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Ultrastructural alterations in the midgut of *Bacillus sphaericus*-treated *Culex pipiens* (Diptera: Culicidae) larvae

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Abstract

The ultrastructure alterations were described in the midgut of *Culex pipiens* (Diptera: Culicidae) larvae following *Bacillus sphaericus* treatment. Two hours post-treatment, the epithelial layer began to have large amount of vacuoles. Explosion in the basement membrane took place 6 h post-treatment. Ten hours post-treatment, the bacterium began to invade the microvilli. At 14 h post-bacterial treatment, epithelial cells were destroyed, microvilli were damaged, and bacteria were present in lumen in large amount. The present study provided the evidence on the main aberrations induced in midgut larvae of *Cx. pipiens* as a result of ingesting *B. sphaericus*.

Keywords: *Culex pipiens*, *Bacillus sphaericus*, Midgut, Larva, TEM

Background

Culex pipiens Linnaeus complex mosquitoes transmit several diseases that affect humans and other animals (Michalski et al. 2010). These species oviposit in stagnant polluted water and populations are increasing and expanding due to creation of favorable habitats caused by urbanization (Bockarie et al. 2009), irrigation, and creation of the Aswan High Dam (Harb et al. 1993). Mosquito control strategy relies heavily on insecticides and particularly pyrethroids. However, widespread of pyrethroid resistance has hindered vector control implementation and sustainability (Shi et al. 2015).

Highly potent mosquitocidal strains of the microbial agent *Bacillus sphaericus* have been applied for the control of mosquito larvae around the world (Mulla et al. 2003). This organism has several advantages, including low environmental toxicity due to the high specificity of *B. sphaericus* toxins, high levels of efficacy and environmental persistence, and the ability to overcome resistance developed against conventional insecticides used worldwide (Nielsen-LeRoux et al. 2001). *B. sphaericus* crystal contains two major polypeptides, a 42-kDa polypeptide and a 51-kDa polypeptide, which are designated BinA and BinB, respectively (Darboux et al. 2007). The mode of action of the toxin complex in susceptible mosquitoes involves highly specific binding to a receptor in the larval midgut (Silva-Filha et al. 1997). The two crystal components act synergistically; the BinB part is responsible for initial binding to the receptor, and the BinA component confers toxicity (Charles et al. 1997). A direct correlation exists between binding affinity and toxicity (Opota et al. 2008).

In mosquitoes, the first physical barrier met by ingested pathogens is the midgut epithelium; it is composed of a single layer of epithelial cells forming a microvillar surface on the luminal side (Hecker 1977).

The present study describes the ultrastructure alterations in the midgut of *Cx. pipiens* larvae following *B. sphaericus* treatment.

Materials and methods

Mosquito rearing

Immature stages of mosquito were collected from a drainage canal in Qualyobia Governorate, Egypt. Fourth instar larvae and emerging adults were identified according to Harbach (2012). Species other than *Cx. pipiens* were discarded. *Cx. pipiens* larvae were reared in the laboratory under controlled conditions of temperature (27 ± 2 °C) and relative humidity (70–80%) and a 12 L: 12D photoperiod. Third instar larvae of the filial generation were used in the study.

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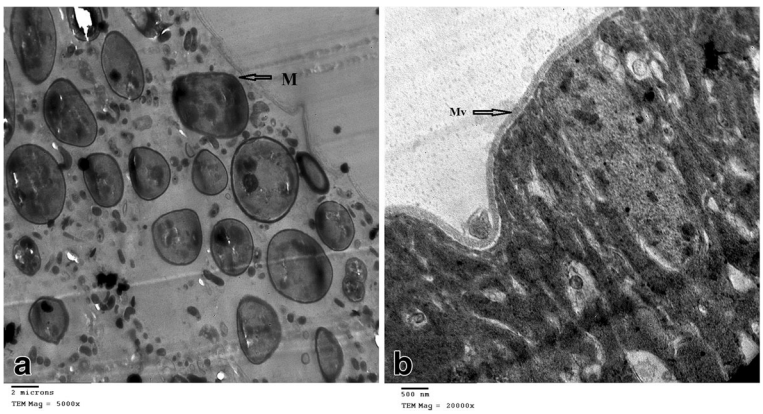

Fig. 1 Midgut ultrastructure of *Culex pipiens* third larval instar showing **a** M: mitochondria and **b** Mv: microvilli

Bacterial strain and treatment

Bacillus sphaericus strain 2362 was provided by Abbot Laboratories, North Chicago, IL, USA. The late third stage instar of *Cx. pipiens* was treated with LC_{50} of *B. sphaericus* (0.07 ppm) (Soliman et al. 2000). Larvae were tested at the periods of 2, 6, 10, and 14 h post-treatment. Individual midgut was dissected and prepared for TEM examination.

Specimen tissue preparation for TEM

Midgut of at least five larvae for each timing were fixed by immersion in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at room temperature and then washed in 0.1 M cacodylate buffer three times, 15 min for each. Specimens were secondarily fixed by immersion in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h and then washed in 0.1 M cacodylate buffer three times, 15 min for each. Fixed and washed specimens were dehydrated through a graded ethanol series (30, 50, 70, 90, 96, and 100%) being kept at room temperature for 15 min during each step of the dehydration process. The absolute alcohol was replaced by propylene oxide or acetone via a stepwise series of ethanol: propylene oxide 2:1, 1:1, and 1:2 and then finally maintained in pure propylene oxide. Dehydrated specimens were embedded in epoxy resin.

Ultra-thin sections (50–80 nm thick) were cut, stained, and examined with a JEOL 1010 Transmission Electron Microscope (Martins et al. 2011).

Results and discussion

Examination of non-treated mosquito midgut larvae revealed that it was composed of a single-layered epithelium surrounded by a network of circular and longitudinal muscle bundles (Fig. 1a, b). The digestive cells had morphological characteristics of enterocyte cells and basolateral membrane. The free surface of enterocytes had a regular array of microvilli. Cells have spherical nuclei. Results of the present study were comparable to those found by Wassim et al. (2014) working on *Cx. pipiens* midgut epithelium.

The morphological structures of *B. sphaericus*-treated Cx. *pipiens* larval midgut showed modifications at the

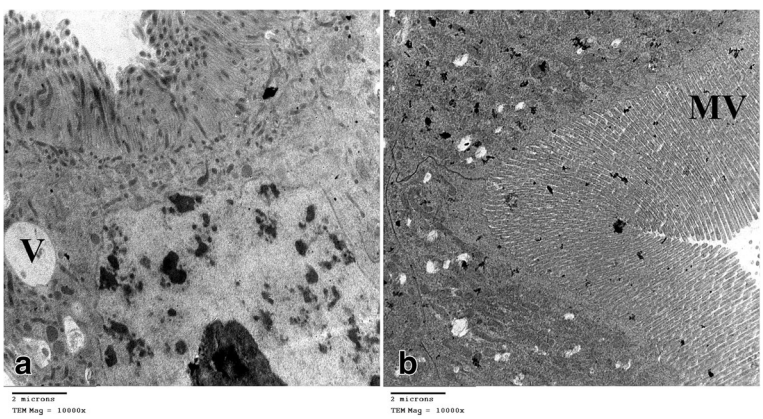

Fig. 2 Midgut ultrastructure of *Culex pipiens* third larval instar 2 h post *Bacillus sphaericus* treatment showing **a** V: vacuoles and **b** Mv: microvilli

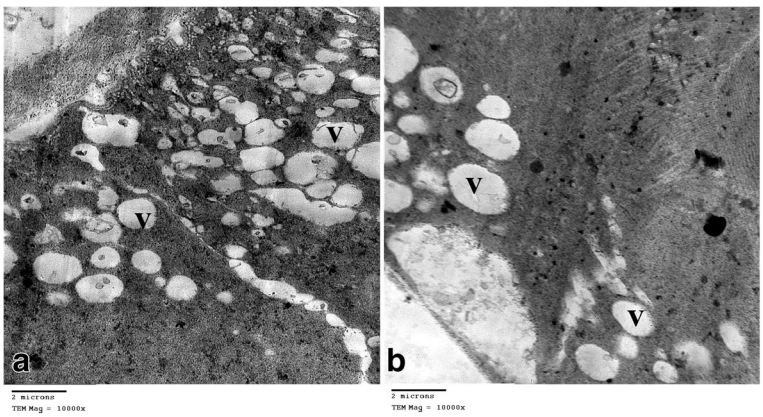

Fig. 3 Midgut ultrastructure of *Bacillus sphaericus*-treated *Culex pipiens* third larval instar 6 h post-treatment showing **a** and **b** V: vacuoles

various timing following *B. shaericus* treatment. Two hours post-treatment, the epithelial layer began to have large amount of vacuoles (Fig. 2a). The microvilli were not affected (Fig. 2b). Examination revealed the presence of vacuoles in lumen cytoplasm. The early appearance of cytological disturbances, following bacterial treatment, could be correlated to the fast digestion of *B. sphaericus* cells in the anterior and central midgut (Labib and Dawoud 2003). Cytoplasmic vacuolization was reported to be one of the major cytotoxic responses of *Cx.* mosquitoes to Bin intoxication (Silva-Filha et al. 1997). *B. sphaericus* bin-induced vacuolization was a transient phenomenon that affects autolysosomes and vacuolization was associated with induction of autophagy in intoxicated cells (Opota et al. 2008). Large vacuoles appeared early in the *Cx. pipiens* mid-gut cells and rough endoplasmic reticula broke into small vesicles.

Six hours post-treatment, the amount of vacuoles increased in the epithelial layer (Fig. 3a, b). This timing showed the onset of abnormalities in the nucleus. Although microvilli were not affected, some positions lost their microvilli. Explosion in the basement membrane made it losing contact, a result that could be explained by the fact that *Cx. pipiens* larval midgut was the primary target of the binary toxin (Bin) present in parasporal inclusions of *B. sphaericus* (Darboux et al. 2001).

Ten hours post-treatment, *B. sphaericus* spores were seen in the lumen (Fig. 4a). Microvilli were still intact (Fig. 4b). However, the bacterium began to invade the microvilli. At this time, the presence of bacteria was evident in the lumen. Bacteria began to enter the epithelial layer. The midgut epithelial cells of mosquito larvae intoxicated with Bin was reported to display several cytopathologies affecting the microvilli, the mitochondria, and the rough endoplasmic reticulum, but the most dramatic feature of Bin intoxication was the appearance of abnormal, electron-clear vacuoles indicating an important cellular stress (Silva-Filha et al. 1997). Bin toxin triggered apoptosis via an intrinsic or mitochondrial pathway in vivo, possibly contributing to larval death (Tangsongcharoen et al. 2015). However,

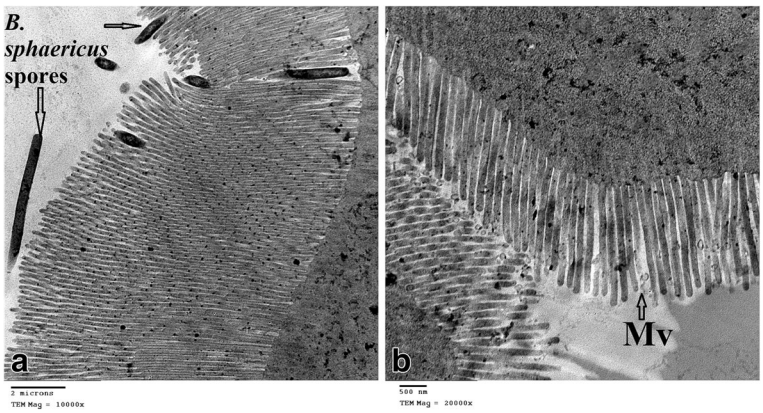

Fig. 4 Midgut ultrastructure of *Bacillus sphaericus*-treated *Culex pipiens* third larval instar 10 h post-treatment showing **a** *B. sphaericus* spores and **b** Mv: microvilli

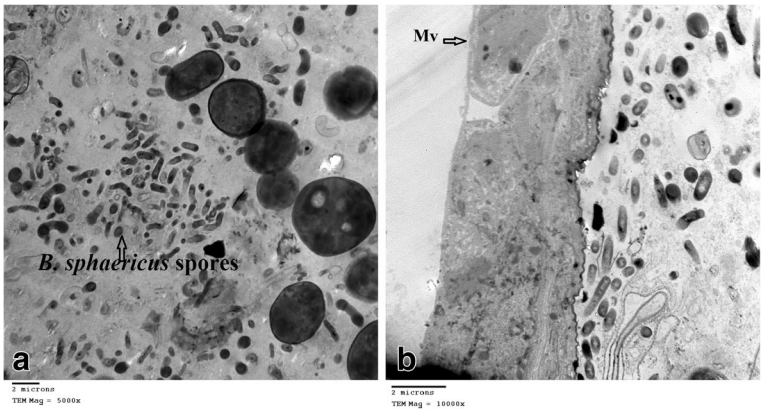

Fig. 5 Midgut ultrastructure of *Bacillus sphaericus*-treated *Culex pipiens* third larval instar 14 h post-treatment showing **a** *B. sphaericus* spores and **b** damaged microvilli

proliferation and differentiation of precursor stem cells could replace the destroyed midgut epithelial cells (Baton and Ranford-Cartwright 2007).

At 14 h post-bacterial treatment, bacteria were present in lumen in large amount (Fig. 5a), the result that agreed with that of Labib and Mohamad (2003) who found that the number of viable spores reached its maximum between 12 and 24 h. Epithelial cells were destroyed. Microvilli were damaged as well (Fig. 5b). Binding to specific receptors on the apical microvilli membrane was considered as the initial step of delta-endotoxin action (Ravoahangimalala et al. 1993). Similarly, Cry4 produced by *B. thuriengiensis israelensis* were proved to bind to the microvilli of the epithelial cells of *Cx. pipiens* posterior midgut and gastric caecae (Yamagiwa et al. 2001). On the contrary, microvilli of the midgut epithelial cells of *Streptomyces griseus*-treated *Culex pipiens autogenicus* larvae were unaltered, and there were no changes in the arrangement of cells in the tissues (Zizka et al. 1989).

Structural alterations in larval midgut following bacterial treatment are evident and enhance as the time proceeds. The present study provided a description of the main aberrations induced in midgut larvae of *Cx. pipiens* as a result of *B. sphaericus* treatment.

Conclusions

The present study provided the evidence on the main aberrations induced in midgut larvae of *Cx. pipiens* as a result of ingesting *B. sphaericus*.

Abbreviations

B.: *Bacillus*; *Cx.*: *Culex*

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Authors' contributions

MKT contributed to the design of the work, mosquito collection and rearing, larval treatment with *B. sphaericus*, larval midgut dissection and examination of morphological structures of B. sphaericus-treated Cx. pipiens larval midgut, and preparing and revising of the manuscript. BAS supervised the work, examined morphological structures of *B. sphaericus-treated Cx. pipiens* larval midgut, and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable for that section.

Consent for publication

Not applicable for that section.

Competing interests

The authors declare that they have no competing interests.

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