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Mosquito-larvicidal activities of toxins from nematode bacterial symbionts and local Saudi Arabian *Bacillus thuringiensis* isolates against the Rift Valley fever vector, *Aedes caspius* (Diptera: Culicidae)

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Abstract.

Mosquito resistance to insecticides have recently enlarged the burden of the majority of mosquito-borne diseases. Tackling of these diseases should mainly rely on the bio-control measures. The current study was conducted to investigate the toxicity of locally isolated mosquitocidal agents *Bacillus thuringiensis* (Bt) isolates, and toxin complexes (TCs) from nematode bacterial symbionts against the 3rd larval instar of the mosquito vector, *Aedes caspius*. Out of sixty-eight *Bacillus thuringiensis* (Bt) isolates, four showed LC_{50} values ranging from 13.8-15.06 ppm, similar to that of the reference *Bacillus thuringiensis israelensis* (Bti-H14) (13.0 ppm). Seventeen isolates were more toxic than the Bti-H14 by 1.3 - 11.1 folds (LC_{50} values 1.17-9.9 ppm). Two isolates were less toxic than Bti-H14, showing LC_{50} values of 39.4 and 24.1 ppm, respectively. In addition, the TCs from the two nematode bacterial symbionts, *Ph. luminescens akhurstii* (HRM1) and *Ph. luminescens akhurstii* (HS1), showed promising larvicidal toxicity, with HS1 2.74 folds more toxic than HRM1. Finally, the larval morphological feature and body size were altered upon HS1-treatment. This study may suggest a new Bt/Bt-TCs complex that may contribute to tackling mosquito-borne diseases. The molecular characterization as well as synergistic effects of the Bt-TCs combination on mosquito larvae is currently under investigation.

Key words: Mosquitoes, larvicidal, *Bacillus thuringiensis*, nematodes, biocontrol.

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1. Introduction

Mosquitoes are the most deadly insect affecting humans and animals worldwide. Over one million deaths every year result from mosquito-borne diseases (Caraballo and King, 2014). The World Health Organization considers mosquitoes as one of the top threats to public health, especially in developing countries (WHO, 2017). Because of the absence of effective drugs for the majority of mosquito-borne diseases, the effective alleviation of this burden should rely mainly on mosquito control programs. The continuous outbreak of mosquitoes and incidence of epidemic diseases (Shanasree and Sumodan, 2019; Rajagopalan, 2019) is a challenge facing public health authorities; many local mosquito species spread all over Saudi Arabia (Al-Khuriji et al., 2007; Ahmed et al., 2011) and transmit many serious diseases (Hawking, 1973; Madani, 2005). *Aedes* transmits many fatal diseases, causing a large number of deaths in humans and animals worldwide including Saudi Arabia (Madani, 2005; Aziz et al., 2014). The second challenge facing the specialists is the resistance of mosquitoes to conventional pesticides (Al-Sarar, 2010; Brouqui et al., 2012). Besides, these chemical pesticides are health and eco-hazardous (Briassoulis, 2001), pollute the environment (Azmi et al., 2009), disrupt the natural-biological control systems, and undesirably affect the food webs and beneficial organisms.

Natural insecticides, for example, essential oils, have been used to repel mosquitoes (Vatandoost et al., 2012); however, they do not kill mosquitoes. On the other hand, entomopathogenic bio-agents have attracted the attention for use in the control measures against mosquito vectors as they are specific to certain species, eco-friendly and safe to non-target organisms, and mosquitoes can hardly develop resistance to them. A successful example is the *Bacillus thuringiensis*, the most effective bio-insecticide for decades (Sanahuja et al., 2011). These bacteria produce parasporal insecticidal proteinaceous

crystals (δ-endotoxins) which cause pores and cell-cytolysis in the midgut epithelium of its target host, resulting in feeding cessation and finally death within 24h (Bravo et al., 2007, Schünemann et al., 2014). Another successful bio-agent is the entomopathogenic nematodes, also provided promising solution as biocontrol agents for integrated pest management (Khan et al., 2018). The two genera *Steinernema* and *Heterorhabditis* penetrate the host's *via* its natural openings and release their symbiotic bacteria, *Xenorhabdus* or *Photorhabdus* respectively into the haemocoel. These bacteria symbionts produce toxins complexes (TCs) that kill their insect host within 24-48h (Ffrench et al., 2007).

The current study was conducted to investigate the mosquito larvicidal activities of locally isolated *B. thuringiensis* isolates from Saudi Arabia, and the two TCs, HRM1 and HS1, of the bacterial symbionts, *Photorhabdus*, isolated from Egypt, as new suggested eco-friendly bio-agents in the battle against the Rift Valley Fever vector, *Aedes caspius* mosquito.

2. Materials and Methods

2.1. Experimental mosquitoes

The experimental mosquitos, *Ae. caspius*, were reared in the insectary of Zoology Department, College of Science, King Saud University, according to Ahmed et al. (1999). Mosquitoes were kept in the insectary for more than 15 generations. Briefly, adults were reared at 28 ± 1°C and 14:10h (light:dark) photoperiod and were allowed access to 10% glucose solution *ad libitum* all time. Female mosquitoes were blood-fed on anesthetized CD mice for egg laying, and hatched larvae were fed on grounded fish food 'Dajana' Gold flakes food (Eu Czech Republic) until pupation. Larvae (3^{rd} instar) were used for bioassay

using *B. thuringiensis* isolates or the toxins of nematode's bacterial symbionts (HS1 & HRM1).

2.2 Collection of environmental samples

Local Bt isolates were recovered from environmental samples collected from 13 different regions throughout Saudi Arabia. Samples were collected from, irrigated public parks, gardens, farms, and the surrounding semi-desert areas. A total of 300 samples were collected from, irrigated public parks, gardens, farms, and the surrounding semi-desert areas for the purposes of this study.

2.3. Processing sample for Bt Isolation

Collected environmental samples were subjected to Bt recovering according to (El-Kersh *et al*., 2012). Briefly, one gram of soil samples was grinded and suspended in 2.0 ml of sterile distilled water. In case of dead insects or plant samples, one gram was softened in 2.0ml sterile saline (0.5%, w/v). In either case, 2 ml from the resulting aliquots was added to 2ml absolute ethanol, vortexed well for 1min, incubated for 45 min at 30°C, with frequent shaking. Ten-fold serial dilutions from the resulting solution were prepared in sterile distilled water. Samples from the appropriate dilutions were added onto a nutrient agar medium supplemented (SNA) with 0.2% yeast extract (Sisco research laboratories, Mumbai, India) and 0.0005% $MnCl_2$ and incubated for 2 - 3 days at 30°C. One single colony of the resulting Bt-like colonies were carefully picked up and re-cultured. This step was repeated several times for producing a pure Bt culture. The resulted Bt colonies were examined for the presence or absence of the parasporal crystals under phase contrast microscope for confirmation. Bt index was calculated for each positive sample according to Xavier et al., (2007). A total of 68 Bt isolates were successfully recovered and purified

by sub-culturing on SNA agar for 48hr and stored in a sterile liquid nutrient broth medium containing 50% glycerol at -20°C according to Xavier et al. (2007).

2.4. Bt phenotypic characterization

Bt Biochemical profiling

The presumptive native Bt isolates were tested by API E20 and API CH50 systems (BioMerieux, Marcyle Etoile, France) according to the manufacturer instructions. Bt isolates were categorized into different biochemical types according to hydrolysis of lecithin, urea or esculin, and acid production from salicin or sucrose. According to (Martin et al., 2010) Bt isolates were cultured on nutrient agar containing 10% egg yolk (Oxoid, Uk) for testing their Lecithinase activity, then incubated at 37°C overnight. Bti-H14 reference strain was used as positive control.

Lecithinase and hemolytic activities

The recovered 68 Bt isolates were phenotypically investigated based on their lecithinase and hemolytic activities. Blood agar media (nutrient agar containing 2% (v/v) sheep erythrocytes) was used to test the production of extracellular hemolysins by Bt isolates. Fresh cultures of Bt isolates [pre-cultured on nutrient agar overnight at 27°C (Ichikawa et al., 2008)] were pin-spot inoculated onto blood agar plates. Then zones of hemolysis surrounding Bt colonies were examined after 24h of incubation at 27°C (Ichikawa et al., 2008). Bti-H14 reference strain was used as positive control.

2.5. Bt morphological characteristics

Bt colonies showing a white, large, nearly circular with fine irregular margins and glossy (or not glossy) appearance were targeted. The Bt index of each positive isolate-sample was

calculated as colony number of Bt isolates divided by total colony-number of spore forming bacilli (El-Kersh et al., 2012). Pure Bt colonies were processed for characterization.

2.6. Bt crystal characteristics

Phase contrast microscopy

Crystals were morphology analyzed under the Phase Contrast Microscope (Gobatto et al., 2010). Briefly, Bt colonies were suspended in sterile distilled water prior to examination under phase contrast microscope (at 100×) for the presence or absence of the parasporal crystals. Based on the crystal shape, Bt colonies were classified into various groups according to Lopez-Pazos et al. (2009). Confirmed Bt-like isolates were sub-cultured in sterile liquid Nutrient Broth containing 50% glycerol and stored at −20°C (Hernandez et al., 2005) until used.

Electron microscopy

Spore or crystal sizes were morphometrically measured using Scanning Electron Microscope (SEM) (El-Kersh et al., 2012). Briefly, the spore-crystal mixtures resulting from the Bt isolates, grown in nutrient agar medium for five days at 30°C, were suspended in 1 ml of ice-cold 1 M NaCl and centrifuged for five min at 13,000 ×g. Then, the mixture was washed trice with sterile distilled water. The resulting pellets were re-suspended in distilled water then, air-dried on cover glasses. Dried samples were then processed for examination and photographing using a FEI-Inspect S50, scanning electron microscope operating at a voltage of 1500 kV at 24,000× magnifications.

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2.7. Bt toxin mixture preparation

An average count of 10^9 cfu/ml of the spores-crystals mixture of the Bt isolates was prepared from SNA plates (72h) by scraping into 10 ml of sterile water (El-Kersh et al., 2012). Dried powder of the spore-crystal mixtures of each larvicidal Bt isolates were prepared in adequate amount by the lactose acetone co-precipitation (Dulmage et al., 1971). Briefly, a pure colony of each larvicidal *Bt* isolates, in parallel with that of the reference Bt-H14 strain, freshly cultured on SNA plate, was inoculated onto sterile tubes containing 5ml broth of Nutrient Yeast Extract Salt Medium, NYSM, (contains per liter: glucose, 5g; peptone 5 g; NaCl, 5 g; beef extract, 3g; yeast extract, 0.5 g; magnesium chloride, 0.020 g; manganese chloride, 0.001g; and calcium chloride, 0.010 g, pH 7.2) and incubated at 37°C overnight. A sample from the resulting growth aliquot was re-cultured on NYSM for 4-6h (~1 McFarland) at 37°C, then; 1ml of the resulting culture was inoculated in sterile 50ml broth of NYSM and incubated on a rotary shaker (200 rpm) at 30°C for 4-6 days. The resulting spore/crystals mixture was recovered by adjusting the pH to 7.0 using 1N HCl, and centrifuged at 8000 rpm for 20 min. The resulting pellet was re-suspended in 6% sterile lactose solution at 1/10 volume of the initial broth and stirred for 30 min. Four volumes of cold acetone were slowly added to the resulting culture followed by stirring for another 30 min, then filtered through Whatsman No.1 filter paper. The resulting mixtures were further re-suspended and filtered for one more time. Finally, the residue on the filter paper was allowed to dry overnight in vacuum desiccators at 25ºC. The obtained white fine powder was collected aseptically and stored at 4°C until used.

2.8. Toxins-complexes extraction from nematode bacterial-symbionts

Two different types of entomopathogenic *Photorhabdus* bacterial symbionts, HRM1 and HS1, of the entomopathogenic nematodes, *Heterorhabditis indica*, isolated from Egypt (El-

Sadawy et al., 2016) were isolated according to Woodring and Kaya (1988). Briefly, 20 infective juveniles were grinded in 5 ml of TSBYE [3% tryptic Soy broth (Difco), 0.5% yeast extract (Difco)]. The resulting mixtures were incubated for 24h at 30°C prior to streaking on NBTA plates [2.3% nutrient agar (Difco), 0.0025% bromothymol blue (Merck), 0.004% 2,3,5-triphenyltetrazolium (Merck)]. The resulting bacteria were kept on NBTA plates at 10°C and subcultured weekly (Sheets et al., 2011). The resulting HRM1 and HS1 *Photorhabdus* bacteria were then suspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM DTT, 10% glycerol, lysozyme (0.6 mg/ml) and bacterial protease inhibitor cocktail (Sigma, St. Louis). To disrupt the bacterial cells, small amounts of glass beads (0.5 mm dia.) were added prior to sonication and centrifuged at 10,000g for 60 min at 4°C. Supernatants (containing Toxins–complexes) were collected, and stored in liquid nitrogen until used for mosquito larvicidal bioassays.

2.9. Larvicidal bioassay

The larvicidal activity of the 23 local *Bt* isolates were tested against the 3rd instar larvae of the Rift Valley fever vector, *Ae. caspius* mosquito in parallel with that of the reference strain *B. thuringiensis israelensis* (Bti-H14) (El-Kersh et al., 2012). Further, the toxic effects of the toxin-complexes (HRM1 and HS1) extracted from the two nematodal *Photorhabdus* bacterial symbionts were also investigated in this study.

Preliminary bioassay

Crystal-spore mixtures of the 68 local Bt isolates were used in parallel with that of the reference strain *B. t. israelensis* (Bti-H14) in preliminary screenings for mosquitocidal activity. Briefly, for each individual isolate, 10 larvae (3rd instar) of *Ae. caspius* were transferred into 10 ml of distilled water in 30 ml plastic cups at 28 ± 1°C. Larval mortality

of Bt isolate or the reference Bt-H14 was scored 24 and 48h post-treatment (Ahmed et al., 2017). Only 23 out of the tested 68 local Bt isolates showed significant larvicidal activity, and hence, were selected for the main bioassay of the current study.

Bioassay of Bt spore-crystals mixture

Five concentrations from each Bt-active toxin powder were used according to (WHO, 2007) with some modifications. Serial concentrations were prepared by suspending a certain weight from each Bt-powder in sterile distilled water. Ten 3rd instars larvae were placed/well (in sterilized standard 12-wells tissue culture test plates) (Nunclone Delta Surface, Thermo Fischer Scientific, Denmark) containing 2ml of de-ionized water. Ten µl of each concentration, or the reference Bti-H14 strain (as positive control) or distilled water (as negative control), were added to each well. Meanwhile, estimating the CFU/µg was carried out using SNA medium-cultured samples from the same serial dilutions of each Bt toxin powder used for bioassay. Bioassays were run for 48h and mortality was observed 24 and 48h post-treatment. Larvae were considered dead unless they respond to gentle prodding with a glass pipette (Brown et al., 1998). Larval mortality % was calculated and corrected; five replicates were used for each concentration (Abbott, 1925).

Bioassay of nematode bacterial TCs

The toxin-complexes (HRM1 and HS1) extracted from the two *Photorhabdus* bacterial types were assayed against 3rd larval instar of *Ae. caspius*. Wide ranges of serial two-fold concentrations were prepared from each type of bacterial toxin-complexes. These toxin-complexes were diluted in deionized water prior to the preliminary bioassays. Based on the results, four ascending concentrations from HRM1 or HS1 toxins were used for carrying

out the main bioassays according to Ahmed et al. (2017). Percentage larval mortality was calculated at 24 and 48h post-treatment.

2.10. Scanning electron microscopy (SEM)

Scanning electron microscopy was utilized to investigate the external morphological alterations on the overall body size of the 3rd instar larvae of control or HS1-treated group. This experiment was conducted upon treating larvae with the HS1 toxin as it showed significant higher toxicity compared to HMR1. Control or treated larvae (alive, but sluggish) were routinely prepared for SEM investigation 48h post-treatment. Briefly, the control or HS1-trated larvae were fixed in 2.5% glutaraldehyde in 0.1 M PBS, followed by 24h fixation. Larvae were then rinsed with 0.1 M PBS trice at 10 min intervals, followed by fixation in 2% osmium tetroxide. Larvae were then washed thoroughly in 0.1 M PBS, dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 95%) for 10 min, then washed in 100% ethanol. Larvae were then treated with 50% ethanol: 50% acetone, 100% acetone, and 50% hexamethyldisilazane (HMDS), each for 15 min. Larvae were then rinsed in 100% HMDS for 15 min, then air-dried for 12hs. Larvae were then coated with gold using a sputter machine (SPI Module Sputter Coater, USA) and processed for observation under the SEM (JSM-6380 LA, Japan). Sizes of larval body parts (in terms of head and thorax widths) were measured under SEM in five replicates for control or treated larvae (n = 5). Resulting images from control or treated larval preparations were processed *via* Adobe Illustrator CC, 2020 Software.

2.11. Statistical analysis

Data of mortality % and body measurements were processed for basic statistical analysis for calculating Means and Standard Errors (SE) of means using MINITAB software

(MINITAB, State College, PA, v: 18.0, 2018). In each experiment, 5 replicates, from 5 different mosquito groups (n = 5), were carried out. Mean mortality values were used for calculating the LC_{50}, LC_{95} and slopes of each treatment (Finney, 1971). Treatments were considered not significantly different in their toxic effect if their confidential limits (95%) of LC_{50} overlapped (Litchfield and Wilcoxin, 1949). Based on Anderson–Darling Normality Test (Morrison, 2002) normality test, data of the body measurements were normally distributed. Thus, a student's t-test was used for comparing size differences between HS1-treated and control larvae in each case.

3. Results

3.1. Geographical distribution of Bt isolates

Sixty eight Bt-like colonies were successfully recovered out of 300 environmental samples (Table 1 and Fig. 1). More than 75% of the recovered isolates were negative for Bt isolates. This may suggest a limited abundance of the organism in several Saudi environmental regions. Only 23 Bt isolates (out of 68 recovered) showed mosquito larvicidal activity. Most of +Ve Bt isolates were recovered from Al-Madina, Yamboa Makkah, Asser and Jezan, while few number were recovered from Abha, Al-Ehsa, Khamis Musheet and Najran. The rest of other regions showed -Ve larvicidal Bt isolates (Table 1).

3.2. Morphological characterization of Bt isolates

Based on the wet-mount Phase contrast microscopy-examination, Bt-like isolates differed in their colony morphology and parasporal crystal shapes within sporangia (Fig. 2A & B and Table 2). The crystals of the obtained 68 native Bt-like isolates were categorized into four types: (i) spherical; (ii) bi-pyramidal; (iii) irregular and (iv) spherical or bipyramidal attached to spores (Table 2). Most of isolates showed the largest number of Irregular

crystals (34%), while the lowest number showed Bi-pyramidal spores (13%) (Table 2). Whereas the Spherical, and triangular, or cubic spores showed 32 and 21% of the Bt-like isolates, respectively (Table 2). Morphologically, most of Bt-like sporangia showed cylindrical shapes and sub-terminal spores, while fewer isolates showed para-central spores (Fig. 2A & B). Bt-like colonies were characterized by slightly raised center with fried egg appearance, white, large, nearly circular, with fine irregular margins and glossy similar to *Bti*-H14 (Fig. 2C & D). Electron microscopy confirmed the morphological characteristics of both crystals and cylindrical spores of the Bt-like isolates (Fig. 2E & F).

3.4. Biochemical characterization of Bt isolates

Extracellular hemolysis

All the 23 mosquito larvicidal Bt isolates showed hemolysis when tested for extracellular hemolysis using Nutrient blood agar (Fig. 3C). The hemolytic activity indicated broad blood hemolysis zones with 94% of the Bt-63 isolates (Fig. 3C). As expected, only 6% of Bt isolates were none-hemolytic.

Lecithisinase production

All the 23 mosquito larvicidal Bt isolates were processed to Lecithinase activity on nutrient agar containing 10% egg yolk (Oxoid, Uk) at 37°C incubation overnight (Fig. 3D). Most of the 68 Bt isolates were positive for lecithinase production. However, all the 23 potentially larvicidal isolates (100%) showed active lecithinase and esculin hydrolysis, but not for salicin.

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Overall-biochemical typing

To reflect the type of bio-insecticidal activity, the biochemical profile of Bt-isolates was carried out. Each of the local 68 Bt-like isolates was tested for identity confirmation and biochemical typing by API E20 API CH50 systems (Van der Auwera et al., 2013). Previous studies (Elkersh et al, 2012) showed that the isolates with positive urease activity were strongly linked with lepidopteran-toxic bipyramidal crystals activity. Whereas, those producing amorphous and/or irregular crystal were linked with mosquito toxicity, and were usually linked with either general low bioactivity, but positive for acid production from starch and lecithinase and/or esculine hydrolysis. The overall biochemical typing showed that all examined larvicidal Bt isolates were motile, hemolytic, and exhibited both lecithinase activity and esculin hydrolysis (100%), but all were (100%) negative for salicin.

3.5. Mosquito larvicidal activities

Bacillus thuringiensis

Twenty-three native Bt isolates showed promising larvicidal activities and subjected to quantitative bioassays for determining LC_{50} and LC_{95} in parallel with the reference Bti-H14 strain (Table 3). Taking into consideration the spore CFU/µg powder (spores have no larvicidal action, but reflect crystals concentration), four native Bt isolates (Bt-26, 57, 58 and 59) showed almost LC_{50} and LC_{95} values similar to those of the reference Bti-H14 strain (LC_{50} values ranged from 13.8 – 15 ppm *Vs* 13.0 of Bti-H14) . Whereas Seventeen isolates were more toxic than the reference Bti-H14 by 1.3 - 11.1 folds (LC_{50} values 1.17-9.9 ppm). The most toxic Bt isolate was Bt-60, it was more toxic by 11.1 folds than Bi-H14 (1.12 *vs* 13.0 ppm). Meanwhile, Bt-56 and 67 showed 33 and 54% weaker toxicity comparing to Bti-H14 (39.4 and 24.1 ppm respectively).

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Nematode bacterial-symbiont (TCs)

The LC_{50} of HS1 toxin complexes was significantly 2.74 folds higher than that of HRM1 24h post-treatment (LC_{50}: 162.3 *vs* 445.5 ppm, respectively) (Table 4). Moreover, the toxicity of HS1 showed significant increase after 48h comparing to 24h post-treatment (LC_{50}: 145.2 *vs* 162.3 ppm respectively), while that of HRM1 was almost similar. On the other hand, based on the LC_{95} 24 post-treatment, HS1 toxicity was 1.37 folds higher than that of HRM1 (145.2 *vs* 199.8 ppm, respectively) (Table 4). These results indicate that the HS1 toxin–complexes need further purification to isolate its active component.

3.6. Larval body morphological alteration

Data showed an overall shrinkage throughout the body of HS1-treated larva compared to the control one (Fig. 4A & B). The thoraces of HS1-treated larvae showed significant mean of 46.4% reduction in size compared with the control ones (540.03 ± 3.53 *vs* 1007.2 ± 1.39 µm, respectively) ($P < 0.05$, $n = 5$, student t-test) (Table 5 and Fig. 4C & D). Moreover, the normal mean thorax size was significant 1.3 folds larger than that of the head in control larvae (1007.2 ± 1.39 *vs* 779 ± 4.3 µm, respectively), while in HS1-treated larvae there was a significant 28.0% reduction (540.03 ± 3.54 *vs* 759.01 ± 5.1µm, respectively. Finally, the size of the head capsule of HS1-treated larvae showed a significant 2.6% reduction compared to that of the control larvae (759.01 ± 5.1 *vs* 779 ± 4.3 µm, respectively). These data clearly indicate a significant shrinkage in the body size of HS1-treated larvae.

4. Discussion

The current study was conducted to investigate the toxic effects of *B. thuringiensis* strains from Saudi Arabia and the toxin complexes, HS1 and HRM1, of the nematode bacterial symbionts, isolated from Egypt (El-Sadawt et al., 2016), against the 3rd larval instars of the

Rift Valley Fever vector, *Ae. caspius* (Al-Afaleq and Hussein, 2012). Initially, it is important to clarify five key points; a) previous studies were conducted as initial steps towards implementing eco-friendly biocontrol agents against the malaria vector, *An. gambiae* (El-Kersh et al., 2016) and the filaria vector, *Cx. pipiens* (Ahmed et al., 2017). b) the current study is a further step towards testing these eco-friendly biocontrol against the Dengue fever vector, *Ae. aegypti* in Saudi Arabia (Aziz et al., 2014). However, we targeted *Ae. caspius*, of the same genus, as an alternative to the Dengue fever vector (Lambrechts et al., 2010) due to the vigorous quarantine banding transporting this vector from the endemic western regions to anywhere of Saudi Arabia. c) the awareness of bad impact of chemical pesticides on human, beneficial organisms and the environment (Azmi et al., 2009) and insect pest-resistance problems (Al-Sarar, 2010; Rosilawati et al., 2019) has led many countries to impose vigorous control on the use of chemical insecticides and to encourage the use of healthy and eco-friendly bio-control agents (Boyce et al., 2013; Rajagopalan, 2018; Naz et al., 2019). In this regard, to the best of our knowledge, these bio-control measures have not been implemented yet in the Middle East and developing countries including Saudi Arabia. Thus, we consider our previous and current studies as real ground-steps towards implementing locally isolated eco-friendly biocontrol agents in the control measures against disease vectors. d). Some previous attempts have successfully recovered local Bt isolates from Saudi Arabia (Assaeedi et al., 2011; Abulreesh et al., 2012), but these isolates targeted lepidopteran pests. Hence, locally isolated *B. thuringiensis* isolates as well as two toxins, HS1 and HRM1, of the nematode bacterial symbiont, *Photorhabdus* isolated from their mutual nematode, *Heterorhabdus* (El-Sadawy, 2016) were tested in the current study as they have previously shown promising mosquitocidal activities against other mosquito species in our labs (El-Kersh et al., 2014; El-Kersh et al., 2016; Ahmed et al., 2017; El-Sadawy et al., 2018). Finally, e) we believe

that these local *Bt* isolates and those Egyptian nematodes bacterial symbionts will be potentially effective when used against the local mosquito vector.

In fact, *B. turingiensis* (Bt) is one of few successful alternatives to chemical pesticides for vector control over the last six decades (Sanahuja et al., 2011) and constitutes 95% of all commercial bio-pesticides nowadays. However, many cases of mosquito resistant to *B. t. israellensis* have been recorded (for example: Wirth et al., 2001; Paul et al., 2005). In the current study, the 23 local Bt isolates showed clearly potential mosquito larvicidal toxicity. Out of them, 17 isolates showed significant higher toxicity than that of the reference *B. t. israellensis* (Bti-H14), and the Bt isolate coded (Bt-60) was 11.1 folds more toxic than the reference Bi-H14. This in fact, may encourage implementing these locally Bt isolates which possess two advantages: a) ensuring effectiveness as they were isolated from the same environment, and b) overcoming the possible mosquito resistance to the commercially available *B. t. israelensis* (Wirth et al., 2001; Paul et al., 2005).

Entomopathogenic nematodes constitute another promising contribution as safe and eco-friendly bio-agent added to the pest control measures (Ahmed et al., 2017, Khan et al., 2018). Upon infection, the infective juveniles of the two well-known entomopathogenic genera, *Steinernema* or *Heterorhabditis*, release their symbiotic bacteria, *Xenorhabdus* or *Photorhabdus*, respectively, into the haemocoel of their insect host. Consequently, the propagation of these bacteria produces virulent toxins that ultimately kill the host within 48h post-infection (Khan et al., 2018). In this context, the current study showed larvicidal activities of the two TCs, HS1 and HRM1 against the 3rd instar larvae of *Ae. caspius* mosquito, and the toxicity of HS1 was 2.74 folds higher than that of HRM1 at 24h post-treatment. These findings are similar to that of a previous study in our lap conducted against

the filaria vector, *Cx. pipiens* (Ahmed et al., 2017), which may indicate that HS1 and HRM1 have promising toxicity against both *Ae. caspius* and *Cx. pipiens*, and that they could be effective candidates in mosquito control measures against these local Rift Valley Fever and filarial vectors respectively.

Moreover, HS1 showed alteration of the internal ultrastructure of midgut of treated *Cx. pipiens* larvae at the sub-cellular level (Ahmed et al., 2017). In the current study, this toxin showed general body shrinkage in HS1-treated larvae of *Ae. caspius*. This, in fact, may indicate that the killing mechanism of the HS1 toxins of the nematode bacterial symbionts may include internal and external alteration accompanied by their toxic effect. Moreover, we managed to synthesize silver nanoparticle (bio-AgNPs) from these nematode TCs that significantly enhanced the larvicidal toxicity (El-Sadawy et al., 2018). Further, the synergistic mixture between Bt toxins and each of the TCs, HS1 and HRM1, has significantly enhanced the overall toxicity against *Cx. pipiens* larvae (unpublished data).

In conclusion, in parallel to the efforts aiming at limiting mosquito vector competence for arboviruses (Kean et al., 2015), the findings of our previous studies and the current study may contribute to the efforts and plans of Saudi Authorities for controlling the local mosquito-borne diseases. The larvicidal activity of the locally isolated *Bt* isolates and the TCs of the nematode bacterial-symbiont, or their synergistic combinations could propose them as promising eco-friend bio-agents in the biocontrol measures against mosquito vector in Saudi Arabia, and may be worldwide in where these mosquito vectors are prevalent. We believe that these local bio-products may contribute to overcoming or suppressing the emergence of mosquito resistance to the commercial insecticides (Al-Sarar, 2010) and the commercially available Bti (Wirth et al., 2001; Paul et al., 2005).

Finally, the molecular characterization of both local Bt δ-endotoxins and nematode bacterial TCs, are currently being investigated in our lab. We therefor believe that this may lead to the production of mosquito larvicidal Bt -TCs- mixture and their bio-AgNPs product that could potentially contribute to tackling mosquito vectors. Hence, it is important to continue searching for new Bt strains and evaluating the larvicidal activity of more toxins extracted from nematode bacterial symbionts, as new potential candidates to be utilized in the bio-control measures against mosquito vectors.

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Table 1**.** Geographical distribution of sampling locations of the potentially larvicidal *B. thuringiensis* isolates, number of total Bt-isolates and number of mosquito larvicidal isolates.

City of collection	**Geographic location (GPS)**	**Total no. of +ve *Bt* isolates**	**No. of Larvicidal *Bt* isolates**
Al-Madinah	24° 28′ 0″ N, 39° 36′ 0″ E	18	12
Yanboa	24° 5′ 0″ N, 38° 0′ 0″ E	5	2
Makkah	21° 30′ 0″ N, 41° 0′ 0″ E	7	2
Taif	21° 16′ 0″ N, 40° 25′ 0″ E	5	0
Qassim	25° 49′ 19.72″ N, 42° 50′ 6.85″ E	4	1
Al-Ehssa	25° 25′ 46″ N, 49° 37′ 19″ E	2	0
Hafr-Elbaten	28° 26′ 3″ N, 45° 57′ 49″ E	4	1
Riyadh region	24° 38′ 0″ N, 46° 43′ 0″ E	9	1
Asseer	19° 0′ 0″ N, 43° 0′ 0″ E	4	2
Khamis Musheet	18° 18′ 0″ N, 42° 44′ 0″ E	1	0
Abhaa	18° 13′ 24″ N, 42° 30′ 26″ E	3	0
Jezan	16° 53′ 21″ N, 42° 33′ 40″ E	5	2
Najran	17° 29′ 30″ N, 44° 7′ 56″ E	1	0
Total		68	23

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Table 2**.** Types of crystals in the 68 local *B. thuringiensis*-like isolates recovered from a total of 300 environmental samples.

Type of crystals	Total number	%
Irregular: (cubic, merged triangular or conical)	23	34
spherical	22	32
Triangular, cubic	14	21
Bi-pyramidal	09	13
Total	68	100

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Table 3. Larvicidal toxicities of 23 potential larvicidal local *B. thuringiensis* isolates against the 3rd instar larvae of *Ae. caspius* mosquito.

Bt-code	**GenBank* Accession No.**	**City of collection**	**Hrs P-T**	**LC_{50} (ppm) (lower to upper)**	**LC_{95} (ppm) (lower to upper)**	**X 10^5 CFU/µg**	**Slope ± SE**
***Bti*-H14 (Reference)**	KJ722438 (Reference)	Reference	24	13.0(11.9-14.2)[a]	28.74(24.4-33.8)	4.5±0.3	4.78± 0.2
			48	4.1(3.5-4.7)	15.1(11.9-19.2)		2.9±0.064
Bt-05	MT279575	Kharj	24	1.3(1.2-1.50)[f]	4.7(3.8-6.05)	1.0±0.1	3.02±0.06
			48	0.5(0.43-0.6)	1.9(1.5-20.50)		3.06±0.16
Bt-07	MT279581	Asseer	24	9.9(9.2-10.7)[b]	19.4(16.77-22.4)	1.0±0.2	5.64±0.26
			48	5.71(5.1-6.4)	15.2(12.5-18.7)		3.85±0.2
Bt-10	MT279582	Qassim	24	3.6(3.03-4.4)[d]	30.0(20.88-44.6)	1.2±0.3	1.8±0.034
			48	1.4(1.07-1.7)	8.8(6.37-12.91)		2.05±0.05
Bt-11	MT292101	Yanboa	24	3.98(3.4-4.6)[d]	20.3(15.6-26.8)	0.65±0.3	2.32±0.03
			48	2.01(1.6-2.4)	11.9(8.6-17.4)		2.12±0.05
Bt-12	MT279583	Yanboa	24	4.8(4.13-5.5)[d]	14.05(11.32-17.5)	0.53±0.4	3.52±0.20

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			48	1.4(1.15-1.7)	6.58(5.04-9.03)		2.52±0.47
Bt-16	MT279593	Asseer	24	3.9(3.46-4.3)[d]	14.5(11.3-18.7)	0.92±0.3	2.9±0.06
			48	1.44(1.3-1.6)	3.41(2.86-4.16)		4.4±0.32
Bt-17	MT279594	Hafr-Elbaten	24	1.32(1.2-1.5)[f]	5.8(4.5-8.01)	8.1±0.4	2.5±0.05
			48	0.6(0.45-0.7)	2.98(2.35-4.23)		2.3±0.082
Bt-26	ND	Madinah	24	14.8(13.4-16.5)[a]	45.7(36.7-57.2)	4.1±0.7	3.36±0.07
			48	9.1(8.1-10.1)[b]	30.13(24.3-37.4)		3.15±0.07
Bt-27	MT292102	Madinah	24	4.6(3.96-5.2)[d]	21.2(16.4-27.7)	5.9±0.4	2.47±0.04
			48	1.2(0.95-1.5)	7.5(5.5-10.80)		2.11±0.06
Bt-28	ND	Makkah	24	5.5(4.70-6.4)[d]	23.3(17.5 31.4)	6.2±0.3	2.62±0.06
			48	2.2(1.6-20.8)	15.9(11.1-23.9)		1.9±0.054
Bt-29	MT292103	Madinah	24	7.2(6.05-8.5)[c]	47.3(34.3-66.1)	0.52±0.1	2.01±0.03
			48	2.31(1.8-2.8)	9.58(7.32-12.85)		2.66±0.09
Bt-42	MT292104	Jezan	24	2.4(2.1-2.6)[e]	8.6(6.83-11.23)	0.93±0.3	2.95±0.06
			48	1.4(1.2-1.6)	6.06(4.7-8.10)		±2.57±0.0

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Bt-44	MT279598	Jezan	24	2.2(1.9-2.5)[e]	10.58(7.77-15.2)	0.63±0.4	2.44±0.05
			48	1.44(0.98-1.3)	5.04(3.97-6.84)		2.55±0.06
Bt-53	MT279599	Madinah	24	2.23(1.9-2.6)[e]	11.78(8.0-19.5)	1.3±0.4	2.3±0.056
			48	1.06(0.94-1.2)	3.93(3.2-5.20)		2.9±0.062
Bt-55	MT292105	Madinah	24	2.27(2.0-2.5)[e]	9.8(7.4-13.20)	1.95±0.3	2.6±0.05
			48	1.43(1.2-1.6)	5.26(4.2-6.70)		2.9±0.072
Bt-56	ND	Madinah	24	39.4(35.5-43.7)[g]	122.7(99.1-152.4)	0.15±0.3	3.33±0.07
			48	25.25(23.2-28.1)	68.6(57.3-82.20)		3.8±0.1
Bt-57	MT292096	Madinah	24	15.0(13.2-17.1)[a]	65.2(47.7-89.5)	1.27±0.3	2.6±0.056
			48	8.76(7.7-9.8)	32.3(25.6-40.9)		2.9±0.065
Bt-58	ND	Madinah	24	13.8(12.2-15.7)[a]	63.6(47.15-86.5)	1.24±0.2	2.5±0.052
			48	7.89(7.06-8.8)	23.5(19.3-28.7)		3.5±0.104
Bt-59	MT292100	Madinah	24	13.8(12.0-15.8)[a]	68.4(48.6-97.01)	1.28±0.2	2.36±0.05
			48	7.24(6.5-8.06)	21.5(17.7-26.3)		3.47±0.09
Bt-60	MT292098	Madinah	24	1.17(1.03-1.3)[f]	4.81(3.81-6.56)	0.13±0.1	2.7±0.057

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			48	0.41(0.28-0.5)	2.76(2.09-4.27)		2.0±0.071
Bt-63	MT279600	Makkah	24	1.28(1.12-1.4)[f]	5.93(4.50-8.40)	0.7±0.2	2.4±0.053
			48	0.7(0.6-0.80)	2.63(2.14-3.50)		2.8±0.08
Bt-67	MT292097	Madinah	24	24.1(21.4-27.1)[g]	87.0(69.4-109.4)	0.6±0.1	2.95±0.06
			48	11.76(9.6-14.3)	43.5(33.4-56.9)		2.89±0.17
Bt-68	MT292099	Madinah	24	1.7(1.5-1.90)[f]	6.7(5.20-9.20)	1.05±0.3	2.7±0.060
			48	1.07(0.95-1.2)	3.7(3.0-4.70)		3.06±0.06

Lethal concentrations (LC_{50} and LC_{95}) were calculated and analysed by Probit analysis for all isolates compared to the reference Bti-H14 according to Finney (1971). Values followed by the different letters are significantly different according to Litchfield and Wilcoxin (1949). LC_{50}: Concentration to kills 50% of test larvae; LC_{95}: Concentration to kills 95% of test larvae; CFU: Colony forming units (No. of bacterial cell) of tested Bt bacteria; Hrs P-T: Hours post-treatment; SE: Standard Error of means (n = 5). Values with different letters (a, b, c, d, e, f & g) are significantly different within strains and compared to the reference bacteria *Bti*-H14 (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). No mortality was recorded in control mosquitoes throughout the experiment. ND: means was not analyzed for the 16S RNA, but proved identity morphological, microscopic, biochemically, and Cry & cyt gene contents (El-Kersh *et al*., 2016).

* El-Kersh et al. (2016), and NCBI GenBank (2020), Website: https://www.ncbi.nlm.nih.gov/nuccore/MT279600/ (10-Apr-2020).

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Table 4. Toxicity of TCs of HS1 and HRM1 against the 3rd larval stage of *Ae. caspius*. Data in the table show means and SE of mortality amongst treated larvae at 24 and 48h post-treatment.

Toxins (TCs)	Hours post-treatment	LC_{50} (ppm) (lower to upper)	LC_{95} (ppm) (lower to upper)	Slope ± SE
HRM1	24	445.5 (248.2-869.8)[a]	199.8 (171.2-238.2)[b]	2.3 ± 0.03
	48	328.0 (251.3-496.5)[a]	150.4 (78.6-188.7)[bcde]	2.25 ± 1.22
HS1	24	162.3(157.40-167.3)[e]	145.2(141.6-148.9)[c]	25.90 ± 5.69
	48	140.2(138.04-142.4)[c]	129.6(127.7-131.4)[d]	33.24 ± 11.03

Lethal concentrations (LC_{50} and LC_{95}) were calculated by Probit analysis according to Finney (1971). Values followed by the different letters are significantly different according to Litchfield and Wilcoxin (1949).

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Table 5. Body size alteration of 3rd larval instar of *Ae. caspius* upon treatment with HS1 toxin. Data in the table show means and SE of head and thorax as an indication of body size at 24h post-treatment.

	Experimental larvae group			
	Control		HS1-Treated	
Body part	Head	Thorax	Head	Thorax
Mean size (µm) ± SE	779.00 ± 4.3	1007.2 ± 1.39	759.01 ± 5.1*	540.03 ± 3.54*
Replicates (n)	5			
P value	< 0.05		< 0.05	

Five larvae from control or HS1-treated group (n = 5) were used for body measurements. Data were normally distributed, and hence, processed for analysis by Students' t-Test. Star (*) represents significantly smaller compared to control one ($P < 0.05$). SE: standard error of mean.

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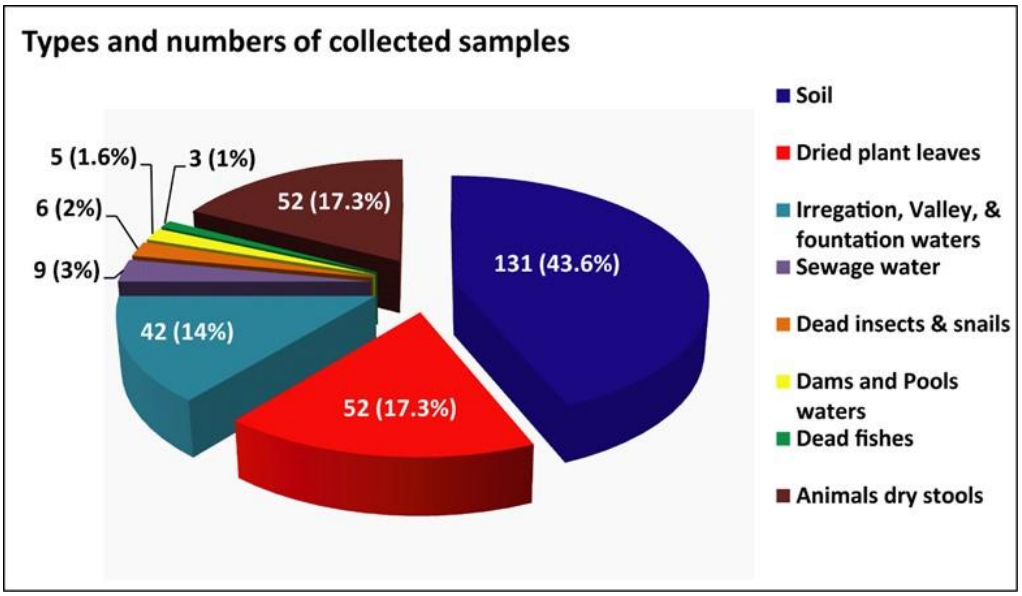

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2 Figure 1. Illustrates types, numbers and % of collected samples (300 samples in total)

3 from all targeted regions throughout Saudi Arabia.

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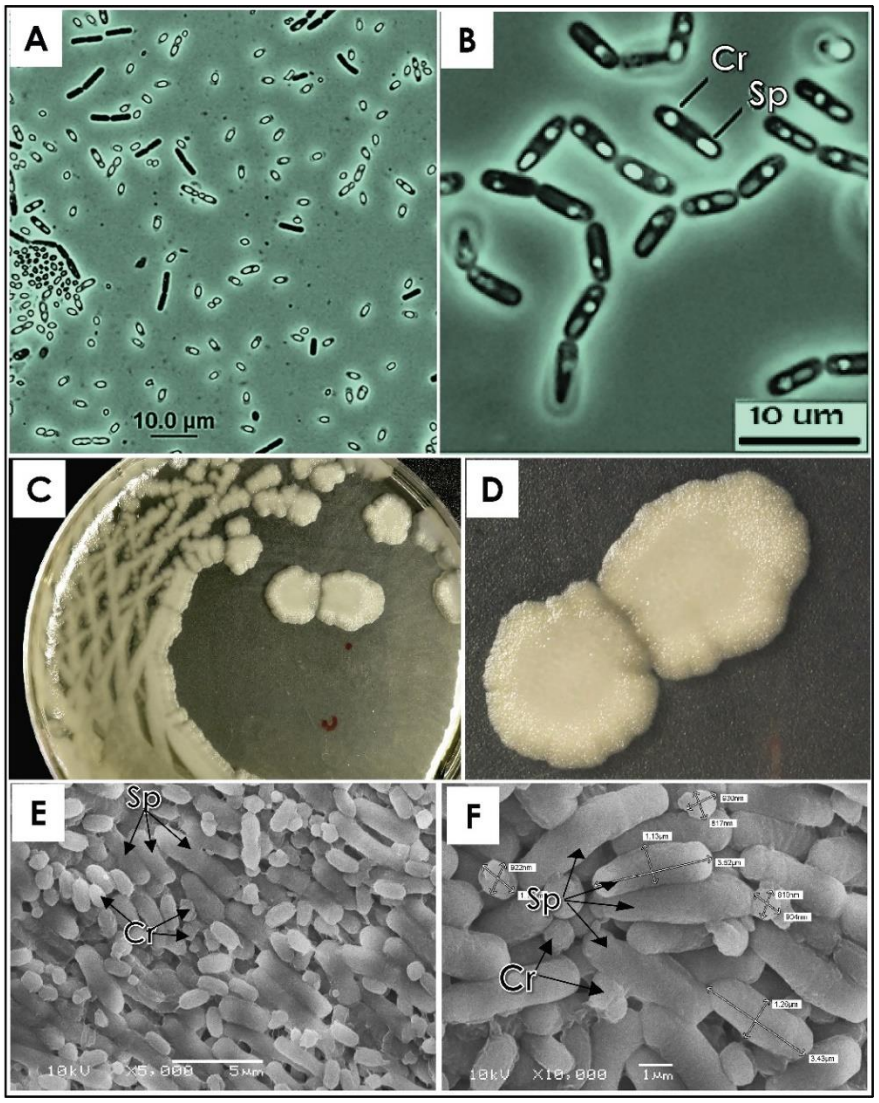

Figure 2. A photo-plate showing morphological features of the native Bt-63 (as an example of the potentially larvicidal *B. thuringiensis* isolates). A & B: Phase Contrast micrographs (×1000) of the native Bt-63 isolate showing spores (Sp) and para-sporal crystals (Cr) which appear brighter. C: growing colonies of Bt-63 seeded in nutrient-supplemented agar media, and D: a magnified colony shows its characteristic slightly raised center with fried egg appearance; white, large, nearly circular with fine irregular margins and glossy similar to Bti-H14 (Photos E and F by AMA). E & F: Scanning Electron Micrograph of Bt-63 shows clearly large numbers of crystals (C) and spores (S).

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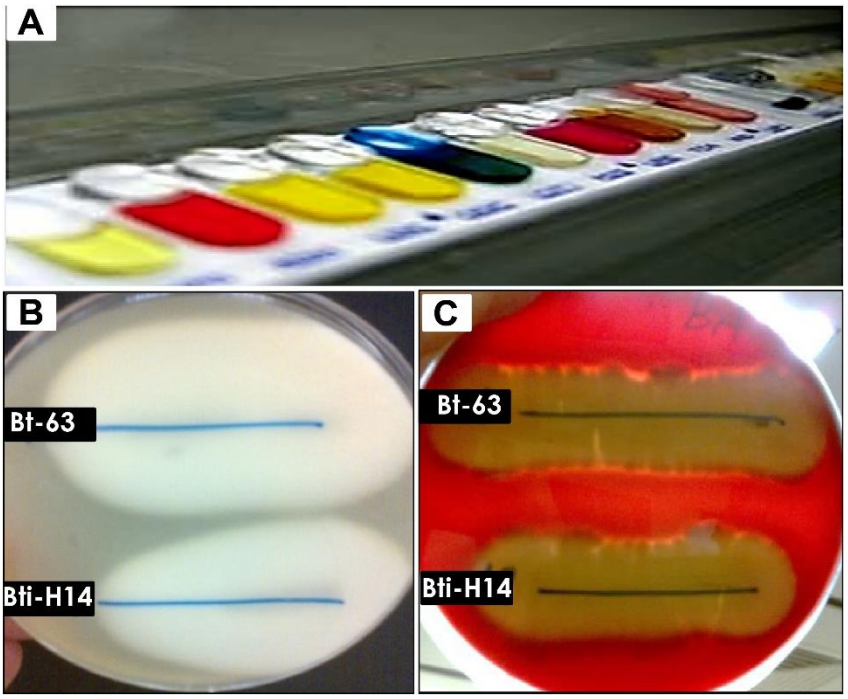

Figure 3. A photo plate showing the API E20 system tests and lecithinase and hemolytic activities of the mosquitocidal Bt isolates. A: An example of API E20 system tests-results, which showed great fluctuations in biochemical results among the tested Bt isolates. B & C: Lecithinase and hemolytic activities of the Bt-63 strain as an example of the native Bt isolates compared to the reference Bti-H14 strain., B: Egg-yolk agar medium seeded with native mosquitocidal Bt-63 isolate which shows larger lecithinase opacity as compared to that of the reference Bti-H14 strain, C: Blood agar medium seeded with the same native Bt-63 strain, which also shows a relatively larger zone of hemolytic activity as compared to that of the reference Bti-H14 strain.

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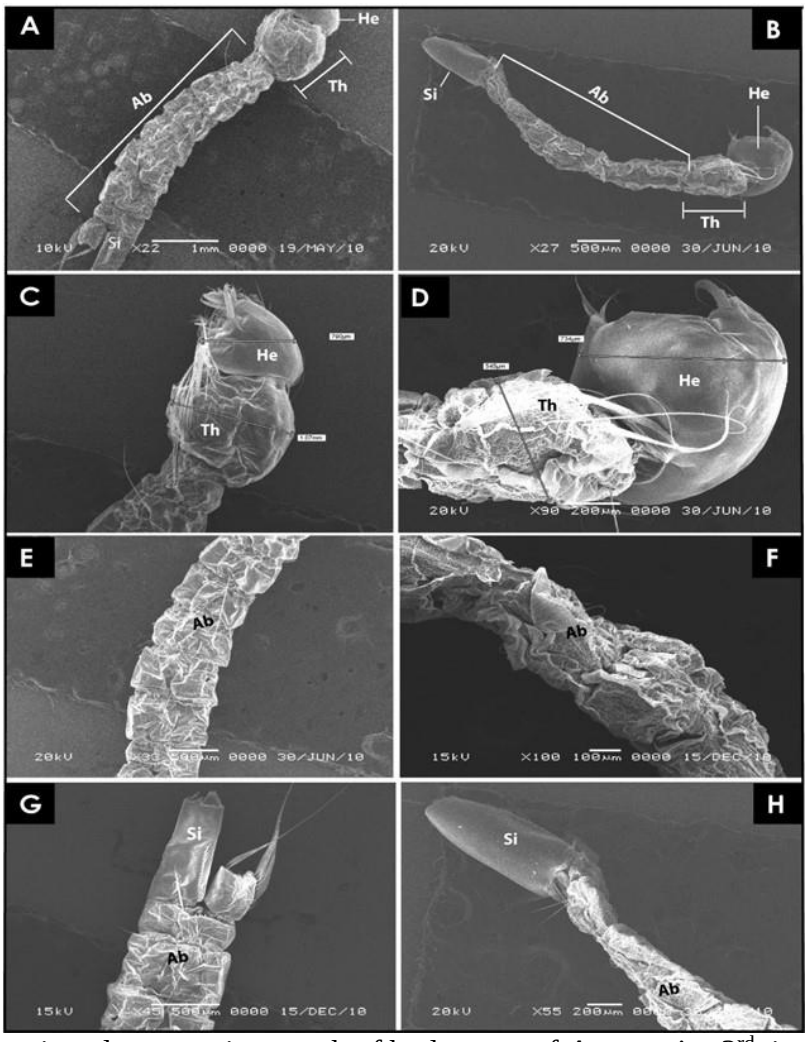

Figure 4. Scanning electron micrograph of body parts of *Ae. caspius* 3rd instar larvae at 48h post-treatment with HS1 toxin compared to control ones. A & B: whole mounts of control and treated larvae, respectively, showing clear shrinkage in thorax (Th) and abdomen (Ab) of treated larva immediately after death. C & D: Head and thorax measurements of control & treated larvae, respectively, showing shrinkage in the thoracic region in treated larvae comparing to its head and to that of the control ones. F & H showing clearly the shrinkage in the abdominal segments of treated larvae comparing to those of the control one (E & G). Thorax and abdomen shrinkage was clear comparing to the head (He) and respiratory siphon (Si). Five different heads and thoraces measurements were performed using five individually different control or infected larvae (n = 5) and used for analysis by students' t-test.