Biological activity and genome composition of a Tunisian isolate of Spodoptera littoralis nucleopolyhedrovirus (SpliNPV-Tun2)

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Abstract

Background: The baculovirus Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) is an entomopathogenic virus utilized as a biological control agent of the Egyptian cotton leaf worm, Spodoptera littoralis. Several studies have focused on the identification of different SpliNPV isolates from a biological and molecular point of view, but few of them conducted in-depth analyses of the genomic composition of these isolates.

Results: Identification of a novel isolate of SpliNPV, termed Tun2, which was purified from infected S. littoralis larvae from Tunisia was reported. This isolate was propagated in vivo and its median lethal concentration (LC50) was determined to be 1.5 × 10^4 occlusion bodies (OBs)/ml for third instar S. littoralis larvae at 7 days of post-infection. OB production in late fourth instar larvae was estimated to be at least 2.7 × 10^9 OBs/g larval weight. The completely sequenced genome of SpliNPV-Tun2 was 137,099 bp in length and contained 132 open reading frames (ORF). It showed a 98.2% nucleotide identity to the Egyptian isolate SpliMNPV-AN1956, with some striking differences; between both genomes, insertion and deletion mutations were noticed in 9 baculovirus core genes, and also in the highly conserved polyhedrin gene. The homologs of ORF 106 and ORF 107 of SpliNPV-AN1956 appeared to be fused to a single ORF 106 in SpliNPV-Tun2, similar to the homologous ORF 110 in SpltNPV-G2.

Conclusion: SpliNPV-Tun2 is proposed as a new variant of SpliNPV and a potential candidate for further evaluation as a biocontrol agent for S. littoralis and probably other Spodoptera species.

Keywords: Egyptian cotton leaf worm, Spodoptera littoralis, Baculoviridae, Alphabaculovirus, Bioassays, Survival time analysis, Illumina sequencing, Genome annotation, Phylogeny, Biological control

Background

The Egyptian cotton leaf worm Spodoptera littoralis (Boisd.) (Lepidoptera: Noctuidae) is considered one of the major pests of cotton, tobacco, and corn in the Mediterranean Area and Asia. Larvae of S. littoralis are polyphagous, causing substantial economic losses in both greenhouse and open field crops on a broad range of ornamental, industrial, and vegetable crops (Martins et al. 2005). Due to the severe damage to various crops, controlling this pest is an important issue for integrated pest management. Up to now, S. littoralis management has mainly focused on chemical insecticides. However, numerous studies have been carried out on the possibility of biological control of the pest. Insect viruses and entomopathogenic bacteria, fungi, and nematodes have been investigated as biological control agents of S. littoralis (Hajek and Shapiro-Ilan, 2018). The Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) is a baculovirus that has been evaluated, registered, and applied for control of S. littoralis, as well as the fall armyworm, Spodoptera frugiperda, and the tobacco cutworm Spodoptera litura in Africa, America and Japan (Abdel-Khalik
et al. 2017; El-Sheikh 2015; Takatsuka et al. 2016). Baculoviruses comprise a large group of double-stranded, circular DNA viruses that infect insects from the orders Lepidoptera, Hymenoptera, and Diptera. Many of these viruses have been investigated because of their potential as biological control agents against agricultural and forest pests (Moscardi 1999). Based on phylogenetic analysis, the Baculoviridae family is separated into 4 genera: Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses, NPVs), Betabaculovirus (lepidopteran-specific granuloviruses, GVVs), Gammabaculovirus (hymenopteran-specific NPVs) and Deltabaculovirus (dipteran-specific NPVs) (Jehle et al. 2006). SpliNPV belongs to the species Spodoptera littoralis nucleopolyhedrovirus of the genus Alphabaculovirus (Harrison et al. 2018).

Different SpliNPV variants have been isolated from cotton leaf worm populations in different countries, and intra-specific variation between isolates was identified by restriction endonuclease or partial gene sequencing (Breitenbach et al. 2013; Cherry and Summers 1985; Kislev and Edelman 1982; Martins et al. 2005). So far, only the Egyptian isolate SpliMNPV-AN1956 has been fully sequenced; its genome is 137,998 bp in length, harbours 132 ORFs, and 15 homologous repeat regions (hrs), and is closely related to the nucleopolyhedrovirus G2 (SpliNPV-G2). Comparisons of the genome sequence of SpliMNPV-AN1956 and SpltNPV-G2 revealed an average of 85% amino acid identity across all genes and high collinearity of the 2 genomes, despite the lack/gain of 16 ORFs (Pang et al. 2001). It was reported that NPVs isolated from Spodoptera spp. have a rather narrow host range (Jakubowska et al. 2010). For example, the Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) infects only larvae of its host S. exigua (Jakubowska et al. 2010), whereas SpliNPV was shown to be infectious also to S. frugiperda, S. exigua, and S. littura (Takatsuka et al. 2016). Recently, a Tunisian isolate, named SpliMNPV-Tun, was detected in 2008 from infected cotton leaf worm caterpillars collected in Tunisian tomato greenhouses and identified as a SpliNPV variant based on the partial polyhedrin (polh) gene sequence (Laarif et al. 2011). Here, the identification of a further SpliNPV isolate, termed Tun2, which was obtained from a S. littoralis colony that was established from collected caterpillars from tomato fields in 2013 is reported. This isolate was tested for its activity towards third instar S. littoralis larvae, and its complete genome was determined to study its relationship to other SpliNPV variants.

Methods

Insects and virus detection

Larvae of S. littoralis were collected in 2013 from tomato fields (Monastir, Central-East, Tunisia) to establish a laboratory colony at the laboratory of entomology at Regional Research Centre in Horticulture and Organic Agriculture (CRRHAB). For colony maintenance, larvae were fed on a semi-artificial diet (Shorey and Gaston 1965) and kept at a temperature of 28 ± 2 °C and 60 ± 5% relative humidity (RH). Adults were kept in cylinders, and egg deposits were collected on filter papers. The filter papers were transferred to Petri dishes until larval hatching. A piece of artificial diet was added to the Petri dishes where larvae were kept until pupation. Occasionally, larvae from the rearing showed symptoms of nucleopolyhedrovirus infection indicating activation of a covert infection of the S. littoralis population. Diseased larvae were removed from the rearing and stored individually at −20 °C.

Occlusion body purification

Baculoviral occlusion bodies (OB) were purified from pooled infected cadavers according to El-Salamouny et al. (2003). Briefly, the cadavers were homogenized in sterile distilled water and the homogenate was filtered through a muslin cloth. The obtained crude OB suspension was washed twice with 0.1% sodium dodecyl sulphate (SDS) and pelleted by low centrifugation. The pellet was resuspended in 50 mM Tris/HCl (pH 8), transferred to a 2-ml Eppendorf reaction vial, and HCl (0.1 M) or Na2CO3 (0.1 M) was added to adjust its pH to 7. Then, the OB suspension was centrifuged through a sucrose gradient and resuspended in H2O. OB concentration was counted using a Neubauer Cell Counting Chamber (0.1 mm depth) and phase contrast light microscopy (Leica DMRBE, Leica, Wetzlar, Germany) (Eberle et al. 2012).

Bioassays

For testing the biological activity of SpliNPV-Tun2, per os infection experiments were conducted with third instar larvae of S. littoralis. For this, 25 larvae were fed with 1.5–2.5 g artificial diet plugs prepared with final concentrations of 105–108 OBs/ml (Shaurub et al. 2014). Untreated control groups consisted of 75 larvae. Each treatment consisted of 3 independent replicates. The mortality data were corrected with untreated control mortality using the formula of Abbott (1925). Calculation of the median lethal concentration (LC50) at 7 days post-infection (dpi) was estimated by Probit analysis using linear regression implemented in the ToxRat 3.2.1 software package (ToxRat Solutions GmbH, Germany). From the same experiment, larval mortality was determined
for each concentration at least at 5 different time points within the time range of 1–14 dpi. Statistical analysis was done with R (version 4) and RStudio (version 1.1393). Survival analysis was conducted with R packages “Survival” (version 2.38) and “Survminer” (version 0.4.3). A test of significant variance between Kaplan–Meier curves was performed by a log-rank test (level of significance, P<0.05).

**OB productivity of *S. littoralis* larvae**

An OB dose of 10⁴ OBs of SpliNPV-Tun2 was pipetted onto cubes of diet of 5 mm³ each and individually offered to early fourth instar larvae of *S. littoralis* (Grzywacz et al. 1998). When the doses were completely ingested within 2 days, a non-contaminated diet was added every second day until 12 dpi. Larvae were harvested at 14 dpi. Three different methods for OB purification were compared; low-speed centrifugation (LSC) (Harrison 2008), sucrose gradient ultracentrifugation (SGU) (El-Salamouny et al. 2003), and sucrose cushion centrifugation (SCC) (Wennmann and Jehle 2014). Purified OBs were counted as described above. OB counting was performed 3 times for each treatment; the obtained concentrations were multiplied with the volume (5 ml), and then normalized with the larva weight. Results were expressed as OBs/g of larval tissue and were used to compute the arithmetic mean of OB/g of each experiment. Differences in the mean number of OB/g were statistically evaluated for a significance value of P ≤ 0.05 using analysis of variance (ANOVA) and the Tukey’s Honestly Significant Difference test (Tukey-HSD) comparison of means with standard R code (R version 3.3.1 in RStudio 3.4.0).

**Viral DNA extraction**

Viral DNA was extracted according to Bernal et al. (2013) with some modification. Occlusion derived virions (ODVs) were released from OBs by mixing 100 µl of the OB suspension (containing about 10⁸ OBs/ml) with 100 µl Na₂CO₃ (0.5 M), 50 µl SDS (10%, w/v) in a final volume of 500 µl. After incubation at 60 °C for 10 min, the suspension was neutralized to pH 7 by adding 0.1 M HCl. Undissolved OBs and other debris were pelleted by centrifugation at 3800 g for 5 min. The supernatant containing the released ODVs was transferred to a fresh vial and treated with 25 µl Proteinase K (10 mg/ml) for 1 h at 50 °C. Viral DNA was extracted twice with Tris/HCl-saturated phenol and once with chloroform by using Phase Lock gel tubes (all purchased from, Carl Roth GmbH + Co., KG, Karlsruhe, Germany), followed by standard ethanol precipitation (Eberle et al. 2012). The DNA pellet was dissolved in 100 µl distilled H₂O.

**PCR amplification and sequencing of the polyhedrin gene**

The PCR amplification of the *polh* gene was chosen according to the specific primers designed by Martins et al. (2005) to amplify a complete SpliNPV *polh* gene (750 bp): 5′-ATG TAT AGT CGC TAC AGT GCC TAC-3′ (forward primer) and 5′-TTA GTA CGC GGG ACC GGT GT-3′ (reverse primer). The PCR mix comprised 34.5 µl of water, 10 µl Green buffer (Promega), 1 µl dNTPs (10 µM each), 1.5 µl Go Taq DNA polymerase (Promega), and 1 µl of each primer (10 µM). Finally, 1 µl of DNA was added to obtain a final volume of 50 µl for each reaction. PCR was initiated at 95 °C for 1 min of denaturation followed by 35 cycles at 95 °C for 30 s, 46 °C for 30 s, 72 °C for 45 s and the final extension at 72 °C for 5 min. The amplification product was visualized by 1% agarose gel electrophoresis at 90 V for 40 min in 1 × TAE buffer after staining with Midori Green DNA strain (NIPPON Genetics Europe). The PCR product was purified with DNA clean and concentrator kit (Zymo Research) according to the manufacturer’s instruction, and both strands were Sanger sequenced. The *polh* sequencing was done for different single infected larvae randomly chosen. Sequences were compiled and then aligned with the complete *polh* gene sequences available in GenBank.

**Genome sequencing**

**Sequencing and raw data processing**

About 50 ng purified DNA was subjected to commercial NexteraXT library preparation and Illumina NextSeq500 sequencing at StarSEQ GmbH company (Mainz, Germany). In total, more than 1.76 million reads of 151 nt in length were obtained. Raw reads were processed by adapter trimming and quality filtering excluding reads with an average phred quality score below 30 (Gueli Alletti et al. 2017). Quality filtered reads with a length shorter than 50 nt were excluded from the analysis for paired reads and 51 nt for unpaired reads. Paired and unpaired reads were kept after all steps of filtering and quality control.

**Genome sequence assembly**

The remaining set of reads was used for de novo sequence assembly as well as mapping against the whole genome sequence of SpliMNPV-AN1956 (GenBank accession number JX454574) (Breitenbach et al. 2013). CLC de novo assembly resulted in multiple contigs (>1000 bp). Contigs were mapped and fit together to a single contig comprising the whole genome. This contig was considered as a first consensus (cons1). In a second approach, all reads were mapped against the SpliMNPV-AN1956 genome using BWA-MEM. From here, a second consensus (cons2) was extracted applying a majority rule (>99%). Both consensus sequences were then aligned
to each other and checked for differences, which mainly occurred in repeated as well as homologous repeat regions (hrs). The alignment was then checked manually for ambiguities and sequence discrepancies. The correction was based on the read coverage supporting one ambiguous region per contig generated by CLC. The cutoff of the adopted corrections was coverage of 20 reads per ambiguous region. One final genome sequence of SpliNPV-Tun2 was generated based on the majority of read coverage and submitted to GenBank (Accession number MG958660).

**Phylogenetic reconstruction**

The 38 core genes of SpliNPV-Tun2 were translated to amino acid sequence, then aligned with core gene amino acid sequences from 88 group II NPVs, 39 group I NPVs, and from CpGV-M and SpliGV-K1 as outgroups using MUSCLE alignment tool v3.8.425 as implemented in Geneious Prime<sup>®</sup> v11 (Biomatters Ltd., Auckland, New Zealand) (Edgar 2004). The concatenated alignments of the amino acid sequences of the 38 baculovirus core genes (Wennmann et al. 2018) were then used to infer a phylogenetic tree using the Minimum Evolution method implemented in MEGA.7 (Kumar et al. 2016).

**Comparison of the SpliNPV-Tun2 genome to other NPVs**

All of the 132 SpliNPV-Tun2 ORFs were tested for sequence similarity using BlastX. A detailed comparison of the similarity with genomes of SpliNPV-AB1956 and SpliNPV-G2 was made. The genome characteristics were compared in terms of length, GC%, ORF number, presence of genes.

**Results**

In 2013, a laboratory colony of *S. littoralis* collected from tomato fields in Monastir (Tunisia) was established. In the reared colony, an occasional occurrence of moribund larvae with symptoms of a nucleopolyhedrosis infection was observed. The purification of viral OBs and DNA, PCR amplification using *polh* specific primers and sequence analysis (data not shown) indicated that the infective agent was a SpliNPV isolate, which was eventually termed SpliNPV-Tun2.

**Virulence and OB yield of SpliNPV-Tun2**

Concentration mortality bioassays with third instar larvae were performed to determine the virulence of SpliNPV-Tun2. The LC<sub>50</sub> value at 7 dpi was estimated to $1.5 \times 10^4$ OB/ml with a 95% confidence interval of $0.2 - 5.6 \times 10^4$ OB/ml ($n = 525$, slope probit line $= 0.42$, Chi<sup>2</sup> $= 8.81$). The survival rates determined at various time points after infection were inversely proportional to the applied OB concentration of $10^3$–$10^8$ OB/ml (Fig. 1). In the uninfected control, a slight decrease in the survival probability with 84% was observed at 14 dpi [95% CI (76.1–92.7%)]. A concentration-dependent decrease in the survival probability was observed in the treatment groups starting from 4 dpi with 96.7% [95% CI (96.0–97.4%)] and reached 7.81% [95% CI (6.48–9.40%)] at 14 dpi. The median mortality was obtained between 7 dpi for applied concentrations of $10^7$ and $10^8$ OB/ml and 10 dpi for the lowest concentrations $10^3$ and $10^6$ OB/ml of SpliNPV-Tun2. To estimate the survival covariance by time and by treatment, the survival was presented by percentage and survival data were normalized with lambda $= 0.57$ (Table 1). The survival time was statistically different depending on the applied virus concentrations. By using the different concentrations of SpliNPV-Tun2 OBs, all treatments produced different survival percentages depending on the time ($F=7.78$, $P$ value $< 0.01$).

![Fig. 1 Kaplan–Meier survival analysis of Spodoptera littoralis L3 larvae infected with different concentrations of SpliNPV-Tun2, ranging from $10^3$ to $10^8$ OB/ml. The untreated control is given as an orange line. Each line contains three independent replicates with 25 larvae each. Survival time is given in days post-infection (dpi). Dash lines represent the median survival time (ST<sub>50</sub>) for high ($10^7$ and $10^8$ OB/ml) and low OB ($10^6$ OB/ml or less) concentrations](image)

| Source | DF | MS  | F value | P value* |
|--------|----|-----|---------|----------|
| Time   | 6  | 28,840.5 | 402.41 | $< 0.001$ |
| Treatments | 11 | 8333.6 | 116.28 | $< 0.001$ |
| Time x treatments | 66 | 557.3 | 7.78 | $< 0.001$ |
| Residual SD | 168 | 71.7 | | |
| Error | 8.466 | | | |

*Two-way factorial ANOVA at $\alpha = 0.05$
OB productivity of late fourth and fifth instar larvae was quantified. The mean weight of larvae with virus infection symptoms was 1548 mg with a standard deviation (s.d.) of 82.5 mg. The OBs were harvested at 14 dpi when infected larvae were seen as highly moribund. Three different standard methods for OB purification were compared, i.e. LSC, SCC, and SGU (Wennmann and Jehle 2014). OB yield was found to be significantly different among LSC, SCC and SGU purification methods (ANOVA, $P \leq 0.05$) [$F(2,6) = 88.11, p < 0.001$]. LSC yielded $2.7 \times 10^9$ OB/g larval weight, followed by SCC $1.3 \times 10^9$ OB/g larval weight, whereas SGU yielded only $5 \times 10^8$ OB/g larval weight. (Fig. 2).

**Genome sequence of SpliNPV-Tun2**

A total of 1,597,175 filtered reads amounting to (90.6%) of the total raw reads were used for the analysis. From the total of the filtered reads, 1,508,620 paired reads and 88,555 unpaired reads could be mapped to the reference genome of SpliNPV-AN1956, whereas about 13,500 reads did not map to SpliNPV-AN1956 but gave BLAST hits with insect or bacterial DNA sequences. The obtained genome consensus sequence of SpliNPV-Tun2 (MG958660) was supported by an average of 720-fold sequencing depth (s.d. = 316). It had a length of 137,099 bp and a GC content of 44.7% (Table 2).

**Phylogenetic reconstruction and genetic distance**

A minimum evolution phylogenetic tree based on the concatenated amino acid sequences of 38 baculovirus core genes of group I and group II NPVs was inferred (Fig. 3). It corroborated the close relationship between SpliNPV-Tun2 and -AN1956. The next neighbour to both isolates was SpliNPV-G2. The SpliNPV isolates and SpliNPV-G2 are only distantly related to other Spodoptera-specific NPVs, such as SeMNPV, SpltNPV-II and Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) (Fig. 3). For baculovirus species demarcation, the Kimura-2-Parameter (K2P) distance of the 38 baculovirus core genes can be used as criterion, according to which, 2 isolates are considered to belong to the same species if their K2P distance is smaller < 0.021 and to different species if the K2P distance is > 0.072 (Wennmann et al. 2018). With a K2P distance of 0.001, SpliNPV-Tun2 and -AN1956 are 2 isolates belonging to the same species *Spodoptera littoralis* nucleopolyhedrovirus. In contrast, SpltNPV-G2 is distant enough (0.099 from the two viruses to constitute a separate species *Spodoptera litura* nucleopolyhedrovirus (Wennmann et al. 2018). The other known *Spodoptera*-specific NPV isolates constitute several other discrete species (Fig. 3).
| ORF name | SpliNPV-Tun2 | SpliNPV-AN1956 | SpliNPV-Tun2 vs SpliNPV-AN1956 | SpliNPV-G2 | SpliNPV-Tun2 vs SpliNPV-G2 |
|----------|--------------|----------------|-------------------------------|------------|---------------------------|
|          | No | Start → end | Size (aa) | Range (%identity) | No | Start → end | Size (aa) | Range (%identity) | No | Start → end | Size (aa) | Range (%identity) |
| polh     | 1  | 1 → 750    | 249      | 248/249 (99%)               | 1  | 1 → 750    | 249      | 249/249 (100%)               |
| pp78/83  | 2  | 747 → 2,396 | 549      | 51/542 (95%)               | 2  | 747 → 2,393 | 548      | 245/345 (71%)               |
| pk-1     | 3  | 2,398 → 3,228 | 756  | 750/927 (99%)               | 3  | 2,392 → 3,204 | 760    | 637/750 (85%)               |
| hoar     | 4  | 3,590 → 5,980 | 519  | 750/927 (99%)               | 4  | 3,519 → 5,714 | 731      | 244/500 (81%)               |
| le-0     | 5  | 6,638 → 6,444 | 596  | 6,420/6,464 (99%)           | 5  | 6027 → 6230 | 67       | 26/35 (74%)                 |
| dutpase  | 6  | 6,764 → 9,892 | 756  | 6,742/9,006 (93%)           | 7  | 6,447 → 8,600 | 717      | 338/443 (76%)               |
| (alt-repeats) | 8  | 9,016 → 10,701 | 756  | 9,006/9,016 (100%)          | 9  | 9,774 → 10,268 | 74        | 105/131 (80%)               |
| odv-e18  | 10 | 10,769 → 11,104 | 756  | 10,749/11,070 (100%)       | 11 | 10,801 → 12,110 | 744      | 456/470 (96%)               |
| odv-ec27 | 11 | 11,113 → 12,54 | 519  | 11,097/12,518 (97%)         | 12 | 12,234 → 12,485 | 83       | 83/83 (100%)                |
| Act146   | 12 | 12,723 → 14,004 | 519  | 12,701/14,064 (99%)        | 13 | 12,651 → 14,368 | 283      | 277/284 (95%)               |
| i.e.-1   | 13 | 14,094 → 14,706 | 519  | 14,079/14,824 (99%)        | 14 | 14,300 → 13,681 | 93       | 87/93 (94%)                 |
| odv-e56  | 14 | 17,058 → 18,167 | 519  | 17,040/18,152 (99%)        | 15 | 17,693 → 18,808 | 370      | 328/351 (93%)               |
| p10      | 15 | 18,182 → 18,733 | 519  | 18,167/18,718 (99%)        | 16 | 18,723 → 18,374 | 183      | 174/183 (95%)               |
| p74      | 16 | 18,787 → 19,101 | 519  | 18,771/19,085 (97%)        | 17 | 18,734 → 18,856 | 105      | 105/105 (100%)              |
| rr1      | 17 | 19,079 → 20,293 | 519  | 19,063/20,034 (97%)        | 18 | 19,734 → 20,179 | 657      | 657/657 (100%)              |
| (10P-I and 6 P-II-like repeats) | 19 | 20,088 → 22,89 | 519  | 20,068/22,044 (97%)        | 20 | 21,766 → 21,879 | 657      | 657/657 (100%)              |
| me53     | 21 | 22,690 → 25,904 | 519  | 22,682/25,902 (99%)        | 22 | 24,586 → 25,785 | 399      | 236/412 (57%)               |
| lef6     | 23 | 26,082 → 26,311 | 519  | 26,117/27,290 (99%)        | 24 | 26,751 → 27,151 | 599      | 267/267 (100%)              |
| dbp      | 25 | 27,166 → 28,123 | 519  | 27,305/28,039 (99%)        | 26 | 27,162 → 28,055 | 297      | 271/304 (98%)               |
| ubiquin/gp37 | 28 | 31,283 → 32,296 | 519  | 31,476/32,489 (99%)        | 29 | 31,305 → 31,304 | 300      | 300/300 (100%)              |
| 39kPP3   | 30 | 32,351 → 33,343 | 519  | 32,541/33,533 (99%)        | 32 | 31,336 → 32,191 | 351      | 309/337 (92%)               |
| lef-11   | 31 | 33,608 → 34,285 | 519  | 33,798/34,478 (99%)        | 34 | 33,075 → 33,509 | 144      | 123/144 (85%)               |
| A38      | 32 | 33,608 → 34,285 | 519  | 33,798/34,478 (99%)        | 35 | 33,479 → 34,141 | 220      | 194/225 (86%)               |
| ORF name | SpiNPV-Tun2 vs SpiNPV-AN1956 | SpiNPV-Tun2 vs SpiNPV-AN1956 | SpiNPV-G2 | SpiNPV-Tun2 vs SpiNPV-G2 |
|----------|-------------------------------|-------------------------------|-----------|--------------------------|
| No       | Start → end                  | Size (aa)                     | Range (%) | Size (aa)                 | Range (%)                  |
| p47      | 32 34,362 → 35,627            | 421                           | 421/421 (100%) | 36 34,211 → 35,479    | 422 371/422 (88%)          |
| lef-12   | 33 35,666 → 36,256            | 198                           | 198/198 (100%) | 37 35,506 → 36,111    | 201 168/198 (85%)          |
| (P-I repeat) | hr3 36,321 → 36,372          | hr3 36,506 → 36,547           | hr3       | 36,102 → 36,301          |
| lef-8    | 34 36,433 → 39,168            | 911                           | 910/911 (99%) | 38 36,316 → 39,072    | 918 830/920 (90%)          |
| bjd     | 35 39,167 → 40,078            | 303                           | 303/303 (100%) | 39 39,071 → 39,979    | 302 303/303 (100%)         |
|          | 36 40,101 → 40,671            | 189                           | 189/189 (100%) | 40 40,001 → 40,570    | 189 189/189 (100%)         |
|          | 37 40,691 → 40,891            | 66                            | 55/66 (83%) | 41 40,590 → 40,754    | 54 54/66 (82%)             |
| chitA    | 38 40,906 → 42,705            | 599                           | 598/599 (99%) | 42 40,767 → 42,461    | 564 56/563 (99%)           |
| (5-P-I repeats) | hr4 42,602 → 43,004 | hr4 42,837 → 43,091           | hr4       | 42,502 → 43,051          |
|          | 39 43,126 → 43,728            | 200                           | 198/200 (99%) | 43 43,021 → 43,635    | 204 130/203 (64%)          |
|          | 40 43,945 → 44,583            | 212                           | 210/212 (99%) | 44 43,779 → 44,405    | 208 167/212 (79%)          |
|          | 41 44,653 → 45,099            | 148                           | 148/148 (100%) | 45 44,475 → 44,888    | 137 123/148 (83%)          |
|          | 42 45,133 → 46,407            | 424                           | 420/425 (96%) | 46 44,926 → 46,194    | 422 241/257 (94%)          |
|          | 43 46,412 → 46,639            | 75                            | 75/75 (100%) | 47 46,199 → 46,426    | 75 71/75 (95%)             |
|          | 44 46,599 → 46,865            | 83                            | 75/83 (90%) | 48 46,386 → 46,640    | 84 71/84 (85%)             |
|          | 45 46,687 → 47,727            | 346                           | 330/330 (100%) | 49 46,474 → 47,532    | 352 306/337 (91%)          |
|          | 46 47,856 → 48,071            | 71                            | 71/71 (100%) | 50 47,653 → 47,868    | 71 57/71 (80%)             |
|          | 47 48,355 → 48,855            | 166                           | 166/166 (100%) | 51 48,172 → 48,693    | 173 154/166 (93%)          |
|          | 48 48,906 → 49,472            | 188                           | 189/189 (100%) | 52 48,722 → 49,246    | 174 104/152 (68%)          |
|          | 49 49,489 → 49,737            | 82                            | 82/82 (93%) | 53 49,271 → 49,519    | 82 57/66 (86%)             |
| cathepsin | 50 49,784 → 50,794            | 336                           | 336/336 (99%) | 54 49,566 → 50,579    | 337 313/337 (93%)          |
| p49      | 51 50,843 → 52,183            | 446                           | 446/446 (100%) | 55 50,628 → 51,947    | 439 376/446 (84%)          |
| fp25k    | 52 52,291 → 52,884            | 197                           | 197/197 (100%) | 57 52,075 → 52,668    | 197 197/197 (100%)         |
| lef-9    | 53 53,042 → 54,538            | 498                           | 498/498 (100%) | 59 52,839 → 54,335    | 498 479/498 (96%)          |
| (8-P-I repeats) | hr5 54,548 → 55,204           | hr5 54,677 → 55,304           | hr5       | 54,401 → 55,642        |
|          | 54 55,105 → 55,392            | 95                            | 98 76/82 (93%) | 55 55,052 → 55,648    | 98 76/82 (93%)             |
|          | 55 55,424 → 55,732            | 102                           | 102/102 (100%) | 56 55,009 → 55,005    | 102 55,992 → 56,166       |
| (3-P-I and 10-P-II-like repeats) | hr6 55,715 → 56,710          | hr6 56,009 → 57,005           | hr6       | 55,992 → 56,166        |
|          | 56 56,760 → 57,793            | 346                           | 342/346 (99%) | 62 56,221 → 57,324    | 367 210/371 (57%)          |
|          | 57 57,944 → 58,948            | 334                           | 334/334 (99%) | 63 57,477 → 58,478    | 333 302/335 (90%)          |
|          | 58 59,434 → 59,030            | 134                           | 134/134 (98%) | 64 58,556 → 58,966    | 136 98/136 (72%)           |
|          | 59 59,030 → 59,434            | 313                           | 313/313 (100%) | 65 58,899 → 59,840    | 313 262/310 (85%)          |
| ORF name | SplNPV-Tun2 | SplNPV-AN1956 | SplNPV-Tun2 vs SplNPV-AN1956 | SplNPV-G2 | SplNPV-Tun2 vs SplNPV-G2 |
|----------|-------------|---------------|-----------------------------|-----------|--------------------------|
|          | No | Start → end | Size (aa) | No | Start → end | Size (aa) | Range (%identity) | No | Start → end | Size (aa) | Range (%identity) |
| 60       | 59,367 | 60,308 | 132 | 60 | 60,558 | 60,974 | 138/138 (100%) | 66 | 59,800 | 60,198 | 123/135 (91%) |
| 61       | 60,25 | 60,558 | 139 | 61 | 60,979 | 62,028 | 346/349 (99%) | 67 | 60,203 | 61,276 | 357/314 (89%) |
| 62       | 60,671 | 61,720 | 800 | 62 | 62,263 | 64,665 | 799/800 (99%) | 68 | 61,552 | 63,924 | 790/698 (80%) |
| 63       | 62,026 | 64,428 | 1020 | 63 | 64,667 | 67,729 | 1019/1020 (99%) | 69 | 63,926 | 66,994 | 1022/924 (90%) |
| 64       | 67,446 | 67,633 | 36 | 64 | 67,683 | 67,871 | 16/16 (100%) | 70 | 67,509 | 68,651 | 380/309 (81%) |
| hr        | 67,605 | 67,800 | 67 | 67,916 | 67,118 | 67 | 67,509 | 67,800 | 67 | 67,362 | 67,581 |
| 65       | 67,907 | 69,094 | 380 | 65 | 68,224 | 69,366 | 380/380 (100%) | 71 | 67,509 | 68,651 | 380/309 (81%) |
| 66       | 69,204 | 69,827 | 207 | 66 | 69,444 | 70,067 | 207/207 (100%) | 72 | 67,272 | 69,353 | 208/160 (70%) |
| 67       | 69,899 | 70,282 | 127 | 67 | 70,139 | 70,522 | 127/127 (100%) | 73 | 69,412 | 69,795 | 127/123 (97%) |
| 68       | 70,305 | 70,559 | 84 | 68 | 70,545 | 70,799 | 84/68 (100%) | 74 | 69,814 | 70,068 | 84/67 (99%) |
| lef-1     | 69 | 70,624 | 71,772 | 382 | 69 | 70,864 | 72,015 | 383/383 (99%) | 75 | 71,307 | 71,669 | 120/84 (68%) |
| vif-1     | 70 | 71,793 | 72,155 | 120 | 70 | 72,036 | 72,398 | 120/119 (99%) | 76 | 71,666 | 72,658 | 330/307 (98%) |
| gp41      | 71 | 72,152 | 73,132 | 326 | 71 | 72,395 | 73,375 | 326/311 (99%) | 77 | 72,633 | 73,331 | 232/206 (87%) |
| ttk-20    | 72 | 73,107 | 73,820 | 237 | 72 | 73,350 | 74,063 | 237/236 (99%) | 78 | 73,210 | 73,803 | 197/160 (79%) |
| vp90      | 73 | 74,377 | 76,807 | 856 | 74 | 74,474 | 77,044 | 856/850 (98%) | 79 | 73,772 | 75,357 | 861/739 (86%) |
| (6 P-I repeats) | hr8 | 76,898 | 78,270 | 259 | hr8 | 77,112 | 77,422 |
| cg30      | 75 | 77,320 | 78,099 | 259 | 75 | 77,534 | 78,313 | 259/259 (100%) | 80 | 76,639 | 77,391 | 250/188 (73%) |
| vp39      | 76 | 78,128 | 79,036 | 302 | 76 | 78,342 | 79,250 | 302/302 (100%) | 81 | 77,450 | 78,358 | 302/296 (98%) |
| lef-4     | 77 | 79,038 | 80,504 | 488 | 77 | 79,252 | 80,721 | 489/479 (98%) | 82 | 78,360 | 79,787 | 475/413 (85%) |
| p33       | 78 | 80,535 | 81,302 | 255 | 78 | 80,751 | 81,518 | 255/253 (99%) | 83 | 79,833 | 80,600 | 255/248 (99%) |
| 89        | 79 | 81,301 | 81,831 | 176 | 79 | 81,517 | 82,050 | 177/174 (77%) | 84 | 80,599 | 81,147 | 182/167 (92%) |
| ovd-v25   | 80 | 81,828 | 82,502 | 226 | 80 | 82,047 | 82,727 | 226/226 (100%) | 85 | 81,144 | 81,827 | 227/215 (95%) |
| DNA helicase | 81 | 82,613 | 86,368 | 1251 | 81 | 82,832 | 86,857 | 1251/1251 (100%) | 86 | 81,918 | 85,625 | 1235/1155 (92%) |
| 38k       | 82 | 86,337 | 86,852 | 171 | 82 | 86,556 | 87,071 | 171/171 (100%) | 87 | 85,594 | 86,106 | 170/165 (96%) |
| lef-5     | 83 | 86,859 | 87,776 | 305 | 83 | 87,079 | 87,906 | 305/304 (99%) | 88 | 86,114 | 87,028 | 304/298 (97%) |
| p6.9      | 84 | 87,672 | 88,568 | 298 | 84 | 87,892 | 88,785 | 297/296 (98%) | 89 | 86,924 | 87,832 | 302/254 (83%) |
| p40       | 85 | 88,589 | 88,858 | 89 | 85 | 88,806 | 89,069 | 87/10 (100%) | 90 | 87,850 | 88,104 | 84/10 (100%) |
| (BP-I repeats) | hr9 | 89,022 | 88,919 | 237 | hr9 | 90,305 | 90,732 |
| p12       | 87 | 90,546 | 90,914 | 122 | 86 | 89,130 | 90,233 | 367/122 (100%) | 92 | 89,657 | 90,022 | 121/97 (80%) |
| p45       | 88 | 90,911 | 92,038 | 375 | 87 | 90,770 | 91,138 | 374/375 (99%) | 93 | 90,019 | 91,140 | 373/356 (95%) |
| ORF name | SpIPNPV-Tun2 | SpIPNPV-AN1956 | SpIPNPV-Tun2 vs SpIPNPV-AN1956 | SpIPNPV-G2 | SpIPNPV-Tun2 vs SpIPNPV-G2 |
|----------|--------------|----------------|--------------------------------|-----------|----------------------------|
|          | No | Start → end | Size (aa) | No | Start → end | Size (aa) | Range (%identity) | No | Start → end | Size (aa) | Range (%identity) |
| vp80     | 89  | 92,058 → 93,992 | 644 | 88  | 91,135 → 92,262 | 375 | 638/648 (98%) | 94  | 91,167 → 93,101 | 644 | 548/630 (87%) |
| odv-ec43 | 90  | 93,989 → 94,156 | 55  | 89  | 92,282 → 94,228 | 648 | 55/55 (100%) | 95  | 93,101 → 93,268 | 55  | 54/55 (98%) |
|          | 91  | 94,182 → 95,267 | 361 | 90  | 94,421 → 95,506 | 361 | 361/361 (100%) | 96  | 93,300 → 94,385 | 361 | 357/361 (99%) |
| odv-e66  | 92  | 95,358 → 95,696 | 112 | 91  | 95,599 → 95,937 | 112 | 112/112 (100%) | 97  | 94,466 → 94,804 | 112 | 103/112 (92%) |
| p13      | 93  | 97,779 → 98,636 | 285 | 93  | 98,017 → 98,874 | 285 | 285/285 (100%) | 98  | 96,785 → 97,744 | 289 | 262/289 (91%) |
| (8P-repeats) | | | | | | | | | | | |
| hr10     | 94  | 98,699...99,009 | | 95  | 99,224 → 99,748 | 174 | | | | | |
|          | 96  | 100,205 → 101,173 | 322 | 97  | 101,215 → 101,913 | 232 | | | | | |
|          | 98  | 101,933 → 103,342 | 469 | 99  | 103,357 → 103,897 | 177 | | | | | |
|          | 100 | 103,972 → 104,169 | 65  | 101 | 104,256 → 105,551 | 431 | | | | | |
| (7P-1 and 24 P-II-like repeats) | | | | | | | | | | | |
| hr11     | 102 | 105,681...107,729 | | 104 | 106,726 → 107,649 | 518 | | | | | |
|          | 106 | 109,998 → 111,158 | 387 | 107 | 111,207 → 111,408 | 67 | | | | | |
|          | 108 | 111,307 → 111,702 | 131 | 109 | 111,704 → 112,936 | 410 | | | | | |
|          | 109 | 112,992 → 113,771 | 259 | 111 | 113,608 → 114,033 | 141 | | | | | |
|          | 112 | 114,024 → 114,739 | 239 | 113 | 115,35 → 118,139 | 929 | | | | | |
| (7P-repeats) | | | | | | | | | | | |
| hr12     | 114 | 117,40...118,157 | | 115 | 118,941 → 119,489 | 182 | | | | | |
|          | 116 | 119,733 → 121,331 | 552 | 117 | 120,536 → 122,234 | 532 | | | | | |

*Note: The table continues with similar entries for other ORFs, each with their respective information.*
Table 2 (continued)

| ORF name | SpliNPV-Tun2 | SpliNPV-AN1956 | SpliNPV-Tun2 vs SpliNPV-AN1956 | SpliNPV-G2 | SpliNPV-Tun2 vs SpliNPV-G2 |
|----------|--------------|----------------|---------------------------------|------------|----------------------------|
|          | No | Start → end | Size (aa) | No | Start → end | Size (aa) | Range (%identity) | No | Start → end | Size (aa) | Range (%identity) |
| f gf     | 117 | 121,458 → 122,189 | 243 | 117 | 122,363 → 123,094 | 243 | 242/243 (99%) | 122 | 119,165 → 119,905 | 246 | 242/243 (99%) |
| pF1      | 118 | 122,214 ← 122,447 | 77 | 118 | 123,121 ← 123,354 | 77 | 77/77 (100%) | 123 | 119,928 ← 120,161 | 77 | 77/77 (100%) |
| 38.7 k   | 119 | 122,452 ← 124,029 | 525 | 119 | 123,359 ← 124,936 | 525 | 525/525 (99%) | 124 | 120,184 ← 121,764 | 526 | 505/507 (99%) |
| lef-1    | 120 | 124,29 ← 125,327 | 345 | 120 | 125,203 ← 126,240 | 345 | 345/345 (99%) | 125 | 125,891 ← 126,919 | 342 | 287/343 (84%) |
| def-1    | 121 | 125,314 ← 126,009 | 231 | 121 | 126,227 ← 126,922 | 231 | 231/231 (100%) | 126 | 126,906 ← 127,601 | 231 | 214/231 (93%) |
| def-2    | 122 | 125,99 ← 126,37 | 126 | 122 | 126,903 ← 127,289 | 128 | 126/128 (98%) | 127 | 127,582 ← 127,950 | 122 | 110/127 (87%) |
| calyx/pep| 123 | 126,367 ← 126,897 | 176 | 123 | 127,286 ← 127,816 | 176 | 176/176 (100%) | 124 | 127,947 ← 128,477 | 176 | 159/176 (90%) |
| pkip     | 124 | 126,907 ← 127,968 | 353 | 124 | 127,826 ← 128,887 | 353 | 353/353 (100%) | 125 | 128,481 ← 129,515 | 344 | 155/168 (92%) |
| arf-1    | 125 | 128,057 ← 128,596 | 179 | 125 | 128,976 ← 129,515 | 179 | 179/179 (100%) | 126 | 129,544 ← 130,161 | 205 | 157/180 (87%) |
| pil-2    | 126 | 129,386 ← 130,651 | 421 | 126 | 129,549 ← 130,277 | 421 | 238/242 (98%) | 127 | 130,199 ← 130,936 | 245 | 215/246 (87%) |
| Ac23     | 127 | 130,679...131,114 | (2P-I and 6P-I-like repeats) | 127 | 130,305 ← 131,570 | 421 | 421/421 (100%) | 128 | 130,910 ← 132,187 | 425 | 371/404 (92%) |
| hr14     | 128 | 131,200 ← 133,242 | 680 | 128 | 131,219 ← 134,155 | 680 | 670/681 (98%) | 129 | 133,451 ← 135,499 | 682 | 608/683 (89%) |
| hr15     | 129 | 133,276 ← 134,004 | 242 | 129 | 134,189 ← 134,917 | 242 | 241/242 (99%) | 130 | 135,545 ← 136,240 | 231 | 150/190 (79%) |
| hr16     | 130 | 134,109 ← 134,885 | 258 | 130 | 135,022 ← 135,798 | 258 | 258/258 (100%) | 131 | 136,338 ← 137,117 | 259 | 223/259 (86%) |
| hr17     | 131 | 134,972 ← 135,394 | 90  | 131 | 135,885 ← 136,295 | 136 | 58/58 (100%) | 132 | 136,923 ← 137,643 | 90  | 58/58 (100%) |
| hr18     | 132 | 135,866 ← 136,750 | 294 | 132 | 136,765 ← 137,649 | 294 | 294/294 (100%) | 133 | 138,104 ← 138,952 | 282 | 138/295 (47%) |

Given are the names of open reading frames (ORF), ORF number (No.), start and end position of the ORFs including direction of transcription (arrow), the translated amino acid (aa) length of the ORF, and the compared range and percent identity of the ORFs.
Main genome differences between SpliNPV-Tun2 and -AN1956

Compared to SpliNPV-AN1956, the new isolate SpliNPV-Tun2 showed insertion and deletion mutations in 62 ORFs, of which 37 ORFs are with predicted function. ORFs with significant changes caused by deletions and insertions are illustrated in (Fig. 4). These differences affect ORFs coding for predicted virus proteins related to virus structure, such as the structural protein PP78/81, the capsid-associated protein VP80 and VP1054, the OB matrix protein (Polyhedrin, POLH), the nucleotide metabolism (Ribonucleotide Reductase, RR1), proteins involved in viral DNA replication (Late Expression Factor 2 (LEF-2) and LEF-10, Protein kinase 1 (PK-1), LEF-5, and the group II Alphabaculovirus-specific HOAR and the BRO-a. Furthermore, a considerable number of amino acid changes were found but will not be further detailed here.

A notable difference is the presence of a tyrosine residue close to the N-terminus fifth amino acid position of the predicted POLH of SpliNPV-Tun2, a residue which is missing in the POLH of SpliNPV-AN1956 (Fig. 4). Another difference between the genome sequences of SpliNPV-Tun2 and -AN1956 is related to ORFs 106 and
Whereas in SpliNPV-AN1956 two ORF 106 and ORF 107 were located from genome position 110,884 < 111,843 (319 aa) and 111,873 < 112,064 (63 aa), respectively, these two ORFs were identified as one single ORF in SpliNPV-Tun2 (ORF 106, genome position 109,998 < 111,161, (387 aa)). The split of the ORF 106 homolog of SpliNPV-Tun2 into two ORFs 106 and 107 in SpliNPV-AN1956 is caused by a missing thymidine residue at genome position 110,991 of SpliNPV-AN1956, causing a frameshift and separation into two ORFs (Fig. 5). Interestingly, a similar homologous ORF 110 (genome position
the ST50 values were similar, suggesting that both isolates—Tun2 (Fig. 5).

region of ORF 107 of aa) would be homologous to the 3′ region of the adjacent ORF 108. In SpltNPV-G2, the 5′ region of ORF 111 (109,007 > 109,165, 52 aa) would be homologous to the 3′ region of ORF 107 of –Tun2 (Fig. 5).

Discussion

A new variant of SpltNPV, termed Tun2, was isolated and characterized by bioassays and genome sequencing. SpltNPV-Tun2 was found in a S. littoralis colony that was derived from larvae collected in tomato fields in Central-East Tunisia, in 2013. Another natural SpltNPV-Tun isolate was found in 2008 from tomato field in Chatt-Mariem (Sousse) (Laarif et al. 2011), suggesting that SpltNPV is present in wild populations of S. littoralis in Tunisia. Though the conditions of bioassays performed with SpltNPV-Tun2 were not fully identical to the bioassays carried out with SpltNPV-Tun, both the LC50 and the ST50 values were similar, suggesting that both isolates may not have significant biological differences.

OB productivity was quantified in the fourth instar larvae, as this instar was identified to be optimal for virus production (Grzywacz et al. 1998). Three different methods for OB purification were tested, of which the low-speed centrifugation (LSC) method (Harrison 2008) producing the highest yields of polyhedral OBs, which corresponded to an OB yield of about 4.2 × 109 OB/larvae. The superiority of LSC for polyhedral OB purification compared to sucrose gradient ultracentrifugation (SGU) and sucrose cushion centrifugation (SCC) was previously noted for isolation of Agrotis segetum nucleopolyhedrovirus by Wennmann and Jehle (2014). LSC and SCC are methods typically used for OB purification from NPVs (Harrison 2008), whereas SGU appears to counter-select for NPV polyhedra but favours purification of GV granules yielding about five times less NPV OBs than the other 2 methods (Wennmann and Jehle 2014).

Whole genome sequencing of SpltNPV-Tun2 revealed its close relationship to SpltNPV-AN1956 (Breitenbach et al. 2013), another isolate of SpltNPV from North Africa, which originated from Egypt and was first described by Abul Nasr (1956). Other isolates of SpltNPV from North Africa and the Mediterranean area were reported by Laarif et al. (2011). Only a few differences between the genome sequence of SpltNPV-Tun2 SpltNPV-AN1956 were noted: (i) the genome of SpltNPV-Tun2 is little shorter, (ii) both genomes contain the same number of ORFs and hrs and are fully collinear to each other, (iii) minor indel mutations could be identified in 34 ORFs as well as in intergenic regions, (iv) genetic changes were noticed in nine baculovirus core genes, and also in the highly conserved polh gene, and (v) the ORFs 106 and 107 of SpltNPV-AN1956 appeared to be fused to a single ORF 106 in SpltNPV-Tun2 but an additional ORF 107 was identified.

Phylogenetic analyses based on the 38 baculovirus core genes have been shown to reflect isolate and species phylogeny of baculovirus evolution and are considered as the most reliable method to infer the phylogenetic position of a given baculovirus (Wennmann et al. 2018). Our Minimum Evolution phylogenetic analysis revealed SpltNPV-AN156 and SpltNPV-G2 as closest neighbours of SpltNPV-Tun2. SpltNPV-G2 is an in vivo cloned genotype of an isolate separated from cadavers of S. littura, cotton leaf worm, in the area of Guangzhou, China (Pang et al. 2001). Breitenbach et al. (2013) found that SpltNPV-AN1956 and SpltNPV-G2 share a highly collinear genome and form a distantly related clade to other NPVs specific for Spodoptera species, such as SeMNPV, SfMNPV, and SpltNPV-II. Our phylogenetic analyses confirm that SpltNPV-Tun2, -AN1956, and SpltNPV-G2 form a clade of Spodoptera-specific NPVs which is separate from other group II alphabaculoviruses isolated from Spodoptera species, such as SeMNPV, SpltNPV-II, and SfMNPV. K2P distances of the 38 core genes clearly indicate that SpltNPV-Tun2 and -AN1956 should be considered as isolates from the same species, whereas SpltNPV-G2 belongs to a separate alphabaculovirus species, as well as all other even more distant NPVs isolated from Spodoptera sp. (Wennmann et al. 2018; Escasa et al. 2019).

Conclusions

Identification and genome sequence of the new isolate SpltNPV-Tun2 originating from Tunisia extended the present knowledge related to the genetic diversity of SpltNPV. With the detailed characterization of its genome, SpltNPV-Tun2 is proposed to be further evaluated as a biological agent for control of S. littoralis and potentially for the fall armyworm, S. frugiperda, and tobacco cutworm S. littura.

Abbreviations

aa: Amino acid; dpi: Days post-inoculation; hrs: Homologous repeat regions; OB: Occlusion body; ODV: Occlusion derived virion; ORF: Open reading frame; PCR: Polymerase chain reaction; LC50: Median lethal concentration; LSC: Low-speed centrifugation; SCC: Sucrose cushion centrifugation; SGU: Sucrose gradient ultracentrifugation; ST50: Median survival time.

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Author contributions
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