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Occurrence and characterization of a nucleopolyhedrovirus from *Spodoptera littoralis* (Lepidoptera: Noctuidae) isolated in the azores

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Abstract

A nucleopolyhedrovirus (SpliMNPV-Az) was isolated from diseased larvae of *Spodoptera littoralis*, collected at the Island of S. Miguel in Azores. The virulence of this isolate was tested against *S. littoralis* larvae in laboratory. LD_{50} against 2nd and 3rd instars were not significantly different, 1.44×10^4, 3.89×10^4 OBs per larvae, respectively, but both were significantly different from that against 4th instar, which was 61.3×10^4 OBs per larvae. The complete codons sequence of SpliMNPV-Az *Polh* gene obtained was 750 bp (NCBI GenBank Accession No. AY600451). This sequence was compared to other 38 polyhedrin genes from NPVs and to 6 granulin genes from GVs and resulted to be identical to the sequence of a SpliMNPV previously published, thus indicating that the natural host of SpliMNPV-Az must be *S. littoralis*. Genetic distances estimated from restriction enzymes profiles showed SpliMNPV-Az is close to the Egyptian SpliMNPV type B, despite some degree of genetic divergence suggested by slight differences observed on *Pst*I profile.
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1. Introduction

The polyphagous insect *Spodoptera littoralis* (Boisduval) damages a wide variety of crops including cotton, tobacco, and corn in countries around the Mediterranean and in Southeast Asia (Balachowsky, 1972; Sneh et al., 1981). The presence of this insect in the Azores was referenced in the middle of the 20th century (Carvalho et al., 1999) and the first damages caused by the larvae in tobacco and sugar beet were reported in the mid-1990s. In a survey for natural enemies of *S. littoralis* at the island of S. Miguel in Azores, about 20–30% of larvae were collected with a viral disease caused by a nucleopolyhedrovirus (Martins et al., 2005). Nucleopolyhedrovirus are characterized by a large circular dsDNA genome package within an enveloped rod-shaped nucleocapsid that is subsequently occluded within a paracrystalline protein occlusion, the polyhedrin. Nucleopolyhedrovirus characterization is based on insect host, but also on biochemical analysis of structural proteins and on DNA-based studies (Bilimoria, 1986). Molecular analysis based on restriction enzyme (REN) patterns was used to assess variability among NPVs isolates (Gettig and McCarthy, 1982; Richards et al., 1999) and to distinguish NPVs from different insects isolated in the same geographic area (Boughton et al., 1999). The sequence of polyhedrin gene has been also largely used despite divergences observed in phylogenies constructed with other genes (Herniou et al., 2001, 2004), which have been attributed to the specific mosaic structure of the polyhedrin gene of *Autographa californica* MNPV (Lange et al., 2004).

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Regarding the *S. littoralis* NPV, a large collection of this virus from Israel was separated based on REN profiles into two distinct genotypes and classified as SlNPV-T and SlNPV-D by Kislev and Edelman (1982) and later as SlNPV-A and SlNPV-B, respectively, by Cherry and Summers (1985). Based on REN, Croizier et al. (1986) showed that NPVs isolated from *S. littoralis* collected in Morocco, Egypt, and France all belong to type B.

In this paper, we characterize a baculovirus isolated from one single larva of *S. littoralis* collected in the field with infection based on ultrastructural morphology, on restriction enzyme genetic patterns, and on the sequence of the coding portion of the polyhedrin gene. Furthermore, the virulence of this isolate against three larval instars of *S. littoralis* was determined.

2. Materials and methods

2.1. Insects

Larvae of *S. littoralis* were reared from surface-sterilized eggs, maintained at room temperature in a 16:8 photoperiod, and fed on artificial diet (Poitout and Bues, 1970), in which fungicide was suppressed.

2.2. Virus isolation and propagation

Virus isolate was obtained from one larva of *S. littoralis* collected with infection at S. Miguel Island in the Azores. Amplification of the virus was performed in fourth instar *S. littoralis* from the laboratorial colony. The larvae were individually placed in 90 mm petri dishes, fed with 500,000 occlusion bodies (OBs) applied on the surface of a 3×3 mm leaf piece of *Rumex* sp., and maintained at 25 °C to develop infection. Occlusion bodies were purified from larvae homogenates according to O'Reilly et al. (1992).

2.3. Ultrastructural studies

For scanning electron microscopy (SEM), pellets of purified Azorean isolate were dehydrated through a graded ethanol series and dried with hexamethyldisilane. The pellet was spread on carbon adhesive tabs, placed on aluminum specimen stubs and coated with palladium/gold on a JEE-400 vacuum evaporator for observation on a JEOL-5410 scanning microscope.

For transmission electron microscopy (TEM), fat bodies and gut tissues were excised from the infected larvae 72 h post-inoculation, bathed overnight at 4 °C in 3% cacodylate (0.1 M) buffered glutaraldehyde, post-fixed in 2% osmium tetroxide for 3 h, dehydrated and embedded in epon. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined in a JEOL 100CXII TEM at 60 kV.

2.4. Extraction of viral DNA

The purified OBs were diluted to 1×10^6 OBs/μl in sodium carbonate to a final concentration of 0.1 M. This mixture was incubated at room temperature for 30 min and then Tris–HCl, pH 8, was added to a final concentration of 0.1 M. The resulting suspension was centrifuged at 3600*g* for 5 min at room temperature. The supernatant, containing the released virions, was incubated in 0.1 M Tris–HCl, pH 8, 0.01 M EDTA, 1% SDS, and 200 μg/ml proteinase K at 56 °C for 4 h. DNA was extracted with phenol–chloroform, ethanol precipitated and resuspended in $0.33 \times$ TE.

2.5. Restriction enzyme digests

Three micrograms of viral DNA were digested with *Ava*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, *Xho*I (Invitrogen Corp.), *Kpn*I (Pharmacia), and *Bgl*I (Takara) following supplier's instructions. Digested DNA together with λ-*Hin*dIII (0.5 μg) was loaded onto a 15×25 cm of 1.2% agarose gel and run at 80 V for 12 h. The gel was stained with ethidium bromide solution and the image under UV light was captured on a GelDoc 2000 Image Analyzer (BioRad Corp.). Quantity One software (Bio-Rad Corp.) was used to estimate the molecular weights of the DNA bands of each restriction enzyme pattern. The resulting restriction enzyme profiles were compared with 85 published digest patterns from NPVs.

2.6. Sequencing of polyhedrin gene

As the NPV isolated from our sample was uncharacterized, we first used a set of degenerate primers for the amplification of a polyhedrin gene fragment, reported to be efficient in amplifying this fragment from a wide range of NPVs (Woo, 2001). The PCR program used for these primers involved an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 59.8 °C, and 30 s at 72 °C; and a final extension step of 5 min at 72 °C. The amplified products (420 bp) were cloned into a TOPO TA Cloning vector (Invitrogen Corp.). Two clones were selected for sequencing. The inserts were amplified from plasmid DNA using T3 and T7 universal primers and sequenced. The sequences obtained were compared with those of the GenBank, using the BLAST network service program (Altschul et al., 1990).

As the sequence of the fragment amplified with the degenerate primers resulted to be identical to a fragment of a polyhedrin gene previously deposited in the GenBank (Accession No. D01017), this last sequence was used to design specific primers to amplify the complete codons of the polyhedrin gene (750 bp). The sequences of the primers were as follows: forward

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