Characterization of a newly established potato tuber moth (Phthorimaea operculella Zeller) cell line

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Background & objectives: Potato tuber moth (PTM), Phthorimaea operculella Zeller is a widely distributed, devastating pest of potatoes attacking the foliage and infest the tubers in both field and store causing serious economic damage. As application of PTM granulovirus (PTM-GV) has shown significant reduction in damage, attempts were made to develop a new cell line from this insect to grow PTM-GV for use as a biopesticide.

Methods: Approximately 100 mg of insect eggs were collected, surface sterilized and crushed gently in a boiling tube aseptically. The tissues were washed with physiological saline, suspended in growth medium and incubated stationary at 28°C. Morphology of cells was studied after staining with Giemsa. Besides karyological and growth curve studies, PCR amplification was also done for rapid amplified polymorphic DNA pattern.

Results: A new cell line from the embryonic tissue of PTM was maintained in Mitsuhashi Maramorosch medium supplemented with 10 per cent foetal bovine serum. It is in the 78th passage level and designated as NIV-PTM-1095. Random amplified polymorphic DNA profile analysis indicated this as a new cell line from potato tuber moth and differed from the profiles of two other lepidopteran cell lines maintained in the laboratory. Three different cell types were observed at the 40th passage level and comprised of epithelial-like cells (77%), fibroblast-like cells (20%) and giant cells (3%). The chromosome number varied from 54-176. The cell line had a cell doubling time of approximately 42 h during the logarithmic phase of growth. The cell line did not support the multiplication of any of the baculoviruses used in the study.

Interpretation & conclusion: Since the new cell line is found to replicate PTM-GV, it may be useful for the propagation of PTM-GV in large scale. Studies to scale up the production of the GV in the cell line and field trials may lead to its widespread use as an eco-friendly biopesticide.

Key words Biopesticide - chromosomes - embryonic cell line - Phthorimaea operculella - PTM-GV - RAPD

In recent years, the development of insect cell lines especially from the order Lepidoptera is on the rise due to their potential application in the fields of agriculture, biotechnology, medicine etc. The emergence of baculovirus expression vector system (BEVS) as a powerful and versatile vector in expression of foreign proteins and the advances made in the field of genetic engineering in recent years has further stimulated interest in developing more and more new lepidopteran cell lines. Many proteins/genes have been successfully expressed in lepidopteran cell lines using BEVS. In the field of agriculture, insect cell lines are being used to mass cultivate insect viruses especially baculoviruses which have the potential as
a bio-pesticide\(^3\). Similarly, The granuloviruses also have the potential as a biopesticide though their full potential has not been exploited yet (personal communication). In India, potato is an important commercial crop and the annual loss due to the potato tuber moth (PTM) in field as well as in storage accounts for several lakhs\(^4\). Efforts to control this pest with natural enemies especially exotic parasites along with other conventional means have not been successful\(^4\). PTM-granulovirus (PTM-GV) is a viable alternative and is being used in the control of this insect pest\(^5\). PTM-GV is presently being produced in PTM larvae, which is labour intensive and expensive. Efforts are being made to propagate PTM-GV in susceptible cell lines and the growth of PTM-GV in a PTM cell line has been reported\(^6\). Though the establishment of a cell line from PTM was reported from India\(^7\), it is not available today for research. An attempt was therefore made to develop new cell lines from this important insect pest to propagate PTM-GV.

**Material & Methods**

*Initiation of primary culture*: A laboratory colony of potato tuber moth was established at the National Institute of Virology, Pune from the eggs procured from the College of Agriculture, Shivajinagar, Pune. Approximately 100 mg of eggs were used to set up one primary culture following the method described by Sudeep et al\(^8\). The eggs were surface sterilized with 70 per cent ethanol and crushed gently in a boiling tube with a sterile homogenizer. The tissue was washed twice with physiological saline, suspended in growth medium and incubated stationary at 28°C.

*Growth medium (GM)*: Mitsuhashi Maramorosch (MM) medium\(^9\) supplemented with 20 per cent foetal bovine serum (FBS) (Gibco BRL, USA) was used as growth medium to initiate the cell line. The concentration of FBS was reduced to 10 per cent after the 18th passage level. The medium was prepared in the laboratory. The chemicals for the salt solution were procured from Sigma Chem. Co., USA while yeastolate and lactalbumin hydrolysate were procured from Difco Laboratories, Detroit, USA.

*Subculturing*: Culture bottles having a confluent monolayer were selected and subcultured. The spent medium from the culture bottles was discarded using a sterile pipette. Three ml fresh growth medium was re-introduced into the culture bottle and the cells were dispersed into the medium with gentle pipetting. The cell suspension was distributed equally into two culture bottles each containing 2 ml growth medium giving a split ratio of 1:2. As the growth rate of cells increased, split ratio was also increased accordingly.

*Morphology*: Morphological studies were carried out after staining the cells with Giemsa\(^8\). Cells were seeded on cover slips in Leighton tubes and cover slips with 75 per cent confluency were selected for staining. Cells were washed with physiological saline and fixed in methanol for 5 min at room temperature. The cells were stained with Giemsa (BDH) stain for 10-13 min, washed once in distilled water, dehydrated in two changes of acetone, cleared in xylene and mounted in DPX.

*Karyology*: Karyological studies were carried out as described earlier\(^10\). Cells grown on coverslips in Leighton tubes were treated with colchicines 12 h before processing the cells. The cells were given hypotonic treatment, washed with physiological saline and fixed in methanol acetic acid mixture (3:1) for 5 min at room temperature. The coverslips were stained with Giemsa stain for 10-13 min, washed once in distilled water, dehydrated in two changes of acetone, cleared in xylene and mounted in DPX.

*Growth curve studies*: Cells grown in Belco tubes were used for growth curve studies. Tubes (n=24) were seeded with cell suspension with a minimum seeding density of approximately 1 million cells per ml. Two tubes were randomly picked up and cell counts were taken from day 0 to 10 using a Neubaur hemocytometer (Fein-Optik, GDR). The results were plotted on a graph and growth rates were determined (data not shown).

*Random amplified polymorphic DNA (RAPD) analysis of cell lines*: DNA from PTM cell line, PTM larvae and two other lepidopteran cell lines *i.e.*, *Helicoverpa armigera* (Ha) cell line and *Spodoptera litura* (Sl) cell line was extracted using DNAzol (Sigma Chem. Co., USA) method as per the manufacturers instructions. Cells from the cultures were dislodged mechanically and pelleted by
centrifugation at 1000 g for 5 min. DNAzol was added into the cell suspension as well as the vial containing the 4-5 insect larvae. The lysis of the cells and larvae was allowed to proceed for 5 min at room temperature and DNA was precipitated by the addition of absolute alcohol (Sigma Chem. Co., USA). The purity and concentration of DNA was determined by optical density at 260/280 nm.

**PCR amplification:** PCR was performed using Invitrogen (USA) PCR ingredients according to manufacturers instructions. Three sets of primers of mammalian aldolase, prolactin receptor and interlukin-1 were utilized for the RAPD pattern11. The PCR mix containing MgCl2 (3 mM), 2.5 µl of 10 X PCR buffer, 11.1µl water, 0.4 µl Taq polymerase (2U), 300 ng of DNA and 2.5 µl of primer (200 pmol each) was prepared in a final volume of 25 µl. The samples were amplified for 39 cycles through initial denaturation at 94°C for 1 min, annealing at 40°C for 1 min and polymerization at 72°C for 2 min, with initial denaturation and final extension for 5 min each at 94°C and 72°C respectively. Each sample (8 µl) was resolved on 1.5 per cent agarose gel at 80 V for 2 h. The analysis was also performed with *H. armigera* and *S. litura* cell lines.

**Results & Discussion**

Of the three primary cultures prepared, only one culture got established as a cell line. Tissue attachment was observed 24 h after seeding. Cell migration or cell division was not observed for nearly six weeks though the tissues were viable. The cultures were replenished with 50 per cent fresh growth medium at a regular interval of 7-10 days. At the end of the 6th week, few multicellular vesicles were observed in the culture bottle. A few patches of cells attached to the culture vessel were observed in the subsequent weeks. The patches slowly increased in size and a monolayer of cells was formed within a month. Initially subculturing was done at an interval of 10-15 days. As the cell growth increased, weekly subculture was made giving a split ratio of 1:6.

The morphological investigation revealed three different cell types with varying percentages. The epithelial-like cells (77%) dominated the cell population followed by fibroblast-like (20%) and giant cells (3%) at the 40th passage level (Fig.1). The epithelial-like cells were mostly uni-nucleate, round or oval in shape and measured 15-30 µm in diameter. The cytoplasm was uniform without any vesicles. The fibroblast-like cells were elongated, cells, mostly uni-nucleated and measured 75-150 µm in length and 15-20 µm in breadth at the widest point. The giant cells were multi-nucleated, irregular in shape and measured 50-170 µm in diameter.

Karyological studies have shown a typical lepidopteran pattern of chromosomes. The chromosomes appeared as darkly stained dot like structures and their number ranged from 54-176 (Fig.2). Cell division was more frequent in epithelial-like cells. In lepidopteran cell cultures, it was demonstrated that the chromosomes vary greatly in number, sometimes exceeding three hundred chromosomes in a single cell8.
Growth curve studies carried out at the 24th passage level indicated five-fold increase in cell number in seven days with a population doubling time of approximately 42 h at the logarithmic phase of growth.

Species specificity: Species identity was determined using RAPD profile analysis which indicated specific bands for PTM cell line and larvae (Fig.3a,b,c) whereas the other cell lines had totally different profiles. The profile analysis clearly distinguished it as a new, unique cell line derived from potato tuber moth and not a contaminant of the other two lepidopteran cell lines maintained in the laboratory.

Potato tuber moth is widely distributed and the most devastating pest of potatoes all over the world. In countries like Tunisia, Algeria and Turkey the losses sometimes can be as high as 86 per cent despite the use of insecticides (personal communication). The International Potato Center in Lima, Peru, recommends the use of granulosis virus as one of the control techniques against this pest as they have found it effective in the control of the pest. As inoculum, infected PTM larvae were pulverized, mixed with water and applied to the plants in the field or to potatoes in storage. The two field trials with PTM-GV recorded 70 to 90 per cent infection of larvae. Also, the foliage damage was reduced significantly in both trials (personal communication).

The major setback in the use of PTM-GV is the production of the virus as in vivo it is extremely labour intensive. Efforts are therefore being made to produce PTM-GV in susceptible cell cultures, which will help in the large scale production of the virus. In India, a PTM cell line was established and reported by Pant et al in 1977, but no information about the cell line is available today. Moreover, the authors have not reported the susceptibility of their cell line to any viruses. We made an attempt to establish new cell lines from this important insect and succeeded in establishing a new cell line from the embryonic tissue. The cell line has been passaged over 78 times in vitro and can be called a continuous cell line. It has been designated as NIV-PTM-1095 and has been characterized using the routine parameters, i.e., morphology, karyology, growth rate etc. The species specificity of the new cell line, which is one of the important parameters in the characterization, was studied using RAPD profile analysis. RAPD is being used as a reliable technique for distinguishing insect cell lines in recent years and this technique is able to differentiate between organisms at the species level. Cross contamination due to mislabeling is quite common in laboratories where a large number of cell lines are maintained simultaneously and RAPD profile analysis has been used successfully to confirm the identity of these cell lines. Our studies indicated different profile for the new cell line, which differed clearly from that of H. armigera and S. litura cell lines, which are presently being used in our laboratory. The RAPD profile with Aldolase gene clearly showed homology with two bands in the profile of the cell line and the host tissue. The additional bands in the
cell line compared with the host tissue may be a reflection of the heteroploid nature of lepidopteran cell lines, resulting in additional genomic DNA in the cell line. McIntosh et al. reviewed similar profile differences in cell lines and their host tissues.

The cell line grows in MM medium, which is inexpensive and ideal for suspension culture. Efforts are also being made to replace FBS with other inexpensive supplements like goat serum to reduce the production cost to a minimum. The new cell line may, therefore, find application in the propagation of PTM-GV, which can be used as an eco-friendly bio-pesticide for the control of this important agricultural pest.

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References


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