Tropical Journal of Applied Natural Sciences
Vol. 2 Issue 2 (2024)

Tropical Journal of Applied Natural Science
ISSN: 2449-2043

HEMATOTOXICOLOGICAL AND MOSQUITO LARVICIDAL STUDIES OF CRYSTAL PROTEINS SECRETED BY BACILLUS THURINGIENSIS AND BACILLUS SPHAERICUS

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Abstract

In Nigeria, mosquitos are transmitting several important human disease etiological agents, including malaria, and causing thousand of death every year. Mosquito control is based mostly on chemical insecticides which may be toxic and cause environmental deprivation. The objective of this study is to evaluate the hematological and larvicidal potentials of *Bacillus thuringiensis* and *Bacillus sphaericus* isolated from the soil. A total of fifty soil samples from garden soil were collected and screened for the presence of *Bacillus thuringiensis* and *Bacillus sphaericus* using pour plate technique. The organisms obtained from mixed culture plates were characterized and identified using their morphological, biochemical and molecular characteristics. Oral administration of the bacteria crystal proteins (Cry) and examination of blood samples from the experimental mice were carried out to assess the effect of the crystal proteins on the hematological indices of the mice. Larvicidal activities of the Cry were carried out by exposing the third instars' of larva stage of *Anopheles gambiae* to different concentrations (12.50, 25.00, 50.00, 100.00 and 200.00 ppm) of the Cry. The study revealed the presence of *Bacillus sphaericus* strain D45 (BsD45), *Bacillus sphaericus* strain DSM396 (BsDSM396), *Bacillus thuringiensis* strain DX3 (BtDX3) and *Bacillus thuringiensis* strain WO15 (BtWO15). The maximum spore counts (3.71 logCfu/ml) and Cry (22.03 mg/L) were revealed from BtDX3 after 96 h. There was no significant ($p > 0.05$) effect of the crystal proteins on the hematological indices of the mice, but slight deviations were observed in the lymphocytes, neutrophils and total white blood cells (WBCs). There was pronounced larvicidal activities of the Cry, mostly from BtDX3 and BtWO15 and these increased significantly ($p < 0.05$) as the concentrations of the Cry increased. The LC_{50} of the study revealed significant ($p < 0.05$) activities of the crystal proteins against the mosquito larvae, of which the Cry from BtDX3 showed the most pronounced activity. Therefore the Cry secreted from the BsD45, BsDSM396, BtDX3 and BtWO15 showed slight deviation only on the WBCs, lymphocytes and neutrophils. There were significant larvicidal activities of the Cry, of which Cry from strains of *Bacillus thuringiensis* (Bt) especially BtDX3 recorded the most pronounced activity.

Keywords: *Bacillus thuringiensis*, hematological indices, *Bacillus sphaericus.* larvicidal potentials.

INTRODUCTION

Mosquito borne diseases, majorly malaria causes extensive morbidity and mortality, and are major economic burden within disease malaria endemic countries, of which Nigeria is one of them (Subbiah and Abidha, 2010). Every year, about 300 million people are estimated to be affected by malaria, a major killer disease, which threatens 2,400 million (about 40%) of the world's population (Smerford *et al.,* 20013; Subbiah and Abidha, 2010). The incidence of mosquito-borne disease is increasing due to uncontrolled urbanization, creating mosquitogenic conditions for the vector mosquito population (Subbiah and Abidha, 2010). Therefore, mosquito control forms an essential component for the control of mosquito-borne diseases.

Malaria is effectively managed through combination of vector control, drugs and management of clinical illness (Subbiah and Abidha, 2010). Malaria vector control relies mostly on the use of an effective insecticide, which is commonly used through indoor residual spraying (IRS) or community based deployment of insecticide impregnated bed nets (ITN) (Subbiah and Abidha, 2010). There are numerous cases of insecticide resistance reported for *Anopheles* species. The emergence of mosquito species resistant to insecticides widely used in malaria control has the potential to impact severely on the control of these disease vector (Coleman *et al.,* 2016). A limited number of resistance mechanisms, including modification of the insecticide's target site, or changes in rates of metabolism involving esterases, glutathione-S-transferases or monooxygenases operate in all insects (Subbiah and Abidha, 2010) the potential for developing resistance in vectors has been apparent since the 1950's but the scale of the problem has been poorly documented (Crickmore *et al.,* 2008; Collins, 2012). Vector control is recognized as an effective tool for controlling tropical diseases. Synthetic insecticides have been used during the past several decades to control varied dipterans pests. However, the use of chemical insecticides has been greatly impeded due to development of physiological resistance in the vectors, environmental pollution, resulting in bio-amplification of food chain contamination and harmful effects on beneficial non-target animals (Subbiah and Abidha, 2010). Therefore, the need for alternate, more effective and environment friendly control agents became urgent.

The last decade has witnessed an increased interest in biological control agents. Many organisms have been investigated as potential agents for mosquito control. Including viruses, fungi, bacterial, protozoa, nematodes, invertebrate predators and fish ((Subbiah and Abidha, 2010).

However, most of these agents were shown to be of little operational use, largely because of the difficulty in multiplying them in large quantities. The discovery of bacteria like *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bs) which is highly toxic to dipterans larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs in the world (Parkins and bell 2008). These bacteria have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organisms including human beings. Also it is innocuous to the environment (Subbiah and Abidha, 2010).

Studies have shown that no evidence has been found that Bt and Bs toxins harm aquatic organisms sharing the breeding sites of these vectors or have adverse effect on the environment (Subbiah and Abidha, 2010). Although Bt toxin is effective specific not recycle in the environment at levels high enough to provide significant residual activity. Bs, on the other hand, has been shown to recycle in the field conditions and exert larvicidal activity for a long period. However, the spores of Bt have the advantage over Bs in that Bt has wider spectrum of activities against *Anopheles culex and Aedes* species due to multiple toxins, while Bs has its effect mainly on *culex,* for lesser extent to *Anopheles and Aedes* species (Subbiah and Abidha, 2010).

Studies have shown that narrow insecticidal range and evolution of insect resistance against some species of Bt and Bs, but more especially Bs have been documented (Pardo-Lopez *et al.,* 2014). An investigation of 24 cases, with each case relating responses of single pest species in one country to an individual Bt toxins, demonstrate that t he practical influence of field-evolved resistance can differ from none to severe, based on the magnitude, incidence and spatial distribution of resistance (Tabashnik *et al.,* 2014). Hence, isolation and characterization of new Bt strains from different regions with novel toxins, which have not been constantly exposed to dipterans are of significance for providing alternatives to these problems. In this regard, the present study will be carried out to explore diversity of Bt and Bs present in different soil samples that will be collected from different sites in Asaba, Delta State, Nigeria. This may yield new Bt strains with novel cry gene sequences which could encode crystal proteins with significant difference in the level of toxicity due to variation in their sequences.

MATERIALS AND METHODS

Reqirements for the Study

Sample collection: Fifty samples of garden soil from different sites was collected from Asaba, Delta State. The soil samples were taken 2 cm below the surface, after scrapping of the surface materials with sterile spatula, and then was finally collected into sterile aluminum foil. All the samples collected were labeled and taken to the laboratory for analysis within 2 h of collection

Sterilization of glass wares: The glass wares that were sterilized using an autoclave at 121^0C for 15 minutes. They were then washed with detergent and rinsed with distilled water. They will be air dried and placed in an inverted position inside the staircase of the hot air oven and set the thermostat at 160^0C for 3 h.

Media preparation: All the media used were prepared in Harmony Laboratory, Asaba. Sterilization will be done by the use of an autoclave, which will provide moist heat to kill the organisms present. Sterilization of media was done at a temperature of 121^0C, 15 PSI for 15 minutes. Work benches were also cleaned using 70% ethanol to ensure adequate sterility.

Procurement of mosquito larvae: The second and third instars of *Anopheles gambiae* were collected from research centers and Institutions and identify appropriately

Procurement of laboratory animals: The albino mice used for this study were collected from veterinary centers. The mice were housed in metal cages and fed prior to the study.

Isolation and Characterization of *Bacillus thuringiensis* and *Bacillus sphaericus*

Isolation of the organism: This was carried out using the method described by Bambang *et al.,* (2015) and Reyaz *et al.,* (2017). One gram of the soil sample was weighed into boiling test tube, 5m of distilled water was added and shake thoroughly and then make up to 10ml using the distilled water (10^{-1}dilution). The boiling tube was kept at 80^0C for 30 minutes and it was allowed to settle. One milliliter of this heat treated suspension was added to four milliliter (4ml) of normal saline (0.85% NaCl), which was give 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 10^{-1}, 5^{-1} and 5^{-5} test tubes were collected and plate on T3 agar medium, and NYSM agar (nutrient agar with 0.5g/l yeast extract, 0.2g/L$mgcl_2$ 0.01g/l $MnCl_2$ and 0.1g/L $CaCl_2$ with 100 mg/ml of streptomycin). These were done in triplicate and incubated invertedly at room temperature (30 ± 2^0C) 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 invertedly at room temperature (30 ± 2^0C) 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: The isolates were characterized and identified using the morphological, biochemical and molecular characteristics (Herssan *et al.,* 2010, Patit *et al.,* 2014, Bambang *et al.,* 2015).

Description of colony: This was carried out by adopting the method of Patil*et al.,* (2014). The appearance of the colonies on the LB agar such as the colonies, sizes, edges, optical properties, elevation and pigmentations would be observed and recorded.

Morphological characteristics: The appearance of the colonies on the study media such as the colonies, sizes, edges, optical properties, elevation and pigmentations were observed and recorded. The morphological features of the isolates such as the Gram reaction, cell morphology, cell arrangement, presence of spores, position of the spores in the sporangioguim, shape of the spores, presence or absence of swollen around the spores, presence or absence of parasporal inclusions and motility were observed and reported using the methods of Herssan *et al.* (2010) and Patil *et al.* (2014).

Gram staining technique: This was carried out using the method of Kanika (2011). In this process, a thin smear of the culture would be prepared on a clean grease free slide, air dried and heat fixed. The smear was flooded with crystal violet solution for 60 seconds and rinsed with water. It was then be covered with Gram's iodine for 60 seconds and rinsed with water. Alcohol (95% w/w ethanol) was used to decolourize the slide content for 10 seconds and rinsed with water. The smear was then be counter stained with safranin solution for 60 seconds, rinsed and air dried. The stained smear with a drop of immersion was then be observed under the light microscope using oil immersion objective lens.

Spore staining: This will be carried out using the method of Kanika(2011). Smear from the colonies of the isolates was carefully and aseptically made, heat fixed and flooded with malachite green. This was heat treated to produce steam for 5 minutes, more was added to keep it wet and

then cooled and rinsed with distilled water. Then the slide was counterstained with safranin for 30 seconds, rinsed with distilled water and air dried. The content of the slide was microscopically examined using oil immersion lens (×100) for the presence of endospores, position of the spore, presence or absence of swollen around the spores.

Motility test: This would be carried out using the method described in Kanika (2011). The medium used would be semi-solid agar. It was prepared by mixing 5.0g of bacteriological agar with 2.0g of nutrient broth in 1 liter of distilled water. Heat was applied to dissolve the agar and 10.0ml amounts was dispensed into test tubes and sterilized by autoclaving. The test tubes were allowed to set in a vertical position. Inoculation was done by making a single stab down the center of the test tube to half the depth of the medium using a standard stabbing needle. The test tubes were incubated at 37˚C for 24h. Motile bacteria would move and give diffused spreading growth that was visible to the naked eye.

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 x100 objective lens (Subbiah and Abidha, 2010).

Biochemical characteristics: The following biochemical tests were carried out:

Indole test: This would be carried out using the method described by Kanika(2011). For this test, tryptophan broth was prepared according to manufacturer's instruction and was inoculated with the isolates and then incubated at 37^{o}C for 24h. 5 drops of Kovac's reagent was added to the broth culture. The presence of indole was revealed by the formation of red layer colouration on the top of the broth culture.

Sugar fermentation test: This was carried out using the method described by Kanika(2011). This test was carried out to know the ability of the isolates to metabolize some sugars (glucose, xylose, ducitol, maltose, arabinose, inositol, mucate and lactose) with the resultant production of acid and gas or either. One litre of 1% (w/v) peptone water was added to 3 ml of 0.2% (w/v)bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then be sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes. The medium would then

be inoculated with the appropriate isolates and the cultures incubated at 37˚C for 48h and were examined for the production of acid and gas. A change in colour from purple to yellow indicated acid production while gas production was assessed by the presence of bubbles in the inverted Durham tubes.

Hydrogen sulphide production: This was carried out using the method described by Kanika(2011). This was performed using triple sugar iron (TSI) agar. The TSI agar was prepared according to the manufacturer's instruction. This was sterilized using autoclave and allowed to cool to 45˚C. The isolate was aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37˚C for 24-48h. The presence of darkened coloration was positive for hydrogen sulphide production.

Methyl Red test: This was carried out as described in the Manual of Microbiology by Kanika (2011). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution was added and mixed thoroughly, and the result was read immediately. Positive tests was give bright red colour while negative tests gave yellow colour.

Voges-Proskauer test: This was carried out as described in the Manual of Microbiology by Kanika (2011). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates was aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48h. After incubation, 1ml of 40% potassium hydroxide (KOH) was added and mixed well and 3ml of 5% solution of α-naphthol would be added and well mixed. Positive reaction would be observed by the development of pink colour within five minutes.

Citrate utilization test: This was carried out as described in the Manual of Microbiology by Kanika (2011). The Simmon's Citrate Agar was prepared according to the manufacturer's direction and the isolates was inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37˚C for 48h. Positive test would be indicated by the appearance of growth with blue colour, while negative test was show no growth and the original green colour was retained.

Catalase test: This was carried out as described in the Manual of Microbiology by Kanika (2011). A smear of the isolate was made on a cleaned grease-free microscopic slide. Then a drop of 30% hydrogen peroxide (H_2O_2) was added on the smear. Prompt effervescence indicated catalase production.

Molecular characterization: This involved DNA extraction, purification, amplification using PCR machine and sequencing of the amplicons using sequenser (Subbiah and Abidha, 2010).

Screening the Isolates for Crytal Proteins Production

Production of mosquitocidal toxins: a loopful of the test organism was inoculated into a 500 ml Evlemeyer flask containing 150 ml of feather meal medium that composed of 0.5g/L NH_4Cl, 0.5g/l NaCl, 0.3g/l K_2 HPO4, 0.4g/l KH_2 PO_4, 0.1g/l $MgCl_2$ $6H_2O$, 0.1g/l yeast extract and 10% chicken feather. This was maintained at pH 7.5, with manual shaking at (30 $\pm$ 2^0C) for 96-120 h. Samples were withdrawn at intervals to determine the optical densities, spore counts (CFU/ml) and toxins (protein mg/l) during crystal proteins production (Patil *et al.*, 2012).

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 80ºC for 15 minutes, serially diluted, then plated on the NYSM agar plates and incubated at 30ºC for 24 h., then the number of colonies on the plate were counted between 30 and 300 CFU/ml (Colony Forming Units) and recorded after every 24 h using electronic colony counter (Patil *et al.*, 2012).

Toxin (Protein mg/l) determination: Five milliliter (5 ml) of the culture medium was centrifuged at 10,000 rpm for 10 minutes and the resulting pellets were washed twice with normal saline (0.85% NaCl) and twice with distilled water. These pellets were then be suspended in 1ml of NaO (50mg/L, pH= 12.5) in order to solubilize protein crystals. After 2 h of incubation at 37ºC, total proteins in the supernatant was measured after re-centrifuged at 10,000 rpm for about 10 minutes using Bradford method (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 generated from mosquito larvicidal production was subjected to ammonium tetraoxosulphate (vi)($NH_4)_2SO_4$) precipitation using 80% ($NH_4)_2$ SO_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.

Effect of the Crystal Proteins on Hematological Parameters of Albino Mice

Hematotoxicological study: A total of ninety (90) albino mice of different sexes were used for this study. The mice were fed on diet specially prepared for the mice (vital grower feed) and were given water throughout the study period. The hematotoxicological study was carried out using the methods of Mozzomo *et al.* (2013) and Freire *et al.* (2014). The mice were divided into five groups (A, B, C, D. and E) consisting of nine adult (four months old) and nine young (one week old) mice. Group A was the control group that was administered only 0.5 ml of distilled water, groups B and C were administered orally by gavage, 0.5ml of the highest concentration (200 ppm) of the crystal proteins from isolates and groups D and E were administered orally by gavage, 0.5 ml of 200 ppm of the crystal proteins from Bs. The mice were maintained in their normal feeds with water for 4 weeks. The mice were monitoring daily for 24, 48 and 72h for lethal toxicity. The hematological parameters such as total white blood cells (WBC), total red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), neutrophils, lymphocytes, monocytes, eosinophils, basophils and total platelets were determined before and after weekly intervals for 4 weeks.

Mosquito Larvicidal Activity of the Crystal Proteins

Preparation of the toxin: The toxins were prepared by dissolving 0.05g, 0.04g, 0.03g, 0.02g and 0.01g respectively of the toxins in 100ml of phosphate buffer saline (PBS) to form 200, 100, 50, 25 and 12.50 ppm respectively

Mosquito larvicidal study: A total of forty (40) third instar larvae of *Anopheles gambiae* was introduced into 200 ml glass beaker containing 100 ml of distilled water with 0.1g of fish meal to prevent starvation. Then, 10 ml of each concentration of the prepared toxins was introduced into the suspension, whereas the control group was not introduced toxin concentrations. Mortality rate was determined at 24, 48 and 72 h respectively.

The LCSO was estimated using probit analysis (Fernando *et al.*, 2010).

Mortality rate of *Anopheles gambiae* larvae was calculated using this formula:

$$mortalityrate: \frac{numberofdeadlarvae}{numberoftotallarvae} x \frac{100}{1}$$

If in the negative control group 5-20% dead larvae are found, Abbott's correction formula was used to correct the mortality rate

Corrected mortality rate: $\frac{mortablityrateoftestgroup - mortalityrateofcontrolgroup}{100\% - mortablityrateofcontrolgroup} x\ 100\%$

Statistical Analysis

The results of the data generated were expressed as mean ± standard deviation (SD). The statistical analysis of valuable data generated from this study was analyzed by one-way Analysis of Variance (ANOVA) to determine the significant difference of the mean values at 95% confidence level. Pair wise comparison of mean was done using student "t" test (Pardo-Lopez *et al*., 2014)

RESULTS

This study showed that bacterial isolates X and Y exhibited similar characteristics in Luria Bertani agar medium but they differed from their positions of endospores and crystal production (Table 1). Isolate X produced endospore that was terminally positioned whereas that of isolate Y was both terminally and subterminally positioned. Also isolate X produced crystals whereas isolate Y showed slight production of crystals. Isolates M and N exhibited similar properties on Luria Bertani agar medium but differed in their colour exhibited on the medium (Table). Isolate M was cream whereas isolate N was slightly cream. Isolates X and Y differed from isolates M and N majorly from their colours on Luria Bertani agar medium and the nature of endosporangium. Isolates X and Y produced

endospores that were terminally/subterminally positioned wherea that of isolates M and N were centrally positioned.

The isolates exhibited similar biochemical reactions to oxidase test (negative), catalase test (positive) casein hydrolysis test (positive), Methyl red test (negative), indole test (negative) they were not able to ferment lactase, mannitol, galactose, and maltose. Isolates X and Y exhibited similar biochemical characteristics and were not able to ferment any of the sugars tested. Isolates M and N were positive to voges-proskauer, test, nitrate reduction test, starch hydrolysis test, citrate utilization test, and were able to ferment glucose, xylose, sucrose and trahalose. They differed from their abilities to ferment sorbitol and dulcitol. Isolate M was slightly positive to sorbitol and dulcitol whereas isolate N showed negative reactions to the two sugar alcohols (Table 2).

The isolates showed reasonable concentration of pure DNA as their ratio of A260/A280 were within 1.80 – 1.90 (Table 3). The cleaned bands of separations of genomic DNA and amplicons from the studied isolates on agarose gel are shown in Figures 1 and 2. The sequencing results of the cleaned amplicons revealed the presence of *Bacillus sphaericus* strain D45 (Bs D45), *Bacillus sphaericus* strain DSM 396 (BsDSM 396), *Bacillus thuringiensis* strain D x 3 (B + D X 3) and *Bacillus thuringiensis* strain WO15 (B+WO15). The identified isolates were able to record 100% query cover and 100% identity.

The study showed significant spore counts of the bacterial isolates during crystal proteins production (Table 5). It was observed that the spore counts increased significantly ($P< 0.05$) in every 24 h intervals. BtDX3 significantly ($P< 0.05$) recorded the highest count after every 24 h interval whereas BsDSM396 recorded the least count

The study recorded significant amount of proteins (toxins) from the studied isolates of which *Bacillus thuringiensis* strains significantly ($P< 0.05$) produced more of the proteins than *Bacillus sphaericus* strains. The proteins production significantly ($p< 0.05$) increased in every 24 h interval with slight retardation after 72 h. *Bacillus thringiensis* strain Dx3 secreted the highest amount of proteins (toxins), followed by *Bacillus thuringiensis* strain

W015, *Bacillus sphaericus* strain D45 and *Bacillussphaericus* strain DSM 396 secreted the least amount of the proteins.

The crystal proteins secreted by the bacterial isolates had no significant ($P > 0.05$) effect on hemoglobin level, total red blood cells (RDC), packed cell volume (PCV), platelet, MCV and MCH of the albino indices. A slight change was observed in the total white blood cell counts (WBCs), neutrophils and monocytes. Also, a minor deviation was observed in the lymphocytes which were seen most among the albino mice administered crystal proteins secreted from *Bacillus thuringiensis* strain D X 3 (B + D x 3).

Statistically, there was no significant difference ($P > 0.05$) between the hematological indices of the control mice and that of the tested mice. It was also observed that both the control and tested mice showed no change or alteration on the amount of eosinophil.

A total of forty (40) larvae were exposed to different concentrations of crystal proteins secreted by the tested bacterial isolates, of which significant deaths were recorded (table 8). The larvicidal activity of the crystal proteins increased significantly ($P<0.05$) as the concentration of the crystal proteins increased. Also, the activities of *Bacillus thuringiensis* strain DX3 (BtDx3) and *Bacillus thuringiensis* strain W015 (BtW015) were significantly ($P<0.05$) higher than that of *B. sphaericus* strain D45 (BsD45) and *B. sphaericus* strain DSM396 (Bs DSM 396) as shown in table 8. Bt Dx3 recorded the highest activity whereas BsDSM 396 recorded the least activity.

This study showed that the concentration required for the crystal proteins to kill 50% of the total larvae exposed to the proteins was recorded lowest from *Bacillus thuringiensis* strain Dx3 (Bt Dx3) followed by *B. thuringiensis* strain W015 (BtW015), BsD45 and BsDSM396 recorded the highest concentration (table 9). The results revealed that the larvicidal activity of BtDx3 was most significantly ($P<0.05$) pronounced against the test *Anopheles gambiae.* Also, the crystal proteins from BtDX3 recorded the highest mortality rare at maximum concentration, followed by BtWO15, BsD45 and BsDSM396 recorded the least mortality rate.

Table 1: Morphological properties of the bacterial isolates

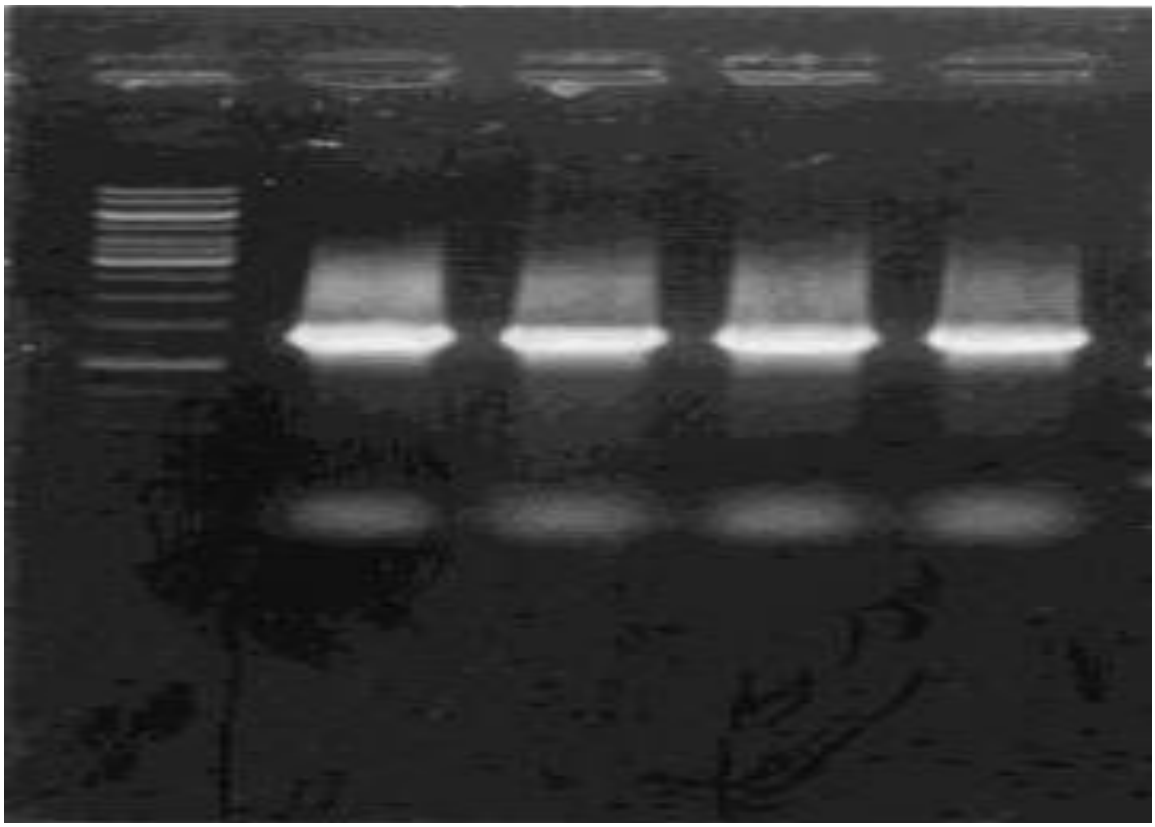
Characteristics	Isolate X	Isolate Y	Isolate M	Isolate N
Colour	white	white	Cream	slightly cream
Margin	Entire	Entire	Entire	Entire
Surface	flat and Smooth	flat and smooth	flat and smooth	flat and smooth
Gram reaction	+	+	+	+
Shape	Rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Single	Single
Endospore	+	+	+	+
Endospore Position	Terminal	Terminal/	central	central

		Subterminal		
Emdosporagim	Bulging	Bulging	not bulging	Not bulging
Motility	+	+	+	+
Crystal production	+	+/-	+	+

Table 2: Biochemical properties of the bacterial isolates

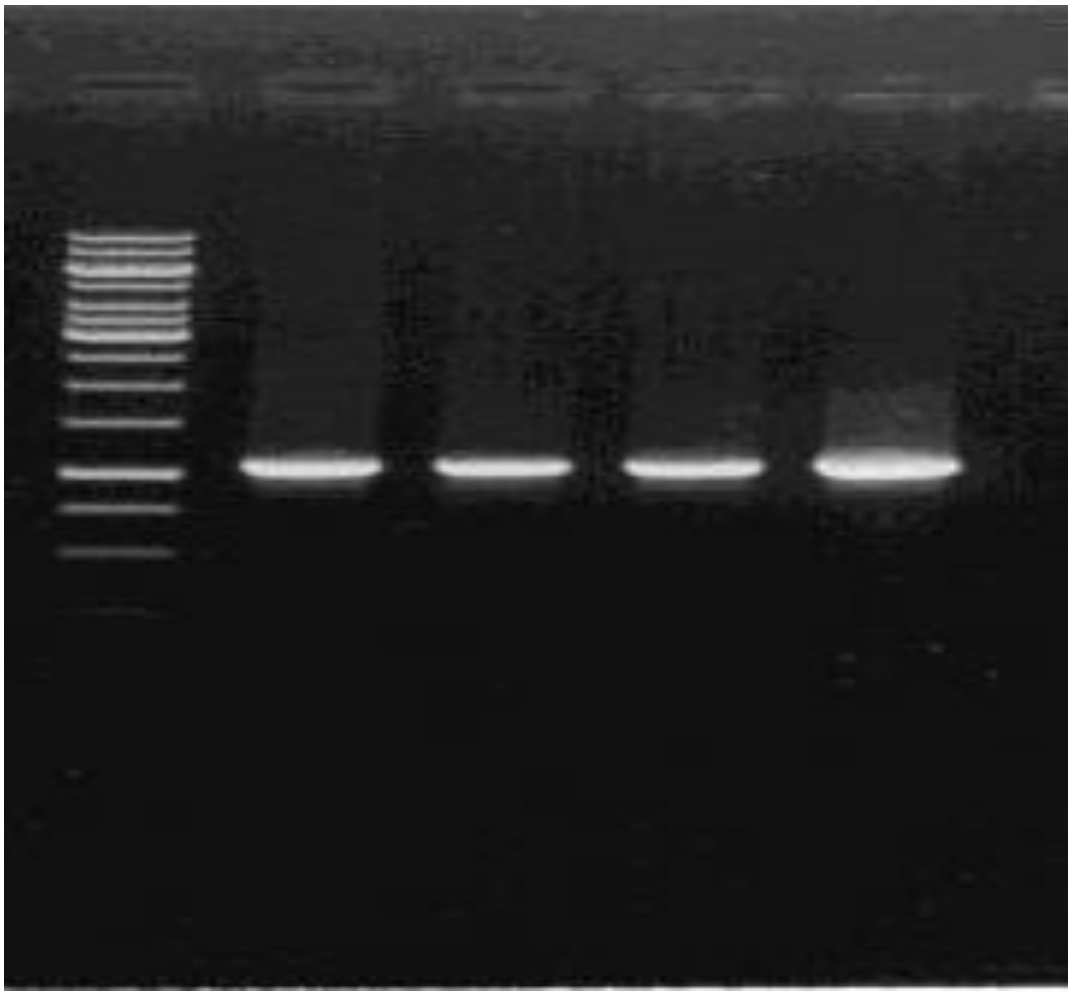
Characteristics	Isolates	isolate X	Isolate M	Isolate N
Oxidase	-	-	-	-
Catalase	+	+	+	+
Methyl red	-	-	-	-
Vogesproskauer	-	-	+	+
Indole	-	-	-	-
Nitrate reduction	-	-	+	+
Urease production	-	-	+/-	+/-
Starch hydrolysis	-	-	+	+
Citrate	+/-	+/-	+	+
Casein	+	+	+	+
Glucose	-	-	+	+
Xylose	-	-	+	+

Lactose	-	-	-	-
Sucrose	-	-	+	+
Mannitol	-	-	-	-
Galactose	-	-	-	-
Maltose	-	-	-	-
Sorbitol	-	-	+/-	-
Dulcitol	-	-	+/-	-
Trahalose	-	-	+/	+



1 kb M N X Y

Figure 1: Agarose gel of the amplified DNA of the isolates



1 kb M N X Y

Figure 2: Agarose gel of the purified amplicons for sequencing

Table 3: Purity of nucleic acids extracted from the bacterial isolates

Isolate	Concentration of nucleic acid (ng/ul)	A_{260}	A_{280}	260/280
X	102.10	2.1460	1.1663	1.84
Y	97.70	2.1140	1.1680	1.81
M	128.40	2.8610	1.5299	1.87
N	119.60	2.6740	1.4454	1.85

Table 4: Molecular identities of the bacterial isolates

Isolate	Max score	Total score	Query cover	Gap	Identity	Accession number	Description
X	2617	2617	100%	0.0	100%	DQ9923492.1	*Bacillus sphaericus* strain D45
Y	2880	2880	100%	0.0	100%	AJ311894.1	*Bacillus sphaericus* strain DSM 396
M	1441	1441	100%	0.0	100%	MK431504.1	*Bacillus thuringiensis* strain DX3
N	6966	6966	100%	0.0	100%	EU282379.a	*Bacillus thuringiensis* strain WO15

Table 5: Spore courts of the bacterial isolates during crystal proteins production

Time (h)	Bsd45 (log CFU/ml)	BsDSM396 (log CFU/ml)	B+Dx3 (log CFU/ml)	B+W015 s(log CFU/ml)
24	3.44±0.02	3.37±0.03	3.49±0.03	3.48± 0.03
48	3.55±0.02	3.50±0.03	3.61±0.02	3.59±0.02
72	3.62±0.01	3.60±0.01	3.69±0.01	3.66±0.01
96	3.64±0.01	3.61±0.01	3.74±0.01	3.67±0.01

BSD45 –*Bacillushaericus*strain D45

BSDSM 396 –*Bacillus sphaericus* strainDSm 396

BtDx3 – *Bacillusthurigiensis* strain Dx3

BtW015 –*Bacillus thurigiensis* strain W015

Table 6: Proteins content of the bacterial isolates

Time (h)	Bs D45 (mg/l)	BsDSM396 (mg/l)	BtDx3 (mg/l)	BtW015 (mg/l)
24	9.47±0.14	8.93±0.05	13.31±0.18	11.99±0.02
48	11.23±0.15	10.55±0.08	17.5±0.10	15.68±0.06
72	14.68±0.04	13.21±0.09	21.04±0.04	19.86±0.13
96	14.94±0.04	13.86±008	22.03±0.09	20.07±0.03

BsD45 - *Bacillus sphaericus* strain D45

BsDSM396 – *Bacillus sphaericus* strain DSM 396

BtDx3 -*Bacillus thuringiensis* strain Dx3

BtW015 - *Bacillus thuringiensis* strain W015

Table 7: Effects of crystal proteins in hematological indices of albino mice

Index	Control	BsD45	BsDsM396	B+Dx3	B+WO15
Hb(g/L)	150.12±0.01	150.08±0.02	150.09±0.01	150.01±0.01	150.05±0.02
Total RBC (10^{12}/L)	7.51±0.01	7.48±0.01	7.50±0.01	7.51±0.01	7.52±0.01
PCV(L/L)	0.45±0.01	0.44±0.01	0.46±0.01	0.46±0.01	0.46±0.01
Total WBCs (10^{9}/L)	7.20±0.01	7.08±0.03	7.14±0.02	7.19±0.03	7.23±0.02
Neutrophil (%)	12.68±0.01	13.02±0.02	12.84±0.04	12.71±0.02	12.76±0.01
Lymphocytes (%)	85.20±0.01	83.68±0.01	83.87±0.02	84.09±0.01	83.98±0.03
Monocytes (%)	2.10±0.02	2.28±0.03	2.26±0.02	2.18±0.02	2.23±0.04
Eosinophi(%)	1.02±0.00	1.02±0.00	1.02±0.00	1.02±0.00	1.02±0.00
Platelet count (10^{9}/L)	858.00±2.00	852.00±1.00	856.00±2.00	856.00±1.00	854.00±1.00
MCV(FL)	55.48±0.02	55.07±0.03	55.15±0.02	55.23±0.01	55.29±0.02

MCH(Pg)	17.88±0.03	17.23±0.03	17.35±0.03	17.41±0.04	17.68±0.04

BsD45 – *Bacillus sphaericus* strain D45

DsDSM396 – *Bacillus sphaericus* strain DSM 396

B+Dx3 – *Bacillus thurigiensis* strain Dx3

B+W015 – *Bacillus thuringiensis* strain WO15

Table 8: Effects of the crystal proteins in *Anopheles gambiae* after 72 h.

Conc (ppm)	BsD45	N = 40 Bs DSM 396	Bt Dx3	Bt W015
12.50	4.67±0.58	3.33±0.58	8.67±0.58	6.67±0.58
25.00	8.33±1.15	6.33±1.15	12.33±1.15	10.67±0.58
50.00	11.67±1.15	9.67±0.58	16.33±0.58	13.33±0.58
100.00	14.33±1.15	12.33±1.15	20.33±1.15	17.33±0.58
200.00	16.67±0.58	14.67±0.58	22.33±1.15	20.00±1.73

Bs D45 - *Bacillus sphaericus* strain D45

Bs DSM 396 - *Bacillus sphaericus* strain DSM 396

Bt Dx3 - *Bacillus thuringiensis* strain Dx3

BtW015 - *Bacillus thuringiensis* strain W015

Table 9: Larvicidal activities of the bacterial crystal proteins

Isolate	LC50	LC 90	Mortality Rate at Max Conc. (%)
BsD45	250	520	41.68
BsDSM396	295	575	36.68
BtDX3	95	380	55.83
BtWO15	200	430	50.00

BsD45= *Bacillus sphaericus* strain D45

BsDSM396 = *Bacillus sphaericus* strain DSM396

BtDX3 = *Bacillus thuringiensis* strain DX3

BtWO15 = *Bacillus thuringiensis* WO15

DISCUSSION

The use of synthetic insecticide as a cardinal approach in controlling mosquito larvae has recorded several limitations ranging from resistance by the target mosquito larvae, effect on non-target organism and distortation of natural ecosystem. In humans and animals, prolonged exposures lead to cancer, immune toxicity, liver damage, reproductive and birth defects (Turusor *et al*., 2002)

Biological insecticides from natural origin that is selectively toxic and environmental friendly will serve as suitable alternative to chemical insecticides. In the present study, the four 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 E l-kersh *et al*. (2016). The presence of amorphous or irregular crystals detected in the tested isolated correlated with high mosquito larvicidal activity as reported by Martins *et al*. (2010). Also the variation in the position of endospores indicates the presence of different species of *Bacillus.*

In addition to the crystal production, the tasted 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 similar conclusion was drawn by Martins et al, (2010).

Traditionally, the laboratory detention of *Bacillus* species has relied on the use of morpological and biochemical characteristics, the introduction of molecular techniques provides more sensitive and rapid technique for detecting this isolates. In this study, the molecular technique which involved sequencing of 16 SrRNA from each isolates revealed the presence of *Bacillus sphaericus* strain D45 (BsD45), *Bacillus sphaericus* strain DSM 396 (BsDSM396), *Bacillus thuringiensis* strain D×3 (BtD×3) and *Bacillus thuringiensis* strain W015 (BtW015). The presence of these species of *Bacillus* has been reported by different researchers (Chang *et al.*, 2007; Keshavarzi, 2008; Martins *et al*., 2014; Dandan *et al.*, 2011; Aileen *et al*., 2013; Elkersh *et al*., 2016) but of different strains. The new strains encountered in the present study suggest genetic variation and diversity among the *Bacillus* isolates.

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 Martins *et al*. (2010). The significant increase in counts in every 24hrs intervals observed during crystal proteins production correlated with the findings of Martins *et al.* (2010). The highest count of *Bacillus thuringiesis* strain D×3 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 o proteins (toxins)

from the studied isolated formed the basic of larvicidal activity of the bacterial isolates. This circulated with the findings of El-Kersh *et al.* (2016).

The significant increase in the proteins production during crystal proteins production circulated with the findings of other researchers (DaCosta *et al.,* 2010; Gobatto *et al.,* 2010; Mahalakshmi *et al.,* 2012; El-Kersh *et al.*, 2016).

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* strains D x 3 (BtDx3) in this study could be attributed to genetic variation and environmental conditions that influence the proteins secretion. Several researchers (Da Costa *et al.,* 2010; Mahalakshmi *et al.,* 2012) reported that environmental conditions influence the level of crystal proteins production.

In order to avoid predisposing humans and other mammals to potential infections, proper evaluation of the safety of the entomopathogens is of utmost importance to draw certainty that actually the organisms to be used in field application are not pathogenic or have any negative effect to humans and other mammals. The non-significant effect on the hemoglobin level, total red blood cells, packed cell volume,

platelet, MCV and MCH observed in the study researchers (Barcellos *et al.,* 2003; Timbre, 2009; Mezzomo *et al.,* 2013). A slight deviation observed in the total white blood cells (WBCs), neutrophils and monocytes correlated with the findings of EL-Kersh *et al.,* (2016). Studies have shown that the inflammatory response in often associated with increased in lymphocytes and neutrophils, and small changes in the number of neutrophils may be biologically significant and reflected in the total leucocytes count (Martins *et al.,* 2010).

The significant death recorded from the larvicidal activities of the crystal proteins secreted from the bacterial isolates correlated with the findings of many researchers (Gobato *et al*., 2010; Mahalakshmi *et al*., 20123; El-Kersh *et al.*, 2016). Many studies have shown that the crystal proteins of *Bacillus thuringiensis* primarily lyse the midgut epithelial cells by inserting into the target membrane and forming pores (Bravo *et al*., 2007). The significant increased in the larvicidal activity of the crystal proteins due to increased in the concentrations of the crystal proteins was also reported by many researchers (Gobato *et al.*, 2010; Mahalakshmi *et al.,* 2012; El-Kersh *et al*., 2016). *Bacillus thuringiensis* strains showed more larvicidal activity than *Bacillus sphaericus* strains. This 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 larvae exposed to the bacteria crystal proteins was recorded lowest from *Bacillus thuringiensis* strain DX3 (BtDX3). This could be due to the amount, nature and potency of toxin secreted by the isolate. Studies have shown that variation in larvicidal 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.,* 2010; El-Kersh *et al.,* 2016).

CONCLUSION

This study has shown the presence of *Bacillus sphaericus* strain D45 (BsD45), *Bacillussphaericus* strain DSM396 (BsDSM396), *Bacillus thuringiensis* strain DX3 (Bt DX3) and *Bacillus thuringiensis* strain WO15 (BtWO15) from the studied soil samples. There were slight deviations on the total white blood cells (WBCs), lymphocytes and neutrophils among the hematological indices tested from the blood samples of the experimented mice administered crystal proteins (Cry) secreted from the bacterial isolates. The Cry exhibited pronounced larvicidal activities against *Anopheles gambiae*, of which Cry from strains of *Bacillus thuringiensis* (Bt) especially BtDX3 recorded the most pronounced activity.

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