Biosafety of crude and formulated *Hyblaea puera* (Cramer) (Lepidoptera: Hyblaeidae), Nucleopolyherovirus (HpNPV) against silkworm *Bombyxmori* (L.), Indian mynah, *Acridotherustristis* (Linn.) and cell lines

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ABSTRACT

An important aspect of an NPV biocide is its safety to different non-target organisms. The safety of the formulated and crude *Hp*NPV against second instar silkworm *Bombyx mori* larvae was tested. Larvae fed with crude virus and formulated *Hp*NPV did not show variation in larval weight, pupation percentage, fresh weight of cocoons, shell weight and fecundity. Bird fed with NPV infected *H. puera* larvae, presence of the viral occlusion bodied was noted in the faecal droppings within an hour of post feeding. Presence of viral particles in the faeces was observed until 24 hrs post-feeding. This suggested that the viral particle did not remain in the body of the bird for long . The test bird behaved normally and remained healthy throughout the observation period of three months suggesting no deleterious effect of *Hp*NPV. Formulated and semi-purified *Hp*NPV was also tested against three cell lines such as human larynx cell line (Hep2), Green monkey kidney cell line (Vero) and *Spodoptera frugiperda* ovary cell line (Sf-9). After inoculation with the virus, the cell lines did not show any symptom of infection. These studies have explicitly proved the safety of *Hp*NPV.

Key words: Baculoviruses, cell lines, *Hyblaea puera*, Nucleopolyhedrovirus, safety

INTRODUCTION

Baculoviruses are naturally occurring pathogens exclusively known from arthropods and mostly from insects. However, as in the case of any novel insecticide, the potential impact of a viral pesticide on the environment has also to be strictly evaluated before it is used for field application. Unlike chemical insecticides, biological agents are capable of replicating and persisting in the environment for many years. The use of pesticides (insecticides, fungicides, weedicides, plant growth regulators and biopesticides including microbial pesticides) are regulated in India under the Insecticides Act, 1968, and Rules framed thereunder. For this purpose the pesticides should be included in the "Schedule" to the Insecticides Act. One of the requirements for registration of nucleopoly-hedrovirus under section 9 (3) of the Insecticides Act, 1968 is the data on safety testing (Pawar, 2002).

The safety of *Hp*NPV to higher organisms has so far not been worked out. It is known that the cultured mammalian cells are ideal for assessing the relative toxicity of a series of compounds (Perchermeier *et al*., 1994) and the rationale behind the use of Vero (Africal green monkey kidney cell line) cells is that these cells can be banked and well characterized. In addition, the continued use of animals is problematic from ethical and economic viewpoints. Sf-9 cells derived from *Spodoptera frugiperda* (ovary) cells are used for virus amplification and plaque assays. Hep2 (Human larynx cell) cells are mostly used in virus and tumourigenity studies. Hence the present investigation was carried out to determine the in vivo and in vitro safety of *Hp*NPV. Silkworm (*Bombyx mori*) was used for the *in vivo* study and three cell lines such as Hep2; Vero and Sf-9 cell lines were used for the *in vitro* study.

MATERIALS AND METHODS

In vivo safety assessment against silkworm

The infectivity of *Hp*NPV to the mulberry silkworm *B. mori* was studied. Semi-purified and formulated *Hp*NPV produced in the laboratory was used for the present study. A culture of the silkworm *B. mori* was raised in the laboratory from the eggs obtained from the Sericulture Department, G.D. Naidu Agriculture University,

Coimbatore, Tamil Nadu. Eggs were allowed to hatch and the emerged larvae were reared upto second instar on mulberry leaves. Separate sets of mulberry leaves were sprayed with semi-purified and formulated *Hp*NPV solution of concentration of 10^8 OBs per mL. The leaves were air dried for 10-15 mins. Thirty larvae (second instar) were allowed to feed on each set of virus treated leaves. A control set was maintained on untreated leaves. Control and treatments replicated thrice with 30 larvae per replication. Data on mortality if any, larval weight, pupation percentage, fresh weight of cocoons, shell weight and fecundity were recorded.

***In vivo* safety assessment against Indian mynah**

Hyblaea puera Nucleopolyhedrovirus was fed to an Indian mynah maintained in the laboratory. The bird was kept under observation for a period of three months.

***In vitro* safety assessment using cell lines**

Cultured mammalian and insect cells are ideal for assessing the relative toxicity of a series of compounds. In this study, three cell lines namely, Sf-9, Hep2 and Vero obtained from National Center for Cell Science, University of Pune Campus, Ganesh Khind, Pune were used. The rationale behind the use of Vero (Africal green monkey kidney) cell line rather than monkey kidney cells is that these cells can be banked and well characterized. In addition, the continued use of animals is not justified from ethical and economic viewpoints. Vero cells are sensitive to infection with SV-40, SV-5, measles, arbovirus, reovirus, rubella, simian adenoviruses, polioviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, vaccinia, and others. Sf-9 cell lines derived from *Spodoptera frugiperda* ovary were used for virus amplification and plaque assays. Hep2 (Human larynx) cell lines are mostly used in virus and tumourigenity studies. The cells were first checked under phase contrast microscope for contamination if any, and excess medium was removed and cells were incubated at appropriate temperature depending on the cell line. All the cells were grown as monolayers in disposable plastic tissue culture flasks. To maintain the cells within the log phase and to prevent them from entering their stationary phase, they were sub-cultured.

Maintenance of Sf9 cell lines

Healthy Sf-9 cells were grown in TNFM Medium (pH- 6.2) containing 10 per cent of Foetal Bovine Serum. The cell culture medium was equilibrated at room temperature before using it. Subculturing of cells was carried out when the cells attained confluence growth on plates, which occurred thrice a week at a passaging ratio of 1:3. For subculturing, the medium was removed and kept undisturbed for 5 minutes. Then the sides of the flask were gently taped from all sides so as to dislodge the cells from the monolayers. The cells were then transferred to fresh tissue culture flask containing medium and serum and were maintained at 28ºC.

Maintenance of Hep2 and Vero cell lines

Hep2 and Vero cells were grown in Minimum Essential Medium (Eagle) (MEM (E) with Earle's BSS (pH-7.2) containing 10 per cent of Fetal Bovine Serum. Metabolism of growing cells in a closed tube results in the production of CO_2 and acidification of the growth liquid. To counteract the pH decrease, a bicarbonate buffering system was employed in the culture medium. To keep the cells at physiologic pH (pH-7.2), phenol red, a pH indicator, which is red at physiologic pH, purple at alkaline and yellow at acidic pH was added. The medium was sterilized by pressure filtration with 0.22µm filters. The media were periodically checked for bacterial and fungal contamination using Nutrient Broth, Sabourauds and Thioglycolate media (HIMEDIA). The old acidic medium was removed and fresh medium was added 2-3 times weekly.

For sub culturing the cells, the medium was removed (old acidic medium), fresh TPVG (Phosphate buffered saline (PBS) with 0.1% trypsin, 0.2% versene and 0.5% glucose) was added. After 1-2 minutes TPVG was removed. The culture flasks were kept at 37ºC for 10 min. In between the flasks were taken out and the sides of the flasks were tapped. After 10 min, fresh medium was added and agitated to dissociate the cells from the surface. Homogenous suspension was made and seeded into new culture flasks. The cell density was estimated and subcultures with a density of 106 cells per flask were made.

Estimation of cell density

The cell density of the master culture was estimated so as to seed the correct cell density

while sub-culturing. The cell density was determined using an improved Neubauer's haemocytometer. After passaging, the cells were taken into a sterile centrifuge tube and centrifuged at 1000g for 3 min. The supernatant was carefully removed without disrupting the cell pellet. The pellet was resuspended by adding 2 mL of fresh medium through the sides of the tubes and mixed well. For enumeration, 0.2 mL of the cells was diluted in 0.7 mL of medium and 0.1 mL of trypan blue (2% w/v, in Phosphate Buffer saline (PBS)) was added to it and mixed well. Trypan blue stain was used to distinguish viable cells from non-viable cells; live healthy cells appear bright, round and refractile and exclude the blue coloured dye; non-viable cells absorb the dye and appear blue in colour. From this, one drop was taken using Pasteur pipette and poured on to the sterile haemocytometer. The cells were counted under phase contrast microscope (x 10 objective). All the cells within the 5 x 5 square grid of the haemocytometer, and touching the middle line of the triple line on the top and left of the squares were enumerated. This count gives the number of cells present in 0.1µl of stock. Counting was repeated thrice to estimate the correct cell densities. Number of cells present in one mL was calculated by the following formula:

Cells per mL = Mean cell number x 10^4 x dilution factor

Freezing and thawing cells

For freezing, the cell density was kept at 4 x 10^6 per mL. Then the cells were centrifuged at 400 rpm for 5 minutes. Supernatant was removed and freezing mixture *i.e.*, 4 mL of medium containing 0.5 mL of serum was added to the cells. The test tube was kept in a beaker containing ice and then 0.5 mL of DMSO (Dimethyl sulphoxide) was added drop by drop. Quickly one mL of the aliquot was added in to freezing vials, the cells were frozen slowly, and initially it was placed in -20ºC for 1 hrs, and then over night at -80ºC and transferred in to liquid nitrogen. The frozen cells were thawed after removing from the liquid nitrogen by gentle agitation in a 37ºC water bath at 30 seconds. The outside of the vial was rinsed with 70 per cent ethanol and aseptically the cells were transferred into centrifuge tube, and 10 mL of medium was added and centrifuged at 400 rpm for 5min, supernatant was removed and the pellets were resuspended in 10 mL of fresh medium. This was transferred into a fresh tissue culture flask, and incubated at 27ºC for Sf-9 cells, for Hep 2 and Vero maintained at 37ºC.

Preparation of virus inoculum

The crude and formulated *Hp*NPV isolated from the laboratory reared *H. puera* larvae were used in these experiments. After gradient centrifugation, 5 mL virus suspension containing 1.3 x 10^9 PIBs/mL was mixed with equal volume of 0.1M sodium carbonate with the suspension of polyhedra and stirred at 28ºC for 30 min. The pH of the suspension was adjusted to less than 8.0 by adding 100mM Tris-HCl, at pH 7.5 and passed through 0.45µm disposable filter. Five dilutions of the filtrate (10^4, 10^5, 10^6, 10^7 and 10^8 OBs per mL) were used as inoculam. The formulated *Hp*NPV was also treated the same way and dilutions were prepared using tissue culture medium. One untreated control was kept. The experiment was replicated thrice.

Inoculation of cells

To screen for *Hp*NPV susceptibility, healthy cells of each cell line (Hep2, Vero and Sf-9) containing 10^6 cells in one mL of growth medium were transferred to 25cm^2 tissue culture flask (Nunclon) and incubated for three days (Sf-9 at 28ºCand Hep 2 and Vero cells at 37ºC). Before infecting the cells with virus, the cell lines were tested under the microscope to confirm all cells are viable. The cell lines attached well and formed an even monolayer that is not too sparse, overcrowded or clumped (If they are too crowded the virus will not replicate efficiently) were selected. From the flasks containing cell lines the medium was removed and 1mL of each virus inoculum was added drop wise, very gently over the cells. In the case Sf-9, the flasks were maintained at 27ºC + 0.5ºC for 1 hrs. Vero and Hep2 were incubated at 37ºC for 1 hrs. The flasks were rocked gently every 5 to 10 mins to ensure an even coverage of virus over the cells. After 1 hrs incubation, the remaining inoculum and culture medium was removed. Using Pasteur pipette the medium was poured on to the other side of the flask so as not to dislodge the cells. The inoculated flasks of Sf 9 cells were closed and maintained at 28ºC while the Hep 2 and Vero cells were kept at 37ºC. After inoculation the cells were examined with photographic ocular inverted phase contrast microscope every 4 hrs interval for cytopathic

effect (CPE) and photographed every 24 hrs up to 5 days.

RESULTS

Safety of *Hp*NPV to silkworm

The results are presented in Table 1. It can be seen that the silkworm infected with *Hp*NPV did not show any symptom of mortality. Larvae fed with crude virus and formulated *Hp*NPV did not show variation with regard to larval weight, fresh weight of cocoons, shell weight, fecundity. Similarly there was no significant difference in larval weight, pupation percentage, weight of cocoons, weight of cocoon shells and fecundity between the treated larvae and the untreated larvae.

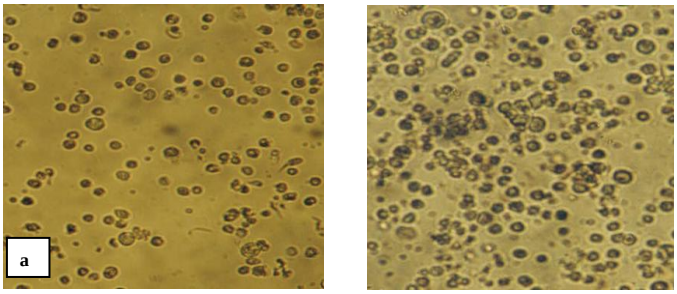

Figure 1. *Spodoptera frugiperda* ovary cell line inoculated with *Hp*NPV. **a)** Cell line before treatment **b**) Cell line 4th day after treatment showing increase in healthy cells

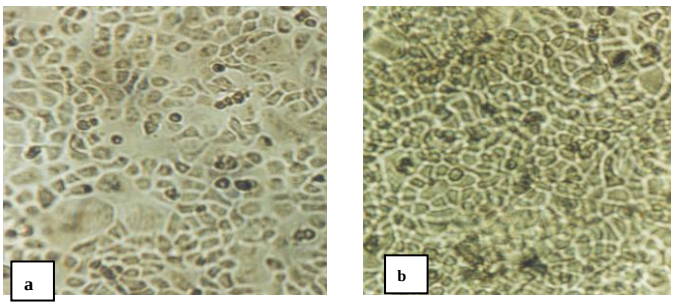

Figure 2. Human larynx cell line (Hep-2) inoculated with *Hp*NPV **a)** Cell line before treatment **b)** Cell line 4th day after treatment showing increase in healthy cells

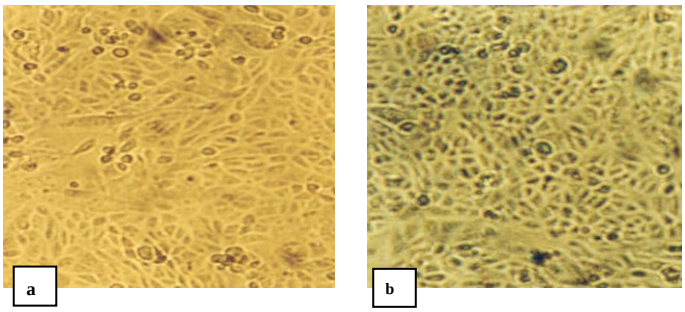

Figure 3. Monkey kidney cell line (Vero) inoculated with *Hp*NPV a) Cell line before treatment b) Cell line 4th day after treatment showing increase in healthy cells

Safety of *Hp*NPV to the bird, Indian mynah

In the case of the bird fed with NPV infected *H. puera* larvae, presence of the viral occlusion bodied was noted in the faecal droppings within I h post feeding. Presence of viral particles in the faeces was observed until 24 hrs post feeding. This suggested that the viral particle did not remain in the body of the bird for long time. After 24 hrs post feeding, no viral presence was observed in the faeces. The test bird behaved normally and remained healthy throughout the observation period of three months suggesting no deleterious effect of *Hp*NPV.

In-vitro safety testing of *Hp*NPV

The *Hp*NPV treated Sf-9 cell lines were periodically observed under the microscope for the presence of occlusion bodies up to five days. But none of the Sf-9 cell lines were found infected (Fig 1) and the number of healthy cells was found increased. The treated cells were not showing any difference compared to the untreated cell lines. The same was the case with the *Hp*NPV treated Hep 2 (Fig 2.) and Vero cell lines (Fig 3.) where no death of cells, giant cell formation or clumping of cells were found.

DISCUSSION

Nucleopolyhedroviruses are exclusively known from arthropods and considered to be host specific entomopathogenic viruses including more than 30 and safe. Safety test of more than 51baculoviruses resulted in a long and complete safety record (Ignoffo, 1973; Burges *et al*., 1980a, 1980b; Groner, 1986). No adverse effect on human health has been observed in any of these investigations indicating that the use of baculovirus is safe and does not cause any health hazards. Even though NPVs are generally considered to be safe to non-target organisms, safety testing is mandatory for their registration as commercial pesticides. Hence, in the present investigation the safety of *Hp*NPV was looked into in detail. Non infectivity of crude *Hp*NPV to lepidopteran forest pests like the caster semi looper, *Achaea janata*, ailanthus webber, *Atteva fabriciella*, Cassia semi looper *Catopsilia crocale*, ailanthus defoliator, *Eligma narcissus*, teak skeletonizer, *Eutectona machaeralis* (Ahamad, 1995), *Helicoverpa armigera*, *Spodoptera litura*, *Amsacta albistriga*, *Bombyx mori* (Rabindra *et al*., 1997) have been reported in the past. The present study has reconfirmed the

host specificity of *Hp*NPV and its safety to silkwom. Several NPVs have been reported to be safety of NPV of the red hairy caterpiller, *Amsacta albistriga* (Walker) (Arctiidae: Lepidoptera) to white mice and poultry birds. In a subacute toxicity evaluation, Kumar and Mathad (1978) reported no allergic effect of NPV of *Mythimma separata* (Walker) to the skin of the albino rats. The NPVs of the grampod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera) and *Spodoptera litura* (Fabricius) (Noctuidae: Lepidoptera) were reported to be innocuous when fed orally to the poultry birds (Narayanan, 1979; Regupathy *et al*., 1978). The present study revealed that *Hp*NPV is safe to the Indian mynah, which may be applicable to all other birds in general. The information is valuable as several birds have been reported to be present in teak plantations when teak defoliator outbreaks safe against mammals and birds by various workers. Narayanan *et al*. (1977, 1978) reported occur. It may be assumed that feeding on *Hp*NPV infected teak defoliator larvae would no way be harmful to the birds. No cytopathic effects were observed in any of the cell lines inoculated with crude as well as formulated *Hp*NPV. The possibility of replication of baculovirus in vertebrate and mammals was investigated by various workers by challenging many vertebrate and human cell lines with OB of many baculoviruses. Although virus uptake of these cells was frequently reported, no evidences of virus replication or cytopathological effects were observed. The few early reports, which stated baculovirus replication in vertebrate cell lines (Himeno *et al*., 1967; McIntosh and Shamy, 1980) could never be demonstrated or confirmed in other laboratories.

Table 1. Safety of *Hp*NPV to *Bombyx mori* larvae

Treatment	Larval weight (g) SD	Pupation%	Weight of cocoon	Weight of cocoon shells	Fecundity
Control leaf	3.19±0.15	87.90±8.8	1.18±0.16	0.17±0.11	599.7±15.6
crude *Hp*NPV treated leaf	3.20±0.19	84.14±8.1	1.14±0.22	0.14±0.02	574.4±31.1
formulated *Hp*NPV treated leaf	3.19±0.21	74.49±5.2	1.15±0.16	0.14±0.03	561.0±3.6
'F' Statistic	0.98^{NS}	0.16^{NS}	0.75^{NS}	0.27^{NS}	0.14^{NS}

NS-Not significant; SD - Standard deviation

Using a recombinant *Ac*MNPV containing the cat gene under the control of the Rous sarcoma virus terminal repeat promoter and the β-galactosidase gene under the control of the very late polyhedrin promoter reporter gene, expression was analysed in different invertebrate and vertebrate cell lines (Carbonell *et al*., 1985; Carbonell and Miller, 1987). No cat or β-galactosidase activity was detected in transfected mouse or human carcinoma cells. On the other hand, recent reports showed that recombinant *Ac*MNPV virus is efficiently taken up by human hepatocytes via an endosomal pathway.

Recombinant *Ac*MNPV carrying the *Escherichia coli* lacZ reporter gene under control of the Rous sarcoma virus promoter and mammalian RNA processing signals showed considerable expression levels in the human liver cell line HepG2, but at very low levels, or not at all, in cell lines from other tissues (Hofmann *et al*., 1995; Boyce and Bucher, 1996). Based on these findings it was suggested that baculovirus might be exploited for liver-directed gene therapy. From the view of baculovirus safety this results also showed that careful attention has to be paid to the promoters used to control heterologous gene expression in recombinant baculoviruses. Safety of baculovirus is a topic, which needs to be reviewed very often. Adverse effects of baculovirus formulation on non-target organisms have been reported at least in few instances. In a recent study lethal effect of NPV of *Lymantria dispar* (LdMNPV) to larvae of coot calm (a molluscan species) *Mulinia lateralis* was reported (Gormly *et al*., 1996).

Recently Elizabeth *et al*., (2001) reported the adverse effect of wettable powder formulations of *Anticarsia gemmatalis* NPV to a general pentatomid predator, *Podisus nigrispinus*, through generations. However, the unformulated, *A. gemmatalis* NPV was specific and safe to the predator. Further it was suggested that the observed effect could be due to some inert components present in the commercial formulation Narayanan (2002). The above studies have explicitly proved the safety of *Hp*NPV.

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Manuscript history

Received : 31.08.2012

Revised : 29.10.2012

Accepted :30.11.2012