Investigation on Pathological Aspects, Mode of Transmission, and Tissue Tropism of *Antheraea proylei*

Nucleopolyhedrovirus Infecting Oak Tasar Silkworm

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Received 7 May 2022; Editorial decision 15 August 2022.

Abstract

The temperate oak tasar silkworm, *Antheraea proylei*, is frequently infested with *Antheraea proylei* nucleopolyhedrovirus (AnprNPV) causing tiger band disease. This disease is one of the key factors that obstructs production and productivity of oak tasar sericulture. The current study aimed to investigate the pathogenicity of AnprNPV, its mode of transmission, and detection of AnprNPV in different tissues. Transmission electron micrographs of AnprNPV showed single rod-shaped bodies and occlusion derived virus (ODV) enclosed within multiple envelopes. The infecting AnprNPV displayed tissue tropism with higher copy numbers detected in the insect fat body and ovary. The virus was observed to multiply in all developmental stages of the silkworm such as egg, larva, pupa, and moth, confirming its ability to spread throughout the silkworm lifecycle. Baculovirus isolated from infected *A. proylei* showed cross-infectivity in other Saturniidae wild silkworm species such as *Antheraea pernyi*, *A. frithi*, and *Samia ricini*, widening their probable host range for infection. Baculoviruses generally display a horizontal mode of transmission, mainly through ingestion of occlusion bodies (OBs); however, the present study revealed a trans-ovum vertical mode of transmission in addition to a horizontal mode. The observations made in this study aid a detailed understanding of the tiger band disease and its causative pathogen AnprNPV, which will support future studies and disease management in oak tasar sericulture.

Key words: Tiger band disease, oak tasar silkworm, vertical transmission, tissue tropism, sericulture

The oak tasar silkworm, *Antheraea proylei* Jolly (Lepidoptera: Saturniidae), is one of the most economically important and commercially exploited wild silkworm species in India. *A. proylei* is frequently infested with *Antheraea proylei* nucleopolyhedrovirus (AnprNPV) causing tiger band disease. This disease is one of the key factors that obstructs production and productivity of oak tasar sericulture. The current study aimed to investigate the pathogenicity of AnprNPV, its mode of transmission, and detection of AnprNPV in different tissues. Transmission electron micrographs of AnprNPV showed single rod-shaped bodies and occlusion derived virus (ODV) enclosed within multiple envelopes. The infecting AnprNPV displayed tissue tropism with higher copy numbers detected in the insect fat body and ovary. The virus was observed to multiply in all developmental stages of the silkworm such as egg, larva, pupa, and moth, confirming its ability to spread throughout the silkworm lifecycle. Baculovirus isolated from infected *A. proylei* showed cross-infectivity in other Saturniidae wild silkworm species such as *Antheraea pernyi*, *A. frithi*, and *Samia ricini*, widening their probable host range for infection. Baculoviruses generally display a horizontal mode of transmission, mainly through ingestion of occlusion bodies (OBs); however, the present study revealed a trans-ovum vertical mode of transmission in addition to a horizontal mode. The observations made in this study aid a detailed understanding of the tiger band disease and its causative pathogen AnprNPV, which will support future studies and disease management in oak tasar sericulture.

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common and dreadful diseases of oak tasar silkworms and causes severe losses to the sericulture industry. Variations in temperature and high humidity are the main predisposing factors for the disease in A. proylei larvae (Berggren and Kading 2018). The extent of damage due to tiger band disease was found to be approximately 70–80% of A. proylei larval and pupal mortality (Shantibala et al. 2018). Tiger band diseased larvae show poor growth and development along with loss of appetite. The disease is characterized by the appearance of black bands/stripes across the body of the silkworm (hence, the name tiger band disease, Supp Fig. 1 [online only]) (Aragão-Silva et al. 2016). Other symptoms include shrinkage and softening of the body eventually leading to death (Shantibala et al. 2018). Field observations indicate that the disease is primarily transmitted horizontally through contaminated leaves and rearing appliances.

The causal organism of tiger band disease has been identified as A. proylei nucleopolyhedrovirus (AnprNPV), which belongs to Alphabaculovirus in the Baculoviridae family (Shantibala et al. 2018). Baculoviruses are insect specific occluded viruses with rod shaped nucleocapsids. Transmission of baculoviruses occurs through the oral or ‘per os’ route when insects consume occlusion bodies (OBs) through food. Food particles contaminated with OBs enter the insect midgut where they are exposed to higher alkaline pH (pH 10–11). The alkalinity of the insect midgut triggers the dissolution of OBs and the release of occlusion derived virions (ODVs) into the midgut lumen. These released virus particles are defined as ODVs (Slack and Arif 2006). It was reported that ODVs are released from OBs within 12 min post entry into the insect midgut (Adams and McClintock 1991). Genetically, these baculoviruses have a double stranded super coiled circular DNA genome with a molecular size ranging from 80 to 180 kb (Shantibala et al. 2018). Based on the phylogenetic information of 29 core genes, biological and morphological characteristics, the family Baculoviridae were subdivided into four genera: Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses), Betabaculovirus (lepidopteran-specific granuloviruses), Gammabaculovirus (hymenopteran-specific nucleopolyhedroviruses) and Deltabaculovirus (Diptera-specific baculoviruses) (Jehele et al. 2006). Over 600 species of insects have been reported to be infected with baculoviruses, including those belonging to the insect orders Lepidoptera, Hymenoptera, and Diptera (Berggren and Kading 2018). The genomes of AnprNPV (Accession no LC375539) and other baculoviruses (Accession no LC375537, LC375538, and DQ486030) infecting Saturniidae silk moths share a high degree of similarity, implying they have evolved from a common ancestry origin. However, a detailed comparative genomics of baculoviruses is necessary to understand the evolution of genes involved primarily in replication, transcription, and other structural protein genes (Hill and Unckless 2017).

With this background, the current study was designed to investigate AnprNPV pathogenicity, mode of transmission, and tissue tropism during infection of A. proylei. Enhanced knowledge on the disease would help us to design appropriate disease control strategies for improving silk production which will lead to increased remuneration of the tribal community associated with the oak tasar silk industry.

Materials and Methods

Virus Studies

Collection of Oak Tasar Silkworms

Fifth instar A. proylei larvae infected with AnprNPV were collected from the sericulture fields of Regional Sericulture Research Station (RSRS), Imphal, Manipur, India, during one autumn season (October, 2018). A total of 500 infected larvae and 500 healthy larvae were collected for the study. Infected larvae were characterized by dark tiger-like stripes across the body of the silkworm, while healthy silkworms did not show any disease symptoms. The sampling site is located in extreme eastern India and has a distinct subtropical climate characterized by hot, humid summers and dry winters. During the autumn season, the minimum temperature was 20.4°C and the maximum temperature was 34.9°C, while the humidity range was 55–85%.

Isolation of Viral Polyhedra

Viral polyhedral bodies were isolated from the infected oak tasar silkworms using a method previously described (Shantibala et al. 2018). In brief, the infected silkworm samples were placed in dark amber colored bottles with distilled water and allowed to putrefy for 15 d. The putrefied samples were treated with ampicillin and chloramphenicol at a conc. of 50 μg/ml before further processing. The treated cadavers were filtered through cheese cloth and subjected to centrifugation at 100 g for 2 min to remove floating debris. The supernatant was collected and further centrifuged at 4,000 g for 15 min to pellet polyhedra. The pellet was washed thrice at room temperature with 0.1% SDS to remove any floating debris. Subsequent centrifugations were performed at 4,000 g for 10 min. The concentration of the purified viral polyhedra was determined using a hemocytometer.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to examine the structure and morphology of virions as per the protocol of Kumar et al. 2011. The purified viral polyhedra/OB sample was pelleted by centrifugation at 5,000 g for 5 min, resuspended with 3% glutaraldehyde, and fixed overnight at room temperature. The samples were concentrated in 1 ml of 0.1M cacodylate buffer and post-fixed with 2% osmium tetroxide (OsO4) for 2 hr at 4°C. Samples were dehydrated using a series of graded ethanol from 70% to 100% and stained for 1 hr with 2% uranyl acetate. The samples were washed with propylene oxide to remove residual ethanol from dehydration. The samples were further embedded in araldite resin and polymerized for 48 hr at 60°C. After polymerization, samples were sectioned, transferred to TEM grids, and stained with lead acetate. The resulting grids were observed under a transmission electron microscope at 80 kV (JEOL JEM 1400 plus, JEOL Ltd., Tokyo, Japan).

Pathogenicity and Mode of Transmission of AnprNPV

Infection Studies with AnprNPV

Lethal Concentration 50 (LC50) of AnprNPV in A. proylei was determined. In brief, 100 µl of concentrations of AnprNPV polyhedral suspensions ranging from 2.2 × 102 OB/ml to 2.2 × 108 OB/ml were smeared on a disc of 4 cm diameter of Q. serrata leaves (the primary food plant of A. proylei) and air dried (Reeta et al. 2008). Before conducting the experiment, Q. serrata leaves were surface sterilized with 70% alcohol to remove any microbial contamination. Three replicates of 30 third instar A. proylei larvae (collected from RSRS, Imphal) were provided food on the air-dried virus smeared leaves. As controls, A. proylei larvae were fed with Q. serrata leaves with the same volume of sterile water. On complete feeding of the leaf disc, the larvae were transferred to fresh Q. serrata twigs inserted in bottles and reared under laboratory conditions. The larvae were reared at 24°C and 70–75% relative humidity under a 16:8 light: dark regime. Larval mortality was recorded daily and the LC50...
value of AnprNPV in *A. proylei* was determined by Probit analysis with Finney’s Probit analysis spreadsheet calculator (https://probitanalysis.wordpress.com/).

**Source of Infection and Surface Disinfection**

To determine the source of infection of AnprNPV, female *A. proylei* moths collected from RSRS, Manipur, India were reared indoors. The mortality of the silkworms was observed and recorded on different days post-infection. The cultures were maintained appropriately by routine feeding and cleaning on a daily basis to remove the excreta. The larvae were reared at 24°C and 70–75% relative humidity under a 16:8 light:dark regime.

**Color-Infection of AnprNPV**

The cross-infectivity of AnprNPV in other wild silkworms was studied by inoculating 3rd instar silkworm (3 replicates; 30 larvae per replication of each spp.) of *S. ricini*, *A. peryyi* and *A. frithi* with AnprNPV. Various concentrations of 100 µl of AnprNPV polyhedral suspensions (2.2 × 10⁸ OB/ml–2.2 × 10² OB/ml) were prepared and applied on leaves which were air dried before being fed to the silkworms. For a control setup, virus un-inoculated 3rd instar silkworm of each spp. (*S. ricini* [Lepidoptera: Saturniidae], *A. peryyi*, and *A. frithi* [Lepidoptera: Saturniidae]) were reared on *Q. serrata* leaves. The silkworms were transferred to twigs inserted in bottles and reared indoors. The mortality of the silkworms was observed and recorded on different days post-infection. The cultures were maintained appropriately by routine feeding and cleaning on a daily basis to remove the excreta. The larvae were reared at 24°C and 70–75% relative humidity under a 16:8 light:dark regime.

**RNA Extraction and cDNA Synthesis**

RNA was extracted from the tissues (midgut, fat body, trachea, Malpighian tubules, ovary, testis, pupa, egg inner content, moth, and silk gland) and different development stages (pupa, infected larvae, moth, egg surface) of infected *A. proylei* collected from sericulture fields. The RNA was extracted using Trizol (RNAiso plus, DSS Takara Bio India Pvt. Ltd., New Delhi, India) using a method previously described (Esvaran et al. 2019) followed by reverse transcription as per the manufacturer’s protocol (PrimeScript 1st strand cDNA synthesis kit, DSS Takara Bio India Pvt. Ltd., New Delhi, India) to generate cDNA. The cDNA was diluted 10-fold and used for normal PCR conventional analysis as well as quantitative Real time PCR (qRT-PCR) analysis.

**Detection AnprNPV Through PCR**

A PCR reaction was carried out to detect AnprNPV in viral infected tissues from different developmental stages of silkworm. The primers were designed based on the whole genome sequence information of AnprNPV (GenBank accession no. LC375539.1). In view of its consistent amplification and being indicated as a gene specific to NPV (Shantibala et al. 2018), the Anpr53 (*he65-like*) gene segment was selected for identification and viral quantification through PCR and qPCR. The Anpr53 gene product, P87 is found to be an integral part of viral capsid associated proteins. This gene family consists of
several nucleopolyhedrovirus capsid protein sequences. The Anpr53 (hek65-like) gene is expressed late in infection and concentrated in infected cell nuclei. BLAST analyses of Anpr53 indicate a 100% homology with NPVs infecting other wild silk moths such as A. pernyi, Samia cynthia (Lepidoptera: Saturniidae), and A. yamamai (Lepidoptera: Saturniidae).

The primers used in this study are listed in Supp Table 1 (online only). The PCR reaction mixture comprised a total volume of 10 μl composed of 1 μl of genomic DNA 0.1 μM each of forward and reverse primers, 5 μl of 2X PCR buffer, EmeraldMaster Mix (EmeraldAmp GT PCR Master Mix, DSS Takara Bio India Pvt. Ltd., New Delhi, India). The samples were subjected to the following thermal profile for amplification: 2 min at 94°C, followed by 30 cycles of [30 s at 94°C, 30 s at 55°C, and 1 min at 72°C] in a BioRad T100 Thermal cycler (Bio-Rad Laboratories India Pvt. Ltd., Gurugram, Haryana). The PCR products were resolved on a 1.2% agarose gel stained with ethidium bromide for visualization of the amplified product.

Real Time qPCR
To quantify the AnprNPV virus from infected A. proylei samples, real time PCR was carried out on extracts as described previously from the different tissues sampled. DNA amplification was carried out using the specifically designed primer (Anpr53) along with a non-template control. The PCR reaction mixture comprised of a total volume of 10 μl with 1 μl of the diluted cDNA, 0.1 μM each of forward and reverse primers, and 5 μl of SYBR Premix Ex Taq II (TliRnaseH Plus, DSS Takara Bio India Pvt. Ltd., New Delhi, India). The samples were subjected to the following thermal profile for amplification: 2 min at 94°C, followed by 30 cycles of [30 s at 94°C, 30 s at 57°C, and 1 min at 72°C] in an Agilent Technologies Stratagene Mx3000P real time PCR machine (Agilent, Santa Clara, CA, USA) with each sample tested in triplicate, and the mean values were used for determining the viral copy number. The unknown samples were compared against a standard curve generated by serial dilutions of the Anpr53 gene cloned into a pJET plasmid (CloneJET PCR Cloning Kit, Thermo Fisher Scientific, Waltham, MA) using the manufacturer’s instructions for the determination of copy numbers.

Results
Transmission Electron Microscopy (TEM)
The TEM image showed the presence of occlusion derived viruses (ODVs). Further, both single and multiple rod-shaped occlusion derived virus (ODV) embedded in a single polyhedral OB was observed. This confirms that the virus purified from infected A. proylei silkworms are ODVs (Fig. 1). The presence of circular and rod-shaped occlusion bodies with single and multiple nucleocapsids per envelope seen in the TEM image indicates that AnprNPV OBs spanned approximately 1.16 × 1.06 μm in size.

Infecitivity, Surface Disinfection, and Vertical Transmission of AnprNPV
The infectivity of the virus was tested by inoculating disease-free 3rd instar A. proylei silkworm with AnprNPV virus isolated from diseased silkworms and recording the number of days of survival and gradual progression up to death of the larvae under different dosages. As expected, larval mortality increased with an increase in the concentration of AnprNPV. The larval mortality after infection was observed to start from day 6, which gradually increased to 100% mortality on day 12. The LC50 value of AnprNPV in A. proylei was estimated to be 4.8 × 10^4 at 95% confidence intervals (R^2 > 0.95, Supp Table 2 [online only], supplementary information). The lower and upper bound LC50 values of AnprNPV in A. proylei were estimated to be 1.1 × 10^2 and 2.0 × 10^3, respectively (Supp Table 2 [online only]). When checked with PCR, DNA from the surface disinfected eggs did not show the presence of AnprNPV, while amplification of virus was observed in non-disinfected egg samples (Fig. 2). Further, the hatched larvae from the non-disinfected sample also displayed the presence of virus.

Tissue Tropism
The presence of AnprNPV in infected A. proylei tissues was tested through PCR amplification. Infected tissues such as midgut, fat body, trachea, Malpighian tubules, ovary, testis, and silk gland showed positive amplification for AnprNPV (Fig. 3). The presence of virus was also noted in various development stages of infected A. proylei such as pupa and moth (Fig. 3). The viral copy number in each of these tissues and developmental stages was quantified using qPCR (Fig. 4). The data showed the presence of the virus and a higher copy number in the fat body (2.34 × 10^11) and ovary (2.07 × 10^11) in comparison with other tissues from the infected silkworm, indicating higher virus propagation in these tissues.

Cross-Infection of AnprNPV
Larvae of S. ricini, A. pernyi, and A. frithi died after exposure to the virus, indicating their susceptibility to AnprNPV infection. The mortality of A. pernyi and A. frithi was observed within 144 h post infection (hpi), while S. ricini mortality was observed within 120 hpi. The LC50 values of AnprNPV in S. ricini, A. pernyi and A. frithi were 3.1 × 10^4, 5.4 × 10^4, and 6.1 × 10^4, respectively (Supp Table 2 [online only]). This corroborated the molecular detection results whereby the presence of AnprNPV was found in Saturniidae silkworms such as S. ricini, A. pernyi, and A. frithi (Fig. 5). Cross infection studies confirmed that AnprNPV can infect other wild silkworms of the Saturniidae family, specifically the silkworm species used in this study.
Discussion

*A. proylei* is one of the most prominent, viable, and commercially exploited silkworm species in India. Since it is reared outdoors, it is frequently infested with different pathogens, one of them being AnprNPV, causing tiger band disease (Shantibala et al. 2018). In the present study, TEM cross-sections revealed the OBs of AnprNPV to be approximately within the size reported in other Alphabaculovirus OBs (Harrison et al. 2018).

Unlike other *Alphabaculoviruses*, lepidopteran NPVs exhibit distinctive tissue tropism in their host insects. The majority of insect NPVs replicate only in the midgut, which is the first target tissue during oral infection. Lepidopteran NPVs, however, establish a transient infection in the midgut before affecting most of the other larval tissues (Katsuma et al. 2012). Similar kinds of observations were found with AnprNPV infection in *A. proylei*. Our RT-qPCR results showed higher replication of AnprNPV in the fat body and ovary followed by trachea (Fig. 4). Viral multiplication was also found in midgut and Malpighian tubules. These results are in support of Katsuma et al. 2012 where poor replication was reported in silk glands, midgut, and Malpighian tubule tissues of NPV infected *Bombyx mori* L. (Lepidoptera: Bombycidae) larvae. Nevertheless, the molecular mechanism involving tissue tropism of baculovirus is largely unknown and may vary between different organisms (Katsuma et al. 2012).

The results of tissue tropism indicate a systemic progression of AnprNPV infection in *A. proylei* larvae. Baculoviruses generally show a biphasic infection with two distinct phenotypes such as occlusion-derived virus (ODV) and budded virus (BV) (Jiang et al. 2021). Primary infection is caused by ODV in the midgut, while the secondary infection is caused by BV, causing systemic spread all over the host (Jiang et al. 2021). Further, AnprNPV was present in all the developmental stages of the silkworm indicating its ability to thrive and propagate throughout all these stages.

Surface disinfection is a commonly used method in the sericulture to remove surface contaminants, which ensures that newly hatched larvae are free from infection. Hence, 0.2% sodium hypochlorite (a common disinfectant) treatment was tested for eggs laid by an AnprNPV-infected mother moth. The surface disinfection with 0.2% sodium hypochlorite solution was found to be effective in removing viral particles from the surface-contaminated eggs. Further, the inner contents from the surface-treated egg samples also did not display the presence of any virus indicating the transmission route of the virus to be trans-ovum vertical transmission in nature. The hatched larvae from the non-disinfected sample also displayed the presence of virus. This might be attributed to the presence of virus on the egg surface, which may have been ingested by the larvae during eclosion.

Baculoviruses adopt a combination of horizontal and vertical transmission strategies, depending on the density of their hosts.
An earlier report on cross-infectivity of A. pernyi nucleopolyhedrovirus showed that it caused 57% mortality in larvae of S. cynthia ricini, whereas, S. cynthia nucleopolyhedrin virus did not kill the larvae of A. pernyi (Qian et al. 2013). To test this hypothesis, bioassays were performed with AnprNPV in Saturniidae silkworms, A. pernyi, A. frithi, and S. ricini. Our experimental results provided evidence for the cross infectivity of AnprNPV in all the wild saturniid silkworms tested in the study (A. pernyi, A. frithi, and S. ricini). This suggests that AnprNPV could be a potential pathogen to the saturniid family of silkmoth, which requires much attention to develop early diagnostic and prophylactic measures to control tiger band disease in the A. proylei rearing fields. The PCR and qRT-PCR techniques used in this study can be utilized to screen field samples for early identification of the pathogen. Further research with respect to baculovirus infection of silkworms can help understand more fully which host pathogen interactions are critical, and the immune response of the host to infection.

This is a first report where the AnprNPV disease occurrence, its etiology along with transmission has been described. Based on the study data, an egg surface decontamination technique was devised which could remove the virus from the surface of the eggs. Currently, the method has been widely popularized in the field to reduce the incidence of diseases and improve the remuneration of farmers (unpublished).

**Acknowledgments**

We thank Central Silk Board, Govt. of India for providing the financial support and infrastructure facilities to carry out research. We would like to thank National Institute of Mental Health & Neurosciences Bangalore, India Common Research Facility, Department of Neuropathology for support in generating the TEM images. We are also thankful to Prof Marian R. Goldsmith, Professor Emerita of University of Rhode Island, USA for English language editing of the manuscript. We thank Department of Biotechnology (DBT), India (Grant: BT/PR15405/TDS/121/14/2015 dt 09.02.2017) and the Swedish Research Council (Grant: 2017-05463) for their support and financial assistance throughout this study.

**Author Contributions**

DK: Concept/ Experimentation/ Manuscript writing; SS: Concept/ Experimentation; GS: Concept/ Experimentation/ Manuscript writing; JK: Data analysis/Scientific Inputs; VS: Scientific Inputs; OT: Concept/ Scientific Inputs/ Experimental design/ Data analysis; KMP: Concept/ Technical advice/ Scientific inputs/Corrections of the Manuscript.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

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