# Natural Occurrence, Infection Dynamics, and Molecular Characterization of Nucleopolyhedrovirus (*Spfr*NPV) in Fall Armyworm, *Spodoptera frugiperda* (J. E. Smith) from Maize Ecosystems in Gujarat, India

**Materials and methods:**

**1.** The visual observations on larval population and infection of NPV on *S. frugiperda*, larvae were randomly recorded from each selected field. Mixed larval population (different instars) of *S. frugiperda* were collected randomly and the larvae, which were sluggish, glossy and flaccid in appearance, had pale pink discoloration on ventral side of the body was observed and collected in sampling containers (**Supplementary file 1; Fig.S2**). The larvae, which were dead and stuck to the foliage upside down with the characteristic symptoms of NPV infection and also healthy larvae, were collected in separate sampling containers.

**2.1 NPV production and Purification**

The virosed larvae were homogenized using a sterile pestle and mortar using 5 ml of sterile distilled water and vortexed for 2 minutes. The suspension was filtered twice through a double-layered muslin cloth and then the filtrate was centrifuged at 500-1500 rpm for 2 minutes to remove the larger particles. The supernatant was carefully transferred and centrifuged at 5000 rpm for 20 minutes to collect the pellets containing polyhedra. The pellets containing polyhedra were re-suspended in sterile distilled water (5 ml) and stored at 4 0C temperature. The polyhedral suspension was observed under phase contrast microscope (40x) and OBs were counted using a Neubauer haemocytometer. The meteorological data recorded during the investigation period were obtained from the Agricultural Research Station, AAU, Derol, Panchmahal and Mahisagar district in order to correlate standard monthly mean weather data with NPV infection percentage. The research involved the collection of data on NPV infection rates, crop stage observations, and a range of relevant weather parameters in maize fields. Subsequently, correlation analysis was conducted to identify potential associations between NPV infection and the developmental stages of the crop. Furthermore, regression analysis was employed to quantify the impact of specific weather parameters on the observed trends in NPV infection, providing a more significance understanding of the intricate relationships. Analysis was carried out through SPSS statistics 21.0 software.

**2.1.1 Purification of polyhedron occlusion bodies (POBs) produced in the cadavers of *Spodoptera frugiperda* nucleopolyhedrovirus (*Sf*NPV) infected larvae.**

The suspension underwent filtration, followed by centrifugation, and the pellets were resuspended in distilled water. Further purification involved isopycnic centrifugation on a 45–60% sucrose gradient with SDS. After centrifugation, the virus band was washed and centrifuged again. Stock suspensions with 109 OBs/ml were prepared and stored at -20°C. The isolated strain was named *Sf*NPV AAUBC1 and AP2, formulated with an emulsifier (5% v/v) and glycerin (20% v/v).

**2.2 DNA extraction from virus**

The total DNA was extracted from larvae exhibiting symptoms of NPV disease using the cetyltrimethylammonium bromide (CTAB) method, following the protocol (Sivakumar et al., 2020). Purified occlusion bodies (OBs) were obtained by first centrifuging for 1 minute at 2500 rpm. The pellet was discarded, and the supernatant was retained as it contained the virus. The supernatant was centrifuged again to allow the OBs to settle on the side walls of the centrifuge vial. The OBs thus obtained were dispersed in double-distilled water and kept at −20 °C. For DNA extraction, the purified OBs suspension was treated with 1% SDS and proteinase K at 20 mg/ml, followed by phenol, chloroform, and isoamyl alcohol (1:1:1), extraction, and DNA precipitation with ethanol. DNA was re-suspended in TE buffer, and the extracted DNA was visualised on a 0.9% agarose gel. Subsequently, all DNA extracts underwent a 1:100 dilution in sterile distilled water (SDW) before being utilised in RCA amplifications. Quantification and adjustment of the isolated DNA concentration to 0.1 g/l were performed using a NanoDropTM 1000 Spectrophotometer from ThermoScientific, USA. Verification of the size and purity of the DNA was achieved by loading 1 µl of the sample DNA and 1 µl of control genomic DNA onto a 0.6% agarose/EtBr gel, along with a 1 kb Plus DNA ladder. This gel electrophoresis confirmed the size and purity of the extracted DNA.

**2.3 PCR amplification of polyhedrin gene of SfNPV strain AAUBC1 and AP2**

The conserved polyhedrin gene (polh) of NPV served as the targeted template DNA in this study. Species-specific primers (forward: 5'-TCTAGGTTCGGT-CATCAAGAAT; reverse: 5'-TTGAACACGAGCGACAGTT) were designed based on the NCBI database for the detection of the polh gene. The PCR master mixture was prepared following the recommended protocol by Thermo Scientific. The PCR reaction mixture (50 μL) consisted of 50 ng of template DNA, 0.25 U of Taq DNA polymerase, 10X Taq buffer, 2.5 mM MgCl2, 2.5 mM of each of four dNTPs, and 1 μL each forward and reverse primer. The reaction was carried out in a thermal cycler (Biorad, Model T100) with initial denaturation at 95 °C for 3 min and 35 cycles at 95 °C for 30 sec, 70 °C for 1 min, 55 °C for 1 min, and a final extension step at 72 °C for 10 min to obtain a PCR product of 700 bp. Visualization of the amplified products was achieved by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. A comparison was made with the GeneRuler 100-bp DNA ladder from Thermo Scientific to confirm the size of the amplified fragments. DNA template sequences were eluted and generated at Barcode Bioscience in Bengaluru, India. Confirmation of the SfNPV identity was established through nucleotide sequence homology, and a matching sequence similarity was conducted using BLAST analysis against the NCBI GenBank database. The sequences were deposited in GenBank with accession numbers PP025827 and PP025828 for the isolated SfNPV strains AAUBC1 and AP2.

**2.8 *In-Silico* docking**

**2.8.1 Preparing protein of Spfr NPV AAUBC1 and AP2 polyhedrin from *S. frugiperda***

A standard protein preparation protocol, as described by (Narramore et al. 2019), was implemented, involving the removal of the co-crystallized ligand, selected water molecules, and cofactors. Energy-minimized ligand molecules were prepared and utilized as input for AutoDock Vina to conduct the docking simulations. The target protein file was prepared using AutoDock 4.2 (MGL Tools 1.5.6), keeping the associated residue with the protein. The graphical user interface program was employed to set the grid box for docking simulations, ensuring it envelops the region of interest in the macromolecule. The docking algorithm provided by AutoDock Vina was used to search for the best-docked conformation between the ligand and protein. During the docking process, a maximum of nine conformers were considered for each ligand. The conformations with the most favorable (least) free binding energy were selected for the analysis of interactions between the target receptor and ligands, conducted using Discovery Studio Visualizer and PyMOL. Ligands were represented in red, and hydrogen bonds and interacting residues were depicted using ball-and-stick model representations.

**2.9 Bioassay**

**2.9.1 Culture**

In order to raise the initial culture of fall armyworm, *S. frugiperda* the larvae were collected from the field and brought to the laboratory of AICRP on Biological Control of Crop Pests, AAU, Anand. It was then transferred to a separate plastic vial (5.5 × 4.5 cm) for rearing and further mass culture. Everyday piece of fresh leaves of maize were fed to the larvae as food. After pupation, pupae were collected and placed in Petri dish. Then the pupae were kept in an oviposition cage (60 × 30 × 30 cm) for the emergence of adults. The young plant of maize was placed in oviposition cages for egg laying. A cotton swab dipped in 5 per cent honey solution was placed in a cage as food for the adults. From the culture of the second generation, the first instar larvae were transferred to a plastic vial (5.5 × 4.5 cm) and small fresh pieces of maize leaf were provided as a natural diet and were allowed to grow up to the second instar. On daily basis, the food was changed and frass was discarded regularly.

**2.9.2 Bioassay**

Aqueous suspensions of viral occlusion bodies (POBs) were prepared at concentrations of 1×104, 1×105, 1×106, 1×107, 1×108, and 1×109 POBs/ml in a Tween 20 solution (0.05% v/v). Ten microliters of the viral suspension were applied to maize leaf discs (3 cm in diameter), dried, and placed individually inside plastic containers (7 × 3 cm). Newly molted 2nd (3-5 days old), 3rd (6-7 days old) and 4th (6-7 days old) instar larvae of *S. frugiperda* were exposed to the treatment. The larvae underwent a 6-hour starvation period and were then introduced to the plastic containers containing the treated leaf discs for feeding. Each treatment involved 30 larvae, and the entire experiment was replicated three times for each concentration. After 24 hours, the larvae were transferred to fresh leaves and kept at a temperature of 26 ± 2°C with a relative humidity of 65–70%. In the control group, larvae were allowed to feed on leaves treated with aqueous Tween 20 (0.05%).