Evaluation of two viral isolates as a potential biocontrol agent against the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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**Abstract**

Two different *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) nucleopolyhedrovirus (SpliNPV) isolates were obtained from natural infected *S. littoralis* larvae in Egypt. The phylogenetic analysis of the partial nucleotide sequence of the polyhedrin gene (*polh*) showed that both isolates, Spli-6 and Spli-7, were closed and had a common ancestor with *S. littoralis* NPV isolate 1263 polyhedrin gene, partial coding sequence with accession number AGE90003.1 that belongs to group II NPVs. This indicates that the natural host of Spli-6 and Spli-7 must be *S. littoralis*. The virulence of these isolates was tested against *S. littoralis* larvae in a laboratory. The LC₅₀ against 1st instar larvae was significantly different, 3 × 10⁴ OBs/ml and 9.5 × 10⁴ OBs/ml for Spli-6 and Spli-7, respectively. However, the LC₅₀ for the mixed infection of both isolates was 3.9 × 10⁴ OBs/ml. On the other hand, the ST₅₀ was 96 h for both isolates, while it was 120 h for the mixed infection, which was higher than that observed of each single isolate. Upon digestion of viral DNA with Scal endonuclease, the restriction profile showed one more fragment of about ~25 kbp and 3 fragments of about ~19, ~5.8, and ~5.3 kbp in Spli-7 isolate compared to Spli-6 as well as the reference strain SpliNPV-AN1956. The differences in the fragment size and number suggest the existence of genotypic variants between both isolates. Spli-6 and Spli-7 proved to possess promising insecticidal properties for the development of virus-based biopesticide for the control of *S. littoralis*.

**Keywords:** Nucleopolyhedrovirus, *Spodoptera littoralis*, Bioassay, Restriction pattern

**Background**

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is one of the most economic insect pests in Egyptian fields. In Egypt, it is considered as a destructive pest that causes economic losses not only for the cotton plants but also for other crops and vegetables (Hosny et al. 1986), orchard trees, and ornamentals (Mohamed et al. 2019). *S. littoralis* is difficult to control due to the resistance developed against synthetic insecticides as well as against *Bacillus thuringiensis* toxins (Ghulam et al. 2017). In addition, the use of chemical insecticide leads to detrimental effects on the natural enemies, pollinators, and all other non-target insects. Moreover, it causes serious toxicological problems to humans, plants, animals, and the whole environment. In order to avoid the risks associated with using chemical pesticides, there is an urgent need to encourage biological control agents as alternatives to chemical pesticides. Many biological agents have been used for the control of insect pests, such as parasitoids, predators, and entomopathogens (bacteria, fungi, and viruses) (El-Gaied et al. 2020 and Atia et al. 2016). Among these biopesticides are baculoviruses, which have been considered to be insect-specific viruses
that have potential for biological control of insect pests in agriculture as well as an effective eukaryotic protein expression system (Elgaied et al. 2017 and Elmenofy et al. 2020).

Baculoviruses are large, double-stranded DNA viruses that infect arthropods and insects, particularly of the order Lepidoptera, but they also infect the orders Coleoptera, Hymenoptera, and Diptera. They are a promising alternative to chemical pesticides. They are able to kill insect larvae within a few days (Agata et al. 2010). It is assumed that baculoviruses are safe and restricted to invertebrates, where they are specific pathogen for insects (Ahmed et al. 2016). Baculoviruses resort to be species- or genus-specific, although there are some exceptions to this rule, like the Autographa californica nucleopolyhedrovirus (Mahendra and Avinash 2012). S. littoralis nucleopolyhedrovirus (SpIPNV) belongs to the family Baculoviridae. It is one of the most promising biocontrol agents against pests. The host range of the virus has been shown to be narrow, likely infecting within Spodoptera spp. with a relatively slow speed of action. The slow speed of action is one of the major drawbacks of using SpIPNV wild-type isolates as an effective biocontrol agent that can compete with broad-spectrum chemical insecticides. In order to solve this problem, developing a new and efficient biocontrol agent is highly important to be used in integrated pest management of S. littoralis.

Therefore, the main objective of this work was to study the molecular and the biological characteristics (toxicity effect) of S. littoralis baculovirus isolates as candidate virus-based biocontrol agents against pests in Egypt.

**Materials and methods**

**Insect and viruses**

*S. littoralis* used for virus propagation and bioassay experiments was derived from the insect-rearing facility of the Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt. The larvae were maintained at 26 °C with 60% RH. Larvae were reared on a semi-artificial diet containing agar-agar, maize meal, wheat germ, brewer’s yeast, ascorbic acid, and Nipagien (methyl 4-hydroxybenzoate) previously described by (Ivaldi-Sender 1974). Viruses used in this study were field-collected baculovirus isolate genus nucleopolyhedrovirus from infected cadavers from Giza district in Egypt.

**Virus propagation in *S. littoralis* larvae**

The *S. littoralis* larvae (4th instar) were placed in boxes (50-well plate) with a small piece of insect medium. Each piece of media was inoculated with $10^5$ of virus occlusion bodies (OBs), then the plates were incubated at 26 °C. The next day, larvae were transferred to virus-free medium and continued rearing until observation of viral infection symptoms (7–10 days post-infection).

**Virus purification from infected larvae**

Infected larvae were collected for virus purification according to Boughton et al. (1999). Using 0.1% SDS, infected larvae were homogenized and the suspension that formed from the grinded larvae was filtered by using a piece of cotton and filter paper. Excess of 0.1% SDS was added until the filter paper became clear. The grinded tissues containing virus occlusion bodies (OBs) were centrifuged at 7000 rpm for 10 min at room temperature. The supernatant was discarded, and the pellet was re-suspended in 1 ml 0.5% SDS/larva using vortex then centrifuged again at 7000 rpm at room temperature for 10 min. The pellets were re-suspended in 0.5 M NaCl by vortex, then centrifuged again at 7000 rpm for 10 min. The pellets were suspended in a small volume of ddH$_2$O (0.5 ml/larvae) by pipetting or gentle vortex. The diluted OBs were counted in the dark field of the Axiovert Zeiss inverted microscope with a × 200 magnification, using a Petroff-Hauser counting chamber (depth 0.01 mm, Hauser Scientific). The purified virus was kept frozen at −20 °C.

**Purification of virus DNA**

Using the method developed by Boughton et al. (1999), viral genomic DNAs were isolated from viral OBs. Briefly, about 300 μl of purified virus OBs was precipitated for 10 min at 7000 rpm, and pellet-containing viral OBs were re-suspended in 200 μl of H$_2$O. 1 M of Na$_2$CO$_3$ (sterile filtrated) in the final concentration of 0.1 M was added then incubated for 1 h at 37 °C in water bath till the solution became clear. The solution was neutralized with autoclaved 1 M HCl to pH 8, and 10% (w/w) SDS solution was added in the final concentration of 1%. Then, proteinase K was added in the final concentration of 250 μg/ml, and the suspension was incubated for 1 h at 37 °C to release the nucleic acids. The samples were washed twice with TE buffer-saturated phenol/ chloroform 1:1 (v/v) and one more time with chloroform to remove the phenolic traces. Viral genomic DNA was precipitated using 2.5 volume ice-cold 96% ethanol and 1/10 volumes 3 M NaAc pH5.2 in ~80 °C and then washed twice with 70% ethanol. The pellet was dried for 5 min at RT, then DNA was eluted in a suitable amount of autoclaved water.

**Restriction profile analysis**

Restriction digestion of the viral DNA was performed to compare the genome pattern of virus isolates, using Scal endonuclease. Briefly, 1–1.5 μg genomic DNA was digested, using the following component: 9 μl of viral DNA, 1 μl of the appropriate enzyme (5 U/μl), 2 μl 10×
buffer, and 7 μl of ddH2O. The mixture then was incubated for 2 h at 37 °C. Electrophoresis was performed overnight, using 0.8% agarose in 1X TAE buffer (40 mM Tris, 20 mM acetate, and 1 mM EDTA, pH 8.6) (Boughton et al. 1999).

PCR amplification of polyhedrin gene
One set of polyhedrin-specific primers, designed and synthesized based on the reference strain SpliMNPV-AN1956 (Accession No. NC_038369) Breitenbach et al. (2013), was used to partially amplify polyhedrin fragment from each virus genomic DNA. Primers denoted Spli-polh-560_F (5′-ATCTGGGCAAACCTATGTAT-3′) and Spli-polh-560_R (CTTGCGGAGACGTGATCCTAT). The PCR reactions were performed in a total reaction volume of 50 μl containing 1 μl of the appropriate upper primer (10 pmol/μl), 1 μl of the appropriate lower primer (10 pmol/μl), 1.5 μl MgCl2 (50 mM), 1.5 μl of dNTPs mixture solution (10 mM), 2 μl of DNA template (0.1–0.5 μg), 5 μl of 10× PCR reaction buffer, and 0.5 μl of Taq DNA polymerase (5 U/μl). The reaction volume was completed to 50 μl, using autoclaved ddH2O. For a typical PCR reaction, the following program was used: an initial 95 °C for 3 min; a total of 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 59 °C for 1 min, and primer extension at 72 °C for 1 min; and a final cycle at 72 °C for 7 min to allow the completion of primer extension. PCR-amplified DNA was analyzed by electrophoresis on a 1% agarose gel prepared in 1× TAE buffer. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Germany). Nucleotide sequences for the purified fragments were performed using the Sanger sequence.

Sequencing and phylogenetic analysis
Analysis of the polyhedrin-deduced amino acid sequences was performed for both isolates, using EditSeq-DNAstar Inc., Expert Sequence Analysis software, Windows 32 Edit Seq 4.00 (1989–1999), ExPasy database, and Clustal Omega for multiple alignment analysis. Blastp search for alignment of the resulted deduced amino acid sequences with the published ones was carried out, using the database of the National Center for Biotechnology Information (NCBI).

The LC50 and ST50
To determine the median lethal concentration (LC50) for each isolate, 6 different concentrations were prepared for each virus suspension as follows: 107, 5 × 106, 105, 5 × 104, 103, and 5 × 102 OBs/ml, in addition to the control solution, which contains ddH2O instead of virus suspension. Two plates (50-well) were used for each experiment in addition to 2 plates for the control treatment. The volume of each virus concentration was completed to 10 ml with water and added to 90 ml larval media (40 °C) that were prepared in advance, then the mixture of media and virus suspension was poured on the plates. After solidification, larvae were added to all plates and incubated for 24 h, then handling error was taken by counting the dead larvae. After 7 days, dead larvae were counted and the LC50 were analyzed, using EPA Probit analysis program (version 1.5). Another way of infection was introduced using a mixed infection by mixing Spli-6 and Spli-7. An equal volume of each isolate OBs was added to the mixture. This mixture was used to prepare serial dilution of virus-infected inoculum that was used to infect larvae in a bioassay experiment as mentioned before. Moreover, the median survival time (ST50) was determined by inoculating 50 individually held larvae in 3 replicates with the calculated LC80 for each tested virus, as well as 2 control plates, which were mixed by water instead of virus suspension. The mortality of the larvae was monitored every 24 h, starting day 3 post-infection until day 7 or larval death. Data analysis was performed, using the Kaplan-Meier survival time estimator analysis (Kaplan-Meier 1958).

Results and discussion
Viruses’ isolation and propagation
In this study, two baculovirus isolates denoted Spli-6 and Spli-7 were characterized. Both isolates were obtained from diseased S. littoralis larvae and exhibited viral-like symptoms which were collected from Giza district in Egypt. Viral symptoms of collected infected S. littoralis larvae were slow motion and visibly swollen by fluid containing the virus; similar results were reported by Toprak et al. 2005 and Ahmed et al. 2016. Both isolates were further propagated, using S. littoralis 4th instar larvae, reared on a semi-artificial diet. Typical viral infection symptoms were detected in infected S. littoralis larvae 7–8 days post-infection, suggesting that these isolates were baculovirus-specific isolates to S. littoralis (Fig. 1). The total counting of polyhedral for each isolate was as follows: 1.1 × 107 (OBs/ml) and 1.7 × 106 (OBs/ml) for Spli-6 and Spli-7, respectively. Using insect-specific viruses is an effective active ingredient for the development of biopesticides in the agricultural field for the control of different insect pests (Cory et al. 2005). The NPVs, belonging to baculoviruses family, are well known for their genetic evolution, higher genetic variation, and differences in their potency. These factors remain conserved even when the baculovirus isolates are purified from the same species of different geographical locations (Cory et al. 2005).

PCR amplification of polyhedrin gene
Viral genomic DNA was purified, using OBs of each isolate (Spli-6 and Spli-7). As shown in Fig. 2a, about 200–500 ng of each purified genomic DNA was examined in agarose gel to check DNA quality. Enough amount of genomic DNA was successfully purified from each
isolate and was used for the downstream work. PCR was performed to partially amplify a specific fragment of polyhedrin gene (\textit{polh}). As shown in Fig. 2b, the amplified fragments give the expected size of \textit{polh} gene of about 560 bp, which subsequently subjected to Sanger nucleotide sequencing. Primers specific for polyhedrin gene were used in this study for Egyptian NPV virus detection and identification by PCR as well as phylogenetic analysis, since it is highly conserved between NPVs (Jehle et al. 2006). Thus, the polyhedrin (\textit{polh}) gene was partially amplified to determine the nucleotide sequence for both isolates to be compared to other NPVs corresponding to sequences available in GenBank.

**Phylogenetic analysis**

The partial coding sequence of the \textit{polh} gene was obtained and compared to another \textit{polh} gene from NPVs, using the \textit{polh}-deduced amino acids. The results of these analyses are shown in Fig. 3. The results showed that the sequence (~500 bp) of both isolates Spli-6 and Spli-7 was closed and had a common ancestor to the sequence of polyhedrin gene, from an Egyptian \textit{Spodoptera littoralis} nucleopolyhedrovirus isolate SpliNPV-4375, with accession no. AAR04375.1, as well as the sequence of \textit{S. littoralis} NPV isolate 1263 polyhedrin (\textit{polh}) gene, partial cds with accession number AGE90003.1. The phylogenetic tree was constructed from the deduced amino acids via the Clustal
Omega (Sievers et al. 2011) used in the mBed algorithm (Blackshields et al. 2010). The phylogenetic tree showed that Spli-6 was more closely related to the isolate Spli-4375 compared to Spli-7; however, both isolates are located in the same cluster, which belongs to nucleopolyhedrovirus group II (NPV II) (Herniou and Jehle 2007). Based on the distance to these viruses, SpliNPV-Spli6 and SpliNPV-Spli7 may be regarded as variant virus isolates from the reference isolate SpliNPV-AN1956 previously published by Breitenbach et al. (2013).

**Analysis of virus DNA**

The genomic DNA of each virus isolate was cleaved by the enzyme Scal, to compare restriction profiles generated among them. The analyses were performed by comparison of additional fragments present in relation to the number of fragments common to both isolates generated by each cleavage. This also was compared to the restriction profile of the reference strain SpliNPV-AN1956 (accession number: JX454574). Digestion with Scal resulted in a characteristic DNA profile for isolate Spli-7, as indicated by the presence of the marker fragments (~25, ~19, 5.8, and 5.3 kbp for isolate Spli-7; Fig. 4, lane C), absent in the corresponding profile of the isolate Spli-6 as well as the reference isolate SpliNPV-AN1956. While it seems that some Scal fragments of about ~13 kbp and 8.5 kbp were missed in the Spli-7 profile, the fragment intensity was low in Spli-7 compared to Spli-6 and the reference strain. Overall, REN profiles contained a number of submolar bands indicating that Spli7 isolate comprised a genotypic variant.

**Median lethal concentration**

In order to test the biological activity of SpliNPV virus isolates, their median lethal concentration (LC50) was determined. The analysis showed that LC50 value of Spli-7 (9.5 × 10^4 OBs/ml) was higher than that of Spli-6 (3 × 10^4 OBs/ml). Interestingly, the calculated LC50 for the mixed infection between both isolates was 3.9 × 10^4 OBs/ml (Table 1). The results showed that Spli-6 had the best virulence then the combination of both isolates and Spli-7 being the lowest based on LC50 values.

**Median survival time**

The ST50 was calculated, using the Kaplan-Meier survival time estimator analysis (Table 2). The calculated ST50 values were 96 h for Spli-6, 96 h for Spli-7, and 120 h for mixed infection. As shown in Fig. 5, the observed survival plots for the tested viruses (Spli-6 and Spli-7) were closed to each other. Non-significant difference was observed between the tested Spli-6 and Spli-7 survival curves, which had the same speed of killing. During the bioassays, no differences were observed in the disease symptoms of the S. littoralis larvae infected either with Spli-6, Spli-7, or the mixed infection. However, the calculated ST50 of the mixed infection was significantly higher than that of each single infection with about 24 h.

Unexpectedly, the calculated LC50 of SpliMNPV isolates against S. littoralis 1st instar larvae were 3.0 × 10^4 OBs/ml and 9.5 × 10^4 OBs/ml for Spli-6 and Spli-7, respectively. They were relatively higher than that observed LC50 (1 × 10^3 OBs/ml) for the 1st instar S. littura.
larvae by using *S. litura* NPV isolate (Trang and Chaudhari 2002). In the same context, according to Kumar et al. (2011), the finding was very close to the LC50 of the 2 different SpltNPV virus-specific isolates, which were $3.5 \times 10^4$ and $2.4 \times 10^5$ OBs/ml against *S. litura* 2nd and 3rd larval instars, respectively. The ST50 for both isolates was 96 h, which was higher than the ST50 of some SpliMNPV isolates presented by Toprak et al. (2005), which ranged from 72–84 h against *S. littoralis* larvae. However, it was less than that observed for SpliMNPV isolates, which ranged from 125 to 360 h (Lacey et al. 2015). On the same context, the calculated ST50 value of isolate Spli-6 and Spli-7 was higher than that of SpltNPV-Pak-BNG that was 84 h but lower than SpltNPV-G1, which was 108 h against the 3rd instar *S. litura* larvae (Ghulam et al. 2017). The high virulence of both isolates (Spli-6 and Spli-7) to *S. littoralis* may suggest their potential as a promising viral bioinsecticide. However, the mixed infection between both isolates did not affect the potency of the isolates based on the virulence parameters (LC50 and ST50) than to each single isolate. This observations suggested that the speed of killing of the tested isolates against the *S. littoralis* larvae may vary based on the collected district and the target insect host. The calculated virulence parameters for both isolates suggested the suitability of the Egyptian environment for collecting new baculovirus isolates that can be used as an active ingredient for the development of new formulated products for effective control of *S. littoralis*. Given that baculoviruses were isolated from Giza district in Egypt, where most of the crops usually received chemical insecticides, it would be expected to find more isolates in the regions where no insecticides are used.

**Conclusion**

In the present study, two different *S. littoralis* nucleopolyhedrovirus (SpliNPV) isolates, denoted as Spli-6 and Spli-7, were obtained from natural infected *S. littoralis* larvae in Egypt. The Spli-6 significantly showed higher virulence toward *S. littoralis* 1st instar larvae as well as the mixed infection. However, both isolates showed the same speed of killing of 96 h based on the

**Table 1** Median lethal concentration (LC50) of 1st instar *S. littoralis* larvae infected either with Spli-6, Spli-7, or mixed viruses 7 days post-infection

| Virus   | No. of larvae | LC50 (OBs ml⁻¹) (CI) | Slope (SE) | X²  |
|---------|---------------|----------------------|------------|-----|
| Spli-6  | 718           | $3.0 \times 10^4$ (2.4 × 10⁴–3.7 × 10⁴) | 2.18 (0.22) | 9.4 |
| Spli-7  | 526           | $9.5 \times 10^4$ (7.5 × 10⁴–1.2 × 10⁵) | 1.68 (0.20) | 7.8 |
| Mixed   | 707           | $3.9 \times 10^4$ (7.1 × 10⁴–3.1 × 10⁵) | 1.58 (0.45) | 9.4 |

The LC50 of probit analysis, as well as the slopes of the probit lines, are given CI 95% confidence interval, SE standard error

**Table 2** Kaplan-Meier estimation of ST50 of *S. littoralis* larvae infected with OBs derived from Spli-6, Spli-7, and mixed viruses

| Virus   | No. of larvae | LC80 (OB/ml) | ST50 CI (lower-upper) |
|---------|---------------|--------------|-----------------------|
| Spli-6  | 103           | $9.05 \times 10^4$ | 96 86–105             |
| Spli-7  | 109           | $3.93 \times 10^5$ | 96 90–101             |
| Mixed   | 97            | $1.75 \times 10^5$ | 120 109–130           |

ST50 and 95% confidence interval (CI) are given in hours post-infection (hpi)
ST_50 value. The phylogenetic analysis of the partial deduced amino acid sequence of polyhedrin gene (polh) showed that both isolates were closed and have a common ancestor with S. littoralis NPV isolate 1263 polyhedrin (polh) gene, partial cds contains accession number AGE90003.1 that belongs to group II NPVs. The high virulence of both isolates (Spli-6 and Spli-7) to S. littoralis may suggest their potential as a promising viral-based bioinsecticide for the control of S. littoralis in Egypt.

Abbreviations
LC50: Median lethal concentration; NPVs: Nucleopolyhedroviruses; OBs: Occlusion bodies; PCR: Polymerase chain reaction; polh: Polyhedrin; REN: Restriction endonuclease; S. littoralis: Spodoptera littoralis; Spli-6: SpliNPV isolate Spli-6; Spli-7: SpliNPV isolate Spli-7; SpliNPV: Spodoptera littoralis nucleopolyhedrovirus; ST_50: Median survival time; TAE: Tris-acetate-EDTA

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Authors’ contributions
WE put the idea, designed the experiments, and wrote and revised the manuscript. LE and EI collected the field samples and restriction pattern analysis as well as shared in manuscript writing. NY, SA, AE, and AE did the molecular biology work as well as the bioassay experiments. AA, MS, and AZ reared S. littoralis larvae and virus isolate propagation. RS, EH, and ST designed the bioassay experiments and analysis of the data. All authors read and approved the final manuscript.

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Competing interests
We declare that we have no competing interests.

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Fig. 5 Survival curves using the StatsDirect statistical analysis software Kaplan-Meier. Graph showing the survival time analysis of SpliNPV isolates: Spli-6, Spli-7, and the mixed infection as observed on infected Spodoptera littoralis larvae.
