Susceptibility of a Cloned Cell Line from *Helicoverpa armigera* to Homologous Nucleopolyhedrovirus

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***Abstract*—A cloned cell line designated B-5 was isolated from the parental HAPO2 cell line, which derived from pupal ovarian tissues of *Helicoverpa armigera*. This cell line was cultured in Grace's medium supplemented with 10% fetal bovine serum (FBS), and continuously subcultured at 4-day intervals. The population doubling time of this cloned cell line during the logarithmic phase was 22.8 h. RAPD and DAF analyses confirmed the identity of the B-5 cell line as *H. armigera* and clearly distinguished it as a new, unique cell line derived from the pupal ovaries. The susceptibility of B-5 cells to the *H. armigera* nucleopolyhedrovirus (HearNPV) was high and a higher yields of occlusion bodies (OBs) were obtained when compared to the parental HAPO2 cells. Infection of HearNPV in B-5 cells was successfully completed and produced the infectious OBs. Ultrastructural studies of infected *H. armigera* tissues revealed evidence of virus infection and OBs formation in the infected fat cells. The results and observations strongly indicated that B-5 cells were fully permissive for HearNPV infection. The data described in this study indicate that B-5 cell line is a highly productive new cloned cell line and will be useful for the production of HearNPV, and other applications in biotechnology.**

***Index Terms*—*Helicoverpa armigera*, nucleopolyhedrovirus, virus replication, insect cell culture, single-cell cloning, cell line characterization**

I. Introduction

The baculovirus HearNPV has been used to control *H. armigera*, one of the most serious insect pests of many economically important crops in the world including Thailand [1]-[4]. NPVs represent a group of insect virus in the Family Baculoviridae [5] whose virions are embedded into polyhedron-shaped occlusion bodies (OBs) or polyhedra in the nuclei of host cells. However, the practical use of HearNPV as bioinsecticides has been rather limited. Several key reasons explain the restricted use of viral insecticides [6], [7]. These include inadequate formulation and application technology, the difficulty of production, the problems of registration and patentability, the slow speed of kill, and the limited host-range of many isolates. The viral insecticides can be produced in both in vivo and in vitro systems. The conventional method used to produce HearNPV for biological control purposes still employs the infection of large numbers of susceptible larvae [2], which is variable potency, high production costs and it is difficult to scale up economically. In vivo production of HearNPV faces a problem of strong cannibalism behavior of *H. armigera* larvae. The larvae must be reared and inoculated individually on an artificial diet. Thus, in vivo mass production of HearNPV is extremely labor intensive, time consuming and adds greatly to the final cost of the product [7]. However, the possibility of using insect cell culture to develop alternative multiplication processes has been proposed [8]. Increasing OBs yield in insect cell culture is the key challenge to enable commercialization of in vitro production of HearNPV bioinsecticide [9].

The successful replication of HearNPV in *H. armigera* (HA) and *H. zea* (HZ) cell lines has been reported [10], [11]. Serial passage of baculoviruses in cell culture causes a change from the many polyhedra (MP) to the few polyhedra (FP) phenotype. FP mutants are characterized by fewer cells containing polyhedra, fewer polyhedra per cell, polyhedra aberrant morphology and fewer or no viruses per polyhedron [12]. Serial passage of HearNPV in HZ cells leads to point mutations in the HearNPV *fp25k* gene, and the relationship between this mutation and the virulence of the virus has also been reported [11], [13]. In contrast, serial passage in HAPO2 cells showed a slower accumulation of FP variants when compared to the use of HZ-AM1 cell line and no mutation was found in the *fp25k* gene [11].

The selection of high producing clones from a heterogeneous cell population is important to increase production yield and stability of HearNPV. In the present

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study, a clonal isolate of the HAPO2 cell line was compared with the parental cell line in cell characters and susceptibility to HearNPV. Ultrastructural studies on virus morphogenesis in the infected larval tissues of *H. armigera* were also described.

II. Materials and Methods

A. Cell Line

The HAPO2 cell line derived from pupal ovarian tissues of *H. armigera* [10], [11] and BCIRL-HZ-AM1 cell line derived from pupal ovarian tissues of *H. zea* [14] were grown as monolayers in 25-cm^2 culture flasks. Cultures were maintained in Grace's medium [15] supplemented with 10% FBS, incubated at 27^{o}C and subcultured at 4-day intervals.

B. Virus

The MP variant of HearNPV was plaque-purified from a wild isolate of Thai HearNPV and designated HearNPV PCB1 [11]. The inoculum was obtained by infecting HAPO2 cell monolayers with budded viruses (BV) from the passage 2 at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU)/cell [11]. The medium containing BV was harvested on day 6 postinfection (pi), and determined the titer by plaque assay on HZ-AM1 cells following standard procedures [16].

C. Single-Cell Cloning

Single-cell cloning by limiting dilution [17] was used to generate individual clones from HAPO2 cells. Cells in the mid-logarithmic phase of growth were collected and diluted to 1 cell/200 µl with Grace's medium supplemented with 10% FBS. The diluted cell suspension was then added to each well of the 96-well plates at a seeding density of a single cell per well. A total of 3 plates were seeded. Cells were allowed to attach for 1 h, then wells were observed under an inverted microscope and marked for presence of single cells. Wells containing single cells were fed with Grace's medium supplemented with 10% FBS, 0.33% lactalbumin hydrolysate and 0.33% yeastolate to support cell proliferation [18], [19], then cell survival and proliferation were observed weekly. After reaching confluency, the selected clones were expanded to wells of 6-well plates and then further expanded to 25-cm^2 culture flasks. The cloned cell lines or clonal cell lines were routinely maintained under conditions described for the parental cell line.

D. Growth Kinetics

The kinetics of cell growth were determined at passages 30 by recording cell densities for 7 days. The culture of cloned cells derived from the parental HAPO2 cell line were seeded at 2×10^5 cells/ml in 5 ml of Grace's medium supplemented with 10% FBS into 25-cm^2 culture flasks. The cell density and viability were determined at 1-day intervals by using a hemocytometer and trypan blue exclusion assay [20]. A cell growth curve was generated from the average of three independent experiments. An exponential regression was used to calculate the population doubling time during the logarithmic phase of growth [21].

E. DNA Extraction

Cells of the selected clones grown in the logarithmic phase were harvested from the culture flask and pellet was washed twice in phosphate-buffered saline (PBS). Total genomic DNA was extracted using PureLink® Genomic DNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Similarly, DNA was also extracted from other insect cell lines maintained in the laboratory. These cell lines were as follows: HZ-AM1 [14], cell lines derived from *H. armigera* pupal ovaries (HAPO1, HAPO2), adult ovaries (HAAO1), and embryonated eggs (HAEE1) [10]. For comparison, DNA was extracted from tissues of *H. armigera* including embryonic tissues derived from the embryonated eggs, ovarian tissues derived from pupal and adult ovaries.

F. Cell Line Characterization

In order to identify a cloned cell line established in this study, two polymerase chain reaction (PCR)-based methods including random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) were performed. Two 10-mer random primers No. 212 (5'-TTG CTG TCC A-3') and No. 273 (5'-GCT GAC CTG T-3') were used for RAPD-PCR as described by [22]. DAF-PCR was performed with primers aldolase 1 (5'-CCG GAG CAG AAG AAG GAG CT-3') and aldolase 2 (5'-CAC ATA CTG GCA GCG CTT CA-3') as described by [23]. Reaction components and conditions were as described previously [24]. Negative controls in which DNA was replaced by sterile distilled water were included in every set of reactions to ensure that there was no carryover contamination. The PCR products were subjected to agarose gel electrophoresis using 1X Tris-Borate-EDTA (TBE) buffer to determine the size, and DNA banding patterns.

G. Virus Susceptibility

Monolayers of the cloned cells were infected with HearNPV at a MOI of 1 PFU/cell, the virus inoculum was allowed to adsorb for 1 h at room temperature and then replaced with fresh medium. Time zero was defined as the time when the inoculum was replaced with fresh medium. The cultures were observed daily for evidence of cytopathic effect (CPE), culture medium was collected and then subjected to plaque assays for determination of BV titers [16]. The yields of OBs derived from infected cells were determined at 7 days pi as described previously [11]. In parallel experiments, the yields of BV and OBs derived from infected parental cells were quantified. PCR analysis was also performed in order to confirm the presence of viral DNA in HearNPV-infected cells. Total DNA was isolated at specified times from virus-infected and mock-infected cells by phenol/chloroform/isoamyl alcohol extraction [16] and subjected to PCR using specific primers for the HearNPV polyhedrin (*polh*) gene. HearNPV DNA isolated from alkaline-treated OBs [11] was used as positive control for PCR amplification.

Reaction components, PCR conditions and agarose gel electrophoresis were as described previously [11].

H. Scanning Electron Microscopy

The mock-infected and HearNPV-infected cells were harvested by using a rubber policeman, pelleted at 1000g and washed twice in PBS. Pellets were resuspended and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, for 2 h at 4 ℃, postfixed in 1% osmium tetroxide for 1 h and washed twice with PBS. Samples were then seeded onto stubs and were allowed to air dry. Then stubs were coated with gold and observed under scanning electron microscope.

I. Transmission Electron Microscopy

Third instar *H. armigera* larvae were fed on formalin-free artificial diet [25] that had been contaminated with OBs derived from HearNPV-infected cells at a concentration of $1x10^6$ OB/cm^2 diet. Three days after inoculation, the larvae were dissected, fat body tissues were removed and then fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, for 2 h at 4 ℃, postfixed in 1% osmium tetroxide for 1 h and were washed twice with phosphate buffer. After washing, the samples were sequentially dehydrated in acetone, infiltrated and embedded in Epon resin. Samples were cut with ultramicrotome, and observed under transmission electron microscope.

III. Results and Discussion

A. Establishment of Cloned Cell Lines

A total of 185 wells containing a single cell were obtained by limiting dilution cloning from the parental HAPO2 cell line. The cells were maintained, observed for cell proliferation and clone formation. However, the healthy-proliferating cells were seen in 104 wells. After reaching confluency, the cells were then plated into individual wells of 6-well plates. Many of the isolates with highly vacuolated cytoplasms eventually died during subculturing. Sixteen clones were selected based on their morphological characteristics and their proliferative ability. These cloned cells were independently expanded into 25-cm^2 culture flasks. Two rapidly growing cloned cell lines, B-5 and D-4, were found to be susceptible to HearNPV. However, the B-5 cell line was more susceptible to HearNPV infection than D-4 cell line, presenting higher infection level and high number of OBs produced per cell. Therefore, B-5 cell line was selected for further studies.

The clone B-5 originated from a single cell after plating the cell suspension of HAPO2 cells into 96-well plates (Fig. 1A). Cell adhered well to the plate within 1 h and cell proliferation occurred within a week of cell cloning (Fig. 1B). Subsequently, cell division was mostly observed 2 weeks after isolation (Fig. 1C), the number of cells started to increase slowly (Fig. 1D). These cells formed adherent monolayers (Fig. 1E), and after 4 weeks of culture in medium under the conditions described above, clone formation was achieved (Fig. 1F).

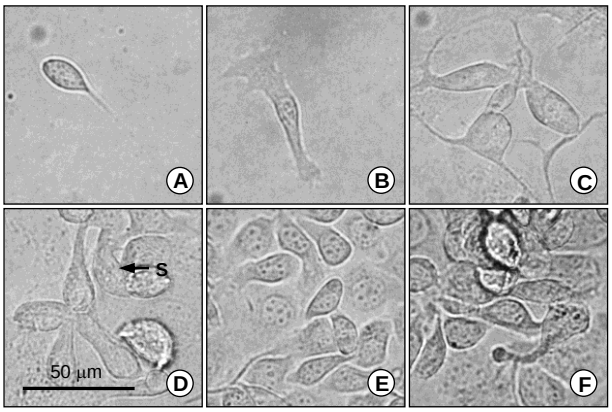

Figure 1. Development of the B-5 cloned cell line derived from single cell cloning of the parental HAPO2 cell line. (A) Single cell after seeding. (B) Cell proliferation. (C) Cell growth and division. (D) A progressive increase in cell number. (E) Adherent cell monolayers. (F) Multilayered superconfluent cultures.

The first successful subculture was obtained after 4 weeks of culturing when cells in the well became confluent. The adherent cells were gently dislodged from the well and were subsequently transferred into 6-well plates. The cells were allowed to grow until they reached 80% confluency. Within 6 weeks, the cloned cells were transferred into a 25-cm^2 culture flask. For routine subculturing, the flasks were tapped gently to detach the cells, then cell suspension was transferred into a new flask. Adherent cells were supplied with fresh medium.

Single-cell cloning is an important step in generating a homogeneous cell line for further study. In the present study, the most successful survival and growth of a single cell was achieved in Grace's medium supplemented with FBS, yeastolate and lactalbumine hydrolysate. The first subculture was obtained 4 weeks after isolation. A use of conditioned medium was found to be unnecessary for encouraging the growth of the isolated single cell. Usually, cell growth depends on cell density, and cell cannot proliferate below critical cell densities [26]. In insect cell culture, growth conditions for maintaining the cloned cell lines from single cell cloning have been described [17], [27]-[29]. Successful cloning requires the addition of conditioned medium [26], [30]. Using yeastolate and lactalbumine hydrolysate accomplishes the same goals as adding conditioned medium. There have been several reports describing the important effects of protein hydrolysates on cell survival and growth [18], [31]-[33]. This study reports the successful cloning of *H. armigera* cell line utilizing the limiting dilution method. The results obtained in this study suggest that fresh medium supplemented with FBS and protein hydrolysates can be used as the cloning medium. Therefore, it is reasonable to suggest that the same method as utilized in establishing *H. armigera* B-5 cell line may be useful in establishing other insect cell lines.

B. Characteristics of Cloned Cell Lines

After the 10^{th} passage, the cells exhibited stable growth and proliferation. Morphological studies carried out at passage 30 showed that the B-5 cell line comprised a majority of spindle-shaped cells while epithelial-like cells

were present in only a minority of the cell population (Fig. 2A-B). The average cell sizes were determined from measurements of 100 cells. The spindle-shaped cells were predominant, and the average cell sizes were 10.21 ± 0.16 μm in width, and 42.87 ± 1.85 μm in length. The epithelial-like cells have a mean diameter of 15.28 ± 0.45 μm. Cell clumping was observed when cultures reached full confluency (Fig. 2C). The morphological characteristics of B-5 cells as revealed by light and scanning electron microscopy were similar. Two basic cell shapes can be distinguished in the B-5 cell line, spindle-shaped cells and epithelial-like cells (Fig. 2A-B, 3A-B). Cells of both types grown in monolayers attached to the culture flask. When the cultures were subconfluent, actively proliferating cells were observed (Fig. 2A, 3A). As cells grown beyond confluency, the morphology of spindle-shaped cells changed slightly, becoming more rounded (Fig. 2B-C, 3B-C). Multicellular aggregates were observed in B-5 cell culture as cell density approached confluency (Fig. 2C, 3C). The observed clumping may result from a low level of contact inhibition, and that may contribute to the observation of high cell density in adherent insect cell cultures. Contact inhibition shown by various mammalian cell lines [34] is low or absent for insect cells, and they tend to aggregate in suspension or in adherent cultures. Therefore, overgrown cells pile up and formed multilayers, or detach from the culture vessel and float in the medium [35].

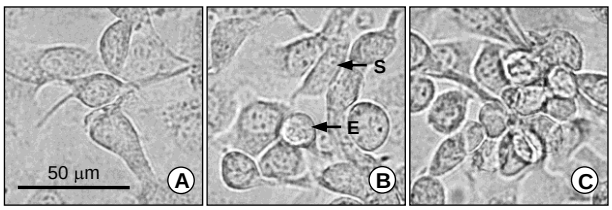

Figure 2. Micrographs of the cell characteristics of B-5 cell line. (A) Subconfluent monolayers. (B). Confluent monolayers. (C) Multicellular aggregates as cell density approached confluency. E, epithelial-like cells; S, spindle-shaped cells.

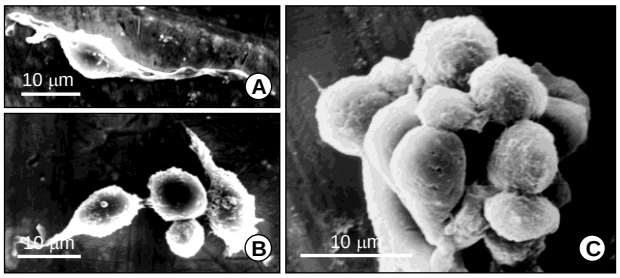

Figure 3. Scanning electron micrographs of the B-5 cell line. (A) Actively proliferating cells in the logarithmic phase. (B) Morphological changes observed in spindle-shaped cells as cell densities were confluent. (C) Cells in the stationary phase formed aggregates.

C. Growth Kinetics

Growth pattern of B-5 cells in monolayer culture was characterized, and the results were compared with those of the parental HAPO2 cells and *H. zea* cells (Fig. 4). Cell density increased by 1 day after seeding, indicating that the seeded cells were able to adapt to the culture environment and preparing for fast growth. By 2-4 days of growth, cells increased exponentially and reached a maximum cell density of 1.6×10^6 cells/ml on day 4, then entered the stationary phase. At the stationary phase, high density culture and the absence of nutrients caused a change in cell shape (Fig. 2B-C, 3B-C) and cells ceased growing (Fig. 4). The growth curves for B-5, HAPO2 and *H. zea* cell lines were similar. From the growth curves, the B-5 cells showed a faster growth rate than that of the parental HAPO2 and *H. zea* cells (Fig. 4) with the population doubling time of 22.8 h. The population doubling times for HAPO2 and *H. zea* cells were 23.2 and 23.7 h, respectively and the maximum cell density were 1.4×10^6 cells/ml and 1.1×10^6 cells/ml, respectively. Insect cell lines derived from the same species but from different tissues tend to differ in their growth characteristics. The population doubling time of B-5 cell line was shorter than that of other *H. armigera* cell lines that have been established from different tissues, NIV-HA-1195 (hemocyte) [36] and HNU-Ha-MG1 (midgut) [37] cell lines. The population doubling time for NIV-HA-1195 and HNU-Ha-MG1 cell lines were 60 h and 58.6 h, respectively. Thus, this newly cloned cell line from *H. armigera* is a fast growing cell line and a promising candidate for future insect-cell based applications.

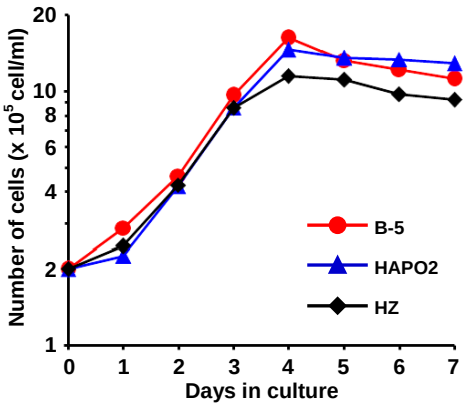

Figure 4. Growth curve of the B-5 cell line in comparison to their parental HAPO2 and *H. zea* cell lines.

D. Characterization of the Cell Lines

Characterization of the cell lines was carried out using RAPD-PCR and DAF-PCR. As shown in Fig. 5, the RAPD analysis showed identical DNA banding profiles for the cloned cell lines (B-5 and D-4) and the parental HAPO2 cell line. These results indicated that the B-5 cell line was derived from the HAPO2 cell line. The HAPO1 and HAPO2 cell lines were independently isolated from *H. armigera* pupal ovaries. The nearly identical DNA banding profiles with only minor differences in band intensity were obtained for these two cell lines, using primers No. 212 and No. 273 (Fig. 5). In contrast, the DNA banding profiles mentioned above were quite distinct from those of other *H. armigera* cell lines including the cell lines from adult ovaries (HAAO1), and embryonated eggs (HAEE1) (Fig. 5). As illustrated by the DNA banding profiles obtained with primers No. 212 and No. 273 (Fig 5), the primer No. 273 was a suitable primer

for distinguishing cell lines derived from *H. armigera*. The primer No. 273 was able to differentiate and confirm the identity of the original sources of the B-5 cell line from *H. armigera*. However, the amplified DNA fragments were different in cell lines from different tissues of *H. armigera*. The major bands identified were shared between the cell lines and their originating host tissues. DNA banding profile differences in cell lines and their host tissues have been reported [38], [39]. The additional bands in the cell line compared with the host tissue may be a reflection of the heteroploid nature of lepidopteran cell lines. In addition, the DNA banding profiles mentioned above were significantly different from those of other cell lines including the cell lines from *H. zea*, *Spodoptera exigua*, *S. frugiperda* and *S. litura* [24]. The efficiency of RAPD-PCR has been previously reported as an effective tool to authenticate cell lines that are taxonomically close [40], [41]. The results of the present study support the use of RAPD-PCR analysis as an effective and inexpensive technique for identification of cell lines originating from different tissues and developmental stages of *H. armigera*.

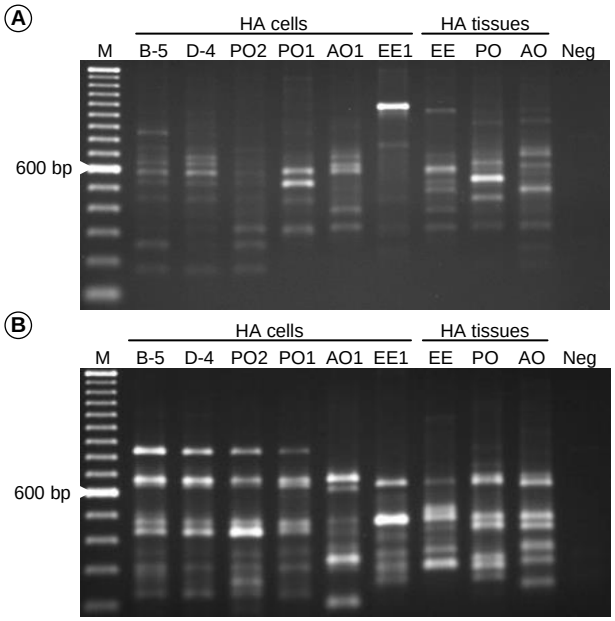

Figure 5. RAPD-PCR profiles of insect cell lines obtained with primers No. 212 and 273. (A) Primer No. 212. (B) Primer No. 273. The PCR products were electrophoresed on a 1% agarose gel along with 100-bp DNA ladder (M) with an arrowhead indicating band of 600 bp. HA, *H. armigera*; AO, adult ovaries; EE, embryonated eggs; PO, pupal ovaries; Neg, negative control.

Species identity of the B-5 cell line was confirmed by using DAF-PCR with aldolase primers. Two cloned cell lines (B-5 and D-4) produced identical patterns to the parental HAPO2 cell line (Fig. 6). Differences were observed among cell lines derived from different tissues of *H. armigera*. Two major bands at approximately 180 and 226 bp were shared by B-5, D-4, HAPO2 cell lines and *H. armigera* pupal ovaries. A major band at approximately 180 bp was also observed in HAAO1 cell line and *H. armigera* adult ovaries, while a major band at approximately 226 bp was also observed in HAPO1, HAEE1 cell lines and *H. armigera* embryonated eggs. Additionally, the B-5 cell line had patterns that were distinct from all other cell lines including the cell lines from *H. zea*, *S. exigua*, *S. frugiperda* and *S. litura* [24]. The same results were obtained when DAF-PCR was repeated at least three times demonstrating the reproducibility of the technique. Therefore, the DAF profile analysis confirmed the identity of the B-5 cell line as *H. armigera* and clearly distinguished it as a new, unique cell line derived from the pupal ovaries. This study also revealed no cross-contamination among the insect cell lines maintained in the laboratory.

The need to identify specific insect cell lines quickly and reliably, using the PCR-based methods such as RAPD and DAF is a crucial element of good quality control both in the laboratory and in industry. These methods are widely used for the identification of insect cell lines as well as for the detection of a cross-contaminated cultures [22], [23], [42], [43].

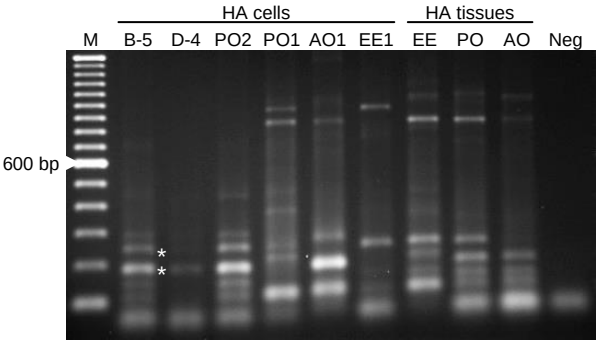

Figure 6. DAF-PCR profiles of insect cell lines obtained with aldolase primers. The PCR products were electrophoresed on a 1% agarose gel along with 100-bp DNA ladder (M) with an arrowhead indicating band of 600 bp. Asterisks indicate the major bands. HA, *H. armigera*; AO, adult ovaries; EE, embryonated eggs; PO, pupal ovaries; Neg, negative control.

The PCR-based methods used in the present study were able to distinguish inter- and intraspecies cell lines. They can be also applied to distinguish the cell lines originated from different tissues and the developmental stages of *H. armigera* (Fig. 5, Fig. 6). This pattern could be reproduced. Availability of this base to laboratories involved in cell culture allows fast and easy identification of individual cell lines by comparison to reference profiles as well as comparison of similar lines from different sources.

E. Susceptibility to HearNPV Infection

For determining the susceptibility of the B-5 cell line to HearNPV, the infectious medium containing BV was used as an inoculum. HearNPV-infected B-5 cells did exhibit altered morphologies at 1 day pi. Typical CPEs like granulated and rounded cells, nuclear hypertrophy and impairment in cell proliferation were observed. The CPEs developed rapidly with subsequent OBs formation. The HearNPV replicated in both epithelial-like cells and spindle-shaped cells and OBs were clearly seen within 1 day pi (Fig. 7). These cells became loosely attached to the substrate. The susceptibility of this cell line to the HearNPV was high and by 4 days pi greater than 90% of the cells contained OBs and some of the infected cells

were dislodged from the culture flask (Fig. 7). At 7 days pi, infected cells were filled with OBs and some cells ruptured. At late stages of infection (8 days pi), almost infected cells lysed and released OBs into culture medium.

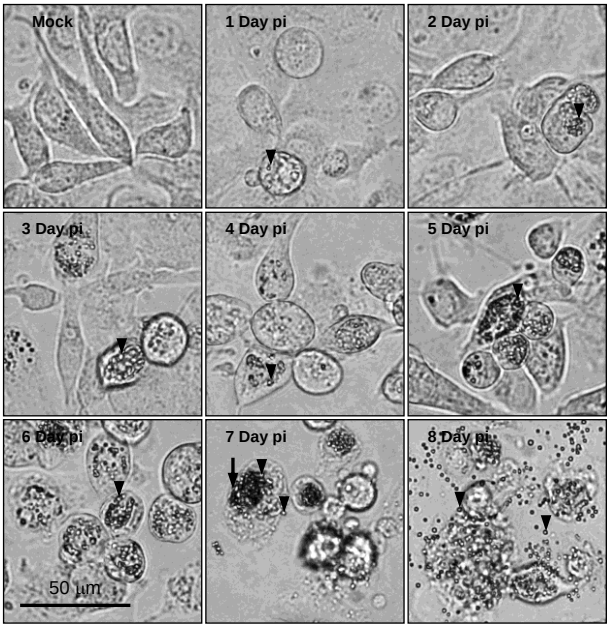

Figure 7. Cytopathology and occlusion bodies (OBs) formation in HearNPV-infected B-5 cells. At 7 days pi, infected cells were filled with OBs (arrowheads) and some cells ruptured (arrow). At 8 days pi, almost infected cells lysed and released OBs into culture medium.

To determine if the B-5 cells were fully permissive and produced progeny virions, culture medium was collected at 1-day intervals until 7 days pi and progeny virus was quantified by plaque assay. For comparison of virus yield, the parental HAPO2 cells were infected and analyzed in parallel. The results revealed that there was no differences in the kinetics of BV production generated from both cells. The highest BV titer from infected B-5 cells and parental HAPO2 cells peaked at 1.1 x 10^7 PFU/ml by 7 days pi. OBs production from the infected B-5 cells and HAPO2 cells were 6.4 x 10^7 OB/ml and 5.9 x 10^7 OB/ml, respectively. These results indicated that B-5 cells were more susceptible to HearNPV infection than its parental HAPO2 cells. Scanning electron microscope observation revealed an increase in cell size by 2 days pi (Fig. 8B). The hypertrophy of infected cells observed by electron microscope could result from accumulation of OBs as observed under light microscope (Fig. 7). OBs were formed and packed in the nuclei which caused the cellular hypertrophy. These OBs were released upon cell lysis into culture medium (Fig. 8C).

To determine the presence of viral DNA in HearNPV-infected cells, a set of primers specific to the HearNPV *polh* [11] was used. The primers successfully amplified the *polh* region, generated a 1029-bp PCR product (Fig. 9). Viral DNA production and accumulation in B-5 cells were detectable as early as 1 day pi. Collectively, these results confirmed successful HearNPV infection. The present study provides more informations on the in vitro production of HearNPV and susceptibility of its homologous cloned cell line.

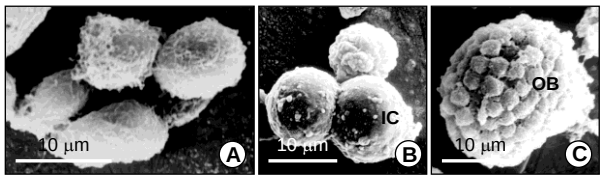

Figure 8. Scanning electron micrographs of HearNPV-infected B-5 cells. (A) Mock-infected cells. (B) At 2 days pi, infected cells (IC) appeared larger. (C) At 7 days pi, some infected cells ruptured to release occlusion bodies (OBs) into culture medium.

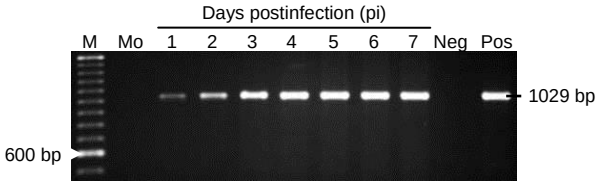

Figure 9. Agarose gel electrophoresis of PCR products amplified by using specific primers for the HearNPV *polh* region. DNA was isolated from mock-infected cells (Mo) and virus-infected cells at various times pi. The PCR products were electrophoresed on 1% agarose gels along with 100-bp DNA ladder (M). Position and size of the PCR products are shown on the right. Neg, negative control; Pos, positive control.

F. Infectivity of OBs in Host Insects

The biological activity of HearNPV in killing the larvae was studied by feeding OBs produced in the infected B-5 cells to third instar *H. armigera* larvae. Transmission electron micrographs revealed evidence of virus infection in the infected fat cells (Fig. 10). Enlarged nuclei and peripheral displacement of the cell chromatin along the nuclear membrane were observed (Fig. 10A). Concomitant with chromatin margination was the appearance of an electron-dense virogenic stroma (Fig. 10B). Subsequently, numerous naked virus rods or nucleocapsids appeared in the nucleus and were closely associated with the virogenic stroma (Fig. 10C). Many virus particles, in addition to single virus rods, were partially or completely enclosed by the inner nuclear membranes. These virions (enveloped nucleocapsids) were scattered throughout the nuclei. A few developing OBs and accumulations of the fibrous substances were seen in some infected cells (Fig. 10C). Several virions were occluded by a crystalline matrix of polyhedrin protein forming a polyhedral inclusion body or OB. During this stage of infection, virions were partially or completely occluded within OBs. Viral envelopes of particles that were partially occluded into OBs were often compressed. Late in the infection, OBs with embedded virions and the polyhedron envelope (PE) were formed (Fig. 10D).

The HAPO2 cell line was established from pupal ovarian tissue of *H. armigera* and the B-5 cell line was clonally derived from HAPO2 cells. The B-5 cell line was confirmed to have originated from *H. armigera* by both RAPD and DAF analyses as shown in Fig. 5 and Fig. 6. The PCR-based methods used in the present study were able to distinguish inter- and intraspecies cell lines,

and also applied to distinguish the cell lines originated from different tissues and developmental stages of *H. armigera*. Infection of HearNPV in B-5 cells was successfully completed as in vitro.

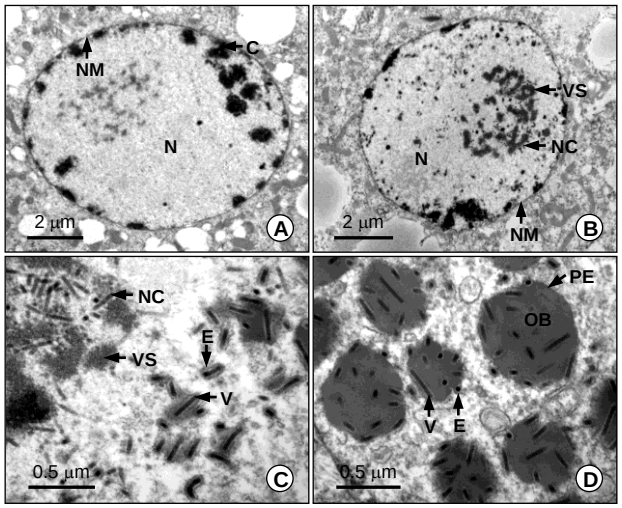

Figure 10. Transmission electron micrographs of HearNPV infection in *H. armigera* larva. (A) Enlarged nucleus and peripheral displacement of the cell chromatin along the nuclear membrane. (B) Nucleocapsids in the virogenic stroma. (C) Virions within and around a dense virogenic stroma. (D) Occlusion body with embedded virions and the polyhedron envelope was formed. C, chromatin; E, envelope; N, nucleus; NC, nucleocapsid; NM, nuclear membrane; OB, occlusion body; PE, polyhedron envelope; V, virion; VS, virogenic stroma.

Insect cell lines are classified into three types based on their permissiveness to support baculovirus replication. Various criteria have been used for the definition of the cell permissiveness [44], [45]. The results and observations obtained in this study strongly indicated that HearNPV can replicate and produce infectious progeny virus in B-5 cells. Therefore, the B-5 cells were fully permissive for HearNPV infection. The criteria for HearNPV replication in this permissive cell line were based on: (a) cytopathology, in which HearNPV-infected B-5 cells did exhibit classical CPEs characteristic of NPV infections, (b) infection kinetic studies, which revealed a higher yields of OBs were obtained when compared to the parental HAPO2 cells, (c) scanning electron microscopic examination of the infected B-5 cells, which revealed ruptured cells and the presence of OBs, and (d) ultrastructural studies of infected *H. armigera* tissues, which revealed evidence of virus infection in the infected fat cells.

Impressive progress has occurred with regard to the development of in vitro production methods of HearNPV. Production methods focus entirely on the most efficient means to produce low-cost, efficacious virus. The ability to produce large amounts of high-potency viral preparations in insect cells is significantly influenced by three key factors, including virus strain, cell line, and medium [9], [46]. A seed stock for both viruses and cells must be developed and determined to be genetically homogeneous. The more stable virus isolates have been derived by plaque-purification that maintain genetic stability, productivity and efficacy [47].

Like virus stocks, cell stocks must be screened for their productivity and growth characteristics. Many insect cell lines are known which potentially can be used for in vitro propagation of HearNPV. These cell lines were obtained from two insect species: *H. armigera* and *H. zea* [10-11]. The most widely used cell line for in vitro studies and production of HearNPV is *H. zea* [9], [46], [48]. However, spontaneous mutation of MP variants together with the relationship between serial passage of HearNPV in *H. zea* cell culture and the appearance of FP mutant has been reported [11], [13]. These phenomena provide strong evidence to suggest that insect cell line is one of the key factor leading to virus genome instability during serial passage of HearNPV in *H. zea* cell culture. Additionally, previous studies have shown that the homologous insect host, HAPO2 cell line, is an important source of virus stability for the scale-up process of HearNPV bioinsecticides. Serial passage in this cell line showed a slower accumulation of FP variants when compared to the use of the heterologous HZ-AM1 cell line [11]. Therefore, the homologous cell lines may be more valid cell stocks for the production of HearNPV than the heterologous cell lines.

Interest in HearNPV as bioinsecticides has led to the search for effective host cell/HearNPV model for in vitro production. The homogeneous in vitro production depends on highly productive cell lines. It is desirable to use a more homogeneous cell population for this model. The current study investigates host cell-virus interaction to determine the influence of a cloned cell line on the virus production yields and its infectivity. A cloned cell line, B-5, derived from the parental HAPO2 cell line was used for production of the MP variant of HearNPV. The B-5 cell line did support replication of HearNPV and had the same pattern of BV production as the parental line. However, the B-5 cell line produced more OBs than the HAPO2 cell line, and these OBs were clearly shown to be infectious in host larvae when given per os.

Several studies have shown that OBs produced in infected insect cells are less potent than OBs produced by infection of insect larvae [13], [48]. In general, infectivity assays of OBs have relied primarily on a mean lethal dose fed to larvae. The insect killing ability of NPV is based on the presence of infectious occlusion derived virus (ODV). ODVs are released from OBs in the insect gut and are responsible for initiating primary infections in the midgut epithelial cells of susceptible hosts. In the present study, infectivity assays were performed at the ultrastructural level by transmission electron microscopy to identify the presence of HearNPV progeny virions. Third instar *H. armigera* larvae were inoculated per os with HearNPV OBs produced in the infected B-5 cells, fat body tissues were taken from the inoculated larvae with typical signs of NPV infection and subjected to electron microscopy. Ultrastructural studies on virus morphogenesis in the infected larval tissues of *H. armigera* revealed that the nuclei contained many nucleocapsids. These nucleocapsids were singly enveloped prior to occlusion in the protein crystalline matrix of the developing OBs. Therefore, B-5

cell/HearNPV model appears to be an effective model for in vitro production of infectious OBs. This model offers several important possibilities in terms of both basic studies and the potential for use of in vitro-produced HearNPV OBs preparations as bioinsecticides.

IV. Conclusions

Considered together, these results indicated that the B-5 cell line possessed higher susceptibility to HearNPV infection than its parental cell line. The ability of B-5 cells to support efficient replication of HearNPV has allowed to study biological characterization of HearNPV in cell culture system and other applications in biotechnology. Further adapting stationary culture of the B-5 cell line to suspension culture is underway to obtain a cost-effective process for large-scale production of HearNPV.

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