Evidence for covert baculovirus infections in a Spodoptera exigua laboratory culture

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A laboratory culture of Spodoptera exigua was examined to assess covert (latent or persistent) baculovirus infections and spontaneous disease outbreaks. Two nucleopolyhedrovirus (NPV) species were found to be reactivated from a covert state in a laboratory culture of S. exigua to fully lethal forms. These were identified as S. exigua multinucleopolyhedivirus (SeMNPV) and Mamestra brassicae NPV (MbNPV) using restriction enzyme analysis of purified viral DNA. Sequence data derived from both overtly and covertly virus-infected insects revealed highly conserved sequences for lef-8, lef-9 and polyhedrin gene sequence (98–100% nucleotide identity to SeMNPV published sequence). By monitoring spontaneous overt infections and quantifying viral DNA (by quantitative-PCR) in asymptomatic individuals over two generations we identified fluctuating trends in viral DNA levels from covert SeMNPV and MbNPV within an S. exigua host population. Virus levels per insect life stage ranged from $3.51 \pm 0.036 \text{ pg}$ (detection limit at 0.06 pg). Bioassays performed with this culture of larvae showed a differential susceptibility to SeMNPV-like or MbNPV-like viruses, with SeMNPV superinfections being extremely virulent. The data presented has broad implications relating to our understanding of transmission patterns of baculovirus in the environment and the role of covert infections in host–pathogen interaction dynamics.

INTRODUCTION

Baculoviruses are pathogenic insect viruses producing infections commonly causing fatalities in Lepidoptera host larvae. Although the horizontal route is thought to be the major pathway for baculovirus transmission (Cory & Myers, 2003), some studies support the idea that their transmission also involves vertical transfer in field populations (reviewed by Kukan, 1999). Individuals exposed to low virus doses may acquire a non-fatal sublethal infection, but transmit the infection vertically to the next generation (Andrealis, 1987; Cory & Myers, 2003). This may affect insect health and fecundity (Rothman & Myers, 1996; Myers et al., 2000; Vilaplana et al., 2008). Insects may also have a covert infection that does not produce visible symptoms. In this state it is suggested that the virus remains either latent in a non-replicating form, or in a persistent state with low-level gene expression (Hughes et al., 1997; Burden et al., 2003). Covert infections may not be critical for maintaining virus populations in nature but could enable the parasite to persist during periods of low host population density especially in seasonal environments (Burden et al., 2003). Another advantage to the virus of being in a covert state is that it could be carried long distances during host migration (Anderson & May, 1981). Interestingly, vertical transmission appears to be frequent in more mobile insect species such as those within the genus Spodoptera (Swaine, 1966; Abul-Nasr et al., 1993; Smits & Vlak 1988; Fuxa & Richter 1991; Vilaplana et al., 2008).

Spontaneous emergence of overt infections from a covertly infected population suggests that the viruses remain replication competent during the quiescent state. Common features of persistent viral infections include the selection of cell subsets ideal for long-term maintenance of the viral genome (Kane & Golovkina 2010). Studies targeting baculoviruses detected the presence of viral DNA in adult tissues such as the fat body (Hughes et al., 1993, 1997) and gonads (Burden et al., 2002; Khurad et al., 2004), which ultimately are involved in transmitting virus to subsequent generations. Spontaneous nucleopolyhedrovirus (NPV) outbreaks have been recorded in laboratory cultures reared under virus-free conditions over several generations. Karpov (1979) reported a Bombyx mori NPV outbreak to be a problem in practical sericulture. Few
studies have been conducted to elucidate the underlying phenomena (Hughes et al., 1997; Fuxa et al., 1999), so little is known about the mechanism(s) involved when a covert virus is triggered into an overt infection (Cory & Myers 2003). Advances in molecular techniques have improved the detection of viral DNA or its transcripts in asymptomatic insects. For instance, a laboratory culture of cabbage moths (Mamestra brassicae) was reported to harbour a covert baculovirus infection without prior exposure to such viruses (Hughes et al., 1993). This virus was activated to an overt state by challenging the host with a heterologous baculovirus and the covert virus identified as M. brassicae NPV (MbNPV). Using PCR the polyhedrin gene was detected in individuals of this population at all ages, indicating this population maintained MbNPV-like persistent virus (Hughes et al., 1997). Similar results were obtained using the immediate early gene 1 as a target (Burden et al., 2006) although the presence of the entire virus genome in the covert state has yet to be demonstrated. A serendipitous observation reported here leads us to conclude that a laboratory population of Spodoptera exigua also contains covert baculoviruses.

RESULTS

Virus reactivation in fourth instar (L4) S. exigua

Inserting whole baculovirus genomes in low-copy-number bacmid vectors in Escherichia coli was used to clone Spodoptera frugiperda multinucleopolyhedrovirus (SfMNPV) (Simón et al., 2008) and S. exigua MNPV (SeMNPV) DNA (Pijlman et al., 2002, 2003). The Se.BAC/G26 genomes derived from cloning SeMNPV-G26 in this study were polyhedrin negative and used to transfect L4 S. exigua by DNA injection into haemocoeys. Insect mortality recorded after injections ranged from 2 to 50% (Fig. 1a, b) in six replicates. Similar variation in mortality was found in groups of insects injected with the wild-type Se-G26 (0–40%). More surprisingly water-injected control and non-treated insects also suffered significant mortalities (10–44% and 5–30%, respectively).

A total of 86 cadavers with symptoms of NPV infection after in vivo transfection were inspected for occlusion bodies (OBs) under a microscope and their DNA analysed by restriction enzyme digestion. Despite Se.BAC/G26 genomes being polyhedrin negative (Fig. 1c), OBs were consistently observed in all samples. The DNA analysis confirmed the viruses (Fig. 2, lanes 3–6) did not contain the bacterial artificial chromosome (BAC) replicon in the original vector (Fig. 2, lane 2). Additional fragments were present in the progeny DNA profiles after in vivo transfection with Se.BAC/G26, suggesting that the SeBAC clones had not simply lost the vector sequences after growth in insects. Three variants were identified in different proportions (Fig. 2c) after digestion of virus DNA with BglIII. Two variants had similar profiles to SeMNPV-G26 and were named Se-OX1 and Se-OX2. These were observed from 73 and four viral deaths, respectively, from a total of the 86 profiled. Another variant (Se-OX3) found in seven insects showed an identical profile to MbNPV, while a mixture of SeMNPV and MbNPV (Fig. 2, lane 6) was found in two cases (Fig. 2c). The Se-OX3 variant had the same profile as MbNPV when digested with PstI, XhoI, EcoRV, EcoRI, Clal and HindIII (data not shown).

Se-OX1 and Se-OX2 variants were observed in larvae injected with Se.BAC/G26, SeMNPV-G26 and water. Se-OX1, Se-OX2 and Se-OX3 were also found in non-challenged insects (non-treated controls), suggesting they had been reactivated spontaneously in those cases.

The L4 S. exigua larvae thought to be virus free were infected with approximately 10⁶ OB ml⁻¹ of MbNPV. Nine individuals succumbed to overt infection, virus was isolated and their DNA profiles after restriction enzyme digestion compared with MbNPV (Fig. 2, lanes 8–16). Seven insects produced viruses with DNA profiles typical of MbNPV but one resembled Se-OX1 (Fig. 2, lane 8) and another a mixed population of MbNPV and SeMNPV (Fig. 2, lane 15).

Quantification of covert viruses in S. exigua breeding cultures

Spontaneous outbreaks of virus disease in S. exigua were indicative of high levels of virus DNA. However, PCR methods where products were detected by electrophoresis failed to detect covert virus consistently when lef-8, lef-9 and rrr1-polh primers were used to test DNA samples from asymptomatic L4 larvae. Hence, a more sensitive approach using quantitative PCR (q-PCR) was optimized. Sensitivity and specificity were tested by using serial dilutions of either SeMNPV or MbNPV DNAs (Table 1). This demonstrated that SeVP80 and MbP87 primers could detect 0.06 pg virus DNA (3 x 10⁻⁶ ng µl⁻¹ in 20 µl reaction) which equated to 490 and 366 for SeMNPV and MbNPV genomes, respectively, with no cross-reactivity (Table 1). Host DNA did not interfere with the PCR (data not shown).

This q-PCR method was used to quantify covert virus DNA throughout insect metamorphosis to correlate it with NPV mortality records in the host population. Twenty breeding couples were used to establish separate F1 populations for rearing 25 adults (Supplementary Fig. S1, available in JGV Online). Four matings produced no offspring and two produced larvae where 96% died from baculovirus infection. None of these could be maintained further. Two other populations (1 and 12) with 8% virus deaths in the larvae (Table 2) were used to produce three F2 populations (1a–c and 12a–c), while others (7a–c, 16a–c and 18a–c) derived from F1 populations with no virus deaths. However, not all breeding pairs produced offspring (Supplementary Fig. S1). Ten samples from each developmental stage (eggs, L₁–L₅ and adults) were screened for virus genomes using q-PCR (Table 2). The mean F₂
viral DNA levels across developmental stages varied from $1.72 \pm 0.020$ to $0.29 \pm 0.036$ pg (Table 2) for five lines, significantly higher than in water controls ($1.80 \pm 0.071 \times 10^{-2}$ pg) ($t$-test pairwise, $P<0.05$). Offspring from line 12b recorded the highest virus DNA levels of $3.51 \pm 0.101 \times 10^5$ pg with 45 positive insects from a total of 80 tested insects ($t$-test pairwise, $P<0.05$). Similar proportions of insects harbouring viral DNA were found across the rest of the lines tested (Table 2) and no trends for prevalence of virus between developmental stages was observed (Supplementary Table S1, available in JGV Online).

Covert viral DNA levels monitored throughout the host development cycle were further studied in the offspring from line 12b as 10 cases of apparent NPV-induced mortality were found in L4 (Table 2). To distinguish SeMNPV- from MbNPV-like virus we used the SeVP80 and MbP87 primer sets with the S. exigua elongation factor-1 alpha (SeEF) primer set as an internal host DNA control. SeMNPV DNA was detected in eggs, L1, L2, L3, L5, pupae and adults. MbNPV DNA was only detected in eggs, L1 and L2 at levels considerably less than SeMNPV, although 50% of eggs were positive. Healthy L4 individuals were not tested for virus. Restriction endonuclease (REN) analysis of larvae that died exclusively showed SeMNPV profiles. When virus DNA levels were normalized to host DNA (SeEF primer set) and log-transformed, maximum viral levels were apparent in eggs and first larval instars (L1; Fig. 3). During the larval stage the ratio of virus : host cell DNA decreased in L2 but increased dramatically in L3, indicating the virus had actively replicated and produced overt infections in L4 instars. L5, pupae and adults recorded the lowest ratios (Fig. 3), corresponding to similar viral DNA values as those quantified for eggs and L1, suggesting viral replication was minimal. Restriction enzyme analysis of virus DNA from 10 L4 larvae resulted in profiles resembling the Se-OX1 genotype.

**Fig. 1.** Percentage NPV mortality following intraheamocoelic injections with SeMNPV DNA in fourth instar (L4) S. exigua larvae. Six repetitions were carried out on different days and included three different treatments: Se-G26, Se.BAC/G26 and water as control (a) or Se.BAC/G26, water and non-treated controls (b). The percentage mortality was calculated from 75 L4 treated larvae at each repetition. The absence or presence of a functional *polyhedrin* gene in the virus genomes used as inocula or generated in the bioassays is shown (c). NA, Not applicable; Rep, replicate.

**Sequencing data from covertly and overtly infected insects**

Sequences for *lef-8* and *lef-9* were generated from 10 insects with covert infection (C1–C10) or overt infection resulting from spontaneous virus reactivation (O1–O10) (Supplementary Fig. S2, available in JGV Online). All 20 individuals had SeMNPV-specific infections. Comparison
of a 533 nt region from lcf-8 showed 100% identity in all samples with Se-G26 (data not shown). For lcf-9 (730 nt), pairwise comparison to Se-G26 showed between 98 and 99% nucleotide identity, due to some nucleotide mismatches that did not affect the gene transcription and functionality. The lcf-8 and lcf-9 sequences showed a 100 and 98% nucleotide identity to the SeMNPV published sequence (GenBank accession no. AF169823.1), respectively.

For the 990 bp rr1-polh intergenic region, a single repeat of 12 bp was found to be a consensus sequence in samples C2, C3, C5, C6, C7, C9, O4, O5, O6 and O10 while it did not appear in samples C1, C4, C8, O1, O2, O3, O6, O8 and G26. The repeat was positioned in the non-coding region between ORF 139 and 1 corresponding to the rr1 and polyhedrin genes at the nucleotide positions 135, 169–135, 181 bp of the GenBank accession number AF169823 genome (Supplementary Fig. S2).

**Biological response to reactivated viruses**

Virus stocks (Se-G26, Se-OX1, Se-OX2, Se-OX3 and MbNPV) were freshly amplified in L4 S. exigua larvae. q-PCR carried out with either SeVP80- or MbP87-specific primers showed no detectable presence of SeMNPV-like viruses in MbNPV and Se-OX3 stocks or MbNPV in SeMNPV preparations (Table 1). Bioassays were used to assess the five virus stocks in early L2 S. exigua larvae. Non-viral deaths occurred in less than 5% across the four replicates for untreated control insects and were not included in subsequent analysis.

Overall mortality response for the five viruses was significantly affected by the dose (F = 24.54, d.f. = 1, P < 0.001), and virus variant species (F = 18.46, d.f. = 4, P < 0.001). As the interaction dose x virus was also significant (F = 5.84, d.f. = 4, P < 0.005) the data were split into SeMNPV-like and MbNPV-like virus groups which showed a different trend from one another (Fig. 4). For the SeMNPV-like virus all viral doses produced over 75% mortality in all four replicates. Neither the dose (F = 3.05, d.f. = 1, P = 0.110) nor the virus variant (F = 0.55, d.f. = 2, P = 0.592) significantly affected the mortality response in this group.

Mortality was found to increase with dose for MbNPV-like viruses (F = 159.72, d.f. = 1, P < 0.001). No significant differences were found between both viruses in pathogenicity (χ² = 10.78, d.f. = 8, P = 0.214) with median lethal concentration (LC₅₀) values of 9015 OB ml⁻¹ (5796–13875) and 13437 OB ml⁻¹ (8628–20685) for Se-OX3 and MbNPV, respectively. The time-to-death responses of these two viruses were not significantly different from one another: Se-OX3 required 69.83 h (62.11–78.06 h) to kill 50% of a treated population, while MbNPV needed 82.94 h (78.99–88.20 h) (Weibull, r = 0.861).

Time-to-mortality curve patterns consistently revealed two different trends for each species group (Fig. 5). SeMNPV-like virus-induced deaths peaked at 40 h post-infection (p.i.) and all insects had succumbed to viral death by 48 h p.i. Deaths caused by MbNPV-like viruses resulted in a distribution with two peaks at 40 and at 80 h p.i., with a different proportion of deaths caused by MbNPV or Se-OX3 (Fig. 5a). To confirm the cause of death, q-PCR was performed for five larvae fed with each virus for each mortality record point. Proportions of specific viral DNA were estimated as a mean of pooled DNA quantities of five larval samples (SeVP80 or MbP87) normalized to the total viral DNA (SeVP80 + MbP87 readings). All dead larvae collected at 40 h p.i. predominantly harboured SeNPV-like virus (r = 0.99), irrespective of the virus that had been inoculated. Dead larvae sampled at later times (80, 88, 96 or 104 h) revealed MbNPV-like virus in different
proportions (Fig. 5c, d), being the only virus detectable in samples collected at 88, 96 and 104 h p.i.

**DISCUSSION**

*S. exigua* larvae injected with polyhedrin-negative SeMNPV genomes amplified using low-copy-number BAC vectors died with symptoms typical of a baculovirus infection but also produced occluded virus. This might have been a consequence of spontaneous BAC vector excision from its insertion site within polyhedrin, thus restoring native gene function. However, analysis of virus genomes revealed restriction fragment length polymorphisms (RFLPs) elsewhere in the virus genome inconsistent with this hypothesis. Further, control insects suffered spontaneous virus infection despite virus-free rearing conditions. This same population of insects was used as negative controls in studies to detect covert infections in *M. brassicae* populations (Burden *et al.*, 2003, 2006) and was considered to be virus free. However, the primers used in the PCR-based tests in these earlier studies were optimized for MbNPV and may have failed to amplify SeMNPV DNA. Covert infections were postulated to explain the different REN profiles of progeny after cross-infections with heterologous NPVs (Longworth & Cunningham, 1968; Fuxa *et al.*, 1992; Table 1.

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**Table 1. Oligonucleotides used for quantification of SeMNPV and MbNPV by q-PCR**

The specificity of the primers was indicated by the generation of a single amplicon with a specific melting temperature and no cross-reactivity to the heterologous virus. Fw, Forward; NA, not applicable; NP, no detectable product for the template range tested; Re, reverse.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (Fw/Re; 5′–3′)</th>
<th>Range of detection(pg)*</th>
<th>Viral genomes</th>
<th>Amplicon melting temp. for specific template (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MbNPV</td>
<td>SeMNPV</td>
</tr>
<tr>
<td>SeVP80</td>
<td>CGAGCGCTTGTGATGAAATAG GAAATTAACGCGCCTCAACG</td>
<td>6 × 10^5–6 × 10^-2</td>
<td>490</td>
<td>NP</td>
</tr>
<tr>
<td>MbP87</td>
<td>GCATTAGGGTGTGCATTACG CATCATTATTAGCTCTGACA</td>
<td>6 × 10^5–6 × 10^-2</td>
<td>366</td>
<td>83</td>
</tr>
<tr>
<td>SeEF</td>
<td>GGCTGTATCTCAAGGTAAGG GTTACACCGAGTGAAGGC</td>
<td>2 × 10^5–2 × 10^-2</td>
<td>NA</td>
<td>NP</td>
</tr>
</tbody>
</table>

*The detection range was determined from amplifications of 10-fold dilution of purified virus DNA.

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**Table 2. Frequency of spontaneous NPV mortality in F1 and F2 offspring, and q-PCR quantification of SeNPV-like persistent DNA for F2 offspring across six lines of *S. exigua* insects generated by breeding two generations (Supplementary Fig. S1)**

NA, Not applicable.

<table>
<thead>
<tr>
<th>Breeding line (F2)</th>
<th>Number of spontaneous overtly infected insects</th>
<th>SeNPV-like persistent viral DNA in F2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1 (parent line)*</td>
<td>F2†</td>
</tr>
<tr>
<td>Lines in which spontaneous NPV infections were recorded in F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 1b</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Line 1c</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Line 12b</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Lines in which spontaneous infections were not recorded in F1</td>
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<td></td>
</tr>
<tr>
<td>Line 7b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Line 16a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Line 18a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Background levels in water controls</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*n*=25.

†n=75.

‡Mean of q-PCR positive insects (n) data pooled from different development stages. Different letters denoted significant differences by the *t*-test (*P*<0.005).
Hughes et al., 1993; Cooper et al., 2003; Kouassi et al., 2009) or a sudden occurrence of overt infection in healthy populations (Fuxa et al., 1999). In this study, production of a different viral progeny was observed after in vivo transfection with Se.BAC genomes or per os OB infections with MbNPV. It was also observed in untreated larvae (water-injected and non-injected controls) so there may have been a stress in rearing conditions during the experiment acting as a trigger. Previously, stress factors such as crowded rearing conditions and high humidity were reported to induce higher prevalence of NPVs in the progeny of a Trichoplusia ni laboratory culture (Steinhaus, 1958; Fuxa & Richter, 1999). However, the mortality induced after in vivo transfections in our studies fluctuated between groups of insects reared under similar conditions. This suggests that despite genetic in-breeding of the S. exigua population, certain individuals are more likely to suffer triggering of covert to overt infection or simply that levels of virus vary between them.

We compared different viruses from S. exigua using REN analysis and DNA sequencing. Interestingly, we observed a degree of genotypic variability among SeNPV-like reactivated virus and a second species resembling MbNPV. Sequencing generated data for lef-8 and lef-9 of SeMNPV-like viruses that not only showed high similarity to the published SeMNPV genome, but no major differences between DNA from overtly and covertly infected insects. Only the rrl-polh intergenic region revealed that 10 out of 20 covert and overt DNA infections contained a 12 bp repeat not present in the wild type initially used for cloning. Although we analysed a very small part of the genome (approximately 2173 bp), these results suggest that the viral genome might not suffer major changes when transformed from a fully lethal form into a covert form or vice versa.

Assuming a high degree of similarity at the nucleotide level between the virus genomes in covert and overt infections we developed a PCR-based technique capable of detecting small quantities of viral DNA specific for any of the identified reactivated NPVs. Regarding sensitivity, we detected as little as 0.06 pg of viral DNA which equates to 490 and 366 genomes of SeMNPV or MbNPV, respectively. Previous studies carried out on different baculovirus species reported a wide range of detection limits for standard PCR [i.e. 10 pg for Plodia interpunctella granulovirus (GV) DNA, Burden et al., 2002; 0.0038 pg for MbNPV DNA, Burden et al., 2003] or nested PCR (0.1 pg for Spodoptera exempta NPV; Vilaplana et al., 2010). In this study, q-PCR enabled us to assess viral load in insects and trends over time, as opposed to expression studies that focus on the activity of the virus using RT-PCR detection.

Vertical transmission of SeMNPV was induced in S. exigua when L5 larvae were fed virus (Smits & Vlak, 1988). A persistent, low-level infection of P. interpunctella GV was established in progeny derived from infected adults (Burden et al., 2002). We assessed the prevalence of covert virus over two generations in S. exigua both of which had seen some insects succumb to a spontaneously reactivated NPV. Across the six lines tested, 5–56% of 80 insects were shown to harbour the virus as a covert infection, with only one line registering NPV-induced deaths. It was possible to demonstrate that levels of viral DNA fluctuated considerably throughout development. In an earlier study, spontaneous NPV-induced deaths were reported to occur in mature larvae but rarely in early instars (Karpov, 1979).
Consistently, in this study the levels of covert virus DNA dramatically increased with larval development up to 10^4-fold, suggesting an extremely active viral replication period that eventually caused the deaths observed at L4. Very low viral levels relative to host DNA were found at later instars, pupae and adults when the virus may not be actively replicating and the host may have suppressed a productive infection.

Larvae from *S. exigua* laboratory culture with a covert infection were subjected to a superinfection with each reactivated virus and two SeMNPV and MbNPV wild types. Un Expectedly, no correlation between dose and mortality was observed for the SeNPV-like virus tested on L2. A dose as low as 9.9 × 10^3 OBs ml^-1 (three OBs per L2) produced over 50% mortality although there was no NPV-induced mortality in controls and virtually all L2 larvae fed with SeNPV-like viruses died by 48 h p.i. Murillo *et al.* (2006) previously estimated the pathogenicity and virulence of the Se-G26 in similar bioassay conditions, with the LD_{50} value of 9.3 × 10^4 OBs ml^-1 (31 OBs per L2) and the mean time to death (MTD) value of 104 h p.i. It seems plausible that the covert virus became active when the larvae were challenged with SeMNPV-like virus derived from an overt infection. Interestingly, the MbNPV-like viruses, Se-OX3 and the reference MbNPV which were indistinguishable by RFLP, presented a close response in terms of their LD_{50} and MTD. Remarkably, MbNPV-like viruses as a group produced a very different response compared with SeMNPV-like viruses. Only approximately 10% of the OB progeny from MbNPV-challenged larvae resulted in SeMNPV-like reactivation detected by q-PCR.

Our study indicates that an *S. exigua* laboratory culture may harbour a covert infection caused by two distinct NPV species. Covert infections have been proposed as the viral strategy in response to variation in transmission opportunities, through fluctuating host population (Burden *et al.*, 2003; Cooper *et al.*, 2003). Theoretically, low levels of covert infections may be explained as an evolved parasite strategy when transmission opportunities vary (Sorrell *et al.*, 2009). However, high prevalence of NPV covert infections has been consistently found in field lepidopteran populations (Burden *et al.*, 2003; Vilaplana *et al.*, 2010). The apparent loss of persistent virus infection observed in some of the experiments in this study is intriguing, but may simply reflect the difficulties in detecting the pathogen, particularly when there is no associated phenotype. In other systems used to study vertical virus transmission, such as *Drosophila melanogaster* and sigma virus, the pathogen renders the host susceptible to CO2 (L’Heritier, 1970; Brun & Plus, 1980), which provides a convenient marker of virus infection. Yampolsky *et al.* (1999) showed that CO2 sensitivity was lost from two female populations, suggesting the virus was no longer present. In *S. exigua*, persistent baculovirus infections may be lost spontaneously in some individuals but in our breeding population it must be maintained in the majority. This putative virus loss may be a consequence of antiviral immunity in the host. Information on host immunity is well developed for *Drosophila* (Wang *et al.*, 2010) but less so for lepidopteran species. This should be a productive area for future studies on persistent baculovirus–host interactions.

![Fig. 5. Mortality of L2 *S. exigua* larvae over time following inoculation with the SeMNPV-like viruses Se-OX1 (●), Se-OX2 (▲), Se-G26 (■) (all dotted lines); the MbNPV-like virus Se-OX3 (□) and MbNPV (○) (both solid lines) (a). Proportion of SeNPV-like (filled bars) and MbNPV-like (open bars) viruses in larvae collected at 40, 80, 88, 96 and 104 h p.i. (means of pooled data of five insects per record time) measured by q-PCR (SeVP80 and MbP87 primers used for SeNPV-like and MbNPV-like virus detection, respectively) for insects inoculated with Se-G26/Se-OX1/Se-OX2 (b), Se-OX3 (c) and MbNPV (d).](http://vir.sgmjournals.org)
**METHODS**

**Virus and insects.** *S. exigua* were reared on diet (Hunter-Fujita et al., 1998) at 25 °C, 16:8 light:dark photoperiod and 60–70% humidity. The SeMNPV (SeMNPV-G26) was originally collected from greenhouses in Almeria (Spain) (Murillo et al., 2006, 2007). MbNPV was used as a comparative control.

**Cloning of the SeMNPV into BAC vector.** A bacmid containing the SeMNPV-G26 genome was constructed by vector insertion at the polyhedrin locus. CsCl-purified viral DNA (2 μg; King & Possee, 1992) and 100 ng pBAC.SanDI vector based on pBAC3.6 (Hitchman, 2002) were digested with SanDI (Stratagene). The reactions were treated for 15 min at 65 °C and the vector dephosphorylated with calf intestinal phosphatase (New England Biolabs). Digested SeMNPV-G26 and gel-purified vector were ligated using 5 U T4 DNA ligase (Promega) at a ratio of 1:20 and dialysed against TE (10 mM Tris/HCl; 1 mM EDTA) at 4 °C. One-tenth of this reaction was used to electroporate Gene-hog cells (Invitrogen), which were amplified on agar plates containing chloramphenicol (12.5 μg ml⁻¹). SeMNPV bacmids were selected from 100 clones and designated Se.BAC/G26.

**Transfection of *S. exigua* larvae with BAC genome DNA.** Se.BAC/G26 DNA was mixed with Lipofectin (300 μg ml⁻¹; Invitrogen) and injected into early L₃ *S. exigua* using a micro-applicator fitted to a syringe and Microfine needle (G30). One hundred nanograms of circular viral DNA in 6 μl was injected into the haemocoel. Injected and control larvae were incubated at 26 ± 2 °C and checked daily for infection. Dead larvae were smeared on slides and inspected under a phase contrast microscope (Nikon Labophot; × 400) for OBs.

**Viral DNA isolation and RFLP analysis.** OBs and virus particles were purified as described by King & Possee (1992). Virus particles were lysed by addition of 0.5 M Na₂CO₃, 0.1% SDS and incubated for 2 h at 65 °C with proteinase K (200 μg ml⁻¹). DNA was purified by phenol/chloroform extractions and ethanol precipitation, resuspended in 30 μl water, digested with BglII, fractionated on 0.6 or 1.2% agarose gel with TBE buffer (reaction buffer, 10 pmoles of each primer and 5 μl of template DNA, 1 × reaction buffer, 10 μmol primers, and 5 μl of template DNA. Standard curves were generated using 7 × 10⁹ dilutions of CsCl-purified virus DNA (6 × 10⁻⁶–0.06 pg). Six water (minus template) controls were included in each batch of 72 samples. The q-PCR was performed in a Rotor Gene 6000 thermal cycler (Corbett Research) and fluorescence recorded on the Green/FAM channel with a gain setting of 5. Cycling conditions were determined for each primer against its target DNA. A common hold step of 2 min at 95 °C was followed by 40 cycles of amplification involving a denaturation step of 30 s at 95 °C, an annealing step of 30 s at 62 °C, and elongation step of 17 s at 72 °C. Melting curves were generated by fluorescence readings over ramped temperatures at the end of cycling between 50 and 99 °C. The specificity of primers was assessed by testing for cross-reactivity against MbNPV, SeMNPV or host DNA. Quantification was by comparison of cycle time at which the fluorescence exceeded the threshold level generating the best fit regression with respect to the standards. Only standard curves in which the regression ratios (R²) exceeded 0.99 were considered sufficiently accurate for determination of persistent virus levels. The readings obtained for the primer set SeEF were used for the standardization of the viral DNA with host DNA.

**Levels of covert virus during *S. exigua* development.** Fifty L₃ *S. exigua* were individually reared on a virus-free diet in a UV-sterilized incubator and sexed at pupation. Twenty couples were mated in paper bags with water feeding pots. Females laid eggs for 2–3 days and then parents were frozen for subsequent analysis. Eggs were placed in 300 ml polystyren containing artificial diet. When neonates emerged, 25 insects were individually reared to adults (offspring F₁) in multi-well plates with diet. Insects were observed daily for signs of NPV infection (Supplementary Fig. S1). The survivors in lines from which some F₁ offspring died of spontaneous overt NPV infections were allowed to produce F₂ offspring. Three more F₁ lines with no deaths were also allowed to generate an F₂. Ten asymptomatic individuals at each development stage of F₂ were frozen for subsequent DNA analysis by q-PCR. Remaining individuals were monitored for NPV deaths. The NPV-killed progeny were stored at −20 °C for subsequent REN analysis as described above.

Total DNA from insect development stages was extracted using a Tepnel robot (Life Sciences) with the Nucleoprep plant DNA kit. Half of each of the L₀, pupae and adult samples were used to avoid column overload, while eggs, L₁, L₂, and L₃ samples were processed in their entirety. Extractions were performed in a 96-well format in tubes that contained a metal ball bearing. Tissue disruption was facilitated by using a bead beater (Bio-Rad) at 1.5 Hz for 2 min once the lysis buffer samples were dispensed. The homogenized samples were incubated in a water bath at 65 °C for 2 h prior to DNA extractions.

**PCR and sequencing of reactivated and persistent virus.** To characterize virus in *S. exigua*, sequence data from three well-conserved genes was assessed (Jehee et al., 2006). Forward (Fw) and reverse (Re) oligonucleotide primers sets were designed targeting three SeMNPV genome regions (GenBank accession no. AF169823): the rrl-polhedrin intergenic region (5′- CGAGCAGTCTTGAGCAACGC-3′ and 5′-TTTCACTGTGCTCGGTAGCCG-3′), lef-9 (5′- GCCAGGTGTGTTTTTGATA-3′ and 5′-AACCTCAAGCACAAC-3′), and lef-8 (5′-AACGAGCTGTGATCTTCCG-3′ and 5′-AGCCAACTCTTCAAGCCGCCAG-3′) for the amplification of 910, 730 and 533 bp fragments, respectively. Each reaction contained 2 μl of viral DNA, 0.3 μl of 2 mM dNTP, 5 μl of 10 × buffer, 1 μl Taq polymerase and 1 μl of primer (10 pm) in a volume of 50 μl. The thermostrapping programme comprised 30 cycles of 94 °C (4 min), 58 °C and 72 °C (1 min each) in a Primus 96 thermocycler (MWG-Biotech). The DNA products were purified and sequenced with primers used for the genome of the DNA plus a second internal primer for the rrl-polh intergenic region (Re 5′-CCATCGTGGTTTGCGTCGTG-3′) and lef-9 (Re 5′-CGTAAACGCCAGCTCGAC-3′). Sequencing reactions were performed according to the supplier’s recommendation and analysed on an Applied Biosystems 3730. Trace files (Fw and Re) were aligned to generate a consensus sequence for each PCR product (Staden et al., 2003). Sequences were aligned and compared using CLUSTAL_X (Thompson et al., 1997).

**Bioassay.** Prior to bioassays fresh stocks of Se-OX1, Se-OX2, Se-OX3 and wild types SeMNPV-G26 and MbNPV were amplified by droplet feeding L₃ *S. exigua* with 10⁹ OBs ml⁻¹. The identity of viral inocula was confirmed by REN and PCR.

Dose–mortality responses of Se-OX1, Se-OX2 and Se-OX3 were measured using bioassays with L₃ *S. exigua* fed on virus droplets (Hughes & Wood, 1981). OBs were counted in a haemocytometer and used immediately to make dilutions in sterile water containing 5% blue food dye (Langdale). The doses were estimated to produce mortalities between 5% and 95%: 9.9 × 10⁵, 2.7 × 10⁶, 8.1 × 10⁶, 2.5 × 10⁷ and 7.4 × 10⁷ OBs ml⁻¹ and 3 × 10⁶, 3 × 10⁷, 3 × 10⁸,
3 × 10^4 and 3 × 10^5 OBs ml^{-1} for SeMNVP-like and MbNPV-like viruses, respectively. L_2 S. exigua were selected by head capsule size, moulded and starved for 16 h prior to virus dosing. Seven groups of larvae containing 30 individuals for each virus treatment were used. Larvae that ingested droplets within 10 min were individually transferred to 25-well plates containing diet and reared at 25 ± 2 °C. Mortality was recorded every 12 h for 7 days. Bioassays were performed on four occasions.

A single dose bioassay was carried out to determine the dose–time response following the same method. A group of 75 L_2 S. exigua was dosed with each virus to produce in excess of 95% mortality. Mortality was recorded every 8 h for 5 days.

Statistical analyses. Dose–mortality responses were subjected to General Linear Models using Minitab (2006) by fitting the data in the minimal possible model for proportionate mortality as response, the virus as fixed factor with five levels and log dose as a covariant. Mortality data not fitting normal distribution were previously subjected to square transformation. Model behaviour was checked by examination of the distribution of residual and fitted values.

To compare relative virulence LC_{50} and MTD were estimated for viruses showing a significantly positive dose–virus response. Probit analyses assuming logic data distribution were used to estimate the LC_{50}. MTD was estimated fitting mortality–time data as a Weibull distribution as right censored data excluding individuals that did not die from virus infection from the analysis (Farrar & Ridgway, 1998).

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REFERENCES


