Efficacy of *Spodoptera litura* multiple nucleopolyhedrovirus after serial passage through the homologous insect larval host

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An originally isolated baculovirus, *Spodoptera litura* multiple nucleopolyhedrovirus (SpltMNPV) was serially passed through the *S. litura* larvae for up to four generations to determine the mean number of occlusion bodies (OBs) harvested per larva and their efficacy in terms of infectivity, feeding cessation and speed of kill of host larvae. The results revealed that the mean number of OBs harvested per larva increased significantly with increase in the dose of SpltMNPV at each passage and the yield was significantly lower in original stock wild-type SpltMNPV (P₀) as compared to serially passed SpltMNPV (P₁, P₂, P₃ and P₄). Laboratory bioassays indicate that median lethal doses (LD$_{50}$), median times to feeding cessation (FT$_{50}$) and median survival times (ST$_{50}$) of P₀, P₁, P₂, P₃ and P₄ were significantly different from each other. The OBs of each passage when tested for their cross-infectivity against *Spodoptera exigua* and *Spilarctia obliqua* revealed significant reduction in their mortality. These results indicate that serially passed SpltMNPV is more host specific and more effective biocontrol agent than the original stock wild-type virus and can be adopted for mass production as a viral pesticide for control of the *S. litura*.

**Keywords:** Baculoviruses, Bioinsecticides, Entomopathogens, Noctuidae

Despite decades of warnings, the inappropriate use of chemical pesticides continues to pose threats to the environment and human health. There have been massive upsurges in pesticide use in recent years and their use is increasingly associated with negative impacts to both human health and the environment. In addition their surging costs are beyond the reach of resource-poor farmers in developing countries. Under these circumstances utilization of natural occurring insect pathogens may prove worthy for insect-pest control. With a rich biodiversity of several entomopathogens, the biocontrol workers in India are developing safe, economical and reliable control of crop pests. Improved access to locally produced, and therefore affordable, biopesticides is a key component of a national Integrated Pest Management (IPM) policy. In this direction, many baculoviruses have been now a day successfully used as biopesticides on a large scale to regulate insect-pest populations owing to their strong pathogenicity, persistence and ecological safety. An important feature of baculoviruses is their capacity to replicate in and spread through host populations under field conditions by horizontal and vertical transmission. The polyhedra which are the form of virus used for insect control are stable, can survive for years if they are in the favourable conditions and cause lethal epizootics in the field which provide added control of pests in nature.

Although, SpltMNPV has been found very effective against *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), greater amount of variation in its efficacy hindered its practical utility in the field conditions. If produced, formulated and applied in appropriate ways, it can provide ecological and effective solutions to pest problems. For instance, in India, virus production by small farmers or village based biopesticide units is straightforward with the nucleus stock of virus obtained from infected larvae under field conditions. Unfortunately, variation within baculoviruses isolates may influence some biological properties, such as the virulence to their specific host as well as its host range. Serial passage of baculoviruses through the homologous larval host drives the evolution of virulence as the pathogen strain with the highest replication rate achieves the greatest numerical representation in the inoculum. A thorough understanding of the interactions of

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nucleopolyhedroviruses (NPVs) with the homologous and heterologous insect hosts is therefore necessary to facilitate their development as biopesticides in order to seek better investment prospects from the public sector to the multinational private sector. In depth investigations were therefore undertaken to assess the pattern of virus production and efficacy of SpltMNPV after serial passage through the homologous insect larval host, *S. litura*, and to check the cross-infectivity against the heterologous insect larval hosts, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) and *Spilosoma obliqua* (Walker) (Lepidoptera: Arctiidae) which is an issue of direct relevance to the environmental assessment studies on the stability of baculoviruses.

**Materials and Methods**

*Host insect Spodoptera litura*—The *S. litura* larvae used in this study was obtained from a laboratory colony, established from a larval population of the insect collected in the region of Jammu during 2011. The culture was maintained at 28±2 °C, 60% RH, and 16L:8D photoperiod on the artificial diet as suggested by Shorey and Hale with certain modifications proposed by Gupta et al. Egg masses were collected and surface-sterilized with 0.01% sodium hypochlorite. The larvae were reared in a plastic container (30 × 20 × 10 cm) lined with a paper towel at the bottom. The diet and the paper were changed every 24 h.

A laboratory cohort of *S. litura* larvae were randomly selected and reared on artificial diet as already described. This was termed as parental group. From this group, 50 larvae were allowed to complete their development and the rest were used in the experiment at 2nd or 3rd instar as desired. At adult emergence, ten randomly selected females were mated to an equal number of males separately in glass jars (30 × 20 × 10 cm) and their resulting progeny were reared for four generations. In each generation, a group of 1000 larvae were randomly selected and used in the experiments.

*Virus preparation*—The multiple nucleopolyhedrovirus used in this study was originally isolated from naturally field-infected *S. litura* larvae collected from tomato fields in Jammu, India. Viral occlusion bodies (OBs) were extracted by homogenizing virus-killed larvae in 0.1% sodium dodecyl sulphate (SDS), followed by filtration through muslin cloth and subsequent pelleting through continuous sucrose gradient centrifugation for 1 h at 50,000 g. After several washes in TE (10 mm Tris-HCl, pH 8, 115 1 mm EDTA) the OBs were resuspended in 0.75 mL of distilled water and stored in aliquots at −20 °C. The virus was quantified using a haemocytometer and phase contrast microscope at x1000 magnification.

**Effect on polyhedra production of SpltMNPV after serial passage through the *S. litura* larvae**—To determine the polyhedra production of SpltMNPV after serial passage through the *S. litura* larvae, five different doses of SpltMNPV viz 3 × 10^3, 1.5 × 10^3, 7.5 × 10^2, 3.75 × 10^2, 1.87 × 10^2 OBs/larva were used in the study at each passage. The polyhedra production of SpltMNPV was compared between the passage zero (P0) and passage through *S. litura* larvae (P1-P4) in the subsequent four generations. The original stock polyhedra were termed herein as passage zero OBs (P0). To obtain the passage one OBs (P1), each 3rd instar *S. litura* larvae were placed in individual Petri dish (80 mm × 15 mm) and were offered a diet plug contaminated with above described virus-doses. For each dose 10µL of viral suspension were pipetted on each diet plug. Larvae, having eaten the entire diet plug within 24 h, were transferred to fresh uncontaminated diet and reared at 28±2 °C, 60% RH, and 16L:8D photoperiod. Larvae that did not consume the entire diet plug were discarded. Similarly, to obtain occlusion bodies (OBs) in subsequent passages viz P2, P3 and P4, the 3rd instar *S. litura* were offered diet plug contaminated with virus inoculum of preceding passage i.e. P1, P2 and P3, respectively. In all passages viral OBs were extracted by homogenizing each individual virus-killed larvae in 0.1% sodium dodecyl sulphate (SDS) and were purified as per Monobullah et al. The OBs were counted using a haemocytometer and phase contrast microscope at x1000 magnification. The purified OBs were stored at −20 °C until further use. The viral doses and a corresponding experimental control group of each passage was replicated three times with 50 larvae per replicate. Control consisted of larvae that were fed on a diet plug inoculated with distilled water.

**Efficacy of SpltMNPV after serial passage through the *S. litura* larvae**—The OBs harvested at each passage were used to determine the efficacy of SpltMNPV after serial passage through the *S. litura* larvae by per os bioassay. The intrinsic biological activities medial lethal dose (LD₅₀), median time to feeding cessation (FT₅₀) and median survival time
Cross infectivity of SpltMNPV against S. exigua and S. obliqua larvae—The cross infectivity of original stock wild-type (P0) and serially passed SpltMNPV (P1, P2, P3, P4) was checked against 2nd instar S. exigua and S. obliqua larvae by per os bioassay. Freshly moulted second-instar larvae of both species were starved for 24 h and then inoculated with a dose of $1.00 \times 10^3$ OBs/larva using artificial diet as described earlier. In each passage 10 µL of viral suspension were pipetted on each diet plug. Diet plugs were air-dried and then placed individually in an acrylic compartment insect rearing system and the experimental set up and conditions were similar as mentioned in the earlier section. Mortality was recorded daily for up to 10 days period and was confirmed by the methods described earlier.

Statistical analyses—All analyses were performed utilizing SPSS version 16. Data on mean number of OBs harvested per larva and their infectivity (cumulative mortality percentage) at each passage were analyzed through nonparametric Kruskal-Wallis H test. Probit analyses were performed for the calculation of median lethal doses (LD$_{50}$ ± 95% C.L.). Median times to feeding cessation (FT$_{50}$s), mean survival times (ST$_{50}$s) and their 95% confidence limits were calculated using log rank test under Kaplan-Meier analyses. Differences of LD$_{50}$s, FT$_{50}$s and ST$_{50}$s among the passages were analysed by Univariate Analysis of Variance.

Results

For field application of baculoviruses it is necessary to understand the interactive behaviour of virus and host in relation to polyhedra production and their efficacy as affected by passage through the homologous insect larval host. In this study the mean number of OBs harvested per larva and their efficacy by per os bioassay was examined with five different doses of SpltMNPV at each passage. The results revealed that the mean no. of OBs harvested per larva increases significantly with increase in the dose of SpltMNPV at each passage. Regardless of the dose, the mean number of OBs harvested per larva was significantly lower in P0 as compared to P1, P2, P3 and P4 (Table 1). No OBs were detected in the larvae that were fed on a diet plug inoculated with distilled water.

The OBs harvested per larva in each dose at each passage were tested for their actual pathogenecity by per os bioassay to 2nd instar S. litura larvae using five different doses. The results revealed significant increase in the cumulative mortality percentage of 2nd instar S. litura larvae with an increase in the viral dose at each passage, with a significant positive correlation (r values; P0 = 0.770; P1 = 0.752; P2 = 0.740; P3 = 0.712; P4 = 0.832) between dose and mortality. Further, at each dose a gradual increase in the cumulative mortality percentage was observed over successive passages (Table 2).

Probit analysis of the mortality data showed median lethal dose (LD$_{50}$) estimates of $2.33 \times 10^2$, $2.07 \times 10^2$, $1.78 \times 10^2$, $1.32 \times 10^2$ and $1.02 \times 10^2$.
OBs/larvae at P0, P1, P2, P3 and P4, respectively (Table 3). At P0 the LD_{50} of SpltMNPV was significantly higher (F = 7.362; df = 4, 70; P < 0.001) than LD_{50} at successive passages i.e. P1, P2, P3 and P4. It was found that the serially passed SpltMNPV was 11-56 times more infective than the original stock virus. The median time to feeding cessation (FT_{50}) values at P0, P1, P2, P3 and P4 were 97, 89, 78,
The feeding time of *S. litura* larvae infected with original stock wild-type virus was significantly higher (F=5.963; df = 4, 70; P < 0.001) than those infected with serially passed virus. The FT50 of serially passed virus was 8-32 times less than the original stock wild-type virus. The ST50 values at P0, P1, P2, P3 and P4 were 185, 169, 153, 142 and 130 h p i., respectively and show a significant decrease (F=6.432; df = 4, 70; P < 0.001) with the successive passages. Regardless of dose the speed of kill of serially passed virus was 8-29 times less than the original stock wild-type virus. These results indicate that the efficacy of SpltMNPV is enhanced after serial passage through the *S. litura* larvae.

The results on the cross infectivity of SpltMNPV against *S. exigua* (χ^2=17.77, P < 0.001) and *S. obliqua* (χ^2 = 16.34, P < 0.001) revealed that larval mortality decreases significantly with the successive passages of SpltMNPV through the *S. litura* larvae (Table 4). From this study it became evident that *S. exigua* and *S. obliqua* are susceptible to SpltMNPV, but serial passage of this virus through its natural host, *S. litura* makes it more host specific and limits its host range.

**Discussion**

The baculoviruses infect host cells, exploit what is there to replicate within them, produce polyhedra, and get transmitted from infected to healthy individuals (horizontal transmission), and also from parents to offspring's (vertical transmission). They are effective and environmentally benign insect control agents and their transmission is a key to their persistence in the environment. The SpltMNPV is used on a large scale in different parts of the country for management of the polyphagous defoliator, *Spodoptera litura* but, greater amount of variation in its efficacy has been reported. In the present study the serially passed SpltMNPV was generated and tested through the homologous insect larval host. It showed a significant improvement in insecticidal properties in laboratory *per os* bioassays as compared to original stock wild-type virus. Mean number of OBs harvested per larva increased significantly in the successive passages. When tested for its actual pathogenicity, it was found that the serially passed SpltMNPV was 11-56 times more infective than the original stock wild-type virus. The harvested OBs resulted in a quicker cessation of feeding and the FT50 of serially passed virus was 8-32 times less than the original stock virus. This finding is of practical utility since prevention of feeding damage is of primary importance. Further, onset of death in 2nd instar *S. litura* larvae was quicker as the ST50 of serially passed virus was 8-29 times less than the original stock wild-type virus. The increase in the OBs yield of a serially passed baculovirus is of great importance for the *in vivo* industrial production of baculoviruses and for evaluating the logistics and economics of virus production in virus factories. The original stock wild-type (P0) and serially passed SpltMNPV (P1, P2, P3, P4) when tested for their cross infectivity against *S. exigua* and *S. obliqua* revealed that larval mortality decreased significantly with the successive passages of SpltMNPV through the *S. litura* larvae. These results indicate that the *S. exigua* and *S. obliqua* are susceptible to SpltMNPV, but serial passage of this virus through its natural host, *S. litura* makes it more host specific and limits its host range.
of kill of host larvae. These findings are of great utility, as secondary transmission under field conditions is important for the success of the program for baculoviruses as biopesticides. It is proposed that for in vivo production systems the nucleus stock of all baculoviruses must be serially passed through the homologous insect larval host for at least 4 generations for greater efficacy and ecological specificity.

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