

Ultrastructural Changes in Hemocyte Cells of Hard tick (*Hyalomma dromedarii*: Ixodidae): A model of *Bacillus thuringiensis* var. *thuringiensis* H14 δ -endotoxin mode of action

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Abstract: The ultrastructure and characteristics of hemocytes of ixodidae tick, *Hyalomma dromedarii*, after treatment with *Bacillus thuringiensis* serovar *thuringiensis* H14 δ -endotoxin were studied. Little information is available on the systemic effects of *Bacillus thuringiensis* toxins in the hemocoel of arthropods. To evaluate the effect of *B. thuringiensis* 43-kDa toxins elicit a toxic response in the hemocoel, it was measured the effect of intrahemocoelic injections of 43-kDa Cry4Ba toxin on the hemocytes (ultrastructure and characteristics) and survival of *Hyalomma dromedarii* engorged female. It was found that of 43-kDa Cry4Ba toxin was highly toxic. In short term (48h) to *Hyalomma dromedarii* engorged female, the results indicated that the complete growth was arrested and death in a dose-dependent manner. On receiving 10 μ l of 157 μ g ml⁻¹ soluble *B. t.* toxin/tick a rapid paralysis, followed by hemocytic disruption and death was occurred. This investigation revealed that a severe damage in the cells membrane and granulocytes of the hemolymph after injection with δ -endotoxin. *Bacillus thuringiensis* var. *thuringiensis* H14 δ -endotoxin, this toxin destroys the granular cell and renders it abnormal. These cells are stored defensin and secreted it in hemolymph plasma after the exposure of the tick to bacterial infection. This explains the mechanism of action of this bacteria *Bacillus thuringiensis* on *H. dromedarii* hard tick. It kills the tick by causing a malfunction of the cellular immune system of the tick. The model proposed for Cry4Ba is that injected with toxins may reach a hemocytic target, as demonstrated by the good correlation between cytotoxicity and toxicity upon injection. In conclusion, the results of This study suggests that *Bacillus thuringiensis* var. *thuringiensis* δ -endotoxins targets are not only present in the gut. This is an important result of biological relevance at the level of *Bacillus thuringiensis* mode of action.

Key words: Ticks • *Hyalomma dromedarii* • Bacteria • *Bacillus thuringiensis thuringiensis* • δ -endotoxin • Hemocyte • TEM

INTRODUCTION

Hemocytes are identified on the basis of their morphology, ultrastructure and physiological function [1]. Two types of phagocytic cells in ticks, the plasmatocytes and granulocytes (spherule cells), have been reported [2, 3]. Hemocytes are the circulating cells of arthropods which that account for as much as 50-60% of the hemolymph, or the circulating fluid content in ticks [4], were functional equivalent to mammalian immune cells. Like that described of immunocytes belonging to other arthropod species, most notably in the insects [5]. The innate immune system is one of the most important factors in the ability of metazoan organisms to survive when challenged by microbes. The innate immune system

comprises cell-mediated and soluble components and is initiated through recognition of Pathogen Associated Molecule Patterns (PAMP) [6, 7]. Hemolymph lectins and other humoral immune factors in the hemolymph have been reported to be produced and secreted into the hemolymph mainly by hemocytes and fat body cells.

Bacillus thuringiensis is opportunistic insect pathogen that was discovered almost a century ago [8]. The salient feature of this species is accumulation of crystalline parasporal inclusions during sporulation. These inclusions are composed of one or more protoxins, known as δ -endotoxins, each of which is specific primarily at the level of insect orders, particularly Lepidoptera, Diptera and Coleoptera [9]. In *Ixodes scapularis* (Acari: Ixodidae), specificity is due in part to the extremely

alkaline midgut environment that is required to solubilize the protoxin into the active form. Solubilized protoxins are activated by midgut proteases and bind to receptors on the epithelial surface. Then, by a process that remains unclear, the toxins appear to insert into the membranes of gut cells, where they form pores that lead to cell lysis [10].

Much of the research on *B. thuringiensis* since its discovery in 1918 and its development for commercial use in the 1950s has been predicated on a mechanism that is quite different from the one we present here. Studies of the mode of action of *B. thuringiensis* in the last 25 years have clarified the molecular specificity of toxin binding and pore formation but have not addressed the subsequent events that lead to insect death [11].

Previous reports have shown that neither Cry1Aa nor Cry1C had any depolarizing effect when applied on the basolateral side of the midgut membrane of *L. dispar*. Cry1C is toxic to various cell lines derived from *Spodoptera frugiperda*, *Manduca sexta*, *Plodia interpunctuella* [12], *Aedes aegypti* and *Anopheles gambiae* [13]. Peyronnet *et al.* [14] showed that neither Cry1Aa nor Cry1C had any depolarizing effect when applied on the basolateral side of the midgut membrane of *L. dispar*. On the other hand, Butko *et al.* [15] demonstrated a membrane perturbing activity of Cry1C in receptor-free phospholipid vesicles. *In vitro* and at low pH, Cry1C undergoes a conformational change that leads to membrane interaction and promotes flux of ions across the lipid bilayer [15]. It is conceivable that, *in vivo*, a transition to a similar conformational state could be triggered by means of another, as-yet-unknown, physiological condition. To date, most *in vivo* studies have focused on this general mode of action. A toxic effect of intrahemocoelic injections of crystals of *B. thuringiensis* was noted in *Pieris brassicae* as early as 2001 [16], but since then all attention has been paid to the toxicity of *B. thuringiensis* toxins after oral intake. Little is known about other putative target organs and cells in insects that are possibly accessible upon injection of the toxin.

In this paper it will describe the cytopathological changes in hemocyte cells of *H. dromedarii* female due to their exposure to *Bacillus thuringiensis* serovar *thuringiensis* δ -endotoxine 43 kDa purified protein derivative using transmission electron microscope (TEM). This work introduces a new proposed mode of action regarding to targeting the hemocytes by *B.t.* δ -endotoxins.

MATERIALS AND METHODS

Ticks: Two hundred fully engorged female of *Hyalomma dromedarii* (Koch 1818) were collected from the ground of camel pens, Burkash village, Giza governorate, Egypt and identified according to Estrada-Pena *et al.* [17]. About 120 female were used for experimental control and 80 female were used for studying the ultrastructural cytopathological changes after exposure to *Bacillus thuringiensis* var. *thuringiensis* δ -endotoxins.

Bacterial Strain: *Bacillus thuringiensis* isolate, serovar *thuringiensis* (H14) used in this study was obtained from National Microbial Bank (Faculty of Agriculture, Ain Shams University, Egypt). The isolate was grown at 28°C on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (15 g) and distilled water (1000 ml).

Fermentation Process: Ten colonies of 24h age *B. t.* were picked up and inoculated in trypticase soya broth (first passage) for 24h at 25°C. The fermentation process was carried out according to Mohd-Saleh *et al.* [18] with a modification in the run fermentation medium by adding a sporulation enhancer salts. The used run fermentation medium was Brain Heart Infusion broth containing salt additives (0.3, MgSO₄.7H₂O; 0.02g, FeSO₄.7H₂O; 0.02.ZnSO₄.7H₂O; 1.0g, CaCO₃) per liter.

Separation of Parasporal Inclusion Crystals: Parasporal inclusions were purified by biphasic separation technique using Na₂SO₄ and carbon tetrachloride according to Pendleton and Morrison [19]. Purified inclusions were washed three times with distilled water via centrifugation at 20,000 g at 4°C for 30 min. The purified parasporal inclusion crystal proteins were precipitated by acetone and were dried by desiccation overnight at room temperature and then stored at 4°C until use.

Solubilization and Extraction of *B.t.* serovar *thuringiensis* (H14) Crystal Proteins: Solubilization of 1g purified parasporal inclusion crystal proteins was performed in 1M NaOH (pH 12) at 25°C for 24h with stirring [20]. The soluble protein solution was adjusted to pH 4 by 1N HCl. The precipitated protein fraction was separated by centrifugation at 25,000 g for 30 min at 4°C; then dissolved in 100 ml phosphate buffer saline (PBS) pH 7.2. The solubilized protein fraction was separated by

centrifugation at 25,000 g for 30 min at 4°C. The total dissolved protein in the supernatant was precipitated in 80% saturated solution of ammonium sulfate anhydrous overnight with stirring at 4°C. The precipitated protein fraction was dissolved in 80 ml distilled water and dialyzed overnight against 2X distilled water 2L each at 4°C. The resulted protein fraction was adjusted to total volume 100 ml. The active protein fraction between 30 and 50 kDa was separated using Vivaspine concentrator 20 by centrifugation at 2000 rpm at 4°C for 2h each. The protein recovery was monitored in each step of purification.

Protein determination and Gel Electrophoresis (PAGE):

Protein concentration was measured by the method of Bradford [21] using bovine serum albumin as standard. SDS-PAGE was performed as described by Laemmli [22], using 4% stacking and 12% resolving gels with Bio-Rad Minigel system at 50 V for 10 min and 100 V for 90 min. After electrophoresis, the gels were stained with 0.1% (W/V) Coomassie blue R250. The molecular masses of the parasporal body proteins were estimated by comparison with those of the standard protein markers. The number and purity of isolated protein fraction were estimated and confirmed by Native PAGE 7.5% as described by Kishimoto *et al.* [23].

Toxicity Test: Three concentrations of *B. t.* var. *thuringiensis* Cry4Ba 43 kDa were used in this experiment, as it was ; 158, 79 and 37 µg ml⁻¹. Each concentration or control treatment was replicated three times and the replicates included ten engorged females. The treatment was applied according to Friesen *et al.* [24].

Prior to inoculation, 10µl/100mg body weight from the three concentrations of *B.t.* Cry4Ba δ-endotoxin or saline (control tick). The toxin and saline were injected individually per tick into the hemocoel through the camerostomal (articulation between the scutum and capitulum). The isolated ticks were in covered plastic cups for 48h after injection. Mortalities were recorded after 24h and 48h. Similar groups were made for testing the effect of 4CryBa toxins by dipping. Calculated mortality rate of females were based on females with brown-black color.

Challenge of *H. drommedarii* female Against *B. t.* δ-endotoxin:

Eighty *H. drommedarii* females were subdivided into two groups. Forty females were injected each as described above with 20µl of *B. t.* var. *thuringiensis* δ-endotoxins 43 kDa protein fraction (37 µg ml⁻¹) for each female, [25]. The other forty females were used as negative control injected with 20µl of saline.

Ticks were transferred to Petri dishes plated with filter paper and incubated under observation in room temperature. After 24 h hemolymph samples were collected from individuals of both groups.

Hemocytes Extraction: Ticks were secured ventrally to a Petri-dish with glue and refrigerated for 15 minutes. Cooling ticks prior to hemolymph collection inhibits gut contraction, thus reducing the chance of breaking the gut and hemolymph contamination, [26]. Incisions (1-2 mm long) were made in the integument with a fine scalpel blade. The exuding hemolymph was collected with capillary tubes and fixed directly in equal volume of 30% glutaraldehyde/ saline (V/V).

Transmission Electron Microscope: Hemolymph processing for transmission electron microscopy was according Kadota *et al.* [27]. Hemolymph was fixed in 3% glutaraldehyde in phosphate buffer saline (PBS) (PH 7.4) and post fixed in 2% osmium tetroxide. The fixed samples were centrifuged at 1.000g for 10m. Samples were Washed two times with (PBS), dehydrated through a graded series of ethanol and infiltrated two times with propylene oxide. Hemolymph in samples were immersed in a different concentration from propylene and resin and embedded in 100% resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with VEOL 100S TEM Japan.

RESULTS

Growth of *Bacillus thuringiensis* var. *thuringiensis* and Their Recovered Toxic Protein During Fermentation:

Bacillus thuringiensis is a Gram +ve endospore former that releases crystalline δ-endotoxins during vegetation of their spores and can be obtained after lyses of the spores.

The obtained spore-crystal complex from *Bt* fermentation was enhanced during the first 12h followed by spontaneous increase in the next 36h. Then it hardly increased in the subsequent 48h (Fig. 1). The medium pH played a significant role in diminishing the growth rate where the pH development resulted in reduction of the growth rate (Fig. 1).

After 48 h, the stationary phase was predominant and the pH markedly increased to 9.5 where sporulation occurred and the crystalline δ-endotoxins was formed.

As shown in Table 1, the obtained spore crystal complex was 16.07 g wet weight L⁻¹. The obtained spore crystal complex was dried by lyophilization to 2.31 g L⁻¹.

Table 1: *Bacillus thuringiensis* serovar *thuringiensis* toxic protein recovery during purification process.

Bacterial material	Recovery weight	Protein recovery %
Spore crystal complex wet weight after fermentation	16 g/l	-----
Spore crystal complex dry weight after fermentation	2.3 g/l	-----
Starting material after lyophilization	7 g	-----
Crystal recovery dry weight	0.1607g/g dry weight	16.07
Dissolved proteins in 1M NaOH pH 12	21.07 mg/g dry weight	2.017
Precipitated toxic protein with 80% NH ₂ SO ₄	15.784 mg/g dry weight	1.578
Purified protein fraction after molecular weight sieve from 50-30 kDa	14.845 mg/g dry weight	1.484

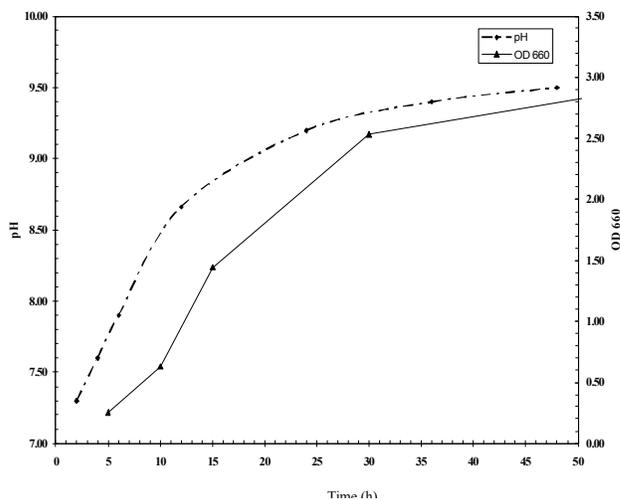


Fig. 1: Growth curve of *Bacillus thuringiensis* in relation to pH of fermentation medium

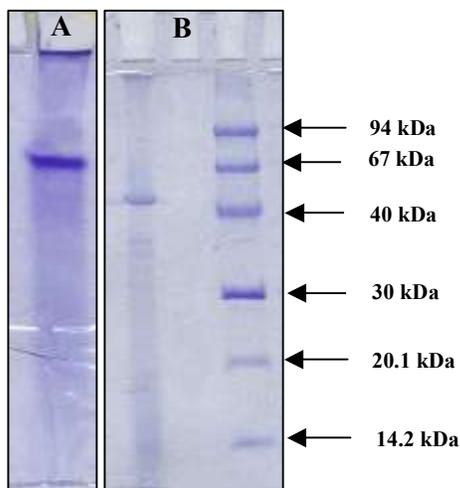


Fig 2: (A) Native electrophoretic analysis by 7.5% PAGE, (B) 12% SDS-PAGE for *B.t.* serovar *thuringiensis* toxic protein fraction lane (1) and molecular weight markers lane (2). Molecular weight markers are lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), phosphorylase b (92 kDa), P-galactosidase (116 kDa) and myosin (200 kDa)

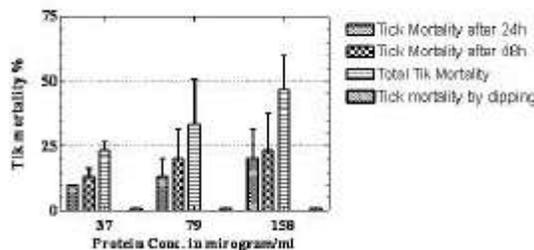


Fig. 3: Tick mortality % within 48 h of toxic protein injection

The recovered crystalline protein represented by biphasic separation system was 16.07% of the total dry weight recovered from the fermentation process. The *B.t.* δ -endotoxin crystalline proteins that were dissolved using 1M NaOH pH 12 were completely dissociated by high pH resulting in the untruncated toxic core protein which represented 2.017% of the total dry weight of spore-crystal complex. The acid precipitation of *B.t.* toxic protein followed by ammonium sulfate precipitation using 80% saturated solution diminished the recovered toxic proteins to 1.578%. After application of dialyses and Vivaspine molecular weight concentrator (50 and 30 kDa cutoffs) the recovered toxic proteins constituted 1.484% of total soluble proteins.

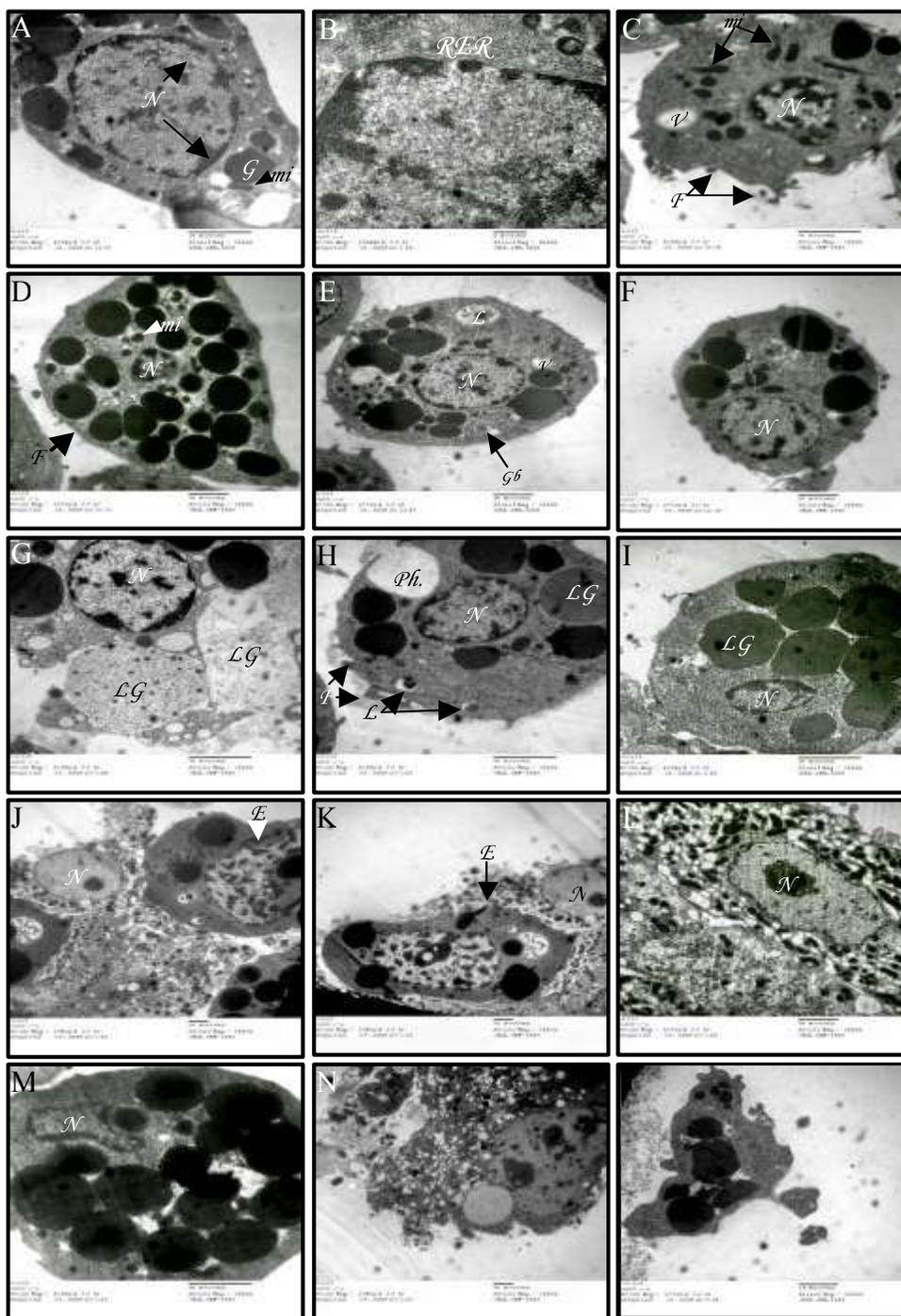


Fig. 4: Ultrastructure of hemocyte morphotypes and cytopathological changes. (A&B) are prohemocytes, (C) non-granular hemocyte (Plasmatocytes), (D) Eosinophilic granular cell (EC), (E,F&H) Basophilic granular cells(BC), (G&I) Granule-rich basophilic cells and (J,K,L,M,N,O) cytopathological changes in all hemocyte cells. **EC**: Eosinophilic granular cell, **F**: Filopodia, **G**: Granules, **G.b.**: Golgi bodies, **L**: Lysosomes, **L.G.**: Lamellate granules, **N**: Nucleus, **Ph.V.**: Phagocytic vacuole, **RER**: Rough Endoplasmic Reticulum and **V**: Vacuole

PAGE of Toxic Protein of *B. thuringiensis* var *thuringiensis*: The native PAGE revealed that the obtained untruncated toxic protein core is composed mainly of single major intact protein molecule (Fig. 2A). By using 12% SDS-PAGE, it was found that the intact toxic protein fraction is approximately 43 kDa (Fig. 2B).

Ticks Mortality Study: It was recorded that *B.t.* Cry4Ba δ -endotoxin did not induce any significant mortality in ticks exposed to the toxin orally through dipping while injection of toxin intrahemocoelily resulted in rapid noticeable mortality after 24h and causing up to 46.66% mortality after 48h where the experiment ceased. The tick mortality was in a dose dependent manner and in direct relationship with incubation time (Fig. 3).

Hemocyte Ultrastructure: According to the classification scheme of insect hemocytes four types of hemocytes were found in the hemolymph semi engorged adult females of *H. dromedarii* [27].

The ultrastructure of hemocytes of females *H. dromedarii* is shown in Fig. 4. Prohemocytes (Ph) appeared as small spherical cells with a large nucleus and low nucleo-cytoplasmic ratio, mitochondria (mi), prominent rough endoplasmic reticulum (RER) the cell cytoplasm is usually homogeneous or occasionally contains few granule-like inclusions (Fig. 4 A,B). Non granular hemocyte (Nc) plasmatocytes have an off-centered nucleus with peripheral chromatin material, numerous mitochondria extensive rough endoplasmic reticulum (RER) in close association with the plasma membrane bearing filopodia (Fig. 4 C).

Eosinophilic granular cells (Ec) are polymorphic shape with electron dense granules, mitochondria and centered nucleus (Fig. 4 D). Basophilic granular cells (Bc) they have prominent thick ectoplasm bearing numerous membrane outpocketings in the form of pseudopodia and they have RER, Mi, ribosomes and glycogen (Fig. 4 E, F). The overwhelmingly abundant round-shaped. Young Bc have fewer lamellate granules but have extensive RER, numerous organelles such as Mi, Golgi bodies, lysosomes vacuoles and filopodia (Fig. 4F,G). Mature BC appeared bloated, with increased density of large granular inclusions tending toward the cells membrane (Fig. 4 G, H,I). Some granules contained an electron-lucent core (Fig. 4G).

Morphological and Cytopathological Changes in Hemocytes of *H. dromedarii* Female: Observation illustrated that, endo-toxin derive from *Bacillus thuringiensis* caused sever damage in hemocytes of

female *H. dromedarii* (Fig. 4). Cell membrane was damaged and completely destroyed, cell contents spread into the plasma of hemolymph (Fig. 4 I, J). The nucleus membrane of eosinophilic granular cells were deformed and became irregular shape. Chromatin material was spread in the nucleus of eosinophilic granular cells (Ec), (Fig. 2 K, I).

DISCUSSION

The four types of hemocytes in engorged *H. dromedarii* females in our study agreed with the classification scheme for insect hemocytes [28], namely: plasmatocyte, type I and II granular hemocytes and prohemocyte-like cells. Ultra-structural data revealed that plasmatocytes and basophilic granular cells might contribute to the attachment and lysosomal degradation of foreign organisms as well as decomposed midgut molecules for foreign origin through their endocytic activity as confirmed by Kuhn and Haug [29] (Fig. 4 C.E.F). Kuhn and Haug [29] reported the role of type I granular hemocytes cells in the coagulation of the hemolymph of *I. Ricinus* following recognition of foreign material. It might also be involved in storing, transporting or metabolising of glycogen starting with blood meal as confirmed by Kuhn and Haug [29] in *I. ricinus*. Shane *et. al.*, [30], found that defensin is stored in the granulocytes of the hemolymph and secreted into the hemolymph upon bacterial insult.

The present study reported for the first time that some *B. thuringiensis* toxins are lethal to hemoplast cells of *H. dromedarii* ticks.

Many authors had been assumed that *B.t.* induce disruption of the midgut epithelium resulted in cessation of feeding and eventual death by starvation. An alternative proposed mechanism of killing is that extensive cell lysis provides spores access to the more favorable environment of the hemocoel, where they germinate and reproduce. This leading to septicemia and death of ticks [30]. However, Broderick *et al.* [32] referred insects mortality to the septicemia caused by accompanied enteric bacteria.

Evidently, the mode of action after injection is quite different from the one that occurs after oral intake of the toxins. According to Kwa *et al.* [33], sensitivity toward Cry1C of cell lines of *S. frugiperda* and *S. exigua* are correlated to the binding of the toxin to a 40-kDa protein.

It is important to record that tick mortality was only in injected groups while the orally exposed ticks was not affected. This may be due to inactivation of *B. t.* Cry4Ba

43-kDa protein by midgut proteases which may not give the toxic protein any chance to interact with hemocytes. While injection of toxic proteins allow it to bind directly with hemocytes.

Controversially to what mentioned by Cerstiaens *et al.* [16], Giannoulis *et al.* [34] about assuming the denaturation, or aggregation of *B. t.* δ -endotoxins in a nonfunctional form and dead *Xenorhabdus nematophila*, that would prevent binding or pore formation, as we recorded that these proteolitic toxins remain in active form and capable to induce cytotoxic effect in tick hemocytes. The cytotoxic effect was represented by drastic cytolysis with complete disruption of intracellular organelles in the hemocytes.

Over exposure of *B.t.* crystalline δ -endotoxin to high alkaline pH resulted into complete solubilization of δ -endotoxin and dissociation of Cry proteins into small peptide leaving single untruncated 43 kDa core (Cry4Ba) [35] with exposed active site that may bind to hemocytes cellular membrane and resulted in pore formation subsequently and cell lysis.

The potent effect of Cry4Ba upon injection in the hemolymph strongly suggests that a target other than the midgut epithelial cells must be present for this delta-endotoxin in the engorged female of *H. dromedarii*.

So, in order to control ticks manifestation biologically we may develop a carrier agents like transgenic plants or cloning of toxin gene in enteric bacteria to deliver the *B.t.* δ -endotoxin directly to the tick hemolymph.

In conclusion, the results of this study suggest that *B. thuringiensis* toxin targets are not only present in the gut. This is an important result of biological relevance at the level of *B. thuringiensis* mode of action. Secondly, insects or other organisms that are susceptible to "injection" (for example, insects or parasitoids that may have ingested the toxin might inject it into their hosts or preys) may be affected. Therefore, the toxic effect described here may be relevant to the more general field of biocontrol, including trophic interactions. The benefit of, or the damage caused by, the phenomenon at the ecological level remains to be investigated. Also, the health protection of workers involved in *B. thuringiensis* spraying, etc. and the nonspecific effects on nontarget species need to be carefully scrutinized.

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