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Deepak T
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Shashank AS
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Dr. Shivaraj Y
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Dr. Asiya Nuzhat FB
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Correspondence
Dr. Shivaraj Y
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Toxic effect of *Bacillus thuringiensis* (Serotype 14) bacteria shows behavioural & histological changes on mosquito larvae

Deepak T, Shashank AS, Dr. Shivaraj Y and Dr. Asiya Nuzhat FB

Abstract
In this current situations the bacteria, *Bacillus thuringiensis* is a gram positive, rod shaped and aerobic soil bacteria. Bt is a bacterium that is not toxic to humans or other mammals but is toxic to certain insects when ingested. Bt works as an insecticide by producing a crystal-shaped protein (Cry toxin) that specifically kills certain insects. These bacteria produces a proteinaceous parasporal crystal in the sporangium at the time of sporulation. These proteins possess insecticidal properties and are called Bt-toxins or delta endotoxins and also called as insecticidal crystal proteins (ICPs). It is not a single toxin but a class of toxic proteins produced from protoxin that are degraded by proteolytic enzyme to form smaller toxic peptides. In this study the mosquito larva are considered as predators which feed on some protozoan's and bacteria, the fixation of concentration levels to larva was 9 gm of (Serotype 14) Bt induced to 10 liter of water which contains 100 mosquito larvae. When these larvae feed on Bacteria, *Bacillus thuringiensis* it show behavioral changes like erratic swimming, slow motility and larva move towards the surface of water and regurgitation because of Bt-crystals effects on enzyme present in the larvae i.e. Cytochrome-c-Oxidases due to oxidative stress and the liberated crystal toxin like Cry-II and Cry-IV bind into the larva gut cell membrane it shows the slight histological changes also and it paralyzes the digestive tract and forming a pore in that regions and results in the death of the larva. This show the toxins nature of *Bacillus thuringiensis* bacteria against the mosquito larvae.

Keywords: *Bacillus thuringiensis* (Serotype) bacteria, behavioural & histological changes, crystal proteins

Introduction
In this present world Mosquitoes are the main vectors conveying the many diseases like Malaria, Dengue, Chikengunia and Yellow fever etc. In our ecological surveys of mosquitoes in India, the occurrence of some species likes *Anaphilies*, *Ades*, *Culex* are found. In these years world facing many deadly diseases caused by some insects. For controlling these insects whole world using some of the chemical insecticides that are currently used to control insect pest are extremely toxic to non-target organisms and in many causes are deleterious to the health of humans and animals, inducing important human diseases such as cancer and immune system disorders. In addition chemical insecticides are recalcitrant breaking down only slowly leading to soil and water pollution. Recent investigations have provided evidences for existence of strains of *Bacillus thuringiensis* processing a highly preferential toxicity to mosquito and black fly larvae.

Bacillus thuringiensis are gram positive, rod shaped spore producing bacteria with entomopathogenic properties. In this worldwide today Bt is naturally found on leaves and in soil and it has been used commercially in organic and other conservative farm type of agriculture since from last fifty years ago. The most important was genetically engineered technique were used and insect-resistant crops express for one to another type Bt insecticidal Cry toxins were used and mostly it has been introduced since from over two decades, the EPA (Environmental Protection Agency) and frequently the scientific bodies have consistently found that Bt and engineered Bt-crops are not harmful to humans life till today. Bt produces insecticidal proteins during sporulation phase as parasporal crystals. These crystals predominantly comprised of one or more proteins (Cry and Cyt toxins), also called delta endotoxins. Cry proteins are parasporal inclusion (crystal) proteins from Bt that exhibit experimentally verifiable toxic effect to a target organism have a significant sequence similarity to a known cry proteins. Similarly, Cyt proteins are parasporal inclusion proteins

from *Bacillus thuringiensis* that exhibits hemolytic (Cytolytic) activity or as obvious sequence similarity to a known Cyt proteins these toxins are highly specific to their target insects and innocuous to human`s, vertebrates and plants and are completely biodegradable. Bt is a viable alternative for the control of insects pests in agriculture and off important human diseases vectors. *Bacillus thuringiensis* is a commonly called as Bt, this was naturally occurring bacterium inside the soil and on plant leaves today. This was first discovered in the year 1901 by Japan, this has been revolutionized how we stop insects from eating our crops in the field. And since from over fifty years ago, Bt has been applied directly to a variety of agricultural crops and plants in home gardens and it has a living pesticide today to control insect pests throughout the globe. In this Bt the proteins are shaped like crystals, and they are commonly called "Crystalline toxins" or "Cry toxins." These Cry toxins persist sluggish until consumed by an insect. And once it has digested, the protein is activated and then it binds to specific receptors in insect guts. The Cry toxins impale the holes in the insect's gut, ultimately it has causing the insides to seepage and the insect to starve. But this shows importantly, in humans do not have the same receptors or gut conditions which the insects as content, and the Cry toxins pass through us with no side effects till today. The studies show that humans digest Cry toxins like any other protein that would be consumed when eating food materials like meat, beans, leafy greens, or tofu. Many types of Cry toxins exist with fluctuating specificity to different insect's body (primarily moths and butterflies, beetles, and flies etc.). The various Cry toxins can be mixed and matched to control pests. *Bacillus thuringiensis* produces 3 classes of insecticidal proteins during its vegetative and sporulation phases, including Cry (Crystal proteins) and Cyt (Cytolytic toxin) proteins during the sporulation phase and Vip (vegetative insecticidal proteins) during the vegetative phase [1]. Cry and Cyttoxins are produced as parasporal crystals [2]. The nomenclature of Cry and Cyt toxins has been revised from function based to sequence-based form [3], and currently, Cry toxins are classified into 300 families in about 75 primary subgroups based on amino acid sequence identities [1]. In this experiment the Cyt toxins present in some *B. thuringiensis* parasporal crystals were first discovered in mosquitocidal *B. thuringiensis*. Sub sp. *israelensis* (Bti) over a time period [4] and other biological insecticide products that are commercially available in the market today these are based on *Serratia entomophila* and *Bacillus sphaericus*, which produce Sep and Bin insecticidal toxins, respectively [5, 6]. In addition, the bacteria Xenorhabdus and Photorhabdus spp. belonging to the family Enterobacteriaceae associated with entomopathogenic nematodes also produce potent insecticidal toxins that could represent additional alternatives for insect control [7].

The study was under taken to evaluate the aquatic toxicity of Cadmium Chloride with special emphasis on behavioural and Oxygen consumption of the freshwater teleost, *C. carpio* exposed to lethal and sub lethal concentrations of commercial grade heavy metal Cadmium Chloride and Cadmium has gained wide interest in the scientific community in recent years due to its potential human health hazards [8] and Histopathological analysis can be considered as a sensitive tool to determine microscopic morphological alternations [9] and The alterations found in these organs are normally easier to identify than functional ones [9]. Bt Cry and Cyt toxins belong to a class bacterial toxins known pore forming toxins (PFT) that are secreted as water soluble proteins undergoing conformational changes in order to insert into or translocate across cell membranes of their hosts. They are two mains groups of (PFT) {i}the α-helical toxins in which α- helix regions from the transmembrane pore and {ii} the β parallel toxins that insert into the membrane by forming a β barrel composed of β sheet hairpins from each monomer. The first class of PFT includes toxins such as the colicins, endotoxins, dipterian toxin and also the Cry III domine toxin. On the other hand aerolysin, α haemolysin, and cholesterol dependent toxins as the perfingolysin and the Cyt toxins belongs to the β barrel toxins in general PFT producing bacteria secrete their toxins and these toxins interact with specific receptors located on the host cell surface. In most cases PFT are activated by host body. After receptor binding including the formation of an oligomeric structure that is insertion component finally, membrane insertion is triggered in most cases by decreases in pH that includes a molten globules state of the protein. In this present investigation which shows that Toxic Effect of *Bacillus thuringiensis* (Serotype) Bacteria shows Behavioural & Histological Changes on Mosquito Larvae.

Materials and Methods

Mosquito larvae collection & treatment

For this study we prepared culture media by adding the mixture of yeast and potato starch in the ratio of 1:3 in a 100 ml of pond water (Heggere pond, Heggere Tumkur) containing hardness off 477µS at the temperature of 25ºC. We exposed this culture medium in an open environment for three days. After 3 days we noticed 3 spices of mosquito's eggs like *Aedes*, *Culex* and *Anophelies*. We collected those eggs and hatched under normal environmental condition separately. Larvae were reared in a group of 300/2 liter of water at 25ºC (±2) in an aquarium, with surface area of height 23cm and width of 50cm. And we feed with yeast and egg albumin with the ratio of 1:1. The amount of food provided in aquarium in each day and it is increased from approximately 20-50 mg. Experiment was carried out with early 4th instars larva were 5 to 6 days (*Ades*) and 4 to 5 days (*Culex*) and 2 to 3 days in *Anophelies*. About 9 gram of powder of *Bacillus thuringiensis* (serotype 14) was suspended in a 100ml of distilled water containing 2.5 mm phosphate buffer and it was added to aquarium containing 100 larvae/ 10 liter of water (larvae are kept for fasting about 6 hours).The larvae of those species were allowed to feed in that contains for 2 hours at 20 ºC and then were collected in a plastic net rinsed with tap water. This experiment is replicated 6 times using 3 different population of mosquitoes.

Bacillus thuringiensis serotype culture

The original isolate Bt was obtained from Murlidhar, Arvee Biotech Tamil Nadu. This isolate was cultured in yeast peptone medium at 30ºC. Fully sporulated culture were harvested after 4 days of incubation in the bacterial incubator and washed in 0.25N of phosphate buffer with the pH of 7.2 and rewashed in distilled water and it was dried by freezing. This results in spore or toxin powder. This powder has potency of 4600 ITU/ mg against the 4th instars larvae of dipterans.

Histological and microscopy examination

In this study at the end of exposure period, mosquito larvae were taken from replicate of one exposed concentration. These larvae were dissected through the abdominal gut cavity

was operated and exploited both the organs like gut epithelium tissue samples of exposed mosquito larvae were excised quickly and fixed in 10% neutral-buffered formalin, (Bouin's solution as a histological fixative for 24 h) [9]. According to the specimens were processed as usual in the recognized method of dehydration, cleared in xylene and finally embedded in paraffin wax before being sectioned at 5 m using a rotary microtome (Leica RM 2235 Germany). The specimens were stained with haematoxylin and eosin. Finally, the prepared sections were examined and photographically enlarged using light microscopy (Hamilton compound photomicroscope).

Results and Discussion

When mosquito larvae feed on bacteria *Bacillus thuringiensis* it shows some behavioral changes like erratic swimming, slow motility and larva move towards surface of the water due to oxidative stress and regurgitation occurs because of the binding of Bt crystals on the midgut of the larva. During this the Bt crystals effects on enzyme Cytochrome-c-Oxidase and liberated crystals toxin like Cry II and Cry IV binds into the larva gut cell membrane and it paralyses the digestive tract and forming a pore in that region and results in the death of the larvae.

In the present study the control mosquito larvae behaved in natural manner i.e., they were active with their well-coordinated movements. They were alert at slightest disturbance, but in the toxic environment mosquito larvae exhibited irregular, erratic and darting swimming movements, hyper excitability, capsizing, attaching to the surface, loss of equilibrium, When these larvae feed on Bacteria, *Bacillus thuringiensis* it show behavioral changes like erratic swimming, slow motility and larva move towards the surface of water and regurgitation because of Bt-crystals effects on enzyme present in the larvae i.e. Cytochrome-c-Oxidases due to oxidative stress and the liberated crystal toxin like Cry-II and Cry-IV bind into the larva gut cell membrane. And the study shows that it shows the slight histological changes also and it paralyzes the digestive tract and forming a pore in that regions and results in the death of the larva. This show the toxins nature of *Bacillus thuringiensis* bacteria against the mosquito larvae.

Mode of action

Bt serotype 14 is highly toxic to different species of mosquitoes like *Aedes, Culex, Anophelies*. These are the vectors of many human diseases. This bacterium produces crystal inclusion composed of Cry II and Cry IV in mosquitoes. The ingested by susceptible larvae dissolve in alkaline gut environment and soluble proteins is proposed that cry toxins are bind to the specific protein receptors in the microvilli of mosquito midgut cells. In contrast Cyt toxins do not bind to protein receptor but directly interact with membrane lipids inserting into the membrane and forming pores or destroying the membrane by a detergent like interaction.

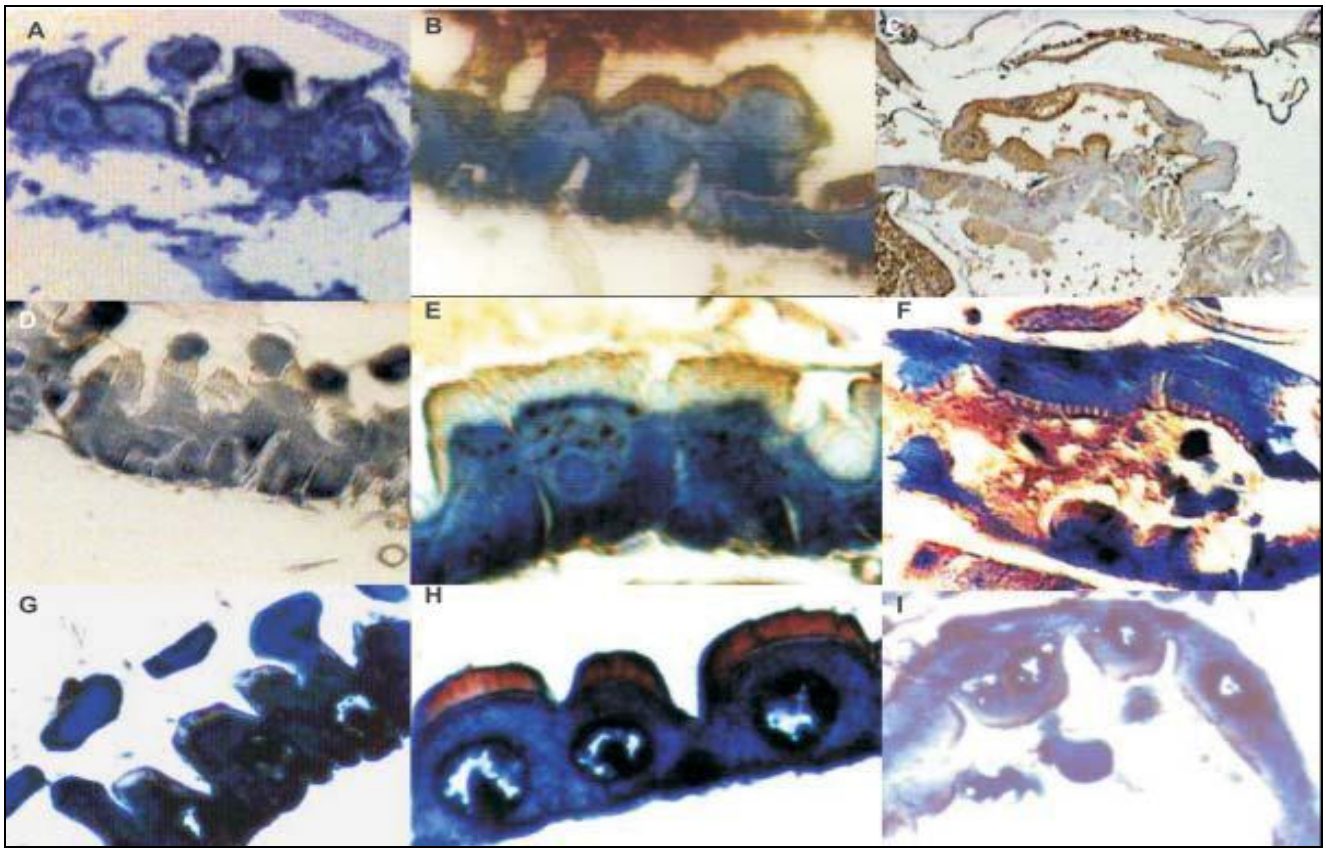

Fig 1: Immuneo histochemical localization of the Cry II toxin of *Bacillus thuringiensis* serotype on the apical microvilli of the midgut epithelial cells of mosquito larvae. *Aedes* (A, B, C), *Anopheles* (D, E, F), and *Culex* (G, H, I) pictures taken 1 hour after exposure to the toxin. Control slides of the posterior midgut omitting the primary antibody (A, D, G). Positive signal in the posterior (B, E, H) and gastric caeca (C, F, I) of the mosquito species. Magnification 40X.

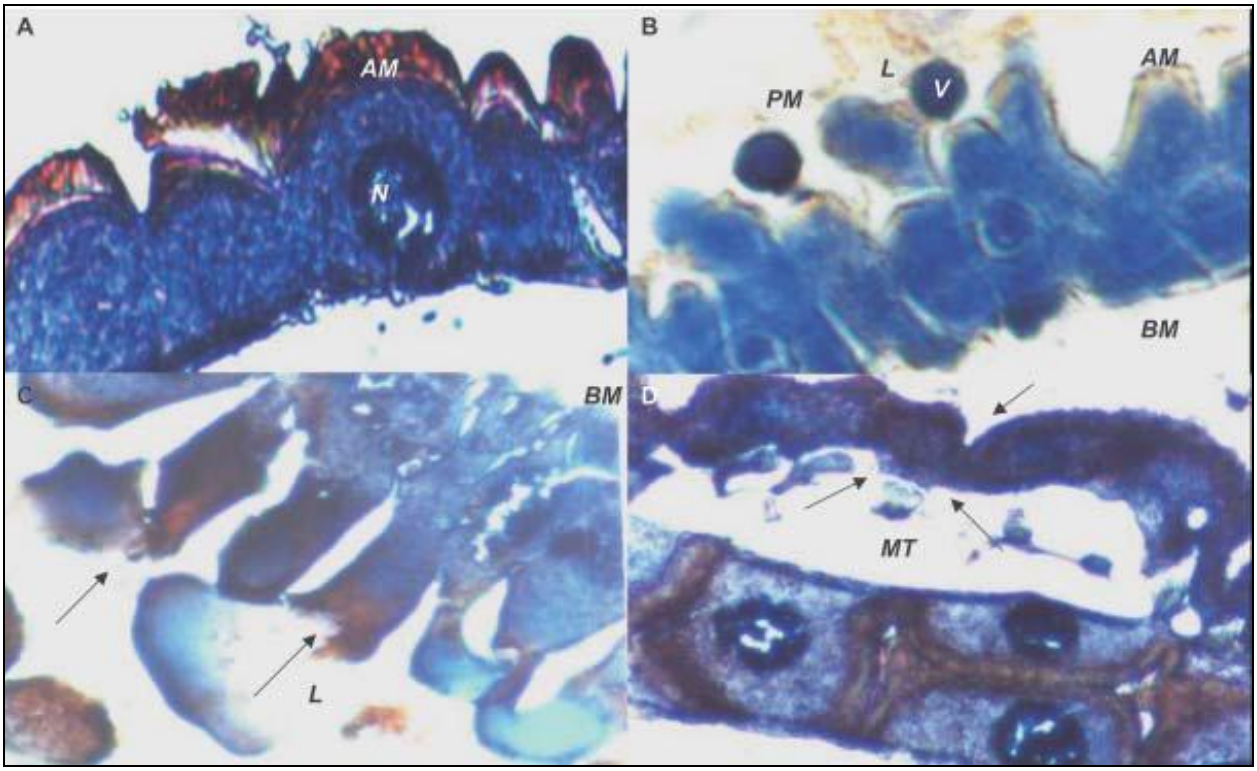

Fig 2: Histopathological effects of the Cry II toxin *Bacillus thuringiensis* serotype in mosquito larvae after 2 hour of exposure to the Cry II toxin. A: vacuolization of the cytoplasm and hypertrophy of the *Culex* epithelial cells and their nuclei; B: vesicle formation in the apical region of cell towards the midgut lumen of the *Anopheles*; C: arrows indicates lysis of columnar cells of *Ades*; D: localization of the protein Cry11Bb on the apical microvilli of the Malpighian tubules of *Culex* larvae 2 h after exposure to the toxin. The arrow indicates the shrunken tubules. AM: apical microvilli; BM: basal membrane; L: midgut lumen; PM: peritrophic membrane; V: vesicles; N: nucleus. Magnification 40X.

A general scheme for the mode of action of Bt toxins, based on recent review and the toxin structure is shown in Fig. 3

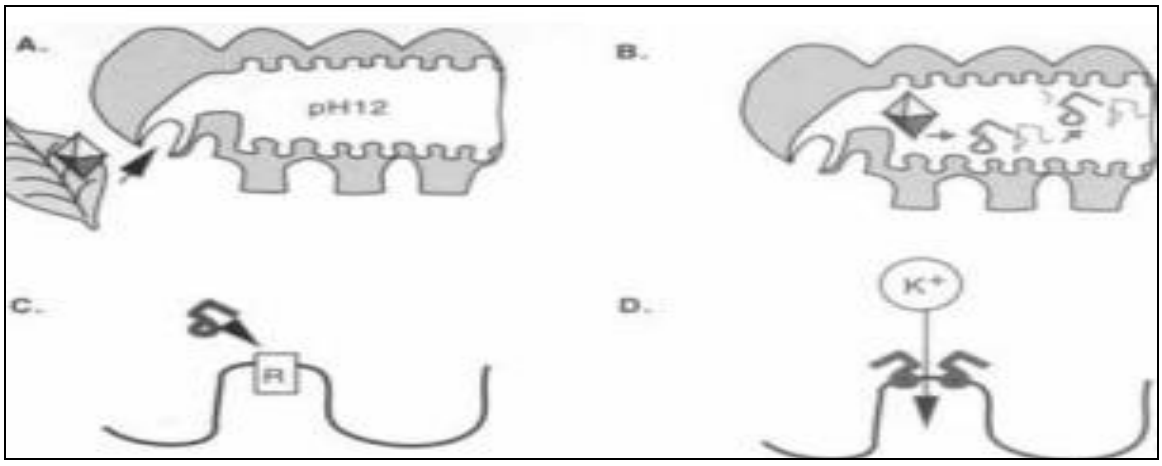

Fig 3: Scheme for the mode of action of Bt toxins. (A) Ingestion; (B) solubilisation and activation; (C) binding to the receptor; (D) formation of the toxic lesion.

A. Ingestion

Bt δ-endotoxins are gut poisons: they must first be eaten by a susceptible insect larva.

B. Solubilisation and proteolytic activation

The proteins are synthesized as insoluble crystalline protoxins which dissolve and are activated in the insect gut before exerting their effects. The gut of most target insects has a very high pH 9 and this is essential for dissolving most Bt protoxins. Which are usually soluble only above pH 9.5. The protoxins are activated by insect gut proteases, which typically cleave some 500 amino acids from the C terminus of 130 kDa protoxins and 28 amino acids from the N terminus, leaving a 65-55 kDa protease-resistant active core comprising the N-terminal half of the protoxin. Alignment of the amino acid sequences of Cry revealed that the 70 kDa proteins (Cry II, and Cry IV) could be considered to be naturally truncated forms of the N-terminal half of 130 kDa Cry proteins.

C. Receptor binding

The specificity of Bt & endotoxins towards particular insects implies the presence of specific receptors in the target tissue. It has become apparent from binding and competition studies (using Cry II toxins and membranes from the gut of susceptible and resistant insects) that there are many different toxic binding proteins. Some of these have been tentatively identified as 120-180 kDa glycoproteins. The role of these binding proteins will be discussed in detail in the following section.

D. Formation of the toxic lesion

After binding to a specific receptor. Located on the brush border membrane of the columnar cells, the toxins insert irreversibly into the plasma membrane of the cell. The next step involves the formation of a pore or lesion in the plasma membrane. This leads to breakdown of the permeability barrier of the membrane, cell lysis, and disruption of gut integrity and finally death of the insect from starvation or septicaemia. Pore-mediated cytolysis is a common strategy adopted by pathogenic bacteria. The effect of pore formation in a plasma membrane will depend on the environment of the cell. It has been proposed that Bt toxins kill cells by pH mediated damage or by osmotic lysis and finally in death of insect.

Cry toxin mode of action

Although widely investigated, the detailed mechanism of Cry toxin mechanism in insect larvae midgut is still controversial [10, 1]. It is generally accepted that Cry toxin recognizes proteins on brush border membranes in a multiple-step binding process to exert toxicity. The crystalline inclusions are cleaved at inter chain disulfide bonds to yield Cry protoxins in suitable gut physicochemical conditions [11]. The soluble protoxin is then proteolytically cleaved by intestinal proteases into an activated form [12, 13]. These activated toxins traverse through the peritrophic matrix [14, 15] and then bind to specific proteins localized on apical brush border membrane microvilli of the insect midgut [16, 17]. The "sequential binding" designates the binding of Cry toxin to receptors such as cadherin and glycosyl phosphatidylinositol (GPI) anchored proteins in a step-by-step fashion [18]. Cadherin binding site close to the brush border was proposed to induce proteolytic cleavage of helix α1 from domain I resulting in the formation of a prepore oligomer [19, 20].

Toxin oligomer was proposed to promote a high binding affinity to GPI-anchored amino peptidase N and alkaline phosphatase [16]. The "sequential binding" model claims that the reversible binding of toxin to APN and ALP occurs prior to the high-affinity binding of toxin with cadherin extracellular domain resulting in a prepore formation of the toxin, allowing toxin oligomer to have highly affinitive binding with APN and ALP [21, 22] Proposed a synergistic effect of cadherin CR peptide for Cry1A oligomer in Manduca sexta, similarly [23], reported a truncated region of APN binding with Cry11Ba with high-affinity works as a synergism for Cry11Ba against Anopheles gambiae larvae [23]. In addition to these proteins, intracellular molecules such as actin, flotillin, prohibitin, and V-ATPase are recognized as Cry-binding molecules [24, 25].

Toxin insertion into the cell membrane forms ion permeable pores, which allows small molecules like inorganic ions and amino acids to pass through the cell membrane; this eventually results in colloid-osmoticlysis of epithelial cells, followed by septicemia and death of the insect [26, 1].Two alternative models, known as the "penknife" and "umbrella" models, propose that the helical α5 and α6 structures in domain I govern the process of membrane insertion [27]. Both of these models are derived from colicin, a well-characterized novel bacterial toxin, with a structure that possesses pore-forming properties similar to Crytoxins [28]. The "penknife" model postulates that only helices α5 and α6 in domain I are inserted into the plasma membrane through a targeting helical "hairpin," segregating the remaining helices lying on the surface of the membrane [29].

In contrast, the widely accepted "umbrella" model proposes that helices α4 and α5 constitute the ribs of an "umbrella," and work as the "hairpin" to insert into the phospholipid bilayer [30]. Domain I is considered to be the major determinant for pore formation, allowing helices α4 and α5 to penetrate into the membrane in an anti-parallel manner [31]. In support of this model, helix α4-loop-α5 was synthesized and shown to be extremely active in assisting toxin insertion into the cell membrane [32]. Furthermore, helix α5 of Cry3A and Cry1Ac were also suggested to be important for toxin assembly and membrane permeation [33]. A significant loss of toxicity was observed from mutagenesis of residues on helix α4 of Cry1Ac, which correlates with its role in formation of an ion channel [34]. A mutant of Serine 176 at the C-terminal end of α-helix 5 of Cry1Ab was identified as playing a crucial role for ion transportaction through the brush border membrane [35]. In addition to the "penknife" and "umbrella" models, a relatively recent "buried dragon" model portrays nearly the whole activated toxin as penetrating into the membrane [36].

According to the "buried dragon" model, α-helix 1 is cleaved prior to membrane insertion, and is the only region of toxin that does not insert into the epithelial membrane [37]. In contrast to the pore-formation model, the cell signaling transduction model [38, 39] postulates that Cry toxin action in Lepidoptera causes cell death by inducing a cell death pathway. Data supporting the cell signaling model comes from studies of Cry toxin action in cultured cells. The model describes an interaction between toxin monomer and cadherin that induces activation of the G protein (Gα) receptor and adenylyl cyclase, which triggers a magnesium-dependent intracellular signaling pathway [39]. The increased cAMP activates the protein kinase A, which trigger sacascade of cell physiologies and causes the programed death of the cell by lysis [39].

Conclusion

The present investigation shows that the effect of Bt shows toxic effect on some larvae of order Dipterans and Leptdopterans. In the environment mosquitoes are the main vector which causes many human diseases. For controlling of these insects many chemical insecticides and pesticides are using. It effects on non-targeting organisms and also effects on human health. For overcoming from this problem usage of microbial insecticides like Bt serotypes are acceptable. These bacteria are used to kill on target organism without affecting any other non-target organisms and also it is used as organic microbial insecticide. These type of microbial insecticides are biodegradable, eco-friendly and easily available. Mosquito larvae feed on protozoan like bacteria *Bacillus thuringiensis*. Serotype 14 results in death. It is helpful in controlling the population of mosquito and resolves the diseases causing from the mosquitoes. *Bacillus thuringiensis* plays an important role in controlling of pests and insects.

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