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A new *Bombyx mori* larval ovarian cell line highly susceptible to nucleopolyhedrovirus

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Abstract

Lepidopteran cell lines constitute the backbone for studying baculoviral biology *in culturo* and for baculovirus vector based recombinant protein expression systems. In the present study, we report establishment of a new continuous cell line designated as DZNU-Bm-1 from larval ovaries of the silkworm, *Bombyx mori*. The cells were grown in MGM-448 insect cell culture medium supplemented with 10% fetal bovine serum (FBS) and 3% heat inactivated *B. mori* haemolymph at 25 ± 1 °C. A large number of attached epithelial-like and round refractive cells migrated from the explants and multiplied in the primary cultures. Both type of cells were subcultured initially for a few passages but after 10 passages the round refractive cells dominated the population, which could be subcultured continuously using MGM-448 medium with 10% FBS. The population doubling time of cell line was about 42 h at 25 ± 1 °C. The cell populations were largely diploids and triploids, while a few tetraploids and hexaploids were also observed. DNA profiles using Inter Simple Sequence Repeat (ISSR)-PCR and Simple Sequence Repeat (SSR) loci established the differences between DZNU-Bm-1 cell line and most widely used BmN cell line and the *B. mori* W-chromosome specific sequences confirmed the origin of DZNU-Bm-1 cell line to be from female silkworm. When cells were infected with free nonoccluded *B. mori* nucleopolyhedrovirus (BmNPV), the cell line was found to be highly susceptible with 92–94% of the cells harbouring BmNPV and having an average of 20–23 OBs/infected cell. We suggest the usefulness of this cell line in BmNPV based baculoviral expression system and also for studying in culturo virus replication.
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Keywords: *Bombyx mori*; Ovarian cell line; DZNU-Bm-1; DNA fingerprinting; BmNPV infection; Baculoviral expression system

1. Introduction

Insect cell lines have become important tools in biological, biotechnological, biopharmaceutical, and biopesticide research. This is true with lepidopteran cells. They are commonly used to study insect viruses (Blissard, 1996) and have been considered for production of certain virus species as biopesticides or for recombinant proteins. Two cell lines from *Spodoptera frugiperda* (fall armyworm) and *Trichoplusia ni* (cabbage looper) are currently in use with baculovirus expression vector (BEV) derived from *Autographa californica* nucleopolyhedrovirus (AcNPV). However there are alternate baculoviruses, which are suitable for cloning of heterologous genes. *Bombyx mori* nucleopolyhedrovirus (BmNPV) is one such baculovirus, which is being used in BEV system for expression of recombinant proteins in susceptible cell lines (Maeda, 1987, 1989; Maeda et al., 1991). Further, cell lines derived from *B. mori* could be genetically engineered to continuously express high levels of foreign protein *in vitro* (Farrell et al., 1998, 1999). These studies show that, like AcNPV–*S. frugiperda*/*T. ni* system, BmNPV–*B. mori* cell lines are equally useful for development of BEV.

There are some established cell lines of *B. mori* originated from embryonic tissues (Chen et al., 1988; Imanishi et al.,

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1988; Inoue and Mitsuhashi, 1984; Ninaki et al., 1988; Pandharipande, 1994) and larval and pupal ovarian tissues (Grace, 1967; Quiot, 1982; Sudeep et al., 2002) but only a few can support replication of BmNPV. Agathos (1993) in his review reported that there is an urgent need for more efficient and more widely applicable cell lines as host for baculovirus replication and foreign gene expression. As it is almost a few decades since the establishment of most commonly used BmN cell line, there is a necessity to introduce a new cell line derived from a commercially successful variety of the silkworm. Such a cell line will be useful in study of BmNPV replication vis-à-vis BmN cell line with respect to effect of strains and effect of the age of the cell lines. Since BmNPV can infect larval ovary of *B. mori* and also transmits to next generation (Khurad et al., 2004), in the present study, a new cell line was developed from ovarian tissue of a commercial variety of the silkworm. We confirmed the origin of the cell line to be silkworm specific by SSR markers and a *B. mori* W-chromosome specific sequence (Nagaraja et al., 2005; Prasad et al., 2005; Reddy et al., 1999). We have also shown that this new cell line is highly susceptible to BmNPV infection.

2. Materials and methods

2.1. Silkworm

Healthy female fifth instar larvae of day one of 'Swarnandhra' variety were collected from the rearing stock maintained at Centre for Sericulture and Biological Pest Management Research (CSBR) of Nagpur University. This is a commercial variety reared in many parts of Andhra Pradesh, Karnataka, and West Bengal, India.

2.2. Primary cultures

About 10–15 female larvae were used to initiate primary culture in a 25 cm^2 Falcon culture flask. Each of the larvae was surface sterilized by submersion in 70% Ethyl alcohol for 2–3 min and dissected under a stereoscopic binocular microscope. The rudiments of ovary from the seventh and eighth segment were removed, washed in Carlson's fluid (Carlson, 1946) thrice and transferred into a cavity block containing culture medium. About 10–15 pairs of pooled ovaries were cut into small pieces and fragments were explanted in culture flask with culture medium. The culture medium used was the MGM-448 (Mitsuhashi, 1984) enriched with 10% fetal bovine serum (FBS) and 3% heat inactivated *B. mori* haemolymph (60 °C for 30 min). The cultures were maintained at 25 ± 1 °C in conditioned medium by replenishing half of the medium once a week.

2.3. Subculturing

The cells were detached by flushing the medium over the monolayer with Pasteur pipette and split into two flasks. Suspension containing cell masses and single cells were subcultured with split ratio of 1:2 using plastic (Falcon) and glass (Abico, Japan) culture flasks.

2.4. Growth analysis

Growth of the cell line was determined at 25th passage by seeding each culture flask with 2×10^5 cells/ml. On alternate day cell suspension was sampled from two culture flasks and cell number was counted using haemocytometer. Cell population doubling time was calculated using the exponential formula of Hayflick (1973). The viability was determined by dye exclusion test using Trypan blue stain. The cells were also adapted to MGM-448 with 10% fetal bovine serum (FBS) and cell growth was estimated.

2.5. Chromosome analysis

Demicolcine at a final concentration of 1 μg/ml was added to the cultures to halt divisions in metaphase and after 24 h cells were harvested, treated with hypotonic solution (0.6% KCl) and fixed in 50% glacial acetic acid for 10 min. Cell smears were stained with Acetorcein. Chromosome number and range were determined by counting 100–150 chromosome spreads.

For Giemsa staining, the cells were lysed in hypotonic solution (0.6% NaCl) and washed repeatedly in fixative (methanol:acetic acid in 3:1 ratio). After the final wash, cell smears were stained with Giemsa (0.7% (w/v) Giemsa in 1:1 methanol:glycerol) for two min, washed in distilled water and air-dried. Chromosome preparation was analysed using Cytovision software (Zeiss).

2.6. DNA profiling

DNA was isolated from DZNU-Bm-1, BmN and NIAS-MaBr-92, *Mamestra brassicae* (Mitsuhashi and Shozawa, 1985) cell lines by standard protocol of Nagaraja and Nagaraju (1995), with the exception that Proteinase K treatment was not given. The quality and quantity of the isolated DNA was confirmed by agarose gel electrophoresis.

SSR- and ISSR-PCR were carried out according to Nagaraju et al. (2001). The details of the primers used are given in Table 1. Briefly, SSR-PCR was performed using a Perkin–Elmer 9700 Thermal cycler in a typical PCR reaction of 10 μl containing 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 100 μM of each dNTPs, 400 nM of SSR primers, 1 unit of Taq polymerase (Perkin–Elmer Cetus) and 20 ng of template DNA with different annealing temperature and $MgCl_2$ concentration (Table 1). Bands were then separated by 2.0% agarose gel electrophoresis. Gel images were captured in Biorad gel documentation system.

For ISSR-PCR, reactions were carried out in a volume of 10 μl containing 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 100 μM of each dNTPs, 400 nM of ISSR primers, 1 unit of Taq polymerase (MBI Fermentas), 2.5 mM $MgCl_2$ and 20 ng of template DNA

Table 1
Primer sequence and PCR conditions of the DNA markers used to ascertain the identity of the DZNU-Bm-1 cell line

Sl. no.	Primer name	Primer sequence	Annealing temperature (°C)	$MgCl_2$ (mM)	Expected size[a] (bp)	Reference
ISSR-PCR						
1	Primer 1	ATAGAGCTGCTGCTGCTGCTGCT	50	2.5		
2	Primer 2	TGAGCGCCGCCGCCGCC	50	2.5		
3	Primer 3	$TGTAAT(GA)_8$	50	2.5		
4	Primer 4	$GCTAGTGCT(CA)_7C$	50	2.5		
SSR-PCR						
1	Bmsat172	F: AGGGATGATGGGTAAAGAGC R: GCAGTAGGCATTTGGAAGGAG	56	3.5	272	Prasad et al. (2005) and Nagaraja et al. (2005)
2	Bmsat173	F: CTCCTTATCCATCCGTTT R: CTCTCGGATCATAGATACG	50	3.0	158	
3	Bmsat110	F: TAGCGAGACTAACGAACAG R: TACTGATAGCGTGGCCTAT	58	2.0	90	
W-specific			60	2.5	427	FAO/IAEA[b]

F, forward primer; R, reverse primer.
[a] Not applicable for ISSR markers.
[b] Satish, V., Sriramana, K., Nagaraju, J., 2005. Molecular technologies to improve the effectiveness of the sterile insect technique. In: FAO/IAEA International Conference on Area-Wide Control of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques. Vienna International Centre, Vienna, Austria, held on 13–18 May 2005.

at an annealing temperature of 50 °C. The PCR products were separated on a 1.0% agarose gel.

Silkworm W-chromosome specific sequence was also amplified (Table 1) similar to ISSR-PCR except for annealing temperature and $MgCl_2$ concentration.

2.7. Virus inoculation

Turbid haemolymph of fifth instar larvae infected with BmNPV (Khurad et al., 2004) was collected through an incision on proleg. After centrifugation (3000 rpm, 10 min) the supernatant was diluted with equal volume of Carlson's fluid, passed through 0.45 μm membrane filter and used as an inoculum. The cultures were inoculated by adding 3–4 drops of the inoculum with a Pasteur pipette in a glass culture flask containing growing cells. The infected cultures were maintained at 25 ± 1 °C and examined every day for cytopathic effects and occurrence of occlusion bodies (OBs) in the nuclei. For the serial passaging of virus, healthy cells were inoculated with the addition of undiluted infected medium from the previous passage of the virus. Infected cells were centrifuged at 3000 rpm for 15 min to harvest the OBs, which were then resuspended in sterile distilled water, washed with 0.5% (w/v) sodium lauryl sulphate and rinsed thrice in distilled water.

3. Results and discussion

3.1. Primary cultures

The migration of cells from the explanted ovarioles started 24 h after setting of cultures. The active proliferation of cells was observed from the beginning and by 28 days the population was comprised of predominantly attached epithelial-like and partially attached small round refractive cells. The explants showed pulsation movement and migration of cells were continued for quite a long time. However, cells having long processes with regular contraction that was reported by Grace (1967) were not observed. Numerous cells migrated from the explants and eventually covered whole area of the culture flask. In one of the cultures, the number of cells increased sufficiently to subculture continuously. Mitsuhashi (2001) has reported that in the primary culture of *B. mori* pupal ovaries, small areas of cell sheets were initially developed, but the cell number declined after three months. In the present study, we observed a continuous growth of the primary cultures.

3.2. Subculturing

After about 36 days of setting the primary culture, first subculture was made and again after 10 days it was subcultured second time. However, thereafter the cell aggregates were formed and the proliferation was very much reduced for a considerable period. The cells grew very slowly from the aggregates, which were attached to the bottom of flask. In small cell masses active proliferation occurred after fifth subculture. The partially adhered refractive cells proliferated more actively than the attached epithelial-like cells and became predominant cell type after fifth subculture. However, they lost adherence nature after ninth subculture corresponding to a year after setting the culture and became suspended freely in the medium (Fig. 1).

3.3. Cell morphology, growth and chromosome number

Currently (March 2006) the cells have passed more than 945 days after the culture was set up and subcultured more

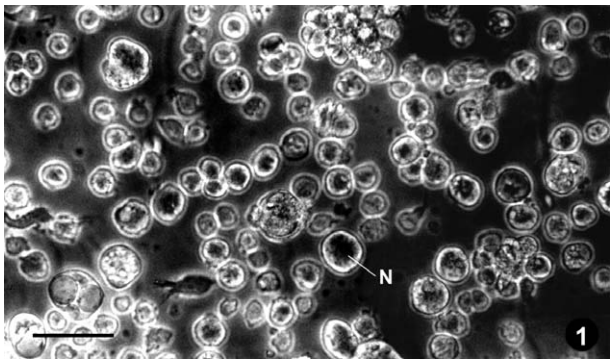

Fig. 1. DZNU-Bm-1 cell line showing freely suspended round refractive cells. N = nucleus. Bar = 60 μm (phase contrast).

than 100 times. The cell population consists of about 53% small round (14.25 ± 0.38 μm), 30% large round (28.0 ± 0.82 μm), 12% spindle shaped (51.0 ± 3.42 μm × 11.0 ± 0.51 μm) and about 5% giant cells (60.0 ± 3.82 μm). The cell growth was stable and high and the population doubling time was about 42 h with high viability (Fig. 2). The cell line was also adapted to grow in MGM-448 medium supplemented with 10% FBS without *B. mori* haemolymph supplementation and is being maintained in it. The chromosome analyses of cells revealed numerous microchromosomes and resembled to BmN cell line. The ploidy level of the cells could be clearly visualized showing the presence of polyploid cells in the culture. The diploid number of *B. mori* chromosomes has been known to be 56. At 25th passage the chromosome number of cells in this cell line was about 51–60. However, some small peaks in the frequency distribution of chromosome number suggested the presence of a considerable number of polyploid cells. Such highly polyploid cells

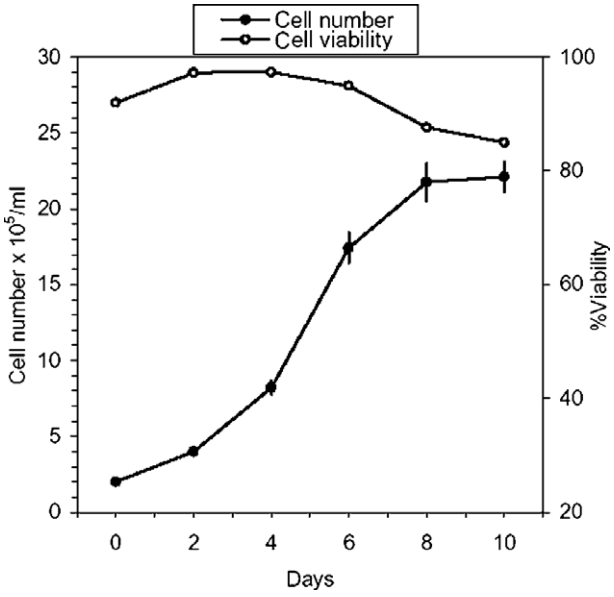

Fig. 2. Growth curve of DZNU-Bm-1 cell line. The cultures were maintained at 25 ± 1 °C in MGM-448 medium supplemented with 10% FBS.

were also observed in BmN cell line. These observations are consistent with the earlier report by Grace (1967) in *B. mori* ovarian cell line containing more than 100 chromosomes. We designated this cell line as DZNU-Bm-1.

3.4. DNA profiling

As DNA fingerprinting profile is a stable characteristic of the cell line irrespective of passage number of the cell lines and also serves as a valuable and reliable technique for identification of insect cell lines both at inter generic and intra generic levels (McIntosh et al., 1996), in the present study we carried out DNA profiling of DZNU-Bm-1 cell line in comparison with BmN and MaBr-92 cell lines.

Three *B. mori* specific SSR loci were used to confirm the identity of the new cell line. Two of these loci are W-chromosome specific (Bmsat172 and Bmsat173), while the other locus (Bmsat110) distinguishes the diapause and non-diapause strains. Bmsat172 and Bmsat173 gave expected female specific allele size of 272 and 158 bp, respectively, in both BmN and DZNU-Bm-1 cell lines (Table 1). A diapause specific Bmsat110 locus that is located on the autosome, amplified a specific product of size 90 bp that was found only in BmN cell line and not in DZNU-Bm-1 cell line (Fig. 3a) distinguishing the two cell lines. Since these SSR loci are specific to *B. mori*, as expected, amplification products were not observed in MaBr-92 cell line.

The ISSR-PCR profiles using four ISSR primers clearly distinguished the DZNU-Bm-1 and BmN cell lines (Fig. 3b). The band-sharing pattern of three of the ISSR-primers showed the differences between the amplified products from these two cell lines, while Primer 4 did not differ between the two cell lines. For example, two products of size 80 (Primer 1) and 150 bp (Primer 2) were specific to DZNU-Bm-1 cell line, while BmN had at least two bands of size 200–220 bp (Primer 1) that were absent in DZNU-Bm-1 cell line. The ISSR-PCR profiles also showed clear distinction of MaBr-92 cell line from the other two *B. mori* cell lines.

We also confirmed the female specificity of DZNU-Bm-1 cell line by amplification of a silkworm W-chromosome specific gene product. A 427 bp amplicon specifically amplified in DZNU-Bm-1 cell line and female *B. mori* genomic DNA, but not from male DNA (Fig. 4). Thus DNA profiling confirmed the origin of DZNU-Bm-1 to be from female silkworm.

3.5. BmNPV infection and serial passage

The DZNU-Bm-1 cells were successfully infected with BmNPV. At early stages of infection, the cytopathic effects such as hypertrophy of nuclei and clumping of cells were prominent. Numerous small and large clumps of cells were observed 16–18 h post inoculation (h.p.i.). At about 36–42 h.p.i. small refractive occlusion bodies (OBs) were appeared in the nuclei of cells. By 66 h.p.i. OBs were matured and were prominently seen in the nuclei of aggre-

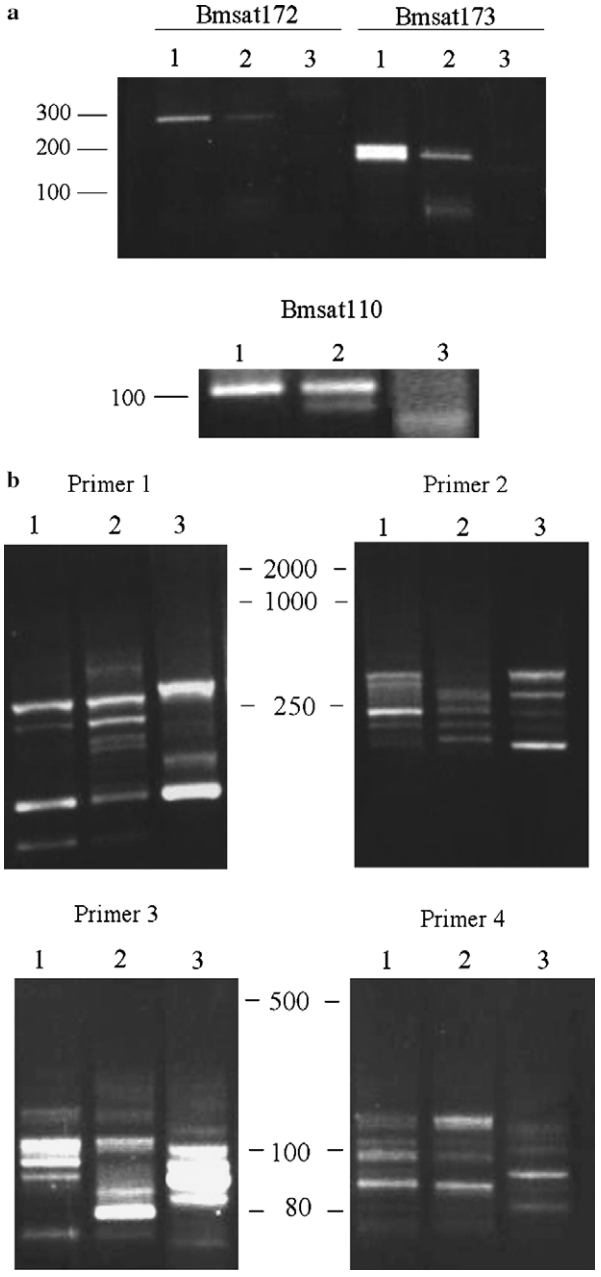

Fig. 3. DNA profiles of DZNU-Bm-1, BmN and NIAS-MaBr-92 cell lines. (a) SSR-PCR profiles of 3 lepidopteran cell lines using three microsatellite loci. Loci names are given above the gel image. Lanes 1–3 represent DNA from a Bm-1, BmN, MaBr-92 cell lines, respectively. (b) ISSR-PCR profiles of 3 lepidopteran cell lines using four ISSR primers. Primer names are indicated above the gel image. Lanes 1–3 represent DNA from Bm-1, BmN, MaBr-92 cell lines, respectively.

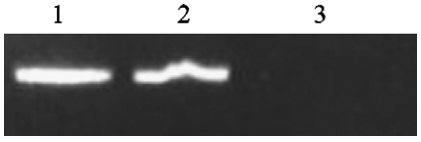

Fig. 4. Amplification of 427 bp *Bombyx mori* W-chromosome specific PCR product demonstrating female specific origin of DZNU-Bm-1 cell line. Lanes 1–3 represent DNA from DZNU-Bm-1 cell line, female *B. mori*, male *B. mori*, respectively.

gated cells (Fig. 5). Raghow and Grace (1974) have made similar observation on viral replication between 16 and 18 h.p.i. and formation of OBs by 40–48 h.p.i. in *B. mori* cells. Some of the cell aggregates were removed from the infected cultures and examined under microscope that exhibited 92–94% infected cells. The cells were loaded with 10–90 OBs/cell in the nuclei depending on the cell size. By 72 h.p.i. lysis of infected cells was initiated and the OBs were released to the medium.

Preliminary study of serial passage of BmNPV in the cell line for five times revealed that the nuclei of infected cells were harbouring on an average 20–23 OBs/cell and the yield of OBs was ranging between 1.0×10^7 and 1.65×10^7/ml when the initial cell number used was about $5.3–9.1 \times 10^5$ cells/ml for inoculation (Table 2). This suggests the high susceptibility of DZNU-Bm-1 cell line to BmNPV infection. The production of BmNPV in the present cell line was also quite high compared to existing available *B. mori* cell lines. Funakoshi and Aizawa (1988) reported around 64–73% cells form OBs in BmN cell line. Recently, Sudeep et al. (2002) and Pant et al. (2002) demonstrated 90 and 70% infection of BmNPV in the larval (BM-1296) and pupal (BM-197) ovarian cell lines of *B. mori* producing about 6.1×10^6 and 5.64×10^6 OBs/ml, respectively. However, they did not mention the initial number of cells inoculated for the production of OBs. Inoue et al. (1988) reported about 13.1–14.2% OBs forming cells in BoMo-15A embryonic cell line, whereas another embryonic cell line, Bm-21E was not found susceptible to the BmNPV (Chen et al., 1988). Pandharipande (1994) reported the susceptibility of the *B. mori* embryonic cell lines to BmNPV but did not mention the percentage of OBs forming cells in them.

The results indicate that DZNU-Bm-1 is a promising candidate for studying the BmNPV replication *in vitro* and can be utilized to express recombinant proteins using BmNPV derived expression vectors. However, further studies using an appropriate BmNPV expression system and comparative BmNPV replication assays are essential to confirm the utility of this cell line vis-à-vis BmN

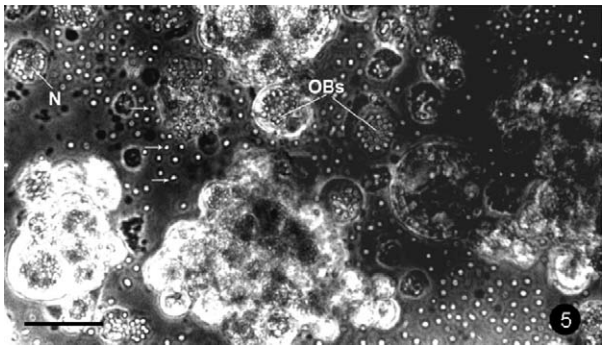

Fig. 5. DZNU-Bm-1 cells infected with *Bombyx mori* nucleopolyhedrovirus 72 h post inoculation showing clumps of infected cells, a few suspended cells containing occlusion bodies (OBs) in the nuclei and some lysed cells and released OBs in the culture medium (arrows). Bar = 60 μm (phase contrast).

Table 2
Five serial passages of *Bombyx mori* nucleopolyhedrovirus (BmNPV) in DZNU-Bm-1 cell line

Virus passage	Days p. i.	Cell passage	Cells per ml ($\times 10^5$)[a]	Percentage infection[b]	Infected cells per ml ($\times 10^5$)[c]	OBs per infected cell[d,f]	OBs per ml ($\times 10^7$)[e]
I	5	29th	5.34	94.18	5.02	23 ± 4	1.0
II	4	32nd	6.12	92.00	5.63	23 ± 3	1.24
III	4	35th	5.85	94.50	5.52	21 ± 2	1.0
IV	4	39th	3.24	86.47	2.80	20 ± 2	0.52
V	4	41st	9.18	91.00	8.35	23 ± 4	1.65

[a] Viable cells added to cultures at the time of inoculation.
[b] The presence of OBs in a cell was the criterion of its infection with BmNPV. Each value is the mean of samples of 300 cells each from two flasks.
[c] Calculated by multiplying the cell number by percentage infection.
[d] Mean number of OBs in infected cells counted randomly under microscope.
[e] Mean value based on samples of two flasks.
[f] Mean ± standard error (SE).

cells as well as other BEV systems like AcNPV–Sf9 combinations.

The cell line is being passaged regularly at an interval of 3–5 days and designated as DZNU-Bm-1. The cell line is now available for use and authors also wish to collaborate with interested researchers to further evaluate and to develop a cell culture system for expression of foreign proteins.

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