosquitoes of the two species *Aedes albopictus* and *Culex pipiens* were tested with *Bacillus thuringiensis* isolated from the soil sample. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of the *Bacillus thuringiensis* isolate were prepared at different concentration; 0.25ml, 0.12ml and 0ml (negative control), and were added to the *Aedes albopictus* and *Culex pipiens* at the same dosage. Results showed that 0ml (negative control) had little effect on both species, 0.25ml had great effect on both species, followed by 0.12ml. In *Culex pipiens* and *Aedes albopictus*, 10^{-5} had the lowest mortality rate, while 10^{-1} had the highest mortality rate. In *Aedes albopictus*, the mortality rate observed at 0.25ml was significantly higher than the mean mortality recorded at 0.12ml at 10^{-3} and 10^{-4} respectively, while the mean mortality of *Culex pipiens* larvae recorded at 0.12 ml and 0.25 ml concentration of *B. thuringiensis* did not differ significantly ($p > 0.05$) in all the dilution series. Conclusively, *Bacillus thuringiensis* can be used as a biological control agent to mosquito larvae, and can help control mosquito borne diseases like dengue, yellow fever, malaria, and filariasis.

Keywords: Biocontrol, mosquito larvae, *Bacillus thuringiensis*, *Aedes albopictus*, *Culex pipiens*

INTRODUCTION

The emergence of drug resistant parasites and insecticide resistant mosquito strains, along with numerous health, environmental, and ecological side effects of many chemical agents, highlighted the need to develop alternative tools that either complement or substitute conventional malaria control approaches. The use of biological means is considered a fundamental part of the recently launched malaria eradication program and has so far shown promising results, although this approach is still in its infancy. The microbial insecticides most widely used in the world are preparations of *Bacillus thuringiensis*. The insecticidal activity of *B. thuringiensis* is due to the protein parasporal inclusions that are produced during sporulation. Insecticides

based on the proteinaceous endotoxin of *B. thuringiensis* constitute part of a more ecologically rational pest control strategy. *B. thuringiensis* sub-speciess. *israelensis* exhibit acute toxicity towards dipteran insects such as larval mosquitoes and black flies [1] and is currently used in mosquito control programs worldwide [2]. The World Health Organization's Onchocerciasis Control Program in West Africa using *B. thuringiensis* toxins has been one of the success stories of international co-operation in the control of infectious diseases program [3]. Due to the importance of *B. thuringiensis* to control several tropical diseases such malaria and dengue, the present study seeks to investigate its mortality efficacy on the larvae of *Aedes albopictus* and *Culex pipiens* mosquito species.

MATERIALS AND METHODS

Identification and Management of Mosquito Larvae

A total of 200 mosquito larvae breed and obtained from the Zoological Garden, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka were used for this experiment. They were of two different species: *Aedes albopictus* and *Culex pipiens*, and were kept in different containers before and during the research. A water container was used to grow the mosquito larvae; a bucket without paint, tar or other chemicals as it will poison the larva or algae. The bucket was filled with tap water, which was treated to make it safe for mosquito larvae otherwise chlorine may prevent algae growth which is the primary food source for the larvae. Water was treated with de-chlorinator to neutralize the chlorine content that may be present in the water. Also, debris that fell in the water adds to the bacterial growth which mosquito larvae will feed on. Bucket was place in a shady area; mosquitoes thrive in dirty, shady water sources. Direct sunlight was avoided as the water may become too warm for the larvae. Mosquitoes came and lay eggs in the water source and the eggs hatched into the larvae within 48 hours.

Collection of Soil Sample and Isolation of *Bacillus thuringiensis*

Two soil samples were collected from the Zoological Garden and along Microbiology Department across the University of Nigeria, Nsukka and were taken to Microbiology Laboratory for identification and authentication. The soil samples were taken 2 – 5 cm below the surface, after scraping of the surface material with sterile spatula. Finally, collected samples were stored in sterile plastic bags at 4 ºC. The sodium acetate heat treatment method was applied to isolate *B. thuringiensis* from environmental soil sample. Approximately, 0.25g of each sample was suspended in 18×180 mm test tubes containing 10 ml nutrient broth with concentrations of sodium acetate 0.12M and 0.25M. The samples were also suspended in nutrient broth without sodium acetate as negative control. Next, suspensions were vortexed vigorously and incubated overnight at 37ºC in a shaking water bath. Afterwards, the samples were pasteurized for 5 minutes at 80 ºC in order to kill vegetative bacterial cells and to eliminate non-spore forming bacterial cells. Following heat treatment, the samples were placed on nutrient agar plates, which were incubated overnight at 35 ºC. Finally, bacterial colonies were separated by their colony morphology. The colonies, which showed *B. thuringiensis* like colony morphology were rough, white and spread out over the plate. These colonies were sub-cultured on nutrient agar plates and incubated for 48h at 35 ºC to check the position of the spore in the bacterial cell by light microscopy. For this purpose, endospore and simple staining methods were carried out [4].

Staining and Procurement of *Bacillus thuringiensis*

Two staining techniques were used in the procurement of *Bacillus thuringiensis*; the gram staining and the spore staining. The gram staining is used to divide bacterial into two broad groups, the gram-positive and the gram-negative bacteria. While the spore of bacillus is heat resistance due to the spore-coat and with the spore staining, a properly stained spore former will show green endospores within a pink sporangium [5]. For the gram staining, a slide was gotten and a drop of water was added using a flamed wire loop. The loop was flamed again and waved briefly in air to cool. Using the flamed loop, a small amount of cell from an isolated colony was collected on a cultured plate. The cell was mixed in a drop of water and spread on the slide to make a thin, uniform smear and the smear was allowed to air-dry. The smear was heat fixed and cooled, crystal violet was added to cover the smear, and it was stained for one minute and washed in tap water. Iodine

was added and allowed to smear for one minute and washed off with water. It was decolorized with alcohol by adding uniformly and quickly to the slide and washed off quickly with water. It was counter-stained for one minute with safranin and washed with water. Oil immersion was added and viewed under ×100 magnification [5]. For spore staining, a clean slide was used to make a heat-fixed smear of the bacterial sample provided; the slide was flooded with malachite green and heated from below with a Bunsen flame till steam was seen to rise from the stain. The dye was allowed to act with the steam rising for one minute. The stain was washed off with tap water and allowed to dry by draw its edge across a bar of the stain rack; the slide was replaced on rack and flooded with water leaving it to act for 30 seconds. It was washed with water and the slide was drained and blotted to dry with absorbent paper and when completely dried, immersion oil was added and slide was observed with ×100 objective [5].

Experimental Protocol

Three different concentrations of *Bacillus thuringiensis* (0.12ml, 0.25ml, and 0ml negative control) were made and stock solution of each concentration was prepared. Serial dilutions were prepared for each solution by arranging the test tubes in the test tube stand, it was labeled in serial number and all the tubes were filled with 9ml of water. 1ml of the original sample suspension from the flask was taken added to into the first tube containing 9ml of water, this gives 1/10 or 10^{-1} dilution and mixed well. 1ml of this dilution was taken and added to the next tube already containing 9ml of water which gives 1/10 dilution of a 1/10 dilution. Thus, these dilutions [(1/10) × (1/10)], which is 1/100 dilution or 10^{-2} dilution. Again, 1ml of this dilution was taken and added to 9ml of water present in the next test tube and this gives 1/1000 dilution or 10^{-3} dilution. In this manner it was diluted up to 10^{-5} dilution. 15 mosquitoes' larvae were put into five containers labeled 10^{-1}, 10^{-2}, to 10^{-5} for 0.12ml, 0.25ml and negative control and *Bacillus thuringiensis* was introduced. 5ml of each *Bacillus thuringiensis* dilutions was added to labeled mosquitoes larvae respectively, a small stick was used to determine whether the mosquito larvae were dead or not. After every one hour, this rod was dipped into the container and brought very close to each and every larva. For the larva that was still alive could respond rapidly by either bending itself or moving away from the stick while for the dead ones no matter how close the stick was brought, there was no response. The result was recorded for every hour for 24 hours. This was done for the three concentrations (0.12ml, 0.25ml, and negative control).

Statistical Analysis

Data was analyzed using Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA) software version 21.0 and compared using analysis of variance (ANOVA). When the effects were significant in the ANOVA, Duncan's New Multiple Range Test (DNMRT) was used to separate concentration and dilution series. Level of significant was kept at $p < 0.05$ (95% confidence interval).

RESULTS AND DISCUSSION

Results

Interaction between dilution series of *B. thuringiensis* and mortality of *C. pipiens*

The mortality observation period recorded among the groups did not show any significant difference ($p > 0.05$). Different preparations of *Bacillus thuringiensis* showed different levels of toxicity to *Culex pipiens* (Figure 1). No significant difference in mean mortality was recorded at 10^{-1} and 10^{-5} dilution series in all the varying concentrations including the negative control ($p > 0.05$). However, mean mortality rate of the larvae in all the dilution series were dose dependent. Mortality observed at 0.12 ml and 0.25 ml was generally higher than the mean mortality of the larvae recorded at the negative control group. The negative control did not record larvae mortality at 10^{-3}, 10^{-4} and 10^{-5} dilution series respectively. The mean mortality of *Culex pipiens* larvae recorded at 0.12 ml and 0.25 ml concentration of *B. thuringiensis* did not differ significantly in all the dilution series. The mean larvae mortality was significantly ($p < 0.05$) decreasing with decrease in dilution series, that is dilution series dependent in all the experimental concentration.

Interaction between dilution series of *B. thuringiensis* and mortality of *A. albopictus*

The mean time duration of observation did not differ among the groups. The effect of varying dilution series of *B. thuringiensis* in the mean mortality of *Aedes albopictus* is presented on table 1. The mean mortality rate of the larvae showed variation among groups at 10^{-1}, 10^{-2} and 10^{-5}, but did not differ significantly ($p > 0.05$). Similar to the result obtained in *Culex pipiens*, no larvae mortality was recorded at 10^{-3}, 10^{-4} and 10^{-5} respectively. Generally, a concentration dose-dependent mean mortality of the larvae was recorded in all the dilution series. The mortality rate observed at 0.25 ml was significantly higher than the mean mortality recorded at 0.12 ml at 10^{-3} and 10^{-4} respectively. In overall, the mean mortality rate recorded in all the groups were dilution series dependent. However, the mean mortality rate recorded in 10^{-1} and 10^{-2} did not differ significantly at 0.12 and 0.25 ml respectively.

Discussion

The result obtained from this shows that *Bacillus thuringiensis* had a greater effect on *Aedes albopictus* larvae than on *Culex pipiens* larvae. However, in *Culex pipiens* and *Aedes albopictus*, 10^{-5} had the lowest mortality rate due to less amount of the bacteria during the serial dilution. In *Culex pipiens* and *Aedes albopictus*, 10^{-1} had the highest mortality rate as shown the figure 1 and table 1 respectively. The findings of Pemba *et al*. [6], is in agreement with the result of this study. Their findings showed that the effectiveness of *Bacillus thuringiensis* was higher on *Aedes albopictus* as compared to *Culex pipiens* larvae. The negative control had little or no effect on the mosquito larvae this is due to lack of sodium acetate in the negative control with no significant difference ($p > 0.05$), which agrees with Lui *et al*. [7] that the control concentration of *Bacillus thuringiensis* had little effect on *Culex pipiens* and *Aedes aegypti*. *Culex* larvae feeds actively up and down the whole depth of shallow water body hence at risk of ingesting lethal dose over a short period of time. On the contrary *Aedes albopictus* feeds on the surface of the water and may not be able to ingest a lethal quantity of toxic particles in the relatively short period of time. These results are in line with the work of Boisvert [8] who stated that, the larvae of *Aedes albopictus* would show higher death rate if the crystals of *Bacillus thuringiensis* were delivered under floating formulation.

CONCLUSION

In conclusion, this study has revealed the effect of *Bacillus thuringiensis* on *Aedes albopictus* and *Culex pipens* at different mortality rate. In the light of this result, *Bacillus thuringiensis* can be used as a biological control agent to mosquito larvae which aids in controlling some mosquito borne diseases like dengue, yellow fever malaria, and filariasis. It is therefore necessary to recommend that further study be done on *Bacillus thuringiensis* on other species of mosquito larvae to extend it effectual use as pesticides and to ask for the use of *B. thuringiensis* in controlling mosquito larvae in Nigeria.

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