International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-005, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Virulence and characteristics of a new nucleopolyhedrovirus strain of *Dendrolimus kikuchii* (Lepidoptera: Lasiocampidae)

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Accepted 19 January, 2019

In present study, a nucleopolyhedrovirus was isolated from the infected larvae of *Dendrolimus kikuchii*, which is a serious pest for a variety of conifers in China. Ultrastructural observation revealed that this virus is a multiple-nucleocapsid nucleopolyhedrovirus (MNPV), which was encapsulated within the envelope of each virion. Based on the morphological characteristics, it was identified as a new virus and named as DkNPV. It exhibited high potential as a biocontrol agent to protect the forest because of the high virulence against the third-instar larvae of *D. kikuchii*. The virus passage in the host after 4 generations, toxicity and ultrastructural morphology were stable.

Key words: *Dendrolimus kikuchii*, multiple-nucleocapsid nucleopolyhedrovirus, *Dendrolimus Kikuchii* Matsumura nuclear polyhedrosis virus (DkNPV), virulence, ultrastructure, biological control.

INTRODUCTION

Dendrolimus kikuchii (Matsumura) (Lepidoptera: Lasiocampidae), is a serious pest for a variety of conifers, including *Pinus kesiya*, *Pinus yunnanensis*, *Pinus massoniana*, *Pinus armandi*, *Pinus fenzeliana*, *Cedrus deodara* and *Keteleeria evelyniana* (Figure 1a). In China, *D. kikuchii* mainly occurs in the south part and chemical pesticides were primarily used as the first control method (Chen, 1990). However, chemical insecti-cides may poison the non-target organisms (e.g., humans, livestock and natural enemy), pollute environment, and induce insecticide-resistant pests. These problems proved that the application of chemical insecticides to be less than an ideal method to controll *D. kikuchii* (Yasuhisa, 2007).

The insect virus is one kind of pathogenic microorganism in nature and may influence the quantity of insect population by causing the epidemic disease of insect. It has high host specificity, so that it is safe to other non- target species and can maintain dynamic balance of insect population for a long term by hori-zontally and vertically spreading epidemic disease in the pest population (Fuxa, 1989; Laird et al., 1990; Heinz et al., 1995). Moreover, it causes no environmental pollution. Therefore, it is realized that the use of virus to control pest insects is an ideal and sustainable means. In 1986, a nucleopolyhedrovirus of *D. kikuchiii*, named as *Dendrolimus Kikuchii* Matsumura Nuclear Polyhedrosis

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Virus (DKMNPV), was isolated and its morphological characteristics and toxicity were described, however, *D. kikuchii* larvae were not sensitive to DKMNPV (Yang et al., 1986). Therefore, the screening of high toxicity and strong stability virus is an imperative work for the biological control of *D. kikuchii*. In present study, a nucleopolyhedrovirus was isolated from diseased *D. kikuchii* larvae in Mile county, Yunnan province, China. Based on its ultrastructural morphological characteristics and the virulence against the third-instar larvae of *D. kikuchii*, it was identified as a new virus and named as DkNPV (multiple-nucleocapsid nucleopolyhedrovirus of *D. kikuchii*). This virus exhibited high virulence against the third-instar larvae of *D. kikuchii*, and high potential as a biocontrol agent to protect the forest from these insects.

MATERIALS AND METHODS

Virus isolation and purification from *D. kikuchii*

Diseased *D. kikuchii* larvae were collected from *P. yunnanensis* forest in Mile, Yunnan Province, China, and kept frozen at -20°C until use. Virus occlusion bodies (OBs) were isolated and purified from the larval cadavers according to the method developed by El-Salamouny (1998). Briefly, 200 g larvae cadavers were homogenized in 500 ml distilled water and filtered through five layers of muslin. The filtrate was subjected to centrifugation alternated between low and high speed several times until the clear off-white OBs were obtained. For further purification, the obtained OBs were centrifuged through sucrose gradient of 40-60% (w/w) at 10,000 g for 30 min at room temperature. The bands containing virus were collected, washed with sterile water, and then centrifuged at 12,000 g for 30 min at 4°C with three replications. The pellets of purified OBs were stored at 4°C for the next study.

Ultrastructural studies of virus isolated from *D. kikuchii*

As to scanning electron microscopy (SEM), the following method was used. Firstly, pellets of purified virus were diluted in distilled water and the concentration of virus was adjusted to 10^9 PIB·ml^{-1} (polyhedral inclusion bodies/ ml^{-1}). Secondly, 20- 30 l of viral suspensions were mounted on a clear coverslip and dried naturally in the ventilate place. After that, the coverslip was placed on an aluminous specimen stub and coated with gold. Finally, the specimen was observed by using a scanning electron microscope (Hitachi 3400N, Japan). One hundred OBs were measured to determine their mean size.

The transmission electron microscopy (TEM) was carried out according to the method described by Luft (1961). Briefly, pellets of purified OBs were fixed directly in 2.5% glutaraldehyde for 2.5 h, dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100%), and then embedded in Epon-Araldite resin. Ultrathin sections were cut with ultractome, slice sizes range from 50 - 60 nm, double strained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope (Hitachi JEM-1230, Japan).

Preparation of *D. kikuchii* larvae

Larvae of *D. kikuchii* were reared from surface-sterilized eggs. Briefly, the pupae of *D. kikuchii* were collected from the *P. yunnanensis* forest and kept in the clear cages (30 × 30 × 40 cm) in the laboratory. Once emergence, adults were provided with 50 gl^{-1} sugar solution as food source and some fresh shoots of *P. armandi* as an oviposition substrate. Eggs were collected and sterilized with 10% methanal for 0.5 h. After hatch, larvae were group reared on fresh needles of the *P. armandi* within the clear plastic jars (15 × 15 × 20 cm) with some holes in the bottom until the third-instars. The above experiments were carried out at 20°C, 50-65% relative humidity and a 18:6 h (day : night) photoperiod (Guo et al., 2007).

Infection studies of isolated virus against the *D. kikuchii* larvae

The infection activity of isolated virus was tested against the third-instars larvae of *D. kikuchii* by following the method developed for Cydia pomonella granulovirus (Huber, 1981) . The pellets of purified virus were diluted in distilled water. Five virus suspensions at different concentrations (2.2×10^4, 2.2×10^5, 2.2×10^6 , 2.2×10^7 and 2.2×10^8 PIB·ml $^{-1}$) were prepared respectively and used for this infection study. For each concentration, fifty larvae were used and 300 µl of viral suspensions were sprayed onto pine needles, which were used as food of *D. kikuchii* larvae and filled into a plastic jar (20 × 20 × 20 cm) with some holes in the bottom. The tested larvae were kept at 20°C, 50-65% relative humidity and a 18:6 h (light : night) photoperiod. As soon as the treated needles were eaten, the tested larvae were provided with fresh, untreated needles, and continuously reared in the plastic jar. Larvae with the typical symptoms of NPV infection such as a bloated body, hanging upside down from the branches and fragile cuticle were recorded as dying of virus infection. Mortality of larvae due to the infection of virus was recorded from the third day and up to the 13th day at a one day interval. Three repetitions were carried out and as control, pine needles were treated with distilled water exactly according to the above method.

Stability studies of isolated virus in toxicity and ultrastructural morphology

The virus has been propagated in the fourth-instars larvae of *D. kikuchii*, for 4 generations. Stability of ultrastructural morphological and the virulence of the virus were tested according to the above method.

Statistical analysis

The median lethal time (LT_{50}), the median lethal concentration (LC_{50}) and the toxicity regression equations of *D. kikuchii* virus were estimated by probit analysis software SPSS package 17.0 (Yu and He, 2003). Death rate and corrected mortality of insect larvae were calculated based on bioassays.

RESULTS

Ultrastructural characteristics of *D. kikuchii* virus

The SEM results showed that OBs of *D. kikuchii* are mostly polyhedral shape, 0.79~2.31 (1.64 ± 0.1) µm [minimum~maximum (mean ±SE)] in diameter (n = 100), with a lots of small holes on their surface, 173.00~254.00 nm × 55.10~116.00 nm in size (Figure 2a). Ultrathin sections revealed that each OB contains many virions, 252.00~359.00 nm × 70.18~200.00 nm in size (Figure

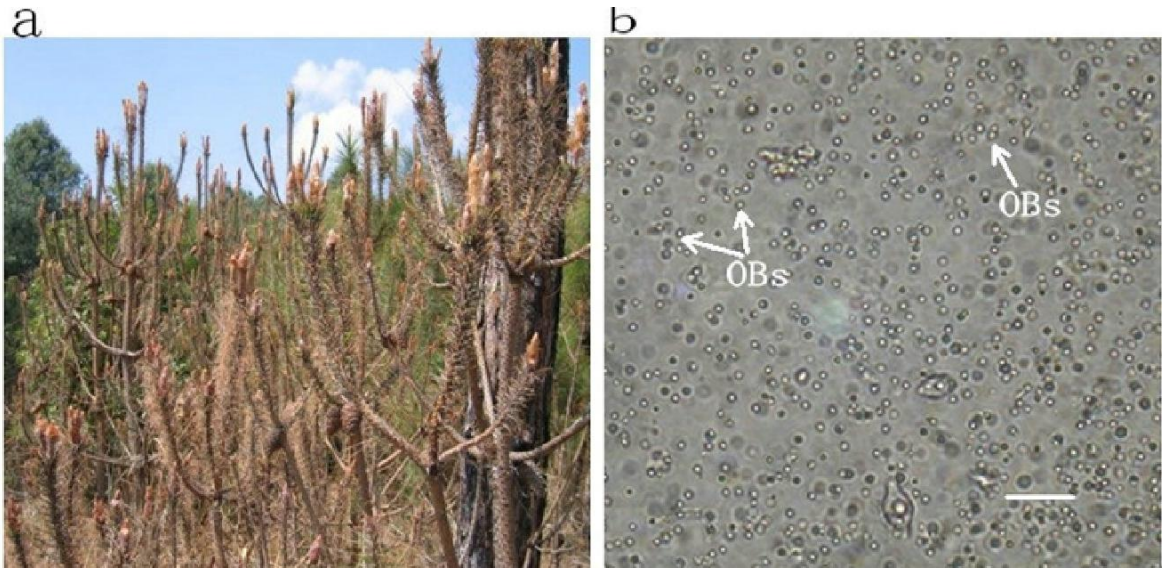

Figure 1. (a) Image showing the co nifers damaged by *D. kikuchii* larvae; (b) OBs in *D. kikuchii* larvae body fluids, and for image (b) the bar represents 20 µm.

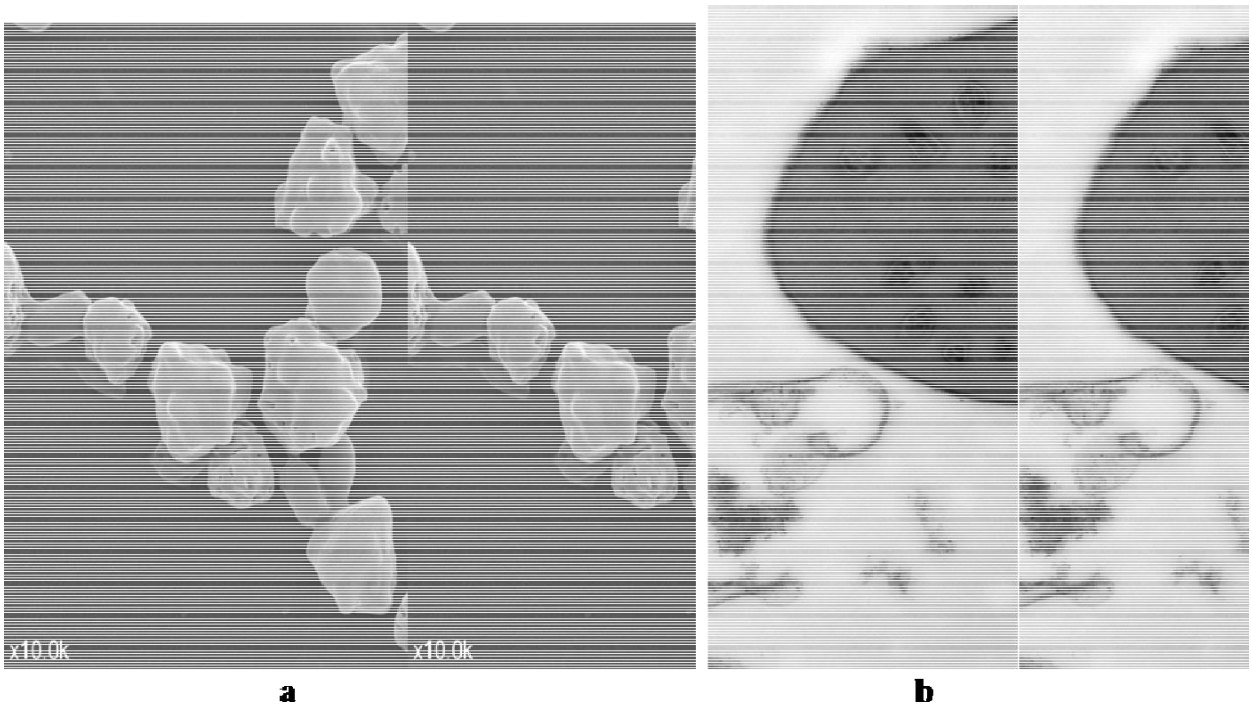

Figure 2. The ultrastucture characteristics of DkNPV. (a) Scanning electron micrograph of the DkNPV OBs; (b) The ultrastructural section of OBs showing the virions (V) inside it. Each virion contains multiple nucleoca psids (N); for images a and b the bar represents 5.00 µm and 500 nm respectively.

2b). The virion is rod-shaped with the truncated or obtuse ends, consists of multiple nucleocapsids (up to 9) within a single viral envelope (Figure 2 b). The size of nucleo-capsid is approximately 25.80 ± 0.86 nm (mean ±SE) in width and 265.00 ± 12.66 nm in length (n = 50) (Figure 2b).

Based on the ultrastru ctural observation, the morphological characteristics a nd the virulence of the *D. kikuchii* virus was identified as a new virus in nucleopolyhedrovirus of Bacu loviridae, and named as DkNPV according to the universal system of virus taxonomy in the 8th ICTV repo t (Hong, 2006).

Table 1. The virulence of DkNPV on 3rd instars larvae of *D. kikuchii.*

Concentration (PIB·mL^{-1})	Regression equation	Correlation coefficient	LT_{50}(d)	LC_{50} (PIB·mL^{-1})
2.2×10^{8}	Y=10.457x+9.219	0.976	6.89	1.72×10^{5}
2.2×10^{7}	Y=10.051x+8.156	0.986	7.18	—
2.2×10^{6}	Y=8.633x+5.487	0.947	8.16	—
2.2×10^{5}	Y=6.536x+2.237	0.980	10.31	—
2.2×10^{4}	Y=6.039x+0.890	0.988	11.13	—

Table 2. The difference between DkNPV and DKMNPV.

Virus name	OBs surface	Both ends of rod-shape	The size of nucleocapsids (nm)	Diameter (μm)	LC_{50} (PIB·mL^{-1})
DkNPV	Lots of small holes	Truncate or obtuse ends	265.00 ± 12.66× 25.80 ± 0.86	0.79~2.31	1.72×10^{5}
DKMNPV	The even surface	Obtuse both ends only	410 ± 10× 40 ± 3	1.3~3.0	$1\times10^{5.9}$

Infection studies of DkNPV against the *D. kikuchii* larvae

The results of infection study showed that DkNPV could infect and kill the third-larvae of *D. kikuchii* (Table 1). LC_{50} was 1.72×10^{5} OBs·ml^{-1}. At the tested five concentrations (from 2.2×10^{8} to 2.2×10^{4} PIB·ml^{-1}), LT_{50} were 6.89, 7.18, 8.16, 10.31 and 11.13 days, respectively (Table 1). A large number of *D. kikuchii* larvae begin to die from the seven day of infection, and peak mortality from 7 to 10 days. A large number of virus was found in *D. kikuchii* larvae with the typical symptoms of NPV infection by optical microscope (Figure 1b). Therefore, DkNPV is virulent to the third-instar larvae of *D. kikuchii.*

Our research demonstrates that the virus has been propagated 4 generations and the fourth-instars larvae of *D. kikuchii* has stable ultrastructural morphological and virulence.

DISCUSSION

In present study, a nucleopolyhedrovirus was isolated from *D. kikuchii*. The main morphological characteristics of this virus is consistent with the NPVs. When it was compared with the reported *D. kikuchii* virus DKMNPV, several significant differences were observed between them (Table 2). It is interesting that lots of small holes were observed on the surface of DkNPV. Firstly, we thought they were caused by the process of sample preparation. Other four samples (HcNPV, BusuNPV, DnNPV and CzNPV) therefore were prepared exactly according to the same method. However, no holes were observed on their OBs surface. One difference lied in small holes on the surface of DkNPV and the even surface of DKMNPV. The second difference existed on the both ends of rod-shape virions. DkNPV had truncate or obtuse in both ends, but the DKMNPV had obtuse both ends only. The third difference was that the size of nucleocapsids of DkNPV was smaller than DKMNPV (265.00 ± 12.66 nm × 25.80 ± 0.86 nm of DkNPV, 410 ± 10 nm × 40 ± 3 nm of DKMNPV). Besides, the diameter of OBs differed widely, the diameter of DKMNPV was 1.3~3 μm, but the diameter of DkNPV was 0.79~2.31 μm. The shape and size of OBs, the size of nucleocapsids and the features of both ends of visions were the important basis for the identification of a baculoviruses (Xie and Hu, 2000). The fourth difference shows the DkNPV is more virulent than DKMNPV against the larvae of *D. kikuchii.* Based on the morphological characteristics, the virulence of this virus and the difference between DkNPV and DKMNPV, it was proposed as a new strain nuclepoyhedrovirus of *D. kikuchii* and named as DkNPV. In the next step, the structure of its nucleic acid and biological characteristics will be further studied to confirm its identification. *Dendrolimus* cytoplasmic polyhedrosis virus (CPV) were used to biologically control the pest of *Dendrolimus* in China since the first DpCPV *(D. punctatus* Cytoplasmic Polyhedrosis virus) was isolated in China in 1973. However, as is well known the time required for CPVs to kill the insect larvae was longer than that for NPVs obviously. The new isolated DkNPV was virulent for the third-instar larvae of *D. kikuchii* and exhibited high infection activity (Table 1). Therefore, this virus was very precious for the biological control of *D. kikuchii*.

ACKNOWLEDGMENTS

We sincerely thank Yan-Li Duan and Jian-Jie Xue for assistance both in field research and virus propagation; Chun-Yan Wang for suggestions of revising the manuscript. This work was supported financially by National Natural Science Foundation of China (No. 30671688), and Forestry Industry Special Foundation of

China (No. 200904029; No. 200704003).

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