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ESCUELA TECNICA SUPERIOR DE INGENIEROS AGRONOMOS | ***NEKAZARITZAKO INGENIARIEN GOI MAILAKO ESKOLA TEKNIKOA***

Analysis of genes potentially involved in Vertical Transmission of Spodoptera exigua multiple nucleopolyhedrovirus

presentado por

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ABSTRACT

The Nucleopolyhedrovirus of *Spodoptera exigua* (Baculoviridae) is the active ingredient of bioinsecticides currently applied in organic production. Baculoviruses are mainly transmitted horizontally from insect to insect, but recent studies on the *S. exigua* host-pathogen system revealed that vertical transmission (from parents to offspring) also occurs. Although the biological mechanisms of the pathway remain mainly unknown, common mutations in the sequence of three genomes of genotypes specialized in vertical transmission were identified, including SeAL1. In this study, we examine the potential role of three genes (*Se5, Se96* and *Se99)* hosting those mutations and, thus hypothetically involved in vertical transmission. For this, three bacmid-based recombinants (SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99) in which these genes had been knocked out were bioassayed in comparison with the wild-type genotype SeAL1. Bioassays were performed to assess the capability of this recombinant to induce sublethal infections in adults. Fourth instar larvae were orally inoculated with sublethal concentration and survivors reared to adult stage and examined for viral DNA presence by q-PCR. Restriction Fragments Length Polymorphism (RFLP) analysis with *Bgl*II was performed to confirm the identity of each virus after an amplification cycle. We observed an extra band of ~7.5 kb in the SeBacAL1Δ99 profile, indicating important modification at a genomic level. The concentrations to kill 50% of treated larvae was determined to be 10^4 OB/ml for all treatments except for SeBacAL1Δ05, for which 10^6 OB/ml was needed. Unexpectedly, we failed to detect virus in adults exposed to any viral treatments. The reason behind sublethal infections were non-detectable is discussed.

RESUMEN

El Nucleopoliedrovirus de *Spodoptera* exigua (Baculoviridae) constituye el ingrediente activo de bioinsecticidas empleados en producción ecológica de hortícolas. Los baculovirus son principalmente transmitidos horizontalmente de insecto a insecto, aunque recientemente en este sistema patógeno-huésped se ha puesto de manifiesto la importancia de la transmisión vertical (adultos a sus descendientes). Aunque los mecanismos moleculares son todavía desconocidos, se han identificado mutaciones en genotipos ligados a la transmisión vertical, incluido el SeAL1. En este estudio, examinamos el posible papel de los genes que albergan dichas diferencias (*Se5*, *Se96* and *Se99)* y que hipotéticamente podrían estar involucrados en la transmisión vertical. Para ello, tres genotipos recombinantes (SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99) en los que estos genes has sido suprimidos son examinados en comparación con el genotipo original SeAL1 mediante bioensayos para evaluar su capacidad para inducir infecciones subletales en adultos. Larvas en cuarto estadio de *S. exigua* fueron inoculadas oralmente con concentraciones subletales de los distintos tratamientos virales. Los insectos supervivientes fueron criados hasta la fase adulta, donde la presencia de ADN viral fue examinada mediante q-PCR. La identidad de cada virus fue comprobada mediante un análisis del ADN viral con la endonucleasa de restricción *Bgl*II. Los perfiles de restricción mostraron una banda extra de ~7.5 kb en SeBacAL1Δ99, indicando una importante modificación a nivel genómico. Las concentraciones subletales determinadas, fueron de 10^4 OB/ml para todos los tratamientos, excepto para SeBacAL1Δ05, para el que fue necesario 100 veces más. No se pudo detectar ADN viral en los adultos expuestos a ningún tipo de tratamiento viral. Las razones por las que las infecciones subletales no pudieron detectarse son discutidas.

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is an important pest both in greenhouses and outdoors crops worldwide. Originally described from Southeast Asia, nowadays this species is distributed in all tropical and subtropical areas of the world (Capinera, 2001). *S. exigua* is markedly polyphagous with a extend host range of more than 200 plant species (Brown et al., 1975). The beet armyworm outbreaks result in damage of economically important vegetables crops such as cucumber, sweet pepper, courgette, beans, watermelon, and open-field crops like sunflower or alfalfa, as well as ornamental and non-commercial plants (Belda, 1994). The injury is produced by the larval stage that feed on leaves, fruits and stems producing large economic losses. In Southern Spain, S. exigua has been considered a key pest in sweet pepper for decades (Caballero et al 2009). Over decades, chemical control was used as the most common method to tackle with this pest. However the systematic spraying of synthetic pesticides lead to the development of resistances in insect populations associated to a lack of efficacy (Ahmad & Arif, 2010). Resistance has been reported to the main ingredients used against this pest including abamectin, cypermethrin, endosulfan or spinosad (Wang et al. 2006; Ahmad & Arif 2010). Other problems associated with broad-spectrum chemical pesticides are the presence of insecticidal residues in fruits exceeding those concentrations legally permitted in harvested fruit (Glass & Egea González, 2012), and the incompatibility with natural enemies used against simultaneous pests (Caballero et al., 2009). All these reasons, couple with the recently enforced regulation on the use of phytosanitary products, have encouraged growers to implement environmental friendly methods in the framework of integrated pest management programs.

Baculoviruses are well known as a good tool for insect pest control (Moscardi, 1999), being the most widely studied insect pathogenic viruses (Inceoglu et al., 2006). Used for biological control since 1900s (Inceoglu et al., 2006), baculoviruses comprise singular advantages respect to chemical insecticides. Namely, they are natural pathogens, highly specific with limited host range. Therefore, they not affect other beneficial organisms, and can persist in the environment for long time. Also they can be mass-produced and commercialized in a similar way to chemical pesticides (Beas-Catena et al., 2014). However, as other biopesticides, baculoviruses are not exempt of some disadvantages for broad commercial use, such as slow speed of kill, short field stability under UV-radiation, and high production costs (Beas-Catena et al., 2014).

Baculovirus have been recorded from hundreds of host species, mainly insect of the orders Lepidoptera, Hymenoptera and Diptera (Cory & Myers, 2003; Herniou et al, 2003). They have a double-stranded, circular, supercoiled genome, varying from 80 to 180 kb size, encoding 90 to 180 genes (Rohrmann, 2013; Serrano et al., 2015). Baculovirus produce occlusion bodies (OBs) that are capable to persist in the environment for long periods of time if they are protected from ultraviolet irradiation and are responsible of spreading infection between hosts. Horizontal transmission is thought to be the main route for baculoviruses spread-out and it occurs when NPV-infected larvae

die and several OBs are liberated to the environment and ingested by susceptible hosts, which become infected (Cory & Myers, 2003). Recent studies have shown that vertical transmission, from covertly infected parents to offspring, is not only possible (Kukan, 1999), but frequent in natural population of insect (Cabodevilla et al., 2011b; Vilaplana et al., 2010). The mechanisms involved in the latency of the virus in the host are still unknown, but evidence indicates that adults survivors to a virus challenge in the larval stage are capable to transmit the infection to their offspring (Burden et al., 2002; Virto, 2016). Vertical transmission is increasingly attracting attention, and its role in the virus ecology has been related to periods in which opportunities for horizontal transmission are unfavorable, due to host population fluctuations (Cory & Myers, 2003; Virto, 2016). OB ingestion does not always result in symptomatic fatal infections, however, sublethal doses can establish covert infections. Two types of covert infections have been reported for NPVs: persistent infection, characterized by a transcriptional activity of the virus at a low level, and latent infection when the virus is essentially dormant (Burden et al., 2003). Molecular techniques advances allowed the detection of both covert and latent infections and the vertical transmission have been investigated in detail. Namely, the polymerase chain reaction (PCR) or reverse transcription polymerase chain reaction (RT-PCR) lead to the detection of latent and persistent infections respectively. Novel and highly sensitive techniques have been developed such as real-time quantitative PCR (qPCR), which is able to detect as low as 5 or 7 copies of viral genomes for hydrolysis probe (TaqMan) and SYBR green based technique (Graham et al., 2015) respectively (Virto, 2016).

The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) has been intensively studied as biological control agent, and has demonstrated excellent insecticidal features. SeMNPV-based biopesticide allowed a level of crop protection against *S. exigua* as efficient as chemicals, and the advantages of high specificity, pathogenicity and virulence (Caballero et al., 2009). Nowadays, three SeMNPV-based biopesticides are registered and allowed to use in Spain: VIREX® (Biocolor, Almería, Spain), Spod-X® (Certis Corp., Columbia, USA), Spexit® (Andermatt Biocontrol, Grossdietwil, Switzerland). Recent studies on SeMNPV vertical transmission and their application on biological control, suggest the possibility of using vertically transmitted genotypes to promote covert infections and reactivation in field application approaches (Virto, 2016). The double transmission means of SeMNPV, combining horizontal and vertical (covert infections) routes, is potentially a novel aspect to be considered to improve the baculovirus-based application efficiency in crops. The introduction of SeMNPV vertically transmitted genotypes in the active ingredient might favor the implementation of inoculative strategies for field applications, favoring the sustainable use of such means to control *S. exigua* pest populations.

Studies on the genotypic variation of the SeMNPV genotypes associated either to vertical or horizontal transmission route revealed that some genotypes may be better adapted to one of both routes. Thézé et al. (2014) identified and compared the whole genome sequence of seven different SeMNPV isolates associated with vertical (VT-SeAL1, VT-SeAL2, VT-SeOx4) or horizontal transmission (HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2A). The great capability of SeAL1 to induce sublethal infection were

consistently found in previous studies, being detectable (qPCR positive) in almost 100% of adults previously inoculated at larval stage (Cabodevilla et al. 2011a). SeAL1 and the rest of vertical transmission associated genotypes shared the same mutations in three genes, different from those of the horizontally transmitted isolates. These mutations were a deletion of 3 bp located at 1382 nt in open reading frame (ORF) 5 (*Se5*), a 3 bp deletion located at 114 nt in ORF96 (*Se96*), and a deletion of 9 bp located at 54 nt in ORF99 (*Se99*). We hypothesize that one or more of these genes would be involved in sublethal infections establishment and vertical transmission, but experimental evidence is lacking.

The aim of this work was to investigate the role of these genes *Se5*, *Se96* and *se99* in the vertical transmission of SeMNPV by using viral recombinant genomes in which each one of these genes had been knocked out (Serrano et al., 2015). Bacterial Artificial Chromosome (BAC)-mid based recombinants, named SeBacAL1, SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99 were compared with the wildtype SeAL1 for their capability in the establishment of sublethal infections. For this purpose, we infect larvae of *S. exigua* with sublethal concentration of each viral treatment, and the survivors adults were analyzed for the detection of the virus by qPCR. Mortality and adults survival rates were compared between viral treatments to assess differential pathogenicity or infectivity and Restriction Fragments Length Polymorphism (RFLP) analysis of the viral DNA were also performed to detect possible rearranges during the infection process.

MATERIAL AND METHODS

1. Insects and viruses

S. exigua larvae used in this study were provided by the insectary of the Universidad Pública de Navarra, where an *S. exigua* insects culture was maintained on a wheat germ-based semisynthetic diet (Greene, Leppla, & Dickerson, 1976) under standard rearing conditions (25 ± 1° C, 50 % ± 5 % RH and a photoperiod of 16:8 h light: dark).

The original SeMNPV isolate used in this study as a reference was SeAL1, known to be capable of being vertically transmitted (Cabodevilla et al, 2011a; Thézé et al., 2014). The rest of virus were DNA-recombinant isolates constructed on the SeAL1 genetic background, by using Bacterial Artificial Chromosome (BAC) recombination methods (Serrano et al., 2015). Briefly, first a BAC-mid was constructed by inserting the complete genome of the wild type to obtain the named SeBacAL1. The recombinants SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99, were performed by double recombination with a helper plasmid resulting in the substitution of the *Se5, Se96* and *Se99* genes by reporter genes (Serrano et al., 2015). OB for all the virus were obtained from the virus collection of the Group of Microbial Bioinsecticides (Instituto de Agrobiotecnología, Universidad Pública de Navarra), and then amplified by inoculation of *S. exigua* fourth instars larvae using the droplet feeding method as describe below.

2. Bioassays

Bioassays were performed to determine the capability of recombinants SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99, to produce sublethal infections in adults of *S. exigua*, as the first step of viral vertical transmission, comparing to the wild type SeAL1. In previous studies performed in our laboratory, sublethal infections with the SeAL1 virus was previously found to be detectable by qPCR in almost all adults inoculated as fourth instar larvae (Cabodevilla et al, 2011a). As the recombinant construction process might affect the virus pathogenicity or other infective traits, firstly we determine the effective concentration causing medium mortality for the three recombinant virus in study. Thus, two types of bioassays were performed: i) a previous experiment to establish the OB concentrations causing between 40-60% NPV-mortality for each viral recombinant, and ii) a vertical transmission bioassay to assess the capacity of the viral recombinants to induce sublethal infections, comparing to the wild type SeAl1.

To test viral mortality induced by viral recombinants, *S. exigua* larvae were inoculate with different viral doses and checked for NPV-mortality. Specific symptoms of the fully overt infections were examined, such as cadaver milky appearance and liquefaction, and OB presence under optical microscope. The inoculation was made by the droplet feeding method (Hughes, van Beek, & Wood, 1986). For that, groups of 24 *S. exigua* fourth instar larvae were selected from mass culture and starved overnight (approx. 16 h). Viral inoculum containing a suspension of either 10^4 or 10^5 OB/ml, 10% sucrose and 0.001% Fluorella Blue (Food diet) was offered to groups of 50 larvae for ten minutes. Since the SeBacAL1Δ05 was reported 10 times less pathogenic than SeAl1, concentration of 10^6 and 10^7 OB/m were tested for this virus (Serrano et al., 2015). After viral exposition, those larvae that had ingested the suspension were individually transferred to 24-well tissue culture plates provided with semi-synthetic diet. Insects were maintained at 25 ±

2°C and 50 ± 5 % RH, and mortality recorded daily until larvae died from polyhedrosis disease or pupated. Control larvae treated with water and Fluorella blue was included. The whole procedure was performed two times.

OBs concentrations selected to killed between 40-60 % of treated larvae for the virus recombinants was 10^4 OB/ ml, whereas for the SeBacAL1Δ05, it was of 10^6 OB/ ml. The inoculation procedure was the same as describe above, and insects were reared in the standard conditions. Mortality was recorded daily until larvae died from polyhedrosis disease or pupated. Surviving pupae were individually reared through to adult stage and the emerged adult individually collected in 1.5 ml vials, and stored at -20ªC for posterior analysis. Bioassay was performed three times.

3. Total DNA extraction

To detect viral sublethal infections in *S.exigua* moths, the total DNA from adult abdomen was extracted, by using the Master Pure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) following manufacturer instructions for tissues samples. Briefly, the abdomens of frozen adults were dissected and individually placed in 2 ml Lysing Matrix tubes (MP Biomedicals) containing ceramic beads. 300 µl tissue lysis solution and 1 µl proteinase K (50 µg/µl) were added per tube. Then, samples were homogenized by MP FastPrep-24 tissue cell homogenizer at 4 m/s for 20 s and incubated for 15 min at constant 1,100 rpm orbital agitation. Samples were split into two aliquots of 150 µl and one of them was treated with 1 µl RNAse (5µg/µl) at 37°C for 30 min incubation. Then, 90 µl of MPC protein precipitation reagent were added and thoroughly mixed. Samples were centrifuged for 13 min at 10000 × *g*. After that, supernatants were recovered and nucleic acids precipitated with 500 µl of isopropanol by centrifugation for 10 min at 13,000 × *g* at 4°C. Pellets were washed twice with 70% ethanol at same conditions, resuspended in 20 µl sterile Mili-Q water and stored at -20°C until used for qPCR. Blank extraction samples containing only water were processed in parallel to control cross-contamination during the extraction procedure.

4. Detection of SeMNPV by qPCR

In order to detect and quantify the presence of viral DNA in the total DNA from insects, Quantitative Polymerase Chain Reaction (qPCR) was performed following the method originally described by Cabodevilla et al., (2011b) and modified by Virto et al., (2013). qPCR based on SYBR Green fluorescence was carried out in 96-well reaction plates in a CFX96 Real-Time System (Bio-Rad). Amplifications were performed in a total reaction volume of 10 µl containing 5 µl of SYBR Premix Ex Taq (2x), 3 µl of sterile MilliQ water and 0.5 µl of both DNApol.F an DNApol.R primers (10 µM) and 1 µl of DNA template. A blank extraction and four non-template reactions were included in each run, as well as a duplicate standard curve, constructed by serial dilutions from 10^{-1} to 10^{-7}, in order to determine the efficiency of reactions. The qPCR protocol consisted of an initial denaturation at 95°C for 30 s, followed by 45 amplification cycles at 95°C for 10 s and 62°C for 30 s. Finally, a melting curve analysis, including a dissociation stage from 65°C to 95°C increasing 0.5°C each 5 s, was added to confirm the specific target products. Data acquisition and analyses were performed using CFX Manager Software (Bio-Rad). DNA quantities were estimated by extrapolation of Cq values from the standard curve. Every DNA sample was performed in triplicate.

5. Viral DNA purification, restriction endonuclease digestion, electrophoresis

Virus-killed larvae from bioassays were collected to confirm viral cause of the infections. For that, cadavers were classified per the viral treatment given and stored at -20°C prior OBs purification and DNA extraction. OBs form viral recombinant previously characterized were used as references. For OBs purification, cadavers were homogenized in 0.1% sodium dodecyl sulfate (SDS), filtered through a muslin cloth and precipitated by centrifugation. Viral DNA was extracted from the OBs by adding 250 µl of water, 50 µl of 10% SDS and 100 µl of 0.5 M Na_2CO_3. The mixture was incubated at 37°C for 5 min in order to dissolve the polyhedron matrix. Undissolved OBs were pelleted by centrifuging at 6,000 ×*g* for 1 min. Supernatant containing the released occlusion-derived virions (ODVs) was incubated with 500 µg/ µl proteinase K at 50°C for 45 min. After that time, 0.1 vol 10% SDS was added and another step of incubation at 50 °C was performed. Viral genomic DNA was extracted with 300 µl of MPC protein precipitation reagent centrifuged at 10,000 x g for 10 min and precipitated by the addition of 850 µl of isopropanol. DNA was washed twice with 70% ethanol and dissolved in 30 µl of sterile milliQ water. For restriction endonuclease digestion 5-17.5 µl of viral genomic DNA were treated with 0.3 µl of *Bgl*II (Takara, Shiga, Japan), adding 2 µl of 10x buffer and the required amount of milliQ water until reach 20 µl of total volume. Mixtures were incubated for 4 hours and loaded in 1% agarose gel with Tris-acetate-EDTA (TAE) (0.04 M Tris-acetate, 0.001 M EDTA). Electrophoresis was performed overnight and the DNA fragments stained with SYBR Safe, were visualized in a UV transiluminator, photographed and examined using the software GeneSnap (SynGen ver. 7.05.02, Sypnoptics Ltd. 1993-2008).

RESULTS

1. DNA viral analysis

The analysis of viral DNA was performed to confirm both identity and genomic integrity of the virus causing larval death after infections of fourth instar larvae of *S. exigua* with the SeAL1 wild type and SeBacAL1, SeBacAL1Δ05, SeBacAL1Δ96 and SeBacAL1Δ99 recombinants. Cadavers of larvae were collected and OBs were purified, DNA extracted and tested by Restriction Fragments Length Polymorphism (RFLP) of viral DNA treated with *Bgl*II (Fig 1). This enzyme was previously found to discriminate SeMNPV isolates and genotypic variants (Muñoz & Caballero, 2001), including the BAC-recombinants (Serrano et al., 2015). RFLP analysis lead to the identification of restriction fragments corresponding to knocked-out genes. The restriction pattern from OBs of previously described BAC-recombinant were used as references. RFLP profiles showed no genetical mutation between reference and newly generated OBs for each of the five treatments, indicating non large delections or DNA recombination events occured during viral infection and OB amplification. However, the SeBacAL1Δ99 profile did not match the *Bgl*II profile previously described (Serrano, 2014). In the current *Bgl*II profile, an extra band of ~7.5 kb was detected compare to the SeBacAL1Δ96 reference (Fig. 1), indicating the possibility of cross-contamination during previous amplifications process or the DNA reorganization events. Therefore, SeBacAL1Δ99 treatment was excluded for subsequent experiments.

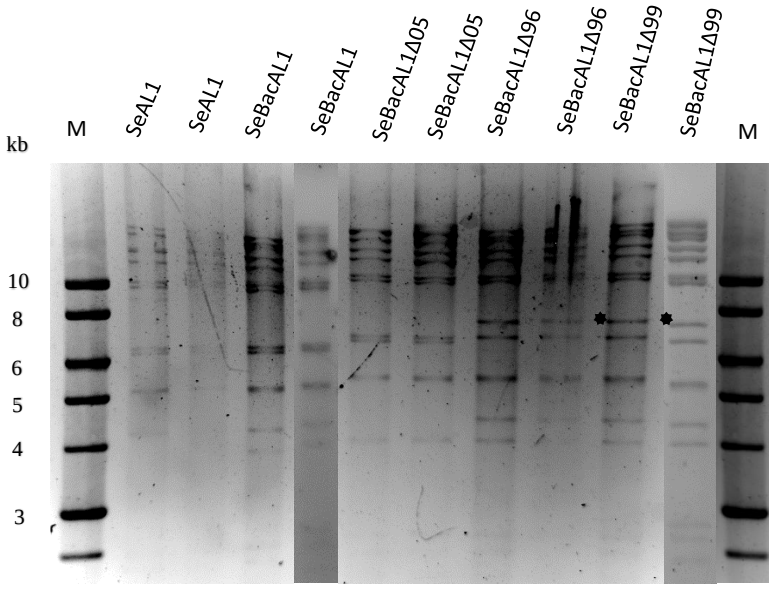

Figure 1. Bgl*II restriction endonuclease profiles of the genomic DNA of the SeAL1 wild type and SeBacAL1, SeBacAL1Δ05, SeBacAL1Δ96, and SeBacAL1Δ99 recombinants from OB of isolates used as references (lanes 1,3, 5, 7, and 9) and the OB generated after viral deaths at bioassay (lanes 2,4,6,8, and 10). Asterisk indicates an extra band detected compare to the originally described SeBacAL1Δ99 profile. Lane M contains 1 kb Marker-Ladder (Stratagene).*

2. Viral sublethal concentration

Since the construction of recombinant viral genomes might had altered viral pathogenicity, a preliminary bioassay was performed to determine the effective concentration to cause mortality rates between 40-60% for each virus. Previous studies found that this mortality ratio in larval challenge with the SeAl1 was related to high levels of sublethal infections in adults of *S. exigua* (Cabodevilla et al., 2011a). In this experiment, two different viral concentrations were provided according to previous studies (Serrano, 2014). NPV- mortality average exceed the 40 % in all tested treatments (Table 1). Overall, the mortality mean increased with OB concentration, but non-significant differences were found statistically (t-Student, P >0.05). Those larval groups treated with 10^4 OB/ml of SeAL1, SeBacAL1, and SeBacAL1Δ96 reached similar mortality proportion of 47.22%, 43.18%, 54.73%, whereas the 57.02%, 86.16%, and 78.01% were calculated for the concentration of 10^5 OB/ml respectively. Low rates of viral mortality (5.88%) were registered in control groups, indicating cross-contamination due to insects manipulation.

Therefore, concentration of 10^4 OBs/ml for SeAL1, SeBacAL1, and SeBacAL1Δ96, and 10^6 OBs/ml for SeBacAL1Δ05 were determined for the subsequent bioassays.

Table 1. Mean percentage of S. exigua larval mortality induced by SeMNPV treatments including the wild type SeAl1, and the recombinant genomes SeBacAL1, SeBacAL1Δ05, SeBacAL1Δ96 for different OB concentrations.

Virus Treatment	OB concentration (OBs/ml)	Treated larvae (N)	Mean mortality (% ± se)
Control	-	37	5.88 ± 5.88
SeAL1	10^4	43	47.22 ± 2.78 a
	10^5	44	57.02 ± 9.65 a
SeBacAL1	10^4	46	43.18 ± 2.27 a
	10^5	33	86.16 ± 7.59 a
SeBacAL1Δ05	10^6	46	59.52 ± 7.14 a
	10^7	45	88.10 ± 7.14 a
SeBacAL1Δ96	10^4	41	54.73 ± 1.79 a
	10^5	40	78.01 ± 16.10 a

Letter a indicate no significant differences between mean values by the t-Student test ($p<0.05$) for each virus treatment.

3. Prevalence of viral infections in adults

Mortality induced after viral challenge varied from 46.8% to 67.3% across treatments during the bioassay (Table 2). Similar mortality average between SeAL1 and SeBacAL1 was estimated (50.53% and 46.84% respectively) indicating that bacmid construction did not affect infective capabilities of the virus. SeBacAL1Δ96 mean mortality (51.76%) was similar to the wild-type virus. Mortality in the knockout SeBacAL1Δ05 was the highest recorded (67.35%) and overtook the mean value expected (40-60%). Regarding survival rates similar averages varying from 38.81% to 42.86% for SeAL1, SeBacAL1, SeBacAL1Δ96 were measured. However, for SeBacAL1Δ05 treated insects, significantly less adults survived due to a higher impact of virus infection. Noticeably, adult survival percentage in control groups was as low as 67.21%, since non-emerging pupae percentage reached 24.07% (Table 2).

Table 2. Viral mortality, non-emerging pupae and adult survival rates after inoculation with SeAl1, SeBacAL1, SeBacAL1Δ05 and SeBacAL1Δ96.

Virus	OB concentration (OBs/ml)	Viral Mortality (%± se)	Non-emerging pupae (%)	Adult survival %(N)
Control	-	3.92 ± 3.92	24.07	67.21 (41)
SeAL1	10^4	50.53 ± 3.67	10.34	38.81 (26)
SeBacAL1	10^4	46.84 ± 3.89	6.25	42.86 (30)
SeBacAL1Δ05	10^6	67.35 ± 8.85	28.57	21.42 (15)
SeBacAL1Δ96	10^4	51.76 ± 3.14	10.00	41.54 (27)

After emergence, almost 30 adult individuals per viral treatment (SeAL1, SeBacAL1, SeBacAL1Δ05 and SeBacAL1Δ96) and control were individually collected and processed for the detection of sublethal infections by q-PCR. First, the detection technique was verified using covertly infected adults from other experiments and the detection limits assessed using ten-fold serial dilution of high quality viral DNA. Tested samples were adult subjected to similar bioassays including all treatments used in this study (SeAL1, SeBacAL1, SeBacAL1Δ05 and SeBacAL1Δ96) as well as non-infected control insects.

The PCR cycle at which a significant increase in terms of Relative Fluorescence Units (RFU: variation in the fluorescence of SYBR Green) is first detected is called quantification cycle (Cq). A standard curve was performed between the Cq values and standard concentration of 10^{-1} to 10^{-7} ng/µl of pure SeMNPV DNA. The regression parameters were assumed acceptable according to R^2 values and the efficiency of reaction (R^2=0.985; slope = - 3.064; 112% efficiency) (Fig. 2). The Cq values for virus specific products defined by the standard curve range between 14.1 and 32.3 (Fig. 3) and the corresponding dissociation curve produced a single pick at 83.5ºC of melting temperature. Thus, adult samples were considered positive when Cq values were comprised between 14.1 and 32.3 and registered a single peak at the expected melting temperature for the dissociation curve. All the samples tested were located between those limits (Fig. 2-3), so that they were confirmed as positive samples.

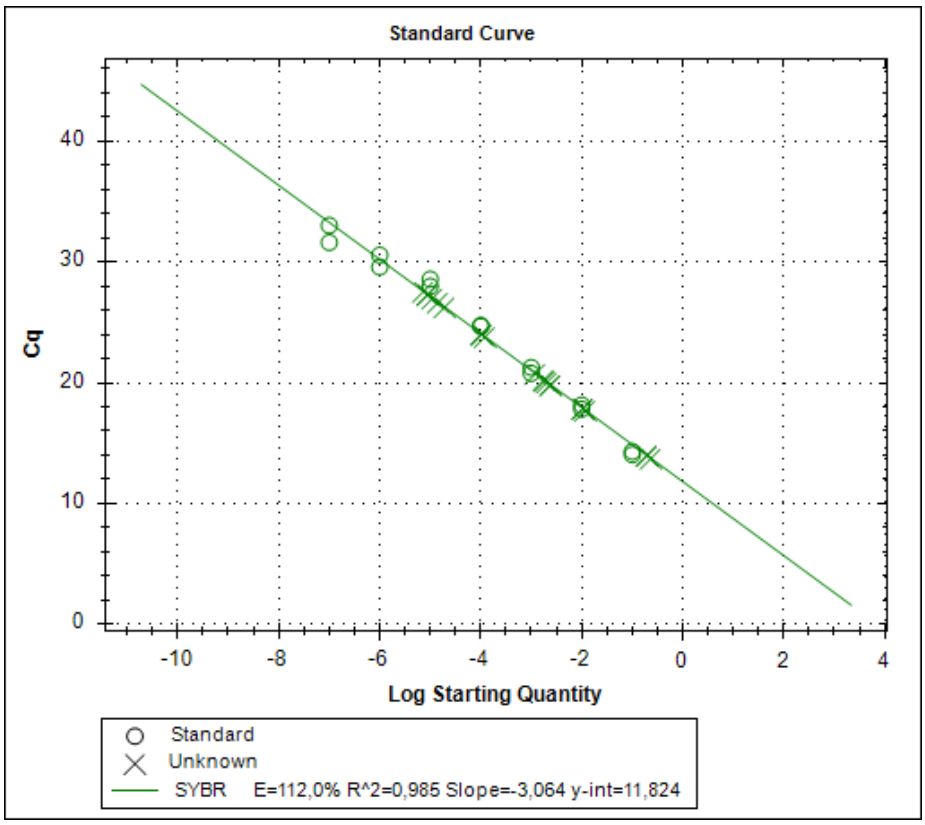

Figure 2. Standard curve estimated for ten-fold SeMNPV viral dilutions (circles) and Cq corresponding values. Extrapolated values from the standard curve for the sample tested were represented (crosses).

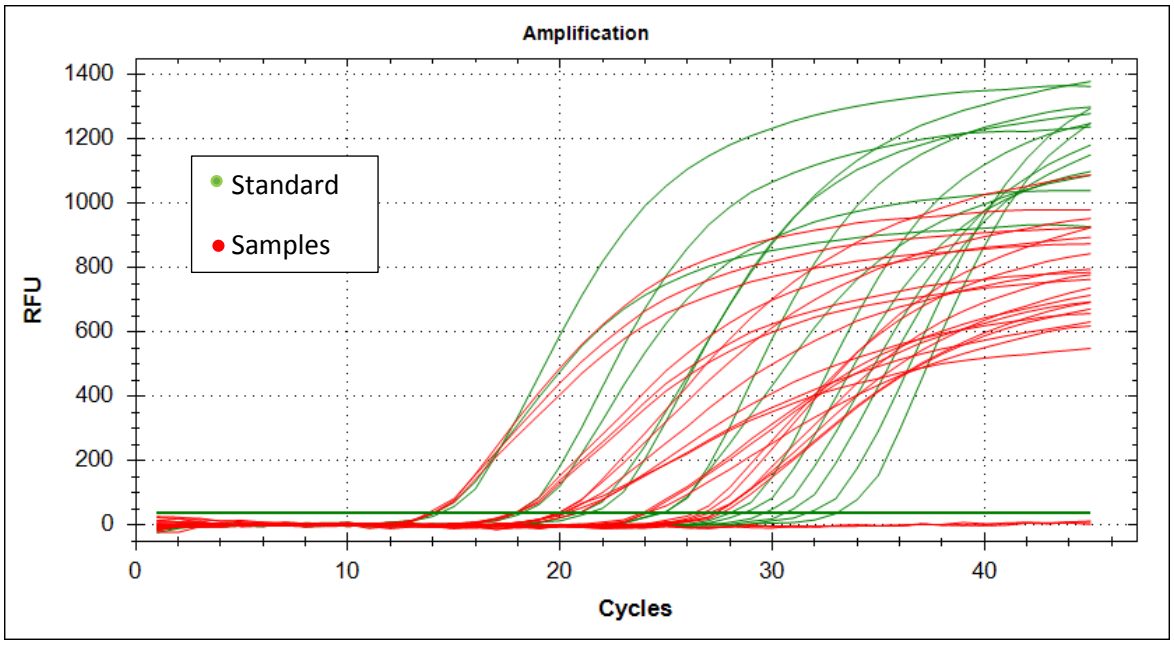

Figure 3. qPCR amplification curves of ten-fold DNA- dilution (green) in duplicate for curve standard construction and viral-DNA positive adult samples (red) in triplicate. The green line indicates RFU quantification value.

In contrast, the amplification patterns during qPCR for those DNA samples obtained in bioassay showed different patterns lacking the exponential phase of DNA amplification. Regression parameters for standard curve were also considered acceptable (R^2=0.980; slope = - 3.133; 108.5% efficiency) (Fig. 4), together with the amplification curves for virus specific products had normal shapes (Fig. 5). Tested adults belonged to all treatments used for bioassays (SeAL1, SeBacAL1, SeBacAL1Δ05 and SeBacAL1Δ96), and non-infected control insects were included. Poor Cq values for tested samples ranging from 31.0 to 37.3, indicate the non-presence of the SeMNPV in DNA samples from the adults bioassayed (Fig. 4, Fig. 5).

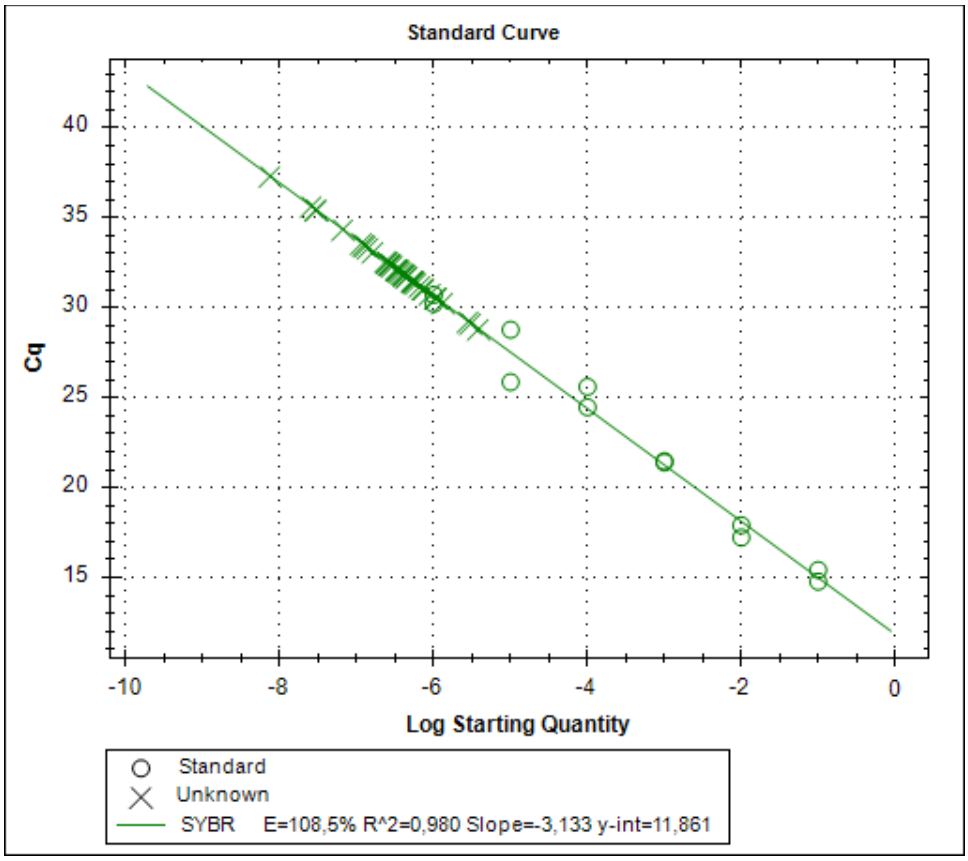

Figure 4. Standard curve estimated for ten-fold SeMNPV viral dilutions (circles). Extrapolated values from the standard curve were represented (crosses) and situated over limit of detection for the adult samples bioassayed.

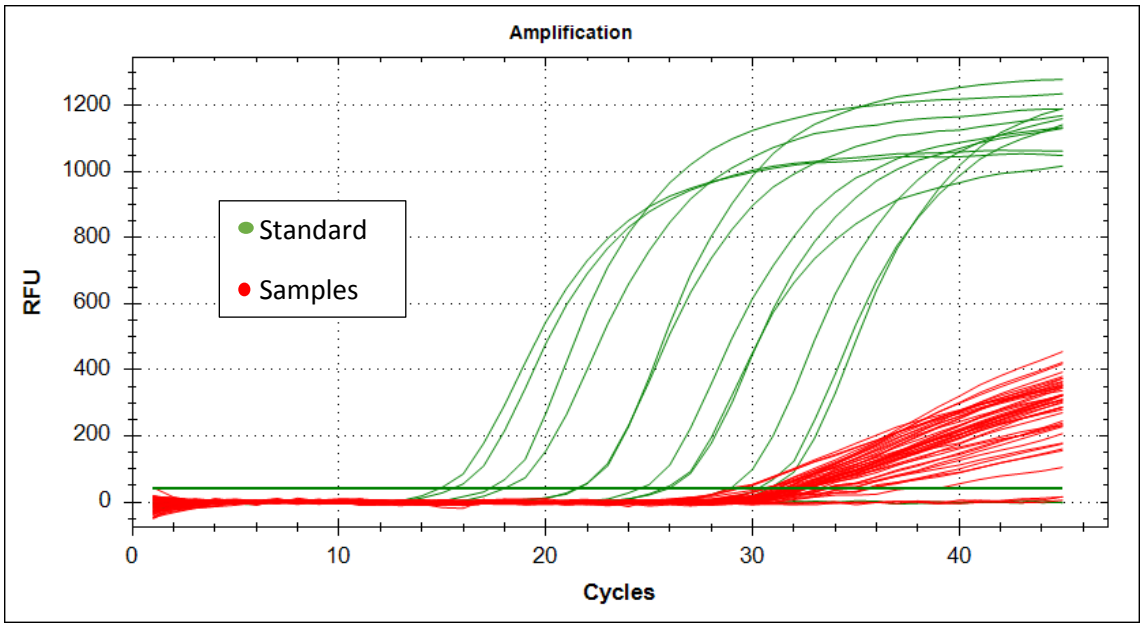

Figure 5. q-PCR amplification curves of ten-fold DNA- dilutions (green) for standard curve construction and DNA samples from adult bioassayed (red). The green line indicates RFU quantification value.

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DISCUSSION

Baculovirus can stablish sublethal infections to transmit the virus from parents to the offspring and persist in the host for long term. However, little is known about the molecular mechanisms that regulate the establishment and persistence of latent and persistent infections. SeMNPV genotypes differed in their ability to induce sublethal infection in the adult stage, indicating that certain genotypes are better adapted to vertical transmission than others (Cabodevilla et al. 2011a). The vertically transmitted genotype SeAL1 produced almost 100% sublethal infection in adults that survived a viral challenge at larval stage (Cabodevilla et al. 2011a). Seven SeMNPV genomes related with vertical and horizontal transmission routes, including the SeAL1, were compared in their nucleotide sequences, and resulted in the identification of three genes (*Se5, Se96* and *Se99*) potentially involved in transmission traits (Thézé et al. 2014, Serrano et al, 2014) . In this study, we investigate the vertical transmission abilities of recombinants in which those genes had been knocked out.

First, the identity of virus after the amplification cycle in the host was determine. DNA analysis of OBs before and after the infection, was carried out by Restriction Fragments Length Polymorphism (RFLP). Previous and post-infection *Bgl*II restriction profiles of viral DNA matched each other and with those previously described (Serrano, 2014) for all virus, except for the SeBacAL1Δ99 recombinant. In this case, an extra band of ~7.5 kb was found in comparison with the corresponding reference. Contrary, this pattern resembled to the one corresponding to SeBacAL1Δ96. These findings may be explained by cross-contamination during viral amplifications passages performed previously to these experiments. Other reason to explain the differences of *Bgl*II profile could be that the current profile is the result of DNA reorganization. During the infection cycle, baculoviruses genetic material may be altered by different events including mutations, gen duplications or DNA deletions or insertions, recombination between virus genotypes, virus and host, or virus and other co-infecting organisms (Cory & Myers, 2003). Occasionally, these rearrangements of genomic material might occur in order to obtain genomic stability, as some variants have been found unstable through viral amplifying passages (Muñoz & Caballero, 2001). Therefore, the SeBacAL1Δ99 was excluded for the rest of the experiments.

Previous studies found that a sublethal dose of ~ 10^4 OB/ml of SeAL1 provided to larvae resulted in 80-100% of prevalence (qPCR+) in adults survivors to the infection (Cabodevilla et al. 2011b). The viral dose needed to kill 40-60% of a treated population was determined for the viral-recombinant SeBacAL1, SeBacAL1Δ05, and SeBacAL1Δ96. An initial experiment was performed inoculating fourth instar larvae of *S. exigua* and 10^4 was selected for SeAL1, SeBacAL1 and SeBacAL1Δ96, whereas 10^6 OB/ml was selected for SeBacAL1Δ05. These results were in line with the concentrations previously used by Serrano (2014), except for SeBacAL1Δ05, for which a concentration of 10^5 OB/ml was employed. Comparison between treatments indicates that there are no-significant differences in pathogenicity between wild-type, SeAL1, and bacmid-based recombinant, SeBacAL1, concluding that the insertion of exogenous DNA (BAC) did not

affect viral pathogenicity. Deletion of *Se5* was found to have an important role in the viral infectivity of SeMNPV and its recombinant, SeBacAL1Δ05, needed to use a higher concentration than the rest of genotypes to reach LC_{50} (Serrano et al., 2015). In line with this, mean mortality for SeBacAL1Δ05 was noticeably higher than for SeAL1 (Table 2). SeBacAL1Δ96 was administered with the same OB concentration as SeBacAL1, and both concentrations produced similar mortalities, accordingly with other studies (Serrano, 2014).

Regarding the prevalence of sublethal infections in adults, bioassays using the previously determined sublethal concentration were performed in fourth instar larvae of *S. exigua*. Mortality rates were similar to those found in the previous experiment, but slightly higher for SeBacAL1Δ05, suggesting that a 10^5 concentration for this treatment would had approached more the 40-60% expected. Adult survival and non-emergence adults rates were calculated in order to detect sublethal effects of the viral infections, since sublethal infections of SeMNPV significantly reduce adult emergence (Cabodevilla et al. 2011b). Adult survival rates were mainly affected by the virus treatment. However, the control group showed higher rates of non-emergence (24.07%) than those found in similar experiments (Virto et al., 2013). Long- term studies of covert infections indicate that NPV can be transmitted in laboratory colonies throughout five generations after viral challenge (Burden et al., 2002). Thus, a covert infection may be present in our laboratory colony explaining unexpected deaths in control groups and low rates of emergence. However, mortality in control larvae found in this study, decrease between subsequent repetitions, suggesting that high rates of non-emerging adults in first experiments were consequence of contamination during insects manipulation.

Finally, the sublethal infection was tested by qPCR in adult stage of insect challenge with the virus. Serrano (2014) reported a similar prevalence for the SeAL1 and the four recombinants studied here, indicating that *Se5, Se96* and *Se99* disruption did not affect the prevalence of virus infection in adults. In this study, we failed to detect SeMNPV in total DNA samples from those adults exposed to virus. However, insect samples from other experiments were tested by qPCR and positive results were found, confirming the value of the method. Also, correct amplification curves, (Fig. 2, Fig. 3) and the regression parameters for standard curves were consistently acceptable, thus we focus the search of the failure on the DNA extraction. Numerous attempts have been made to adjust DNA extraction conditions, including Proteinase K concentration, time and speed of incubation, protein precipitation reagent concentration, precipitation conditions, and resuspension volume of mili-Q water for DNA. Unfortunately, none of these modifications resulted in the improvement of viral DNA amplification in insect samples. For subsequent studies, it would be of interest to analyse samples using different target genes capable to amplify other regions of the SeMNPV DNA, in order to discard genome modifications preventing the primers hybridization.

Biopesticides based on baculovirus are a feasible alternative to chemical pesticides, and a valuable tool in integrated pest management programs, that promotes a sustainable agriculture. Vertical transmission may be an interesting feature to improve pest control

strategies, and the establishment of covert infections in populations that may eventually trigger fatal disease in larvae causing damages in the crops. SeMNPV transgenerational transmission might reduce the number of applications of baculovirus-based insecticides, improving their effectivity in field and the risk of resistance development. Unfortunately, genetic factors associated with vertical transmission mechanisms are unclear yet. These findings will contribute to understand molecular mechanisms of vertical transmission pathway, where these genes might have an important role. Further studies would be needed to determine whether the disruption of *Se5*, *Se96* or *Se99* genes alter the viral expression and the efficiency of virus transmission to the offspring.

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