EFFECT OF AGROTIS SEGETUM NUCLEOPOLYHEDROVIRUS VIRAL ENHANCING FACTOR 1 (Vef 1) ON THE LETHALITY OF AUTOGRAPHA CALIFORNICA MULTIPLE NUCLEOPOLYHEDROVIRUS AGAINST EGYPTIAN COTTON LEAFWORM (*Spodoptera littoralis*)

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Baculoviruses are a group of rod-shaped insect viruses that belong to the family *Baculoviridae*. They are insect specific pathogens that commonly used as bioinsecticides for insect pests control specially order Lepidoptera, Hymenoptera and Diptera. In addition, baculoviruses have been used for recombinant protein expression in insect cells for industrial and pharmaceutical purposes (Bonning and Nusawardani, 2007; Van Oers *et al.*, 2015). Generally, baculoviruses have a very narrow host range and most of them are restricted to only one insect host species. For baculoviruses to compete with the chemical pesticides, their host range has to be expanded to control multiple host insects.

The Egyptian cotton leafworm, *Spodoptera littoralis*, is considered as one of the most destructive agricultural insect pest that attacks a broad range of economically important crops not only in Egypt but also in Africa, Southern Europe, and the Middle East (Ellis, 2004; Abd El-Kareem *et al.*, 2010).

Previous studies have shown a resistant mechanism of *S. littoralis* larvae toward Autographa californica multiple nucleopolyhedrovirus (AcMNPV) infection *per os*. However, it is highly sensitive to infection with budded virus (BV) *via* hemolymph injection (Bishop *et al*., 1988; Rivkin *et al.*, 2006). It was proven that humoral and cellular immune responses are involved in the resistance mechanism of *S. littoralis* against AcMNPV *via* oral infection (Rivkin *et al*., 2006).

Interestingly, enhancins (enhancing proteins) were found to increase the infectivity of some baculoviruses in non-natural hosts especially order Lepidoptera (Derksen and Granados, 1988). Enhancins are characterized as metalloproteases that can enhance virus midgut penetration through degrading the mucins which is the major proteins component of the peritrophic membrane. Additionally, by expanding the combination capability of viral envelope and cells plasma membrane (Wang *et al*., 1994; Bischoff and Slavicek, 1997).

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Previously, it was demonstrated that co-infection using occlusion bodies of both Nucleopolyhedrovirus (NPV) and Granulovirus (GV) enhanced the virulence and infectivity of NPV to its main host due to the presence of enhancin proteins in GV (Tanada, 1959; Yamamoro and Tanada, 1978). The Pseudaletia unipuncta granulovirus (PsunGV) showed the first significant synergistic effect of Pseudaletia unipuncta nucleopolyhedrovirus (PsunNPV) against its natural host *P. unipuncta* larvae (Tanada, 1959). Recently, successful improvement of AcMNPV recombinants virulence were observed by fusing truncated Agrotis segetum granulovirus enhancin or truncated Cydia pomonella granulovirus into AcMNPV genome (Yang *et al.*, 2017).

The complete genome sequence of Agrotis segetum nucleopolyhedrovirus type-A (AgseNPV-A), revealed the presence of three putative enhancin genes known as viral enhancing factor 1, 2 and 3 denoted vef1, vef2 and vef3, respectively, with a putative late promoter for all three genes (Jakubowska *et al.*, 2006). In this study, a recombinant AcMNPV expressing vef1 under the p10 promoter, originated from AgseNPV, was generated to examine whether the expression of enhancin protein vef1 of AgseNPV can enhance the lethality of the AcMNPV against *S. littoralis* larvae *per os.*

MATERIALS AND METHODS

Insects, insect cell line and viruses

Laboratory colony of the cotton leafworm, *Spodoptera littoralis*, was maintained in the insect rearing laboratory of Agricultural Genetic Engineering Research Institute (AGERI), Giza, Egypt. Larvae were reared at 26°C on a semi-synthetic diet described by Levinson and Navon (1969). *Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9 (Sf9) insect cells were cultured at 27°C in EX-CELL ® 420 Serum-Free Medium (Sigma-Aldrich) contains 10 µg/ml Gentamycin. The AcMNPV wild type (clone C6) was used for recombinant virus construction and the AgseNPV-A genomic DNA was used as template for vef1 gene amplification. Titers of AcMNPV budded virus (BV) and the recombinant virus were determined using endpoint dilution assay in Sf9 cells according to O'Reilly *et al.* (1992).

PCR of vef1 and polyhedrin genes

Agrotis segetum nucleopolyhedrovirus (accession number NC_007921) was used for PCR amplification of vef1 gene using two specific primers denoted; vef1_F: tttctcgagtttatgagtgaaaccattgcca with XhoI restriction site at its 5' terminus (under lined), and vef1_R tttggtacctttttattctataattttccg with KpnI site at its 5' terminus (under lined). The AcMNPV polyhedrin gene (*polh*) was amplified using a forward primer polh-F (tttgtcgactttatgccggattattcata) includes

SalI restriction site at its 5' end (under lined), and a reverse primer polh-R (tttctgcagttttaatacgccggaccag) includes PstI restriction site at its 5' end (under lined). The PCR reaction in a total volume of 50 µl was performed using the following component; 1 µl of the forward-primer (10 pmol/µl), 1 µl of the revers-primer (10 pmol/µl), 2 µl $MgCl_2$ (25 mM), 1.5 µl of dNTPs mixture solution (10 mM), 3 µl of viral genomic DNA (0.1-0.5 µg), 10 µl of 5X PCR reaction buffer and 0.5 µl of Goflexi Taq DNA polymerase (5 U/µl) (Promega). Then ddH_2O was added to complete the reaction volume to 50 µl. The following PCR program was used for each reaction; initiation at 95°C for 3 min; a total of 35 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 1-3 min, based on the fragment length, and final cycle at 72°C for 5 for completion of primer extension. Subsequently, the amplified genes (Vef1 & Polh) were subjected for sequencing to be verified with the published sequence data.

Construction of the recombinant AcMNPV

The AgseNPV vef1 gene (ORF75) was released from pGEM-T vector using XhoI/KpnI restriction enzymes and subsequently cloned into the baculovirus cloning vector pFasBAC-Dual vector, previously digested by XhoI/KpnI restriction enzymes, under p10 promoter generating the pFBD-vef1 plasmid.

Subsequently, the amplified *polh* (ORF1) of AcMNPV was cloned into pGEM-T vector and then released as SalI/PstI fragment, which was subsequentially purified and introduced into the pFBD-vef1 plasmid, downstream of polh promoter, previously digested by SalI/PstI to construct the bacmid cloning vector pFBD-vef1-polh. The generated bacmid cloning vector was transformed into DH10Bac competent cells (Thermo scientific) to allow transposition into AcMNPV bacmid. The recombinant bacmid harboring vef1/polh cassette was then selected following the manufacturer's instructions (Bac-to-Bac Baculovirus expression system, Thermoscientific) by PCR using two specific primers flanking the transposition site.

Production of recombinant virus Occlusion Bodies (OBs)

Sf9 cells (1×10^6/35-mm in 6-well plates) were transfected with 1 µg of the generated bacmid using 10 µl Cellfectin II transfection reagent (Invitrogen Life Technologies). At 96 hours post transfection (hpt), the Budded Virus (BV)-enriched culture supernatants were harvested after centrifugation at 3,000 rpm for 10 min to precipitate infected cells pellets. The BV-enriched culture supernatant collected after cell transfection was used for Sf9 cells infection making viral stock P1. Titers of BV were determined after two more virus passages using Tissue Culture Infectious Dose 50% (TCID50) endpoint dilution assay in Sf9 cells following the standard protocol

(O'Reilly *et al*., 1992). The produced viral stock P3 was used for re-infection of Sf9 cells (1.5 x 10^7 cells) with multiplicity of infection (MOI) of 10 in 25 cm^2 flasks. Budded viruses of the generated recombinant virus were used to inject 20 fourth instar larvae of *S. littoralis via* hemolymph (1-2 µl BVs) using Microliter Syringes (25 µl) (HAMILTON, Switzerland). All larvae were reared on a semi artificial diet until larval death or pupation.

Viral OBs purification

Infected larvae cadavers were collected and subjected to OBs purification. Briefly, the cadavers were homogenized in 0.5% sodium dodecyl sulphate (SDS) solution and the crude suspension was filtered through a piece of cotton wool which was subsequently washed with additional volumes of 0.5% SDS. OBs were pelleted by centrifugation at 7000 rpm for 10 min. The pellets were washed by re-suspension twice in 0.1% SDS and once in 0.5 M NaCl and pelleted by centrifugation at 7000 rpm after each washing step according to O'Reilly *et al.*, (1992). OBs were resuspended in ddH_2O and kept frozen at -20°C. Purified OBs were subjected for counting using Petroff-Hauser counting chamber (depth 0.01 mm, Hausser Scientific) using the dark-field of Axiovert inverted microscope (Carl Ziss, Germany).

Biological analysis

Neonates of *S. littoralis* larvae were inoculated with the generated recombinant virus carrying the vef1 gene in two different virus concentrations; (1.5 x 10^4 Obs/ml) and (1.5 x 10^5 obs/ml). SpliNPV was used with a final concentration of 1.5 x 10^4 ob/ml in addition to untreated animals as a control. Fifty neonate of *S. littoralis* larvae were exposed to each virus treatment mixed with the semi-artificial diet to reach the virus final concentrations. Larvae were individually placed in each well of a 50-well bioassay boxes (Raster boxes, *Neolab*-Heidelberg, Germany), and all boxes were kept in 25°C incubator with a 16/8-h light/dark photoperiod. Mortality was scored on the next day of treatment to exclude larvae that died from handling, and after 7 days post infection (dpi). Larvae that died by symptoms-like viral infection were collected and stored individually at -20°C for downstream analysis. Each infection experiment was repeated three times using the same number of larvae. Calculation of significant variation using the mean mortality between treatments was performed by One-way ANOVA with post-hoc Tukey HSD Test Calculator R statistical package.

Total RNA extraction and RT-PCR

Reverse transcription PCR (RT-PCR) was performed using total RNA isolated from infected Sf9 cells with the recombinant virus in a time course of infection 0, 6, 12, 24, 48 and 72 hours post infection (hpi). Total RNA was extracted from infected cells using SV Total RNA extraction kit according to manufacturer's protocol (Promega). First-

strand cDNA synthesis was performed using 500 ng total RNA as a template for each time-point. The reaction was performed in 20 µl reaction volume containing; 1 µl Oligonucleotide $(dT)_{12-18}$ (500 µg/ml), 500 ng total RNA, 2 µl 10 mM dNTPs mixture solution and sterile ddH_2O to 20 µl. The mixture was heated to 65°C for 5 min and quickly chilled on ice. Then, the following contents were added: 4 µl 5X First-strand buffer, 2 µl 0.1 M DTT, 1 µl RNase OUT™ Recombinant Ribonuclease Inhibitor (40 units/µl). The reaction was incubated at 42°C for 2 min, and 1 µl (200 units) of cloned SuperScript™ II was added to the mixture and further incubated at 42°C for 50 min. Finally, the reaction was inactivated by heating to 80°C for 5 min. The synthesized first-strand cDNA was used as a template for vef1 gene amplification using two specific primers (vef1_F and vef1_R). The RT-PCR was performed according to standard conditions (Sambrook and Russell, 2001) in a total reaction volume of 50 µl as descriped above. The PCR-amplified DNA fragment was analyzed by electrophoresis on a 1% agarose gel prepared in 0.5 X TBE buffer (44.5 mM Tris-base, 44.5 mM Boric acid and 1 mM EDTA) and stained with ethidium bromide before analysis under UV-Transilumenator.

RT-PCR of infected S. littoralis larvae per os

Neonates and early 4th instar of *S. littoralis* larvae were subjected for infection *per os* using the recombinant virus OBs that were purified from injected larvae. Neonates of *S. littoralis* were reared on semiartificial diet that was mixed with fixed concentration of recombinant virus (10^5 ob/ml). Larvae were kept until larvae mortality or pupation. For 4th instar larvae, each individual larva was inoculated using a fixed amount of virus OBs (10^5 OB) using a small piece of medium. Larvae that completely ingested the virus dose within 24 h were transferred to fresh virus-free diets and reared individually at 26°C. Fifty larva were used for each experiment and each experiment was repeated three times using the same number of larvae. For each group, RT-PCR was carried out using cDNA synthesized from total RNA extracted from a group of five larval midguts infected with the recombinant AcMNPV harboring vef1 gene using different time points at 3, 4, 5, 6 days p.i. RT-PCR was performed using one set of specific primers (vef1_F and vef1_R) that amplify the vef1 gene.

RESULTS AND DISCUSSION

Previous studies demonstrated that *Spodoptera littoralis* larvae showed resistance to *Autographa californica* nucleopolyherovirus (AcMNPV) infection *per os*, however they showed high susceptibility *via* haemocoelic injection using BVs (Bishop *et al*., 1988; Du *et al*., 1999; Rivkin *et al.*, 2006). This phenomenon was recently attributed to the ability of *Spodoptera littoralis* larvae to develop an immune response that limit

AcMNPV infection at the gut and at the haemocyte level. The cellular and the humoral aspects, are suggested to be involved in this immune response subsequently limited virus infection *per os* (Rivkin *et al.*, 2006). In the same context, the AcMNPV lacking immediate-early gene IE0 has showed to develop infectivity toward *S. littoralis* larvae *via* intrahaemocoelic injection using virus BVs (Lu *et al*., 2003; Lu *et al*., 2005). Although these studies elucidate some plausible reasons for larvae resistance mechanisms; however, they did not contribute in a significant development of AcMNPV infection toward *S. littoralis* larvae *per os* (Lu *et al*., 2003).

Generation of vAcMNPV-enAg1 recombinant virus

In the present study, the *vef1* enhancin gene has been cloned under the late promoter (p10) in AcMNPV genome to facilitate enhancin protein expression at a late stage of viral infection. This expriment was performed in order to examine the synergetic effect of the recombinant virus carrying the enhancing gene toward *S. littoralis* larvae.

Gene encoding for AgseNPV VEF1 was PCR amplified using two specific primers (Ag-Vef_F/Ag-Vef_R) using AgseNPV genomic DNA as a template. The PCR product of 2633 bp for vef1 ORF was purified and cloned into the p10 locus of pFastBac-Dual vector under the control of the p10 promoter. The resulted plasmid denoted pFBD-*vef1*. The gene encoding for polyhedrin ORF of AcMNPV was PCR amplified using polh-F/polh-R specific primers and AcMNPV DNA (Clone C6) as a template giving the expected size of 733 bp. This fragment then cloned into pFBD-vef1 cassette under the control of its native polyhedrin promoter generating the backbone plasmid pFBD-vef-polh. The recombinant plasmid pFBD-vef-polh was selected by antibiotic resistance and the insertions of *vef1* and *polh* genes were further confirmed by enzyme restriction digestion and nucleotide sequencing. The generated recombinant plasmid was used for the transposition of vef1 and *polh* cassette into the AcMNPV genome using Bac-to-Bac baculovirus expression system (Fig. 1).

The generated recombinant bacmid was selected by antibiotic resistance and the insertions of heterologous genes were further confirmed by PCR analyses using M13 forward/ vef1 forward & M13 Forward/M13 Reverse primers sets as shown in Fig. (2A). In Fig. (2B), it shows the positive colonies which give the expected amplicon of 1400 bp corresponding to the successful transposition of the cassette into the bacmid. In contrast, negative colonies carrying the empty bacmid construction showed amplicons of 300 bp. The resultant recombinant bacmid denoted vAcMNPV-enAg1.

The full genome sequence analysis of the AgseNPV-A has showed the presence of three enhancin genes (vefs) with late promoter motifs suggesting expres-

sion in the late phase of virus life cycle (Slavicek and Popham, 2005), parallel with assembly of the Occlusion Derived Viruses (ODVs).

The prediction of different VEFs protein analyses showed that VEF1 of AgseNPV-A has two domains peptidase M60 and Mucin_bdg. Peptidase M60, is a zinc-requiring metalloprotease; peptidase family M60 domain targets complex host glycoproteins, such as mucins. In addition, the Mucin_bdg, is the putative binding domain for the substrates of enhancin, and other similar metallopeptidases. This analysis suggested that the VEF1 might bind to the midgut mucin leading to the disruption of the larva peritrophic membrane (PM). The prediction of VEFs of AgseNPV-A showed that VEF2 and VEF3 do not have any extra domains indicating that they may have better synergetic properties compared to VEF1. Therefore, a recombinant AcMNPV-en1Ag harboring only AgseNPV enhancin gene (vef1) was constructed in order to investigate the possible impact of VEF1 on PM of S. littoralis larvae when treated with the recombinant AcMNPV-en1Ag *per os* compared to the wild type AcMNPV.

The effect of enhancins to improve the efficacy of baculovirus infection *via* disruption of larvae midgut peritrophic membrane has been described in many GVs and NPVs. For example, the deletion of en1 or en2 of LdMNPV resulted in an approximately 2-fold reduction in virus efficacy (Popham *et al.*, 2001). However, the complete deletion of both genes (en1 and en2) showed 12-fold reduction in virus potency in comparison to the wild type virus (Bischoff and Slavicek, 1997; Popham *et al.*, 2001). *Mamestra configurata* nucleopolyhedrovirus (MacoNPV-A) enhancin showed to have degradation activity toward *M. configurata* insect intestinal mucins McIIM4 but not to McIIM2 suggesting that MacoNPV-A enhancin is able to degrade major structural PM proteins (Toprak *et al.*, 2012).

The activity of enhancin proteins has been consequently attributed to two mechanisms: a) enhancement of cell fusion between virus BVs and plasma membrane of host midgut cells (Kozuma and Hukuhara, 1994), b) dissolution of peritrophic major proteins *via* metalloprotease activity of enhancins (Lepore *et al*., 1996; Wang and Granados, 1997). Previously, it was demonstrated that enhancins of *Trichoplusia ni* granulovirus can improve the infectivity of AcMNPV against neonates and fourth instar *T. ni* larvae. The results suggested that the enhancement in infection efficiency most likely caused by the disruption of the insect PM (Gallo *et al*., 1991).

Recently, in an attempt to increase the infectivity of AcMNPV against *Spodoptera* exigua larvae, a recombinant AcMNPV virus expressing a truncated enhancin of *Agrotis segetum* GV and *Cydia pomonella* GV in OBs was generated. The results of the bioassay revealed that the recombinant viruses were 3- to 5-

fold lower than that of the control virus using LC_{50} analysis. These results showed relative enhancement of the recombinant virus harboring enhancin protein toward the permissive host *S. exigua* (Yang *et al.*, 2017). However, enhancin genes used in this experiment have been originated from different virus species (AgseGV and CpGV), with no infectious properties toward *S. exigua* larvae.

Light microscopy visualization of cells infected with vAcMNPV-enAg1

The spreading of the recombinant virus vAcMNPV-enAg1 in Sf9 insect cells was analyzed *via* the detection of the cytopathic effect of infected cells nuclei using the dark field of the inverted fluorescent microscopy 72 hpi (Fig. 3). These results suggested the successful syntheses, transfection and expression of polyhedrin protein using the generated recombinant virus vAcMNPV-enAg1. The observed occluded bodies generated from the recombinant virus showed less number ~5 fold compared to the produced number from infected cells using the wild type AcMNPV. This observation may due to that the wide type virus has a replication advantage in Sf9 cells compared to the recombinant virus.

RT-PCR analysis of vAcMNPV-enAg1 transcripts in infected Sf9 cells

Semi-quantitative (RT-PCR) was carried out using cDNA synthesized using total RNA extracted from Sf9 cells infected with vAcMNPV-enAg1. The RT-PCR analysis was applied using different time points at 0, 6, 12, 24, 48 and 72 hpi. Using vef1 specific oligonucleotides, a single band was detected with the predicted size of about 2.6 kbp corresponding to the vef1 transcripts (Fig. 4). Enhancin vef1 transcription was detected at 24 hpi and increased until 72 hpi. The detection of vef1 transcripts at the late stage of vAcMNPV-enAg1 virus infection confirmed the successful transcription from the late promoter p10.

The AgseNPV vef1 gene (ORF75) is 2634 bp in length and encodes an 878-amino-acids protein with a predicted molecular weight of 100 and 130 Da. Other VEFs such as the AgseNPV-VEF-2 showed the greatest amino acid identity percentage with AgseNPV VEF1 (43%), however the least identity with 11% similarity was observed with PsunGV-VEF-4 (Slavicek, 2012). Different reports elucidated that late promoter elements (A/TTAAG) have been detected upstream of all enhancin genes identified in various baculovirus genomes up to date such as; PsunGV enhancin (Roelvink, *et al.*, 1995), LdMNPV enhancins (en1 and en2) (Kuzio *et al.*, 1999), and AgseNPV enhancins (vef1, vef2 and vef3) (Jakubowska *et al.*, 2006). Similarly, using RT-PCR the LdMNPV showed that enhancin-specific transcript was detected in infected Ld652Y cells late at 48 and 72 hpi (Bischoff and Slavicek, 1997). The presence of the late gene promoter motif(s) suggested that enhancin is likely expressed at late phase of viral infection parallel with the assembly of ODVs.

RT-PCR analysis of vAcMNPV-enAg1 transcripts in infected larvae

In order to investigate whether died larvae, that infected with the generated recombinant virus, were due to viral infection, RT-PCR was carried out using cDNA synthesized from total RNA extracted from a group of five midgut of *S. littoralis* larvae infected with vAcMNPV-enAg1 or AcMNPV. The RT-PCR analysis included 4-time points of infection at 3, 4, 5 and 6 days p.i. and two sets of specific primers were used for *vef1* and *polh* genes. No transcripts were detected from those animals infected *per os* even with vAcMNPV-enAg1 or AcMNPV wild type (data not shown).

Biological activity of vAcMNPV-enAg1

In order to examine the lethality of the generated recombinant virus vAcMNPV-enAg1 against *S. littoralis* larvae, neonates were fed using vAcMNPV-enAg1 OBs (previously purified from injected larvae with BVs). The controls were larvae fed with the wild type AcMNPV OBs, SpliNPV OBs as well as untreated larvae. All experiments were performed using 45-50 larvae for each experimental group. For infected neonates using vAcMNPV-enAg1 *per os*, 7/48 larvae fed with vAcMNPV-enAg1 OBs died 4-7 days post infection (p.i). Three larvae died out of 47 which were inoculated with AcMNPV OBs 7 days p.i. and the rest of larvae remain healthy and continue until pupation. However, 38/47 larvae died during the same period of larvae treated using SpliNPV OBs. Interestingly, no clear signs of viral infection (liquefaction and/or melanization) was observed on collected died larvae that was treated even with vAcMNPV-enAg1 or AcMNPV OBs. In addition, no polyhedra were observed in dead larvae infected *per os* using vAcMNPV-enAg1 or AcMNPV Obs by examination of a semi-purified larva filtrate under light microscopy. The differences between the mortality results of virus treatments were analyzed after 7 days p.i. by one-way analysis of variance (ANOVA), and the differences between the treatments were scored by post hoc Tukey's test (level of significance, $P < 0.05$). Using the first virus concentration (1.5×10^4 ob/ml), the p-value (2.6958e-07) corresponing to the F-statistic (461.4) of one-way ANOVA is lower than 0.05, suggesting that the one or more treatments are significantly different. The lowest mortality was observed for the AcMNPV treatment (6.4%, SE: 0.33) compared to the vAcMNPV-enAg1 treatment (10.4%, SE:1.15) and SpliNPV treatment (80.8%, SE: 0.88). No significant difference was observed between AcMNPV and vAcMNPV-enAg1 treatments due to low number of died larvae (*p*-value = 0.55) (Fig. 5A). The highest mean mortality was observed using SpliNPV treatment (80.8%, SE: 0.88), where the mortality was statistically different from that of AcMNPV (P= 0.001) and vAcMNPV-enAg1 (P = 0.001) treatments.

By using the second virus concentration (1.5×10^5 ob/ml) which is 10-fold higher than that of the first

concentration (1.5 x 10^4 ob/ml), the p-value (2.3253e-07) corresponing to the F-statistic (484.8) of one-way ANOVA is lower than 0.05, suggesting that some treatments are significantly different. The lowest mortality was also observed for the AcMNPV treatment (9.1%, SE: 1.0) compared to the vAcMNPV-enAg1 treatment (12.5.4%, SE: 0.33) and SpliNPV treatment (94.9%, SE: 1.45). The mortality rate for all treatments showed slightly improvement, however no significant difference was observed between AcMNPV and vAcMNPV-enAg1 treatments due to low number of died larvae (*p*-value = 0.53) (Fig. 5B). The highest mean mortality was observed using species specific SpliNPV treatment (94.9%, SE: 0.1.45), where the mortality was statistically different from that of AcMNPV (P= 0.001) and vAcMNPV-enAg1 (P = 0.001) treatments. These results showed no significant improvement of *S. littoralis* larvae mortality rate which was treated with the recombinant vAcMNPV-enAg1 expressing the vef1 enhancin protein *per os.*

The generated recombinant virus vAcMNPV-enAs1 showed biological activity after injection *via* the haemocel of 4^{th} instar larave, which suggested that the integration of vef1 gene into AcMNPV did not affect the infection properties of the virus. However, the generated recombinant virus could not to produce progeny OBs, after larvae oral infection, and did not show clear viral infection symptoms on neonates of *S. littoralis* 7 days p.i. There was no significant difference between infected neonates of *S. littoralis* using the recombinant virus carrying vef1 gene and the wild type AcMNPV after oral infection using virus OBs. However, the mortality rate was 8-10 fold higher than that using the species specific SpliNPV. This observation suggested that the integration of enhancin gene (vef1) of AgseNPV-A into AcMNPV genome did not significantly improve the infection capability of AcMNPV against *S. littoralis* neonates *per os*, which was proved using RT-PCR of mRNA isolated from infected larvae midguts.

The most plausible reasons for these observations might be due to the vAcMNPV-enAs1 inability to increase the amount of initial infection in the host insect midgut or to enhance the fusion efficiency of BVs with midgut cells. Another reason that may explain this phenomenon, is that AcMNPV may does not generate the appropriate *per os* infection factor (PIF) needed to infect *S. littoralis* larvae, since recently the construction of a series of pifs-substituted pseudotyped baculoviruses, showed that baculoviruses have strict host specificities (Song *et al.*, 2016). In addition, the complete genome sequence of SpliNPV (isolate AN-1956), the species-specific virus isolate, showed low identity of pif genes with AcMNPV homologs as follow; 40, 56 and 49 (%) for pif-1, pif-2 and pif-3, respectively, suggesting that PIF protein could be one of the major factor affecting the infection capacity of AcMNPV against *S. littoralis per os*.

It would be of interest to determine whether AgseNPV enhancin, as expressed in the AcMNPV-enAg1 recombinant virus, interacts with the intestinal mucin component of *S. littoralis* peritrophic matrix in a way similar to that of GV enhancins as demonstrated by Wang and Granados, (1997). Furthermore, approaching this line of investigation using swapping of pif genes from SpliNPV to AcMNPV followed by determination of AcMNPV mutants' biological activity against *S. littoralis* would be interesting as well. This will tell whether PIF proteins of SpliNPV are able to help the mutants of AcMNPV to penetrate the peritrophic membrane of *S. littoralis* larvae, and thus can overcome larvae immune resistance system.

SUMMARY

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) cannot infect the Egyptian cotton leafworm, *Spodoptera littoralis* larvae *per os*. However, successful infection can be observed *via* larvae haemocoel injection using AcMNPV BVs. Baculovirus enhancins, known also as viral enhancing factors (VEFs), have shown to enhance infectivity of some baculoviruses toward non-natural hosts. In the present study, a recombinant AcMNPV (vAcMNPV-enAg1) that expresses the Viral Enhancing Factor 1 (vef1) originated from an enhancin-rich Polish isolate of *Agrotis segetum* nucleopolyhedrovirus (AgseNPV-A) was constructed. The vef1 gene was expressed under the control of the p10 promoter in the presence of the polyhedrin gene. The recombinant vAcMNPV-enAg1 was generated to examine the possible enhancement of AcMNPV lethality against *S. littoralis* larvae *per os.* Using RT-PCR, successful transcription of vef-1 mRNA was detected in a time course intervals, and occlusion bodies production was determined in Sf9 cells infected with vAcMNPV-enAg1. However, no transcripts were detected in mRNA isolated from mid-guts of larvae infected using vAcMNPV-enAg1. In addition, the production of the recombinant virus OBs was 5-fold lower than that of the wild type AcMNPV. Biological analysis of the progeny OBs isolated from injected *S. littoralis* larvae using the recombinant virus did not show significant improvement of virus lethality against *S. littoralis* neonates *per os* compared to the wild type AcMNPV. Hence, the results suggested that the failure of *S. littoralis* infection using AcMNPV *per os* is more likely unrelated to enhancin activity.

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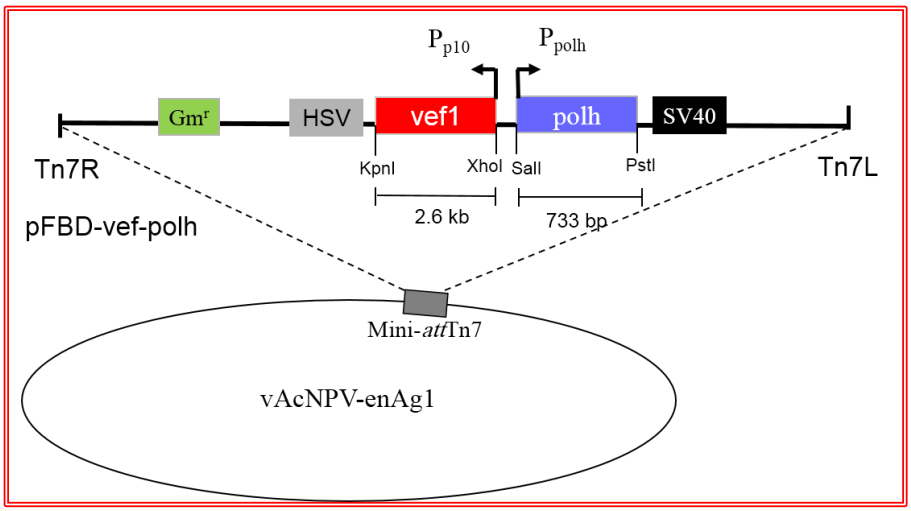

Fig. (1): Schematic representation for the construction of vAcMNPV-enAg1. The bacmid cloning vector pFBD-*vef-polh* harboring the AgseNPV vef1 (2.6 kbp) downstream of p10 promoter, and the AcMNPV polyhedrin gene (733 bp) downstream of its native polyhedrin promoter. The restriction sites used for cloning are present in both ends of each gene fragment. The figure shows the integration of pFBD-*vef-polh* cassette into the Mini-*att*T7 transposition site in the generated recombinant virus vAcMNPV-enAg1.

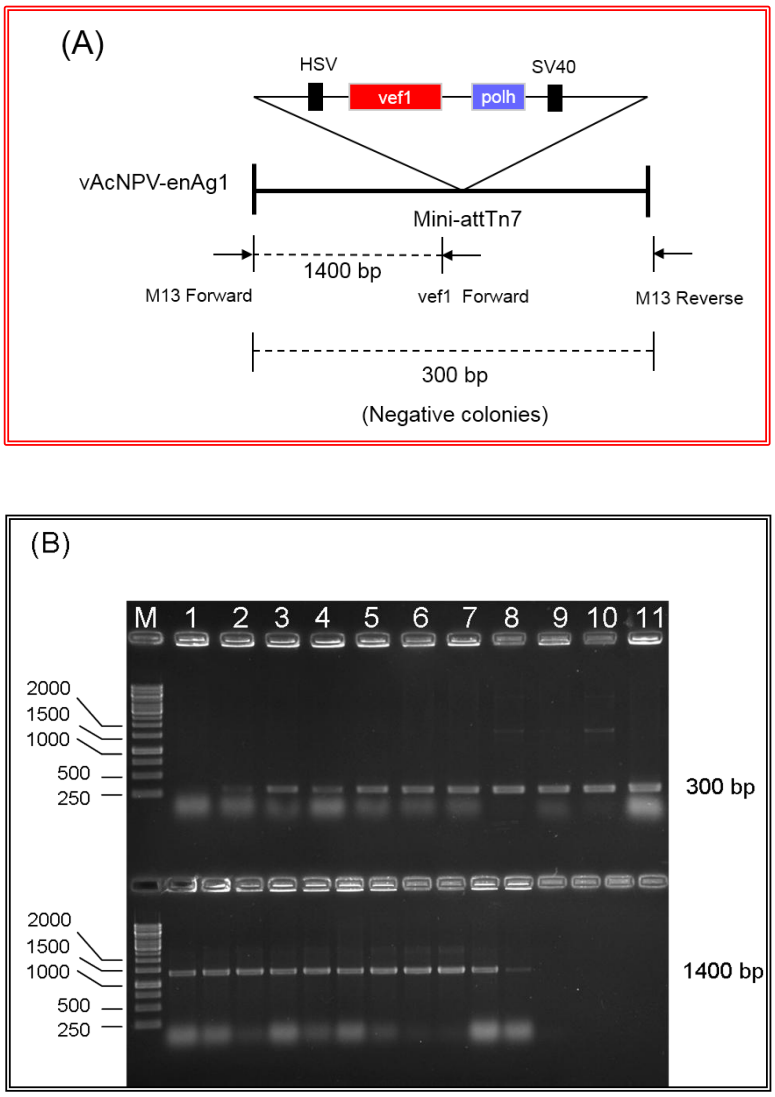

Fig. (2): Screening of the recombinant bacmid vAcMNPV-enAg1 using PCR.

(A) Schematic representation of the generated vAcMNPV-enAg1 virus contains the *att*Tn7 transposition site. Black arrows show M13 Forward, vef1 Forward and M13 Reverse primers sets used for the PCR screening of the recombinant bacmid with the expected sizes (bp).

(B) Upper gel: shows PCR of negative colonies (blue colonies) carrying only empty bacmid with PCR fragment size of 300 bp. Lower gel: shows positive colonies (white colonies) carrying recombinant bacmid with the expected PCR fragment size of 1400 bp. M: 1 Kb DNA ladder marker.

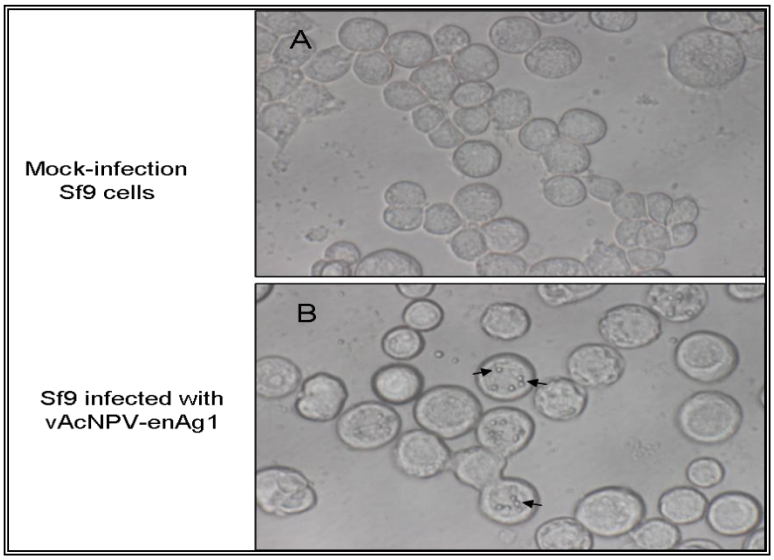

Fig. (3): Detection of polyhedra formation (Occlusion Bodies) in Sf9 insect cells infected with the recombinant vAcMNPV-enAg1 using Inverted Phase Contrast Microscope (400X). (A) Healthy Sf9 cells (mock infection). (B) Infected Sf9 cells using vAcMNPV-enAg1 recombinant virus. Black arrows show the spreading of virus occlusion bodies inside the infected Sf9 cells nuclei 72hpi.

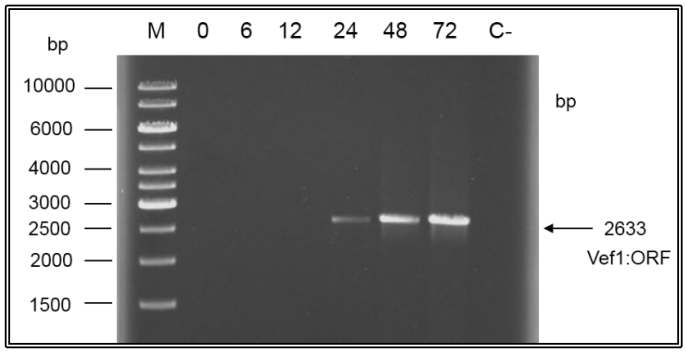

Fig. (4): Analysis of AgseNPV enhancing gene (vef1) transcription using semi-quantitative RT-PCR. The RT-PCR analysis was performed on infected Sf9 cells at 0, 6, 12, 24, 48 and 72 hours post infection (hpi) as shown on the top of the gels, by using vef1 specific primer pairs. The arrow show the detected RT-PCR product of vef1 gene (2633 bp) using the synthesized cDNA as a template with its predicted size (bp). (C-) denoted for negative control where the reverse transcriptase was omitted. M: 1 Kb DNA standard marker.

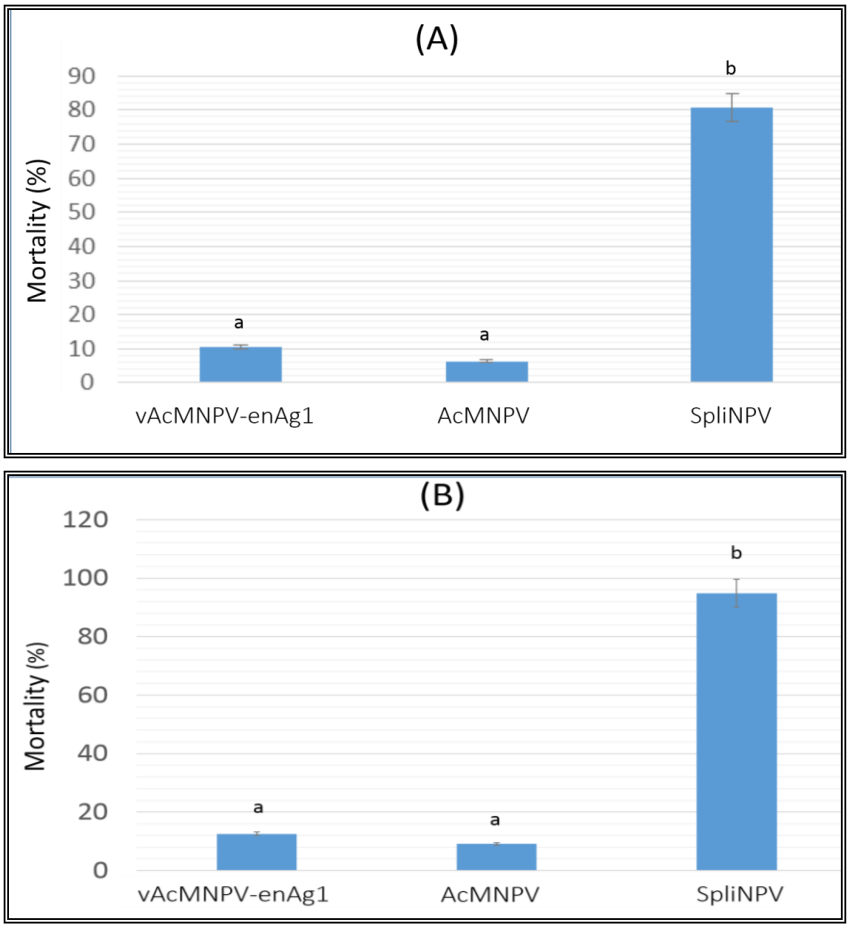

Fig. (5): Comparison of the mortality rates of *S. littoralis* neonates 7 days p.i. using viruses concentration of (A) 1.5 x 10^4 OB/ml and (B) 1.5 x 10^5 OB/ml of the mutant vAcMNPV-enAg1, AcMNPV and SpliNPV. Different superscript letters indicate statistical differences between treatments ($P< 0.05$) using one-way analysis of variance [ANOVA] followed by Tukey's honest significant difference [HSD] test. Standard errors are represent on the top of each bar.