

# Evidence for Covert Baculovirus Infections in the Field-Collected *Spodoptera litura* Larvae

Sudawan Chaeychomsri

Central Laboratory and Greenhouse Complex, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University,  
Kamphaeng Saen, Nakhon Pathom, Thailand  
Email: rdisuc@ku.ac.th

Win Chaeychomsri and Jindawan Siruntawinetti

Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand  
Email: {fsciwcc, fscijws}@ku.ac.th

**Abstract**—A total of 55 *Spodoptera litura* larvae collected from marigold fields were reared on an artificial diet in the laboratory and determined for the presence of covert baculovirus infections. Polymerase Chain Reaction (PCR) analysis by using primers specific to the highly conserved baculovirus genes, *granulin* and *polyhedrin*, combined with transmission electron microscopy confirmed the presence of granulovirus (GV) and nucleopolyhedrovirus (NPV). Eight out of 55 larvae (14.5%) were PCR positive for *S. litura* granulovirus (SpltGV), while one larva (1.8%) was PCR positive for *S. litura* multicapsid nucleopolyhedrovirus (SpltNPV). Electron microscopic observations of the PCR-positive samples revealed the presence of a single baculovirus infection, either GV or NPV. The identification of SpltGV and SpltNPV in the field-collected *S. litura* larvae indicated that the viruses were found to be present in this population. The findings in the present study suggest that the persistent baculoviruses in the field populations of *S. litura* may be activated by some stressors rather than by exposure to another baculovirus. It can be speculated that such covert baculovirus infections may lead to the decline in insect populations and also can lead to a collapse of laboratory-reared insect colonies for in vivo production of bioinsecticides.

**Index Terms**—*Spodoptera litura*, nucleopolyhedrovirus, granulovirus, PCR, electron microscopy

## I. INTRODUCTION

The members of the family Baculoviridae are classified as a group of arthropod-specific viruses with rod-shaped nucleocapsids [1]. Based on morphological, biological characteristics, and phylogenetic features, the members of this family are classified into four genera, as follows: Alphabaculovirus (lepidopteran-specific nucleopolyhedrovirus, NPV), Betabaculovirus (lepidopteran-specific granulovirus, GV), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV) [2]. Alphabaculovirus can be further subdivided into groups I and II according to the phylogenetic analysis of the *lef-8*, *lef-9* and *polyhedrin* (*polh*)/*granulin* (*gran*) genes [3],

whereas Betabaculovirus is classified into three types based on the host tissue tropism [4]. Typically, the baculovirus virions (enveloped nucleocapsids) are present as two types, Occlusion-Derived Virions (ODVs) and Budded Virions (BVs). ODVs are responsible for oral infection of insect hosts and horizontal transmission, while BVs are responsible for the systemic cell to cell infection within infected insects. The ODVs of NPV and GV are embedded in a crystalline protein matrix to form Occlusion Bodies (OBs), which protect the virions against adverse environmental conditions. NPV produces many virions embedded in large polyhedral OBs called polyhedra or Polyhedral Inclusion Bodies (PIBs), whereas GV produces a single virion embedded in a smaller ovocylindrical OBs called granules or capsules. The crystalline protein matrix in either form is composed of a single polypeptide which is known as polyhedrin or granulin in NPV or GV, respectively [5].

As baculoviruses are obligate pathogens, these viruses can only be propagated in living cells, so it is necessary to propagate them either in larvae or cultured cells of susceptible insects. For this reason, the commercial baculovirus products used in insect pest control are currently produced using in vivo production systems. The process for baculovirus production involves mass rearing and infecting of host larvae, and then harvesting virus from the dead larvae [6]. It is labour-intensive and has to be managed carefully to avoid contamination with other unwanted organisms. Insect pathogens, especially insect pathogenic viruses, can contaminate host-insect cultures, destroy the rearing colonies, and cause serious production problems [7]. Epizootics caused by baculoviruses are generally lethal and can reduce dramatically their host population. However, baculoviruses have also been found to cause covert infections in the field and laboratory host populations [8]-[12]. Covert infections (also known as inapparent, sublethal, silent or occult infections) are characterized by the absence of visible signs of disease [11]. The apparently healthy insects, collected in the field and reared in the laboratory, may suddenly succumb to a virus infection affecting most, if not all, of the individuals in a colony.

Manuscript received April 3, 2020; revised October 28, 2020.

In Thailand, the baculovirus *Spodoptera litura* NPV (SpltnPV) has been developed as bioinsecticide and used to control the common cutworm, *S. litura*, in cut flower and orchid [13]. The production process for SpltnPV is an *in vivo* process, that relies on the successful mass-rearing of the host insects which are then infected with the virus. The main limitations of the *in vivo* mass production systems in Thailand have been reported [13]. One of the main limitations of this process is the rearing of sufficient healthy host insects.

In large-scale production systems both lethal and sub-lethal insect disease effects may cause serious damage; thus monitoring, prevention and control of diseases are essential [14]. Therefore, the objective of the present study was to determine the incidence of covert infections of baculoviruses in the field-collected *S. litura* larvae. Primers specific to the SpltnGV *gran* and SpltnPV *polh* genes were used for Polymerase Chain Reaction (PCR) to detect the presence of SpltnGV and SpltnPV, respectively. Ultrastructural studies on virus replication and morphogenesis in the infected larvae of *S. litura* were also described.

## II. MATERIALS AND METHODS

### A. Insects

The larvae of *S. litura* were originally collected from marigold fields in Kamphaeng Saen District, Nakhon Pathom, Thailand, and maintained in the insectary facilities of the Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Thailand. In the laboratory the field-collected larvae were transferred individually to the sterile petri dishes (90 mm dia × 15 mm deep) containing a piece of mung bean-based artificial diet [15]. The larvae were reared at 27±1°C, 60±10% Relative Humidity (RH), 12L:12D photoperiod and observed daily for the signs of baculovirus infection. The larvae exhibiting signs of baculovirus infection were collected and subjected to PCR analysis. The infection was also confirmed by transmission electron microscopy.

### B. DNA Extraction from Insects

In order to detect baculovirus infection, each larva was dissected, just prior to death, in a sterile petri dish (90 mm dia × 15 mm deep) containing Phosphate Buffered Saline (PBS) under a stereo microscope (Olympus Corporation, Tokyo, Japan) with sterile tools in a laminar flow cabinet. Prior to the DNA extraction of the larvae, the fat body tissues were removed, fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, PA, USA) in 0.1 M phosphate buffer pH 7.2 at 4°C and processed for transmission electron microscopy as described below. The remaining tissues in PBS were then pooled into a sterile 1.5-mL tube, kept on ice and subjected to DNA extraction.

The total DNA was extracted from larval tissues using the phenol/chloroform method. The larval tissues in PBS were pelleted by centrifugation at 2,500 × g for 5 min. Subsequently, the supernatant was aspirated and

discarded. A total volume of 1 mL PBS was added to the pellet and larval sample was homogenized using a microtube pestle. The homogenate was centrifuged to pellet tissue debris at 2,500 × g for 5 min and the supernatant was transferred into a new tube. The supernatant was passed through a sterile 100-µm nylon mesh filter into a sterile tube which was centrifuged at 10,000 g for 5 min. Then the supernatant was discarded. Subsequently, 500 µL of 0.1% Sodium Dodecyl Sulfate (SDS) was added and the suspension was repeatedly washed with SDS followed by centrifugation at 10,000 g for 5 min until the supernatant was clear. The final pellet was resuspended in 200 µL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and mixed with an equal volume of 2X alkaline buffer (0.2 M Na<sub>2</sub>CO<sub>3</sub>, 0.02 M EDTA, 0.34 M NaCl) for 30 min on ice to dissolve baculovirus OBs and release ODVs. The undissolved materials were removed by centrifugation at 10,000 g for 1 min. The supernatant was incubated with proteinase K in the presence of SDS overnight at 37°C. After incubation with RNase A at 37°C for 1 h, viral DNA was then purified once with TE-saturated phenol and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). A final extraction with chloroform/isoamyl alcohol (24:1) was used to remove residual phenol, followed by ethanol precipitation. DNA was pelleted by centrifugation and the precipitate was rinsed twice with 70% ethanol, dried under vacuum, and finally suspended in 100 µL of sterile MilliQ water (Millipore Corporation, Molsheim, France). The extracted DNA was subsequently used for PCR amplification of the SpltnGV *gran* and SpltnPV *polh* genes. DNA was also extracted from the healthy-looking larvae as described above and used for comparative purposes.

### C. PCR Amplification and RFLP Analysis of Viral DNA

PCR was performed with specific primers designed from nucleotide sequence of SpltnGV *gran* and SpltnPV *polh* obtained from the sequences available in the public databases. The sequence accession number of the SpltnGV *gran* region used in this study was NC\_009503 [16], while accession number of the sequences of the SpltnPV *polh* gene used in this study was AF325155 [17]. Primers SGF (5'-ATG GGA TAT AAT AGA TCA TT-3') and SGR (5'-TTA GTA CGC GGG TCC AGT-3') were used to amplify the entire *gran* coding region, whereas primers LTF (5'-ATG TAT AGT CGT TAT AGT GC-3') and LTR (5'-TTA ATA CGC GGG ACC CGT GT-3') were used to amplify across the coding region of the *polh*. The amplification of the target genes was done with 50 µL final volumes in 0.2-ml PCR tubes. The reaction mixture in each tube consisted of 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1.0 U Taq DNA polymerase (all from Invitrogen Corporation, CA, USA), and 5 pmol of each primer. As template DNA, 10 µL DNA sample was used. All the primers used in this study were synthesized by Invitrogen (Invitrogen Corporation). PCR amplification of the SpltnGV *gran* and SpltnPV *polh* genes was carried out using the same conditions in a DNA thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA). The amplification conditions included an initial

denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 56 °C, extension for 1 min at 72 °C, and then a final extension for 5 min at 72 °C. Sterile MilliQ water for the replacement of the template DNA was used as a negative control. The PCR products were visualized by electrophoresis of 10 µL of the reaction on UltraPure™ agarose gels (Invitrogen Corporation) using 1X Tris-Borate-EDTA (TBE) buffer and stained with ethidium bromide. The 100-bp ladder (Invitrogen Corporation) was used as a molecular weight marker. To determine the presence of amplified DNA, ethidium bromide-stained gels were visualized through UV illumination and digital images were captured using Syngene G:Box-gel documentation and analysis system (Syngene, Cambridge, UK). The size of the resulting PCR products was calculated against the 100-bp ladder, using GeneTools automatic image analysis software (Syngene). The PCR products were further analysed and confirmed the successful amplification of baculovirus DNA by restriction enzyme digestions.

In order to confirm that the amplified PCR products were the SpltGV *gran* and SpltNPV *polh* genes, the PCR products were further used for Restriction Fragment Length Polymorphism (RFLP) analysis. The restriction sites used to identify both genes were searched from the sequences available in the public databases. The PCR products were separately digested with the appropriate restriction endonucleases (New England Biolabs, MA, USA) according to the manufacturer's protocols. The digested fragments were subjected to agarose gel electrophoresis and the size of fragments obtained from digestions was calculated as described above.

#### D. Transmission Electron Microscopy

All glutaraldehyde-fixed fat body tissues were washed twice with phosphate buffer and then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences), for 1 h at 4 °C. After washing twice with phosphate buffer, the samples were sequentially dehydrated in acetone, infiltrated and embedded in Epon resin (Electron Microscopy Sciences). Samples were sectioned using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted onto 100-mesh copper grids (Agar Scientific Ltd, Essex, UK), double-stained with uranyl acetate and lead citrate. Ultrathin sections were observed under a JEOL JEM 1230 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV.

### III. RESULTS

#### A. Detection of Baculovirus by PCR

A total of 55 *S. litura* larvae of various instars were collected from marigold fields (Fig. 1A) and reared individually in the laboratory (Fig. 1B). None of these larvae were observed with signs of baculovirus infection during the process of collecting insects in the field. After seven days of maintaining larvae in the laboratory, occurrence of baculovirus infection was observed in the field-collected larvae. The larvae showing phenotypical

signs of baculovirus infection, such as sluggish behavior, swollen appearance of abdominal segments and larval body (Fig. 1C), were picked up out of several healthy-looking larvae (Fig. 1B). Nine larvae with signs of baculovirus infection were subjected to baculovirus detection.



Figure 1. Laboratory rearing of field-collected *S. litura* larvae. (A) *S. litura* larvae collected from marigold fields. (B) Healthy-looking larva. (C) Larva showing phenotypical signs of baculovirus infection.

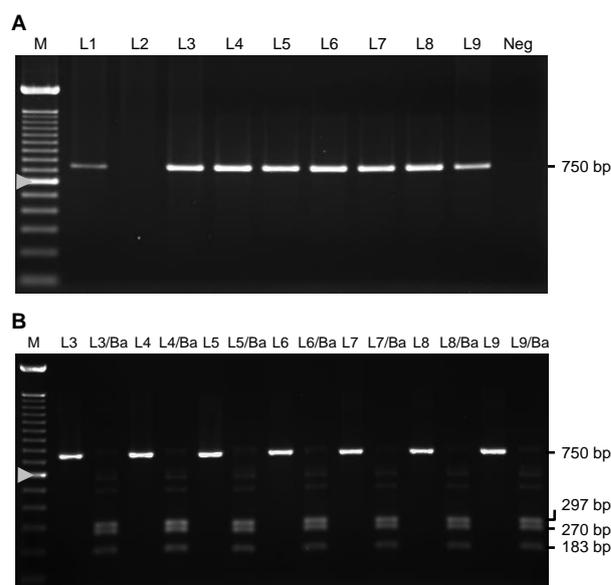


Figure 2. Agarose gel electrophoresis of PCR products amplified by using specific primers for the SpltGV *gran* region. DNA was isolated from nine larvae with signs of baculovirus infection (L1-9). (A) PCR products of the SpltGV *gran*. (B) PCR-RFLP patterns of the SpltGV *gran* digested with *Bam*HI (*Ba*). The PCR and PCR-RFLP products were electrophoresed on a 1% agarose gel along with 100-bp DNA ladder (M) with an arrowhead indicating the band of 600 bp. Position and size of the PCR and PCR-RFLP products are shown on the right. Neg, negative control.

Detection of SpltGV and SpltNPV was performed by DNA purification from a single larva followed by PCR amplification with specific primers for the SpltGV *gran* and SpltNPV *polh* fragments. The PCR results confirmed the phenotypical observation of baculovirus infection of the collected *S. litura* larvae (Fig. 1C). Eight out of 55 larvae (14.5%) were positive for SpltGV by PCR (Fig. 2A), while the remaining one larva (1.8%) was positive for SpltNPV (Fig. 3A). All SpltGV-positive samples were negative for SpltNPV. SpltGV *gran* presented weak but detectable amplification in sample L1, whereas no amplification occurred in sample L2 (Fig. 2A). However, sample L2 was successfully amplified by using SpltNPV *polh*-specific primers (Fig. 3A). Both primer sets yielded PCR products of the same size of approximately 750 bp. The sizes of the DNA fragments in the two genes detected by agarose gel electrophoresis were consistent with the results of primer design.

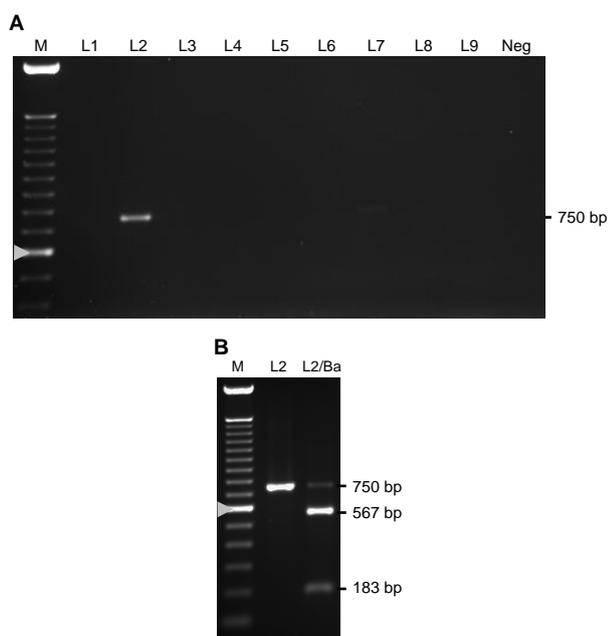


Figure 3. Agarose gel electrophoresis of PCR products amplified by using specific primers for the SpltNPV *polh* region. DNA was isolated from nine larvae with signs of baculovirus infection (L1-9). (A) PCR products of the SpltNPV *polh*. (B) PCR-RFLP pattern of the SpltNPV *polh* digested with *Bam*HI (Ba). The PCR and PCR-RFLP products were electrophoresed on a 1% agarose gel along with 100-bp DNA ladder (M) with an arrowhead indicating the band of 600 bp. Position and size of the PCR and PCR-RFLP products are shown on the right. Neg, negative control.

The specificity of the amplified products was confirmed by restriction enzyme digestion. The resulting SpltGV *gran* PCR products from larvae were digested with *Bam*HI, and the calculated sizes of the fragments after digestion were 183, 270 and 297 bp (Fig. 2B) according to the SpltGV *gran* sequence (accession number NC\_009503) [16]. An identical low-intensity RFLP profile was also observed in sample L1 when the SpltGV *gran* PCR product was digested with *Bam*HI (data not shown). Similarly, subsequent digestion of the SpltNPV *polh* PCR products with *Bam*HI resulted in two scorable bands. The calculated sizes of the fragments after digestion were 183 and 567 bp (Fig. 3B) according

to the SpltNPV *polh* sequence (accession number AF325155) [17]. The same results were obtained when PCR-RFLP was repeated at least three times demonstrating the reproducibility of the technique. The larvae were also examined for baculovirus infection by transmission electron microscopy.

Five out of the healthy-looking larvae (approximately 10%) were subjected to baculovirus detection by PCR targeting the SpltGV *gran* and SpltNPV *polh* regions, and the results revealed that these larvae were not infected with baculovirus (Fig. 4). All offspring derived from the remaining larvae survived without any apparent signs of baculovirus infection. The observations of the covert infection were carried out up to three generations.

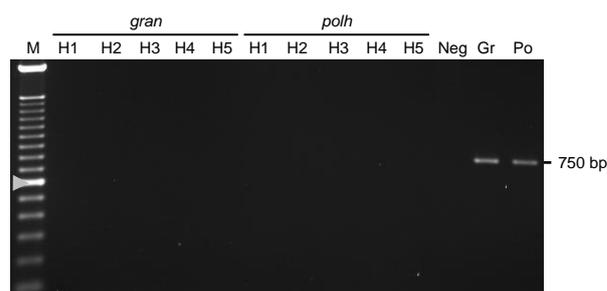


Figure 4. Agarose gel electrophoresis of PCR products amplified by using specific primers for the SpltGV *gran* and SpltNPV *polh* regions. DNA was isolated from five healthy-looking larvae (H1-5). The PCR products were electrophoresed on a 1% agarose gel along with 100-bp DNA ladder (M) with an arrowhead indicating the band of 600 bp. Position and size of the PCR products are shown on the right. Neg, negative control. Gr, positive control for the SpltGV *gran*. Po, positive control for the SpltNPV *polh*.

### B. Transmission Electron Microscopy

In order to confirm the presence of SpltGV and SpltNPV, the PCR-positive samples were subjected to transmission electron microscopy. Electron micrographs revealed evidence of virus infection in the fat body tissues (Fig. 5, Fig. 6). For granulovirus, observations of infected fat body cells revealed the ovocylindrical granulovirus granules with irregular form and size (Fig. 5). All of the granules of the *S. litura* granulovirus observed in this study contained a single nucleocapsid, and the envelope could be clearly seen around each nucleocapsid.

Observations of infected fat cells from SpltGV-positive samples showed different stages of virus infection. Infected cells contained hypertrophic nuclei occupied by nucleocapsids and virions within and around the virogenic stroma (Fig. 5A). The balloon-like swollen mitochondria with fragmented cristae were observed in the cytoplasm (Fig. 5B). The cellular hypertrophy was accompanied by disruption of the nuclear membrane in some regions (Fig. 5C). Consequently, nuclear and cytoplasmic components were mixed throughout the cell (Fig. 5D). At this stage, numerous mature granules with entirely wrapped by the electron-dense layers were observed in both nucleus and cytoplasm of infected cells (Fig. 5E). As there was only one virion per OB, therefore, this virus was identified as GV and named SpltGV according to its host.

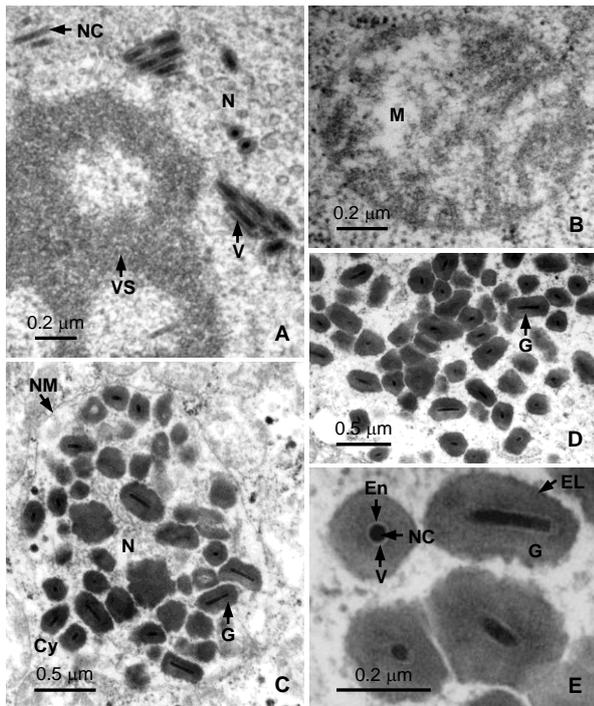


Figure 5. Transmission electron micrographs of SpltGV infection in *S. litura* larvae. (A) Nucleocapsids and virions within and around the virogenic stroma in the nucleus of infected fat body cell. (B) Swollen mitochondrion with fragmented cristae. (C) Enlarged nucleus with partially disrupted nuclear membrane containing several granules. (D) The presence of granules in both nucleus and cytoplasm after nuclear membrane disruption. (E) Mature granules with a single virion embedded. Cy, cytoplasm; EL, electron-dense layer; En, envelope; G, granule; M, mitochondrion; N, nucleus; NC, nucleocapsid; NM, nuclear membrane; V, virion; VS, virogenic stroma.

Electron microscopy of infected fat cells from SpltNPV-positive sample revealed the presence of naked and enveloped nucleocapsids within and around the virogenic stroma in the nuclei (Fig. 6A). The infection resulted in change of mitochondria that appeared swollen and rounded (Fig. 6B). The rod-shaped nucleocapsids were enclosed singly or multiply within an envelope and embedded within a crystalline matrix of polyhedrin forming a polyhedral OB. The infected cells had swollen nuclei with intact nuclear membrane containing several OBs (Fig. 6C). Mature OBs with embedded virions were entirely surrounded by the polyhedron envelope (PE) (Fig. 6D). The presence of OBs that contained several virions (Fig. 6E) and varied in both shape and size indicated that virus from SpltNPV-positive sample was NPV rather than GV. Based on the number of nucleocapsids within an envelope, this virus was categorized as a multicapsid NPV and named SpltNPV according to its host.

#### IV. DISCUSSION

Covert infections of insect pests have long been recognized and have been detected in both laboratory colonies and natural insect populations [11]. Little is known about whether sublethal, persistent virus infection actually occurs in field populations, the rate at which triggering occurs, what causes it, or whether this has any impact on the development of virus epizootics [18]. Studies on covert infections are necessary to understand

the transmission and epizootics of baculoviruses. PCR has been used extensively to identify baculovirus infections because of the sensitivity of the method [19]. PCR-based techniques used for detection of covert infection have targeted genes that are required for genome replication (*dpol*, *lef-8*), or the assembly of structures such as viral capsids (*vp39*, *vp80*) or OBs (*gran*, *polh*) [11].

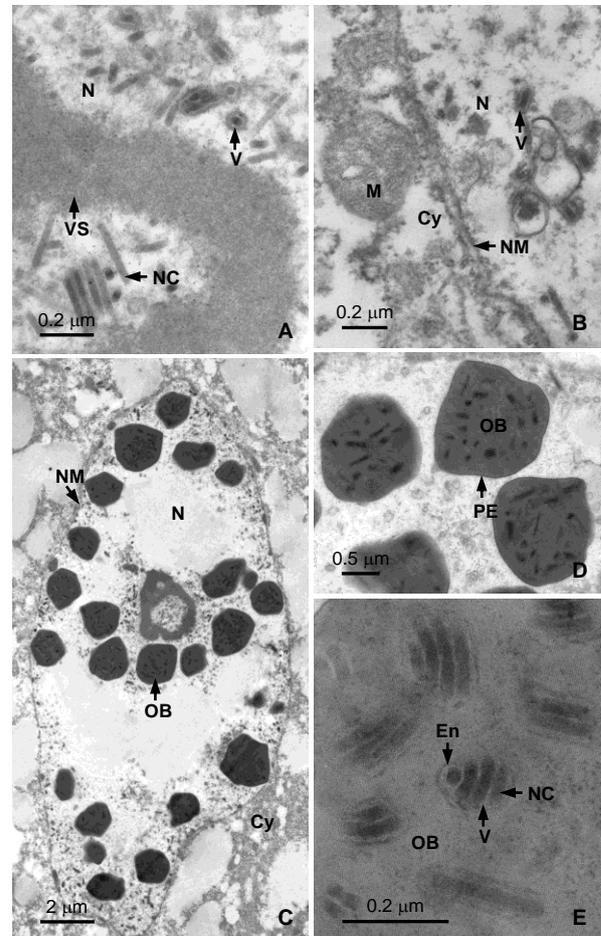


Figure 6. Transmission electron micrographs of SpltNPV infection in *S. litura* larvae. (A) Nucleocapsids and virions within and around the virogenic stroma in the nucleus of infected fat body cell. (B) Swollen mitochondrion with fragmented cristae. (C) Enlarged nucleus with intact nuclear membrane containing several OBs. (D) Mature OBs surrounded by PE. (E) Mature OB containing bundles of rod-shaped virions. Cy, cytoplasm; En, envelope; M, mitochondrion; N, nucleus; NC, nucleocapsid; NM, nuclear membrane; OB, occlusion body; PE, polyhedron envelope; V, virion; VS, virogenic stroma.

In this study, the covert infections of baculoviruses in the field-collected *S. litura* larvae were determined by PCR analysis and electron microscopy. PCR primers were designed targeting the *gran* and *polh* genes in SpltGV and SpltNPV, respectively. PCR results showed that amplification occurred in all DNA samples derived from larvae showing phenotypical signs of baculovirus infection. PCR analysis of the samples from the field-collected populations of *S. litura* indicated that SpltGV was much more prevalent than SpltNPV. Overall, 14.5% of larvae collected from natural infestations of *S. litura* on marigold plants harbored a persistent GV infection, whereas 1.8% of larvae harbored a persistent NPV

infection. No amplification occurred in samples from the healthy-looking larvae and no death was recorded in offspring derived from the remaining larvae. All PCR-positive samples revealed the presence of a single baculovirus infection, either GV or NPV, mixed covert infections with GV plus NPV were not detected (Fig. 2, Fig. 3). The identification of baculoviruses in these samples indicates that the viruses are present in natural populations of this insect, although the origin of viruses remains unclear.

*S. litura* larvae have been shown to be susceptible and permissive for GV [16], [20] and NPV [17], [21]. SpltGV has been reported to cause a persistent sublethal infection in laboratory populations of *S. litura* [20]. The offspring mortality in survivors increased up to three subsequent generations. A significant reduction in fecundity of moths emerging from treated larvae was also noticed. SpltNPV has also been reported to cause covert infections in populations of laboratory stock and field-collected *S. litura* [22]. The prevalence of covert infections in the laboratory stock (20.0%) was considerably lower than that found in the field-collected samples (22.6%). According to the above mentioned study, the prevalence of covert SpltNPV infection in field-collected populations is considerably higher than the 1.8% prevalence reported in this study. It is clear that the SpltNPV was prevalent in natural populations of *S. litura*, although the prevalence of infection was low. The results from the present study confirm that the covert infections of baculoviruses can occur as low-level persistent infections in apparently healthy populations.

Although PCR-based methods can be used to screen for the presence of covert baculovirus infections, it has less utility for ultrastructural studies of baculoviruses. The present study could also provide information on the covert infections of baculoviruses in the field-collected *S. litura* larvae by electron microscopy. As in most GV and NPV infections in lepidopterans, the main tissues infected were the fat body tissues [23]-[29]. For transmission electron microscopy, fat body tissues were collected in parallel from the same larvae that were used to extract DNA samples. Observations of tissue sections from PCR-positive samples were made, and positive agreement between PCR and electron microscopy was evident. Representative electron micrographs of fat body cells infected with GV shown in Fig. 5 revealed high levels of virus infection. The cytopathological study showed that at the time of tissue sampling numerous granules were seen in the host fat body cells, and the integrity of the nuclear membrane was lost during the replication process. Many cells with ruptured nuclear membrane were observed and granules were found in the cytoplasm. Electron microscopy confirmed typical GV morphology with ovocylindrical granules, each granule contained a single rod-shaped virion. In the case of NPV, the infected cells exhibited the typical NPV infection. Nucleocapsids and virions were localized in the regions of the enlarged nuclei that were tightly packed with OBs. Deposition of a crystalline polyhedrin matrix around and between a number of bundles resulted in the formation of an OB

containing multiple virions. The infected nuclei of infected fat body cells with intact nuclear membrane contained large number of NPV OBs. There was no evidence of OBs in the cytoplasm. In addition, electron microscopy supported the PCR findings and showed that the covert baculovirus infections identified in this study involved in a single infection. Mixed infection by GV and NPV was not observed. Although mixed infection by GV and NPV has been reported in *Hyphantria cunea* when inoculated simultaneously with GV and NPV [30]. The two viruses were found in adjacent cells in the fat body tissues but there was no infection with both viruses within the same cell. At present there is no evidence that spontaneous mixed infections with GV and NPV occur in natural insect populations. Thus, it may be almost impossible to find evidence of mixed virus infections from a single naturally-infected larva.

Although SpltGV and SpltNPV exhibited a similar gross pathology in infected larvae, examinations of sectioned tissues with the electron microscope showed that they differed in their morphological and pathological characteristics. The different characteristics between SpltGV and SpltNPV identified in this study can be summarized as follows: (i) the number of nucleocapsids in an envelope. GV has one nucleocapsid per envelope, whereas NPV has one to many nucleocapsids per envelope; (ii) the number of virions in an OB. Each GV OB contains a single virion, whereas OB of NPV contains many virions; (iii) the shape of the OB. GV produces ovocylindrical OB, whereas NPV produces polyhedral OB; (iv) the size of the OB. GV OB is smaller than the NPV OB; (v) the crystalline matrix of the OB. The major component of the GV OB is granulin, whereas the matrix protein of the NPV OB is polyhedrin (as identified by PCR followed by restriction endonuclease digestion of the PCR products); and (vi) accumulation of OBs in the infected cells. GV OBs can be found in both the nucleus and cytoplasm, whereas NPV OBs can be found only in the nucleus.

The persistent infections can be triggered into the lethal overt state by various environmental stimuli, parasitization and infection by a second pathogen [11], [18]. As infection by a second pathogen represents a major threat to host survival. Several reports have described the activation of persistent baculovirus into overt infection in the presence of a second baculovirus [8], [12], [22], [31], [32]. Challenging the persistently infected larvae with either the closely related baculovirus, or the distantly related baculovirus resulted in the persistent virus being triggered into an overt infection in most cases [33]. Baculoviruses have been found to be reactivated from a covert state to fully lethal forms in both laboratory colonies [32] and field-collected populations [31]. In this study, however, the activation of covert infections was not likely to be caused by a second baculovirus or superinfection. The larvae were not treated with baculoviruses. The overt infections occurred spontaneously and the disease developed rapidly after maintaining the field-collected *S. litura* for seven days in the laboratory. This demonstrates that the activation of

persistent viruses into overt infections was not simply due to the larvae have ingested another baculovirus. Thus, environmental stimuli and stressors would be expected to be the important role. Many studies have shown that the activation of persistent infection may be triggered by larval rearing conditions such as overcrowding, high humidity, dietary changes and fluctuations in temperature [34]-[36]. In the case of this study, overcrowding during the rearing period may not be the cause of stress since the field-collected *S. litura* larvae were reared individually. It appears that the activation of persistent infection is dependent on other types of stress. Changing the food constitutes is one of the common types of stress. The stress induced in an insect by plant chemicals may lead to greater susceptibility to disease [35]. There is evidence that the virus infection can be activated to produce lethal disease when *Pieris brassicae cheiranthi* was fed on cabbage instead of its normal food, nasturtium (*Tropaeolum majus*) [36]. In the wild, *S. litura* larvae consume a wide variety of crops. Thus, replacing their natural food plants with mung bean-based artificial diet [15] for laboratory rearing may cause stress in the larvae. However, detailed laboratory and field investigations are necessary to understand more fully the interactions between insects and plants.

One main source of stress is a change in the larval environmental temperature. Temperature change is likely to influence and cause stress that activates the covert infection to an overt form in insect [37]. Air temperature in the field where the larvae were collected was about 40 °C during daytime (in March 2017), and those larvae were then subsequently maintained in the laboratory at 27°C. The difference in temperature between field at the time of collection and laboratory was as high as 13°C. Under these circumstances, laboratory conditions imposed on *S. litura* larvae collected in the field could have caused stress, resulting in activation of the the persistent baculoviruses into an overt infection. Another possible cause of stress may be larval rearing containers. *S. litura* is a serious crop pest with a strong migratory ability. Its populations increase in large numbers and move across fields like an army [38]. When larvae were reared in petri dishes, they had very limited space to move, possibly causing them to be stressed out.

Activation of baculovirus infections by various stressors has been described and documented in several insect-virus systems [11], [18]. However, the results of this study suggest that the persistent baculoviruses in the field-collected *S. litura* larvae may be triggered by different stressors, including changes in nutrient, changes in temperature and space limitation of larval rearing containers. Further experiments are needed to determine whether covert baculovirus infections can be triggered either directly by these stressors or indirectly through the association with other factors.

## V. CONCLUSION

The present study provides evidence for covert infection of *S. litura* by SpltGV and SpltNPV. The transition from covert to overt infections occurred

spontaneously in the absence of any other baculovirus infections after maintaining the field-collected larvae in the laboratory. Thus, positive identification of SpltGV and SpltNPV in this insect provides strong support for the possibility of the spontaneous outbreaks of baculovirus disease that occurred in laboratory insect colonies. Molecular screening of the field-collected populations of *S. litura* can help prevent the collapse of the insect population resulting from covert baculovirus infections.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Sudawan Chaeychomsri, Win Chaeychomsri, and Jindawan Siruntawinetti conceived and designed the experiments; Sudawan Chaeychomsri, Win Chaeychomsri, and Jindawan Siruntawinetti conducted the research; Sudawan Chaeychomsri and Win Chaeychomsri analyzed the data; Sudawan Chaeychomsri wrote the paper with support from Win Chaeychomsri and Jindawan Siruntawinetti; all authors had approved the final version.

## ACKNOWLEDGMENT

This work was supported by Kasetsart University Research and Development Institute, Kasetsart University, Thailand.

## REFERENCES

- [1] E. A. Herniou, *et al.*, "Family baculoviridae," in *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*, A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, Eds., San Diego: Elsevier Academic Press, 2012, pp. 163-173.
- [2] R. L. Harrison, *et al.*, "ICTV virus taxonomy profile: Baculoviridae," *J. Gen. Virol.*, vol. 99, pp. 1185-1186, Sep. 2018.
- [3] P. M. Zanutto, B. D. Kessing, and J. E. Maruniak, "Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations," *J. Invertebr. Pathol.*, vol. 62, pp. 147-164, Sep. 1993.
- [4] A. Laarif, A. B. Ammar, M. Trabelsi, and M. H. B. Hamouda, "Histopathology and morphogenesis of the granulovirus of the potato tuber moth *Phthorimaea operculella*," *Tunis. J. Plant Prot.*, vol. 1, pp. 115-124, Dec. 2006.
- [5] B. A. Federici, "Baculovirus pathogenesis," in *The Baculoviruses*, L. K. Miller, Ed., New York: Plenum Press, 1997, pp. 33-59.
- [6] R. Harrison and K. Hoover, "Baculoviruses and other occluded insect viruses," in *Insect Pathology*, F. E. Vega and H. K. Kaya, Eds., San Diego: Academic Press, 2012, pp. 73-132.
- [7] L. K. Etzel and E. F. Legner, "Culture and colonization," in *Handbook of Biological Control: Principles and Applications*, T. S. Bellows and T. W. Fisher, Eds., San Diego: Academic Press, 1999, pp. 125-197.
- [8] J. P. Burden, C. P. Nixon, A. E. Hodgkinson, R. D. Possee, S. M. Sait, L. A. King, and R. S. Hails, "Covert infections as a mechanism for long-term persistence of baculoviruses," *Ecol. Lett.*, vol. 6, pp. 524-531, June 2003.
- [9] L. Vilaplana, K. Wilson, E. M. Redman, and J. S. Cory, "Pathogen persistence in migratory insects: High levels of vertically-transmitted virus infection in field populations of the African armyworm," *Evol. Ecol.*, vol. 24, pp. 147-160, Jan. 2010.
- [10] A. E. Hatem, H. K. Aldebis, and E. V. Osuna, "Effects of the *Spodoptera littoralis* granulovirus on the development and

- reproduction of cotton leafworm *S. littoralis*," *Biol. Control*, vol. 59, pp. 192-199, Nov. 2011.
- [11] T. Williams, C. Virto, R. Murillo, and P. Caballero, "Covert infection of insects by baculoviruses," *Front. Microbiol.*, vol. 8, p. 1337, July 2017.
- [12] A. Larem, S. B. Tiba, E. Fritsch, K. Undorf-Spahn, J. T. Wennmann, and J. A. Jehle, "Effects of a covert infection with *Phthorimaea operculella* granulovirus in insect populations of *Phthorimaea operculella*," *Viruses*, vol. 11, p. 337, April 2019.
- [13] H. Warburton, U. Ketunuti, and D. Grzywacz, "A survey of the supply, production and use of microbial pesticides in Thailand," NRI report 2723, Natural Resources Institute, University of Greenwich, Chatham, UK, 2002.
- [14] J. Eilenberg, J. M. Vlask, C. Nielsen-LeRoux, S. Cappellozza, and A. B. Jensen, "Diseases in insects produced for food and feed," *J. Insects as Food Feed*, vol. 1, pp. 87-102, Feb. 2015.
- [15] M. Abdullah, O. Sarnthoy, and S. Chaeychomsri, "Comparative study of artificial diet and soybean leaves on growth, development and fecundity of beet armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae)," *Kasetsart J. (Nat. Sci.)*, vol. 34, pp. 339-344, July 2000.
- [16] Y. Wang, J. Y. Choi, J. Y. Roh, S. D. Woo, B. R. Jin, and Y. H. Je, "Molecular and phylogenetic characterization of *Spodoptera litura* granulovirus," *J. Microbiol.*, vol. 46, pp. 704-708, Dec. 2008.
- [17] Y. Pang, et al., "Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome," *Virology*, vol. 287, pp. 391-404, 2001.
- [18] J. S. Cory and J. H. Myers, "The ecology and evolution of insect baculoviruses," *Annu. Rev. Ecol. Evol. Syst.*, vol. 34, pp. 239-272, July 2003.
- [19] C. M. Thorne, I. S. Otvos, N. Conder, and D. B. Levin, "Development and evaluation of methods to detect nucleopolyhedroviruses in larvae of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough)," *Appl. Environ. Microbiol.*, vol. 73, pp. 1101-1106, Feb. 2007.
- [20] R. K. Gupta, M. Amin, K. Bali, M. Monobrullah, and P. Jasrotia, "Vertical transmission of sublethal granulovirus infection in the tobacco caterpillar *Spodoptera litura*," *Phytoparasitica*, vol. 38, pp. 209-216, July 2010.
- [21] S. D. Woo, Y. H. Je, and B. R. Jin, "Characterization of the polyhedrin gene of *Spodoptera litura* nucleopolyhedrovirus isolated in Korea," *J. Asia Pac. Entomol.*, vol. 8, pp. 257-262, Sep. 2005.
- [22] L. N. Kouassi, K. Tsuda, C. Goto, S. Mukawa, Y. Sakamaki, K. Kusigemati, and M. Nakamura, "Prevalence of latent virus in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) and its activation by a heterologous virus," *Appl. Entomol. Zool.*, vol. 44, pp. 95-102, Feb. 2009.
- [23] M. D. Summers and J. D. Paschke, "Alkali-liberated granulosis virus of *Trichoplusia ni* I. Density gradient purification of virus components and some of their in vitro chemical and physical properties," *J. Invertebr. Pathol.*, vol. 16, pp. 227-240, Sep. 1970.
- [24] R. Wäger and G. Benz, "Histochemical studies on nucleic acid metabolism in granulosis-infected *Carpocapsa pomonella*," *J. Invertebr. Pathol.*, vol. 17, pp. 107-115, Jan. 1971.
- [25] A. Sciocco-Cap, A. D. Parola, A. V. Goldberg, P. D. Ghiringhelli, and V. Romanowski, "Characterization of a granulovirus isolated from *Epinotia aporema* Wals. (Lepidoptera: Tortricidae) larvae," *Appl. Environ. Microbiol.*, vol. 67, pp. 3702-3706, Aug. 2001.
- [26] A. V. Goldberg, V. Romanowski, B. A. Federici, and A. Sciocco-Cap, "Effects of the Epap granulovirus on its host, *Epinotia aporema* (Lepidoptera: Tortricidae)," *J. Invertebr. Pathol.*, vol. 80, pp. 148-159, July 2002.
- [27] G. Feng, Q. Yu, C. Hu, Y. Wang, G. Yuan, Q. Chen, K. Yang, and Y. Pang, "Apoptosis is induced in the haemolymph and fat body of *Spodoptera exigua* larvae upon oral inoculation with *Spodoptera litura* nucleopolyhedrovirus," *J. Gen. Virol.*, vol. 88, pp. 2185-2193, Aug. 2007.
- [28] L. A. Lacey, D. F. Hoffmann, and B. A. Federici, "Histopathology and effect on development of the PhopGV on larvae of the potato tuber moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae)," *J. Invertebr. Pathol.*, vol. 108, pp. 52-55, Sep. 2011.
- [29] M. T. Asri, S. R. C. Sy, B. T. Rahardjo, and S. B. Sumitro, "The spreading of *Spodoptera litura* multiple nucleopolyhedrovirus in midgut of third instar of *Spodoptera litura* larvae," *IOSR J. Agric. Vet. Sci.*, vol. 3, pp. 40-43, Jun. 2013.
- [30] H. Watanabe and M. Kobayashi, "Histopathology of a granulosis in the larva of the fall webworm, *Hyphantria cunea*," *J. Invertebr. Pathol.*, vol. 16, pp. 71-79, July 1970.
- [31] D. Cooper, J. S. Cory, D. A. Theilmann, and J. H. Myers, "Nucleopolyhedroviruses of forest and western tent caterpillars: Cross-infectivity and evidence for activation of latent virus in high-density field populations," *Ecol. Entomol.*, vol. 28, pp. 41-50, Feb. 2003.
- [32] R. Murillo, M. S. Hussey, and R. D. Possee, "Evidence for covert baculovirus infections in a *Spodoptera exigua* laboratory culture," *J. Gen. Virol.*, vol. 92, pp. 1061-1070, May 2011.
- [33] D. S. Hughes, R. D. Possee, and L. A. King, "Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects," *Virology*, vol. 194, pp. 608-615, June 1993.
- [34] J. R. Fuxa, J. Z. Sun, E. H. Weidner, and L. R. LaMotte, "Stressors and rearing diseases of *Trichoplusia ni*: Evidence of vertical transmission of NPV and CPV," *J. Invertebr. Pathol.*, vol. 74, pp. 149-155, Sep. 1999.
- [35] J. S. Cory, R. S. Hails, and S. M. Sait, "Baculovirus ecology," in *The Baculoviruses*, L. K. Miller, Ed., New York: Plenum Press, 1997, pp. 301-339.
- [36] W. A. L. David and B. O. C. Gardiner, "Breeding *Pieris brassicae* apparently free from granulosis virus," *J. Invertebr. Pathol.*, vol. 8, pp. 325-333, Sep. 1966.
- [37] B. Okamura, "Hidden infections and changing environments," *Integr. Comp. Biol.*, vol. 56, pp. 620-629, Apr. 2016.
- [38] M. Ahmad, M. I. Arif, and M. Ahmad, "Occurrence of insecticide resistance in field populations of *Spodoptera litura* (Lepidoptera: Noctuidae) in Pakistan," *Crop Prot.*, vol. 26, pp. 809-817, June 2007.

Copyright © 2020 by the authors. This is an open access article distributed under the Creative Commons Attribution License ([CC BY-NC-ND 4.0](https://creativecommons.org/licenses/by-nc-nd/4.0/)), which permits use, distribution and reproduction in any medium, provided that the article is properly cited, the use is non-commercial and no modifications or adaptations are made.



**Sudawan Chaeychomsri** received Doctoral Degree in Agricultural Science (Insect Virus Molecular Biology) from Nagoya University, Japan. Her current research interests focus on insect cell culture and insect virus molecular biology.



**Win Chaeychomsri** received Doctoral Degree in Agricultural Biotechnology (Animal Biotechnology) from Kasetsart University, Thailand. He is an outstanding lecturer in research and innovation. His current research interests focus on parasitology, immunology and animal biotechnology.



**Jindawan Siruntawineti** received Doctoral Degree in Agricultural Science from University of Tsukuba, Japan. Her current research interests focus on applied biochemistry, cell and molecular biology, immunology, and animal physiology.