PLAQUE ASSAY FOR *SPODOPTERA EXIGUA* AND *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUSES IN A NEWLY ESTABLISHED CELL LINE OF THE BEET ARMYWORM, *SPODOPTERA EXIGUA*

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Summary. – The nuclear polyhedrosis viruses of *Spodoptera exigua* (SeNPV) and *Autographa californica* (AcNPV) produced plaques in a newly established cell line of the beet armyworm, *Spodoptera exigua.* Plaques were composed of infected cells containing many polyhedra and were visible without any staining procedure. Dose-response assays showed a direct correlation between the number of plaques and the inoculum size. Growth kinetic studies of the two viruses, using the developed plaque assay system, revealed that the release of extracellular viruses began 6 hrs post infection (p.i.) and the titer reached a plateau 48 hrs p.i. The sensitivity of this plaque assay system was 100 times greater for the heterologous AcNPV than for the homologous SeNPV.

Key words: nuclear polyhedrosis virus; *Spodoptera exigua* cell line; plaque assay

Introduction

Baculoviruses constitute a complex group of large, enveloped, rod-shaped, double-stranded DNA viruses with genomes of 90 to 230 kbp. Of these, the nuclear polyhedrosis virus (NPV) is a member of the subfamily *Eubaculovirinae* (Francki *et al.*, 1991). This virus is considered to be a promising agent for control of insect pests in agriculture due to its high pathogenicity, restricted host range and safety for man, animals, plants and other non-target organisms (Maramorosch and Sherman, 1985).

The beet armyworm, *S. exigua,* is widely disseminated pest that causes severe damage to ornamental and vegetable crops (Trumble and Baker, 1984). It is difficult to control this insect due to its increased resistance to chemical insecticides. Thus SeNPV is a candidate for effective control alternative to conventional tools (Gelernter *et al.*, 1986; Smits and Vlak, 1988; Gaballero *et al.*, 1992).

SeNPV possesses very narrow host range both *in vivo* and *in vitro* (Vlak *et al.*, 1981; Gelernter and Federici, 1986*a*; Hara *et al.*, 1994). Recently Hara *et al.* (1993) achieved successful replication of SeNPV in continuous cell lines newly established from *S. exigua*, and the growth kinetics of the virus growth in these cell lines were analyzed by an end-point dilution method (Hara *et al.*, 1994).

In this paper, we established a plaque assay system for titration of SeNPV in our new cell line of *S. exigua*. We also show that the developed plaque assay method is available for infectivity titration of AcNPV.

Materials and Methods

Cell line. A continuous cell line from *S. exigua*, designated Se3FH (Hara *et al.*, 1993), was used in this study. Cells were grown in IPL-41 medium (Dougherty *et al.*, 1981) supplemented with 10% heat-inactivated foetal bovine serum (FBS). Cells were routinely seeded into 25 cm^2 tissue culture flasks (Falcon, 3013) at a density of 5×10^5 cells/ml in 4 ml medium, incubated at 27 °C, and subcultured at 4-day intervals.

Viruses. The SeNPV Isahaya isolate was originally obtained from Mr. K. Yokomizo (Nagasaki Agricultural and Foresty Experiment Station, Nagasaki, Japan). The virus was propagated in laboratory-reared *S. exigua* larvae as described by Hara *et al.* (1993). Early fourth-instar larvae of *S. exigua* were fed on artificial diet surface-contaminated with 1.0×10^6 polyhedral inclusion bodies (PIBs) per larva. Four days after inoculation, haemolymph,

containing extracellular virus, from several living larvae were pooled and centrifuged at 8,000 rpm for 10 mins. The supernatant was diluted 1:100 in IPL-41 medium and passed through a 0.45 μm membrane filter. The dilution (0.1 ml) was incubated with cells (0.4 ml/well) in exponential phase in multiwell tissue culture plate (Falcon 3014, cell density 6×10^5/ml). Glutathion (0.6 mg/ml), for preventing melanization, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, Gibco) were added. Three days after incubation, the infectious medium was harvested and centrifuged at 3,000 rpm for 10 mins. The supernatant (passage 1 virus) was stored at -80 °C until use.

AcNPV was supplied by Dr. S. Maeda (Faculty of Agriculture, Tottori University, Tottori, Japan). The virus was grown in Se3FH cells and the infectious medium was stored as described above.

Plaque assay. Se3FH cells in logarithmic phase were seeded into multiwell tissue culture plate (Falcon, 3014) at a density of 0.5 to 1.25×10^6 cells/ml (0.4 ml/well) in IPL-41 medium supplemented with 10% FBS and antibiotics. 0.1 ml of an appropriate virus dilution was added to each well. The plate was centrifuged at 1,800 rpm for 60 mins at 27 °C and the medium was removed. Finally, the cells were overlaid with 0.2 to 0.5 ml/well of overlay medium at 36 °C and incubated at 27 °C. Plaques were scored by microscopic examination 6 to 7 days p.i.

The overlay medium consisted of 9 parts of IPL-41 medium, containing 10% FBS and antibiotics, and 1 part of solidifying agent. The following three agents were tested for this purpose: (1) 0.75% Seaplaque agarose (FMC BioProduct), (2) 0.5% Agar Powder (Nakarai Tesque Inc.), (3) 0.5% Seakem agarose (Marine Colloids Inc.).

Plaque purification of the passage 1 virus was done on Se3FH cells. A single plaque was picked up by a Pasteur pipette from wells containing ≤ 10 plaques each. The plaque was suspended into 100 μl of IPL-41 medium and pipetted gently to release the virus. The suspension was centrifuged at 3,000 rpm for 10 mins and the resulting supernatant was diluted 1:100 in IPL-41 medium and applied to repurification of the virus. The repurified virus was stored at -80 °C until use.

Virus growth kinetics. Se3FH cells (0.6×10^6 ml) in logarithmic phase were distributed to wells of multiwell tissue culture plate (0.4 ml/well). The virus (0.1 ml/well) was then added to cells at a multiplicity of infection of 1.0 PFU per cell. The virus adsorption proceeded for 1 hr with centrifuging at 1,800 rpm at 27 °C. Subsequently, the cells were washed with culture medium and resuspended in fresh medium. At appropriate intervals, samples of infected cultures were taken and centrifuged at 3,000 rpm for 10 mins. The supernatant was stored at -80 °C until titration. The infectivity virus titers were determined by plaque assay as described above.

Results

SeNPV produced plaques clearly visible on the confluent monolayer of *S. exigua* Se3FH cells (Fig. 1A). Since the plaques were mostly composed of cells containing many polyhedra, they were visible without any staining procedure and enabled easier quantification and effective cloning of the virus. Se3FH cells also supported the replication of AcNPV and the virus produced visible plaques in Se3FH cells (Fig. 1B).

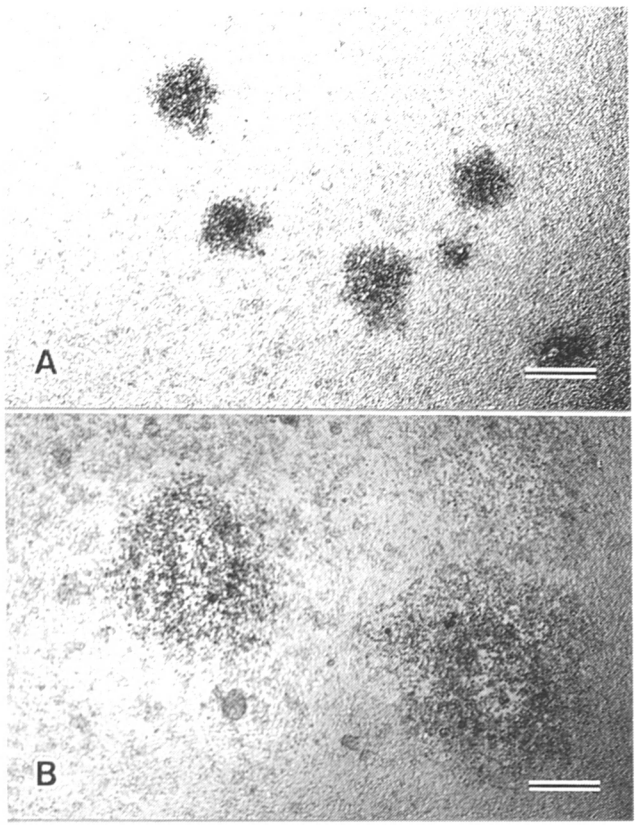

Fig. 1
Phase-contrast photographs of plaques of SeNPV (A) and AcNPV (B) in S. exigua Se3FH cells (Bar = 500 μm)

Table 1 shows the effect of cell density and volume on overlay medium on the formation of plaques. When four densities of the cells were tested, the largest number of plaques were obtained at a cell density of 1.0×10^6 cells/ml.

Table 1. Effects of cell concentration and volume of overlay medium on the number of SeNPV plaques formed on S. exigua Se3FH cells

Volume of overlay per well	Number of plaques[a]			
	Cell concentration in well (x 10^6/ml)			
	0.5	0.75	1.0	1.25
0.2 ml	10 ± 2	10 ± 4	23 ± 3	24 ± 6
0.3 ml	13 ± 4	6 ± 4	25 ± 6	17 ± 5
0.4 ml	9 ± 3	7 ± 3	17 ± 9	10 ± 5
0.5 ml	7 ± 4	4 ± 1	11 ± 3	7 ± 2

[a]Average of the number of plaques counted 6 days p.i. ± standard error.

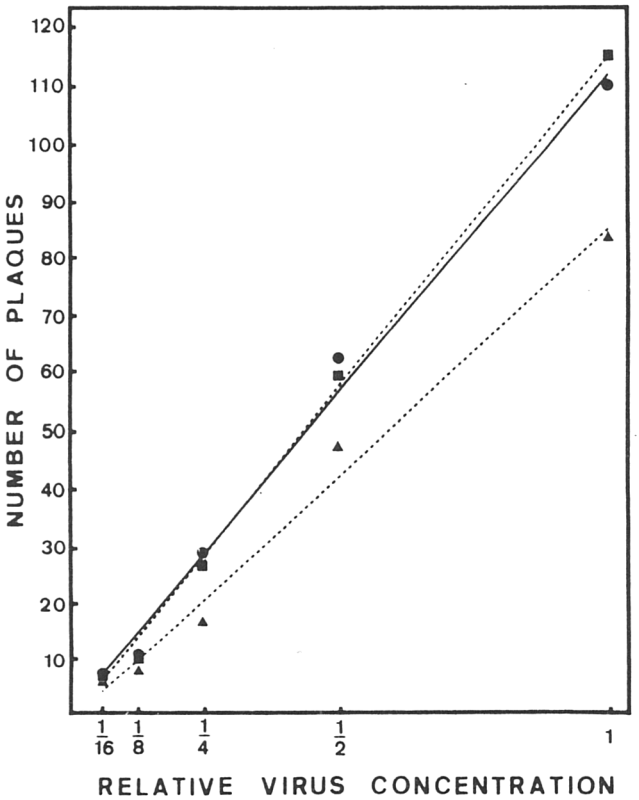

Fig. 2
Dose-response curves of SeNPV in S. exigua Se3FH cells
0.75% Seaplaque agarose (●), 0.5% Agar powder (■), 0.5% Seakem agarose (▲).

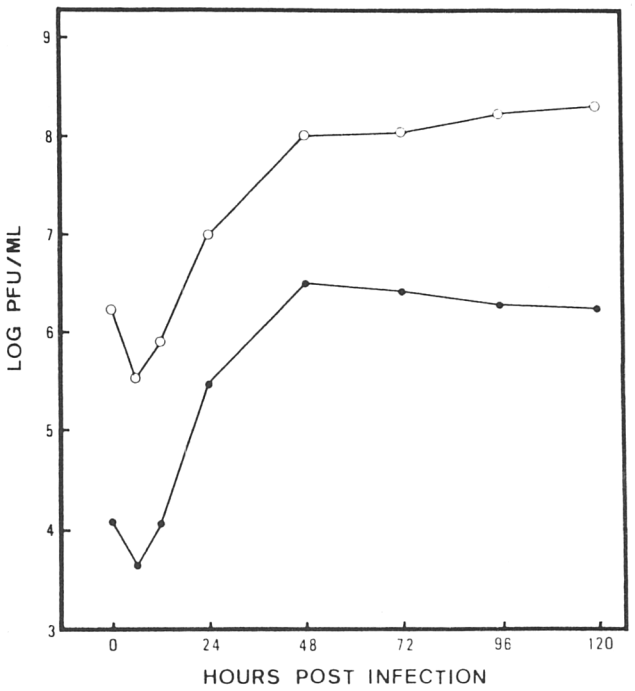

Fig 3
Replication of SeNPV and AcNPV in S. exigua Se3FH cells
SeNPV (●), AcNPV (○). The extracellular virus was monitored by plaque assay.

The plaques had a diameter of 0.48 ± 0.01 mm after 6 days of incubation. At the lower cell densities, the formation of cell monolayer was incomplete and there was a marked decrease in the number of plaques. The volume of overlay medium also greatly affected the formation of plaques. The largest number of plaques was produced, when the culture was covered with overlay medium of 0.3 ml/well. The use of larger overlay volume (≥ 0.4 ml) resulted in a decreased number of plaques.

With three different solidifying agents, dose-response studies were conducted to compare the sensitivities of plaque assays. As shown in Fig. 2, the number of plaques formed was proportional to the relative virus concentration in all tests. There was no significant difference in plaque number between agarose and agar.

A clone of the SeNPV, designated SeNPV-I1, was successfully isolated by plaque purification in the present study. Fig. 3 shows the comparative growth of SeNPV-I1 and AcNPV in Se3FH cells. The three phases (decrease, increase, and stationary phases) were clearly observed in growth curves of the both viruses. After the decrease phase of 6 hrs, extracellular titer of SeNPV-I1 began to increase and reached a maximum of $10^{6.5}$ PFU/ml 48 hrs p.i.

In the case of AcNPV, the titer of extracellular virus began to increase 6 hrs p.i. and reached the stationary phase 48 hrs p.i. The sensitivity of this plaque assay system was 100 times grater for the heterologous AcNPV than for the homologous virus.

Discussion

In the present study, we developed a plaque assay system for SeNPV, using a newly established cell line (Hara *et al.*, 1993) of the beet armyworm. This is the first report of the plaque formation of SeNPV on a cell line from the homologous host. Our data show that this plaque assay method is highly effective for titration and isolation of the virus, although several factors, including the property and the volume of overlay materials and the cell density, affect its sensitivity. The highest sensitivity was obtained when the test was done at the cell density of 1.0×10^6/ml with 0.3 ml of overlay medium containing 0.75% Seaplaque agarose.

Se3FH cells supported the replication of AcNPV as well and the virus produced clear plaques. The successful replication of AcNPV in *S. exigua* cells was consistent with the results reported by Gelernter and Federici (1986*a*). Moreover, the *in vivo* infection of AcNPV in *S. exigua* has been reported by several investigators, indicating broad host

range of AcNPV (Gelernter and Federici, 1986*b*; Smits and Vlak, 1988; Kondo *et al.*, 1994). The growth pattern of AcNPV in Se3FH cells was similar to that of SeNPV. In AcNPV infected *S. frugiperda* or *Trichoplusiani* cells, the virus had about 20 to 30 hrs exponential phase (Volkman *et al.*, 1976; Carstens *et al.*, 1979). Our results showed that AcNPV exhibited in *S. exigua* cells a relatively long exponential phase, namely about 40 hrs.

In this study, the sensitivity of plaque assay system was 100 times greater for the heterologous AcNPV than for the homologous SeNPV. In the test of infection rate of Se3FH cells to SeNPV, about 70% of Se3FH cells were susceptible to SeNPV (Hara *et al.*, 1993). However, Se3FH cells were more susceptible to heterologous AcNPV than to homologous SeNPV (data not shown). Thus, we assume that the higher sensitivity of this plaque assay system for AcNPV as compared to SeNPV was due to different virus susceptibility of Se3FH cells.

There have been several reports of genotypic variants in SeNPV isolated from beet armyworm larvae in California, Netherland, Egypt, Spain, and Japan (Hunter and Hall, 1968; Vlak *et al.*, 1981; Caballero *et al.*, 1992; Hara *et al.*, 1993; Kondo *et al.*, 1994). The plaque assay technique developed in this study facilitated the cloning of a Japanese isolate of SeNPV. The availability of the cloned SeNPV may allow the genotypic analysis of virus DNA using restriction endonucleases.

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