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Full Length Research Paper

The gene expression of caspasses is up-regulated during the signaling response of *Aedes caspius* against larvicidal bacteria

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Our current knowledge on the key molecular mechanisms and cognate signaling transduction, by the δ-endotoxin-mediated mosquitoticidal effects, associated with exposure to *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* (*Bs*), is limited. Moreover, this observed mosquitocidal activity that is related to program cell death is largely unknown. Therefore, in an attempt to answer this question, the current study was primarily sought to provide evidence as to the molecular mechanism of mortality in *Bt*/*Bs* infected *Aedes caspius* mosquito larvae. Thus, the impact of *Bt* and *Bs* treatment on the expression of some selected apoptosis-related caspase genes in *A. caspius* mosquito larvae was investigated, via quantitative reverse-transcriptase PCR (qRT-PCR). Mosquito larvae were collected from natural water niches. Larvae were grown to adult stage and were subsequently identified as *A. caspius* at Natural History Museum, London, UK. Remarkably, light and transmission electron microscopy studies of the midgut epithelial tissues revealed that both *Bt* and *Bs* brought about significant histopathological effects. Moreover, this treatment resulted in severe destruction at the sub-cellular organelle level for the mitochondria. Interestingly, qRT-PCR studies revealed that the treatment of *A. caspius* mosquito larvae with both *Bt* and *Bs* caused a significant up-regulation in the transcription level of all caspase genes under study, namely: *CASPS17*, *CASPS18*, *CASPS19*, *CASPS20* and *CASPS21*. The results are discussed in the light of our current understanding of the signaling transduction pathway of apoptosis in insects and mosquitoes and the putative role of caspases gene expression in response to the treatment of *A. caspius* mosquito larvae with larvicidal bacteria.

Key words: *Aedes caspius, Bacillus thuringenesis*, *Bacillus sphaericus*, apoptosis, caspase, larvicidal bacteria.

INTRODUCTION

Mosquito-borne diseases are major causes of death and morbidity worldwide. Mosquitoes of the *Aedes* genus are considered disease vectors as they are responsible for the transmission of a number of significant human viral and parasitic pathogens (Knight and Stone, 1977; Tolle, 2009). In particular, *Aedes caspius* is a vector for rift valley fever; *Aedes aegypti* is a vector for yellow fever and dengue, while *Aedes albopictus* is a vector for dengue and West Nile virus (Tolle, 2009). In the current absence of effective vaccines (Pierson et al., 2008) or specific antivirals (Perera et al., 2008) for the majority of these diseases, effective alleviation of this burden has essentially

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relied on mosquito control programs (Lounibos, 2002). To this end, the harmful effects of chemicals on non-target populations, ever-growing resistance to chemical insecticides along with the recent resurgence of different mosquito-borne diseases have induced scientists to explore alternative and simple sustainable methods of mosquito control primarily employing non-chemical based control measures, which include the use of larvicidal entomopathogenic bacteria such as *Bacillus* spp (Federici et al*.,* 2007).

The genus *Bacillus* constitutes a diverse group of rod shaped, gram-positive bacteria with entomopathogenic properties, characterized by their ability to produce robust endospores as a survival mechanism in response to adverse environmental conditions (Gibson and Gordon, 1974). One of the most important applications of bacilli is their utilization in the production of biopesticides, which are currently considered the safest insecticides and constitute safe and environmentally friendly substitute of the harmful chemically synthesized insecticides. Two *Bacillus* species with mosquitocidal activities namely: *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* (*Bs*) strains, are now produced commercially as bioinsecticides and used extensively in the field for the control of insect pests and vectors of human disease (Poopath and Tyagi, 2004; Afify et al., 2009).

Bt and Bs are considered as soil bacteria characterized by their ability to produce crystalline prasporal inclusions called "δ-endotoxin proteins" during sporulation. The crystalline inclusions, along with the spores, have a great potential to control a number of insect pests of different orders, lepidoptera, diptera and coleoptera and also other invertebrates such as nematodes (Vidyarthi et al., 2002). Therefore, they represent a valuable tool for Integrated Pest Management (IPM). *Bt* is one of the most important microbial biopesticides produced in the world, accounting for 1 to 2% of the global insecticide market (Schnepf et al., 1998), and is responsible for over 90% of all biopesticide sales (Glare and O'Callaghan, 2000). The widespread occurrence of this bacterium and interest in the use of *Bt* and *Bs* products as an alternative to chemical insecticides stimulated the isolation of native *Bt* strains in many parts of the world (Uribe et al., 2003; Gao et al., 2008; Afify et al., 2009). In this context, both *Bs* and *Bt* have been successfully used for the biological control of mosquito-borne diseases such as malaria (Barbazan et al., 1998; Mulla et al., 2001; Haq et al., 2004).

Significant efforts has been devoted by the scientific community to the study of the biological mode of action of insecticidal and nematocidal activities associated with *Bs* and *Bt* Ingestion often results in midgut paralysis and cessation of feeding (Heimpel and Angus, 1959). In *Bt*, the best known virulence factors are the specific protein toxins predominantly comprised of one or more proteins (Cry and Cyt toxins) formed as crystalline inclusions during sporulation. In *Bs*, these toxins are named binary (Bin) and mosquitocidal (Mtx) toxins. These toxins target, and cause the destruction of, the epithelial cells lining the midgut (Bravo et al., 2007). Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores. The powerful endotoxins are initially produced by those *Bacilli* as inactive protoxins. They are partially hydrolyzed and converted by some proteases in the midgut of the susceptible insect species to the active bioinsecticidal toxins that are lethal to the target insects (Afify et al., 2009). Like other pore-forming toxins that affect mammals, Cry toxins interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding resulting in the formation of a pre-pore oligomeric structure that is insertion competent (Bravo et al., 2007). For some insects, the action of these toxins alone is sufficient for mortality, whereas for others, the spore must also be present and death can be caused by septicaemia (Heimpel and Angus, 1959).

Apoptosis, a type of programmed cell death (PCD), is a highly regulated process that is considered as a key pathway involved in normal processes such as development, tissue homeostasis and DNA damage responses, as well as pathological processes including cancer, ischemia, neurological diseases and defense against pathogens like viruses (Thompson, 1995; Vaux and Korsmeyer, 1999). The key components of the machinery that carries out this evolutionarily conserved process are caspases. Caspases are a conserved family of cysteine proteases that play important roles in apoptosis and in other cellular processes. These enzymes are expressed as zymogens and are activated by multiple stimuli upon receiving an apoptotic signal (Li and Yuan, 2008). There are two types of caspases, initiator and effector, which carry out different functions in apoptosis. A death insult first results in activation of one or more members of the initiator class, which then cleave and activate members of the effector class. The effector caspases cleave many cellular targets and dismantle the cell (Li and Yuan, 2008).

Significant research has been carried out on the molecular and biochemical characterization of the δ-endotoxins produced by these mosquitocidal bacteria and their mode of action. However, our current knowledge on the key molecular mechanisms and cognate signaling, mediated by the observed endotoxin-mediated mosquitocidal activities, associated with exposure to *Bs* and *Bt*, is limited. Moreover, whether this observed mosquitocidal activity is related to program cell death or not, is largely unknown. Therefore, to answer these questions the current study was primarily sought to provide evidence as to the molecular mechanism of mortality in *Bt*/*Bs* infected *A. caspius* mosquito larvae. Thus, the impact of *Bt* and *Bs* treatment on the expression of some selected apoptosis-related caspase genes in *A. caspius* mosquito larvae was investigated, via quantitative reverse-transcriptase PCR (qRT-PCR). The results are discussed in the light of our current understanding of the signaling transduction pathway of apoptosis in insects and the putative role of

Table 1. List of primers and annealing temperatures used for expression analysis of caspases genes of *A. aegypti* Roche Lightcycler® 480 real-time PCR.

Gene	Forward (F) and reverse (R) primers (5'- 3')	Description	Vectorbase Gene ID*/ GenBank accession no.**	Annealing temperature (°C)
CASPS17	*F:* TGCCATTGATGAGAAGAGAATTTGAG *R:* GCCTACTTGTCCCGTGTTACC	Caspase-1	AAEL005955	60
CASPS18	*F:* CTGTCTTGTGGTAGTTGTGATGTC *R:* CGGATGCTTGTGATTCTTCTTCTC	Caspase-1	AAEL003439	60
CASPS19	*F*: CTCGCCGTGTGACATCATAAC *R*: AAGCAAGGAAGTTCTCGTTTCTC	Caspase-1	AAEL003444	60
CASPS20	*F*:GCGGATTGCCTGATGGTATTC *R*: ATGCTTGGACTATGAACAACTTCG	Caspase-1	AAEL014658	60
CASP21	*F*: CGATTGTAATAAAACGGTTCCTAGTCC *R*: CTATTGACATTTCTGGCATCTCTCTTAG	Caspase-1	AAEL017498	60
ACTIN6	*F*: AAGGCTAACCGTGAGAAGATGAC *R*: GATTGGGACAGTGTGGGAGAC	Housekeeping	DQ124691	60

*,http://aaegypti.vectorbase.org/index.phpandhttp://cegg.unige.ch/Insecta/immunodb/; **, http://www.ncbi.nlm.nih.gov/Genbank/

caspases gene expression in response to the treatment of *A. caspius* mosquito larvae with larvicidal bacteria.

MATERIALS AND METHODS

Field collection of mosquito

Field trips have been made to the different places in the Eastern region (Ehsaa') in Saudi Arabia to collect *A. caspius* mosquitoes. Mosquitoes were collected from salty water lakes and pools in the desert places away from palm farms. Salinity, temperature and pH of water were monitored.

Establishment of experimental mosquitoes

Field-collected *A. caspius* mosquitoes were identified according to standard classification keys (Mattingly and Knight, 1956) and confirmed by the Natural History Museum (London, UK). Mosquitoes were reared in the insectary of Zoology Department, College of Science, King Saudi University, under standard conditions as previously outlined (Ahmed et al., 2010). Adults emerging within a 24 h period were maintained in rearing cages (30 x 30 x 30 cm each) with continuous access to a 10% glucose solution (w/v). At least 10 generations were produced prior to use for the experimental purposes. After adult emergence, third instar larvae were employed in all experimental procedures. To maintain a stock of mosquito colony, they were routinely fed upon the blood of an anaesthetized CD mouse in order to lay eggs for new generations. International protocols governing the ethical treatment of animals were followed.

Light and transmission electron microscopy of *A. caspius* larvae

After treatment with *Bt* or *Bs* LC_{50} doses, 3rd instar *A. caspius* larvae were studied for morphological alterations under light and scanning electron microscopy (JEOL Ltd., model JEM-100CX II). Light microscopic studies were carried out according to Ahmed et al. (2010). Briefly, the midgut sections were fixed overnight in cold 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) and post-fixed for 1 h at room temperature in 1% OsO4 in the 100 mM phosphate buffer. The tissues then were dehydrated through an ethanol series, treated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin 10 µM sections were mounted on slides, stained with hematoxylin and eosin or 1% toluidine blue-Borax (Sigma-Aldrich) and mounted with Paramount (Fisher) and examined by light microscopy using a Zeiss Axioskop 50 compound microscope (Carl Zeiss, Inc., Thornwood, NY). For transmission electron microscopy studies, larval midguts were dissected for 18 h post-treatment just before death, fixed, sectioned and stained routinely as previously described (Hayat, 1970) with some modifications. Briefly, the midgut sections were fixed overnight in cold 0.8% glutaraldehyde, 4% Paraformaldehyde in 0.1 M sodium cacodylate, (pH 7.0) and post-fixed for 4 h at 4°C in 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.0). Tissues then were dehydrated through an ethanol series, treated with propylene oxide and embedded in Epon-Araldite resin (1:1). Ultrathin 4 µm sections were mounted on slides, stained with 2% uranyl acetate for 30 min and then incubated in lead citrate for 10 min. The samples were analyzed with a transmission electron microscope (JEOL Ltd., model JEM-100CX II) at 80 kV.

Real-time RT-PCR technique

The expression levels of selected caspases genes in response to *Bt* and *Bs* treatment in *A. caspius* were examined at 6, 12 and 24 h post treatment (hpt). Thus, gene-specific primers, designed based on *A. aegypti* gene sequences (http://aaegypti.vectorbase.org/index.php), were employed. The sequences of the specific sets of primers (Bryant et al., 2008), utilized for the amplification of each cDNA and the annealing temperature employed, are summarized in Table 1. Then, total RNA was purified from 3rd instar larvae using Norgen Biotek kit, according to the manufacturer's instructions (Norgen Biotek, Canada). Purity and integrity of the isolated total RNA were verified using formaldehyde denaturing gel electrophoresis (Sambrook et al., 1989). The absence of genomic DNA in the total RNA prepa-rations was confirmed by both agarose gel electrophoresis as well as the detection of no PCR product when

Table 2. Physical conditions of breading water of collected mosquito larvae from Eastern Province (Ehsaa' region) in Saudi Arabia.

Place	Temperature (°C)	Salinity (ppm)	pH
Al-Sho'bah 1	24.11	> 12800	7.4
Al-Sho'bah 2	24.00	5478.4	7.5
Al-Zeraqe	22.00	7040	7.5
Al-Tohaymeyah	23.7	2400	7.7

specific primers for the housekeeping gene *ACTIN6* were used. The first strand cDNA synthesis was performed with 5 µg of total RNA using 100 ng of oligo-p $(dT)_{12-18}$ primer and SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. Expression levels were normalized to *ACTIN6* gene expression, which was used as an internal housekeeping control. Real-time quantification was performed in the LightCycler® 480 Instrument with 96-well plate (Roche Diagnostics) using the SYBR green dye. PCR mixtures (final volume of 20 µl) contained 10 µl of LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), 5 µ of a 1:100 dilution of the cDNA and 250 mM of each primer (Table 1). The cycling conditions included an initial heat-denaturing step at 95°C for 10 min, 40 cycles at 95°C for 10 s, annealing at 60°C for all the primers, and product elongation and signal acquisition (single mode) at 72°C for 10 s. Following amplification, the melting curves were determined in a three-segment cycle of 95°C for 0 s, 65°C for 15 s and 95°C for 0 s at the continuous acquisition mode. The temperature transition rates were set at 20°C/s except for segment three of the melting curve analysis where it was set to 0.1°C/s. Water was used as the template for negative control amplifications included with each PCR run. Serial dilutions of each cDNA (10^{-1}-10^{-6}) were used to generate a quantitative PCR standard curve to calculate the corresponding PCR efficiencies. Results reported were obtained from three biological replicates and PCR runs were repeated twice.

Data analysis

Data were analysed using the Roche LightCycler® 480 software Version 3.5. Crossing point (C_P) was calculated by the Second Derivate Maximum method. The amount of the target mRNA was examined and normalized to the *ACTIN6* gene mRNA. The relative expression level (Fold change) was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the relative level of each mRNA normalized to the *ACTIN6* gene was calculated using the following equation:

$$\text{Fold change} = 2^{Cp \text{ target (control)} - Cp \text{ target (treatment)}} / 2^{Cp \textit{ ACTIN6} \text{ (control)} - Cp \textit{ ACTIN6} \text{ (treatment)}}$$

The relative gene expression data were subjected to Student's *t-test* in order to identify significant differences between samples treated with larvicidal bacteria compared to untreated control. Treatments were considered statistically significant when at $P \leq 0.05$ using a paired two-tailed t-test comparing the treated cells and the untreated controls. Statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA, v. 13.1, 2001).

DNA sequencing

Polymerase chain reaction (PCR) products of caspases genes were gel-purified using GE Healthcare Kit according to manufacturer's instructions (GE Healthcare, Sweden). DNA sequencing was conducted in Sequencing Core Facility at King Faisal Specialized Hospital & Research Centre (Riyadh, KSA), using Sanger Sequencing Technology on ABI Prism 3730XL (Applied Biosystems/Sanger) according to the dideoxy chain-termination method (Sanger et al., 1977). The obtained sequences were manually-cleaned and pairwise alignment against corresponding annotated caspase genes database (http://aaegypti.vectorbase.org/index.php) was performed with BioEdit Sequence Alignment Editor (Hall, 1999)

RESULTS

Salinity and pH measurements of larvae-breeding waters

Mosquito larvae were collected from different places throughout the Eastern Province in Saudi Arabia, namely, Ehsaa' and Dammam regions. It was noticed that *A. caspius* is rarely breading in fresh water but abundantly breading in salty water. Thus, salinity, pH and temperature of water of each place were recorded (Tables 2 and 3). Based on the obtained results, it was concluded that collected *A. Caspius* larvae were capable of breeding in water of different salinity ranging from 2400 to 12800 ppm.

Histopathological effects of Bt and Bs on midguts of *A. caspius* larvae

In order to investigate the impact of *Bt* and *Bs* treatments on *A. caspius*, 3rd instar larvae were examined for 24 h post treatment (hpt) by both light and transmission electron microscopic (TEM) techniques (Figure 1). Light microscopic examination results revealed the destructive effects of both *Bt* and *Bs* on midgut tissues of *A. caspius* larvae (Figure 1B and C) compared to the unexposed midgut control (Figure 1A). Notably, midgut epithelial cells were significantly destroyed by bacterial toxins at 24 hpt (Figures 1B and C), whereas cells of the untreated control retained their structural integrity (Figure 1A). The severe destruction of midgut epithelium tissue allows the bacteria to pass to haemolymph causing cessation of feeding, septicemia and finally death (Gill et al., 1992). In order to examine the impact of larvicidal bacterial treatment on sub-cellular organelles, TEM was employed. Strikingly, TEM results clearly indicate that, the treatment with larvicidal bacteria is associated with severe damage at the mitochondrial level (Figures 2D - F). The observed mitochondrial damage, visualized as white spots, was equally obtained with both Bt (Figure 2E) and Bs (Figure 2F) treatment at 24 hpt as compared to untreated control (Figure 2D).

Expression of apoptosis-related caspase genes

The impact of the treatment of *A. caspius* larvae on the

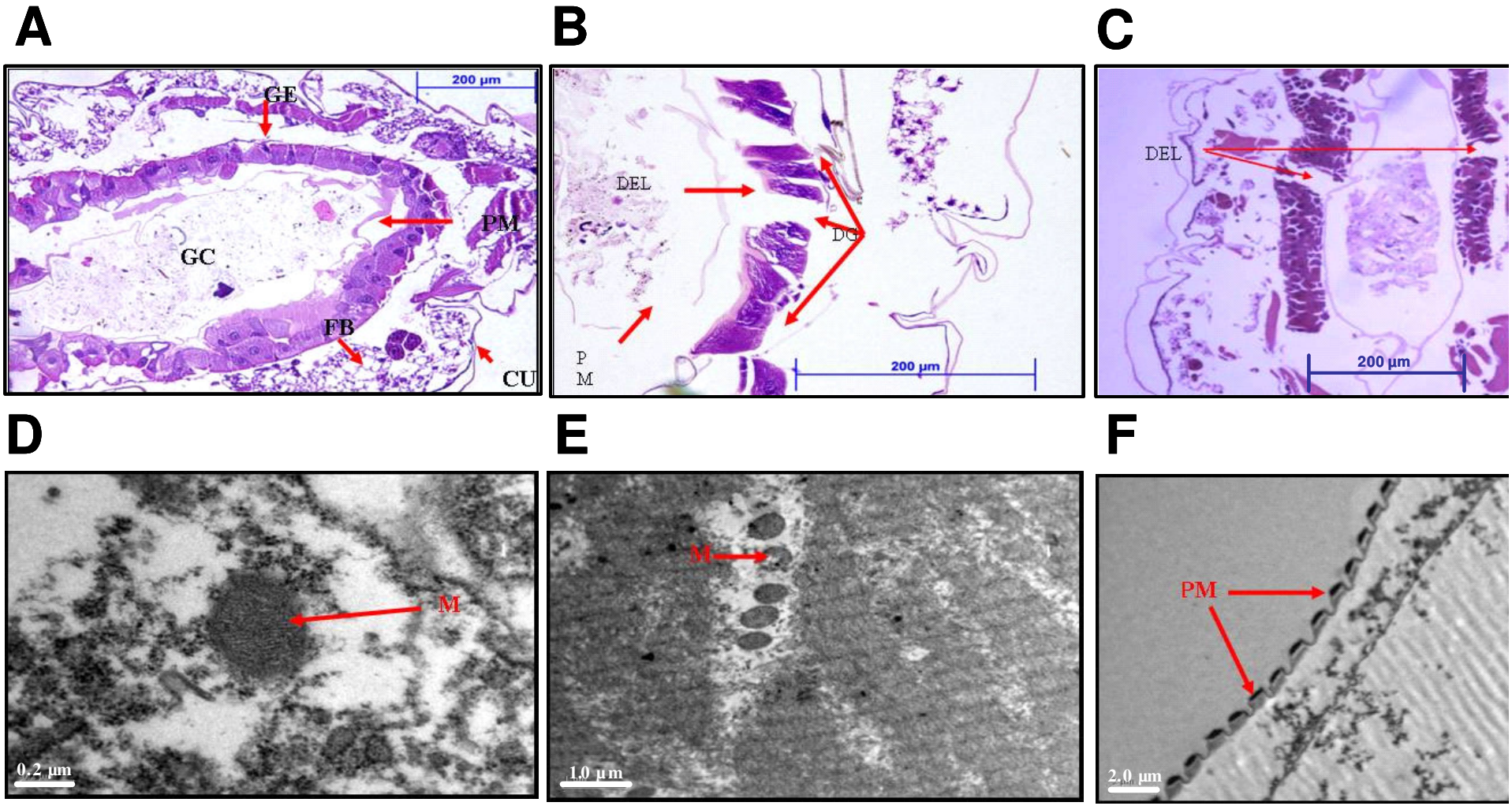

Figure 1. Histopathological effects of *B. thuringiensis* and *B. sphericus* treatment on the midgut epithelial tissue of *A. caspius* larvae after 24 h post-treatment. (A to C), Light microscopy of cross-section of normal gut epithelium from untreated control larvae showing normal gut epithelium (GE) and nutritional gut contents (GC) filling the gut lumen (GL); (B) and (C), longitudinal sections of *Bt*-treated (B) and *Bs*-treated larvae depicting damaged gut epithelium (DGL) and damaged gut (DG). Thin 10 µM sections were examined by light microscopy; (D to F) Transmission electron microscopic images depicting normal mitochondrial structures in untreated larvae as compared to *Bt*- (E) and *Bs*-treated (F) larvae with evident damage to the mitochondria (arrows). Ultrathin 4 µm sections were analyzed with transmission electron microscope model JEOL JEM-100CX II at 80 kV. M, mitochondria; PM, plasma membrane; FB, fat body.

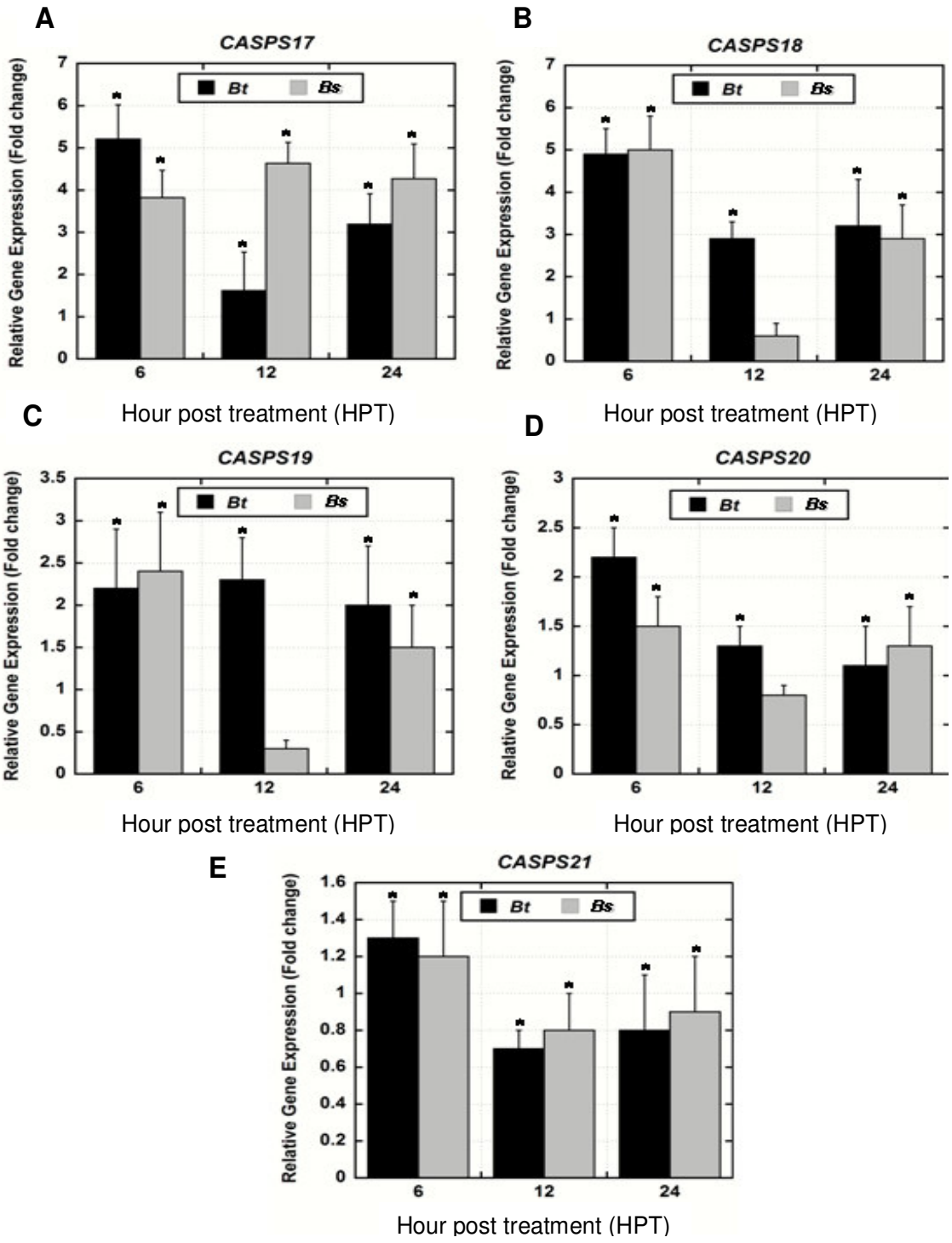

Figure 2. LightCycler® 480 real-time PCR analyses of apoptosis-related genes in mosquito larvae treated with *Bt* or *Bs* for 6, 12 and 24 h. RNA was isolated, reverse transcribed and used for real-time PCR analysis with primers listed in Table 1. Relative expression was determined with the $E^{-\Delta\Delta Cp}$ method using the PCR efficiencies determined with the standard curve included in each run. Each data point represents the results obtained from three independent batches of cDNA made from each stage or tissue. (A) *CASPS17* gene expression; (B) *CASPS18* gene expression; (C) *CASPS19* gene expression; (D) *CASPS20* gene expression; (E) *CASPS21* gene expression. Expression of target genes is normalized to the reference housekeeping gene *ACTIN6* and is presented as mean ± the standard error. Asterisks (*) indicate that, the treated samples are statistically different from the untreated control samples ($P < 0.05$) using a paired t-test.

Table 3. Physical conditions of breeding water of collected mosquito larvae from Qateef region Eastern Province (Qateef region in Saudi Arabia.

Place	Temperature (°C)	Salinity (ppm)	pH
Salasel	18.25	7040	8.5
Seehat	22.00	7200	7.6
Al-Qateef	22.00	6016	7.9
Al-Dammam	18.00	5568	8.00

transcription level of five selected apoptosis-related caspase genes (*CASPS17*, *CASPS18*, *CASPS19*, *CASPS20* and *CASPS21*) was investigated via real-time RT-PCR using the LightCycler® 480 (Roche Diagnostics) (Figure 2). Thus, primers specific for *A. aegypti* caspase genes, were designed and employed (Bryant et al., 2008; http://aaegypti.vectorbase.org/index.php) (Table 3). Additionally, specific primers for an actin cDNA (*ACTIN6*) were used as an internal control (Table 3). After gel purification, the identity of PCR products for each of the genes tested, was confirmed by DNA sequencing and pairwise alignment against the corresponding annotated gene database (http://aaegypti.vectorbase.org/index.php) using BioEdit Sequence Alignment Editor (Hall, 1999) (Data not shown).

The expression of *CASPS17* in *Bt* and *Bs* treated mosquito larvae and untreated control at 6, 12 and 24 hpt is illustrated in Figure 2A. *CASPS17* gene exhibited significantly ($P \leq 0.05$) up-regulated expression at all time points throughout the observation period (Figure 2A). The highest level of *CASPS17* relative gene expression in *Bt* treated larvae compared to untreated control was observed at 6 hpt (5.21 ± 0.81). After the peak at 6 hpt, *CASPS17* gene expression dropped to 1.62 ± 0.91 at 12 hpt, followed by another increase to 3.19 ± 0.72 at 24 hpt. Similarly, *CASPS17* exhibited a significantly ($P \leq 0.05$) increased expression profile in *Bs* treated mosquito larvae when compared with untreated control, initially increasing from 3.82 ± 0.65 to 4.63 ± 0.5 at 6 and 12 hpt, respectively. This difference between *Bs* treated and untreated control was reduced with fold change of 4.27 ± 0.82.

CASPS18 expression was significantly ($P \leq 0.05$) increased at all time points in *Bt* treated when compared with untreated mosquito larvae (Figure 2B). The fold changes were 4.9 ± 0.60, 2.9 ± 0.40, 3.2 ± 1.10 for 6, 12 and 24 hpt, respectively. By contrast, *CASPS18* expression was significantly up-regulated throughout the observation period in *Bs* treated mosquito larvae except for time point 12 hpt, where it exhibited insignificant fold change in treated when compared with untreated mosquito larvae. The fold changes were 5.0 ± 0.80, 0.6 ± 0.30, 2.9 ± 0.80 for 6, 12 and 24 hpt, respectively.

CASPS19 gene expression exhibited significant ($P \leq 0.05$) and quasi-steady up-regulation in *Bt* treated mosquito larvae throughout the time course experiment (Figure 2C). The fold changes were 2.2 ± 0.70, 2.3 ± 0.50, 2.0 ± 0.70 for 6, 12 and 24 hpt, respectively. Similarly, in *Bs* treated mosquito larvae, *CASPS19* expression level was significantly high at 6 and 12 hpt with fold changes of 2.4 ± 0.70 and 1.5 ± 0.50, respectively, decreasing to insignificant level at 12 hpt with fold change of 0.30 ± 0.10 when compared with untreated control (Figure 2C).

CASPS20 expression level significantly ($P \leq 0.05$) peaked at 6 hpt reaching a fold change value of 2.2 ± 0.30 in *Bt* treated when compared with untreated control (Figure 2D). Subsequently, the difference between *Bt* treated and untreated control was reduced, but remained significant ($P \leq 0.05$), to 1.30 ± 0.20 and 1.10 ± 0.40 by 12 and 24 hpt, respectively (Figure 2D). Significant fold changes for *CASPS20* were equally attained with *Bs* treated when compared with untreated mosquito larvae with values of 1.50 ± 0.30 and 1.30 ± 0.40 at 6 and 24 hpt, respectively. However, it dropped to insignificant levels when compared with untreated control at 12 hpt with fold change of 0.8±0.10 (Figure 2D).

Significant increases ($P \leq 0.05$) in *CASPS21* gene expression were observed for both *Bt* and *Bs* treated mosquito larvae throughout the observation period (Figure 2E). The highest levels of *CASPS21* gene expression in *Bt* and *Bs* treated mosquito larvae when compared with untreated control were similarly observed at 6 hpt with fold changes of 1.30 ± 0.20 and 1.20 ± 0.30, respectively. The difference between *Bt* treated mosquito larvae and untreated control was subsequently reduced to 0.70 ± 0.10 and 0.80 ± 0.30 by 12 and 24 hpt, respectively (Figure 2E). Similarly, *Bs* treated mosquito larvae and untreated control was subsequently reduced to 0.80 ± 0.20 and 0.90 ± 0.30 by 12 and 24 hpt, respectively.

DISCUSSION

The primary objective of the current study was to shed some light as to the molecular and biochemical mechanisms of mortality in *Bt*/*Bs* treated *A. caspius* mosquito larvae. Thus, histopathology results carried out via light and transmission electron microscopy (TEM) revealed that both *Bt* and *Bs* treatments brought about a massive destruction to the larval midgut epithelial tissues in *A. caspius* as compared to untreated control (Figures 1A - C). Moreover, this observed cellular damage was associated with a severe damage at the sub-cellular organelle level to the mitochondria when compared with untreated control (Figures 1d to f). These finding indicate that the observed cellular destruction might take place via the breach of the mosquito immune response by the bacterial cell and/or spores. In this context, it has previously been reported that injecting *A. caspius* with *Bt* or *Bs* resulted in a strong induction of humoral activity against *Bs* but not against *Bt*. Moreover, the induced activity was not effective against *Bt* (Ahmed et al., 2010). This observation

raises the question as to why the humoral activity raised by the innate immune response fails to be induced against *Bt* in particular. The innate immune system recognizes conserved molecular patterns that are present on the pathogen's surface, such as LPS, peptidoglycans and β-(1,3)-glucans (Medzhitov and Janeway, 2002). Upon recognition, the recognition receptors stimulate immune responses by activating humoral activities, proteolytic cascades in the haemolymph and intracellular signaling pathways in immune-responsive tissues (Kurata, 2004). Ingested spores are able to germinate in the gut (Du and Nickerson, 1996; Salamitou et al., 2000), although this environment is hostile to ingested cells or germinated spores. One explanation as to why *Bt* is capable of combating this environment, whereby preventing the elicitation of the humoral activity, is that *Bt* is capable of synthesizing a modified cell surface that helps protect it from AMPs synthesized by the host or competing bacteria (Abi Khattar et al., 2009). To be able to compete with other gut bacteria, *Bt* produces its own AMPs and bacteriocins (Gohar et al., 2008; Cherif et al., 2008) and it is likely that it also expresses a number of drug efflux transporters to combat antibiotics produced by competitors (Gohar et al., 2008). Having survived the gut environment, *Bt* must attack the host through virulence factors, such as specific protein endotoxins, formed as crystalline inclusions during sporulation.

These toxins target and cause the destruction of the epithelial cells lining the midgut and often associated with midgut paralysis and cessation of feeding (Bravo et al., 2007). This, in fact may explain the high toxicity of *Bt* against mosquitoes and being an effective biocontrol agent in the field of mosquito control (Schnepf et al., 1998; Glare and O'Callaghan, 2000). This is confirmed by the observed massive histological damage in the gut epithelium and mitochondrial structure of *A. caspius* mosquito larvae when compared with untreated control (Figures 1A- F) (Clark et al., 2005).

Whether the observed mosquitocidal activity of *Bt* and *Bs* is related to apoptosis or programmed cell death (PCD) is largely unknown. Thus, we have investigated the impact of *Bt* and *Bs* treatment on the expression of some selected apoptosis-related caspase genes in *A. caspius* mosquito larvae, via quantitative reverse-transcriptase PCR (qRT-PCR) (Figures 2A to E). The exp-erimental results presented here provide evidence that, there is a strong correlation between the treatment of mosquito larvae with *Bt* and *Bs,* and the induction of selected apoptosis-related caspase genes (Figures 2A to E). The results clearly indicated that, the treatment with larvicidal bacteria brought about statistically significant ($P \leq 0.05$) up-regulation in the relative gene expression levels of *CASPS17*, *CASPS18*, *CASPS19*, *CASPS20* and *CASPS21* (Figures 2A to E).

Apoptosis has been established as a component of the innate immune response in baculovirus infections of lepidopteron insects. In addition, cross-talk exists between innate immunity pathways and apoptosis pathways in insects (Clem, 2005). For example in mosquitoes, apoptosis occurs during *Plasmodium* and arbovirus infection in the midgut, suggesting that apoptosis plays a role in mosquito innate immunity (Clem, 2005; and references therein). Previously, it has been shown that, the stimulation of mosquito immunity results in the induction of apoptotic pathways in the developing ovarian follicles in *A. caspius* (Ahmed at al., 2010). The key components of the machinery that carries out this evolutionarily conserved process are caspases. These enzymes are initially synthesized as inactive zymogens, which are cleaved upon activation into large (p20) and small (p10) subunits, releasing an N-terminal prodomain of varying length. The p20 and p10 subunits heterodimerize to form the active site and two of these heterodimers associate further to form the active caspase holoenzyme. Upon receiving an apoptotic signal, initiator caspases are activated, which have the ability to auto-activate with the assistance of adaptor proteins (Bryant et al., 2010). In this context, the expression level of potential apoptosis regulators at various developmental stages (early and late larvae, pupae, adults, females, midgut and adult body minus the midgut) throughout the life cycle of *A. aegypti*, has previously been examined, via qRT-PCR (Bryant et al., 2008). Interestingly, caspases were expressed at the highest levels in the midgut of *A. aegypti* as compared to the other tissues or stages (Bryant et al., 2008).

In mosquitoes, there are reports that numerous viral infections cause cell death pathology resembling apoptosis in the midgut epithelial and salivary glands of mosquitoes such as *A. aegypti*, *A. albopictus*, *Culex pipiens quinquefasciatus* and *Culex pipiens pipiens* (Bryant et al. 2008). A valuable body of evidence, in support of our findings, clearly indicates that a number of apoptosis-related genes, as well as other immune response genes, are expressed in hemocytes of *A. aegypti* and *Armigeres subalbatus* infected with bacterial pathogens (Bartholomay et al., 2004). Moreover, it has been documented that parasites are capable of eliciting elicit pathology resemb-ling apoptosis in mosquito vectors. Thus, *Plasmodium* sp. infections have been shown to cause apoptosis and activation of caspases in the midgut cells of *A. aegypti*, *Anopheles stephensi* and *An. Gambiae* (Bryant et al., 2008).

Taken together, our results provide solid evidence that the apoptosis-related gene expression constitutes a key event in the signal transduction pathway associated with the treatment of *A. caspius* mosquito larvae with larvicidal bacterial namely, *Bt* and *Bs*.

Abbreviations

Bt, *Bacillus thuringiensis*; **Bs,** *Bacillus sphaericus*; **PCD,** programmed cell death.

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