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Toxicity Evaluation and Genetic Improvement of *Bacillus thuringiensis* Isolated from Different Regions in Assiut, Egypt against Mosquito Larvae

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Abstract

This study aims to evaluate the toxicity and genetic improvement of *Bacillus thuringiensis* isolates. Isolates were obtained from soil, insect and water samples from different regions of Assiut, Egypt for biological control of mosquito larvae. *B. thuringiensis* colonies were identified based on morphological and then by PCR which detect the *Cry* toxic genes in the isolates. Bioassays were performed to evaluate the toxicity of different strains of *B. thuringiensis* against mosquito larvae such as (*Culex* spp). In general, 36 *B. thuringiensis* isolates were obtained (31 from soil, 4 from insects, and 1 from water). And they were all toxic to mosquito larvae with different mortality percentages from 7 to 97% after 48 hours. Isolate Am2 recorded the highest mortality percentage 97% and Mn3 lowest mortality percentage 7%. PCR revealed that Am2 isolate which caused the highest mortality encodes three different types of *Cry* toxins, *Cyt1AA, Cry1Ac* and *Cry2Aa*. This isolate Am2 was examined by scanning electron microscopy to observe the shape of the *Cry* proteins. The results showed that the Am2 isolate contained of spherical and cuboidal toxic proteins. Then UV-mutagenesis was performed on the Am2 isolate to improve its toxicity. Out of 30 obtained UV-mutants, only one mutant showed improvement in the mortality of mosquito larvae since it caused a mortality rate of 100%. The results of the present study revealed the larvicidal efficacy of *B. thuringiensis* (Am2) isolate found in the soil of Assiut, could be used in biological control program of mosquito larvae.

Keywords: *B. thuringiensis*, *Cry* Genes, *Cyt* Genes, UV- mutagenesis, Scanning Electron Microscope

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INTRODUCTION

Mosquitoes are among the most important insects that transmit diseases to humans, as they infect them with diseases due to their transmission of viruses and parasites.[1,2] The mosquito transmits the virus that causes several diseases, including dengue fever, yellow fever, and chikungunya.[3] Where they are found in places where humans and animals live and breed in places that contain ponds with stagnant water and swamps, as they multiply rapidly and the resulting eggs are resistant to drought and withstand harsh conditions until the appropriate conditions are available to complete the life cycle of the insect, so there is difficulty in controlling them.[4]

The current strategy for controlling insect vectors of different infectious diseases is based on the use of programs of chemical insecticides, the development of resistance is a threat to the program's efficacy or by eliminating breeding sites.[5,6] However, the frequent use of these chemicals has led to limiting the effectiveness, environmental pollution, toxicity in humans and animals and the development of resistance in mosquito populations.[7-9] Therefore, it is necessary to search for alternative and more harmless control methods including biological methods such as the use of entomopathogenic bacteria, in which the bacterium *B. thuringiensis* represents a very promising alternative.[10]

*B. thuringiensis*is is a Gram-positive bacteria as well as facultative in that they are aerobic or anaerobic and produce spores in the form of protein crystals that are toxic against many orders of insects including *B. thuringiensis subsp. israelensis* (Diptera), *B. thuringiensis subsp. tenebrionis* (Coleoptera), *B. thuringiensis subsp. kurstaki, B. thuringiensis subsp. aizawai* (Lepidoptera) are examples of subspecies with specific bacterium against at variance groups of insects.[11] These crystals are δ-endotoxins proteins, including *Cry* and *Cyt* proteins.[12] These toxic proteins are encoded by the *Cry* and *Cyt* genes during the sporulation.[1,13] More than 700 *Cry* genes have been sequenced and classified into at least 70 groups named *Cry1, Cry2, Cry3 ... Cry70,* whose corresponding insect toxicity.[13,14]

The severity of toxicity to the insect also depends on the combinations of different proteins expressed by the *Cry* genes.[15,16] Some strains can encode for more than one toxic protein, a well-known example is *B. thuringiensis var. israelensis*, which contains six proteins that are toxic to Diptera, this strain contains a plasmid called megaplasmid pBtoxis that encodes six different toxic proteins to mosquito larvae.[17,18] It was observed in some strains that were isolated from different places that they contain different combinations of genes and have specialized toxicity to different insects order.[19,20] These toxic proteins bind to receptors located in the lining membrane of the intestine of the mosquito larvae, where they feed on crystalline proteins, which leads to an imbalance of ions that leads to cell rupture and death of the larva.[21-23]

The main objective of this study was to collect different isolates of *B. thuringiensis* from different locations in Assiut governorate, Egypt that cause mortality to mosquito larvae, molecularly characterize its *cry* genes, and enhance the toxicity by UV- mutagenesis.

MATERIALS AND METHODS

Isolation of *B. thuringiensis*

Samples were collected from different areas and different sources (soil, water and insects) of Assiut governorate, Egypt. Samples were collected at a depth of 10 cm below soil surface using cylindrical sampler and kept in polyethylene bags. All samples were immediately transported to the laboratory and stored in a refrigerator at 4°C until used for the isolation of *B. thuringiensis*.

B. thuringiensis was isolated according to previous studies with some modifications.[19,24] One gram of soil sample was taken and mixed in 10 ml of sterilized distilled water. 100 µl of each sample was plated to a petri dish containing the nutrient agar supplemented with penicillin G (100 mg/L). Insect samples were cut into small pieces and mixed with 2 ml of sterilized distilled water then 100 µl were transferred to petri plates containing the selective media. And then, 100 µl of the water samples were transferred directly to petri plates. Then all the plates were placed in the incubation

for 48 hours and incubated at a temperature of 28°C. The colonies showed round shapes with wavy edges and no pigment morphology were inoculated in a nutrient broth and selected for further studies.

B. thuringiensis Bioassay Activity

To test the ability of different *B. thuringiensis* isolates to kill the mosquito larvae, all the *B. thuringiensis* isolates were incubated in nutrient broth for five days at 28°C and 180 rpm until the sporulation was observed. The concentration of each isolate was measured and adjusted to be equal at a concentration of OD_{600}= 0.2. The sporulated isolate were added to 10 ml of sterile water that contains 10 mosquito larvae and incubated at of 28 ± 2°C. The number of deaths mosquito larvae was recorded after 48 hours of the treatment.[25] The treatment was carried out in three replicates for each isolate. For comparison, an additional replicate containing the commercial bio-insecticide named Dacron 54% WP (SAFA TARIM. A. S. Turky) was used as a positive control.

Detection of the Presence of Toxic Genes within the Genome of the Isolates by PCR

Different *Cry* toxic proteins are produced by different *B. thuringiensis* strains.[1,26-28] PCR technique was used to screen the presence of some toxic genes in collected *B. thuringiensis* isolates. Five primer pairs were newly designed to target 5 different *Cry* and *Cyt* genes (*Cry11AA, Cyt1AA, Cry1Ac, Cry2Aa* and *Cry4AA*) from *B. thuringiensis* (Table 1). All gene sequences were

obtained from NCBI (www.ncbi.nlm.nih.gov). Sequences of DNA primers were manually selected by considering, sequence location, GC content, number of nucleotides and simulated with In-Silico PCR amplification website (http://insilico.ehu.es/PCR/). There is no probability of complementarity between one primer with other or bases in the same primer.[29]

DNA isolation was performed by the boiling method.[24,30] PCR was performed in a final volume of 20 µl containing 10 µl of GoTaq blue master mix (Promega, Madison, WI, USA), 1 µl DNA sample, 0.2 µl of each primer and 8.5 µl of water, nuclease-free. The amplification reactions were carried out in a thermocycler (Sensoquest, Biomedical Electronics, Germany) under the following initial denaturation at 93°C for 5 min, followed by 30 cycles of, 30 sec of denaturation at 95°C, 1 min for annealing at 55 - 58°C and 2 min for the extension at 72°C, then followed by 5 min final extension at 72°C and a final hold at 4°C. Then 1% agarose gel and 1X TAE were used to perform the electrophoresis. The gel was stained with ethidium bromide, visualized under UV rays and photographed. The molecular sizes of DNA fragments were measured by comparison with a 100-bp molecular marker (Hyperladder 100 bp, Bioline, Meridian Bioscience, UK).

Scanning Electron Microscope

To observe the crystalline proteins under the electron microscope, isolate Am2 was grown on nutrient agar medium for 5 days at 28°C until sporulation. The spores were washed three times by cacodylate buffer for 13 minutes and post fixed

Table 1. Primers used in the PCR to amplify Cry and Cyt genes of *B. thuringiensis* that cause toxic activity against mosquito, showing the primer sequences, the size of the target fragment, and the annealing temperature

Gene	Accession No.	Nucleotide sequence (5'-3')	Fragment size (bp)	Temp. (°C)	Activity
cry11AA	NC_010076	CCAGGTTCTCAACCTGCTAC CTCCCTGCTAGGATTCCGTC	325	56	Dip
cyt1AA	NC_010076	CCCCTCAATCAACAGCAAGG CTCACTACAGCACCCATCGG	241	56	Dip
cry1Ac	NC_020249	GAGTGGGAAGCAGATCCTAC CCAAGAGAACATAGGAGCTC	1020	55	Lep
cry2Aa	NZ_CP013056	GGTAGTGGACCACAGCAGAC GTAGAGGTAGCAACGCCCTC	338	58	Lep/Dip
cry4AA	NC_010076	CCCAGGGGTAGCAGAACTGG	802	58	Dip

in 1% osmium tetroxide for two hours. The sample was washed by cacodylate buffer for 13 minutes for three times and then dried by an ascending series of ethanol 30, 50, 70 and 90% for two hours and 100% for two days and then transferred to amyl acetate for two days. The sample was dried by using liquid carbon dioxide. The sample was stuck on metallic blocks using silver paint. By using gold sputter coating apparatus, the sample was evenly gold' coated in a thickness of 15 nm. The sample was examined in the electron microscope unit at Assiut University, Egypt by uSIng JEOL JSM 5400 LV scanning electron microscope 15- 25 kV at a voltage of 15 kV and magnification of 5000x.[31,32]

Induction of Mutagenesis with Ultraviolet Rays (UV)

The most mortal *B. thuringiensis* isolate to mosquito larvae was selected to improve its efficiency by UV-mutagenesis. 1 ml of an overnight culture was poured and spread on the petri plates that contain nutrient agar medium. The plates were exposed to UV source at different time intervals (0 sec as control), 20, 40, 80, 120, 160, 200, 240 and 280sec). The plates were covered in a black polyethylene bag and incubated at 28°C for 48h to get first generation mutants. The plates that were exposed to the time causing the death of half the colonies (LC$_{50}$) were used to isolate the mutants.[33] And all the selected mutants were screened for their mortality by the bioassay test which was carried out in three replicates.

RESULTS AND DISCUSSION

Isolation of *B. thuringiensis* isolates from different Locations and Sources

Around 100 bacterial isolates with similar morphological characteristics to *B. thuringiensis* (Table 2) were obtained from 9 different locations (Assiut university farm, Menqabad, Al-Ghanaim, Posra, Arab almadabigh, Sedfa, Sahel Salim, Al-Fath, West country) and different sources (water, soil and insects). The 100 bacterial isolates were morphologically screened, and colonies showed round shapes with wavy edges and no pigment morphology were furthermore checked by inoculation on a selection media containing penicillin G (100 mg/L).

Soil is the main natural reservoir for *B. thuringiensis* bacteria and is currently the preferred substrate for the isolation of *Bacillus* species.[34-36] However, *B. thuringiensis* was also found in insects and water samples.[37-42] Our results showed that soil samples are wealthy with *B. thuringiensis* isolates since we could isolate from soil 31 different isolate.

Table 2. Different isolates code, location, type of sample, number of bacterial isolates, and number of *B. thuringiensis* isolates

Isolates code	Sample's location	Type of sample	No. of Bacterial isolates	No. of isolates caused Mortality
Am2, Am3, Am4, Am4.2	Arab Almadabigh	Soil	5	4
Laq		Water	10	1
Ar1, Ar7, Ar9, Ar6, Ar7.2, Ar5, Ar8, Ar3, Ar4, Ar2	Assiut University Farm	Soil	30	10
Be3, Sp4.2, Sp5		Insects	8	3
Ps1, Ps5, Ps6	Posra	Soil	6	3
Gh5.2, Gh3, Gh2, Gh1, Gh6.2, Gh5	Al-Ghanaim	Soil	8	7
Mn8, Mn9, Mn10, Mn3	Menqabad	Soil	10	4
Wc1	West Country	Soil	5	1
St3	Sedfa	Soil	6	1
Ft5	Al-Fath	Soil	7	1
Ss2	Sahel Salim	Soil	4	1
Btc	Egypt Microbial Culture Collection (EMCC)	Insects	1	1
	Total		100	36

Isolates Toxicity Evaluation

After the morphological screening, all the isolates were examined for their mortality to mosquito larvae. Out of the 100 isolates, only 36 isolates showed a mortality effect on mosquito larvae (Table 2), so these isolates were selected for further investigations. The rest of the isolates (64 isolates) did not give a mortality rate (results are not shown), so they were excluded from further experiments.

According to the mortality rate of mosquito larvae (Figure 1), the isolates were divided into 3 different levels (high, intermediate, and low). The potency of the tested isolates after 48 hours indicated that 9 isolates were highly toxic (Am2, Ar1, Ps1, Ar7, Gh5.2, Ar9, Be3, Ar6 and Ar7.2) with a mortality rate ranging from 70 to 97%; 12 intermediate isolates (Btc, Ar8, Mn8, Gh3, Ar5, St3, Sp4.2, Ar3, Ar4, Gh2, Am3 and Gh1) causing 40-57%; the rest of the isolates, 15 isolates, (Laq, Gh6.2, Ft5, Ar2, Ps5, Gh5, Ss2, Wc1, Ps6, Mn9, Sp5, Am4, Mn10, Am4.2 and Mn3), showed low mortality rate ranged from 7 to 37%.

Am2 isolate showed the highest mortality rate (97%) among all the isolates, while Mn3 isolate showed the lowest one (7%) after 48h of incubation.

The results illustrated in (Figure 1) showed that, the used bio-pesticide recorded a very low mortality rate (27%) compared to bacterial isolates especially the Am2 isolate which showed the highest mortality rate.

Previous studies indicated that it is possible to distinguish between different isolates with the mortality rate of mosquito larvae, which can be used to develop biological control tools to help control diseases caused by mosquitoes.[14,43,44] The results showed that *B. thuringiensis* isolated from soil samples were high toxicity and more abundant than those from other sources (insects and water), these result were also found by Asokan and Puttaswamy[45] who also isolated different *B. thuringiensis* isolates from different sources.

Detection of the Presence of *B. thuringiensis* Toxin Genes by PCR

Polymerase chain reaction (PCR) was performed to determine the presence of different *Cry* genes (*Cry11AA, Cyt1AA, Cry1Ac, Cry2Aa* and *Cry4AA*) in the 36 selected isolates using specific primers illustrated in (Table 1). The results of the PCR screening were illustrated in (Figure 2) and (Table 3).

The presence of *Cry11AA* gene was only detected in three isolates (Ar6, Sp4.2 and Laq isolate). While Cyt1AA gene was found in 6 isolates (Am2, Ar7, Gh5.2, Sp4.2, Laq, and Ss2 isolate). Results also showed that the *Cry1Ac* gene are present in four isolates (Am2, Mn9, Am4 and Am4.2 isolate). Four tested isolates (Am2, Ar1, Be3, and Laq isolate) produced *Cry2Aa* gene. *Cry4AA* gene was detected in only one isolate (Ps6).
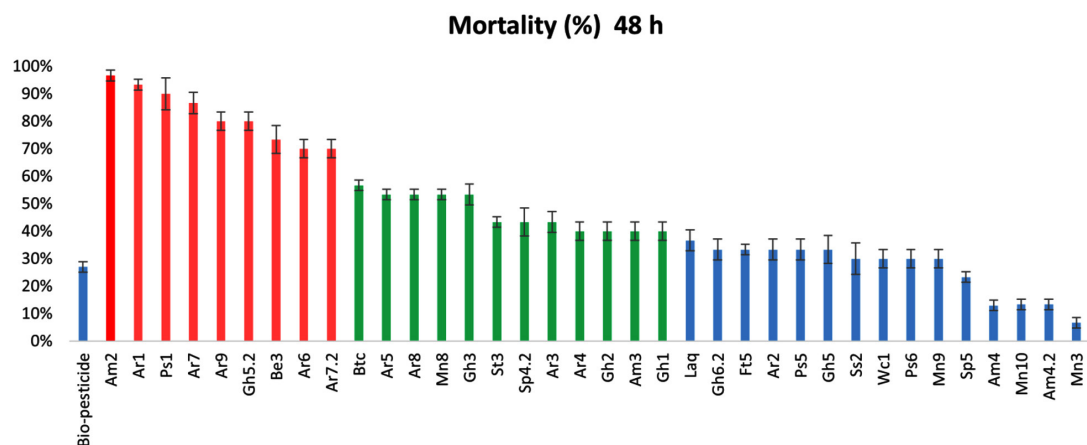

Figure 1. Mortality rate caused by *B. thuringiensis* isolates on mosquito larvae

Based on the results of the PCR amplification listed in Table 3, Am2 isolate amplified three different types of toxic genes (*Cyt1AA, Cry1Ac* and *Cry2Aa*) and caused the highest mortality (97%). On the other hand, Laq isolate also showed the presence of three different types of toxic genes (*Cry11AA, Cyt1AA* and *Cry2Aa*) but caused low mortality (37%). Both Am2 and Laq isolates contained 3 toxic genes, but they showed different mortality rates this could be due the differences in the detected genes.

The *Cyt1AA* gene was more frequent among all the 5 tested genes and was present in six different isolates. *Cyt1AA* was found to cause high mortality since Ar7 and Gh5.2 isolates showed only the presence of *Cyt1AA* gene and caused a mortality percent of 87% and 80%, respectively. On the other hand, lepidopteran-specific *Cry1Ac* gene causes the least mortality rate, since it was detected individually in three isolates (Mn9, Am4 and Am4.2) and the maximum mortality rate was 30%, thus it can be concluded that the *Cry1Ac* gene has no major effect on the mortality. The results showed the lowest frequent amplified gene was *Cry4AA* since it was detected in only one isolate (Ps6) which showed a mortality rate with 30%.
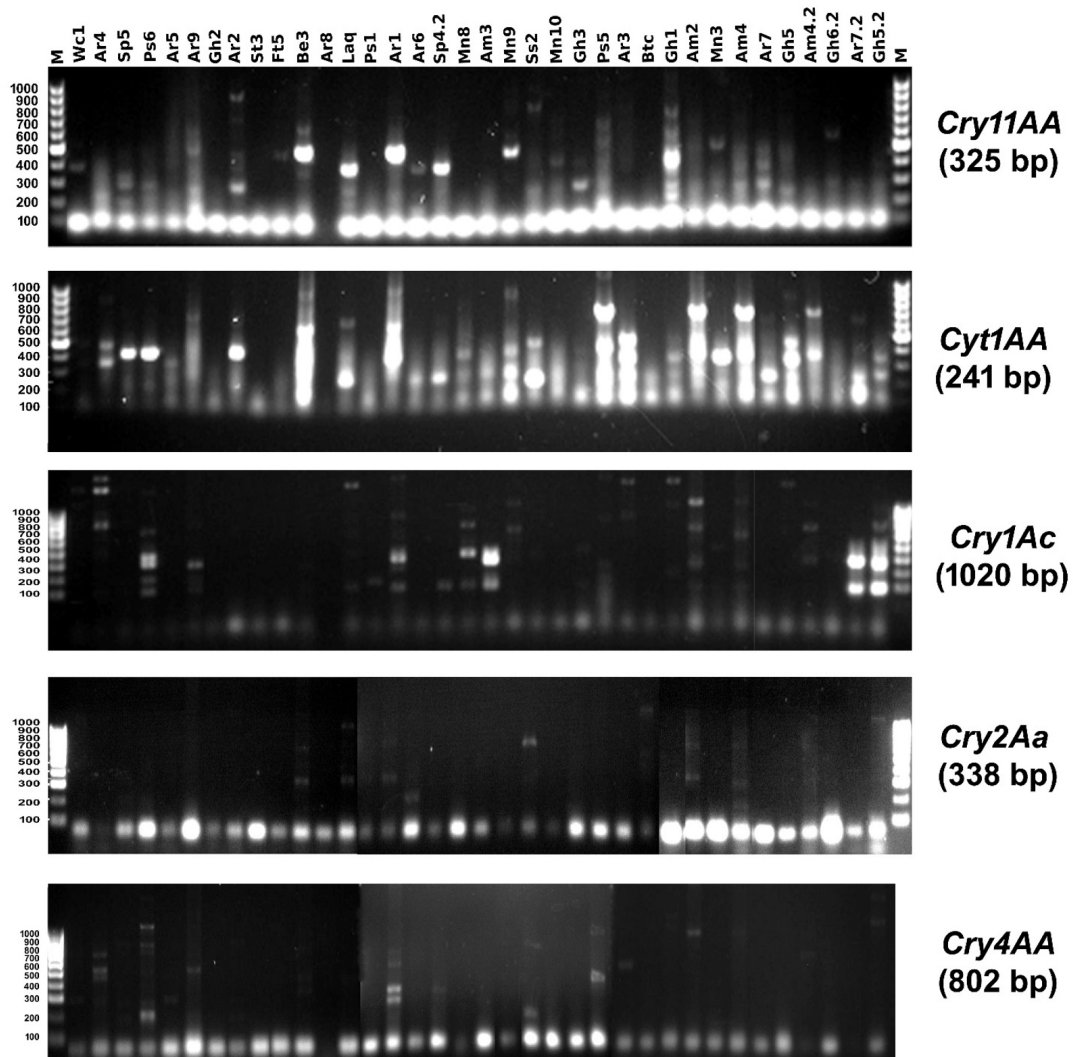

Figure 2. PCR amplification of the different Cry genes on the different isolates

Table 3. Genes detected in the *B. thuringiensis* isolates

Code of Isolate	Gene					Mortality (%) 48 h	Total
	Cry11AA	Cyt1AA	Cry1Ac	Cry2Aa	Cry4AA		
Am2	-	+	+	+	-	97%	3
Ar1	-	-	-	+	-	93%	1
Ps1	-	-	-	-	-	90%	0
Ar7	-	+	-	-	-	87%	1
Ar9	-	-	-	-	-	80%	0
Gh5.2	-	+	-	-	-	80%	1
Be3	-	-	-	+	-	73%	1
Ar6	+	-	-	-	-	70%	1
Ar7.2	-	-	-	-	-	70%	0
Btc	-	-	-	-	-	57%	0
Ar5	-	-	-	-	-	53%	0
Ar8	-	-	-	-	-	53%	0
Mn8	-	-	-	-	-	53%	0
Gh3	-	-	-	-	-	53%	0
St3	-	-	-	-	-	43%	0
Sp4.2	+	+	-	-	-	43%	2
Ar3	-	-	-	-	-	43%	0
Ar4	-	-	-	-	-	40%	0
Gh2	-	-	-	-	-	40%	0
Am3	-	-	-	-	-	40%	0
Gh1	-	-	-	-	-	40%	0
Laq	+	+	-	+	-	37%	3
Gh6.2	-	-	-	-	-	33%	0
Ft5	-	-	-	-	-	33%	0
Ar2	-	-	-	-	-	33%	0
Ps5	-	-	-	-	-	33%	0
Gh5	-	-	-	-	-	33%	0
Ss2	-	+	-	-	-	30%	1
Wc1	-	-	-	-	-	30%	0
Ps6	-	-	-	-	+	30%	1
Mn9	-	-	+	-	-	30%	1
Sp5	-	-	-	-	-	23%	0
Am4	-	-	+	-	-	13%	1
Mn10	-	-	-	-	-	13%	0
Am4.2	-	-	+	-	-	13%	1
Mn3	-	-	-	-	-	7%	0
total	3	6	4	4	1		

+: amplified the gene; -: did not amplify the gene

In this study, 23 isolates did not show any presence of any tested toxic genes, but these isolates still have toxicity on mosquito larvae. This indicates the presence of other toxic genes that were not included in this study. Among those isolates, Ps1 isolate showed the highest mortality percentage 90%. On the other hand, Mn3 isolate showed the least mortality percentage 7%.

The absence of the PCR product in some isolates was detected was also obtained by[46,47] that found 89 out of 215 field-collected strains of *B. thuringiensis* did not produce PCR products with universal primers.

PCR was used as a basic parameter to detect the different types of toxic proteins of the tested isolates and their activity against mosquito

larvae.[13,48] The genes/toxins *Cry11AA, Cyt1AA, Cry2Aa* and *Cry4AA* are associated with poisoning against mosquito larvae.[1,26-28] The *Cry1Ac* gene is associated with toxicity against Lepidoptera larvae.[49]

Results in Figure 2 showed that, most of the isolates did not produce a positive single band with the specific primers, and an extra band/bands were amplified. Similar findings were reported by[50,51] who suggested that, isolates containing novel *Cry* genes may give PCR products different in size relative to the standard or may completely lack PCR products. This could be because two or three *Cry* genes might be positioned next to each other, forming an operon.[52]

Scanning Electronic Microscopy (SEM)

Am2 isolate which caused the highest mortality 97% were preliminarily screened with phase contrast microscopy 100X magnification to observe the presence of shape of the *Cry* crystals. Scanning electron microscopy showed crystal architecture of cuboidal shapes for the toxic proteins and confirmed the presence of rod-shaped bacterial cells, the morphological characteristics of *B. thuringiensis* (Figure 3).

A previous study,[53-55] reported different shapes of crystals protein cuboidal, spherical, irregular, bipyramidal, balloon shaped and flat square all these crystal structures were found in different isolates of *B. thuringiensis* and isolated from different regions. In the present study, Am2
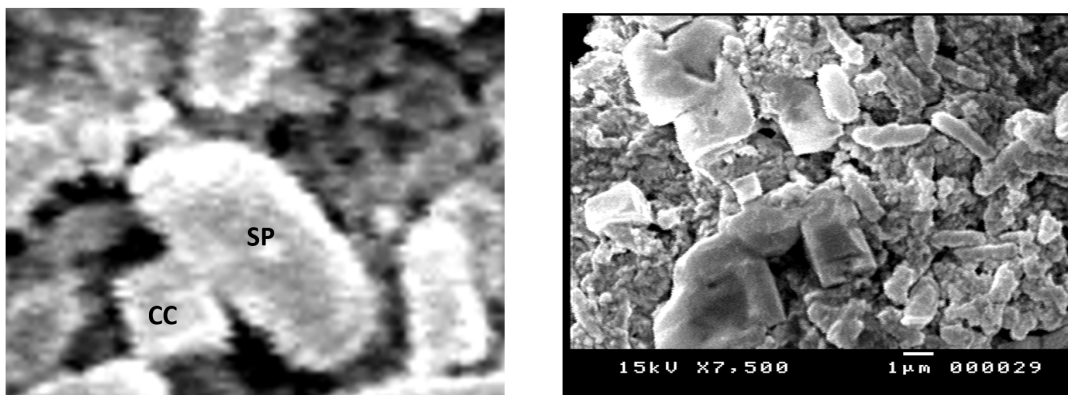

Figure 3. Scanning electronic microscopy (SEM) of *B. thuringiensis* isolates, showing the characterized parasporal inclusion shapes. Sp: spore and crystal cuboidal (CC)
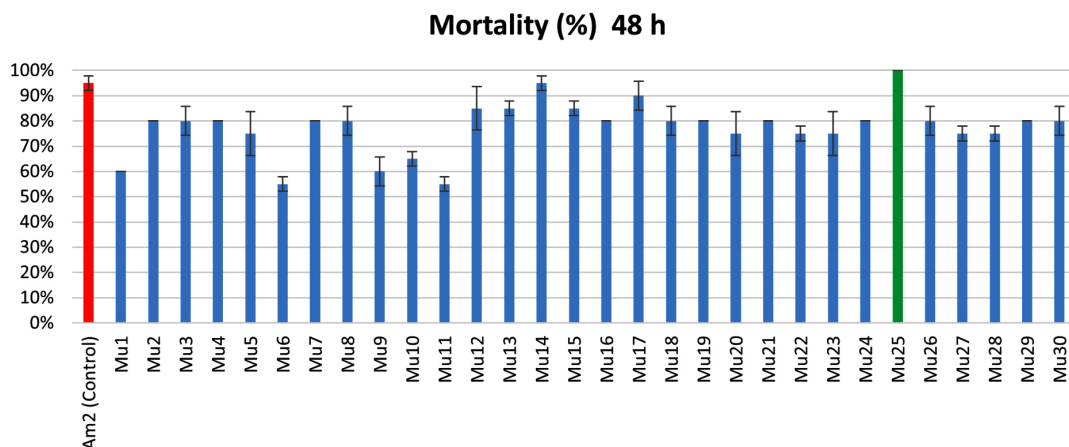

Figure 4. Mortality rate of different obtained mutants against mosquito larvae

isolate amplified three different types of toxic genes *Cyt1AA, Cry1Ac* and *Cry2Aa* and the scanning electron microscopy showed crystal structure of cuboidal shape for the toxic proteins. *Cry* proteins related to some crystal structures.[46,56,57] In this study, Am2 isolate demonstrated cuboidal crystals related to amplify *Cry2Aa* protein.[56,58]

Genetic Improvement with Mutagenesis by Ultraviolet rays (UV) The radiation of sunlight is crucial in the decrease of biological activity of pesticides of *B. thuringiensis* due to ultraviolet rays that break down spores and their internal toxins.[59-61] Previous studies were obtained in *B. thuringiensis* mutants that were produced by successive rounds of ultraviolet rays, the mutants were more UV resistant and some of them were lost encoding their toxin *Cry* genes due to mutations.[33,62,63]

UV-mutagenesis was performed to improve the toxicity of the best killing BT isolate (Am2). Am2 isolate was exposed to UV irradiation for different time intervals (0 sec (as control), 20, 40, 80, 120, 160, 200, 240 and 280sec). The mutants were selected from plates that showed a survivability rate of 50% after exposure (280 sec of UV irradiation).

Thirty mutants were obtained from the mutagenesis of the wild Am2 isolate. All the mutants were screened for their mortality. Out of 30 mutants, only one mutant Mu25 showed better mortality than the wild type Am2 isolate. The improved Mu25 mutant showed toxicity to mosquito larvae with a mortality rate of 100% after 48 hours (Figure 4).

Results in Figure (4) showed that, the toxicity of the isolates was decreased in all the isolates after the UV-mutagenesis. These results were also found by[64,65] since they found that, insecticidal activity of *B. thuringiensis* were negatively affected by UV rays, which leads to a decrease in toxicity and a decrease in stability. Only one mutant Mu25 was improved and caused a high mortality for mosquito larvae after UV-mutagenesis.

CONCLUSION

In this study, the genetic variability was obvious among different isolates and caused variation in their toxicity against mosquito larvae. The isolate Am2 amplified the highest number of different types of genes (*Cyt1AA, Cry1Ac* and *Cry2Aa*) and caused high mortality rates against mosquito larvae. The *Cyt1AA* gene toxin has predominant efficiency against dipterous according to.[19,66] The *Cry2Aa* protein demonstrated form cuboidal crystal in Am2 isolate under scanning electron microscopy according to.[58,67,68] UV-mutagenesis showed high toxicity and stability in mutant Mu25 against mosquito larvae. Further studies will be performed to investigate the effect of UV mutagenesis on different *cry* gene sequences. Moreover, the gene expression levels of different *cry* gene will be determined by qRT-PCR and SDS-PAGE.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION
AEH and AEE conceptualized the study. AR conducted the experiment. AEH and AR wrote original draft. AAA and GAM revised original draft. AEH wrote, reviewed and edited the manuscript. AEH, AEE, AAA and GAM performed supervision. All authors read and approved the final manuscript for publication.

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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