CIDAL ACTIVITY OF PROTEINS SECRETED BY BACILLUS THURINGENSIS AGAINST ASCARIS LUMBRICOIDES

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ABSTRACT

Ascarisis is the infection caused by *Ascaris lumbricoides* which can be transmitted through the injection of food, water and vegetables contaminated with the egg *of Ascaris.* This study was undertaken to evaluate the cidal activity of proteins secreted *by Bacillus thuringiensis* against *Ascaris lumbricoides*. A total of fifty soil samples from garden soil were collected and screened for the presence of *Bacillus tkuringiensis* using pour plate technique. The organisms obtained from mixed culture plates were characterized and identified using their morphological and biochemical characteristics. Cidal activities of the proteins were carried out by exposing 10 of the *Ascaris lumbricoides* to different concentrations (50.00, 100.00, 150.00, 200.00 and 250.00 ppm) of the secreted proteins. The study revealed the presence *of Bacillus thuringiensis* isolates; Bt M, Bt N and Bt Y. The maximum spore count was 4.93 logCfu/ml after 96 h. There were pronounced cidal activities of the secreted proteins, mostly from Bt M, and these increased significantly ($p< 0.05$) as the concentrations of the secreted proteins increased. The LC_{50} of the study revealed significant ($p< 0.05$) activities of the secreted proteins against the *Ascaris lumbricoides,* of which the secreted proteins from Bt M showed the most pronounced activity. Therefore the secreted proteins from the BsD45, BsDSM396, BtDX3 and BtWO15 showed significant larvicidal activities against *Ascaris lumbricoides*, of which secreted proteins from isolate *of Bacillus thuringiensis* (Bt) M recorded the most pronounced activity.

INTRODUCTION

Bacillus thuringiensis is an ubiquitous Gram positive rod shaped and sprorulating bacterium have been isolated worldwide from many habitats, including soil, insects, stored-products, dust and deciduous and coniferous leaves (Carozzi *et al.,* 2001; Schnepf *et al*., 2008). A typical method of isolation involves heat treatment to select for spores, sometimes with an acetate enrichment step (Li *et al.,* 2000), antibiotic selection or non-selective agar media (Chilcott and Wigley, 2003). Despite the need for information on genetic diversity of indigenous strains of *B. thuringiensis* for potential use in bio control programs, studies addressing these issues are scarce. Some workers tested commercial preparations of *B. thuringiensis* (Dipel) on crop pests against cutworms (*Agrotis* spp.) and stalk borer (*B. fusca*) in cereals and potato tuber-worm on tomatoes (Alemayehu *et al.,* 2003). A similar study was carried out on *B. thuringiensis* var kurstaki isolates against African bollworm, *Helicoverpa armigera* (Alemayehu *et al.,* 2003). Results from these studies were not encouraging from a biocontrol perspective, but it has been shown that standard *B. thuringiensis* subspecies israelensis were active against *Anophele gambiae.*

Bacillus thuringiensis is characterized by its ability to produce crystalline inclusions during sporulation and exhibiting highly specific insecticidal activity against different insect orders, including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera and Mallophaga (Hofte and Whiteley 2009; Freire *et al*., 2014; Nwosu and Ubaoji, 2020; Sawicka and Egbuna, 2020). The toxicity of Bt culture lies in its ability to produce the crystalline protein, this observation led to the development of bio insecticides based on Bt for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera (Freire *et* al., 2014; Mezzomo *et al*., 2015). Nowadays, Bt isolates are reported also active against certain nematodes, mites and protozoa. It is already a useful alternative or supplement to synthetic chemical pesticide for applications in commercial agriculture, forest management, and mosquito control, and also a key source of genes for transgenic expression to transfer pest resistance in plants. Due to this economic interest, numerous approaches have been developed to enhance the production of Bt bio insecticides. The insecticidal activity of Bt is known to depend not only on the activity of the bacterial culture itself, but also on abiotic factors, such as the medium composition and cultivation strategy (Marvier *et al.,* 2007)

MATERIALS AND METHODS

Study Area: This study was conducted in Uli Community Ihaila L.G.A, Anambra State, Nigeria. Uli is a town situated at the extreme southeast of Ihiala L.G.A. Its closet neighboring towns are Ihaila town, Amorka, Ubulu, Ozara, Egbuoma and Ohakpu. The town extends Westland to Conference Rivers of Amamiri and Enyinja, and across Usham Lake down to the lower Niger region. Its coordinates at $5^0$47'N, 6^0 52'E and 5.783^{0}N, 6.687^0 E, occupying a land mass of 99 square miles. The climate of this region majorly tropical rainforest, characterized with this season (rainy and dry season) and average temperature of 32^{0}C and 25^{0}C respectively. People leaving within region are basically trader and farmers.

Sample Collection, Handling and Transportation: Sterile soil auger was aseptically used to collect soil samples from 3-5 cm depth, into sterile container. The sample was covered and carefully transported to the laboratory for the bacteriological analysis.

Isolation and Characterization of *Bacillus thuringiensis*

Isolation of the organism: This was carried out using the method described by Mezzomo *et al,* (2015) and Reyaz *et al,* (2017). One gram of the soil sample was weighed into boiling test tube, 5 ml of distilled water was added and shake thoroughly and then make up to 10 ml using the distilled water. The boiling tube was kept at 80°C for 30 minutes and it was allowed to settle. One milliliter of this heat treated suspension was added to four milliliter (4 ml) of normal saline (0.85% NaCl), which gave 5^{-1} dilution. From 5^{-1} dilution test tube, a five-fold serial dilution was carried out to obtain 5^{-5} dilution. One milliliter aliquots from 5^{-1} and 5^{-5} test tubes were aseptically collected and plated on T3 agar medium, and NYSM agar (nutrient agar with 0.5g/l yeast extract, 0.2 g/L $MgCl_2$, 0.0l g/L $MnCl_2$ and 0.1 g/L $CaCo_3$ with 100 mg/ml of streptomycin). These were done in triplicate and incubated in inverted position at room temperature (30 ± 2°C) for 3 days.

Purification of the isolates: The best growing colonies from the culture plates, with prominence characteristics was aseptically picked using sterile wire loop and aseptically streaked on, NYSM agar, and nutrient agar plates. The plates were incubated in inverted position at room temperature (30 ± 2°C) for 48 h. The purity of the sub-cultured isolates was checked microscopically be examining their cells using Gram staining technique (Herssan *et al.,* 2010).

Characterization and identification of the isolates: The isolates were characterized and identified using the morphological and biochemical characteristics (Herssan *et al,* 2010, Patit *et al,* 2014, Mezzomo *et al,* 2015; Reyaz *et al*., 2017).

Detection of crystalline inclusions: The culture smears was prepared, heat fixed and stained with Coomassie Brilliant Blue Stain (0.133% Coomassie Brilliant Blue G250 in 50% acetic acid). Then, the smear was washed softly in running tap and observed microscopically using oil immersion objective lens (Herssan, 2010).

Estimation of spore counts: The progress of bio-pesticide production was monitored by measuring the spore count at 24 h intervals. One milliliter (1ml) sample was collected in a sterile test tube and was heat treated at 80°C for 15 minutes, serially diluted, then plated on the NYSM agar plates and incubated at 30°C for 24 h., then the number of colonies on the plate were counted and recorded after every 24 h for 5 days using electronic colony counter (Patil *et al,* 2014).

Precipitation of the toxins: This was carried out using the method described by Fernando *et al.* (2010). The supernatant that was obtained from mosquito larvicidal production was precipitated using 80% NH_4SO_4 solution. The precipitate was obtained by filtering the solution using what man N01 filter paper. The crystals obtained on the filter paper were air dried.

Preparation of the toxin: The toxins were prepared by dissolving 0.05g, 0.04g, 0.03g, 0.02g and 0.0l g respectively of the toxins in 200 ml of phosphate buffer saline (PBS) to form 250, 200, 150, 100 and 50 ppm respectively. Ten of the test organisms *(Ascaris lumbricoides)* were

introduced into the different concentrations of the toxins and the extracts. These were observed for 3 days. The number of death in each set-up were manually counted and recorded.

Statistical Analysis: the data generated from the study were presented in tables and percentage. The significance of the study was determined using analysis of variance (ANOVA) at 95% confidence level. Student "T" test was used for the pair wise comparison (Iheukwumere *et al*., 2018).

RESULTS

The study revealed that the isolates exhibited similar morphological characteristics but differs in their colouration with isolate Y which showed slight cream on NYSM. The isolates were Gram positive rod with centrally positioned endospore. They were motile and enable to produce crystals. Their colonies were flat with entire edge and smooth surface. The isolates shown in Table 2 exhibited similar biochemical characteristics to catalase, oxidase, methyl red, Voges proskauer, indole, nitrate reduction, citrate, galactose, maltose, glucose, xylose, sucrose, lactose tests. They differ in their abilities to utilize sugars and sugar alcohols. Isolate M slightly utilizes mannitol whereas other isolates were negative. Isolate N was slightly positive to sorbitol whereas other isolates were negative. Isolate M and Y were slightly positive to dulcitol whereas isolate N was negative. The bacterial isolates showed significant spore counts after 96h. The spore counts significantly ($p<0.05$) increased as the time increased. *Bacillus thuringiensis* M recorded the highest spore count whereas *Bacillus thuringiensis* Y recorded the least count. The bacterial isolates showed pronounced secretion of crystal proteins, which was significantly ($p<0.05$) most in Bt N. The amount of proteins (crystal proteins) produced significantly ($p<0.05$) increased in every 24 h interval. The study revealed significant cidal effect of the crystal protein on the *Ascaris lumbricoides* after 72 h. There was significant ($p< 0.05$) increased in the cidal activity of the crystal proteins as the concentration of the crystal protein increased. It was also observed that the crystal proteins secreted by Bt M had the most significant activity when compared to Bt N and Bt Y.

Table 1: Morphological properties of the bacterial isolates

Parameter	isolate M	isolate N	isolate Y
Colour	Cream on NYSM	Cream on NYSM	slightly cream on NYSM
Margin	Entire	Entire	Entire
Surface	Flat and Smooth	Flat and smooth	Flat and smooth
Gram Reaction	+	+	+
Shape	Rod	Rod	Rod
Cell arrangement	Singly	Singly	Singly
Endospore	+	+	+
Endospore Position	Central	Central	Central
Endosporangium	Not bulging	Not bulging	Not bulging
Motility	+	+	+
Crystal Production	+	+	+

Table 2: Biochemical characteristics of the bacterial isolates

Parameter	isolates M	isolates N	isolates Y
Oxidase	–	–	–
Catalase	+	+	+
Methyl Red	–	–	–
Voges Proskauer	+	+	+
Indole	–	–	–
Nitrate Reduction	+	+	+
Citrate	+	+	+
Glucose	+	+	+
Xylose	+	+	+
Sucrose	+	+	+
Lactose	–	–	–
Mannitol	+/_	_	_
Galactose	–	–	–
Maltose			
Sorbitol	_	+/_	_
Dulcitol	+/_	_	+/_
Probable Isolate	*Bacillus thuringiensis*	*Bacillus thuringiensis*	*Bacillus thuringiensis*

Table 3: Spore Counts of the bacterial isolates during crystal proteins production

Time (h)	BtM	BtN	BtY
24	4.62	4.58	4.53
48	4.74	4.67	4.61
72	4.86	4.79	4.76
96	4.93	4.87	4.82

Table 4: Proteins content of the bacterial isolates

Time (h)	Bt M	Bt N	Bt Y
24	9.24 – 0.11	13.08 + 0.02	11.03+ 0.04
48	11.85+ 0.04	17.08+ 0.03	14.05 + 0.04
72	14.10 + 0.02	19.82 + 0.02	17.01 + 0.03
96	14.12 + 0.03	19.86 + 0.05	17.02 + 0.05

Table 5: Effects of Crystal Protein on *Ascaris lumbricoide* after 72 h

	N = 10		
Conc.(ppm)	Bt M	Bt N	Bt Y
50	2	4	2
100	5	7	4
150	7	10	5
200	9	10	7
250	10	10	10

Table 6: Activity of the crystal proteins on the Ascaris lumbricoides

	Ascaris lumbricoides		
Parameter	Bt M(ppm)	Bt N(ppm)	Bt Y(ppm)
LC_{50}	100	65	125
LC_{70}	150	100	170
LC_{90}	200	135	225

DISCUSSION

Bacterial entomopathogens majorly *Bacillus thuringiensis* is one of the most advanced bio-rational rector control alternatives to synthetic insecticides (Sawicka and Egbuna, 2020). In the present study, the three bacterial isolates used, exhibited similar morphological characteristics and variation in crystal production which suggested the level of their toxicity to vectors. Similar observation was made by Pardo-Lopez *et al.* (2013). The presence of amorphous or irregular crystals detected in the tested isolated correlated with high *Ascaris lumbricoides* cidal activity as reported by Li *et al.* (2000). Also the variation in the position of endospores indicates the presence of different species of *Bacillus* (Schnepf *et al*., 2008). In addition to the crystal production, the tested isolates exhibited variation in some of their biochemical characteristics. The inability of some of the isolates to ferment sugar, and sugar alcohols suggested the presence of non-fermentative *Bacillus* species. The variation in the abilities of the fermentative *Bacillus* species to utilize different sugars and sugar alcohols suggested variation in their strains which corroborated with the report of Martins *et al*. (2008).

The significant spore counts of the bacterial isolates obtained during crystal proteins production could be attributed to the adaptive potentials and ability of the bacterial isolates to grow and

multiply in unfavorable conditions. Similar conclusion was drawn by Herssan *et al.* (2010). The significant increase in counts in every 24 h intervals observed during crystal proteins production correlated with the findings of Martins *et al.* (2008). The highest counts of *Bacillus thuringiensis* M recorded in the study could be attributed to the proliferation rate of the organism as the organism was able to thrive favorably in temperature, moisture content, pH and oxygen capacity of the growth medium. The significant amount of proteins (toxins) from the studied isolates formed the basic of larvicidal activity of the bacterial isolates. This correlated with the findings of Mezzomo et *al.* (2015).

The slight retardation in proteins (toxin) production after 72 h observed in the study could be attributed to the fact that the bacterial isolates might have reached their optimum level of proteins production and started diminishing when the conditions for the proteins production was no longer favourable. The highest secretion of proteins recorded in *Bacillus thuringiensis* M in this study could be attributed to genetic variation and environmental conditions that influence the proteins secretion. Masson *et al.* (2002) and Mitlner *et al.* (2004) reported that environmental conditions influence the level of crystal proteins production.

The significant death recorded from the cidal activities of the crystal proteins secreted from the bacterial isolates correlated with the findings of many researchers (Pardo-Lopez *et al,* 2013; Patil *et al.,* 2014; Mezzomo *et at.,* 2015). Many studies have shown that the crystal proteins of *Bacillus thuringiensis* primarily lysed the midgut epithelial cells by inserting into the target membrane and forming pores (Bravo *et al.,* 2007). The significant increase in the cidal activity of the crystal proteins due to increase in the concentrations of the crystal proteins were also reported by many researchers (Pardo-Lopez *et al,* 2013; Patil *et al.,* 2014; Mezzomo *et at.,* 2015). The highest cidal activity of crystal proteins secreted by *Bacillus thuringiensis* M in this study could be attributed to the amount and potency of crystal proteins secreted by the organisms. Studies have shown that cry toxins interact with specific receptors located on the host cell surface and activated by host proteases (Bravo *et al,* 2007). The concentration required for the crystal proteins to kill 50% of the total *Ascaris lumbricoides* exposed to the bacteria crystal proteins was recorded highest from *Bacillus thuringiensis* M. This could be due to the amount, nature and potency of toxin secreted by the isolate. Studies have shown that variation in activities of bacterial toxins could be attributed to the type of toxins, whether cry, cyt, vip or Bin toxins or the shape of the toxins, whether triangular, spherical or pyramidal crystals (Martins *et al.,* 2008; Mezzomo *et al.,* 2015).

CONCLUSION

This study has shown the presence of *Bacillus thuringiensis* strains M, N and Y in the studied soil samples. The Cry protein exhibited pronounced cidal activities against *Ascaris lumbricoides* of which Cry protein from *Bacillus thuringiensis* (Bt) M recorded the most pronounced activity and the Cry protein from the *Bacillus thuringiensis* Y recorded the least.

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