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Detection, characterization and virulence analysis of nucleopolyhedrovirus isolated from the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

Yasmein A. El Sayed¹, Samy Sayed², Aml Magdy³ and Wael Elmenofy^{4*}

Abstract

Background: In the present study, detection, characterization and virulence analysis of a field collected nucleopolyhedrovirus isolated from the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) was carried out. The obtained isolate, named SpliNPV-YW, was collected from diseased *S. littoralis* larvae in El-Menoufia governorate, Egypt.

Results: Transmission electron microscopy showed the presence of typical occlusion bodies with average size of $(1.06 \times 1.19 \mu\text{m})$. Upon digestion using two different endonucleases, *Pst*I and *Sca*I, no clear difference was detected in the collected isolate (SpliNPV-YW) DNA genome pattern compared to the reference strain SpliNPV-AN1956. The evolutionary analysis of the polyhedrin gene's partial nucleotide sequence revealed that SpliNPV-YW isolate was closed and had a genetic origin with the NPV isolate SpliMNPV-A26-5 that belongs to group II NPVs with identity of 99.7%. The median lethal concentration (LC_{50}) and the median lethal time (LT_{50}) values were estimated for second and fourth larval instars of *S. littoralis*. The LC_{50} values were 2.8×10^4 OB/ml for second larval instar and 5.2×10^5 OB/ml for fourth larval instar after 10 days of treatment. Regarding the speed of killing of the viral isolate, the results showed that the LT_{50} value for the second instar larvae ($LT_{50} = 5.5$ days) was lower than that of the fourth instar larvae ($LT_{50} = 6.2$ days) at concentrations of 4.3×10^{10} (ob/ml) and 1.2×10^{11} (ob/ml) for second and fourth instar larvae, respectively.

Conclusions: Host specificity and virulence characteristics make SpliNPV-YW isolate a good potential to be utilized as a candidate biopesticide for the control of *S. littoralis* population in Egypt.

Keywords: *Spodoptera littoralis*, Nucleopolyhedrovirus, Bioassay, Biological control, Phylogenetic tree

Background

Chemical pesticides are widely regarded as environmentally unacceptable, despite their widespread use in different countries all over the world. As a result, social pressure is dramatically increased to gradually replace them with environmental friendly biopesticides that

are safe for both humans and nontarget organisms (Erlandson 2008). The cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is a highly dangerous insect pests that can infest different plant families. In Egypt and during the last decades, cotton plants, as well as other economic crops, have dramatically suffered significant from yield losses as a result of such insect pest infestation (Moussa et al., 1960). Due to the uncontrollable using of chemical insecticide by farmers worldwide, *S. littoralis* populations have developed accelerated resistance to different chemical pesticides such as: organophosphate, carbamates, and

*Correspondence: wael.elmenofy@ageri.sci.eg

⁴ Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza 12619, Egypt

Full list of author information is available at the end of the article

pyrethroid pesticides. In addition, *S. littoralis* was also uncontrollable completely based on other bioagents such as *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) (Ghulam et al. 2017). Alternative methods for the control of *S. littoralis* that are consistent with integrated pest management (IPM) approach have been identified as a natural way to lessen the environmental impact of crop protection techniques (Tanada and Kaya 1993). Because of its safety to the ecosystem, mammals, natural enemies of pests and plants, baculoviruses are considered as the most widely explored insect virus family in terms of its development as a viral-based bioinsecticide. These viruses are typically highly selective, causing no harm to other insect species. As a result, baculoviruses are ideal control agents to be used in agriculture, forests, and pastures as part of IPM programs. The Baculoviruses have circular genome with double-stranded DNA that range in length from 80 to 180 kbp and encoding for up to 180 proteins. The virus produces in the nuclei of infected host cells. Nowadays, with the development of next generation sequencing tools (NGS), more than 90 baculovirus genomes have been fully sequenced. It is highly suggested that these bioagents can be used as microbial insecticides for the effective and sustainable control of different insect pests (Wang and Hu 2019). *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) is taxonomically belongs to the lepidopteran-specific baculoviruses Group II. The virus's host range has been determined to be limited, with the virus most likely infecting *Spodoptera* spp. at a slow rate of infection.

One of the most problematic aspects of employing SpliNPV natural isolates as a biocontrol agent capable of competing with wide synthetic pesticides for the effective management of *S. littoralis* is the lack of genetic diversity as well as their slow speed of action (Wang and Hu 2019). Therefore, and in order to address this issue, the development of a new and effective biocontrol product to be used in IPM programs of *S. littoralis* is critical. As a result, the primary goal of this research was to investigate the biological activity and molecular properties of a baculovirus isolate collected from diseased *S. littoralis* larvae from pepper cultivar in Egypt as a promising candidate virus-based bio-agent.

Methods

Insect

Larvae of the cotton leaf worm, *S. littoralis*, were received from the Plant Protection Institute, Agriculture Research Center's insect rearing facility. The larvae

were reared on a semi-artificial diet reported by Martins et al. (2005) at 27 °C and 60% RH.

Virus propagation

The nucleopolyhedrovirus characterized in this study was a field collected isolate from pepper cultivar in El-Menoufia Governorate, whereas *S. littoralis* larvae showed viral infection symptoms.

Diseased *S. littoralis* larvae were brought to the laboratory and examined by a light microscope for the presence of occlusion bodies (obs). The late second instar *S. littoralis* larvae were placed in 50-well plate, with a small piece of the insect medium, then, 1–10 viral diluted obs was prepared from previously field collected samples and used to inoculate each piece of medium (5 µl/each). The larvae were kept on virus-free medium and reared at 26 °C until appearance of infection symptoms or larvae death.

Viral occlusion bodies' purification

The diseased *S. littoralis* larvae were collected for occlusion bodies' purification using the methods of Boughton et al. (1999). In brief, the diseased larvae were grinded in 0.1% SDS and subsequently obs were purified via piece of cotton, followed by pelleting of viral obs. The pelleted obs were washed twice using 0.5% SDS and one time using 0.5% NaCl. The purified obs were re-suspended in suitable amount of ddH₂O and kept at – 20 °C for downstream work.

Transmission electron microscopic (TEM)

For ultra-structural analyses, isolated viral obs were subjected for transmission electron microscope (TEM) examination according to Bozzola and Russell (1992). Purified viral obs were examined under TEM. Viral obs were fixed for 24 h at 4 °C in 2.5 percent (v/v) glutaraldehyde and 0.05 M phosphate buffer (pH 7.2) before being post fixed for 2 h in 0.5% (v/v) aqueous osmium tetroxide with the same buffer. After that the sample was dehydrated in a series of graded alcohol and embedded in epoxy resin. The uranyl acetate and lead citrate were used to stain ultrathin sections prepared as 75–90 µm thickness. Then, the samples examined at TEM lab FA-CURP by transmission electron microscope JEOL (JEM-1400 TEM), Research Park, Faculty of Agriculture, Cairo University, Egypt.

Extraction of virus genomic DNA

Viral genomic DNA was purified from viral obs using 0.1 M Na₂CO₃ as described by Boughton et al (1999). The final mixture includes 1% and proteinase K in a final concentration of 250 µg/ml. Viral genomic DNA was purified using phenol–chloroform extraction and ethanol

precipitation. The DNA pellet was eluted in about 50 μ l of autoclaved ddH₂O.

Restriction profile analysis

Purified viral genomic DNA was subjected for restriction digestion analysis using two different restriction enzymes; *Pst*I and *Sca*I. For restriction profile separation and visualization, digested DNA fragments were separated overnight via 0.8% agarose gel according to Boughton et al. (1999).

PCR amplification of polyhedrin gene (polh)

Two specific primers, synthesized based on SpliMNPV-AN1956 isolate (accession No. YP_009505893), were used to amplify small polyhedrin gene fragment of about 560 bp for nucleotide sequencing. The conditions of PCR amplification as follow: incubation was performed for 3 min at 95 °C, followed by 30 cycles at 95 °C for 1 min and at 56 °C for 1 min. The PCR amplicon was electrophoresed in 1% agarose gel, purified and cloned into GenJET PCR cloning vector, following the manufacturer instruction (Thermo Fisher). The ligation mixture was used directly for transformation into DH10 β competent cells. The positive plasmid carrying polyhedrin gene fragment was subjected directly to nucleotide sequences with the Sanger sequence.

Phylogenetic analysis

Retrieve of the published polyhedrin gene nucleotide sequences was carried out with the Blastn of the National Center for Biotechnology Information (NCBI). The parameters of the probability distribution were estimated using the maximum likelihood method. Sixteen partial polyhedrin nucleotide sequences were used to construct the phylogenetic tree using the Molecular Evolutionary Genetics Analysis (MEGA-X) (Kumar et al. 2018).

Determination of virus LC₅₀ and ST₅₀ values

Laboratory bioassay experiments were conducted using purified viral obs. Firstly, the viral obs were enumerated using Neubaur Hemocytometer (Alves and Moraes 1998) and five concentrations (10³, 10⁴, 10⁵, 10⁶ and 10⁷ OB/ml) were prepared from the virus obs mother suspension by serial dilution, which were used against both of second and fourth larval instars of *S. littoralis* with the method of diet surface treatment. The two tested larval instars were starved for 8 h prior to virus feeding, then put in plastic cups contain contaminated semi-artificial diet each with 50 μ l of the tested virus concentration prepared previously. Two days post infection, the larvae were transferred to clean cups contain fresh diet and dead larvae were daily monitored until day 10 post treatment. The control plates contained the diet were treated

with distilled water instead of virus suspension. Thirty larvae for each concentration were triplicate in addition to larvae for the control plates. In addition, the LT₅₀ value was calculated by infection of 5 day's old *S. littoralis* larvae using LC₈₀ value. Mortality was monitored two times daily until larval death or pupation.

Statistical analysis

Mortality data were analyzed by the Ldp Line software according to Finney (1971), in which the recorded mortality data between 20 and 80% with the virus concentrations after 10 days of infection were used to calculate LC₅₀ values. In addition, LT₅₀ values for the second and fourth larval instars of *S. littoralis* were also determined at the concentrations caused mortalities between 20 and 80%

Results

Virus isolation and propagation

The SpliNPV isolate, named SpliNPV-YW, was isolated from diseased *S. littoralis* larvae exhibited viral-like symptoms previously obtained from El-Menoufia Governorate Egypt. Slow motion and larval enlargement by virus-containing fluid were observed in infected *S. littoralis*. The SpliNPV-YW was propagated using 25 *S. littoralis* larvae. As shown in Fig. 1, infected *S. littoralis* larvae showed viral infection symptoms 10 days p.i, indicating that this isolate was baculovirus-specific to *S. littoralis*. The purified obs were subjected for counting under the dark field using the inverted microscopy. The total counting of the SpliNPV-YW obs were found to be 1.2 \times 10¹⁰ obs/ml.

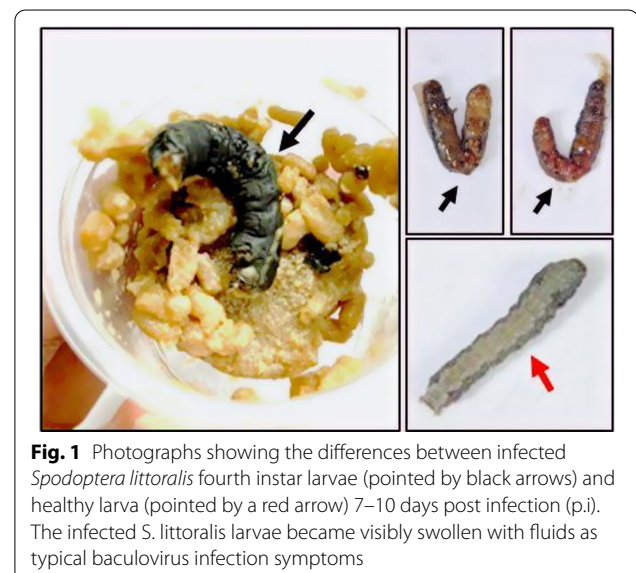


Fig. 1 Photographs showing the differences between infected *Spodoptera littoralis* fourth instar larvae (pointed by black arrows) and healthy larva (pointed by a red arrow) 7–10 days post infection (p.i). The infected *S. littoralis* larvae became visibly swollen with fluids as typical baculovirus infection symptoms

Transmission electron microscope

The TEM results revealed several single and multiple nucleocapsid scattered in the obs. They were bacilliform shaped measuring about 233 nm in length and 70 nm in width. The size of the obs was around $1.06 \times 1.19 \mu\text{m}$ (Fig. 2).

Restriction endonuclease analysis (REN)

Analysis of the SpliNPV-YW genomic DNA pattern was carried out to verify the heterogeneity of SpliNPV-YW profile compared to the reference strain SpliNPV-AN1956. As shown in Fig. 3, upon digestion with *Pst*I and *Sca*I, the genomic DNA patterns were compared to the SpliNPV-AN1956 digested with the same enzymes. Regardless of the enzyme, no clear polymorphism pattern was observed between both. This may be attributed to the high similarity between both isolates.

PCR amplification of *polh* gene

Using polyhedrin specific PCR primers, the PCR amplicon showed that the fragment size of the polyhedrin gene was 560 bp (Fig. 4). Obtained fragment was subjected to Sanger nucleotide sequencing and deposited in the GenBank with the accession number of OM256472.

Phylogenetic analysis

The nucleotide sequence of the *polh* gene as a baculovirus core gene was compared to the reference strain of SpliMNPV-AN1956 and other NPVs in the GenBank database for building the phylogenetic tree. As shown in

Fig. 5, the obtained SpliNPV-YW nucleotide sequence of *polh* gene was much closed and had a common ancestor with the sequence of SpliMNPV-A26-5 isolate (Identity: 99.7%) (Accession No. AY706717.1), and SpliMNPV-AN1956 (Identity: 99.4%) (Accession No. YP_009505893) (Breitenbach et al. 2013).

Insect bioassay

The virulence of SpliNPV-YW isolate was bio-assayed against the second and fourth larvae instars of *S. littoralis*, using five different concentrations (10^3 , 10^4 , 10^5 , 10^6 and 10^7 OB/ml) by diet surface treatment method. The calculated LC_{50} value was 2.8×10^4 OB/ml for second instar larvae, and 5.1×10^5 OB/ml for fourth instar larvae 10 days p.i (Table 1). In order to examine the viral speed of mortality, the time required to kill 50% of the two tested instars was also calculated. As shown in Table 2, the LT_{50} values were 5.5 days at concentration of 4.3×10^{10} ob/ml and 6.2 days at concentration of 1.2×10^{11} ob/ml for second and fourth larval instars, respectively.

Discussion

This work was performed in order to investigate the molecular characteristics and the biological activity of *S. littoralis* NPV isolated from *S. littoralis* infected larvae. Upon purification of viral obs, the purified obs were subjected to examination under transmission electron microscope analysis (TEM). The results showed that the obs size located in the same range of all

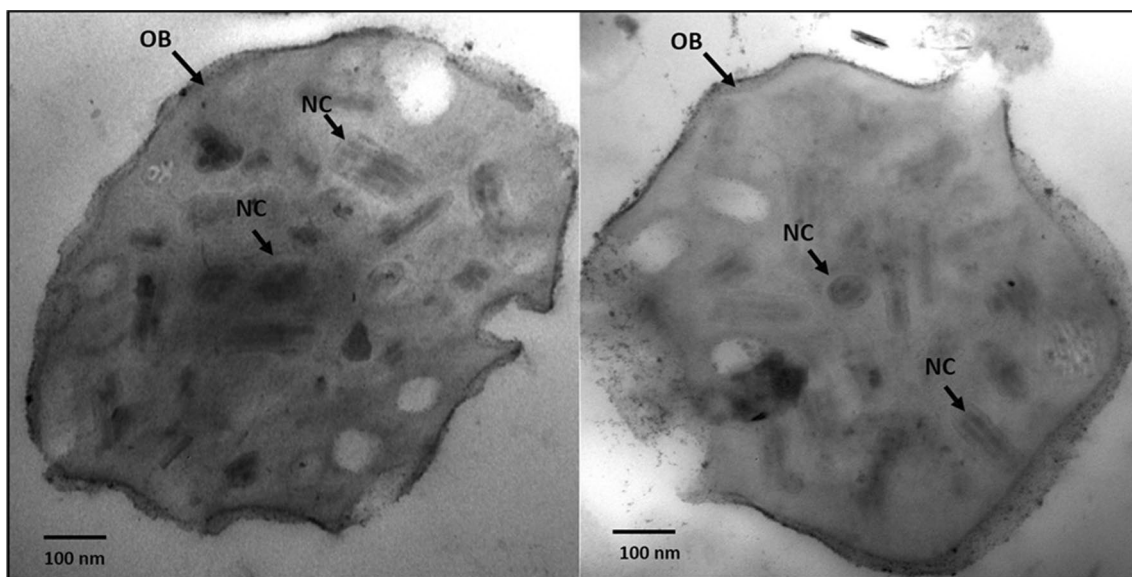
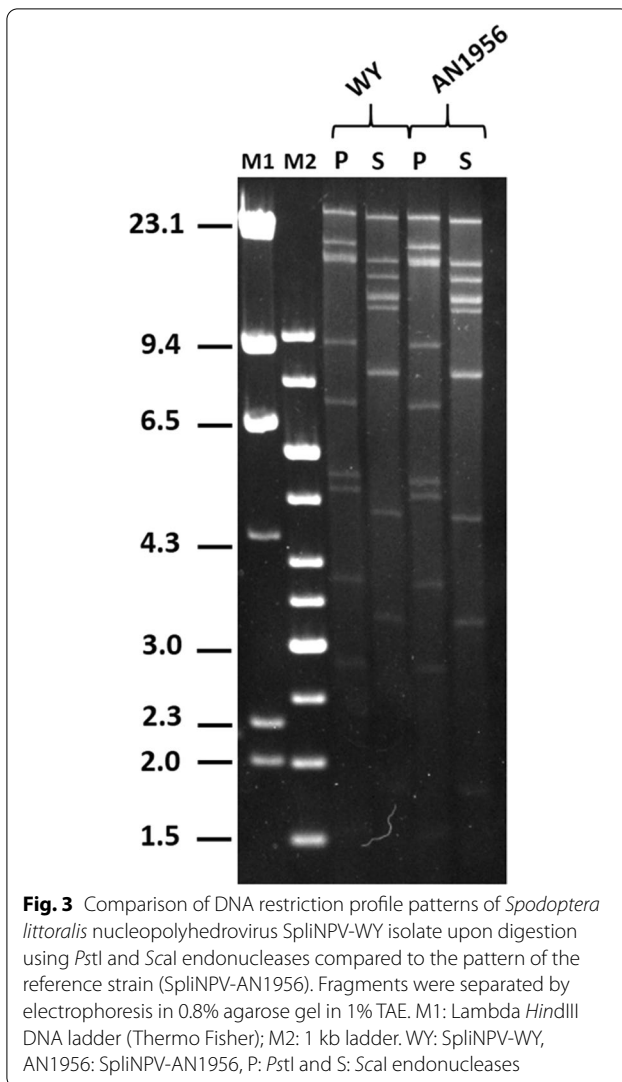
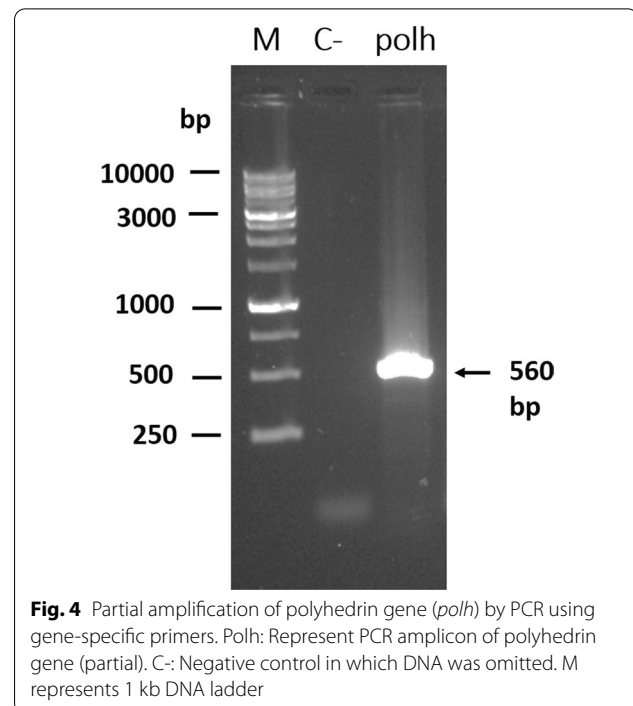


Fig. 2 Transmission electron micrographs of ultrathin section of obs obtained from nucleopolyhedrovirus infected *Spodoptera littoralis* larvae (magnification = 148000 \times), showed single and multiple nucleocapsid (NC) scattered in the Occlusion Body (OB)



baculovirus isolates obs sizes whereas all known baculovirus isolates had a rod shaped nucleocapsid with 200–450 nm (in length) and 30–100 nm (in width). The present results confirmed the identity of the baculovirus isolate SpliNPV-WY as nucleopolyhedrovirus. The restriction pattern generated either by *Pst*I and *Sca*I for the viral isolate genomic DNA was similar to the corresponding pattern of the Egyptian isolate SpliNPV-1956 (Breitenbach et al. 2013), as well as the SINPV type B (Takatsuka et al. 2003). The single nucleotide polymorphisms (SNPs) may represent the genetic variation between these isolates. Hence, we propose that this isolate is SpliNPV-1956 variant and is a type B SINPV closely related to the Egyptian isolates. The reduction of genetic variability might dramatically affects the diversity and a population's ability to adapt, subsequently the likelihood of extinction will be increases for the smaller populations (Allendorf and Lundquist



2003). As previously reported, based on the evolutionary relationships of polyhedrin genes and on deferential relative rate of evolution, the NPVs were divided into two groups: I and II. The partial sequence of the polyhedrin gene was compared to other polyhedrin genes from NPVs to estimate the evolution pattern of the SpliMNPV-YW isolate. The generation of the phylogenetic tree of NPVs *polh* gene indicated that SpliMNPV-YW was closed to SpliMNPV-A26-5 isolate (Identity: 99.7%) and by identity of 99.4% with SpliNPV-1956 isolate, which confirming that it is belongs to the nucleopolyhedrovirus group II (NPV II) (Herniou and Jehle 2007). According to the data obtained from the phylogenetic analysis, the obtained SpliNPV-YW may be considered as a variant isolate of the SpliNPV-AN1956. However, this finding should be interpreted with caution, as subsequent research has revealed that the *polh* gene has likely undergone horizontal transfer across various NPVs (Herniou et al. 2001). On the other hand, the calculated LC_{50} value was 2.8×10^4 OB/ml for second instar larvae, and 5.1×10^5 OB/ml for fourth instar larvae 10 days p.i. The LC_{50} for the investigated isolate (SpliNPV-YW) was lower than that previously estimated for the 2 SpliNPV isolates (3×10^4 OBs/ml and 9.5×10^4 OBs/ml) collected from different districts in Egypt (Elmenofy et al. 2020). Moreover, the LC_{50} value for the current isolate was lower than that of isolate SpliNPV-Cab3 which was 1.1×10^5 OBs/ml (Elmenofy et al. 2021). The discrepancies in

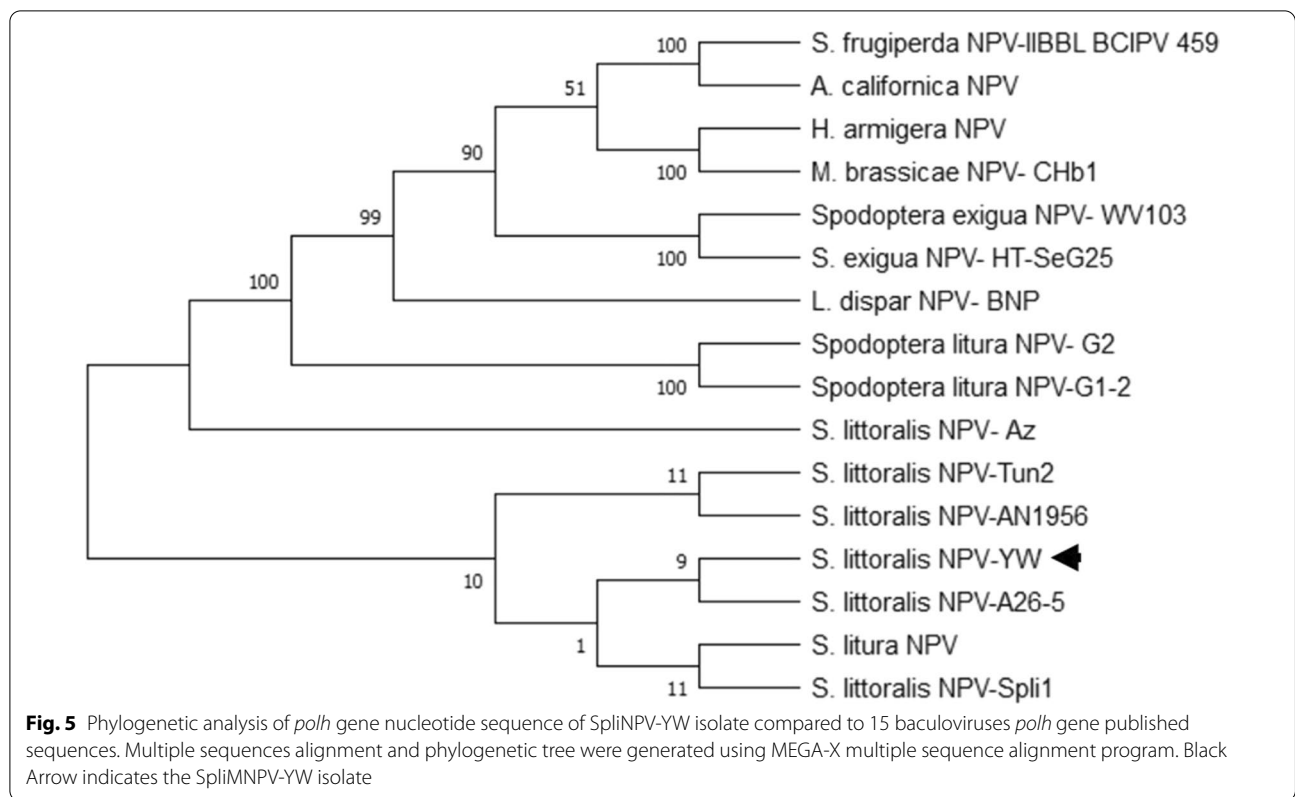


Table 1 Median lethal concentration (LC₅₀) of SpliNPV-WY virus infection against second and fourth larvae instar of *Spodoptera littoralis* after 10 days SpliNPV-WY viral infection

The second instar larvae			The fourth instar larvae		
LC ₅₀ (CI) (ob/ml)	Slope ± SE	χ ²	LC ₅₀ (CI) (ob/ml)	Slope ± SE	χ ²
2.8 × 10 ⁴ (1.5 × 10 ² – 3.1 × 10 ⁵)	0.2292 ± 0.0465	0.3064	5.1 × 10 ⁵ (3.3 × 10 ⁴ – 2.3 × 10 ⁶)	0.2579 ± 0.0442	1.1097

Table 2 Median lethal time (LT₅₀) of *Spodoptera littoralis* calculated at the concentrations of 4.3 × 10¹⁰ and 1.2 × 10¹¹ ob/ml for second and fourth larvae instars, respectively

The second instar larvae (at concentration 4.3 × 10 ¹⁰ (ob/ml))			The fourth instar larvae (at concentration 1.2 × 10 ¹¹ (ob/ml))		
LT ₅₀ (CI) days	Slope ± SE	χ ²	LT ₅₀ (CI) days	Slope ± SE	χ ²
5.5 (5–5.9)	4.4114 ± 0.4808	2.87	6.2 (5.7–6.7)	4.1944 ± 0.4736	4.64

susceptibilities could be attributed to variances in the quantity of virions in occlusion bodies, the method of surface treatment, larval instar, and the insect’s eating habits (Seufi 2008). Interestingly, it was closed to the LC₅₀ of *Spodoptera litura* NPV isolate (1 × 10³ OBs/ml) previously examined against 1st larval instar of *S.*

litura after 7 days of treatment (Trang and Chaudhari 2002). Previously, Kumar et al. (2011) examined the LC₅₀ of *Spodoptera litura* NPV isolate against second (3.5 × 10⁴ obs/ml) and 3rd (2.4 × 10⁵ obs/ml) larval instars of *S. litura* after 7 days of treatment, which were close to the SpliNPV-YW values under investigation. In contrast, the calculated LC₅₀ value for SpliNPV-YW isolate showed to be higher than that observed by Seufi (2008), who determined the efficacy of SpliMNPV, an Egyptian isolate, against the second instar of *S. littoralis* larvae was 1.2 × 10³ obs/ml. On the same context, the SpliNPV-YW isolate speed of killing against the two tested instars was also determined via determination of LT₅₀ value. The LT₅₀ value for second instar larvae was 5.5 days at concentration of 4.3 × 10¹⁰ ob/ml and was 6.2 days at concentration of 1.2 × 10¹¹ ob/ml for fourth instar larvae. It is clear from the obtained LT₅₀ value that second instar larvae were highly sensitive to tested

viral concentrations than fourth instar ones and this was evidenced by low LC_{50} and LT_{50} values. This result indicated that the investigated SpliNPV-YW isolate significantly had a better speed of kill against second instar larvae than fourth instar larvae. In the current study, a virus isolate derived from diseased *S. littoralis* larvae was analyzed. Even though no clear variation was observed, as inferred by the absence of submolar bands in restriction profile analysis, more research is needed for determination of more genotypes from Egypt. Furthermore, determining the genetic characteristics of the viral genotypes via Next Generation Sequencing (NGS) and their relationship with insect host should be utilized in their application as a viral-based biopesticides. The present research might give more information not only on viral genetic characterization and its host, but also on how to define pest management for better biological control, such as reintroducing additional NPV types to enhance genetic variety and potency regarding the target host.

Conclusions

It was concluded that *S. littoralis* nucleopolyhedrovirus isolate named SpliNPV-YW was obtained from field diseased *S. littoralis* larvae previously collected from El-Menoufia Governorate Egypt. The viral isolate exhibited viral-like symptoms upon infection of *S. littoralis* fourth instar larvae. The ultra-structural analyses of the viral isolate using transmission electron microscope showed typical nucleopolyhedrovirus shape with several single and multiple nucleocapsid scattered in the viral occlusion bodies. The SpliNPV-YW, although it did not show clear difference in genome pattern upon digestion with two different endonucleases (PstI and ScaI), it showed significantly high virulence toward *S. littoralis* second instar larvae than fourth instar larvae. So, it was concluded that the oldest the *S. littoralis* larvae, the least susceptibility to the SpliNPV-YW isolate was noticed. Further studies under field conditions are needed.

Abbreviations

SpliNPV: *Spodoptera littoralis* Nucleopolyhedrovirus; obs: Occlusion bodies; LT_{50} : Median lethal time; LC_{50} : Median lethal concentration; h: Hours; Min: Minute; hpi: Hours post infection; pi: Post infection; REN: Restriction endonuclease.

Acknowledgements

Not applicable.

Author contributions

WE conceived the idea and designed the study. AM performed wet lab. experimentation. WE and SS provided technical and financial assistance for the study. WE did the virus counting, propagation and purification. YS did the insect rearing, the bioassay experiments in addition to the statistical analysis. WE and SS wrote the manuscript and technically proofread the manuscript. All authors have read and approved the final version of the manuscript.

Funding

This study was funded by the Agricultural Genetic Engineering Research Institute, ARC, Giza, Egypt. This funder provided chemicals, reagents and equipment used in this study. Also, this study was financed by Taif University Researchers Supporting Project number (TURSP -2020/92), Taif university, Taif, Saudi Arabia. This funder provided materials used for insect rearing and virus propagation, nucleotide sequencing and oligos synthesis used for PCR analysis.

Availability of data and materials

All data generated or analyzed in this work are available in the 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 not to have any competing interests regarding the publication of this work.

Author details

¹Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza 12311, Egypt. ²Department of Science and Technology, University College-Ranyah, Taif University, B.O. Box 11099, Taif 21944, Saudi Arabia. ³Faculty of Science, Cairo University, Giza, Egypt. ⁴Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza 12619, Egypt.

Received: 19 February 2022 Accepted: 13 June 2022

Published online: 21 June 2022

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