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In vitro evaluation of *Bacillus thuringiensis* larvicide effect on *Anopheles subpictus* larvae

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Abstract
Microbial larvacide effects of *Bacillus thuringiensis* isolates on mosquito (*Anopheles subpictus*) larvae was investigated *in vitro*. The isolates obtained were coded as BTA, BTB and BTC. The bacilli load were determined as MacFallan Standard 0.5(1.0×10^8 cell), 1.0 (3.0×10^8 cell) and 2.0 (6×10^8 cell) and time ranging from 0 – 360 mins. The results obtained showed a significant ($p > 0.5$ and $r = 0.1$) correlation between the percentage mortality rate of the mosquito larvae, the bacilli load, time of incubation and the isolates type. The positive increase in larvae (percentage) mortality rate against different bacilli concentrations at various times (minutes) were as follows: 0.5 MacFallan (30:12%, 120:44%, 240:68%), 1.0 MacFallan (30:18%, 120:52%, 240: 72%) and 2.0 MacFallan (30: 23%, 120: 68%, 240:92%). Secondly, each isolate demonstrateted slightly different and insignificant ($p>0.1$) mortality rate; example at 0.5 MacFallan, isolate BTA, BTB and BTC were seen to have (30:4%, 6% and 5%) while at 120:44%, 32%, 46% respectively. Results obtained has shown that proper formulation of *B. thuringiensis* (var) would be used as a biopesticide to control mosquito larvae at breeding sites, hence the control of malaria and other mosquito vector borne diseases in the public health sector.

Keywords: *Bacillus thuringiensis*, mosquito, larvae, soil, larvacide

1. Introduction
Biopesticide formulations have been used for many decades as alternatives to chemical formulated pesticides globally because of its environmental safety [1].
The greatest microbial biopesticide formulation comes from the use of *Bacillus thuringeinsis* subspecies *isrealensis* against mosquito infested environment [2] The commercial preparations of *B. thuringiensis* subspecies *isrealensis* insecticide has reduced in many part of the world mosquito vector borne related diseases such as malaria, filariasis, dengue fever, yellow fever, west Nile virus and the Chikungunya virus [3].
The insecticidal properties of Bt depends on its ability to synthesis three proteinous endotoxins crystal known as δ-endotoxins such as crystal (cry) cytolytic (cyt) and parasporal toxins at sporulation as parasporal crystalline inclusions [4-6].
The crystal δ-endotoxins proteins exhibit host specificity mode of action with no consequence to human [7-9].
Therefore, the research attempted to evaluate in-vitro the effective larvacidal rate of the *B. thuringiensis* load versus the mosquito larvae mortality. The results obtained indicated that there is a significant correlation between the *B. thuringiensis* load, time of exposure and mosquito larvae mortality.
Secondly, it demonstrated that proper formulation of *B. thuringiensis* could be used as a biolarvacide to control mosquito larvae at breeding sites thereby controlling malaria and other mosquito vector borne diseases in the public health sector.

2. Materials and Methods
2.1 Samples collection
Three (3) soil samples were collected from three different ecological composition (namely; highly organic compost soil, less organic top soil and forest soil, bagged and transported to the laboratory for microbiological assay.
A twenty litre (20L) capacity transparent polyvenyl (PVC) container containing 18.0L of clean water (pH7 and temperature of 22 ±2^{o}C) was strategically placed 10m and monitored outside the laboratory for laying of eggs and subsequent development of mosquito larvae.

Active larvae (Wrigglers) were collected using a tea bag catching net into a normal saline (0.85%v/w) for *Bacillus thuringiensis* biotoxicity assay. The adult mosquito was identified as *Anopheles subpictus* by resident Entomologists in the Department of Animal and Environmental Biology.

2.2 Isolation and identification of *B. thuringiensis*

Sodium acetate heat treatment method described by [10, 11] was adopted. In the method, 25g of each soil sample was suspended in 100ml nutrient broth supplement with 0.25mL (pH6.8), sodium acetate. Control soil samples were also treated without sodium acetate. Each of the suspensions was vigorously vortexed and incubated at 37ºC for18 hrs on a shaking water bath. The soil suspension was further pasteurized for five (5) mins at 80ºC to destroy vegetative and non-spore forming cells. The suspended spore were seeded on nutrient agar and incubated at 35ºC for 48hrs. Developed discrete colonies were picked and purified by subculturing. Pure *B. thuringiensis* cultures were examined by light microscope for the presence of endospore using simple staining techniques.

2.3 Preparation of *B. thuringiensis* suspension

The BtA, BtB and BtC suspensions were prepared as described by [12]. Three different concentration of each *B. thuringiensis* suspension were made and standardize as MacFallan 0.5 (1.0×10^8cell), 1.0 (3.0×10^8cells) and 2.0 (6.0×10^8cells) and suspended in 250mL sterile deionized water at pH7.1.

2.4 *B. thuringiensis* bio toxicity assay

Two hundred (200mL) milliliter sterile deionized water was put into 1000mL capacity transparent PVC containers. Then one hundred viable *Anopheles subpictus* larvae randomly selected for stock were trapped and transferred into water at pH7.1 and temperature (28 $\pm$ 2ºC). After five (5) mins (Time allowed for adaptation of the larvae) 200mL of *B. thuringiensis* suspension was introduced aseptically into the mosquito larva suspension and incubated on a shaker. The set up was done in triplicate for each *B. thuringiensis* concentration and the control that has no *B. thuringiensis* cells The bio toxicity and mortality (death) rate of the larvae were accessed every 30mins for a total of 360mins. This was achieved by probing the larvae at the bottom of the container with a sterile glass rod for life signs by wriggling or tumbling away from the rod.

2.5 Statistical analysis

The data collected were subjected to mean calculation, percentage determination and correlation analysis of variance (ANOVA) using Statistical Analysis System, Generalized Linear Model (SASGLM, SAS version 8.02 [13]. Results are discussed based on the various statistical conclusions and recommendations put forward accordingly.

3. Results

Isolates of *B. thuringiensis* sp coded as BtA, BtB and BtC were obatined from each soil type. The percentage occurance is represented in Table 1. The highly organic soil (type A) with high carbon content has 70% Bt load. This was followed by dark forest soil (type C) presumed also to be rich in carbon having 68% and the low organic top soil (type B) having 66%.

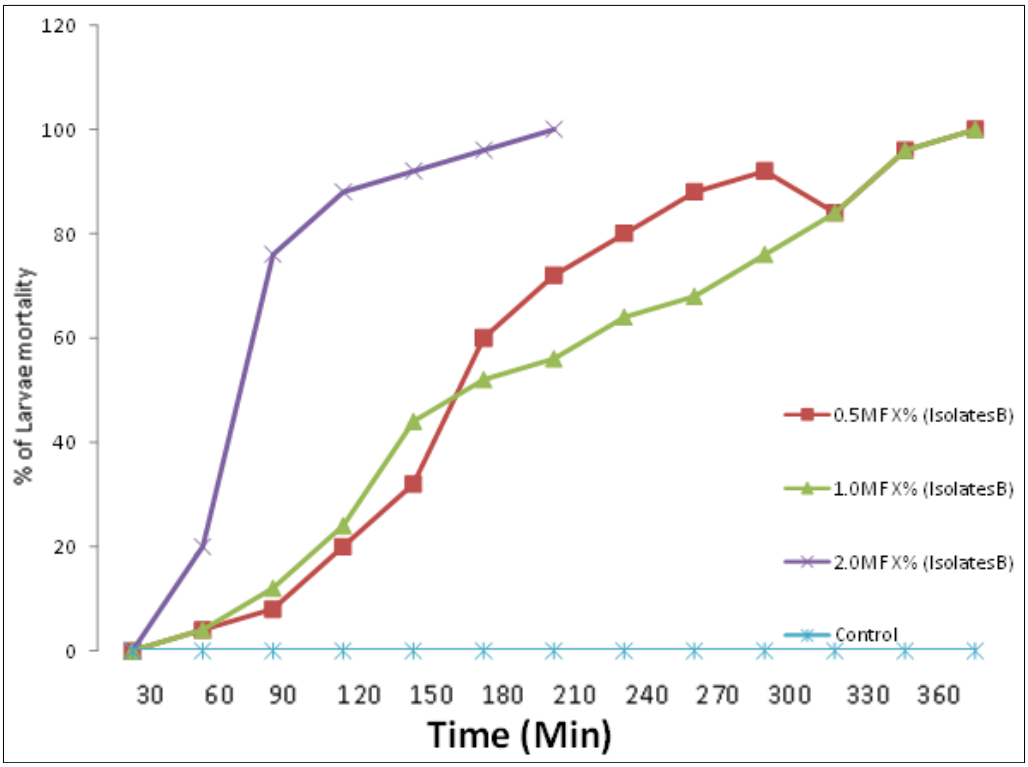
Table 1: Percentage abundance of *Bacillus thuringiensis* and soil type

S/N	Soil type	Type	% abundance
1	> Organic	A	70
2	< organic	B	66
3	Forest	C	68

The larvacidal activities and larvae mortality rate expressed in percentage as mean of triplicate readings for each *B. thuringiensis* concentration is presented in figures 1, 2 and 3 respectively.

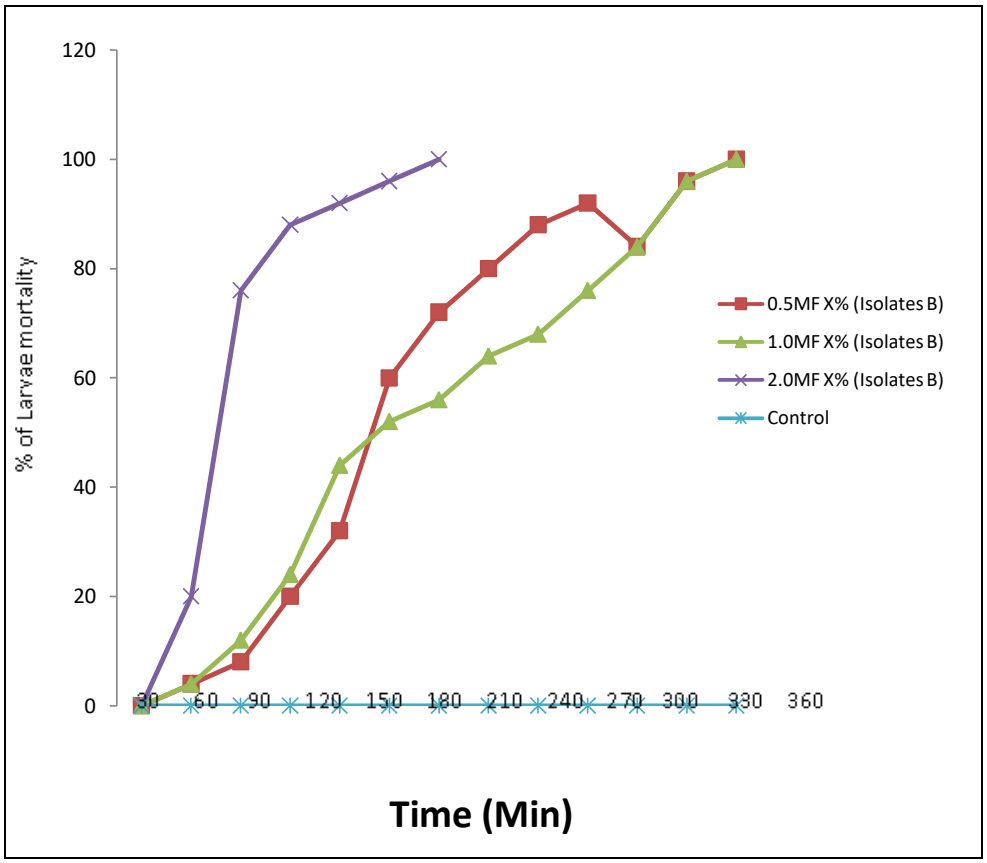
The results obtained clearly indicated that the larvae mortality rate was positively ($P > 0.5$) significant and related ($r = 0.5$) to the increase in *B. thuringiensis* concentration and time. For instance, at 0.5 MacFallan standard representing 1.0×10^8 cells concentration and the time of 30mins, BtA, BtB and BtC were 12%, 18% and 23% respectively. Secondly, it became clear that Bt A, B and C at each concentration demonstrated less and non-significant ($P < 0.1$) relationship to time. For example, at 30mins an average lethal rate remained at BtA = 4%, BtB = 4% and BtC = 4%. Similarly at 120 mins the percentage mortality was constant as follows: BtA =44%, BtB = 44% and BtC = 43.5%.

Thirdly, the results also demonstrated that the higher the *B. thuringiensis* concentration, the shorter the lethal and toxicity period. For instance at 2.00 MacFallan standard representing 6.0×10^8cell load at 120mins the mortality rate were BtA=64%, BtB=96% and BtC=96%.



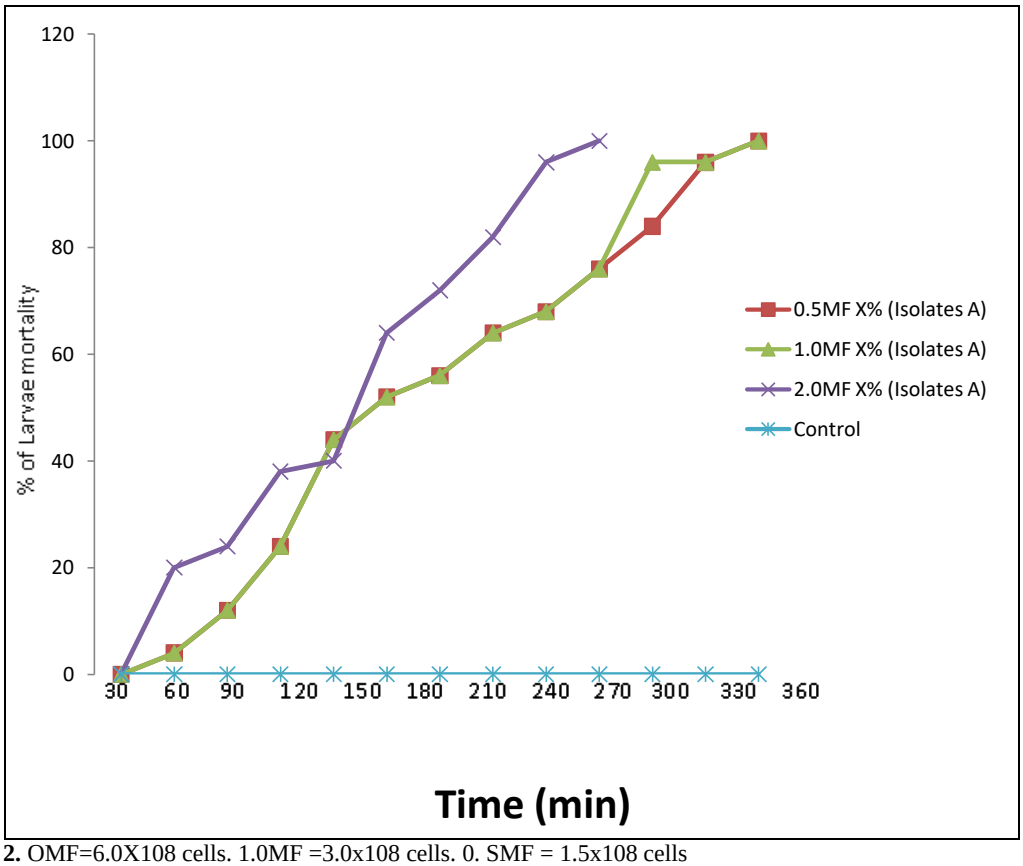
2. OMF=6.0X108 cells. 1.0MF =3.0x108 cells. 0. SMF = 1.5x108 cells

Fig 1: Larvicidal activity of Bacillus thurinqiensison mosquito's larvae at 0.5, 1.0 and 2.0 Mc Farlane (Isolate A)



2. OMF=6.0X108 cells. 1.0MF =3.0x108 cells. 0. SMF = 1.5x108 cells

Fig 2: Larvicidal activity of Bacillus thurinqiensison mosquito's larvae at 0.5, 1.0 and 2.0 Mc Farlane (Isolate B)



2. OMF=6.0X108 cells. 1.0MF =3.0x108 cells. 0. SMF = 1.5x108 cells

Fig 3: Larvicidal activity of Bacillus thurinqiensison mosquitoes larvae at 0.5, 1.0 and 2.0 Mc Farlane (Isolate C)

4. Discussion

Bacillus thuringiensis isolates BtA, BtB, BtC obtained from the various soil types adopted in this investigation agrees with the report presented by [14]. They authors, reported that *B. thuringiensis* could be obtained worldwide from great diversity of ecosystem including the ones adopted in this investigation.

The adopted isolates (BtA, BtB, BtC) were confirmed by the work described by [10, 11]. These authors in their different reports stated that acetate heat treatment of soil sample inhibit the growth of vegetative and non-spore formers but promotes the germination of spore former *B. thuringiensis* species. The organic rich soils provided the needed carbon source that brought about the high diversity and high percentage load of *B. thuringiensis*.

The isolated *B. thuringiensis* species had caused significant ($P > 0.5$) mortality hence a reduction of the larvae population. Individual concentration of *B. thuringiensis* had a dramatic lethal effect on the larvae population versus exposure time. However, respective *B. thuringiensis* isolates showed little or no significant differencial toxicity potential [15]. The condition occurred because the ingestion rate of each *B. thuringiensis* type depends rather on the concentration over a determined time

The biotoxicity potential of *B. thuringiensis* species has been demonstrated in many studies in recent past. The ingestion of *B. thuringiensis* into the gut of mosquito larvae activated the proteinous δ-endotoxin crystal (Cry-protein) [16]. The release of the protein molecules into the larvae gut dissolve the gut lining membrane creating an osmotic imbalance of solute in the midget [9]. This condition brings about a leakage in the gut and the subsequent death of the larvae. Consequently, the lethal time and potency were observed to depend on the suspension concentration of *B. thuringiensis*, meaning that the higher the level of *B. thuringiensis* ingestion, the higher the lethal toxicity and mortality rate of the exposed larvae.

5. Conclusion

In conclusion, the study has shown that soil with high organic content contained high Bt load. Also the toxicity potential of *Bacillus thuringiensis* as a biopesticide against mosquito larvae is a function of its cell load over time. Therefore proper formulation of *B. thuringiensis* concentration can be used in many environments to control the breeding of mosquitoes and other insect borne disease vectors.

6. References

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