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Identification and Characterization of a New Nucleopolyhedrovirus Strain of *Neodiprion zhejiangensis* Zhou & Xiao (Hymenoptera: Diprionidae)

Qinghua Wang, Yuzhu Wang, Ruizhen Wang and Yongan Zhang*

The Key Laboratory of Forest Protection, State Forestry Administration of China, Research Institute of Forest Ecology, Environment and [rotection, Chinese Academy of Forestry, Beijing, China.

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Baculovirus is an ideal pathogenic microbial pesticide for the control of pests in agriculture and forestry. This study isolated and purified a new viral strain that can infect the larvae of *Neodiprion zhejiangensis* Zhou & Xiao, named NezhNPV. Ultrastructure studies indicated that the virus is a single nucleocapsid nucleopolyhedrovirus with a diameter of 0.47-0.91 ìm. Biological activity assays showed that NezhNPV is highly virulent in the third instar larvae of *N. zhejiangensis*, with an LC_{50} of 1.15×10^4 polyhedral inclusion bodies (PIB)/ml. The LT_{50} of the virus increased with decreasing viral concentrations. When the viral concentrations were 7×10^7, 7×10^6, 7×10^5, 7×10^4, and 7×10^3 PIB/ml, the LT_{50} values were 3.70, 4.12, 4.56, 5.08, and 6.24 d, respectively. Interestingly, unlike the Hymenoptera virus, which only infects the midgut, this virus can infect various tissues of parasites, such as the fat body, trachea, and epidermal cells.

Key words: *Neodiprion zhejiangensis* Zhou & Xiao, Nucleopolyhedrovirus, biological control, ultrastructure, pathology.

The pine sawfly class (diprion) is one of the major classes of insect pests of pine forests in the world and is mainly active in Europe, North America, Japan, and China[1]. The main types of pests include *Neodiprion sertifer*, *N. lecontei*, *N. pinetum*, *European Gilpinia hercyniae*, *Diprion nanhuaensis*, *Diprion sp.*, *Pristiphora erichsonii*, and *N. zhejiangensis*[1]. In China, the pine sawfly often causes periodic outbreaks, with a population density of up to one million insects/plant, and pine needles are often consumed by the pests, which produces an appearance of fire damage and seriously affects the normal growth and development of the trees[2]. *N. zhejiangensis* causes severe damage in many regions of China. This insect reproduces rapidly, with three to four generations a year. Each female insect can lay 14-21 eggs, and parthenogenesis may occur. Overlapping generations are evident. The larvae live long and have a strong environmental adaptability. Under optimum conditions, outbreaks can easily occur once they are triggered[3]. Chemical pesticides can quickly reduce the population density in a short time, but they also cause a series of problems such as environmental pollution, which is becoming increasingly serious. The nucleocapsid nucleopolyhedrovirus for insects is a biological pesticide recommended by the World Health Organization (WHO) and the Food and Agriculture *Organization (FAO)* of the United Nations (UN) [4]. Its advantages include that it is highly specific to the host and causes no harm to humans, plants, and the natural enemies of the pests. It is one of the best biological agents for the effective and sustainable control of the pine sawfly.

Pine sawfly viruses are mainly nucleopolyhedroviruses, which can infect the epithelial cells in the midgut of larvae, resulting in

* To whom all correspondence should be addressed.
Tel.: 86-10-62889509; Fax: 86-10-62884972;
E-mail: zhangya9520@126.com

a loss of appetite of the parasites and eventually causing death[5]. The infected larvae can release the virus into the environment, resulting in the horizontal and vertical spread of the virus, which leads to insect epidemics and thereby effectively controls the parasite population over a long-term period. Currently, the *N. sertifer* nucleopolyhedrovirus (NeseNPV) and the *N. lecontei* nucleopolyhedrovirus (NeleNPV) are popular in Europe, Asia, Africa, and North America[6, 7]. Both pine sawfly viruses have been registered in the United States with the trade names of Neochek-S and Lecontvirus, respectively[8, 9]. Studies have shown that Lecontvirus is highly infective in the pine sawfly. The pests die 4-7 days after infection with the virus, and the virus can be spread within the population, thereby forming a pandemic. Li et al. reported the biological activity and sensitivity of the *N. abietis* nucleopolyhedrovirus (NeabNPV) in 2005. Their study showed that this virus is highly toxic to *N. abietis*, and based on their field tests, the third instar larvae were most sensitive to the virus[10].

In this study, a strain of nucleopolyhedrovirus was first isolated and purified from *N. zhejiangensis*. This virus is highly virulent in the third instar larvae of the sawfly. It is not only replicated in the midgut cells but also able to infect the fat body, tracheal and epidermal cells. This viral strain is expected to become an ideal biological factor to control *N. zhejiangensis*.

MATERIALS AND METHODS

Insects

The pupae of *N. zhejiangensis* were collected from the Research Institute of Forestry, Chinese Academy of Forestry, in the Haidian District of Beijing. The pupae were incubated in the laboratory under the following conditions: a temperature of 26±2°C, relative humidity of 60±10%, and a light:dark cycle of 16:8 hours until the emergence of adults. After eclosion, the adults were placed in an insectary cage (30×30×40 cm) with the same temperature, relative humidity, and light cycle as above, and fresh pine needles from the *Pinus thunbergii* Parl were placed on a clean sheet of paper, which served as the oviposition matrix. After oviposition, the pine needles were collected and disinfected with a 10% formaldehyde solution for 0.5 h followed by rinsing with sterile water and then drying, so that the larvae could hatch. The newly hatched larvae were incubated in a cage with fresh pine needles after surface disinfection. Fresh pine needles were disinfected by treatment with a 0.25% sodium hypochlorite solution for 5 min, followed by rinsing with sterile water and drying. The third instar larvae were used in the experiment.

Purification of viral occlusion bodies (OBs)

The fifth and sixth instar larvae of *N. zhejiangensis* were collected from *P. wallichiana* at the Research Institute of Forestry, Chinese Academy of Forestry. The virus was purified according to the method used by El-Salamouny (1998) with modifications[11]. Briefly, 100 g of sawfly bodies was ground, added to 200 ml distilled water of 0.3% SDS, and then filtered through double gauze sandwiched with cotton. After the filtrate was centrifuged at 800 rpm for 5 min, the precipitate was discarded, and the supernatant was obtained. After the supernatant was centrifuged at 4°C, 3000 rpm for 20 min, the supernatant was discarded, and the precipitate was obtained.The step was repeated three times. The precipitate was resuspended with sterile water followed by centrifugation at 5000 rpm for 20 min, and the precipitate was obtained, while the supernatant was discarded. To further purify the virus, the precipitate was resuspended with an appropriate amount of sterile water and then applied onto a 40-60% sucrose density gradient, followed by centrifugation at 10,000 rpm for 30 min. The suspension band containing the virus was collected and washed three times with sterile water. The concentration of the virus suspension was determined using a haemocytometer. The preparation was stored at 4°C for later use.

Microscopic observation

Observation with a scanning electron microscope was performed using the following method. A 1.5-2 ml virus suspension containing 10^8 polyhedral inclusion bodies (PIB/ml) was centrifuged at 3000 rpm for 10 min, and the obtained precipitate was fixed with 2.5% glutaraldehyde for 2 h. Then, it was washed three times with 0.1 mol/L phosphate buffer, fixed with 1% osmium tetroxide for 2 h, and then washed three times with 0.1 mol/L phosphate buffer. The obtained samples were dried with a critical point drying apparatus (Leica

EM CPD300) and coated with gold using an ion sputter coater (EiKoIB-3). Finally, the samples were observed using a scanning electron microscope (Hitachi SU8010).

Observation using a transmission electron microscope (TEM) was performed according to the method described by Luft[12]. Briefly, the sample was fixed, dehydrated with an acetone gradient (30, 50, 60, 70, and 100%), and then embedded and polymerised in the epoxy resin SPURR. Ultra-thin sections of the samples were obtained using a *Leica UC7* ultramicrotome, and double staining was then performed with uranyl acetate and lead citrate. Finally, observations were performed, and the images were captured using a transmission electron microscope (JEOL JEM-1400).

Biological activity

After fasting for 12 h, the selected healthy and active third instar larvae of *N. zhejiangensis* were fed with fresh pine needles coated with the virus. Viruses in the concentrations of 7.0×10^7, 7.0×10^6, 7.0×10^5, 7.0×10^4, and 7×10^3 PIB/ml were used to infect the experimental insects. The viruses were applied to clean fresh pine needles from *P. thunbergii* Parl. using a clean brush and 300 ìl of polyhedral suspension for each treatment. Starting from the second day after the infection, the number of larvae were recorded until death or pupation (the dead larvae showing significant symptoms of nucleopolyhedrovirus infection, such as swelling or loss of contact with or an inverted position on the pine needles, were recorded as deaths due to the virus; deaths due to other pathogens, such as bacterial and fungal infections, were not recorded, and the dead larvae were discarded). Each treatment included a total of 30 larvae and was repeated three times. Sterile water was used as a control.

Sectioning of pathological tissues

The 7.0×10^6 PIB/ml concentration of NezhNPV virus was selected to inoculate the third instar larvae of *N. zhejiangensis*. At 0, 24, 48, 72, 96, and 120 h, the experimental insects were collected and fixed with 10% formalin overnight. Conventional tissue sectioning was performed according to the method described by Wang *et al.*[13]. After embedding in paraffin, the tissue was sectioned with a thickness of 2 µm and stained with haematoxylin-eosin. Observation was conducted and images were captured with a light microscope (Olympus).

Statistical analysis

SPSS 19.0 statistical software was used for probit analysis and the computation of the virulence regression equation, median lethal time (LT_{50}), and median lethal concentration (LC_{50}) for *N. zhejiangensis*.

RESULTS

Ultrastructural characteristics of NezhNPV

Scanning electron microscopy showed that the occlusion bodies of the virus mainly consisted of a polygonal shape with an irregular pattern (Fig. 1a). The diameter was 0.47-0.91 ìm (n=100), with the average diameter of 0.76±0.1 ìm. The surface was uneven, with elongated and round holes on the surface of a few polyhedra (Fig. 1b-c). TEM observations showed that the derivative virions of the inclusion bodies were rod-shaped and scattered within the inclusion bodies with an irregular arrangement. Each virion contained a nucleocapsid as a single-embedded nuclear polyhedrosis virus (SNPV). Each inclusion body contained 2-16 virions. The baculovirus particles were obtuse at both ends, with a length of 83-201 nm and a width of 32-48 nm (Fig. 1d). These results indicate that the *N. zhejiangensis* virus is a new rod-shaped nucleopolyhedrovirus, which we called NezhNPV, according to the conventional classification method for viruses.

Effects of the virus on *N. zhejiangensis* larvae

After the *N. zhejiangensis* larvae were infected by NezhNPV, the symptoms of nucleopolyhedrovirus were observed: the body was shiny and swollen (Fig. S1a), and in the late infection stage, the skin of the larvae was thin and tended to be broken, leading to parasite liquefaction (Fig. S1b). Table 1 shows that the time to lethality for NezhNPV in the third instar larvae of *N. zhejiangensis* increased with decreasing concentrations. The LT_{50} values of the five assigned concentrations (from 7×10^7 to 7×10^3 PIB/ml) were 3.70, 4.12, 4.56, 5.08, and 6.24 days. The LC_{50} was 1.15×10^4 PIB/ml. These results indicate that NezhNPV is highly virulent in the third instar larvae of *N. zhejiangensis*.

Pathological observation of *N. zhejiangensis* infected with NezhNPV

When fourth instar larvae of *N. zhejiangensis* were fed with the virus, they showed no obvious symptoms in the midgut, fat body, dermal cells, or tracheal cells (Fig. 2a-c). At 72 h after infection, the *N. zhejiangensis* larvae exhibited symptoms including a reduced number of fat bodies, swollen nuclei, slightly swollen trachea, and overall swelling in the midgut region (Fig. 2d-f). The symptoms were most obvious at 120 h after infection, showing an overall disordered structure, pink staining substances in the tissues (NezhNPV), a relaxed epidermal structure, disruption and disintegration of the midgut cells, a significantly decreased number of fat bodies, and swelling in the muscles (Fig. 2g-I). NezhNPV infection was mainly observed in the midgut, fat body, epidermal, and tracheal cells, of which the fat body cells showed the most obvious pathological changes.

DISCUSSION

In this study, a new nucleopolyhedrovirus strain that can infect *N. zhejiangensis* larvae was isolated, which showed the same morphological characteristics as other NPVs. Interestingly, one or two circular and elongated holes were observed on the surface of NezhNPV (Fig. 1b-c). This feature is different from pine sawfly NPVs from other sources. The holes on the surface of the polyhedra were not likely caused by the relocation of the virions in the process of sample preparation because these holes were not observed in viruses that underwent the same processes, including *D. nanhuaensis* NPV (DnNPV), NeseNPV, and NeleNPV. In addition, a comparison of the reported NPVs from other sawfly species showed some differences in ultrastructure (Table 2)[1].First, the diameters of the polyhedron were different. The diameters of polyhedrons were

Table 1. The virulence of NezhNPV in third instar larvae of *N. zhejiangensis*

Concentration (PIB·ml^{-1})	Regression equation	Correlation coefficient	LT_{50} (d)	LC_{50} (PIB·ml^{-1})
7×10^{7}	Y=-10.240+18.024x	0.975	3.7	11515.873
7×10^{6}	Y=-7.353+11.957x	0.919	4.121	
7×10^{5}	Y=-6.204+9.417x	0.927	4.558	
7×10^{4}	Y=-5.497+7.783x	0.914	5.084	
7×10^{3}	Y=-5.181+6.514x	0.925	6.242	

Table 2. The Characteristics of NPVs from different sawfly species

Host insect	Baculovirus	Isolation place and time	Morphological Features
N. zhejiangensis	NPV	Beijing, China in 2015	The OBs are mostly polyhedral in shape, with a diameter of 0.47-0.91 μm and 1-2 small holes on their surface. The virion is rod-shaped with obtuse ends consisting of a single nucleocapsid. The size of the nucleocapsid is 25-40 nm×110-200 nm.
Diprion nanhuaensis	NPV	Yunnan, China in 2005	The OBs are 0.5-1.0 μm in diameter. The virion is a single nucleocapsid with truncated ends. The size of the nucleocapsid is 25-30 nm×125-200 nm. No holes were observed on the surface.
Diprion sp.	NPV	Guodong, China in 1981	The OBs are 0.8-1.6 μm in diameter. The virion is 58 nm×290 nm. No holes were observed on the surface.
N. sertifer	NPV	Unknown	The virion is a single nucleocapsid. No holes were observed on the surface.
N. lecontei	NPV	Ontario, Canada in 1950	No holes were observed on the surface.

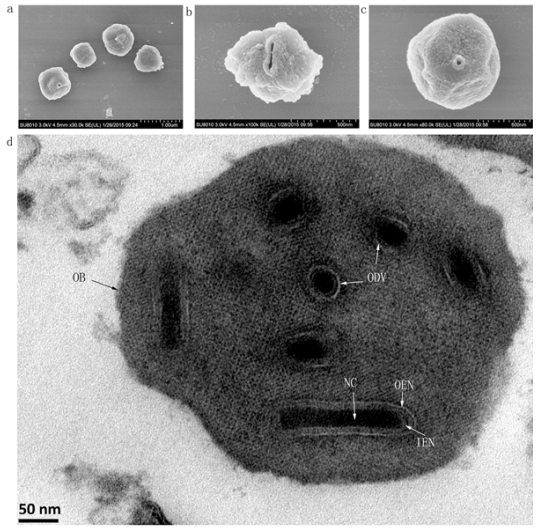

Fig. 1. The ultrastructural characteristics of OBs from NezhNPV. The structures of OBs (a), with elongated and round holes (b, c) are shown by scanning electron microscopy (d). The section of an OB containing virions is displayed by transmission electron microscopy. Each ODV includes a single nucleocapsid (NC). OB: occlusion body, ODV: occlusion-derived virus, IEN: inner layer of the envelope, OEN: outer layer of the envelope

0.47-0.91 ìm for NezhNPV, 0.5-1.0 ìm for DnNPV, and 0.8-1.6 ìm for *Diprion sp.* NPV. Second, DnNPV baculovirus particles exhibited truncated ends, and rounded ends were observed in NezhNPV virions. Third, the sizes of the virions were 25-30 nm×125-200 nm for DnNPV baculovirus, 58 nm×290 nm for *Diprion sp.* NPV, and 32-48 nm×83-201 nm for NezhNPV. Finally, their virulence levels were different. Lecontvirus is highly infective in *N. lecontei*, and the infected insects die within 4-7 d. In 2005, Li et al. reported that NeabNPV at a concentration of 10^7 PIB/ml required 5 d to kill the second and third instar larvae of *N. abietis* and 10-12 d to kill the fourth and fifth instar larvae [10]. The LC_{50} of NezhNPV for the third instar larvae of *N. zhejiangensis* was 1.15×10^4 PIB/ml. When the concentrations were 7×10^7, 7×10^6, 7×10^5, 7×10^4, and 7×10^3 PIB/ml, the LT_{50} values were 3.70, 4.12, 4.56, 5.08, and 6.24 d, respectively. DnNPV has some pathogenic effects on *N. xiangyunicus* and *D. nanhuaensis*. Further investigations are required to determine whether NezhNPV can infect a variety of pine sawfly species.

Unlike most lepidopteran baculoviruses, Hymenoptera baculovirus can only replicate in the

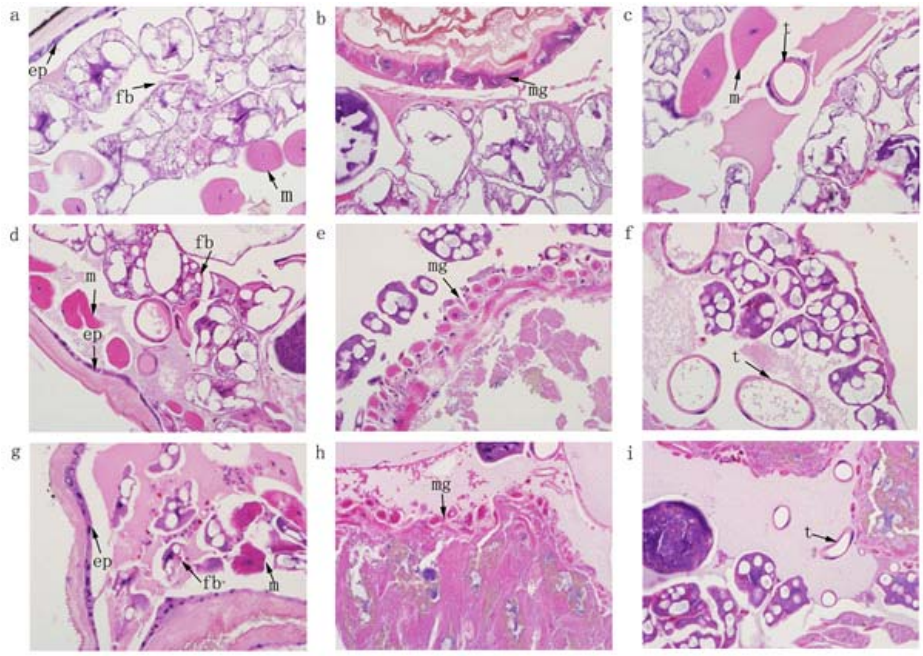

Fig. 2. Selected tissues of the third instar stage of *N. zhejiangensis* infected with NezhNPV at different time points (Haematoxylin-eosin staining). a,b and c, normal larval tissues. d,e and f tissues infected by NezhNPV at 72 h; g, h and I, infected tissues at 120 h. All images are magnified 100 times. fb-fat body, ep-epidermis, t-trachea, mg-midgut, and m-muscle

epithelial cells in the midgut of larvae. The gregarious nature of pine sawflies result in their contact with excrement from the midgut after infection, which accelerates the rapid spread of the virus in the pine sawfly, leading to death within 4-7 d after infection[5, 7,14]. A study of a series of pathological changes in the *N. zhejiangensis* after infection with NezhNPV showed that the pathological characteristics of the infection were similar to those of NPV infection in lepidopterans, with infection not only in the midgut but also in the fat body, epidermal, and tracheal cells (Fig. 2). These findings showed that NezhNPV can substantially replicate inside the body of insects. The lepidopteran NPV first invades the host at the midgut and then enters the haemocoel after proliferation, thereby infecting other sensitive tissues. In NezhNPV infection, a silty substance (NezhNPV) was first observed in the midgut, indicating that NezhNPV was first replicated in the midgut. The next host tissue that is infected by NezhNPV still requires further study.

In conclusion, a newly discovered viral strain, NezhNPV, isolated in this study is significant in its application for the prevention of *N. zhejiangensis*, which is a very valuable resource for this pathogen. In addition, the infectious approach of NezhNPV is different from that of other pine sawfly NPVs.

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