ESTABLISHMENT OF CELL LINE DERIVED FROM EMBRYONIC TISSUE OF THE DIAMONDBACK MOTH, *Plutella xylostella* (L.)

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ABSTRACT

A new cell line designated KMITL-PX-E1 has been established from embryonic tissue of the diamondback moth, *Plutella xylostella* (L.). The primary cultures were seeded in TNM-FH medium supplemented with 20% fetal bovine serum and incubated at 28 °C. After the 18th passage, the cultures were adapted and maintained in TNM-FH medium supplemented with 10% fetal bovine serum. The cell line consisted of a mixture of two cell types, spindle-shaped cells and epithelial-like cells, both of them grew as attached monolayer. The population doubling time at the 2nd and the 18th passages were 110.59 and 61.22 hrs, respectively. The chromosome numbers varied from 6-124. Random amplified polymorphic DNA profile analysis showed that the diamondback moth cell line (KMITL-PX-E1) profile differed from the profiles of the beet armyworm (SE-1) and the American cotton bollworm (KMITL-HA-E1) cell lines maintained in the laboratory. KMITL-PX-E1 cell line supported replication of *Autographa californica* (*Ac*MNPV) and *Exoa scandens* cytoplasmic polyhedrosis virus (*Es*CPV). However, this cell line was sensitive to *Bacillus thuringiensis* toxin.

KEYWORDS: *Plutella xylostella* cell line, diamondback moth, nucleopolyhedrovirus, cytoplasmic polyhedrosis virus, Random amplified polymorphic DNA

1. INTRODUCTION

The *in vitro* insect tissues cultures offer considerable potential for studying various aspects of the pathogenic cycles of protozoan, bacteria, and viral agents having insect vectors or reservoir. Insect cell culture is recognized as an emerging technology for the production of biologicals, including recombinant proteins and biological insecticides [1]. Viral production by cell culture is widely used for the propose of biological control [2]. Cell lines are normally established from pupae [3] and embryonic tissue of the diamondback moth, *Plutella xylostella* (L.) [4], however, this report describes the establishment of new cell line derived from embryonic tissue of this moth which is the important pest in Thailand.

2. MATERIALS AND METHODS

2.1 Cell line development

Primary cultures were initiated from 2 to 3 day old eggs of *P. xylostella* which had been reared in the laboratory for several generations. Approximately 200-300 eggs were used to set up one primary culture. The eggs were sterilized with 10% formaldehyde, 10% chlorox and 70% ethanol for 10 mins, respectively. After washing the eggs twice with distilled water, they

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were rinsed two times with incomplete medium. The eggs were crushed and homogenized in TNM-FH medium, supplemented with 20% fetal bovine serum (FBS). The suspension was then placed in a 25 cm^2 plastic tissue culture flask containing 1 ml of the growth medium. The cells were maintained overnight in an incubator at 28 ^oC and later, 1 ml of the growth medium was added every day until the total volume of growth medium in the flask was 5 ml. The culture medium was added or replaced with 1 ml of fresh medium on each culture at 5 day intervals until enough cells were available for subculture.

2.2 Subcultures

Cells were dislodged from the flask surfaces by repeatedly surging the medium with pipette until a uniform cell suspension was obtained. For subculture, 2.5 ml of suspended cells were transferred to a new 25 cm^2 flask containing 2.5 ml of fresh medium and incubated at 28 ^oC.

2.3 Cell morphology

The cell cultures were observed using a phase contrast microscopy at 400x magnification to observe cell morphology.

2.4 Measurement of cell growth

The cultured cells in a 25 cm^2 flask at log phase were adjusted to $2x10^5$ cells/ml. For testing the effect of different pH of media on cell growth, media were prepared by adjusting at pH 6.2, 6.4 and 6.6, respectively. For the evaluation of the doubling time of this cell line, cell samples were taken daily for seven days, and the cells were counted under a microscope in a hemacytometer after staining with trypan blue.

2.5 Karyotype analysis

Karyological studies were carried out as described by Lee and Hou [4] and Petcharawan *et al.* [5]. Cells in the logarithmic growth phase were incubated for 3 hrs in medium containing 20 µg/ml of colcemid (GIBCO). Cultures (3 days after being subcultured) were incubated with colcemid (20 µg/ml) at a 28 ^oC for 3 hrs. The cells were detached and centrifuged at 3,500 rpm for 10 mins. The pellets were suspended and submerged in 0.56% KCl hypotonic solution for 30 mins. After centrifugation, the cells were fixed with methanol and glacial acetic acid (3:1). The fixed cells were dropped vertically onto glass slides. After air drying, the cells were stained with 7% Giemsa solution for 15 mins and the chromosome numbers were counted under a light microscope.

2.6 Characterization using RAPD markers

P. xylostella adults from Animal Cell Culture Laboratory, Department of Applied Biology, Faculty of Science, King Mongkut' s Institute of Technology, Thailand were used in this study. Cell lines of *P. xylostella* (KMITL-PX-E1), *Spodoptera exigua* (UCR-SE-1) and *Helicoverpa armigera* (KMITL-HA-E1) were used. Extraction, purification and quantification of DNA from *P. xylostella* adults and cell cultures were performed according to methods described previously [6-7].

PCR amplification in 50 µl reaction mixtures contained 5 µl of DNA (10 ng/µl), 5 µl of 10x PCR buffer, 3 µl of $MgCl_2$ (25 m*M*), 1 µl of deoxynucleotide triphosphate (10 m*M*), 0.5 µl of *Taq* DNA polymerase (5 U/µl), 2.5 µl of primer and 33 µl of sterile water.

The samples were amplified for 40 cycles under the following conditions: denaturing at 94 ^oC for 1 min, annealing at 36 ^oC for 1 min and extension at 72 ^oC for 1 min, with initial denaturation at 94 ^oC for 4 mins and final extension at 72 ^oC for 7 mins. Three 10-mer RAPD primers used for PCR were A02 (5'-TGCCGAGCTG-3'), A04 (5'-AATCGGGCTG-3') and B01 (5'-GTTTCGCTC-3'). These primers were purchased from Bioservice Unit (BSU), Thailand. Aliquots of 5 µl of amplified products mixed with 2 µl of loading buffer were analyzed in 1.5% agarose gels and run at 50 V/cm for 1 h and 15 mins in TBE buffer.

The similarity index of Nei and Li [8] was used to compare patterns between cell lines and *P. xylostella* adults. This index reflects the extent of band sharing calculated as $2 N_{AB}/(N_A + N_B)$ where N_{AB} is the number of bands common to individuals A and B. N_A and N_B are the total number of bands in individuals A and B, respectively. The value produced by this index ranges from 0, representing no band sharing, to 1 representing complete identity.

2.7 Cell susceptibility to viruses

The viruses tested in these studies include nucleopolyhedroviruses (*Ha*SNPV, *Ac*MNPV, *Gm*MNPV and *Se*MNPV) and occluded virions released by alkaline treatment of polyhedra of cytoplasmic polyhedrosis viruses (*Bm*CPV, *Cf*CPV, *Es*CPV and *Ha*CPV). To screen virus susceptibility, KMITL-PX-E1 cells were seeded in 25 cm^2 tissue culture flask at $2x10^5$cells/ml in 5 ml of TNM-FH medium and were allowed to attach overnight. After an overnight of cell attachment, the medium was removed and replaced with 1 ml of viral inoculum and incubated at room temperature for 1 h. At the end of this adsorption period, the viral inoculum was discarded and the cells were washed twice with 1 ml of incomplete medium. The cells were then incubated in 5 ml of TNM-FH medium supplemented with 10% FBS and incubated at 28 ^oC for 7 days. Cytopathic effect (CPE) and occlusion bodies production were observed.

2.8 Response of KMITL-PX-E1 to *Bacillus thuringiensis* toxins

For studying the response of KMITL-PX-E1 to *Bacillus thuringiensis* toxins, three commercial formulations were tested: Thuricide® (var. *kurstaki*) from Thermo Ecotek Corpolation (Ontario, Canada), Teknar-HPD® (var. *israelensis*) from Zaecon Canada Inc. (Mississauga, Ontario, Canada) and Trident ® (var. *tenebrionis*) from Sandoz Agro Canada. Evaluation tests of cell response to *Bt* toxins were performed in 96-well plates. In summary, the toxin of each formulation was dissolved, treated and used in the toxicity test as described by Thomas and Ella [9] and Chapentier *et al*. [10]. The cell lysis was monitored under microscope after incubating in toxin solution for 24 hrs and the tissue culture toxicity dose 50% ($TCTD_{50}$) was calculated with Kärber formula [11].

3. RESULTS

3.1 Cell line development

The embryonic tissues of *P. xylostella* began to attach to the culture flask after incubating for 2-3 days. The explant shrank gradually and cell mass liberated from it. Then, more cells migrated from the explant and a number of cells were mitotic. At this point, cell size and morphology varied (Figure 1a). The early migrated cells were mostly spindle in shape with prominent networks attaching to the bottom of the culture flask. These networks were muscle-like and nerve-like structures. The spherical, spindle, and vacuolated cells were seen in the primary culture (Figure 1a).

3.2 Subcultures

In the first two subcultures, cell growth was slow, an average duration of 30 days between a subculture and other. Subcultures were maintained in TNM-FH medium supplemented with 20% FBS, pH 6.2 in the first five passages, then the 20% FBS concentration was gradually reduced to 15% from the 6^{th} passage to the 17^{th} passage. The FBS concentration was reduced from 15% to 10% starting from the 18^{th} passage. The cell line derived from embryonic tissue of *P. xylostella* was designated as KMITL-PX-E1.

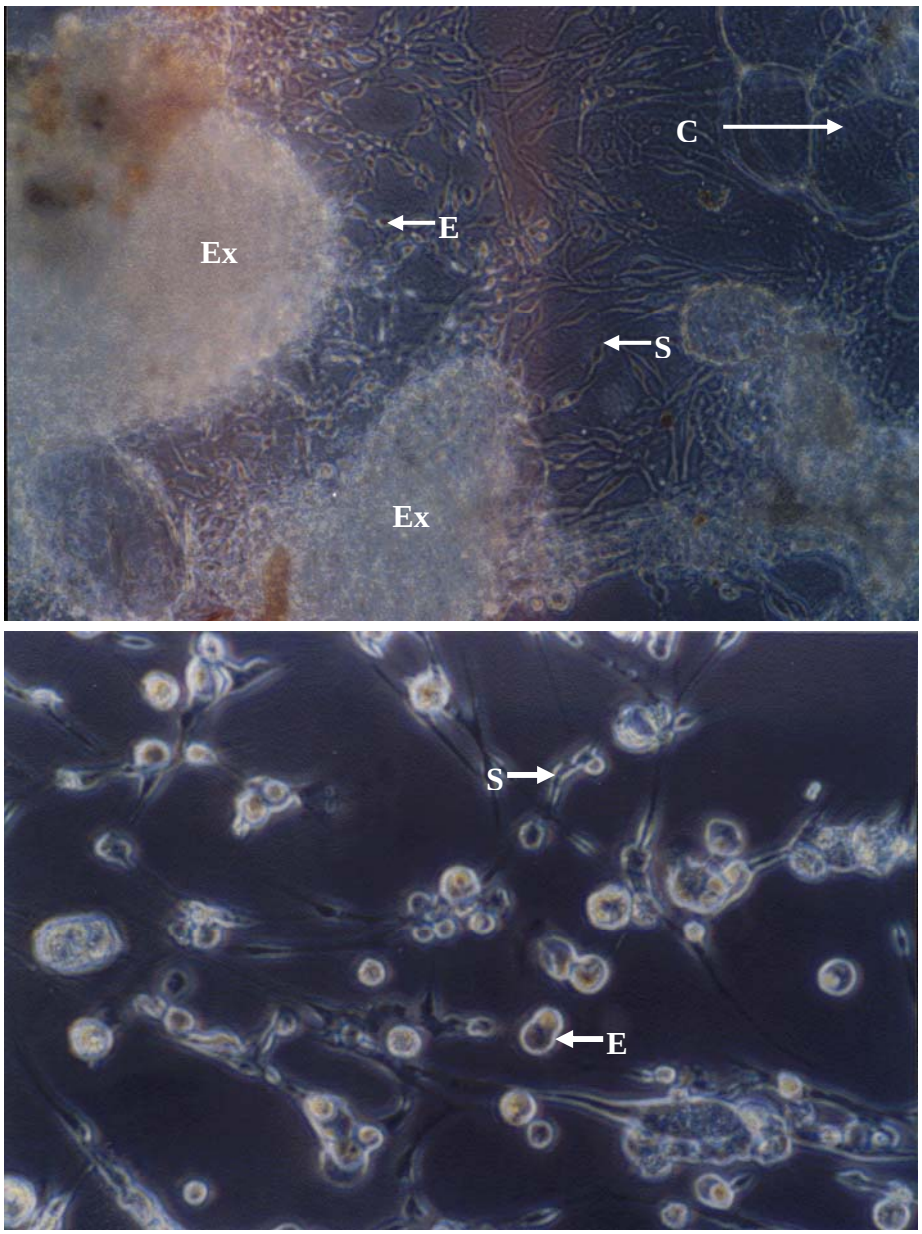


Figure 1 Phase-contrast micrographs of primary culture of *P. xylostella* embryos at 30 days post seeding (a), magnification 200 x at 60 days post seeding (b), magnification 400x (E, epithelial-like cell; Ex, explant tissue; C, companion cell; S, spindle-shaped cell)

3.3 Cell morphology

Cell forms in the primary cultures varied, consisting of three major morphologically different cell types (companion cell, spindle-shaped cell and epithelial-like cell) (Figure 1b); nevertheless, after the 3rd subculture, the cells in the monolayer were predominantly epitheloid and spindle-shaped cells (Figure 2).

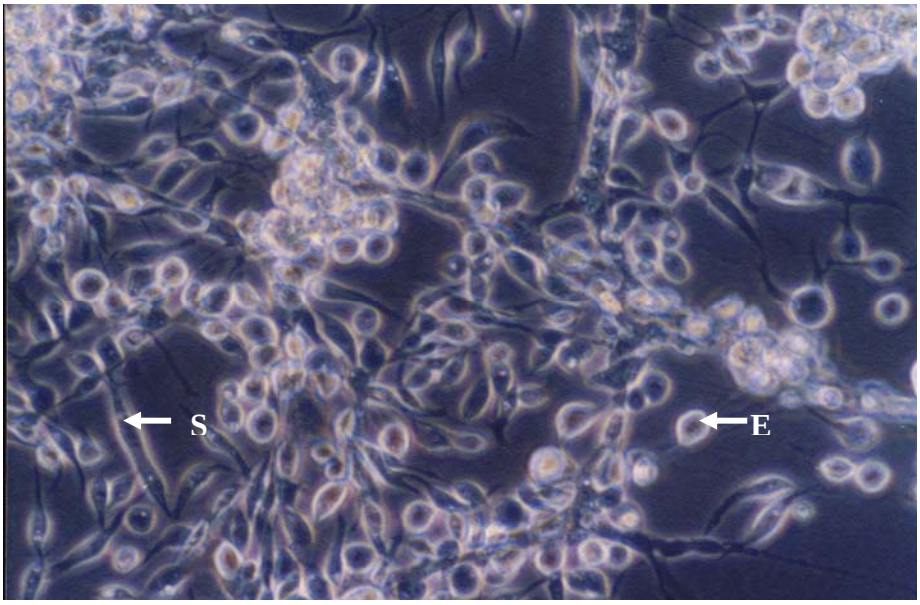


Figure 2 Monolayer of KMITL-PX-E1 showing the predominantly epitheloid (E) and spindle-shaped (S) cell population of the 3 rd passage, magnification 400x

3.4 Measurement of cell growth

The optimal pH of medium for the growth of KMITL-PX-E1 cell line was 6.2 (Figure 3). The cell population doubling time of the 2nd passage and the 18th passage incubated at 28 ^{o}C were 110.59 hrs and 61.22 hrs, respectively (Figure 4).

3.5 Karyotype analysis

A representative chromosomal spread from KMITL-PX-E1 cells shows the typical round shape of Lepidopteran chromosome (Figure 5a). The cell was polyploidy and the distribution of chromosome numbers varied widely from 6-124 (Figure 5b).

3.6 Characterization using RAPD markers

The species specificity of the new cell line, which is one of the important parameters in the characterization, was studied using RAPD profile analysis. This study indicated different profile for the new cell line, which differed clearly from *S. exigua* (UCR-SE-1) and *H. armigera* (KMITL-HA-E1) cell lines, which are presently being used in the laboratory.

Figures 6a-c show RAPD-PCR results comparing among KMITL-PX-E1, UCR-SE-1, KMITL-HA-E1 cell lines and *P. xylostella* adult using primers A02, A04 and B01, respectively. The profiles generated by primers A04 and B01 appear to be clearly distinct ion from each other when cell lines from different species were compared (Figures 6b-c). However, the profile generated by primer A02 appears to be the allele sharing between KMITL-PX-E1 and KMITL-HA-E1 cell lines, the similarity coefficient was 0.36 when the similarity was compared. Coefficient between KMITL-PX-E1 and *P. xylostella* adult using

primers A02, A04 and B01 gave the similarity coefficients of 0.57, 0.50 and 0.33, respectively.

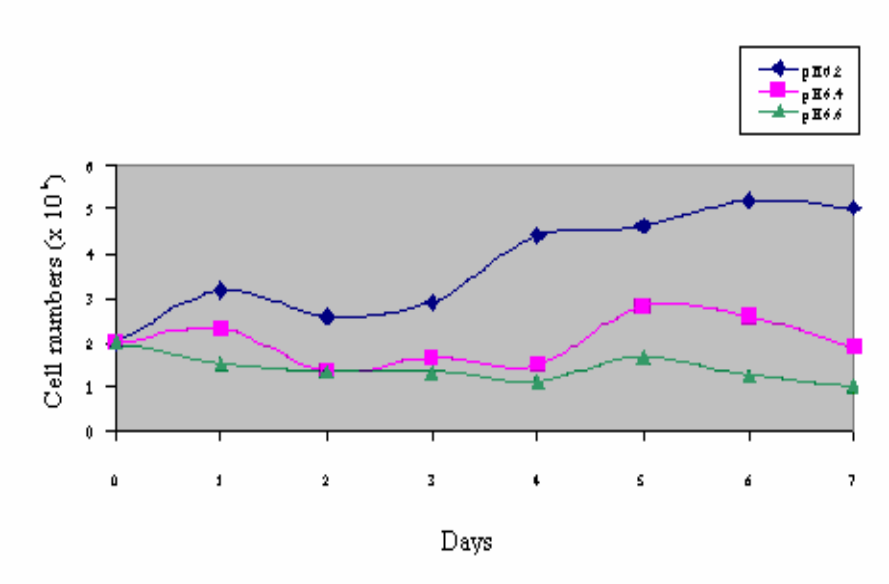


Figure 3 Growth curves of KMITL-PX-E1 cells at the 11th passage in different pH of media

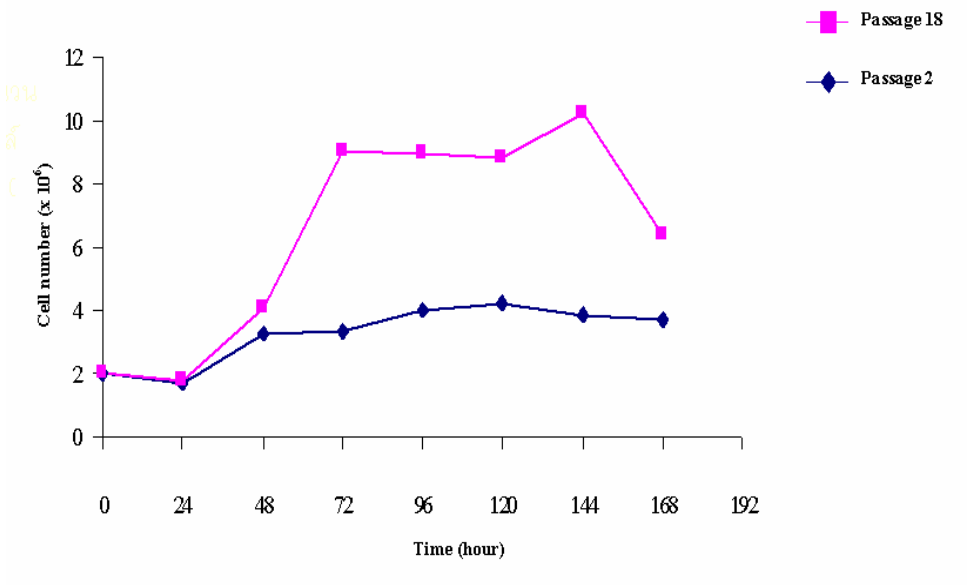
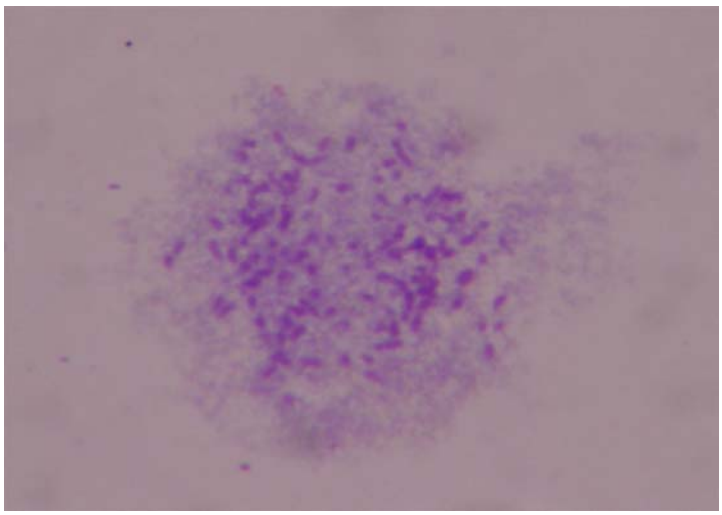
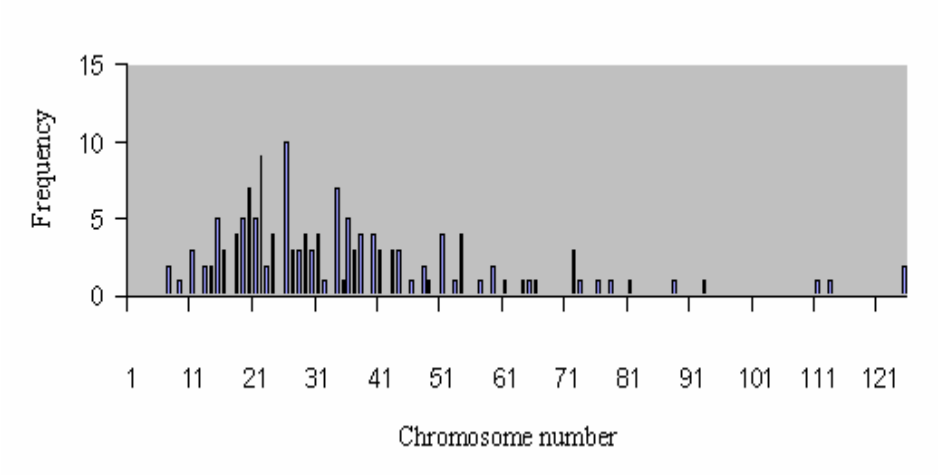


Figure 4 Growth curves of KMITL-PX-E1 cells at the 2nd and the 18th passages



(a)



(b)

Figure 5 A representative mitotic chromosomal spread (100 x) and the distribution of chromosome numbers at the 11th passage of KMITL-PX-E1 cells

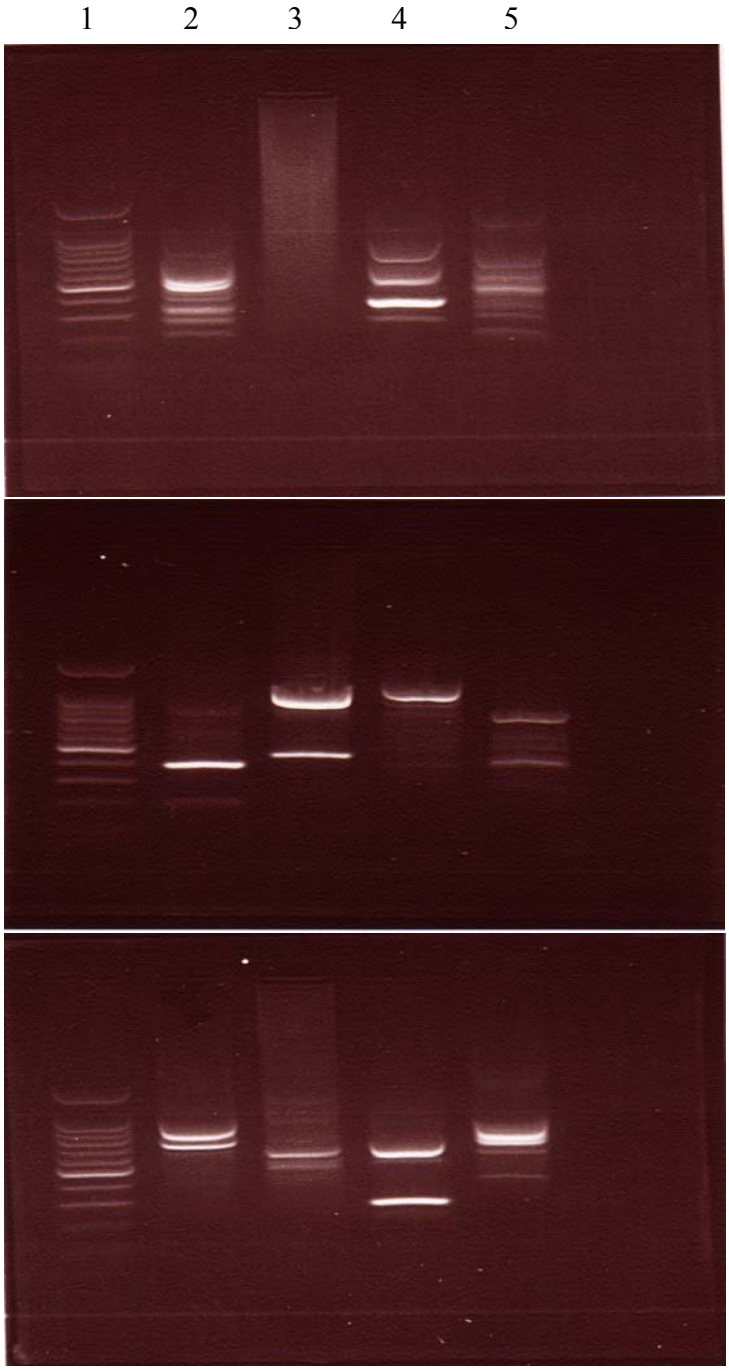


Figure 6 RAPD profile of different cell lines and insect, using primer A02 (a), A04 (b) and B01 (c). Lane 1: 100 bp step ladder, Lane 2: KMITL-PX-E1 cell line, Lane 3: UCR-SE-1 cell line, Lane 4: KMITL-HA-E1 cell line, Lane 5: *P. xylostella* adult

3.7 Cell susceptibility to viruses

The KMITL-PX-E1 cell line was susceptible to *Ac*MNPV (Figure 7) and *Es*CPV (Figure 8). Polyhedra were noted at 3 days post-infection for both of them.

3.8 Response of KMITL-PX-E1 cell line to *Bacillus thuringiensis* toxins

Tissue culture toxicity dose 50 ($TCTD_{50}$) of *B. thuringiensis* toxins on KMITL-PX-E1 cell line comparison to the reactions of other cell lines from *Spodoptera exigua* (UCR-SE-1) and *Helicoverpa armigera* (KMITL-HA-E1) are stated in Table 1. The results demonstrated the sensitivity of KMITL-PX-E1 cell line to the solubilized crystal toxins from commercial preparations of *B. thuringiensis*. However, the Btk in the Thuricide® was as expected to be the most effective one.

Table 1 Cytotoxicity of *B. thuringiensis* toxins on different cell lines

B. thuringiensis toxins	$TCTD_{50}$ (µg/ml) *		
	KMITL-PX-E1	UCR-SE-1	KMITL-HA-E1
Thuricide®	0.128 [a]	0.644 [a]	1.019 [a]
Teknar-HDP®	10.900 [b]	11.864 [b]	26.490 [b]
Trident®	14.242 [c]	14.610 [c]	36.638 [c]

* means within column followed by the same superscripts are not significantly different at p = 0.01 using Duncan's new multiple range test.

4. DISCUSSION

Many continuous cell lines have been established from various tissues of lepidopteran insects mostly for the purpose of study or the large-scale production of insect viruses. Although, more than 400 cell lines have been established in insect cell cultures [12], only two of them designated as BCTRL-PX2-HNU3 and PX-1187 were derived from pupae and embryonic tissue of *P. xylostella*, respectively [3-4]. The present cell line should be the third cell line established from embryonic tissue of this species because embryos consisted of cells that will differentiate into various tissues. Therefore, various types of cells will migrate from embryo fragments. The result of primary culture may vary depending on the stage of embryogenesis used. In general, the ratio of undifferentiated cells is higher in the early stage embryo, and these cells are considered to have larger potential to proliferate. However, it is not easy to get sufficient numbers of such cells because early stage embryos are small and consist of fewer cells. In contrast, in matured embryos, most tissues have been differentiated already, and the ratio of undifferentiated cells is relatively small [12]. The population of KMITL-PX-E1 composed of various cell types, similar observation had been reported by Lee and Hou [4]. The chromosome numbers of this cell line ranged from 6-124, comparable to 20-180 in the PX-1187 cell line derived from embryos of *P. xylostella*, 45-185 in the NIV-HA-197 [13] and 48-228 in the KMITL-HA-E1, cell lines derived from the embryos of *H. armigera* [5] The karyotype of lepidopteran cell line are always polyploidy or heteroploid and the chromosome number is distributed over a wide range [12].

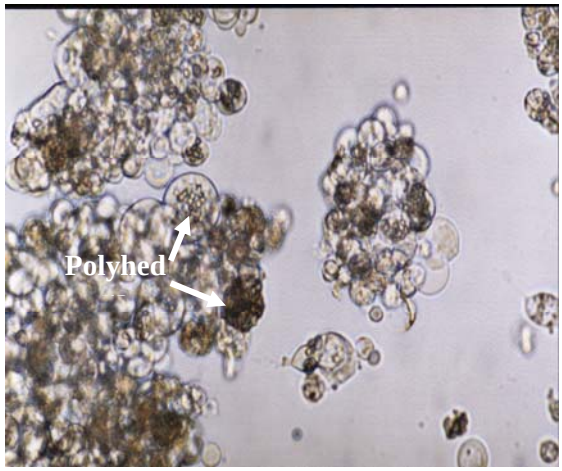


Figure 7 Micrograph of KMITL-PX-E1 cells 6-days post infection with *Ac*MNPV, magnification 400x

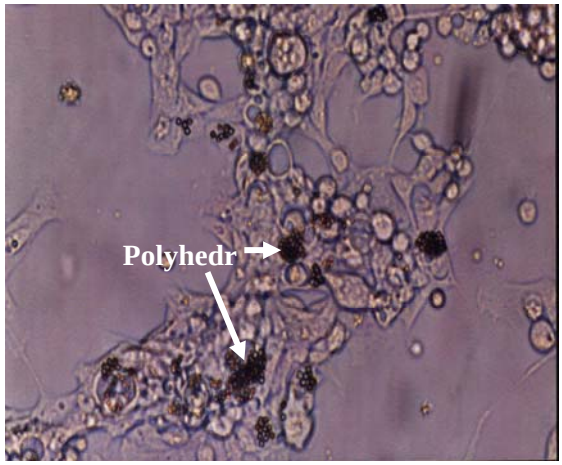


Figure 8 KMITL-PX-E1 cells 4-days post inoculation with *Es*CPV, magnification 400x

The growth of primary cultures began after a relatively long time. The population doubling times of KMITL-PX-E1 at the 2nd passage (110.59 hrs) and the 18th passage (61.22 hrs) were longer than PX-1187 cell line (27 hrs). The optimal cell growth was obtained at 28 ^{o}C in TNM-FH medium containing 10% FBS, pH 6.2. However, PX-1187 cell line growth was obtained between 25 ^{o}C and 28 ^{o}C in TNM-FH medium containing 10% FBS, pH 6.4 [4].

Species identity was determined using RAPD profile analysis which indicated specific bands for KMITL-PX-E1 and adult (Figures 6a-c), whereas the other cell lines had totally different profiles. The profile analysis clearly distinguished this cell line as a new cell line derived from *P. xylostella* and was not contaminant of the other two lepidopteran cell lines maintained in the laboratory.

This cell line was highly susceptibility to *Ac*MNPV (Figure 7) and *Es*CPV (Figure 8). It was not surprising that the *Ac*MNPV replicated in this cell line because the *Ac*MNPV has a wide host range [5, 14].

The preliminary study of the response of KMITL-PX-E1 cell line to some *B. thuringiensis* toxins, demonstrated that this cell line was sensitive to three commercial formulations of *B. thuringiensis* toxin. However, the Btk toxin was higher toxic to KMITL-PX-E1 cell line than the others.

The cell line is a good tool for *in vitro* testing of cell line susceptible to insect viruses, bacterial and entomophagous fungal toxins as well as for cytotoxicity testing of bioactive compounds from plants and animals.

REFERENCES

[1] Agathos, S.N. **1994** Large scale insect cell production. In: Maramorosch, K. and McIntosh, A.H. *Insect Cell Biotechnology*. CRC Press, Tokyo, pp. 89-103.

[2] Grace, T.D.C. **1982** Development of insect cell culture. *In*: Maramorosch, K. and Mitsuhashi, J. *Invertebrate cell culture*, *applications*. Academic Press, New York, pp.1-8.

[3] Chen, Q. and Dai, X. **1987** Replication of *Pieris rapae* granulosis virus in an insect cell line. *In*: Kuroda, Y., Kursta, E. and Maramorosch, K. *Invertebrate and fish tissue culture*. Japan Scientific Societies Press, Tokyo, pp. 170-172.

[4] Lee, S.H. and Hou, R.F. **1992** Establishment of a cell line derived from embryos of the diamondback moth, *Plutella xylostella* (L.), *J. Invertebr. Pathol*., *59*, 174-177.

[5] Petcharawan, O., Charoensak, S. and Belloncik, S. **2005** Establishment and characterization of cell line from embryonic tissue of the American cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *In*: Qian, Y. and Ziniu, Y. *Study on plant pest and diseases biological control and biotechnology*. Heilongjiang Science and Technology Press, pp. 107-121.

[6] Haymer, D.S., McInnis, D.O. and Arcangeli, L. **1992** Genetic variation between strains of the Mediterranean fruit fly, *Ceratitis capitata*, detected by DNA fingerprinting, *Genome*, *35*, 528-533.

[7] Lery, X., Larue, B., Cossette, J. and Charpentier, G. **2003** Characterization and authentication of insect cell lines using RAPD markers, *Insect Biochem. Molec. Biol*., *33*, 1035-1041.

[8] Nei, M. and Li, W. **1979** Mathematical model for studying genetic variation in terms of restriction endonucleases, *Proc. Natl. Acad. Sci*., *76*, 5269-5273.

[9] Thomas, W.E. and Ellar, D.J. **1983** *Bacillus thuringiensis* var *ieraelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*, *J. Cell Sci*., *60*, 181-197.

[10] Charpentier, G., Tian, L., Cossette, J., Lery, X. and Belloncik, S. **2002** Characterization of cell lines developed from the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera : Chrysomelidae), *In Vitro Cell Dev. Biol*., *38*, 73-78.

[11] Kärber, G. **1931** Beitrag zur kolliktiven behandlung pharmakologischer reihenversuche Naunyn-Schwiedebergs, *Arch. Exp. Pathol. Pharmakol*., *162*, 680-687.

[12] Mitsuhashi, J. **2002** Invertebrate tissue culture methods. Springer, Tokyo, 446 pp.

[13] Sudeep, A.B., Mourya, D.T., Shouche, Y.S., Pidiyar, V. and Pant, U. **2002** A new cell line from the embryonic tissue of *Helicoverpa armigera* HBN. (Lepidoptera: Noctuidae), *In Vitro Cell Dev. Biol. Anim*., *38*, 262-264.

[14] Sieburth, P.J. and Maruniak, J.E. **1988** Susceptibility of and established cell line of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) to three nuclear polyhedrosis viruses, *J. Invertebr. Pathol*., *52*, 453-458.