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Evaluation of the Larvicidal Activity of *Bacillus Thuringiensis* Isolated from Soil Samples Against *Aedes aegypti*

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Abstract

Application of chemical insecticides poses a wide range of problems in the environment. In addition to this, these chemicals are non specific and are implicated as carcinogens. All these necessitated the search for an eco-friendly biological alternative for the control of insect pests and vectors in environment such as the use of *Bacillus thuringiensis* known to habour insecticidal crystals. Bioassay to assess the insecticidal activity of the isolates was carried out on *Aedes aegypti* larvae using 3 different concentrations of spore crystal mixture (100, 75 and 50 ppm) alongside one control. In each case, 10 larvae were exposed in triplicates for *Aedes aegypti.* The mortality range of *Aedes aegyti* was between 40.00% and 100.00%. The concentrations of the spore crystal mixture which kill 50% of the exposed populations in standard bioassays (LC_{50}) were determined by probit analyses. The LC_{50} using was between 118.03ppm and 55.79ppm. The results of this research shows that the isolates from the soils in Zaria can serve as biocontrol agent for the control of mosquito by targeting their larvae stage hence controlling the diseases they spread.

Keywords

Larvicidal, Bacillus Thuringiensis, Aedes Aegyti, Biocontrol, LC_{50}

1. Introduction

Bacillus thuringiensis belongs to the family of the *Bacillaceae;* which Taxonomists consider as sub-species of *Bacillus* cereus because they are closely related [9, 14, 16]. According to [18], the genotypic and phenotypic characteristics of *Bacillus thuringiensis* are very similar to *Bacillus cereus*; the only difference between these two species being the formation of large proteinaceous parasporal inclusions observed in *Bacillus thuringiensis.*

Around the world, the medical and economic burden caused by vector-borne diseases continues to grow as current control measures fail to cope. There is an urgent need to identify new control strategies that will remain effective, even in the face of growing insecticide and drug resistance [1].

Repetitive use of man-made insecticides for mosquito control disrupts natural biological control systems and lead to reappearance of mosquito populations. It also resulted in the development of resistance, detrimental effects on non-target organisms and human health problems and subsequently this initiated a search for alternative control measures [11].

Mosquitoes, which are vectors for many diseases belong to the family Culicidae in the insect order of true-flies or two-winged flies called Diptera. During these blood meals, a female mosquito transmits agents of diseases to man.

Aedes aegypti (Linnaeus) belongs to the family Culicidae and the order Diptera. It is the vector of diseases such as dengue fever, chikungunya and yellow fever dengue hemorrhagic fever, other viral diseases, lymphatic filariasis. It is also been implicated in the transmission of Zika virus which is

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suspected of causing a neurological disorder called the Guillain Barre syndrome. The female *A. aegypti* preferably lay eggs in artificial collections of water. The hatched larvae undergo growth and metamorphosis.

Insect pests of crops and forest plants and vectors of disease of human beings and other animals are serious threat for agriculture and public health. Worldwide, about US $8000 billion is spent for insecticides and estimates reveal that US $2700 can be substituted by the biopesticide *B. thuringiensis*. Besides exorbitant cost, and resistance and resurgence of the different pests, the chemical pesticides are the single main cause of health and environmental hazard. The situation demands the safer pesticides and biopesticides are the most desired alternatives. Bacteria, especially *B. thuringiensis* and *B. sphaericus* are the most potent and successful group of organisms for effective control of insect pests and vectors of diseases [8].

Owing to the non-specificity of chemical insecticides, both target and non target organisms are often impacted upon with undesirable consequences. As a result, organisms that play an important role in the environment could be detrimentally affected. Key insect pollinators and other resourceful life forms could be wiped out [5]. Both surface and underground water bodies could become heavily contaminated with insecticide residues. This could pose a serious threat to aquatic life forms. [10]. These problems are further compounded by exorbitant cost of procurement and almost total lack of proper knowledge of their application among the general populace [5].

B. thuringiensis has certain advantages for exploitation as biopesticide viz. *B. thuringiensis* can be used directly and as transgenic microbes and plants, being a prokaryote there is no dominant or recessive allele, highly vulnerable to genetic manipulation and the toxin gene is coded by single gene (monocistronic), *B. thuringiensis* is fermentation friendly and therefore commercially exploitable and it is host specific or has narrow host range [8].

The predominant type of δ-endotoxins found in *B. thuringiensis* is the *Cry* proteins, a crystal protein encoded by *Cry* genes. The accumulation of *Cry* protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells [3, 4].

Each Crystal protein has its own insecticidal spectrum. The crystal protein of *B. thuringiensis* acts as a protoxins*,* for this protoxins to become active; a suspectible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, enzymes in midgut (proteases) convert the protoxins into active toxins. This active toxin then binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). This pore formation leads to gut paralysis. Finally, insect larvae stop feeding and die from lethal septicemia [2, 12].

Present study was envisaged to isolate and identify the *B. thuringiensis* of indigenous soils of Zaria, Kaduna state, Nigeria and characterized the polymorphic crystal producing strains, which was exploited for biological control of mosquito insect-pests which are disease vectors.

2. Materials and Methods

2.1. Collection of Samples

Samples of organic matter-rich soil were collected from various sites in Zaria. From each location, samples of about l0g were collected from five spots. Samples were taken from one inch below the surface by scrapping off surface material with a spatula. The samples were brought to the laboratory and stored under appropriate storage conditions.

2.2. Isolation Procedure

Bacillus thurigiensis were isolated according to the method described by Travers *et al.* (1987). For each sample, 0.5 g soil was added in 10 ml of LB medium (Tryptone 10g /L, yeast extract 5g/L, NaCl 5g/L) to which 0.25M sodium acetate was added and incubated in shaking incubator at 30°C and 250 rpm for 4 hours. From each sample 2ml was taken and heat shocked in a water bath at 80°C for 20 minutes. Serial dilutions of treated samples were prepared and spread on T3 agar ((Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, $MnCl_2$ 0.005g/L, Sodium phosphate 0.05M and Agar15g/L) and incubated for 2days at 26°C. Colonies with *B. thuringiensis* like morphology (entire margin, off white color, dry and rich growth of colony) were picked up at random and purified by streaking them on LB agar plates (Tryptone 3g/L, Yeast extract 1.5g/L, Tryptose 2g/L, $MnCl_2$ 0.005g/L, NaH_2 PO_4 6.9g/L, Na_2HPO_4 8.9g/L and Agar15g/L).

2.3. Characterization of *Bacillus thuringiensis*

The suspected isolates of *Bacillus thuringiensis* were identified by following the diagnostic plan specified in Bergey's Manual of Determinative Bacteriology [15]. The isolate were characterized using Gram reaction and endospore staining, motility, growth above 45 °C, catalase activity, Voges Proskauer test and confirmed using Microgen™ Bacillus-ID.

2.4. Preparation of Spore Crystal Mixture

To obtain the spore-crystal mixtures, single colonies from

overnight LB plates were inoculated into 10ml T3 sporulation medium (per litre 3g of tryptone, 2g of tryptose, 1.5g of yeast extract, 0.05m sodium phosphate (pH 6.8) and 0.005g MnCl$_2$) and cultured for 60 hours in a shaker incubator at 30°C. Spores and crystals were harvested by centrifugation at 7000g for 10 minutes and washed twice with distilled water, the spore crystal mixture were stored at low temperature until required [6, 13].

2.5. Collection and Counting of Mosquito Larva

The third instar larvae (L3) of *Aedes aegypti* were collected from discarded containers and stagnant water around the university. Early third instar (L3) Larvae of *Aedes aegypti* used in the bioassay were selected using a modified pasteur pipette and placing 10 early L3 larvae into each test tube and the volume was made up to 10 ml using distilled water.

2.6. Determination of the Larvicidal Activity of *Bacillus thuringiensis* Against *Aedes aegypti* Larvae

The mosquitocidal assay was performed using three concentrations of spores and crystal mixture (100ppm, 75ppm and 50ppm). Each concentration was replicated three times and one test tube with 10 (L3) larvae was used as the control. Mortality of the treated larvae were recorded after 24 hours [17].

2.7. Data Analysis

Results were presented in tables, graphs and charts where applicable. ANOVA was used to compare the mean mortality of the isolates at different concentration of spore crystal mixture of each of the isolates. Using probit analysis, the mean lethal concentration, LC$_{50}$ was determined, for each of the isolates tested. Discussion and conclusions were made afterwards.

3. Results and Discussion

Chemical insecticides provide many benefits to food production and human health and have proven very effective at increasing agriculture and forestry productivities. However, they also pose some hazards such as contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistant to the chemical insecticides [20].

A total of 30 soil samples were collected. From these 30 soil samples, 12 *Bacillus thuringiensis* were isolated and characterized biochemically.

The bioassay for the larvicidal activity of the isolates on *Aedes aegyti* larvae using different concentrations of the spore crystal mixture shows that the higher the concentration of spore crystal mixture, the more the larvicidal activity.

Isolate L3 had the highest activity (100% mortality) at 100ppm concentration of spore crystal mixture, followed by L1 (93.33% mortality) at the same concentration. Isolate D2 shows the lowest activity (40.00%) at 100ppm concentration (Figure 1). The differences observed between the mean mortality of the isolates at different concentration of the spore crystal mixture were statistically significant (Table 1).
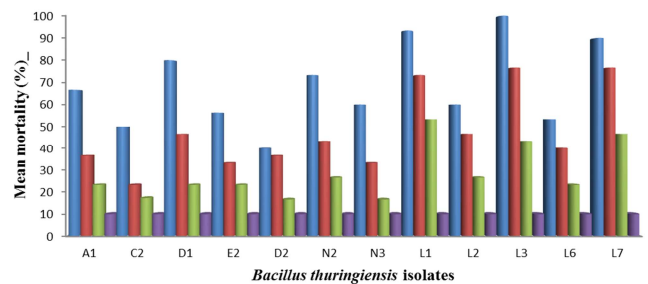

Figure 1. Mean mortality of *Aedes aegypti* to different concentrations of *Bacillus thuringiensis* spore crystal mixture after 24 hours of exposure.

Table 1. Mortality of *Aedes aegypti* larvae on exposure to various concentrations of twelve isolates of *Bacillus thuringiensis*.

| Isolates | Conc. (ppm) | No. of larvae | | Mean mortality(95% CI ±SE) | ANOVA | |
		Exposed*	Dead		F-Value	P-Value
A1	0 (Control)	10	3	1.00^c (1.00-1.00±0.00)		
	50	10	7	2.33^{bc} (-0.52-5.18±0.66)		
	75	10	11	3.67^b (2.22-5.11±0.34)	35.4222	5.744x10^{-5}
	100	10	20	6.67^a (5.22-8.11±0.34)		
C2	0 (Control)	10	3	1.00^b (1.00-1.00±0.00)		
	50	10	6	2.00^b (-0.48-4.48±0.58)		
	75	10	7	2.33^b (0.88-3.77±0.34)	26.1948	1.725x10^{-4}
	100	10	15	5.00^a (5.00-5.00±0.00)		
D1	0 (Control)	10	3	1.00^d (1.00-1.00±0.00)		
	50	10	7	2.33^c (0.88-3.77±0.34)		
	75	10	14	4.67^b (1.81-7.52±0.66)	42.4804	2.923x10^{-5}
	100	10	24	8.00^a (5.51-10.48±0.58)		
E2	0 (Control)	10	3	1.00^c (1.00-1.00±0.00)		
	50	10	7	2.33^b (0.88-3.77±0.88)		
	75	10	10	3.33^a (1.88-4.77±0.34)	19.0021	5.359x10^{-4}
	100	10	16	5.33^a (3.88-6.77±0.34)		

| Isolates | Conc. (ppm) | No. of larvae | | Mean mortality(95% CI ±SE) | ANOVA | |
		Exposed*	Dead		F-Value	P-Value
D2	0 (Control)	10	3	1.00[c] (1.00-1.00±0.00)		
	50	10	5	1.67[b] (0.22-3.11±0.34)		
	75	10	11	3.67[a] (2.22-5.11±0.34)	24.9854	2.044x10^{-4}
	100	10	12	4.00[a] (1.51-6.48±0.58)		
N2	0 (Control)	10	3	1.00[d] (1.00-1.00±0.00)		
	50	10	8	2.67[c] (1.22-4.11±0.34)		
	75	10	13	4.33[b] (2.88-5.77±0.34)	43.8119	2.604x10^{-5}
	100	10	22	7.33[a] (4.47-10.18±0.66)		
N3	0 (Control)	10	3	1.00[b] (1.00-1.00±0.00)		
	50	10	5	1.67[b] (0.22-3.11±0.34)		
	75	10	10	3.33[a] (1.88-4.77±0.34)	16.2346	9.182x10^{-4}
	100	10	18	6.00[a] (1.70-10.29±1.00)		
L1	0 (Control)	10	3	1.00[d] (1.00-1.00±0.00)		
	50	10	16	5.33[c] (3.88-6.77±0.34)		
	75	10	22	7.33[b] (5.88-8.77±0.34)	76.2826	3.159x10^{-6}
	100	10	28	9.33[a] (6.47-12.18±0.66)		
L2	0 (Control)	10	3	1.00[c] (1.00-1.00±0.00)		
	50	10	8	2.67[b] (1.22-4.11±0.34)		
	75	10	14	4.67[ab] (1.81-7.52±0.66)	21.857	3.287x10^{-4}
	100	10	18	6.00[a] (3.51-8.48±0.58)		
L3	0 (Control)	10	3	1.00[d] (1.00-1.00±0.00)		
	50	10	13	4.33[c] (2.88-5.77±0.34)		
	75	10	23	7.67[b] (4.81-10.52±0.66)	111.707	7.204x10^{-7}
	100	10	30	10.00[a] (10.00-10.00±0.00)		
L6	0 (Control)	10	3	1.00[b] (1.00-1.00±0.00)		
	50	10	7	2.33[ab] (-1.47-6.13±0.88)		
	75	10	12	4.00[ab] (-0.96-8.96±1.16)	5.6209	0.022
	100	10	16	5.33[a] (2.47-8.18±0.66)		
L7	0 (Control)	10	3	1.00[c] (1.00-1.00±0.00)		
	50	10	14	4.67[b] (1.81-7.52±0.66)		
	75	10	23	7.67[a] (6.22-9.11±0.34)	56.9693	9.661x10^{-6}
	100	10	27	9.00[a] (6.51-11.48±0.58)		

Means followed by the same superscript within the same isolate are not significantly different (P>0.05).
* 10 larvae were exposed in triplicate giving a total of 30.

The probit analysis was used to assess the potency of the toxin on living organisms (as such it was used to assess the potency of the spore crystal mixture of the isolates on mosquito larvae) the probit table was used to get the Empirical Probit of kill for each of the concentrations using their corresponding mortality. The empirical probit of kill was plotted against the log of concentration to derive the regression equation. The coefficient of regression (R^2) was also generated from the graph. The regression equation was used to calculate the median lethal concentration (LC_{50})

which is the concentration that will kill 50% of the exposed population. The table below shows the median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Aedes aegypti* larvae obtained by probit analysis.

The concentrations of the spore crystal mixture which kill 50% of the exposed populations in standard bioassays (LC_{50}) were determined by probit analyses with triplicate bioassay. These concentrations range between 52.17ppm and 125.89ppm (Table 2).

Table 2. Median lethal concentration (LC_{50}) of twelve isolates of *Bacillus thuringiensis* against *Aedes aegypti* larvae obtained by probit analysis.

Isolates	Conc. (ppm)	Log of conc	Mort. (%)	LC_{50} ppm
A1	100	2.00	67	88.78
	75	1.87	37	
	50	1.67	23	
	0	-	10	
C2	100	2.00	50	125
	75	1.87	23	
	50	1.67	20	
	0	-	10	
D1	100	2.00	80	76.54
	75	1.87	47	
	50	1.67	23	
	0	-	10	

Isolates	Conc. (ppm)	Log of conc	Mort. (%)	LC$_{50}$ ppm
E2	100	2.00	53	92.59
	75	1.87	53	
	50	1.67	23	
	0	-	10	
D2	100	2.00	40	118.03
	75	1.87	37	
	50	1.67	17	
	0	-	0	
N2	100	2.00	73	80.40
	75	1.87	43	
	50	1.67	27	
	0	-	10	
N3	100	2.00	60	95.56
	75	1.87	33	
	50	1.67	17	
	0	-	10	
L1	100	2.00	93	52.17
	75	1.87	78	
	50	1.67	53	
	0	-	10	
L2	100	2.00	60	88.94
	75	1.87	47	
	50	1.67	27	
	0	-	10	
L3	100	2.00	100	55.79
	75	1.87	77	
	50	1.67	43	
	0	-	10	
L6	100	2.00	53	102.35
	75	1.87	40	
	50	1.67	23	
	0	-	10	
L7	100	2.00	90	54.62
	75	1.87	77	
	50	1.67	47	
	0	-	10	

Conc. = concentration, Log of conc = logarithm of concentration, Mort. = mortality, LC$_{50}$ % = Median Lethal concentration.
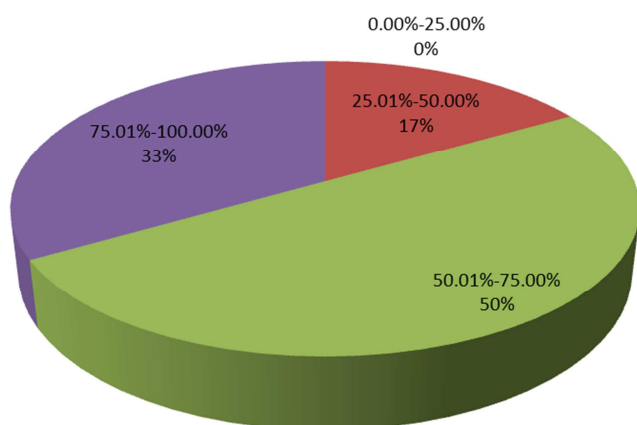

Figure 2. Distribution of the insecticidal activity (%) of 100ppm spore crystal mixture of the isolates to *Aedes aegypti* larvae.

Based on the distribution of the insecticidal activity of the isolates *Aedes aegypti*; 0% of the isolates had activity between 0.00-25.00%, 17% had activity between 25.01%- 50.00%, activity between 75.00%-100.0% was seen in 33% of the isolates, while activity between 50.01%-75.00% was seen in 50% (Figure 2).

4. Conclusion

The search for alternative biocontrol agents such as *Bacillus thuringiensis*-based biopesticides is increasingly attracting interest. This bacterium produces parasporal crystalline inclusions (Cry proteins) which is toxic to many important agricultural pests. The Cry proteins are encoded by *cry* genes and so far, many *cry* genes have been identified in different *B. thuringiensis* strain collections. *Cry2* and *cry4* are toxic to dipteran insects [7, 21].

Bioassay to screen for the larvicidal activity of the isolates against *aegypti* larvae showed that the isolates are promising biocontrol agents for the control of these mosquitoes. The

bioassay also showed that most of the *Bacillus thuringiensis* isolates from the soil harbor dipteral-active c*ry* hence they are good biocontrol agents for mosquitoes.

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