Int. J. Indust. Entomol. Vol. 11, No. 2, 2005, pp. 135~138

[Note]

International Journal of *Industrial Entomology*

PCR-Based Detection of Densovirus Infection in Silkworm (*Bombyx mori* L.)

Chengxiang Hou, Muwang Li*, Zhongzheng Gui, Anying Xu and Xijie Guo

Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, 212018, P. R. China.

(Received 13 September 2005; Accepted 25 November 2005)

Two pairs of DNA primers were designed for the detection of the Zhenjiang (China) strain of *Bombyx mori* densonucleosis virus (BmDNV-Z). These primers were designed from the nucleotide sequence of major structural protein gene (putative VD1-ORF2). PCR amplification was attempted from different issues (including silk gland, blood, skin and midgut) and feces of the silkworm which infected wit BmDNV-Z were amplified by PCR. Both of the primers gave expected size of in the DNA bands from midgut and feces, but not in the DNA of silk gland, blood and skin. The two bands were sequenced, and their sequence were same as the sequence designed for. BmDNV-Z could be successfully detected in single silkworm after it was infected for 12 hrs, and could not be detected before 9 hrs after infected.

Key words: Silkworm (*Bombyx mori* L.), Densonucleosis virus, PCR, Diagnose

Introduction

Bombyx mori densonucleosis virus (BmDNVs) belong to the genus Iteravirus of the family Parvoviridae (Lü, 1998). It was first isolated from silkworms with flacherie disease in Ina City in Japan. BmDNVs, which cause serious economic damage to sericulture, were discovered in 1976. They comprise several strains, such as Ina virus, Saku virus, Yamanashi virus and Zhenjiang (China) virus (DNV-Z or DNV-3). As distinguished by Watanabe *et al.* (1986), Ina virus belongs to the DNV-1 type, and Saku virus and Yamanashi virus belong to the DNV-2 type. Iwashita *et al.* (1983) discussed the chemical characteristics of DNV-Z, and Qian *et al.* (1985) demonstrated that it is similar to Saku virus. BmDNVs multiply only in the nuclei of the columnar cells of the larval midgut epithelium (Guo *et al.*, 1985; Seki *et al.*, 1983). These nonenveloped, icosahedral viruses package a linear, single-stranded DNA of about 4 – 6 kb with terminal hairpins that are required for virus replication.

The nucleotide sequence and genome organization of BmDNV-1 were the first to be reported for DNVs (Bando *et al.*, 1987a, b, 1990). Xu *et al.* (2004) cloned the major structural protein gene of DNV-Z and expressed it in *E. coli*. The whole genome of DNV-Z had been sequenced recently (Yao *et al.*, 2005).

BmDNV could not form visible capsid in silkworm, and it could not be detected by microscope. Its symptom is difficult to differentiate from symptom of *Bombyx mori* flacheric virus and bacteria that parasitize in the silkworm midgut. Normally, serological methods were adopted to diagnose it (Qian *et al.*, 1989) but it is a little difficult to purify the antigen, and the antiserum has finite period of validity. Since the Genome of BmDNV-Z had been published and its major structural protein gene had been cloned, it would be exact and quick to detect this disease in silkworm using molecular methods. In this research, PCR primers were designed based on the sequence of major structural protein gene, and this virus was detected in the silkworm according to different time after they were infected the virus.

Materials and Methods

Silkworm strains

Jingsong, widely used in Chinese sericulture, susceptible to DNV-Z, was maintained by single pair mating in Sericultural Research Institute, Chinese Academy of Agricul-

*To whom correspondence should be addressed.
Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, 212018, Jiangsu province P. R. China. Tel: +86-511-5616575; Fax: +86-511-5622507;
E-mail: cangene@ pub.zj.jsinfo.net

tural Sciences (SRI-CAAS).

Virus inoculum

Dried silkworm midgut containing DNV-Z was ground with distilled water until it turned into a dense solution. The solution was filtered with gauze, and then centrifuged at 3500 rpm for 20 min. After adding an equal volume of 7% acetic acid to the supernatant, the solution was incubated at 25°C for 40 min, followed by adjustment to pH 7.0 and progressive dilution to 0.5% solutions of tissue. Bioassays were conducted at 25°C. The fourth newly exuviated larvae were fed for 24 hrs with mulberry leaves which had been smeared with a DNV-Z suspension, followed by rearing on uncontaminated fresh mulberry leaves. These silkworms were dissected at different time after inoculated BmDNV-Z. The feces of the infected silkworm were collected at different time too.

DNA extraction

Silkworm issues (silk glands, blood, skin or midgut) was frozen quickly in liquid nitrogen and ground into powder, then the powder was suspended in DNA extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 100 μg/ml proteinase K. Half gram of silkworm feces was ground and suspended in 1 ml DNA extraction buffer. After digestion with proteinase K at 55°C for 3 – 5 hrs, phenol / chloroform extraction was carried out and DNA was recovered by isopropanol precipitation. Purified DNA was dissolved in 0.1 × TE buffer (pH 8.0). DNA concentration was measured spectrophotometerically (BioPhotometer, Eppendorf).

Primer design and PCR amplification

Two pairs of primers were designed from the main structural protein gene sequences of putative VD1-ORF2 (Xu *et al.*, 2004; Gene bank: AY236978) using the program Primer Premier 5.00 (Premier Biosoft International, Palo, Alto, Calif. http://www.premierbiosoft.com).

Primer-1 could amplify 1500 bp of band from BmDNV-Z genomic DNV: sense, 5'-ATTATGGGTAGAGTACTTGGCT-3', anti-sense, 5'-TTATTTATTGAAAACCAACAAGCCT-3'.

Primer-2 could amplify 563 bp of band from BmDNV-Z genomic DNV:

Sense, 5'-GTATCCCGACTCAACACTCT-3', anti-sense, 5'-TCCACTGCCTGTAACTTCTT-3'.

PCR reactions were performed using a Flexigene Cycler (Techne, England). PCR cycles included (1) a 3 min at 95 °C initial denaturation step, (2) 35 cycles performed as follows: 94°C for 45 sec, 56°C for 1 min, 72°C for 1.5 min, (3) a final elongation step of 10 min extension at 72°C. The PCR was performed in a final volume of 20 μl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, approximately 20 ng of template DNA, 1 U of Taq polymerase (Takara) and distilled deionized water. PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed using a Tanon gel image system.

Results and Discussion

Detection of BmDNV-Z in different issues of infected silkworm

The silkworms were dissected after they were infected by DNV-Z for 72 hrs and DNAs from different issues were amplified by the two primers. The DNAs from preserved midgut (containing DNV-Z) (provided by silkworm pathology lab of SRI-CAAS), midgut and feces of infected silkworm could be amplified by these two primers, but DNAs from other issues (blood, silk glands and skin) of infected silkworm and midgut of the uninfected silkworm could not be amplified (Fig. 1), indicating that BmDNV-Z could only multiply in the silkworm midgut. These results were same as the results of Guo *et al.* (1985) and Seki *et al.* (1983). Primer-1 could amplify the proper 1500 bp of DNA band and primer-2 could amplify the expected 563 bp of DNA band. These two DNA bands were cloned using pMD18-T vector (Takara) and were sequenced respectively, their sequences were blasted in NCBI website using blast 2 sequence method, they were found to be same as the designed sequence except 2 bases in the band amplified from primer-1 and one base in the DNA band amplified from primer-2 (Fig. 3 showed the result the band of primer-2). These results demonstrated that the amplified bands were all from DNV-Z genome. Two bases in the band amplified from primer-1and one base in the band amplified from primer-2 were not accorded with the sequences from NCBI. These errors maybe come from the sequencing.

Detection of BmDNV-Z in silkworm midgut of different time after infected

The silkworm midguts were dissected at different time after infected, and their DNAs were amplified using these two primers. BmDNV-Z could be successfully detected after the silkworms were infected for 12 hrs even single silkworm midgut was detected, and could not be detected before 9 hrs after infected.

The development of rapid and sensitive diagnostic tools using molecular approaches will be useful in detecting BmDNV-Z. PCR method is sensitive in detecting BmDNV. BmDNV-Z could be detected in the silkworm

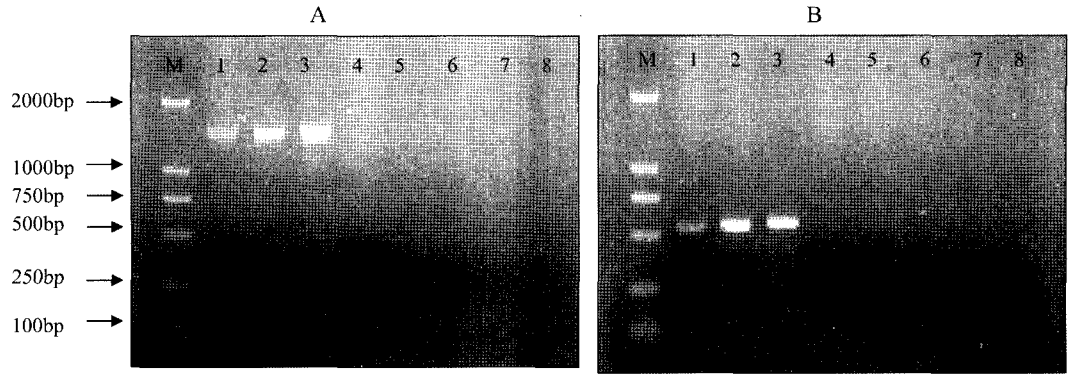

Fig. 1. PCR detection of BmDNV-Z in different issues and feces in the silkworms infected BmDNV-Z after 3 days. A, Primer-1; B, Primer-2.
M, Molecular marker; 1, Preserved midgut containing DNV-Z; 2, Infected silkworm feces; 3, Infected silkworm midgut; 4, Infected silkworm silk glands; 5, Infected silkworm blood; 6, Infected silkworm skin; 7, Uninfected silkworm midgut (control); 8, Water as template

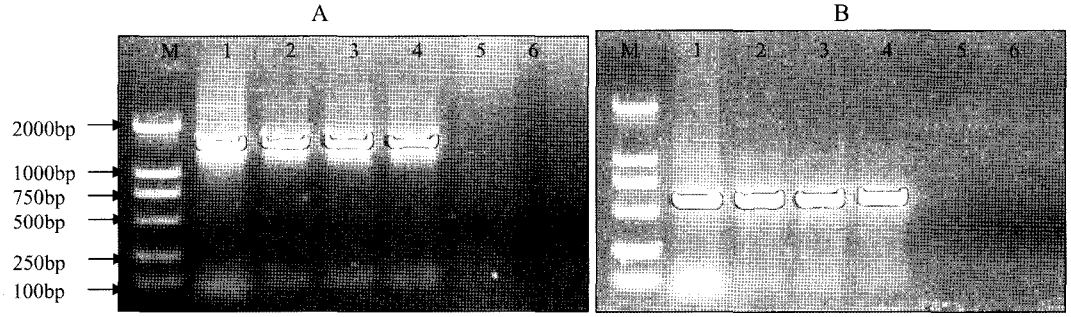

Fig. 2. PCR detection of BmDNV-Z in the silkworm midguts after different time of infecting BmDNV-Z. A, Primer-1; B,Primer-2. M, Molecular marker; 1, 48 hrs after infecting BmDNV-Z; 2, 36 hrs after infecting BmDNV-Z; 3, 24 hrs after infecting BmDNV-Z; 4, 12 hrs after infecting BmDNV-Z; 5, 9 hrs after infecting BmDNV-Z; 6,6 hrs after infecting BmDNV-Z.

```
DNVNCBI : TCCACTGCCTGTAACTTCTTTTATATACAAATATCCTGTATTTCCTGTAGTAGTAGTTTGACGATTAACAGCTTCTGCACTTCC : 84
primer2 : TCCACTGCCTGTAACTTCTTTTATATACAAATATCCTGTATTTCCTGTAGTAGTAGTTTGACGATTAACAGCTTCTGCACTTCC : 84

DNVNCBI : AGCAGTGAGGAAATTAGTATGACTTCTATCAATTGTAAAAGAATTTCCAACATCTCTTTCTTGACATGGTATAATTACTACATT : 168
primer2 : AGCAGTGAGGAAATTAGTATGACTTCTATCAATTGTAAAAGAATTTCCAACATCTCTTTCTTGACATGGTATAATTACTACATT : 168

DNVNCBI : TTCAAAACTTTCAGTCATACTATCGGCTCCAGCATTTCTAGTTAATGGTACTAGTTGTTTAAGTTGACCATGATTTTGTCTTGA : 252
primer2 : TTCAAAACTTTCAGTCATACTATCGGCTCCAGCATTTCTAGTTAATGGTACTAATTGTTTAAGTTGACCATGATTTTGTCTTGA : 252

DNVNCBI : ACATAAATTTTCATATGGTATACCAATAGGAGTTAAACCTCCATCAGCACTTTCTTCATATAAGCGATATCCCCATCCAGTAAA : 336
primer2 : ACATAAATTTTCATATGGTATACCAATAGGAGTTAAACCTCCATCAGCACTTTCTTCATATAAGCGATATCCCCATCCAGTAAA : 336

DNVNCBI : AGAATCTTCAGGCTCCCACATAACTATCTTAGAGTTTTGTACAAAGGAGCTTACTTCATTAATAGAATTACCAGTAGTTAAATT : 420
primer2 : AGAATCTTCAGGCTCCCACATAACTATCTTAGAGTTTTGTACAAAGGAGCTTACTTCATTAATAGAATTACCAGTAGTTAAATT : 420

DNVNCBI : ATCTGATAATACTATAAAATTGCTAATTCTAACTGGTCCTGTTTCAATATCAAATCTACTGAATCTAACATCAGATAAATATGG : 504
primer2 : ATCTGATAATACTATAAAATTGCTAATTCTAACTGGTCCTGTTTCAATATCAAATCTACTGAATCTAACATCAGATAAATATGG : 504

DNVNCBI : TTCTAAAAATCGTAATCTATTAGTAAAATATGCACTTAAAGAGTGTTGAGTCGGGATAC : 563
primer2 : TTCTAAAAATCGTAATCTATTAGTAAAATATGCACTTAAAGAGTGTTGAGTCGGGATAC : 563
```

Fig. 3. Blast results of sequence from primer-2 and the origin sequence from NCBI.
Only one base (No 222) was different from the sequence deposited in NCBI.

midgut after 12 hrs of infecting, but it is a little later than the serological method, which could detected BmDNV-Z after 8 hr of infecting (Qian *et al.*, 1989). Although serological methods could detect this virus quickly, antiserum

is difficult to prepare and need many days. Using PCR methods would be economical and convenient in detecting this virus, and could be completed in any lab with a PCR apparatus.

Acknowledgements

This project was supported by National Natural Science Foundation of China. (No. 30400327) and Innovation Program of the Chinese Academy of Agricultural Sciences (No. 2004-30).

References

Bando, H., H. Choi, Y. Ito and S. Kawase (1990) Terminal structure of a densovirus implies a hairpin transfer replication which is similar to the model for AAV. *Virology* **179**, 57-63.

Bando, H., J. Kusuda and S. Kawase (1987a). Molecular cloning and characterization of *Bombyx* densovirus genomic DNA. *Arch. Virology* **93**, 139-146

Bando, H., J. Kusuda, T. Gojobori, T. Maruyama and S. Kawase (1987b) Organization and nucleotide sequence of a densovirus genome imply a host-dependent evolution of parvoviruses. *J. Virology* **61**, 553-560.

Guo, X. J., Y. J. Qian and X. F. Hu (1985) Studies on thr infection site by the densonucleosis virus in the diseased silkworm (*Bombyx mori*) in China. *Acta Sericologica Sinica* **11**, 93-98.

Iwashita, Y. and Y. S. Chao (1983) The development of a densonucleosis virus isolated from silkworm larvae, *Bombyx mori*, of China. *Acta Sericologica Sinica* **9**, 219-222.

Lü, H. S. (1998) Molecular biology of insect viruses. China agricultural scientech press. Beijing.

Qian, Y. J., X. F. Hu and Y. X. Wang (1989) Serological diagnoses of the densonucleosis disease of *Bombyx mori*. *Acta Sericologica Sinica* **15**, 217-222.

Qian, Y. J., X. J. Guo, X. F. Hu and H. Watanabe (1985) The serological relationship of *Bombyx mori* densonucleosis virus from China and Japan. *Acta Sericologica Sinica* **11**, 241-242.

Seki, H. and Y. Iwashita (1983) Histopathological features and pathogenicity of a densonucleosis virus of the silkworm, *Bombyx mori*, isolated from sericultural farms in Yamanashi prefecture. *J. Seric. Sci. Jpn* **52**, 400-405.

Watanabe, H., S. Kawase, T. Shimizu and H. Seki (1986) Difference in serological characteristics of densonucleosis viruses in the silkworm *Bombyx mori*. *J. Seric. Sci. Jpn* **55**, 75-76.

Xu, G. Z., Z. F. Zhang, G. X. Qin, K.W. Huang and X. J. Guo (2004) Cloning of the major structural protein gene of densovirus (Zhenjiang Isolate) in silkworm, *Bombyx mori* and its expression in *Escherichia coli*. *Chinese Journal of Virology* **20**, 279-282.

Yao, Q., Y. J. Wang, K. P. Chen, G. T. Gao, J. Lu, X. D. Tang and X. Han (2005) *Bombyx mori* densovirus 3 isolate VD1, complete genome. *GenBank Accession number DQ017268.* Bombyx mori densovirus 3 isolate VD2, complete genome. *GenBank Accession number DQ017269.*