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Vertical transmission of nucleopolyhedrovirus in the silkworm, *Bombyx mori* L.

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Abstract

Nucleopolyhedrovirus (NPV) was tested for vertical transmission in the silkworm, *Bombyx mori*. Fifth instar larvae were exposed to four different dosages of BmNPV (830, 1300, 1800, and 2000 OBs/larva) and a dosage of about 2000 OBs/larva was found suitable for obtaining infected adults. Histopathological studies revealed the infection in susceptible tissues and organs initially, and at later stages of infection cycles the spermatocytes and nurse cells in the young oocytes were infected in the larval rudiments of testis and ovary, respectively. The mating of infected females with uninfected males resulted in significant reduction in fecundity ($P < 0.01$) and hatching of eggs ($P < 0.001$) due to transovarial transmission of BmNPV. Mating tests of uninfected females and infected males also confirmed venereal transmission as there was a significant reduction in hatching of eggs ($P < 0.01$). Further, among the F_1 hybrid offspring (infected female × uninfected male) that were infected transovarially, larval progeny died at first and second instar stages, whereas those infected venereally developed acute lethal infection late and died by the end of third and fourth instar stage. PCR amplification and sequencing of 473 bp of immediate early-1 (*ie-1*) gene of BmNPV isolated from the viral-infected parent and the F_1 offspring confirmed that the viral infection is vertically transmitted to the progeny.

Keywords: Silkworm; *Bombyx mori* NPV; Nucleopolyhedrovirus; Vertical transmission; Immediate early-1 gene

1. Introduction

Bombyx mori has long been reared as a beneficial insect in the sericulture industry and as an experimental laboratory insect. A major disease problem next to pebrine (*Nosema bombycis* infection) in mass rearing of this insect in tropical countries like India is Grasserie, a polyhedrosis disease caused by *B. mori* nucleopolyhedrovirus (BmNPV). The appearance of BmNPV occlusion bodies (OBs) in the blood cells of infected silkworm was first described independently by Maestri (1856) and Cornalia (1856). Historical writings have described catastrophic outbreaks of polyhedrosis disease in regions of Asia and Europe where *B. mori* were reared on a large scale (Benz, 1986). Usually high temperature and humidity prevalent in tropical regions is conducive to proliferation of polyhedrosis disease. It is known to occur in all larval instars and more commonly in fourth and fifth instars during all seasons causing 20–50% cocoon crop losses in India (Chitra et al., 1975; Nataraju et al., 1998; Samson et al., 1990; Shivaprakasam and Rabindra, 1995; Vidya, 1960).

Disease is a condition in which a state of physiological equilibrium of an organism with its environment becomes unbalanced due to noninfectious or infectious causes (Fuxa and Tanada, 1987). At present pebrine-free eggs of silkworm races are used in the sericulture industry. The female moths are inspected for pebrine infection after oviposition and if they are *N. bombycis* infected, all their eggs are discarded. For

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other pathogens, the decontamination of egg surface by formaldehyde was mentioned as particularly important to prevent the transovum transmission of nucleopolyhedrovirus not only in *B. mori* (Krishnaswami et al., 1973) but also in rearing of other pest insects like *Trichoplusia ni* (Henneberry and Kishaba, 1966). During rearing, other precautionary measures such as use of disinfectants, removal of diseased insects and general cleanliness and sanitation techniques advocated by Krishnaswami et al. (1973) and Krishnaswami (1978) are followed by the sericulturists and researchers.

In spite of attempting disease prevention measures, we observed the incidence of BmNPV infection in larvae during rearing at fifth instar stage quite frequently, although the extent of the problem varied from one batch to the other. Kukan (1999) reviewed evidence that virus could be transmitted from parent to progeny in lepidopterans and could be found in caterpillars reared from surface decontaminated eggs. To investigate such possibility of vertical transmission of BmNPV in *B. mori*, the present study was conducted. The objectives were to determine the transmissibility of BmNPV via parents to progeny, which is one of the most important conditions for the sericulture industry.

2. Materials and methods

2.1. Silkworm rearing

The eggs of commercial crossbreed PM (Multivoltine) $\times$ NB_4D_2 (Bivoltine) of *B. mori* were obtained from Government Grainage Centre, Directorate of Sericulture, Maharashtra. The hatched larvae were reared in the decontaminated rearing house using disinfected rearing appliances. The feeding, cleaning, and sanitation schedule was followed according to Krishnaswami (1978).

2.2. Diagnosis of disease

The larvae were observed daily to identify the NPV infected ones as per the signs and symptoms of disease. The larvae that died were dissected and tissues were examined as soon as possible with the naked eye and tissue smears under light microscopy. If viral polyhedral occlusion bodies (OBs) were observed, they were dissolved in 1 N NaOH and tested under phase contrast microscope to determine their viral origin (Thomas, 1974). Some of the smears were stained with Azan stain (Humason, 1962) after due fixation. The OBs stained dark red.

2.3. Isolation and purification of OBs

Larvae with symptoms of NPV infection were isolated from the stock culture and triturated in distilled water. The homogenate was filtered through muslin cloth. The filtrate was subjected to repeated centrifugation until clear white OBs were obtained. They were also obtained directly by bleeding the haemolymph of infected larvae. The purified OBs were stored in refrigerator until their use.

2.4. Larval inoculation, histopathology, and mating of the adults

About 1500 newly moulted fifth instar healthy larvae were starved for 6 h and divided into five groups, each containing 300 larvae. The larvae from four groups were inoculated individually per os via ingestion of a 2-cm^2 piece of mulberry leaf coated with 10 μl suspension of OBs of different concentrations (8.3×10^4, 1.3×10^5, 1.8×10^5, and 2.0×10^5 OBs/ml). The first group was inoculated with 830, second with 1300, third group with 1800, fourth group was inoculated with 2000 OBs/larva, and fifth group was fed with a piece of leaf dipped in distilled water and used as a control.

The inoculated larvae that consumed the entire dosage and controls were reared further on fresh mulberry leaves as per the feeding, cleaning, and sanitation schedule till the onset of spinning of cocoons. A few larvae from each group were sacrificed daily for histopathological study. The midgut, fat body, testes, and ovaries of inoculated insects were dissected under a stereoscopic binocular microscope and were fixed in Bouin's fixative for 24 h, dehydrated in alcohol series, cleared in xylene and embedded in paraffin wax. Embedded materials were sectioned at 6 μm and stained with Azan stain (Humason, 1962).

The mortality due to infection at larval and pupal stages was recorded. The moths emerged from each group were provisionally regarded as infected. Those that emerged from the pupae of the group inoculated with 2000 OBs/larva were selected for the mating experiment. Infected females (IF) were paired with healthy (uninfected) males (HM) for transovarial transmission studies and healthy (uninfected) females (HF) were paired with infected males (IM) for venereal transmission. In addition to this IF and IM were also paired to assess the severity of transmission. HF and HM were paired as controls. After oviposition, all the moths, both male and female were macerated and the whole wet mounts were examined for NPV infection under phase contrast microscope. The egg batches from experimental uninfected pairs were discarded.

2.5. Diagnosis of infection in the progeny

Eighteen to twenty hours after oviposition, test eggs were surface sterilized by immersing in 2% formaldehyde for 15 min at room temperature, washed several times with tap water and finally rinsed with distilled

water. Those eggs were given cold acid treatment (HCl of 1.1 specific gravity at 25 °C for 60–80 min) to terminate the egg diapause and washed in running water to remove traces of HCl. The newly hatched larvae were reared on fresh mulberry leaves and their mortality due to transmission of infection was recorded at each succeeding larval stage. The larval progeny died due to NPV infection were homogenized individually in distilled water and OBs were isolated and counted using haemocytometer.

2.6. Viral DNA extraction, PCR amplification, and sequencing

The viral DNA was isolated from the OBs collected from parent and F_1 offspring according to Summers and Smith (1987) with slight modifications. Briefly, the OBs were digested by proteinase K for about 4 h and DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated in 2.5 volumes of ethanol, washed with 70% ethanol, and resuspended in 10 mM Tris–1 mM EDTA. Twenty nanograms of viral DNA was used to amplify the 473-bp product of the immediate early-1 (*ie-1*) gene sequence (GenBank Accession No. X58442, Huybrechts et al., 1992) using the following primers:

Forward —5′-CCAAACGACTATGACGCAAATTAA TTTT-3′

Reverse —5′-TTGTTAAATTGGCCCACCACACTTT GT-3′

The DNA was also extracted from the freshly hatched larvae of F_1 obtained from the cross of HF and HM, as described previously (Nagaraja and Nagaraju, 1995) and used as a negative control in PCR amplification with *ie-1* primers.

The PCR amplification was performed in 10 mM Tris–HCl, pH 8.3 (50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.01% Triton X-100), 1 mM dNTPs, with 5 pmol of forward and reverse primers and 0.5 U of *Taq* Polymerase (IMMOLASE DNA Polymerase from Bioline, Germany) per reaction. Thermal cycling was carried out on a thermal cycler (PTC 100, MJ Research, Watertown, MA) using the following conditions: initial denaturation of 5 min at 94 °C; 35 cycles of 30 s at 94 °C; 30 s at 52 °C; and 1 min at 72 °C; and final extension of 10 min at 72 °C. The PCR products were separated in 1.0% agarose gel along with 100 bp ladder (NEB, Beverly, MA).

For DNA sequencing, 50 ng of PCR product was used in a sequencing reaction that contained 8 μl of Ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA) and 5 pmol of forward primer. The cycling conditions used were as follows: 25 cycles of 96 °C for 10 s, 50 °C 5 s, and 60 °C 4 min. Samples were ethanol precipitated, washed with 70% ethanol, and resuspended in Hi-Di formamide (Applied Biosystems). The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences obtained were checked against National Centre for Biological Information (NCBI) database using BLASTN search algorithm (Altschul et al., 1997).

3. Results

3.1. Diagnosis of disease

The diseased larvae became obvious in the rearing trays and on mountages during cocoon spinning due to their shiny white translucent skin, elevated inter-segmental membrane and swelled body segments. At an advanced stage of infection the body wall ruptured and white turbid haemolymph containing a large number of OBs oozed out. The cadavers were white at the time of death and became dark within 3–5 h as they putrefied.

3.2. Inoculation of virus

The OBs of BmNPV isolated from the haemolymph of infected larvae were uncontaminated and hence used for inoculation of larvae. The peroral inoculation of OBs to fifth instars at dosage of 2000 OBs/larva resulted in death of about 50% of the larvae and pupae prior to adult eclosion, and all of the adults were infected. At lower dosages of 830, 1300, and 1800 OBs/larva many individuals survived to adulthood and about 92, 96, and 96% of these adults were infected, respectively (Table 1).

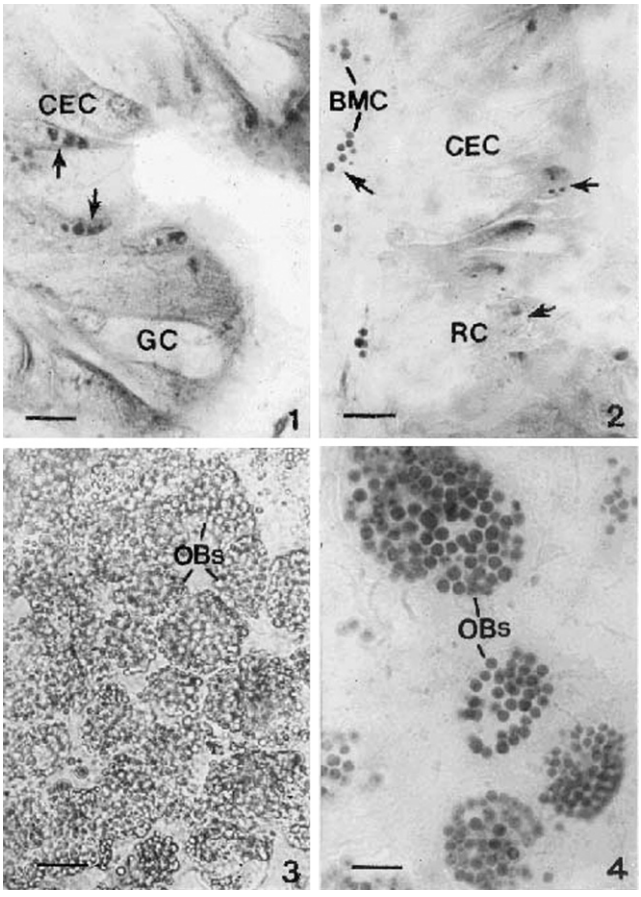
3.3. Histopathology

Histopathological observations revealed the hypertrophy of the nuclei of midgut columnar epithelial cells

Table 1
Effect of BmNPV infection on larval mortality, pupation, and moth infectivity of silkworm, *B. mori* after inoculation at fifth instar stage

Inoculum (OBs/larva)	No. of larvae observed	Mortality due to NPV infection		No. of moths emerged	No. of moths examined for infection	% NPV infection
		No. of larvae	No. of pupae			
Control	200	00	00	200	25	00
830	200	04	14	182	25	92
1300	200	35	39	126	25	96
1800	200	40	43	117	25	96
2000	200	46	60	94	25	100

2 h post inoculation (p.i.), indicating the initiation of NPV infection. The infection progressed further and most of the cells were harboring a few OBs in their nuclei and by the 7th day a few regenerative and basal membrane cells also became infected, however goblet cells remained uninfected (Figs. 1 and 2). About 4 days p.i. haemocytes and fat body cells exhibited the infection and by the 7th day almost all the haemocytes and fat body cells contained a large number of OBs in the nuclei in case of severe infection (Figs. 3 and 4). The infection of gonadal rudiments occurred quite late at about 7 days p.i. In testes, the wall of the follicle and a few primary and secondary spermatocytes in the cysts exhibited OBs in the nuclei (Figs. 5 and 6). In ovaries, the nuclei of nurse cells in the young oocyte follicle contained OBs (Figs. 7 and 8).



Figs. 1–4. BmNPV infection in the midgut, haemocytes, and fat body of fifth instar larva of *Bombyx mori*. (Fig. 1) Columnar epithelial cells (CEC) of the midgut showing few OBs (arrows) in the nuclei 4 days post inoculation. Note goblet cells (GC) remained uninfected. Azan stain. Bar = 25 μm. (Fig. 2) Columnar epithelial cells (CEC), regenerative cells (RC), and basal membrane cells (BMS) showing OBs in the nuclei (arrows) 7 days post inoculation. Azan stain. Bar = 25 μm. (Fig. 3) Infected haemocytes filled with OBs 7 days post inoculation. Phase contrast. Bar = 25 μm. (Fig. 4) Infected fat body cells showing darkly stained OBs in the nuclei 7 days post inoculation. Azan stain. Bar = 20 μm.

3.4. Mating and transmissibility of NPV

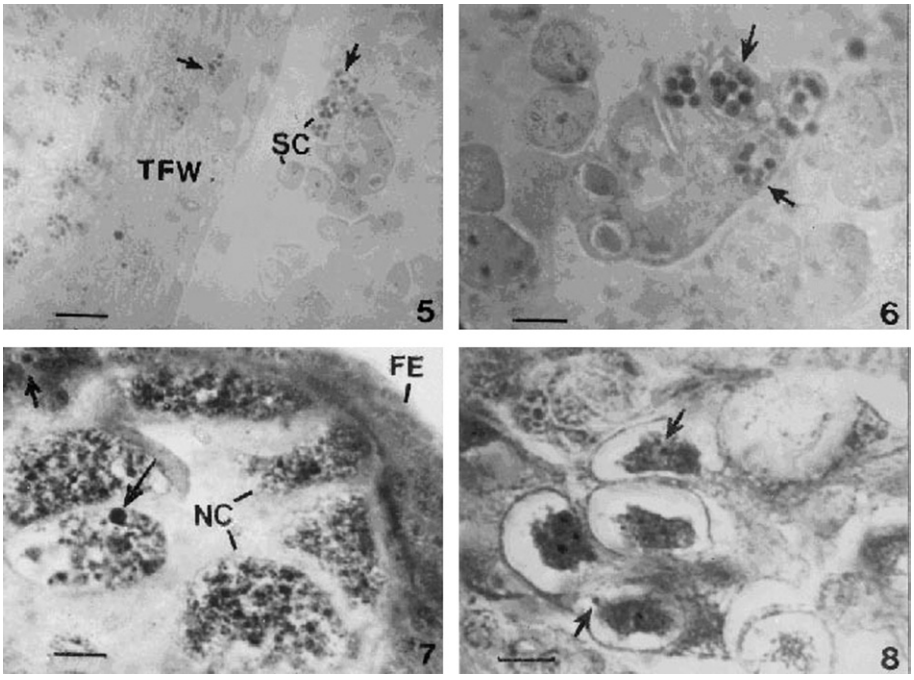
The reduction in the fecundity of infected female (IF) mated with infected male (IM) and healthy (uninfected) male (HM) was more significant ($P < 0.01$) than the healthy (uninfected) female (HF) mated with IM ($P < 0.05$). Moreover, the number of hatched eggs was significantly lower in all the three matings (Table 2). The prevalence of NPV was recorded in the larval progeny of all the matings, however 100% death of larval progeny of IF × IM mating occurred at first instar stage only. The progeny of IF × HM mating died at first (78%) and second (22%) instar stages, suggesting NPV transmission via eggs, whereas the progeny of HF × IM pairing died at third (57%) and fourth (43%) instar stages indicating the venereal transmission of NPV. These results indicate the vertical transmission of BmNPV in the progeny of all the three matings, only the degree of infection and expression of disease in the larval stage varied which also reflected on the recovery of OBs from larval progeny of IF × IM, IF × HM, and HF × IM matings (Table 2).

3.5. PCR amplification and sequencing of ie-1 gene fragment of BmNPV from infected parent and F_1 offspring

DNA isolated from the polyhedra collected from both parent and offspring yielded 473-bp *ie-1* product upon amplification as expected, while the negative control did not result in any amplification (Fig. 9). Further, sequencing of the *ie-1* PCR product confirmed that the amplification product was of BmNPV origin. No differences in *ie-1* nucleotide sequence of BmNPV isolated from parent and F_1 offspring were observed (Fig. 10). The BLAST results showed >97% similarity with the published BmNPV *ie-1* sequence (GenBank Accession No. X58442). These results confirm that the BmNPV recovered from F_1 offspring are derived from parent through vertical transmission.

4. Discussion

In silkworm, *B. mori* the most common mode of entry of NPV is per os as in the case of other lepidopteran insects. Individual feeding of a piece of leaf coated with 10 μl suspension of 2×10^5 OBs/ml ensured the ingestion of a dosage of about 2000 OBs/larva which was sufficient to initiate the infection since about 50% mortality occurred prior to eclosion of adult moths. Usually a successful infection depends on the ingestion of sufficient virus to initiate replication in the host. The virus up take is controlled by larval behavior, feeding rate, and the concentration and distribution of the viral dosage (Hunter et al., 1984).



Figs. 5–8. BmNPV infection in the rudiments of testis and ovary of fifth instar larva of *Bombyx mori*. (Fig. 5) Cells of testis follicle wall (TFW) and the cyst containing spermatocytes (SC) showing darkly stained OBs (arrows) in the nuclei 7 days post inoculation. Azan stain. Bar = 50 μm. (Fig. 6) Magnified view of BmNPV-infected spermatocytes in the cyst containing OBs (arrows). Azan stain. Bar = 20 μm. (Fig. 7) Young oocyte follicle enveloped with follicular epithelium (FE) showing OBs (arrows) in the nuclei of nurse cells (NC) 7 days post inoculation. Azan stain. Bar = 30 μm. (Fig. 8) Another oocyte follicle showing vacuolated nuclei of nurse cells containing few OBs (arrows) 7 days post inoculation. Azan stain. Bar = 30 μm.

Table 2
Assay on transovarial and venereal transmission of BmNPV from infected parents to the progeny of silkworm, *B. mori*

Progeny stage assayed	Paired moths			
	HF × HM	IF × IM	IF × HM	HF × IM
No. of eggs laid[#]	580 ± 47	360 ± 34**	395 ± 22**	438 ± 27*
No. of eggs hatched[#]	514 ± 75	136 ± 38***	182 ± 13***	227 ± 59**
% progeny mortality at each instar stage due to NPV infection	00	100 (1st instar)	78 (1st instar) 22 (2nd instar)	57 (3rd instar) 43 (4th instar)
No. of OBs/larva ($\times 10^7$)	00	0.90	1.21	2.32

HF, healthy female; HM, healthy male; IF, infected female; IM, infected male.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
[#] Mean ± SE.

Histopathology of midgut, fat body, and haemocytes confirms the initiation of infection cycles in the susceptible cells and organs of *B. mori*. Benz (1963) reported that non occluded virions of the virus usually initiate infection cycles in tissues and organs like haemocytes, fat body, hypodermis, tracheal matrix, muscle, nerve ganglia, and pericardial cells in the other Lepidoptera. In *B. mori* in addition to these, larval gonadal tissues both testes and ovaries were found to be infected at varying degree at later stages of infection.

The lightly and moderately infected larvae exhibited a few infected nurse cell nuclei of the young oocyte follicles in the ovarian rudiments. In male larvae the nuclei of the external epithelial lining of testicular follicle and spermatocytes inside the cyst were also infected containing OBs in them. These observations clearly indicate that young oocytes leading to maturation and spermatocytes leading to reduction division harboring virions in them and in survivors the infection persisted in the eggs and spermatozoa during pupal and adult stages.

The mating of IF with IM and HM resulted in significantly ($P < 0.01$) low fecundity, that may be attributed to improper vitellogensis in the infected oocytes. Furthermore, after decontamination of egg-surface, larval progeny at first instar stage exhibited viral infection in them and died at first and second instar stages,

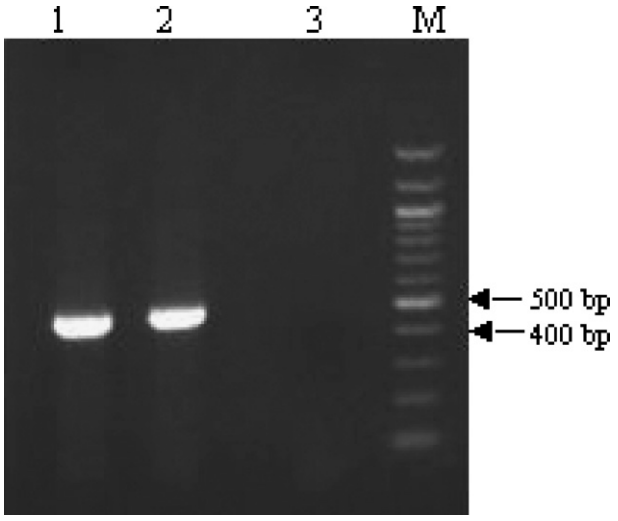


Fig. 9. PCR amplification of 473 bp *ie-1* gene of BmNPV isolated from: lane 1, infected parent; lane 2, F_1 offspring of infected parent; and lane 3, negative control (DNA from freshly hatched F_1 larvae of healthy parent). M, 100 bp size marker.

suggesting transovarial transmission of virus. The possibility of venereal transmission was also confirmed when progeny of HF and IM pairing exhibited the virus infection in the larval progeny analogous to the latent form and death occurring at later stage of their development (third and fourth instars). Although the transovarial and venereal transmission of NPV in insect populations are questionable (Payne, 1982), there are some clear evidence of transovarial transmission of viruses. Recently, Fuxa et al. (1999, 2002) confirmed transovarial transmission of TnNPV, TnCPV, and AcMNPV in *Trichoplusia ni* population. Diallo et al. (2000) and Gokhale et al. (2002) have reported the vertical transmission of yellow fever and dengue-2 viruses in *Aedes aegypti* and *A. albopictus* mosquitoes, respectively, via eggs of infected females in the progeny population.

Ridhards et al. (1998) and Kukan (1999) reviewed that dispersal of baculoviruses by adults may occur following vertical transmission to larvae either inside the egg (transovarial) or to its surface (transovum). However, they pointed out that the dominant source of virus among generations is probably through environmental contamination. The present study based on demonstration of viral DNA as shown by PCR amplification and sequencing of a viral gene in the offspring derived from the infected parent confirms that the larvae infected by virus at sub-lethal levels can survive and the moths derived from such larvae can successfully transmit the virus infection vertically to their offspring. Hence, it is important to confirm the viral-free nature of the eggs preferably by PCR-based techniques before they are used for seed multiplication purpose or for large-scale distribution to farmers. However, further studies focusing on sensitivity of the PCR assay are needed to put this into application. For domesticated silkworm,

```
F1 offspring        CGCGTCGTAC ACCAGTGCTC CGACTCCGTC CCGGGCGTCG TTCGACAACG
Infected Parent     CGCGTCGTAC ACCAGTGCTC CGACTCCGTC CCGGGCGTCG TTCGACAACG

F1 offspring        GCTATTCAGA GTTTTGTGAT AAACAACAGC CCAACGACTA TTTGAATTAT
Infected Parent     GCTATTCAGA GTTTTGTGAT AAACAACAGC CCAACGACTA TTTGAATTAT

F1 offspring        TATAACAATC CCACGCCGGA TGGAGCCGAC ACGGTAGTAT CTGACAGCGA
Infected Parent     TATAACAATC CCACGCCGGA TGGAGCCGAC ACGGTAGTAT CTGACAGCGA

F1 offspring        GACTGCAGCA GCTTCAAACT TTTTGGCAAG TGTCAATTCG TTAACTGATG
Infected Parent     GACTGCAGCA GCTTCAAACT TTTTGGCAAG TGTCAATTCG TTAACTGATG

F1 offspring        ATAACGATAT AATGGAATGT TTGCTCAAGA CCACTGATAG TCTCGGAGAA
Infected Parent     ATAACGATAT AATGGAATGT TTGCTCAAGA CCACTGATAG TCTCGGAGAA

F1 offspring        GCAGTTAGTT CTGCTTATTA TTCGGAATCC CTTGAGCTGC CTGTTGCGGA
Infected Parent     GCAGTTAGTT CTGCTTATTA TTCGGAATCC CTTGAGCTGC CTGTTGCGGA

F1 offspring        TCAACCATCG CCCAGTTCTG CTTATAATGC GGAATCTTTT GAGCATCCTG
Infected Parent     TCAACCATCG CCCAGTTCTG CTTATAATGC GGAATCTTTT GAGCATCCTG

F1 offspring        TTGATGTGAA CCAACCATCG GCAGCTGGAA CTAAACGGAA GCTGGACGAA
Infected Parent     TTGATGTGAA CCAACCATCG GCAGCTGGAA CTAAACGGAA GCTGGACGAA

F1 offspring        TACTTGGACG ATTCACAAAG TGTGGTGGCC C
Infected Parent     TACTTGGACG ATTCACAAAG TGTGGTGGCC C
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Fig. 10. BmNPV *ie-1* 473 bp sequence from BmNPV isolated from infected females (IF) and F_1 offspring of IF × Healthy male (HM).

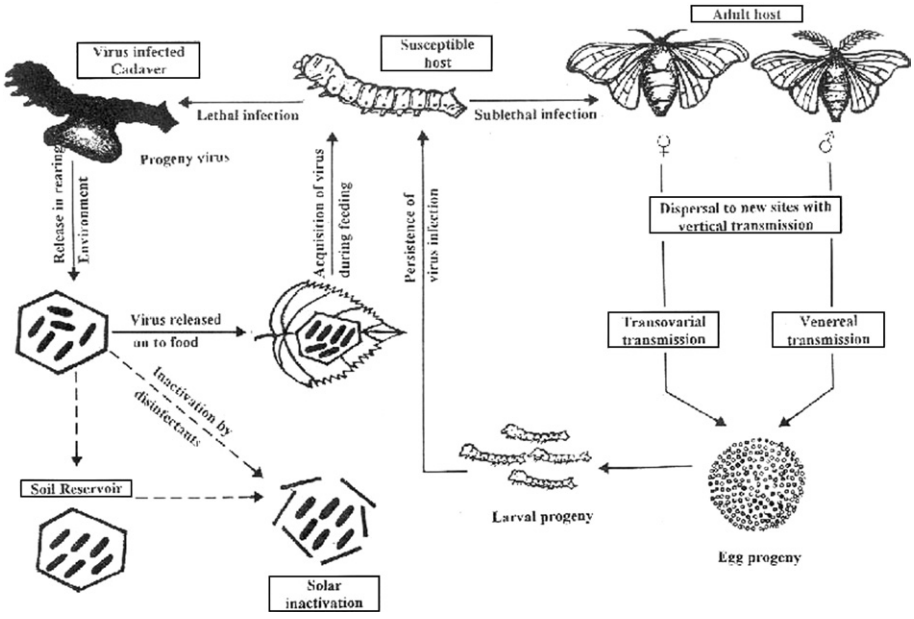

Fig. 11. Schematic representation of BmNPV transmission routes in the silkworm, *Bombyx mori.*

B. mori even though the sericulturists take precautionary measures before and during rearing such as decontamination of rearing house and appliances and use of disinfectants to destroy the OBs of NPV released by infected silkworms of the previous crop, some OBs may escape inactivation and become a source of infection to the next generation (Fig. 11). So even if the adult survivors of the sublethally infected larvae are used in the sericulture industry for egg production, the unfavorable effect on cocoon production might be quite large due to vertical transmission of NPV both transovarially and venereally.

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