Virulence and genetic characterization of six baculovirus strains isolated from different populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

Fall armyworm (FAW), *Spodoptera frugiperda* (Smith, 1797), is a polyphagous, voracious, and economically important agricultural pest. Biological control of FAW is a strategy that must be further explored. This study evaluated six baculovirus strains isolated from infected FAW larvae from Mexico, Argentina, Honduras, and the United States. Five alphabaculoviruses (SfNPV-An2, SfNPV-Arg, SfNPV-Fx, SfNPV-Ho, and SfNPV-Sin) and one betabaculovirus (SfGV-RV) were tested against FAW larvae, showing a wide diversity of virulence levels among strains when their estimated LC50s were compared, being SfNPV-Arg, SfNPV-Ho and SfNPV-Fx more virulent than SfNPV-An2, SfNPV-Sin, and SfGV-RV. To determine any virulence difference in vitro studies of these isolates, Sf9 cell cultures were used. Interestingly, only ODVs from four of the test SfNPV strains showed infectivity on Sf9 cell cultures, and some differences in virulence were observed. Genomic restriction analyses and partial sequences of *lef-8*, *lef-9*, and *polh/granulin* genes showed little variability among alphabaculoviruses, both, among them and with previously reported sequences. However, sequences from SfGV-RV were closer to previously reported sequences from the SfGV-VG008 strain than the SfGV-Arg and SfGV-VG014 strains. The great difference in the in vivo virulence was not correlated with great similarity among the isolates. The characterization of these six baculovirus isolates offers the basis for exploring their potential as biological control agents against *S. frugiperda*, as well the initial studies on their specific infection mechanisms, evolution, and ecology.

Keywords *Spodoptera frugiperda* · Baculovirus · Virulence · Phylogeny · Genetic comparison

Introduction

Maize (*Zea mays* L. subsp. *mays*) (Poaceae) is one of the most important staple crops worldwide (Moya-Raygoza et al. 2018), and it is usually grown as an extensive...
monoculture system to satisfy human and animal consumption demand. At the same time, monoculture agricultural systems induce population increase of pests (Altieri et al. 2018). The fall armyworm (FWA), Spodoptera frugiperda (Lepidoptera: Noctuidae) (Smith, 1797), is a polyphagous, voracious, and economically important pest in North and South America (Cokela et al. 2021; Lee et al. 2020; Martínez-Jaime et al. 2018), affecting a wide variety of plant species such as maize, sorghum, cotton, and soybeans (Montezano et al. 2018), among many other crops. Management of FAW usually relies on the use of insecticides. However, resistance against chemical treatments has risen (Gutierrez-Moreno et al. 2019; Yu 1991; Yu et al. 2003). Therefore, sustainable integrated pest management strategies must be explored, such as the use of biological control agents. Entomopathogens, parasitoids, and predators offer promising specific alternatives as they do not represent a risk to human health, the environment, or to other beneficial insects (Lacey et al. 2015; Melo et al. 2016; Tavares et al. 2010; Vega 2018; Wasim et al. 2009).

Baculoviruses belong to the family Baculoviridae (Harrison et al. 2018). These viruses are highly selective pathogens to insects from orders Lepidoptera, Hymenoptera, and Diptera (Fuxa 2004; Kong et al. 2018). They are a diverse group of viruses with supercoiled, circular double-stranded DNA genomes, ranging in size from 80 to 180 kb and encoding between 90 and 180 genes. The two commonly found virion phenotypes in baculovirus are occlusion-derived virions (ODV) and budded virions (BV) (Blissard and Rohrmann 1990; Blissard and Theilmann 2018; Herniou et al. 2011; Jehle et al. 2006). Occlusion bodies (OBs) enclose the viral particles and allow viruses to survive in the environment. They are composed of a crystalline matrix of protein, either polyhedrin in nucleopolyhedroviruses (NPV) and granulin in granuloviruses (GV) that occlude virions (Bilimoria 1991; Kelly et al. 2016). The Baculoviridae family is divided into four following genera: Alphabaculovirus (Lepidoptera specific NPVs; group I and II), Betabaculovirus (Lepidoptera specific GVs), Gammabaculovirus (Hymenoptera specific NPVs), and Deltabaculovirus (Diptera specific NPVs) (Carstens and Ball 2009; Jehle et al. 2006).

Autographa californica multiple NPV (AcMNPV) polyhedrin (polh) gene was the first baculovirus gene sequenced (Hooft van Iddekinge et al. 1983; Rohrmann et al. 1981) and used for phylogenetic studies (Cowan et al. 1994; Zanotto et al. 1993). However, as genome sequences became available, some studies showed that different genes present different phylogenies (Harrison and Bonning 2004; Herniou et al. 2003), implying that to understand the evolution of baculoviruses, some genes were more valuable than others. This led to the definition of “core genes” and the selection of a set of conserved genes that together could offer robust phylogenetic results (Herniou et al. 2001, 2003; Lange et al. 2004).

Another important aspect to consider in basic studies of baculoviruses as well as biological control agents is their potential large-scale, in vitro production, which has been possible since the establishment of the first cell line from pupal tissues of Antheraea eucalypti (Lepidoptera: Saturniidae) (Grace 1962), followed by more insect cell lines (Echalier 1971; Goblirsch et al. 2013; Grasela et al. 2012; Lynn and Hung 1986; Smagghe et al. 2009). These include the established IPLB-Sf21 (Sf21) pupal cell line of S. frugiperda (Vaughn et al. 1977) and the Sf9, a monoclonal cell line derived from Sf21 (Pasumarthry and Murhammer 1994).

Several baculoviruses with activity towards S. frugiperda have been isolated and studied throughout the American continent (Barrera et al. 2011; Barreto 2005; Berretta et al. 1998; Escribano et al. 1999; Fuxa 1987; García-Banderas et al. 2020; Gómez et al. 2010; Ordóñez-García et al. 2020; Vieira et al. 2012; Yaser et al. 2009). The Food and Plant Biotechnology laboratory of the Life Sciences Division, Campus Irapuato-Salamanca of the University of Guanajuato, Mexico, has a collection of six isolates of SfNPVs, with some molecular variations and different biological activities towards S. frugiperda (Rangel-Núñez et al. 2014; Ríos-Velasco et al. 2011; Ríos-Velasco et al. 2012). In this study we characterize those isolates with activity towards FAW larvae at the biological and phylogenetic level.

Materials and methods

Virus strains

Five SfNPVs and one SfGV isolate were previously isolated from S. frugiperda or soil at different locations. SfNPV-An2 was isolated in Coahuila, Mexico: SfNPV-Arg in Argentina; SfNPV-Fx in the United States; SfNPV-Ho in Honduras, and SfNPV-Sin in Sinaloa, Mexico. The granulovirus SfGV-RV was isolated in Coahuila, Mexico. The six isolates were previously characterized using restriction endonuclease patterns. The isolates SfNPV-Arg, SfNPV-Fx, and SfNPV-Ho were previously characterized by their virulence, establishing their lethal media concentration (LC50) on S. frugiperda (Rangel-Núñez et al. 2014; Ríos-Velasco et al. 2011, 2012).

Virus propagation

Viral particles were amplified using larvae of S. frugiperda. Larvae were maintained in a semi-artificial diet containing 100 mL distilled water, 12.5 g bacteriological agar, 120 g corn flour, 50 g yeast, 5 g wheat germ, 25 g ground corn spike, 2.5 g sorbic acid, 5 g ascorbic acid,
3.125 g methylvparaben, 8.75 g salt mixture, 62.5 g soybean, 3.125 mL formaldehyde 37%, 0.75 mg streptomycin, and 18.75 g Vandersant vitamin mixture. They were maintained under insectarium conditions (60 ± 10% relative humidity, 26 ± 2 °C and 16:8 h of light: dark photoperiod) in an environmental chamber (PERCIVAL) (Rangel-Núñez et al. 2014). Insect infection was carried out by diet surface contamination, using 500 µl of 1 × 10^6 OB/mL and 10 2nd instar FAW larvae per container. Larvae were placed individually within a grid on the diet and subsequently incubated for 6–7 days under the previously described conditions. The OBs from infected larvae were purified through sucrose density gradients by ultracentrifugation in a SW-32 rotor (Optia XPN-100, Beckman Coulter) at 24,000 rpm for 90 min. The concentrations of viral OBs were quantified with a hemacytometer and stored in aliquots of 500 µl of distilled water at 4 °C until required.

**In vivo virulence of baculovirus isolates**

The LC₅₀s were estimated on FAW first instar larvae for the SfNPV-Sin, SfNPV-An₂, and SfGV-RV isolates and compared with those previously determined for isolates SfNPV-Arg, SfNPV-Fx, and SfNPV-Ho (Rangel-Núñez et al. 2014). Bioassays were performed using 20 larvae per concentration, spreading ten larvae per petri dish containing the semi-artificial diet. Concentrations for NPV and GV isolates were quantified in a hemocytometer. The highest concentrations per bioassay were for SfNPV-Sin, 1.5 × 10^4 OB/mm² of diet; for SfNPV-An₂, 3.2 × 10^2 OB/mm², and for SfGV-RV, 3.1 × 10^4 OB/mm². These concentrations were selected based on preliminary bioassays. For each isolate, six concentrations were calculated, using a dilution factor between 0.5 and 0.75. Larval mortality was documented after 5 days of incubation, and the results were analyzed using Probit analysis. A mean LC₅₀ was estimated for each isolate, 2 × 10⁸ purified OBs were suspended in 300 µL of an alkaline buffer (0.1 M Na₂CO₃, 0.1 M NaCl, pH 10.8) and incubated at 50 rpm shaking for 10 min. Released virions were suspended in cell culture media SF900 and sterilized by filtration (Lynn 2003). Filtrate was inoculated on cell line cultures and monitored for 10 days using an inverted microscope (Zeiss Primo Vert). Supernatants from positive infections containing BV were used to keep on the in vitro virus culture. Subsequent cell cultures started with 1 × 10⁶ cells in 25 cm² culture bottles and incubated for 24 h. These synchronized cell cultures were reinfected with a MOI of 10 and monitored for 10 days to detect any virulence difference of each isolate, in terms of the number of cells showing OBs and the lysing period, when OBs were released.

**Genetic characterization**

Restriction pattern analysis of isolates genomes and sequences of three core genes were used to characterize each of the six baculovirus isolates, by comparing those sequences, both, among them, and with some previously reported.

**DNA extraction**

The OBs from infected larvae were purified as described above. The purified OBs were resuspended in a TE buffer solution (0.01 M Tris–HCl, 0.001 M EDTA, pH 7.6) and an alkali solution (0.1 M Na₂CO₃, 0.1 M NaCl, pH 10.8) for 15 min in agitation. Virions were purified through sucrose density gradients at 28,000 rpm for 40 min. Virions were then suspended in buffer (10 mM Tris, 5 mM EDTA, 0.5% SDS) and incubated for 15 min at 60 °C. Then, 100 µg of proteinase K (Invitrogen) was added and incubated again for 30 min at 60 °C. The mixture was washed with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 14,000 rpm for 10 min; then the aqueous phase was mixed with one volume of cold isopropanol and centrifuged at 14,000 rpm for 10 min. The pellet was washed with 70% ethanol, centrifuged again, and solubilized in sterile distilled water (Del Rincón-Castro and Ibarra 1997). DNA was quantified in a Nanodrop (Thermo Scientific) and its integrity corroborated by electrophoresis in 1% agarose gels.

**Genomic restriction pattern analysis**

Once integrity of the genomic DNA from the NPVs strains was corroborated, they were subjected to endonuclease digestion using BamHI and EcoRI restriction enzymes (Invitrogen), followed by electrophoresis in 0.8% agarose gels. Digestion mixtures included 1 µg of genomic DNA, 1 µL restriction enzyme, 2 µL 10X enzyme buffer, and water to complete 20 µL. Mixtures were incubated at 37 °C for 2 h, and restriction patterns were visualized in agarose gel electrophoresis carried out at 25 V for 13 h. Patterns were recorded in a gel documentation system (Gel Doc™ EZ Image, Bio Rad) and compared visually between each other.
Differential bands between the isolates were detected by comparing three replicates electrophoresed under different conditions and DNA concentrations.

**PCR amplification of core genes**

Fragments of _lef-8_, _lef-9_, and _polh/gran_ genes were amplified from genomic DNA extracted from the six baculovirus isolates by PCR, using degenerated primers (Table 1) (Jehle et al. 2006; Lange et al. 2004). These primers included universal primer tails (M13 Rev, M13 Fw, and BGH Rev) to facilitate direct sequencing of amplicons. The specific primers for gene _lef-8_ should amplify a 702-bp fragment, while _lef-9_ should amplify 295 bp, and _polh_ gene amplifies a 540-bp fragment. Amplification of _lef-8_ and _lef-9_ gene fragments were performed by PCR touchdown (initial denaturalization at 95 °C for 3 min: 15 cycles decreasing the alignment temperature − 1 °C each cycle, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; plus 20 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; and final extension at 72 °C for 7 min). Gene _polh_ was amplified by conventional PCR (initial denaturalization 95 °C for 4 min; 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; with a final extension at 72 °C for 10 min). The PCR products were purified with the Pure Link PCR purification Kit (Invitrogen), and the amplicons were sequenced on the Illumina platform at MACROGEN Co. (Korea).

**Phylogenetic analysis**

Nucleotide sequences of _lef-8_, _lef-9_, and _polh_ were downloaded from the NCBI GenBank from the following reported genomes: SfNPV-3AP2 (EF033042), SfNPV-19 (EU258200), SfNPV-459 (MK503924), SfNPV-ColA (KF891883), SfNPV-ArgM (MW162628), SfNPV-281 (MK503923), SfNPV-B (HM595733), SfGV-VG008 (KM371112), SfGV-Arg (MH170055), and SfGV-VG014 (KJ698693, KJ698695 and KJ698691 for genes _lef-8_, _lef-9_, and _polh_, respectively). Nucleotide sequences were compared to the partial sequences of the six FAW baculoviruses used in the present study.

SeqMan 5.0 software was used to assemble the sequences (DNASTAR Inc.). The nucleotide sequence alignment was performed in the Mega X program (Kumar et al. 2018) using the Muscle algorithm and fitting to the size of the fragments obtained from the sequencing, to be later concatenated in the Mesquite software (version 3.5.1). The phylogenetic analysis was completed in the Mega X software using the neighbor-joining method (Saitou and Nei 1987). The nucleotide substitution model applied was p-distance. Gaps were treated as missing data. Bootstrap analyses (using 1000 replications) were used to assess the confidence in the branching order.

**Results**

**In vivo virulence of baculovirus strains**

Once bioassays used to evaluate the baculovirus strains on FAW 1st instar larvae, Probit analyses estimated a wide variability of _LC50_ values. The SfNPV-Sin strain showed an _LC50_ of 1619.24 OB/mm² of diet, while the SfNPV-An2 isolate showed an _LC50_ of 21.24 OB/mm², and the SfGV-RV isolate showed an _LC50_ of 3500 OB/mm² (Table 2). The virulence of the other three isolates included in the present study was previously estimated (RangelNúñez et al. 2014), and resulted in an _LC50_ of 1.15 OB/mm² for SfNPV-Arg, 3.42 OB/mm² for SfNPV-Fx, and 4.36 OB/mm² for SfNPV-Ho (Table 2). The isolate SfNPV-Sin turned out to be 1408 times less virulent than the most virulent strain (SfNPV-Arg). Strains SfNPV-Fx, SfNPV-Ho, and SfNPV-Arg showed no statistical difference, but a highly significant difference was observed between these three strains when compared with the SfNPV-An2 and SfNPV-Sin, which showed very low virulence. Comparisons with the SfGV-RV strain has no meaning, as OBs from NPVs and GVs are not comparable in size and virion content. All statistical

| Primer      | Sequencea | Universal primer | bp b | Source          |
|-------------|-----------|-----------------|------|-----------------|
| _lef-8_ forward | CAGGAAACAGCTATGACCAYGGHGRATGAC | M13 Rev | 702 | Lange et al. (2004) |
| _lef-8_ reverse  | GTAAAAGCAGCGGCGCAGYRTAGRTGCYTCSCGC | M13 Fw |  | |
| _lef-9_ forward  | CAGGAAACAGCAATGACAAAYGGITAYGCG | M13 Rev | 295 | Lange et al. (2004) |
| _lef-9_ reverse  | GATAAACAGCCGGCCAGTTGGTDCCRTCRARTC | M13 Fw |  | |
| Polh/gran forward | TAGAAGGCACACCTGAGGNGRNGCARGAYCCTNTT | BGH Rev | 540 | Jehle et al. (2006) |
| polh/gran reverse | CAGGAAACAGCTATGACCDDGGNGCRAAYTCYT | M13 Rev |  | |

a Underlined nucleotides indicate standard sequencing M13 forward, M13 reverse, T7 and BGHrev primers (this part of the primer allows for the direct sequencing of polymerase chain reaction products); degenerate baculovirus primers are not underlined

b Expected size of the amplification product
requirements were fulfilled, as previously described (Ibarra and Federici 1987).

**In vitro virulence of baculovirus strains**

When Sf9 cell cultures were inoculated with released ODVs from OBs of each isolate, only SfNPV-An2, SfNPV-Arg, SfNPV-Fx, and SfNPV-Ho were able to infect, replicate, and develop OBs within the cell. Isolate SfNPV-Sin showed no infectivity, after several trials, and SfGV-RV was not expected to be infectious, as it occurred, but still it was tested. The infected cells developed characteristic cell changes from the baculovirus infection, such as nuclear hypertrophy, development of virogenic stroma, presence of OBs in the nuclei, and cellular lysis. Additionally, the supernatants from positive isolates were infective to subsequent cultures.

Synchronized cultures, inoculated with the same MOI, showed signs of infection at day three post-infection (PI). On day 10 PI a significant number of infected cells was evident in all isolates (Fig. 1). Almost all the cells infected with isolate SfNPV-An2 showed OBs in their nuclei, but no lysed cell were observed at this time, while cells infected with SfNPV-Ho showed all the cell with OBs in their nuclei. All cells infected with SfNPV-Arg showed OBs in their nuclei and some lysis, showing some released OBs. In contrast, cells infected with SfNPV-Fx showed only some cells with OBs, because a great majority were already lysed, and many free OBs were apparent (Fig. 1).

**Genomic restriction pattern analysis**

Electrophoretic patterns obtained from the digestion of the NPV strains genomes, using the BamHI and EcoRI endonucleases are shown in Fig. 2. Differential bands are
marked with an arrowhead. When the genomic DNA from each isolate was digested with BamHI (Fig. 2B), simple patterns were obtained. Identical patterns were shown by isolates SfNPV-An2, SfNPV-Arg, Fx: SfNPV-Fx, Ho: SfNPV-Ho, and Sin: SfNPV-Sin. MWM molecular weight marker. Differential bands are pointed by an arrowhead.

Fig. 2 Genomic restriction analysis of the five SfNPV isolates. A Genomes digested with EcoRI. B Genomes digested with BamHI. An2: SfNPV-An2, Ar: SfNPV-Arg; Fx: SfNPV-Fx, Ho: SfNPV-Ho, and Sin: SfNPV-Sin. MWM molecular weight marker. Differential bands are pointed by an arrowhead.

The sequencing of the six baculovirus resulted in amplicons of approximately 702, 295, and 540 bp from lef-8, lef-9, and polh/gran, respectively. After the sequence editing, the sequence alignments sizes were 613 pb for gene lef-8, 163 pb for lef-9, and 486 pb for polh.

Using the SfNPV-3AP2 isolate as a standard SfNPV reference strain, sequence alignment showed that the nucleotide homologies of lef-8 ORFs of SfNPVs were above 98.7%. The homologies of the deduced amino acid sequences of all isolates were 100%. The aligned sequences of lef-9 did not show changes in nucleotides or amino acids. In polh, differences were not present in the nucleotide or amino acid sequences.

Sequencing of lef-8, lef-9, and polh genes

The sequences of lef-8, lef-9, and polh/gran genes coming from the six strains were submitted to GenBank under the access numbers MK501795 to MK501800 (lef-8), MK507900 to MK507905 (lef-9), and MK558035 to MK558040 (polh/gran). Seven reference sequences of the lef-8, lef-9, and polh genes from SfNPVs and three reference sequences of the lef-8, lef-9, and gran genes from SFGV were downloaded from the NCBI GenBank for sequence comparison.

The sequencing of the six baculovirus resulted in amplicons of approximately 702, 295, and 540 bp from lef-8, lef-9, and polh/gran, respectively. After the sequence editing, the sequence alignments sizes were 613 pb for gene lef-8, 163 pb for lef-9, and 486 pb for polh.

For the studied betabaculovirus strain, the SfGV-VG008 isolate was used as a standard SfGV reference. Sequence alignment showed that the nucleotide homologies of lef-8 ORFs of SfNPVs were above 98.25%. The homologies of the deduced amino acid sequences of all isolates were 100%. The aligned sequences of lef-9 did not show changes in nucleotides or amino acids. In polh, differences were not present in the nucleotide or amino acid sequences.
deduced amino acid sequences of all isolates were 99.03%.
Sequence alignment showed that the nucleotide homologies of \textit{lef-9} ORFs of \textit{SfGVs} were above 97.32%. The homologies of the deduced amino acid sequences of all isolates were above 98.84%. Sequence alignment showed that the nucleotide homologies of \textit{gran} ORFs of \textit{SfGVs} were above 98.27%. The homologies of the deduced amino acid sequences of all isolates were above 98.84%.

**Phylogenetic analysis**

The phylogenetic analysis used a total of 1371 positions of concatenated nucleotide sequences from \textit{lef-8}, \textit{lef-9}, and \textit{polh} genes of the five nucleopolyhedroviruses (\textit{SfNPV-Arg}, \textit{SfNPV-Ho}, \textit{SfNPV-Fx}, \textit{SfNPV-An2}, and \textit{SfNPV-Sin}) and the granulovirus \textit{SfGV-RV}, as well as several \textit{SfNPVs} and \textit{SfGVs} downloaded from the NCBI GenBank which were used as references, for a total of 17 compared sequences. The optimal tree (Fig. 3) with the sum of branch length was 0.67489023. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic reconstruction showed a great similarity between all the compared \textit{SfNPVs} sequences. Specifically, an identical identity between strain \textit{SfNPV-Arg} and \textit{SfNPV-Sin}, along with a reference strain in the same clade is observed. Similar, but in a different clade, appears the \textit{SfNPV-Fx} strain along two reference sequences. \textit{SfNPV-An2} and \textit{SfNPV-Ho} share the same clade, whose sequences were identical, as well as two reference sequences. Of course, \textit{SfGV-RV} is in a totally different clade, whose most similar reference strain was \textit{SfGV-VG008}. Other reference strains showed more differences. The sequences from the gammadbaculovirus \textit{Neodiprion lecontei} NPV was used as an outgroup.

**Discussion**

This research was focused on the comparison of six baculovirus strains isolated from FAW larvae in four different countries. Virulence and some genetic features were the main characteristics to compare. Although virulence was highly variable, genetic features showed only limited differences between them.

Virulence of isolates \textit{SfNPV-Sin}, \textit{SfNPV-An2}, and \textit{SfGV-RV} was compared with other baculovirus isolates involved in the present study (Rangel-Núñez et al. 2014). Although the isolate \textit{SfNPV-An2} showed the highest virulence among the three isolates estimated here, it was still 18 times less virulent than the \textit{SfNPV-Arg} isolate. This great difference in virulence contrasts with other reports (Rowley et al. 2010) where \textit{SfNPV} isolates showed more consistent levels of virulence. Trying to explain such a big difference in virulence, it is important to notice that those isolates showing the lowest virulence (\textit{SfNPV-Sin} and \textit{SfNPV-An2}) were originally isolated within the Mexican territory and tested on a Mexican population of FAW. This may indicate that
Mexican NPV strains and Mexican FAW populations have co-evolved during a significant long period of time, developing natural resistance by Mexican FAW populations towards their NPVs. It would be interesting to test these isolates on Argentinian and/or Honduran FAW populations to prove this hypothesis. About the virulence of the SfGV-RV isolate, no comparison was done as NPV OBs not only are much bigger than GV OBs but also NPV OBs contain a great number of virions per OB, as compared with the single virion of a GV OB. However, if SfGV-RV is compared with other SfGV strains, the former is about 100 times less virulent than the latter (Cuartas et al. 2014). The same hypothesis can be postulated to explain this phenomenon, based on some genetic differences.

On the other hand, the in vitro virulence of the six strains tested on Sf9 cell cultures showed some differences, too, but not at the same level than those found in the in vivo bioassays. First, there is an unsolved question about why hemolymph from infected larvae failed to infect Sf9 cell cultures, while ODVs were able to infect them. It is important to notice that, when infection of Sf9 cells was finally achieved with ODVs, such infections occurred in a very limited number of cells, showing low infectivity, similar to results shown in some pioneering reports (Volkman et al. 1976; Volkman and Summers 1977). Subsequent cultures used supernatants containing BVs from the established cultures. Still, only four out of six isolates were able to replicate in vitro as SfNPV-Sin was unable to grow, which also showed very little virulence on larvae. Also, the SfGV-RV strain did not grow in cultured cells, although this result was expected, as very few cases of stale GV in vitro cultures have been achieved (Ma et al. 2019).

Differences were observed mostly on the length of the infection process. Three replicates of synchronous cultures, with the same cell concentration and the same inoculum, consistently showed that infection with isolate SfNPV-AN$_2$ was slower than infections with isolates SfNPV-Arg and SfNPV-Fx. To some extent, these results may explain those obtained in the in vivo bioassays.

With reference to the genetic characterization of the isolates, both, the genomic restriction analysis, and the sequence comparison of the three selected genes, they showed a high degree of similarity between the isolates. Minor differences were observed in terms of additional or missing bands in the restriction patterns of the NPVs tested. The great difference in the in vivo virulence between isolates SfNPV-Sin and SfNPV-Arg contrasted with the almost identical restriction patterns (only one differential band). In fact, these results confirmed that SfNPV-Sin was actually an SfNPV.

Other reports have reported restriction patterns of SfNPV isolates from other American countries such as Colombia, Nicaragua, and some Caribbean islands, additional to strains with the same geographical origin as ours (Argentina, Honduras, Mexico and, United States) (Barrera et al. 2011; Berretta et al. 1998; Escribano et al. 1999; Rangel-Núñez et al. 2014; Ríos-Velasco et al. 2012), showing more difference than those found in this report. Interestingly, some of those differences were correlated to the virulence level of those strains, opposite to our results.

On the other hand, sequencing of specific baculovirus genes have been widely used to establish phylogenetic relationships between different species and strains, using highly conserved genes (Gani et al. 2017; Kaur et al. 2014). Sequences of lef-8, lef-9, and polh/granulin genes have been recommended to be used as a minimum information to detect phylogenetic relationships between strains (Jehle et al. 2006). The size of the sequences obtained from the six isolates used in this report agree with other reports (Jehle et al. 2006; Lange et al. 2004); however, minimal variations were detected in the lef8 and lef9 genes, and no variation in the polh gene, among the SfNPVs studied here and when compared with reported sequences. Interestingly, identical sequences were detected when SfNPV-Arg and SfNPV-Sin were compared. Again, as observed in the genomic restriction analysis, a highly virulent strain shows identical sequences with the low virulent strain SfNPV-Sin, both from totally different origin. The same result was observed between the SfNPV-AN$_2$ and the SfNPV-Ho strains. These results are not surprising as a previous report shows little variability on the lef8 and polh genes, when 40 SfNPV isolates from the USDA-ARS collection were compared (Rowley et al. 2010). Therefore, sequence variation on those genes cannot be used to identify neither the geographical origin nor the virulence of each strain. Genomic sequencing may clarify some of these questions. This study agrees with previously reported results by other authors regarding the high specificity exhibited by SfNPVs (Popham et al. 2021), which creates interest for deeper studies on virus-host interaction and their specificity mechanisms. The genotypic and phenotypic variability found between the studied SfNPVs isolates, mostly those related to the evident difference in virulence among isolates, emphasizes the need for sequencing their genomes to compare virulence variability at the genomic level. All this information would make it possible to determine in the future which of these American isolates of SfNPV are the most suitable to be developed for biological control programs against the FAW.

Conclusions and future prospects

Results shown here and in other reports confirm that SfNPVs and SfGVs can be found all across the American continent, which is the geographical origin of FAW. Attempts to find genetic variation among the six strains included in
the present work were unsuccessful as only minimal variation was observed. However, a great variability in virulence was evident among the strains, which might be explained by the geographical relationship between the strain native to Mexico and the Mexican population used in the bioassays. If so, exotic strains should be the best choice to use in biological control programs against FAW. For the recent invasions of FAW to other continents, genetic characterization of those populations should be made, to identify their specific American origin. Due to the wide extent of FAW hosts, large-scale control programs should be designed, especially in places with new invasions, to try to halt their spread, and baculoviruses have shown their great potential to be included in those programs.

Once highly virulent strains are selected, the main limitation is their mass production. In vivo production is the first option, which requires the rearing of great numbers of FAW larvae to be infected and processed as a bioinsecticide, in spite of its labor-intensive requirements. However, most of the newly invaded regions are in countries where hand labor is not as expensive as in industrial countries, making plausible this alternative of control. More studies are required and production strategies should be developed, before one or more of these strains are usable in the field.

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Author contributions

Bioassays, analysis of genes, cell line infections [IZS]; manuscript writing [IAHA]; data analysis of the manuscript [JEI]; data analysis, correction, and revision of manuscript [JEI]; project design, correction, and revision of manuscript [MCRC].

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Declarations

Conflict of interests

Authors declare there are no conflicts of interest.

Ethical statement

This manuscript is in compliance with ethical standards. This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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