Molecular characterization and biological activity of native *Spodoptera littoralis* nucleopolyhedrovirus isolate

Wael Elmenofy[1]* , Lamiaa El-Gaied[1], Norhan Yasser[1], Engy Osman[2], Samy Sayed[3], Reem M. Abd El-Maksoud[1], El Shaimaa Naguib[4] and Aziza Abdelal[4]

Abstract

Background: *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) is considered a promising biocontrol agent that can be used for the effective control of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), which is an economic pest on many cultivated plants and crops in tropical and sub-tropical regions. The genome of the SpliNPV-AN1956 isolate has been fully sequenced, providing a reference strain for comparison of new isolates. In this study, identification, biological activity, and genetic characterization of a field collected SpliNPV isolate were carried out.

Results: The examination of viral occlusion bodies (OBs) by TEM showed a typical baculovirus OBs of type nucleopolyhedrovirus (NPV) with polyhedral structures. The phylogenetic analysis of the late expression factor- 5 (lef-5) gene as well as the restriction profile analysis confirmed the identity of SpliNPV as a variant isolate. Digestion with *Sca*I endonuclease showed that 4 fragments of 50, 35, 27, and 13 kb were detected but 3.2 kb was absent in SpliNPV-Cab3 pattern compared to the reference strain. Meanwhile, upon digestion with *Pst*I and *Hin*dIII endonucleases, no differences were observed in both isolates' pattern. Moreover, the virulence evaluation against *S. littoralis* 1st instar larvae indicated that LC_{50} value of SpliMNPV-Cab3 was higher than that estimated for the reference strain. Also, ST_{50} of SpliNPV-Cab3 (120 h) was significantly different with that of the reference strain (132 h).

Conclusion: The host specificity based on virulence parameters make SpliNPV-Cab3 isolate a potential candidate to be involved in the integrated pest management strategies for the control of *S. littoralis* population with a prospect to biopesticide development.

Keywords: *Spodoptera littoralis*, Nucleopolyhedrovirus, Transmission electron microscope, Biological activity, Restriction patterns

Background

Different bio-insecticides such as fungi, bacteria, and viruses have been used for many years for insect pest control. Among these bio-insecticides are baculoviruses (Mahendra and Avinash, 2012). Baculoviruses belong to the family Baculoviridae which constitutes large number of DNA viruses that specifically infect insects. Baculoviruses are characterized by having rod-shaped virions ranged in length of 250–400 nm and 30–70 nm in diameter. They have two different phenotypes: occlusion-derived virus (ODV) and budded virus (BV); those are responsible for initiation of infection and spreading of viral infection within the host, respectively. Virions are embedded in a protective proteinaceous matrix that forms polyhedral or occlusion bodies (OBs), the primary infectious form of the virus (Graves et al., 2019).

* Correspondence: wael.elmenofy@ageri.sci.eg; elmenofy10@hotmail.com
[1]Agricultural Genetic Engineering Research Institute, ARC, 9 Gamma St, Giza, Egypt
Full list of author information is available at the end of the article

The Baculoviridae family taxonomically is sub-divided into four genera; Alpha- and Betabaculovirus which are specific for Lepidoptera, Deltabaculovirus specific for Diptera, and Gammabaculovirus specific for Hymenoptera (Harrison et al., 2018). Such viruses are highly specific to their hosts and thus do not cause any pollution to the environment and do not leave residues on fruits which are an important issue of most people (Wang and Hu, 2019). Therefore, Baculoviruses have been recommended by Food and Agriculture Organization (FAO)/World Health Organization (WHO)/as a promising bio-pesticide for the control of different insect pests worldwide. Recently, over 50 baculoviral products have been used as an effective biocontrol agent worldwide against more than 600 insects' species (Wang and Hu, 2019). In addition to use of baculovirus as a biocontrol agent, it was engineered as a protein expression vector. The baculovirus expression vector system is nowadays widely used for production of different recombinant protein in insect cells (Elgaied et al., 2017).

Naturally, the ingested virus OBs are dissolved in the insect midguts under the alkaline conditions to release ODVs, which initiate the infection of midgut columnar epithelial cells. Budded viruses (BVs) are then produced and released from infected midgut epithelial cells to infect other insect tissues which cause systemic infection within the body of the larva (Abbas, 2020). Baculoviruses can encode large number of genes and have adapted mechanisms to regulate the immunity, physiology, and behavior of insect hosts to favor their own distribution and propagation (Wang and Hu, 2019).

The cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is considered as a worldwide critical pest (Mohamed *et al.* 2019). In Egypt, it is a destructive phytophagous of cotton and other field crops, including vegetables, orchard trees, and ornamentals (Hatem et al., 2009). During the last few years, the uncontrollable use of chemical pesticides against *S. littoralis* and other lepidopterous insect pests resulted in the generation of resistance phenomena against many known pesticides, in addition to the pollution of the environment and its toxic effect on animal, human, and plants in contrast to biopesticides (Mahendra and Avinash, 2012). In order to solve these problems, developing of a new and efficient virus-based bioinsecticides is highly important for the effective control of *S. littoralis*. This study aimed to fully characterize a new field collected SpliNPV genotypic variant and to determine its biological activity against *S. littoralis*, as a candidate virus-based bioinsecticide for controlling *S. littoralis* population.

Methods
Virus isolates and insect
Spodoptera littoralis was obtained from the insect rearing facility of the Faculty of Agriculture, Cairo University, Egypt. The larvae were kept at 26±1°C with 60% relative humidity and reared on the artificial diet previously described by Ivaldi-Sender (1974). Neonates and 4th larval instar were used for bioassay experiments and virus propagation, respectively. Virus used in this study is a field collected baculovirus isolate genus nucleopolyhedrovirus from infected dead larva collected from cabbage leaves. In addition, SpliNPV-AN1956 strain was used as a reference.

Virus propagation
For virus propagation, the semi artificial diet described by Ivaldi-Sender (1974) was used. Semi-artificial diet was prepared using the following components: maize meal, Agar-Agar, brewer's yeast, wheat germ, Ascorpic acid, and Nipagien (hydroxybenzoic acid methyl ester). After preparation and diet solidification, tiny cubes of the diet were put into each well of Raster boxes (50-well plates). About 10 µl of each virus suspension was prepared and added on each cube. The *S. littoralis* larvae (4th instar) were placed in every well, then plates were incubated at 26±1°C. One day later, larvae were transferred to virus-free semi artificial diet and reared till observation of viral infection symptoms (about a week post infection). Virus suspension was prepared according to the polyhedral counting (OBs/ml) for each isolate.

Virus occlusion bodies' counting
Viral OBs' purification was carried out according to Boughton et al. (1999). Briefly, infected *S. littoralis* larvae were collected and homogenized using 0.1% SDS, followed by filtration using a piece of cotton and filter paper. After centrifugation of the suspension, the pellets were re-suspended in 0.5 M NaCl and the final pellet contains OBs was re-suspended in suitable volume of ddH2O. The diluted OBs were counted under the dark field, using inverted microscope with ×200 magnification (Axio-VertA1, Zeiss, Germany). Counting was performed using a Petroff-Hauser counting chamber (depth 0.01 mm, Hausser Scientific). The purified virus OBs were kept frozen at −20°C, subsequently, examined under light microscope as well as electron microscope.

EM examination
In order to examine the viral OBs under a transmission electron microscope (TEM), the purified OB suspensions was loaded on carbon coated grib, followed by staining with 2% phosphor tangistic acid before examination under TEM (JEOL model JEM-1200EX II). The sample images were visualized and photographed at different magnification illustrated. The TEM photos provided by a scale were used to measure the sizes of the OBs.

Viral DNA purification

The SpliNPV genomic DNA was isolated from the purified viral OBs according to the method described by Boughton et al. (1999). Briefly, the purified OBs were dissolved, using 1M of Na_2CO_3 to release virions (ODV). Subsequently, ODV were treated with 10% (w/w) SDS, followed by adding 1%. Proteinase K allow genomic DNA to be released. The nucleic acids were collected from cells debris by washing 2 times, using TE-buffer saturated phenol/chloroform 1:1 (v/v). The viral genomic DNA was precipitated using 96% ethanol and 1:10 volume of 3M NaAc pH 5.2. After washing with 70% ethanol, the purified genomic DNA was eluted in suitable amount of ddH2O.

Digestion of viral DNA

For comparison of genome pattern of the SpliNPV isolate with the reference strain, restriction digestion was performed, using *Hin*dIII, *Pst*I, and *Sca*I endonucleases at 37°C for 2. Electrophoresis was performed for the digested samples overnight using 0.8 % agarose in 1X TAE buffer (Boughton et al., 1999).

PCR amplification and sequencing of *lef-5* gene

In order to partially amplify *lef-5* gene, one set of specific primer pair were used. Primers used denoted LEF-5 (ssr1) 5′-AGTCATAAAATCATCGTCGGCG-3′ and LEF-5 (ssr1) 5′-GATTCTCACACGGCGCTCTC-3′. The PCR reactions were performed in a total volume of 25 μl containing the following components: 12.5 μl of EmeraldAmp® GT PCR Master Mix - Takara Bio, 1 μl of each forward and reverse primers, 2 μl of viral genomic DNA template (500ng). Volume was completed to 25 μl using autoclaved ddH$_2$O. The PCR program was initiated at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, 60°C for 1 min and primer extension at 72°C for 45 s. The primer extension was completed at 72 °C for 7 min. PCR-amplicon was electrophoresed in a 1% agarose gel prepared in 1X TAE buffer. The PCR amplicon was visualized using UV-transilluminator. The PCR fragments were purified from agarose gel using Qiaquick PCR purification kit (Qiagene, Germany). Sequence of the purified fragments was performed using Sanger sequence.

Phylogenetic analysis

The partial *lef-5* gene sequence was subjected for alignment with the published ones, using Blast search data base of the National Centre for Biotechnology Information (NCBI). Analysis of the deduced amino acid sequence was carried out, using EditSeq-DNAstar Inc., sequence analysis software, Windows 32 Edit Seq 4.00 (1989–1999). The multiple sequence alignment analysis and the phylogenetic tree were achieved, using Clustal Omega for multiple alignment analysis (The EMBL-EBI search and sequence analysis tools APIs in 2019).

Bioassay

The virulence of both examined and reference isolates was determined, using median lethal concentration (LC_{50}) and median survival time (ST_{50}). LC_{50} was determined by exposure of the 1st instar *S. littoralis* larvae to serial dilutions of virus OBs suspensions of 1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, and 5×10^5 (OBs/ml), in addition to diet mixed with ddH2O instead of virus suspension as a negative control. Each concentration from both isolates was replicated 5 times where each replicate contained 20 larvae. For estimation of LC_{50}, the total dead larvae after 7 days of infection were counted. In order to determine viruses' speed of kill, the ST_{50} was estimated.by inoculating 30 individuals of *S. littoralis* (5 days old) with 3 replicates and calculated LC_{85} for each tested viral isolate. Control plates were mixed with water instead of virus suspension. Mortality was observed 12-h intervals starting at the 3rd after infection till the 10th day or larval death.

Statistical analysis

The LC_{50} and slopes of tested viral isolates were estimated using EPA Probit analysis program (Version 1.5) (Robertson and Preisler, 1992). Also, the ST_{50} was estimated. Data analysis was performed, using the Kaplan-Meier survival time estimator analysis (Kaplan-Meier, 1958).

Results

SpliNPV-Cab3 OBs EM examination

Typical viral infection symptoms were detected 5–7 days post infection, using purified viral OBs suggested the specificity of the collected isolate to *S. littoralis* larvae. The viral OBs were examined under a light and transmission electron microscope (TEM). Microscopic examination revealed clear polyhedra in all surfaces. Furthermore, transmission electron microscopic analysis revealed that the examined viral OBs are a typical baculovirus OBs of type nucleopolyhedrovirus (NPV) with polyhedral structures (Fig. 1).

Restriction analysis of SpliNPV-Cab3

The analysis was carried out by comparing additional fragments present in relation to number of common fragments of examined isolates generated by restriction endonucleases. The results showed some differences among the restriction patterns of the SpliNPV-Cab3 isolate analyzed by digestion, using *Sca*I endonuclease (Fig. 2). Besides 15 *Sca*I fragments common in both isolates, there were one more fragment of approximate molecular weight of 50 kb and three
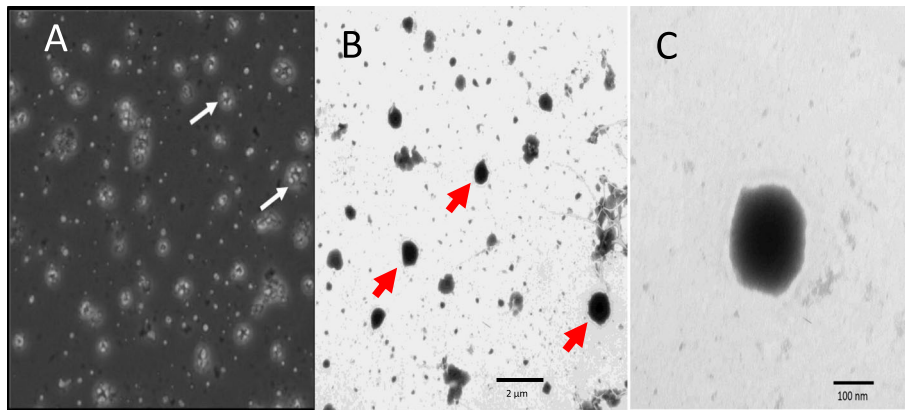
Fig. 1 Electron micrographs of *Spodoptera littoralis* SpliMNPV-Cab3 OBs. **a** Light microscope examination showing the SpliNPV-Cab3 occlusion bodies. **b** Transmission electron micrograph showing the SpliNPV-Cab3 occlusion bodies as indicated by red arrows. Bar = 2 μm. **c** Viral occlusion body showing typical NPV polyhedral shape. Bar = 100 nm
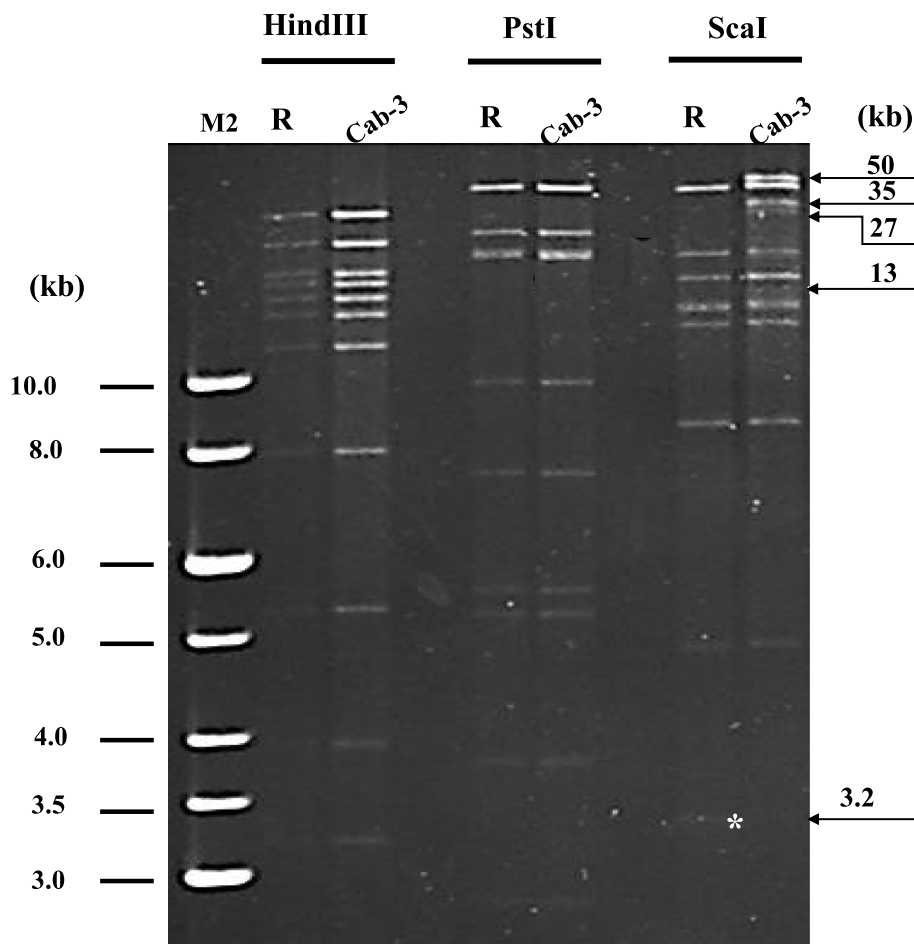
Fig. 2 Restriction fragment pattern of SpltNPV-Cab3 and SpltNPV-AN1956 DNA using *Hind*III, *Pst*I, and *Sca*I endonucleases. Restriction pattern of both SpliMNPV-Cab3 (Cab-3) and SpliMNPV-AN1956 (R). M1 represents lambda *Hind*III ladder. M2 represents 1kb DNA ladder. Fragments are indicated by kilo base (kb)

different fragments of about 35 kb, 27 kb, and 13 kb were detected in SpliNPV-Cab3 pattern. However, a fragment of about 3.2 kb was absent in SpliNPV-Cab3 compared to the reference strain *Sca*I-pattern. Upon digestion using *Pst*I and *Hin*dIII endonucleases, no differences were observed in both isolates pattern.

Phylogenetic analysis

The partial coding sequence of the *lef-5* as a baculovirus core gene was obtained for SpliNPV-Cap3 (Accession No: MW713059) and compared with the reference strain, SpliMNPV-AN1956 (Accession No: NC_038369) and other NPVs located in GenBank database using the *lef-5* deduced amino acids. The phylogenic tree was constructed from the deduced amino acids via the Clustal Omega (Sievers *et al.*, 2011), using the mBed algorithm (Blackshields *et al.*, 2010).

As shown in Fig. 3, the SpliNPV-Cap3 deduced amino acids sequence of lef-5 was closed and had a common ancestor to the sequence of SpliMNPV-Tun2 (Identity: 95%) (Accession No.AYU75273), as well as SpliMNPV-AN1956 (Identity: 95%) previously published by Breitenbach et al. (2013) (Accession No. YP_009505893), which belongs to Group II NPVs. Based on the distance to these viruses, SpliNPV-Cab3 may be regarded as a variant virus isolate of SpliNPV-AN1956.
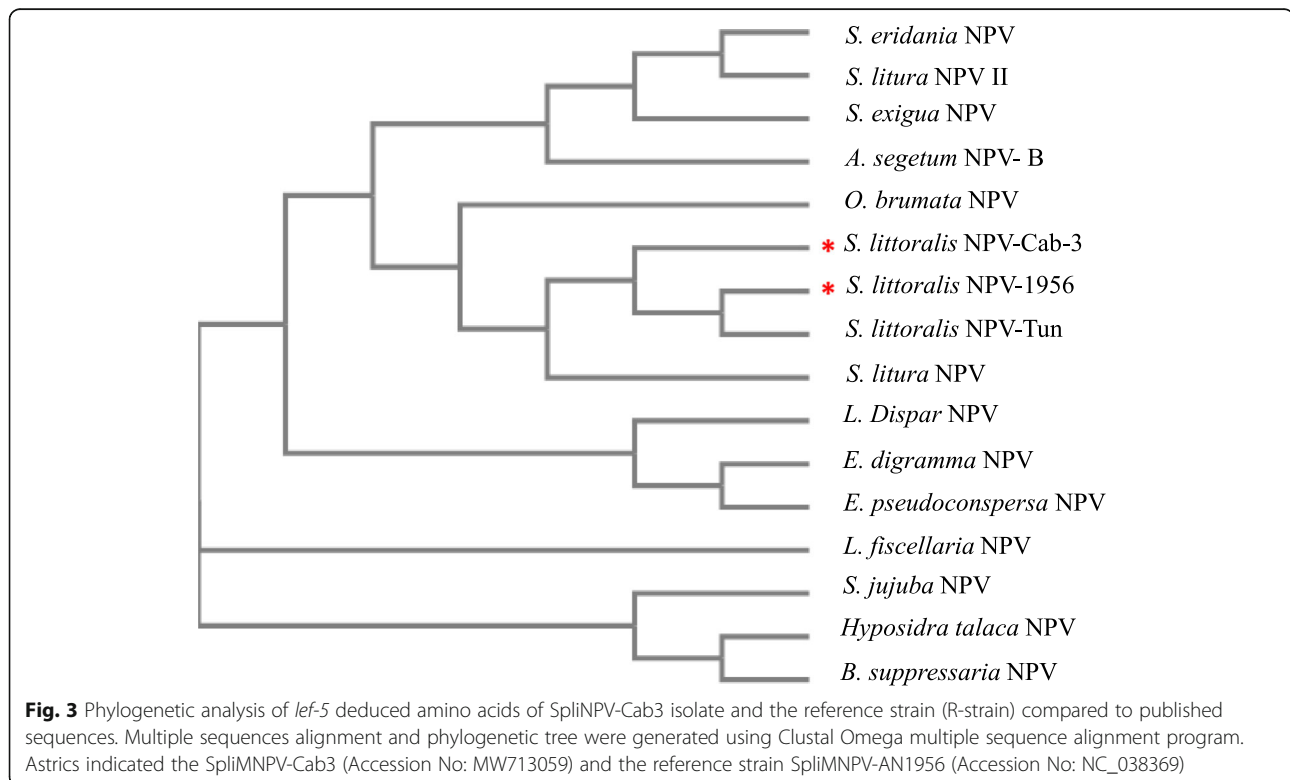
Median lethal concentration (LC$_{50}$)

Biological activity of SpliMNPV-Cab3 isolate was tested via measuring their median lethal concentration (LC$_{50}$) against *S. littoralis* 1st instar larvae. The LC$_{50}$ values and slopes are given in Table 1. All Wald chi-square (χ^2) tests (df=1) were significant at P <0.0001 according to Robertson and Preisler (1992). Analysis showed that LC$_{50}$ value of SpliMNPV-Cab3 was 1.1×10^5 OBs/ml, which was significantly higher than that estimated for the reference strain (1.6×10^4 OBs/ml) (t=8.403; df=1,8; P <0.001).

Median survival time (ST$_{50}$)

The ST$_{50}$ of tested isolate SpliNPV-Cab3 (120 h) was significantly different than the reference strain (132 h). The calculated ST$_{50}$ value was 120 h for SpliMNP-Cab3, which was lower speed of kill than the reference strain (132 h) (t =3.211; df =1,4; P <0.033) (Table 2). There was no obvious difference in the disease symptoms on the infected *S. littoralis* larvae either with SpliNPV-Cab3 or the reference strain during the bioassay.

Discussion

Development of effective biopesticides for the sustainable control of *S. littoralis* is highly important to control such insect pest which is considered as one of the most economic phytophagous insect pests (Wang and Hu 2019). The wild types of baculovirus isolates with high

Fig. 3 Phylogenetic analysis of *lef-5* deduced amino acids of SpliNPV-Cab3 isolate and the reference strain (R-strain) compared to published sequences. Multiple sequences alignment and phylogenetic tree were generated using Clustal Omega multiple sequence alignment program. Astrics indicated the SpliMNPV-Cab3 (Accession No: MW713059) and the reference strain SpliMNPV-AN1956 (Accession No: NC_038369)

Table 1 Median lethal concentration (LC$_{50}$) values for 1st larval instar of *Spodoptera littoralis* infected with OBs derived from SpliMNPV isolates 7 days post infection.

Virus isolate	No. of larvae	LC$_{50}$ (CI)	Slope ± SE	χ^2 values
Cab-3	744	1.1×10^5 (8.7×10^4 to 1.4×10^5)	1.5 ± 0.14	3.85
R-Strain	945	1.6×10^4 (3.5×10^3 to 3.5×10^4)	1.5 ± 0.32	19.6

Cab3 SpliNPV-Cab3 isolate, *R-Strain* reference isolate (SpliNPV-AN1956), *CI* 95% confidence intervals limits

infectivity (low LC$_{50}$), high virulence (low ST$_{50}$), and high OBs production could be an ideal component for pest management in an approach to control the *S. littoralis* population. In this study, a new field collected SpliNPV isolate was characterized by means of restriction endonuclease digestion, phylogenic analysis, and biologically in terms of LC$_{50}$ and ST$_{50}$. The light microscopic and TEM study of the examined isolate revealed typical baculovirus occlusion bodies of nucleopolyhedrovirus with polyhedral structure shape and sizes ranging from 900 to 1500 nm (Blissard and Rohrmann, 1990).

For the genetic variation between SpliMNPV-Cab3 and the reference strain SpliMNPV-AN 1956 with *Sca*1 enzyme, different sub-molar bands were observed in SpliMNPV-Cab3, which were missing in SpliMNPV-AN1956. However, upon digestion with *Pst*I and *Hin*dIII endonucleases, both isolates' profiles were identical. The combination resulting of the DNA analyses with the 3 restriction enzymes indicates that this isolate may comprised genotypic variant of the Egyptian isolate SpliMNPV-AN1956. These data suggested that the characterized SpliMNPV-Cab3 isolate belongs to the Egyptian SpliNPV-B that was studied and identified based on the genetic variation of NPVs infecting *S. littoralis* (Takatsuka et al., 2003).

In addition, and in order to track the evolution pattern of the SpliMNPV-Cab3 isolate, the partial coding sequence of the *lef-5* gene was obtained and compared to other *lef-5* genes from NPVs, using the lef-5 deduced amino acids. The deduced amino acid sequences of Lef 5 protein showed that SpliMNPV-Cab3 is closed to the isolate SpliNPV-1956 as well as SpliNPV-Tun2, suggested that it is a member of the group Nucleopolyhedrovirus group II (NPV II) (Herniou and Jehle, 2007). The NPVs were classed into groups I and II based on the building of the phylogenetic relationships of

polyhedrin genes. However, the *Polh* gene probably has suffered horizontal transfer between different NPVs and therefore is suboptimal for phylogenetic reconstructions (Herniou et al., 2001). Therefore, partial coding sequence of *lef-5* core gene was used to reconstruct the phylogenetic relationships of *lef-5* genes. According to our reconstruction, SpliMNPV-Cab3 could be placed in Group II, which is in accordance with the findings of Bulach et al. (1999) obtained for other isolates of SpliMNPV.

In regard to the biological activity of the examined isolates, the obtained LC$_{50}$ for SpliNPV-Cab3 was slightly higher than that estimated LC$_{50}$ for other 2 field collected SpliNPV isolates; those were 3×10^4 OBs/ml and 9.5×10^4 OBs/ml for isolates Spli-6 and Spli-7, respectively (Elmenofy et al., 2020). As well as *Spodoptera litura* NPV isolate, which was 1×10^3 OBs/ml against *S. litura* against 1st larval instar (Trang and Chaudhari, 2002). However, it was very close to Kumar et al. (2011) results who found that the LC$_{50}$ of two different SpltNPV virus-specific isolates were 3.5×10^4 and 2.4×10^5 (OBs/ml) against 2nd and 3rd larval instars of *S. litura*, respectively. In the same context, the estimated LC$_{50}$ for SpliNPV-Cap3 isolate was higher than that obtained by Seufi (2008), who examined the activity of SpliMNPV, an Egyptian isolate, against *S. littoralis* 2nd larvae (1.2×10^3 OBs/ml). On the other hand, the LC$_{50}$ of an Egyptian isolate of *S. littoralis* NPV denoted Egy-SlNPV against *S. littoralis* larvae were 1.28×10^6 (OBs/ml) and 2.53×10^7 (OBs/ml) for the 2nd and 4th larval instars of *S. littoralis*, respectively (Ahmed et al., 2016). These results confirmed that the earlier larval instar of *S. littoralis* larvae were more sensitive to Egy-SlNPV than the elder ones. These differences in susceptibilities may be due to the variation in the number of virions included in occlusion bodies, method of surface treatment, larval age, and the feeding habit of the insect (Seufi, 2008).

The calculated ST$_{50}$ for SpliNPV-Cab3 isolate was 120h compared to the reference strain, which was 132h. This observation elucidated that the SpliNPV-Cab3 isolate significantly had a speed of kill slightly faster than SpliNPV-AN1956. On the same context, the obtained ST$_{50}$ showed that SpliNPV-Cab3 isolate speed of kill was lower than the ST$_{50}$ of other SpliMNPV isolates estimated by Toprak et al. (2005), which varied between 72

Table 2 Kaplan-Meier estimation of ST$_{50}$ for *Spodoptera littoralis* larvae infected with OBs derived from two SpliNPV strains in a single infection

Virus isolate	Nr. larvae	LC$_{85}$ (OB/ml)	ST$_{50}$ (h)	CI (lower-upper)
Cab-3	102	5.3×10^5	120	115 – 125
R-Strain	101	7.2×10^4	132	129 – 137

Cab3 SpliNPV-Cab3 isolate, *R-Strain* reference isolate (SpliNPV-AN1956), *CI* 95% confidence intervals limits

and 84 h. Also, the calculated ST_{50} value of isolate SpliNPV-Cab3 was 120 h, which was higher than the ST_{50} of SpltNPV-Pak-BNG that was 84 h. This was compared with Japanese SpltNPV-G1 isolate originated from *S. littoralis* in which the ST_{50} was 108 h for 3rd larval instar of *S. litura* (Ali et al. 2018). These findings suggested that the speed of kill of the tested isolates against *S. littoralis* larvae may vary according to the collected location and instars of the target insect host.

Conclusion

The restriction pattern and the phylogenetic analysis showed that SpliNPV-Cab3 indigenous isolate may be regarded as a variant isolate of SpliNPV-AN1956. The SpliNPV-Cab3 isolate achieved significantly higher LC_{50} value than that of the reference strain. Meanwhile, ST_{50} of SpliNPV-Cab3 (120h) was significantly lower with that of the reference strain (132 h). Therefore, this indigenous strain could be involved in integrated pest management programs for the control of *S. littoralis* larvae. Furthermore, addition of some reagents to the virus-based formula such as optical brightener could be a useful tool to reduce the quantity of viral inoculum OBs required to achieve a high degree of *S. littoralis* control, in addition to reduce the effects of UV-Irradiation on virus OBs. Other investigations regarding virus-host interaction could help to examine the effect of introduction of multiple isolates on the control of such insect pest.

Abbreviations
SpliNPV: *Spodoptera littoralis* nucleopolyhedrovirus; OBs: Occlusion bodies; LC_{50}: Median lethal concentration; ST_{50}: Median survival time; TEM: Transmission electron microscope

Authors' contributions
WE designed the study and performed the statistical analysis. EO collected the field infected insect samples and did the virus pre-propagation experiments and purification. LA did the virus purification, virus examination under light microscope and electron microscope, and wrote the original draft. NY and RA did the molecular biology work as well as the bioassay experiments. EN reared *S. littoralis* larvae under directed supervision of AA; in addition, they shared in virus isolates' propagation. All authors read and approved the final manuscript. WE and SS proofread the manuscript.

Availability of data and materials
All data generated or analyzed in this study are available in this published manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This study does not contain any individual person's data.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Agricultural Genetic Engineering Research Institute, ARC, 9 Gamma St, Giza, Egypt. [2]Department of Genetics, Faculty of Agriculture, Cairo University, Giza 12613, Egypt. [3]Department of Science and Technology, University College-Ranyah, Taif University, B.O. Box 11099, Taif 21944, Saudi Arabia. [4]Plant Protection Institute, Agricultural Research Center, Douki, Giza, Egypt.

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