# DEVELOPMENT AND EVALUATION OF DIAGNOSTIC TOOLS FOR *Nucleopolyhedroviruses* (NPVs) INFECTING MAJOR LEPIDOPTERAN PESTS OF LEGUME CROPS IN THE SEMI-ARID TROPICS

**DOCTOR OF PHILOSOPHY**

**IN**

**BIOCHEMISTRY**

**BY**

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**ACHARYA NAGARJUNA UNIVERSITY Dr. M. R. APPAROW CAMPUS**

**NUZVID - 521 201, ANDHRA PRADESH, INDIA**

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*Thesis submitted to*

**ACHARYA NAGARJUNA UNIVERSITY**

*in partial fulfillment of the requirement for the award of the degree of*

**DOCTOR OF PHILOSOPHY**

*in*

**BIOCHEMISTRY**

*by*

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# *CERTIFICATE*

*This is to certify that the thesis entitled* ***"Development and Evaluation of Diagnostic Tools for Nucleopolyhedroviruses (NPVs) Infecting Major Lepidopteran Pests of Legume Crops in the Semi-Arid Tropics"*** *submitted to "**ACHARYA NAGARJUNA UNIVERSITY**" in partial fulfilment of the requirement for the award of the degree of "**DOCTOR OF PHILOSOPHY**" in "**BIOCHEMISTRY**" is a record of the bonafide research work carried out at **ICRISAT** by **Mr. SRIDHAR KUMAR CHITNENI** under my guidance and supervision. No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.*

***Date :***

***(G. V. RANGA RAO)***
***CO-RESEARCH DIRECTOR***

# *DECLARATION*

*I, **SRIDHAR KUMAR CHITNENI** hereby declare that the thesis entitled **"Development and Evaluation of Diagnostic Tools for Nucleopolyhedroviruses (NPVs) Infecting Major Lepidopteran Pests of Legume Crops in the Semi-Arid Tropics"** submitted to "**ACHARYA NAGARJUNA UNIVERSITY**" in partial fulfilment of the requirement for the award of the degree of "**DOCTOR OF PHILOSOPHY**" in "**BIOCHEMISTRY**" is the result of the original research work done by me. It is further declared that the thesis or any part there of has not been published earlier in any manner.*

*Date :* ***( SRIDHAR KUMAR CHITNENI )***

*Dedicated to My Beloved Teachers*
*Dr. G.V.Ranga Rao*
*and*
*Dr. P. Lava Kumar*

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# ABSTRACT


Investigations were carried out towards the "Development and evaluation of diagnostic tools for *Nucleopolyhedroviruses* (NPVs) infecting major lepidopteran pests of legume crops in the semi-arid tropics" during 2005-2008 at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru centre.

Nucleopolyhedroviruses (NPVs) were isolated from three major lepidopteran pests of legume crops during natural epizootic conditions at ICRISAT farms. They are: 1) From *Helicoverpa armigera* (Legume pod borer) (Hubner) (Lepidoptera: Noctuidae) larvae on pigeopea and chickpea crops; 2) From *Spodoptera litura* (Tobacco caterpillar) (Fabricious) (Lepidoptera: Noctuidae) and 3) From *Amsacta albistriga* (Red hairy caterpillar) (Walker) (Lepidoptera: Arctiidae) larvae on Groundnut crop and their mass multiplication was standardized at ICRISAT-NPV production laboratory. During mass multiplication of *H. armigera* NPV (HaNPV), there was a significant difference in parameters like POBs/ml and POBs/larvae which showed that NPV multiplied on field collected larvae recorded significantly higher yield ($5.35 \pm 0.31 \times 10^9$ POBs/larva) compared to laboratory reared larvae ($5.18 \pm 0.45 \times 10^9$ POBs/larva). While $5.73 \pm 0.17 \times 10^9$ POBs/larva for *S. litura* NPV (SlNPV) and $7.90 \pm 0.54 \times 10^9$ POBs/larva for *A. albistriga* NPV (AmalNPV) were recorded when multiplied on laboratory reared larvae emerged from field collected egg masses.

Under scanning electron microscope (SEM) the POBs of NPVs appeared as crystalline structures of variable shapes (irregular) of size 0.5 to 2.5µm (HaNPV), 0.9 to 2.92µm (SlNPV) and 1.0 to 2.0µm (AmalNPV) in diameter. Under transmission electron microscope (TEM) the cross-sectioned POB revealed multiple nucleocapsids in each envelop, which were of bacilliform shaped structures of 277.7 × 41.6nm (HaNPV), 285.7 × 34.2nm (SlNPV) and 228.5 × 22.8nm (AmalNPV) in size. The POBs of HaNPV and AmalNPV contained 2 to 6 and SlNPV contained 5 to 7 nucleocapsids per envelope.

Purification protocol for polyhedrin protein of NPVs was standardized by initial heat inactivation of endogenous proteases; alkali disruption of POBs to release virions and then ultracentrifugation to pellet virions. Further purification was achieved by either of the following approaches: (i) in one approach polyhedrin was further purified through centrifugation by layering on 10-40% liner sucrose gradient; and (ii) in second approach through precipitation of polyhedrin at isoelectric pH. In 10-40% linear sucrose gradient centrifugation, the polyhedrin formed one diffused light scattered zone in 10% sucrose region. In isoelectric precipitation method the polyhedrin of all the three NPVs was precipitated at pH between 5.5 and 5.6. In 12% SDS-PAGE analysis, the molecular weight of major polyhedrin of three NPVs revealed that 31.65kDa (± 0.00) of HaNPV, 31.29kDa (± 0.00) of SlNPV and 31.67kDa (± 0.295) of AmalNPV respectively. In addition, these preparations contained some minor molecular weight peptides of about 7-27kDa and a high molecular weight peptide of about 60-70kDa fragment. This has revealed that three NPVs have 6-8 minor polypeptides.

Polyclonal antibodies were raised in New Zealand White rabbits against polyhedrin protein of NPVs isolated in the present study. The concentration (500µg) of polyhedrin of NPVs used for immunization gave an antibody titer of 1:5000 dilution, 18 weeks after immunization. In western immunoblotting all three antibodies were specifically reacted with polyhedrin (31kDa) and did not cross-reacted with healthy larval proteins indicated that the antibodies are highly specific to polyhedrin. In addition to the major polyhedrin (31 kDa), the polyclonal antibodies recognized some minor low molecular weight polypeptides of about 11-27 kDa and high molecular weight peptides of about 43.6-99.14kDa proteins when sufficient amounts of samples were loaded in to wells. Some of these proteins could not be aligned with those polypeptides in silver stained gels of polyhedrin preparations examined previously. The antibodies were highly specific to polyhedrin protein and did not cross-reacted with healthy larval proteins but, each antiserum had shown different degrees of cross reactivity with heterologous polyhedrins in direct antigen coating (DAC) enzyme-linked immunosorbent assay (ELISA) and western immunoblotting.

Various immunochemical tools were developed using the polyclonal antibodies raised against the poly occlusion body (POB) protein (polyhedrin) and evaluated for the detection and quantification of NPV in insect larvae and viral insecticide preparations. Indirect immunofluorescence assay and western immunoblot assay were developed for detection of POBs in homogenates of NPV-infected larvae. DAC-ELISA and indirect competitive (IC)-ELISA were developed for detection and quantification of polyhedrin protein in insect extracts. The sensitivity of DAC-ELISA is 30ng/ml of polyhedrin in 5µg/ml of insect total protein extracts. But in DAC-ELISA there was competition between insect and viral proteins for binding to the ELISA plate surface reducing the sensitivity of the assay. To eliminate this, IC-ELISA was developed, which has sensitivity of 0.156µg/ml of polyhedrin in 25 or 50µg/ml of alkali dissolved total insect protein extracts. The 50% competitive inhibition ($IC_{50}$) values for HaNPV polyhedrin polyclonal antiserum were calculated to be 1.10µg/ml of homologous polyhedrin and heterologous polyhedrins were calculated to be 2.0µg/ml of SlNPV polyhedrin and 2.20µg/ml of AmalNPV polyhedrin. For SlNPV-polyhedrin polyclonal antiserum, $IC_{50}$ was calculated to be 1.26µg/ml of homologous polyhedrin and heterologous polyhedrins were calculated to be 2.25µg/ml of HaNPV polyhedrin and 2.85µg/ml of AmalNPV polyhedrin. For AmalNPV-polyhedrin polyclonal antiserum, $IC_{50}$ was calculated to be 1.19µg/ml of homologous polyhedrin and heterologous polyhedrins were calculated to be 1.82µg/ml of Ha NPV polyhedrin and 2.32µg/ml of SlNPV polyhedrin. The percent cross-reactivity of each antiserum with their homologous polyhedrins was calculated to be 100% while with heterologous polyhedrins the antisera showed differential cross-reactivity. The HaNPV- polyhedrin polyclonal antiserum has showed 54.72% and 50.0% of cross-reactivity with SlNPV and AmalNPV polyhedrins. The SlNPV- polyhedrin polyclonal antiserum showed 56.0% and 43.85% of cross-reactivity with HaNPV and AmalNPV polyhedrins. Similarly, AmalNPV-polyhedrin polyclonal antiserum showed 65.38% and 51.29% of cross-reactivity with HaNPV and SlNPV polyhedrins. In recovery experiments, 25 and 50µg/ml of insect body proteins did not show interference with artificially spiked polyhedrin and the percent of amount of polyhedrin spiked in to 25 or 50µg/ml of larval protein extract was 82.1 to 116.8 %.

Among these tools the DAC-ELISA is a rapid and highly sensitive tool, which can detect low levels of NPV at early stages of infection in larvae as well as latent infection in pupae. While competitive-ELISA, western immunoblotting and indirect immunofluorescence tools were highly specific but not much sensitive than DAC-ELISA to detect low levels of NPV infection. Both DAC-ELISA and IC-ELISA tools were sensitive to the analysis of alkali dissolved protein extracts of POBs or infected larval extracts than direct POBs or larval extracts. Whereas, western immunoblotting and indirect immunofluorescene tools were specific to both. As part of the quality control during mass production of NPVs used for commercial viral insecticide preparations at ICRISAT, Patancheru, India, the present study developed some sensitive immunochemical methods such as DAC and IC-ELISA and evaluated their performance in quantification of POBs in commercial NPV preparations. A simple purification protocol was standardized for extraction of total polyhedrin from NPV preparations of $6 \times 10^9$ to $2.34 \times 10^7$ POBs/ml. The purity of the extracted polyhedrin was assayed in SDS-PAGE and evaluated in both DAC as well as IC-ELISA with sensitivity of $4.68 \times 10^7$ POBs/ml (0.015LE/ml). The ELISA results were comparable to light microscope counting of POBs.

Application of ELISA and western immunoblot assay in bioassay experiments during optimization of conditions for the productivity and quality of NPVs suggested that 4$^{th}$ instar larvae is suitable for *H. armigera* and 5$^{th}$ instar larvae suitable for *S. litura* and *A. albistriga* for virus inoculation, and virus harvesting 9 days after inoculation from both live and dead larvae was better to get the maximum virus yield as well as to reduce bacterial contamination. Application of ELISA tools at field level evaluation of efficacy of NPV against *H. armigera* on pigeonpea crop showed that the concentration of NPV (250 LE/ ha) used for field spray was successfully infected the field population. The infection was initiated in field population on 3 days post application (dpa) and the per cent of infection in field sampled larvae was peaked at 8 and 9 dpa and started declining on 10 dpa.

A double round PCR protocol was standardized using degenerate primer set to isolate the full-length polyhedrin gene of NPV isolated from *H. armigera.* This resulted in ~ 750 bp product which was cloned and sequenced. Gene sequencing analysis of selected clones resulted in 744 bp nucleotide long ORF with a predicted coding capacity for a polypeptide of 247 amino acids. In BLASTX search the sequence showed homology with baculovirus occlusion body protein domain of known polyhedrin and granulin proteins from the GenBank data base. The sequence was deposited in GenBank with a public accession number of EU047914.

The nucleotide sequence of HaNPV-P polyhedrin gene had a high homology with polyhedrins of several NPVs. Among which, it was showing maximum homology of 98.2% with *Mamestra configurata* NPV, 98% with *Mamestra brassicae* NPV, 96.1% with *Leucania seperata* NPV and 90.6% with *Panolis flammea* NPV. At the same time with minimum homology of 72.4% was noticed with WsNPV. Similarly, the amino acid sequence of HaNPV-P polyhedrin protein was showed maximum homology of 95.5% with *Mamestra configurata* NPV and *Mamestra brassicae* NPV, 93.9% *Panolis flammea* NPV and 93.5% with *Leucania seperata* NPV and minimum homology of 79.4% with *Wiseana signata* NPV and 81.8% with *Spodoptera littoralis* NPV. Phylogenetic analysis at nucleotide as well as amino acid levels showed that the virus belongs to group-II NPVs and the virus was named as *H. armigera* NPV, Patancheru strain (HaNPV-P). This is the 1$^{st}$ report from the Indian subcontinent and 8$^{th}$ report worldwide to be described the complete polyhedrin gene of a NPV isolated from *H. armigera*.

In the present investigation the HaNPV-P strain was distinguished from other NPVs by developing PCR-RFLP marker based on its unique restriction site present in the amplified portion of the polyhedrin gene. Restriction mapping analysis of HaNPV-P polyhedrin gene along with other known published polyhedrin sequences showed that one unique restriction sites present at particular nucleotide positions in polyhedrin gene of HaNPV-P and some other NPVs. This has showed that *Xho-I* restriction site at nucleotide position 131 was found in NPV from *M. brassicae* and *M. configurata*, and the same restriction site at position 671 was found in NPV of *L. seperata*. Whereas in HaNPV-P, the *Xho*-I site was found at

both 131 and 671 base pairs. But, the *Xho*-I site was not found in any of the HaNPV polyhedrin gene sequences deposited earlier in the GenBank. This indicates that the HaNPV-P is a unique strain among earlier reported HaNPV isolates.

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| A | Absorbance |
| ALP | Alkalinephosphatase |
| AV | Ascovirus |
| BC | Buffer control |
| BLAST | Basic local elignment search tool |
| bp | Base pair |
| BV | Budded virus |
| $CaCl_2$ | Calcium chloride |
| CFA | Complete Freund's adjuvant |
| CFU | Colony forming units |
| CIB | Central insecticide board |
| cm | Centimeter |
| CPV | Cytoplasmic polyhedrosis virus |
| $CsCl_2$ | Cesium chloride |
| Da | Daltons |
| DAC-ELISA | Direct antigen coating enzyme linked immunosorbant assay |
| DAS-ELISA | Double antibody sandwiched enzyme linked immunosorbant assay |
| DAS | Dilute alkaline saline |
| $dH_2o$ | Distilled water |
| DNA | Deoxy ribonucleic acid |
| dNTP | Deoxy nucleotide triphosphate |
| dpa | Days post application |
| dpi | Days post inoculation |
| ds | Double stranded |
| DTT | Dithiothreitol |

| | |
|---|---|
| EDTA | Ethylene diamine tetra acetic acid |
| EM | Electron microscope |
| EP | Electro–eluted polyhedrin |
| EPP | Entire polyhedral particles |
| EPV | Entomopoxvirus |
| *et al* | All other |
| etc | Et cetera |
| ETL | Economic Threshold Level |
| FA | Fluorescent antibody |
| FAO | Food and agriculture organization |
| FITC | Fluorescein isothiocyanate |
| g | Gram |
| gp64 | Glycoprotein 64 |
| h | Hours |
| HA | Hemagglutination |
| ha | Hectare |
| HC | Healthy control |
| HI | Hemagglutination inhibition |
| HLP | Healthy larval proteins |
| *i.e* | That is |
| $IC_{50}$ | Fifty percent competitive inhibitions |
| IC-ELISA | Indirect competitive enzyme linked immunosorbant assay |
| ICTV | International committee on taxonomy of viruses |
| IFA | Incomplete Freund's adjuvant |
| IgG | Immunoglobulin G |
| im | intramuscularly |
| IPM | Integrated pest management |
| IPP | Isoelectric precipitated polyhedrin |
| kb | Kilo basepair |
| kDa | Kilo dalton |
| km | Kilometer |

| | |
|---|---|
| KOH | Potassium hydroxide |
| KV | Kilo volts |
| l | Liter |
| LB | Luria Broth |
| $LC_{50}$ | Lethal concentration required to cause 50 per cent mortality |
| $LD_{50}$ | Lethal dose required to cause 50 per cent mortality |
| LE | Larval equivalent |
| LOVAL | Larvae-occluded virus, alkali liberated |
| LSD | Least significant difference |
| $LT_{50}$ | Lethal time required to cause 50 per cent mortality |
| m | Meter |
| M | Molarity |
| MBV | Monodon baculovirus |
| mg | Milligram |
| mg $ml^{-1}$ | Milligram per milliliter |
| $MgCl_2$ | Magnesium chloride |
| min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| MNPV | Multiple enveloped nucleocapsid nucleopolyhedrovirus |
| N | Normality |
| $Na_2CO_3$ | Sodium carbonate |
| NaCl | Sodium chloride |
| ng | Nanogram |
| NGOs | Non government organizations |
| nm | Nanometer |
| NOV | Non occluded virus |
| OB | Occlusion body |
| ºC | Degree Celsius |
| ORF | Open reading frame |

| | |
|---|---|
| PAGE | Polyacrylamide gel electrophoresis |
| PAP | Peroxidase anti-peroxidase |
| PBS | Phosphate-buffered–saline |
| PCR | Polymerase chain reaction |
| PDBC | Project directorate on biological control |
| PFU | Plaque forming units |
| pH | Negative logarithm of $H^+$ ion |
| PI | Isoelectric point |
| POBs | Polyhedral occlusion bodies |
| PVP | Polyvinyl pyrrolidone |
| $R^2$ | Regression equation |
| RBD | Randomized block design |
| REN | Restriction endonuclease |
| RFLP | Restriction fragment length polymorphism |
| RHC | Red hairy caterpillar |
| RIA | Radioimmunoassay |
| RNA | Ribonucleicacid |
| rpm | Revolutions per minute |
| SAT | Semi-arid tropics |
| SDS | Sodium dodecyl sulphate |
| SE | Standard error |
| SEM | Scanning electron microscope |
| SNPV | Single enveloped nucleocapsid nucleopolyhedrovirus |
| spp | Species |
| Sq.mm | Square millimeter |
| $ST_{50}$ | Survival time required to cause 50 per cent mortality |
| TBE | Tris boricacid EDTA |
| TE buffer | Tris EDTA buffer |
| TEM | Transmission electron microscope |
| TEMED | N,N,N,N-tetra methylethylene diamine |
| Tris | Tris (hydroxymethyl) amino methane |

| | |
|---|---|
| U | Units |
| UA | Uranyl acetate |
| UK | United kingdom |
| USA | United States of America |
| USDA | United States Department of Agriculture |
| US-EPA | United States Environmental protection agency |
| UV | Ultraviolet |
| V | Volts |
| v/v | Volume / volume |
| viz | Namely |
| vol | Volume |
| VP | Viral protein |
| w/v | Weight / volume |
| WHO | World health organization |
| $ | Dollar |
| % | Per cent |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| @ | At the rate of |

# CHAPTER - I

# *Introduction*

# CHAPTER- I

# INTRODUCTION

Legume crops are important sources of human food and animal feed, and also helps in managing the soil fertility through biological nitrogen fixation, thus plays a vital role in sustainable agriculture (Maiti, 2001). Since their protein content is high, these are principal sources of dietary protein for millions of people, especially for vegetarians in the Indian subcontinent and other parts of the world. Among legume crops, groundnut (peanut, *Arachis hypogaea* L.), chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L.) are important in the semi-arid tropics (SAT), where one-sixth of the world population lives. Unpredictable weather, limited and erratic rainfall, and nutrient poor soils, occurrence of pests and diseases are the major constraints limiting the productivity of the crops in the SAT regions. Among the various insect pests, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), *Spodoptera litura* (Fabricious) (Lepidoptera: Noctuidae) and *Amsacta albistriga* (Walker) (Lepidoptera: Arctiidae) are major constraints to the production of groundnut, pigeonpea and chickpea in the SAT.

*H. armigera* popularly known as legume pod borer or cotton bollworm is widely distributed in Africa, Australia, South East Asia, New Zealand and Mediterranean Europe (Sharma, 2005). It is a polyphagous pest and attacks a great variety of food, fibre, oil seed, fodder, and horticultural crops of more than 300 plant species (Arora *et al.,* 2005). In India, it has been recorded from over 20 crop and 180 wild plant species (Sharma, 2005). This species causes extensive damage to high value crops such as cotton, pigeonpea, chickpea,

groundnut, soybean, pepper, tomato, tobacco, maize, sorghum, sunflower and okra (Fitt, 1989; King, 1994). Global crop losses due to *Helicoverpa* species exceed US$ 5 billion per annum, despite the use of US$ 1 billion worth of pesticides for its control. Whereas, in the semi-arid and dry tropics, losses due to *H. armigera* on cotton, legumes, vegetables and cereals alone exceed US$ 1 billion and an additional cost of >US$ 500 million is incurred on pesticides (Russell *et al.,* 1998; Sharma, 2001). In India, the extent of losses in chickpea and pigeonpea have been estimated at over $300 million per annum (Reed and Pawar, 1982). Total losses in both pulses and cotton exceed $530 million per annum, and the insecticides applied for *Helicoverpa* control cost nearly $127.5 million on cotton and pulses (Reed and Pawar, 1982).

*S. litura,* well known as tobacco caterpillar or tobacco cut worm, is an important polyphagous pest in India. It is widely distributed in India, entire South and South-East Asia, Australia and Pacific Islands (CAB, 1967). It is known to cause economic damage on tobacco, groundnut, cole crops, taro (*Colacasia*) etc. Ayyanna *et al.* (1982) has reported it as major pest in groundnut in AP, India. It is reported to feed on 112 species of plants belonging to 44 families worldwide and reported from 60 species of cultivated crops and wild hosts in India (Mousa *et al.,* 1960, Thobbi, 1961). Patel *et al.* (1971) reported that two, four and eight larvae per plant reduced yield by 23, 44 and 50% respectively, on tobacco. In controlled experiments on soyabeans in India, crops chemically protected from *S. litura* and other pests yielded over 42% more compared to unsprayed (Srivastava *et al.,* 1972). On *Colocasia esculenta*, an average of five $4^{th}$-instar larvae per plant reduced yield by 10 %, while 2.3 and 1.5 larvae per plant reduced yield of brinjal and *Capsicum* in glasshouses by 10% (Nakasuji and Matsuzaki, 1977).

*A. albistriga,* commonly known as red hairy caterpillar (RHC), is a pest of several rainy season crops in Asia especially on groundnut in South India (Nagarajan *et al.,* 1957; Nagarajan and Ramachandran, 1958; Narayana and Ranga Rao, 1959; Mukundan, 1964;

Venkataraman *et al.,* 1970; Saroja *et al.,* 1971; Paramasivam *et al.,* 1973). *A. albistriga* is a common pest in south India; this pest is frequent in Coimbatore, Madurai and Pollachi districts of Tamil Nadu (Kuppuswamy *et al.,* 1965) and adjoining areas (Nagarajan and Ramachandran, 1958). Other plants recorded as hosts of this insect are finger millet, cowpea, castor, cholam, cotton (Nagarajan *et al.,* 1957); sorghum, pearlmillet, maize, soybean, horsegram, greengram, blackgram, clusterbean, pigeonpea, sesame, jute, sunnhemp and several weeds (Nagarajan and Ramachandran, 1958). The RHC infestation is sporadic, but the devastation is widespread. In certain years it can become serious and cause heavy losses (75%). But there are well-marked locations in Tamil Nadu, Andhra Pradesh and Karnataka where it appears regularly in rainy season (Venkataramanan *et al.,* 1970; Sandhu and Brar, 1977; Siva Rao *et al.,* 1977). When an outbreak of this pest occurs, a total crop loss over a large area is not uncommon (Nagarajan *et al.,* 1957).

So far, use of synthetic insecticides has been the major approach for controlling these pests on different crops in India and most of the developing countries. Chemical control is one of the effective and quicker methods in reducing pest population, where farmer obtains spectacular results within a short period. However, over reliance and indiscriminate unscientific use of pesticides for longer periods resulted in a series of problems, mainly risk of environmental contamination, loss of biodiversity, development of insecticide resistant pest populations, resurgence, outbreaks of the secondary pests, increase in inputs on chemicals and toxicological hazards due to accumulation of pesticide residues in food chain *etc.,* ultimately contributing not only to inefficient insect control, but also environmental and health hazards (Armes *et.al.,* 1992, Kranthi *et al*., 2002). Therefore, there is an urgent need to rationalize use of chemical pesticides for the management of insect pests. Growing public concern over potential health hazards of synthetic pesticides and also steep increase in cost of cultivation have led to the exploration of alternative and eco-friendly pest management tactics, such as Integrated Pest Management (IPM). IPM combines cultural, biological and chemical measures

in the most effective, environmentally sound and socially acceptable way of managing diseases, pests and weeds. IPM aims at suppressing the pest population by combining available eco-friendly methods in a harmonious way with emphasis on farm health and net returns. In an attempt to overcome the present crisis and to find alternatives to synthetic insecticides, the application of 'biopesticides' as eco-friendly measure for pest suppression has came up as one of the effective tools in IPM approach.

Biopesticides are developed from natural plant or animal origin, which can intervene in the life cycle of insect pests in such a way that the crop damage is minimized. The biological agents employed for this purpose, include parasites, predators and disease causing fungi, bacteria, nematodes and viruses, which are the natural enemies/pathogens of pests. More than three thousand microorganisms, comprising viruses, bacteria, fungi, protozoa and nematodes, have been reported as insect pathogens. Of these, microbial pathogens gained significance for use as biopesticides primarily due to ease in production and application. Many species of insect pathogenic microorganisms have been exploited as biopesticides, and some species have been developed into commercial formulations that are being used in many countries. At present the world market for the biopesticides exceeds US$ 125 million per annum, which is expected to increase to US$ 300-500 million by 2010 (Dhaliwal and Arora, 2001).

Among microbial insecticides, the insect pathogenic viruses such as baculoviruses are attractive alternatives for biological control under IPM and have been used for more than 20 years with great success (Zhang, 1989). There are several advantages of using insect viruses for pest control: these are highly host specific and are known to be completely safe to humans, animals and non-target beneficial insects such as bees, predatory insects and parasitoids (Monobrullah & Nagata 1999, Nakai *et al.,* 2003, Ashour *et al.,* 2007). In addition, these are highly compatible with other methods of pest control and are well suited for use in integrated pest management (IPM) programs. Another important reason for the

interest in baculoviruses as potential insect control agents is that they are relatively easy to visualize and monitor using a light microscope.

Several viruses belonging to 18 different families are known to infect invertebrates and insects (Fauquet *et al.,* 2004). However, biopesticide development is concerned almost exclusively with members of one family, the *Baculoviridae* because of their common occurrence in most important insect pests primarily in the order of Lepidoptera and acts as natural regulators of pest population dynamics (Blissard *et al* 2000, Gelernter and Federici, 1990; Caballero *et.al.,* 1992a; Weiser, 1987). Baculoviruses are enveloped rod shaped nucleocapsids having circular, double stranded DNA genome (Blissard and Rohrman, 1990, Volkman *et al.,* 1995). The infectious virus particles are embedded in proteinaceous occlusion bodies (OBs) specially designed to survive outside their hosts for horizontal transmission and persistence in the environment for several years until the availability of susceptible host at particular life–stage from a given locality for significant period of time to maintain a continuous cycle of infection (Jacques, 1975; Rohrmann, 1986). Based on the occlusion body (OB) morphology and virion phenotype, baculoviruses are placed in two genera (Blissard *et al*., 2000, Fauquet *et al.,* 2004). Subgroup-A viruses called *Nucleopolyhedroviruses* (NPVs) (Rohrmann, 1999). The occlusion bodies in this subgroup are large (0.13-15 µm) and polyhedral shape called POBs. The virions in this subgroup contain either a single nucleocapsid (SNPV) or many nucleocapsids (MNPV) per envelope. Both SNPVs and MNPVs form nuclear occlusions late in infection in which many virions are embedded per occlusion body. Sub group-B viruses, the *Granuloviruses* (GVs) (Winstanley and O.Reilly, 1999) the occlusion bodies in this subgroup are small (0.3-05 µm) and ovicylindrical shaped, called granules. The virion has only one nucleocapsid per envelope and one virion per inclusion body. Baculoviruses infect more than 600 species of insects, mostly Lepidoptera, including many important pest species but also various insect species in

Hymenoptera (31 species), Diptera (27 species) and Coleoptera (5 species) as well as from the crustacean order Decapoda (shrimp) (Martignoni and Iwai, 1986b; Adams and Bonami, 1991; Couch, 1974). Baculoviruses do not infect any non-arthropod species. This high host specificity is one attraction of baculoviruses as biopesticides.

Among the 633 potential baculovirus species compiled by the International Committee on Taxonomy of Viruses (ICTV) (Fauquet *et al.,* 2004), 15 NPVs were categorized as assigned species where as 483 NPVs are tentative species. The GV contains 5 assigned and 131 tentative species. In the USA, NPV was first produced as viral insecticide against *Heliothis* species and registered by the Environmental Protection Agency (EPA) for agricultural use in the year 1973 (Ignoffo and Couch, 1981). Since then, several isolates and strains of NPV have been used to develop commercial biopesticides in USA, Australia, India, China and Thailand. Although the NPVs have distinct advantages to fit suitably in IPM module, some difficulties exist with its production and storage.

The reliability of the product is crucial in ensuring acceptance and sustained use by the farmers. The issue of erratic performance of viral biocontrol agents has been recognized as a significant factor in the limited successful commercialisation (Lisansky, 1997). It has been widely perceived that viral agents have not achieved a level of efficacy comparable with that of chemicals or other biopesticides such as *Bacillus thuringiensis* (Berliner). Many of the viral products available in the markets in developing countries were characterized as weak, with poor efficacy, questionable quality control (Harris, 1997) and are failing to meet acceptable standards (Kern and Vaagt, 1996). Unless this matter is addressed effectively, there is serious danger in these countries that poor quality products with their inevitable failures will erode the farmers confidence in microbial control products like NPV and significantly retard the promotion of this potential technology.

Mass production of NPV insecticide is simple and widely produced even at farmer level. Healthy larvae reared in the laboratory or collected from the fields are fed with low dose

of NPV and the virus produced in the insect is harvested and its concentration is estimated by counting polyocclusion bodies (POBs) using a light microscope fitted with hemocytometer. Recently local production and utilization of NPV gained momentum in India through participation of scientists, farmers, NGOs and state agricultural and extension departments. Although, commercial production, quality and storage were still contentious issues, NPV is multiplied on field collected larvae and being applied on crops. Multiplying NPV on field-collected larvae was found to be easier and cost effective compared to laboratory-reared larvae, but efficacy and quality of which may be effected due to contaminants such as bacteria and fungi.

The effectiveness of the viral insecticide is critically dependent on concentration of POB, which is expressed as LE (Larval Equivalent). Generally, a standard stock preparation consists of 1LE, i.e. $6 \times 10^9$ POBs/ml. Microbial pesticides including NPVs and GVs have now been brought under the ambit of the Central Insecticide Act, 1968. Commercialisation of microbial pesticides is possible only after registration with the Central Insecticide Board (CIB) in India, a body constituted under the insecticides act that regulated their manufacture and use. Section 9(3b) of the act specifies a minimum quantity of active ingredient in formulation.

While NPV insecticide production methods have been well established in many developing countries, the microscopic counting procedure used to screen the larvae for NPV infection and quality control of the viral insecticide lots has low-detection efficiency, unknown specificity and is laborious and requires considerable skill (Wigley, 1976). Because of this many NPV products produced have poor efficacy and found to be ineffective under field conditions. To over come this problem and for effective production of viral insecticides, it is necessary to have an efficient strategy for virus production, combined with rapid and specific diagnostic and quality control tools (Shieh, 1989). Development of appropriate, sensitive and reliable serological tools (Kőhler & Milstein, 1975; Kelly *et al.,* 1978b; Towbin *et al.,* 1979;

Crook and Payne, 1980; Smith & Summers, 1981; Zhang and Kaupp, 1988 and Lu *et al.,* 1995) are not available at this stage and will go a long way in the quality control of insect viruses in developing countries. Once developed, the tools would be of immense value to public and private entrepreneurs, such as state biopesticide production laboratories and regulatory agencies. In addition to this, the highly standardized, accurate, and sensitive diagnostic tools for NPV detection in field-collected larvae would be beneficial to pest management personnel, because early detection of NPV disease could make it possible to predict the occurrence of an imminent epizootic and thus alter the pest control tactics to be employed (Volkman and Falcon, 1982).

In addition to the development of immunochemical tools for diagnosis and quality control of NPVs, molecular level identification and evaluation of phylogenetic status of a particular baculovirus species is also important for establishment of purity of seed stock or master stock. The information is very limited in India on molecular level identification and evaluation of phylogenetic status of commercial baculovirus preparations against major insect pests. Other potential methods are available for confirming preservation of the particular strain includes DNA sequencing and the use of polymerase chain reaction (PCR) with specific primers for variable and highly conserved regions. These methods are not available for several NPVs and not used routinely. Detailed protocols and expected results will be required if these methods are pursued. Once the master stock is proved to be particular strain it will be used for production batches. The master stock will be replenished with the production batch once they are shown to be identical in conserved gene sequence. Given the in-field production process all batches must be subjected to appropriate quality assurance

protocols, including an analysis and characterization of the active ingredient to ensure consistency between production batches.

Therefore, to address the diagnostic and quality control issues pertaining to successful production of NPV against these major insect pests, the present research was undertaken to develop and evaluate the immunochemical tools for quantitative estimation of NPVs in commercial lots; to apply these tools in diagnosis of NPV infection at field level and to characterize and determine the phylogenetic status of NPV used for commercial viral insecticide preparations at ICRISAT, Patancheru, India.

## The objectives of this study are:

1. Development and evaluation of immunochemical tools for diagnosis and quality control of NPV infecting *Helicoverpa armigera, Spodoptera litura* and *Amsacta albistrga.*

2. Applications of immunochemical tools for quantitative estimation of NPVs in insects and viral insecticide preparations.

3. Characterization of polyhedrin gene and development of a PCR-based assay and determination of phylogenetic status of *Nucleopolyhedrovirus* infecting *Helicoverpa armigera*.

## Work plan:

### To achieve the above objectives the following work plan has been developed

1.1 Isolation of *Nucleopolyhedrovisrus* (NPV) from *Helicovera armigera, Spodoptera litura* and *Amsacta albistriga* insect species at ICRISAT farms and their mass multiplication in laboratory conditions.

1.2 Electron microscopic studies (SEM and TEM) of polyocclusion bodies (POBs) purified from infected larvae.

1.3 Standardization of POB protein (polyhedrin) purification from HaNPV, SlNPV and AmalNPV.

1.4 Production of polyclonal antibodies against polyhedrin in New Zealand White inbred rabbits and their characterization.

1.5 Development of western immunoassay and indirect immunofluorescence assay for detection of NPV in insect extracts.

1.6 Development and evaluation of direct antigen coating (DAC)-enzyme-linked immunosorbent assay (ELISA) and Indirect Competitive (IC)-ELISA for detection and quantification of polyhedrin in NPV infected insect extracts and commercial NPV preparations.

2.1 Application of ELISA tools at field level evaluation of NPV infection through detection and quantification of polyhedrin in larvae days after field application against *Helicoverpa armigera* on pigeonpea crop.

2.2 Application of ELISA and Western immunoassay in optimization of inoculation age of larvae and harvesting time of virus for obtaining maximum virus yield with low levels of bacterial contaminants.

3.1 Design of degenerate oligomers and amplification of polyhedrin gene of NPVs.

3.2 Purification of amplified polyhedrin gene and cloning in to pJET1 cloning vector.

3.3 Sequencing of cloned polyhedrin gene using pJET1 forward and reverse primers.

3.4 Phylogenetic analysis of NPV (from *H.armigera*) based on polyhedrin gene at nucleotide and amino acid levels using Lasergene software (DNASTAR, Madison, USA), MgAlign tool of Lasergene software and CLC workbench 3 software (CLC Bio).

3.5 Development of PCR based RFLP marker for identification and differentiation of NPV isolated from *H.armigera* (HaNPV-P, Patancheru strain).

# CHAPTER - II

# *Review of Literature*

# CHAPTER - II
# REVIEW OF LITERATURE

The literature pertaining to the related work has been reviewed and presented in this chapter.

## 2.1 Major Lepidopteran Pests of Legume Crops in the Semi-Arid Tropics:

The important legume crops of semi-arid tropics (SAT) such as chickpea, pigeonpea, groundnut, mung bean, urd bean and cowpea are susceptible to a number of insect pests. Among the various insect pests, three lepidopteran species *Helicoverpa armigera* (Hubner) (Lepidoptra: Noctuidae), *Spodoptera litura* (Fabricious) (Lepidoptera: Noctuidae) and *Amsacta albistriga* (Walker) (Lepidoptera: Arctiidae) are major constraints to the production of legumes in the semi-arid tropics (Figs 1 and 2). In this section the literature related to the distribution, host range, biology, pest status and crop losses caused by these three species were detailed as follows:

### 2.1.1 *Helicoverpa armigera* (Hubner) (Lepidoptra: Noctuidae):

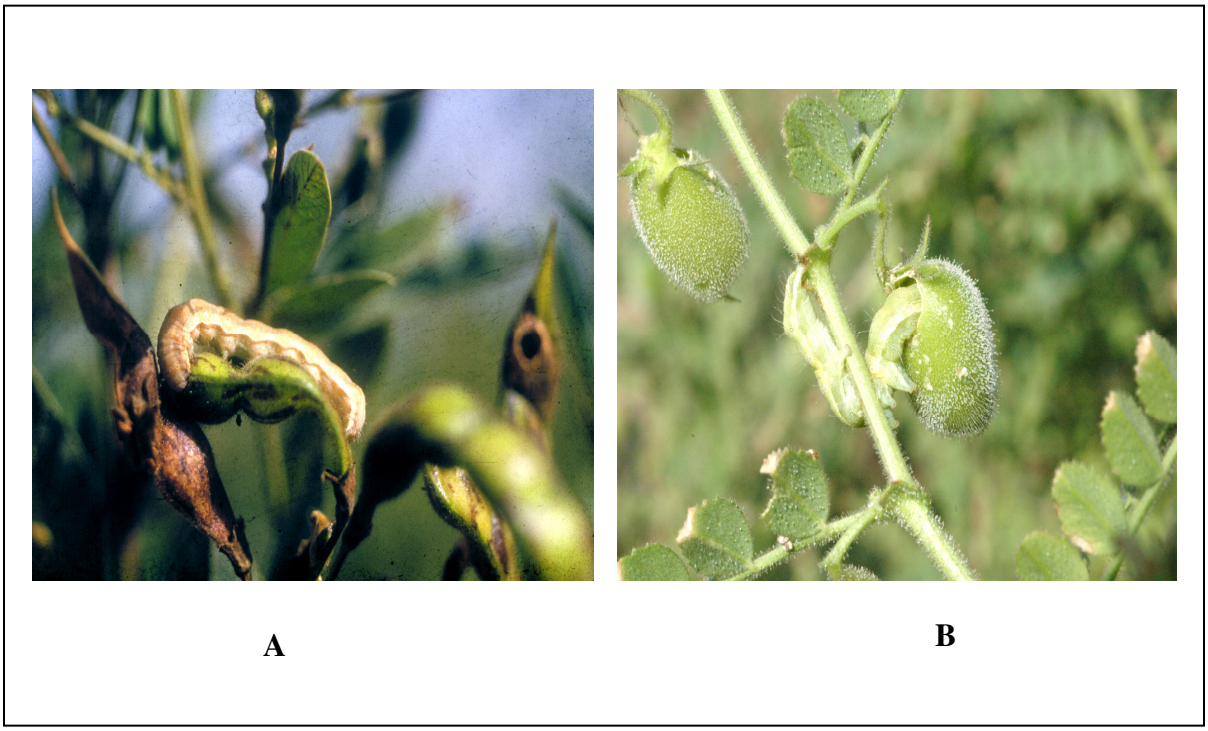
*Helicoverpa armigera* popularly known as legume pod borer or cotton bollworm is widely distributed in Africa, Australia, South East Asia, New Zealand and Mediterranean Europe (Sharma, 2005). It is a polyphagous pest attacks more than 300 plant species (Arora *et al.,* 2005). In India, it has been recorded on over 20 crops and 180 wild species (Sharma, 2005), representing a great

variety of food, fibre, oilseed, fodder, and horticultural crops (Fitt, 1991; Manjunath *et al.,* 1989). Extensive damage to high value crops such as cotton, pigeonpea, chickpea, groundnut, soybean, pepper, tomato, tobacco, maize, sorghum, sunflower, and okra account for most of the revenue loss (Fitt, 1989; King, 1994). Its preference for nitrogen rich reproductive organs such as flowering/fruiting parts of cotton, pulses, tomato and corn confers a high socio-economic cost to its depredations in subsistence agriculture in the tropics (Sharma, 2005). *H. armigera* significance as a pest is based on its biotic potential such as high degree of polyphagy, high mobility, facultative diapause, high fecundity and multi generation (Fitt, 1989). Its biology and population dynamics follows different patterns in various agro-climatic zones. It completes seven or more generations a year in the southern states of India, and 3-4 generations in the northern states of India. It is a holometabolous insect; its developmental stages include eggs (2 to5 days), six larval instars (15 to 24 days), pupa (6 to 30 days at $15^0$C) and adult (1 to 23 days for males and 5 to 28 days for females). Its life cycle on several crops has been studied at several locations by many workers (Reed, 1965; Singh and Singh, 1975; Doss, 1979; Jayaraj, 1982; Rajagopal and Channa Basavanna, 1982).

Various stages of the life cycle are outlined in Fig 6. The developmental period of the various stages depend upon the weather conditions and food (Bhatt and Patel, 2001). The importance of *H. armigera* is largely due to its well-developed survival strategies, diapause, and dispersal, which enable it to exploit food sources separated by unfavorable times and distance. This also enables it to escape the natural enemies. It exhibits a facultative diapause, which enables it to survive the adverse weather conditions in both winter as well as in summer (Ditman and Cory, 1931; Roome, 1979; Hackett and Gatehouse 1982a; Jayaraj, 1982; King, 1994; Masaki, 1980). It is a facultative migrant, and responds largely to local environmental cues and undertakes short or long distance flight in the direction largely governed by prevailing weather systems (Fitt, 1989). Larval stage attracts the maximum importance due to its relevance to the crop damage. The habit of particularly damaging many fruiting structures makes it a highly destructive crop pest. For example

on cotton, 2 to 3 larvae per plant can destroy all the bolls within 15 days, on pigeonpea, one larva per plant reduces 4.95 green pods, 7.05 dry pods, 18.01 grains, 3.79 g pod weight, and 2.05 g grain weight per plant (Sharma, 2005).

**Fig 1: Larva of legume pod borer (*Helicoverpa armigera*) feeding on pigeonpea (A) and chickpea (B) pods**



**A** **B**

A unit increase in larvae per plant results in 2.61 and 4.93% increase in pod damage at the green and dry stages, respectively (Meenakshisundaram and Gujar, 1998). Global crop losses due to *Helicoverpa* species exceed US$ 5 billion per annum, despite the use of US$ 1 billion worth of pesticides. Whereas, in the semi-arid and dry tropics, losses due to *H. armigera* on cotton, legumes, vegetables and cereals alone exceed US$ 1 billion and an additional cost of >US$ 500 million is incurred on pesticides (Russell *et al.,* 1998; Sharma, 2001). In India, the extent of losses in pulses such as chickpea and pigeonpea have been estimated at over $300 million per annum and total losses in both pulses and cotton exceed $530 million per annum, and the insecticides applied for *Helicoverpa* control cost nearly $127.5 million on cotton and pulses (Reed and Pawar, 1982). In Queensland, Australia, the cost of crop loss and control has been estimated to be A$ 16 million (Alcock and Twine, 1981) and A$ 25 million (Twine, 1989). In the tropics, total losses due to *H. armigera* on cotton, legumes, vegetables, and cereals may exceed 1000 million, and the cost of insecticides used to control *H. armigera* may be nearly $500 million (Reed and Pawar, 1982). In Africa (Tanzania), the loss of cotton exceeds $20 million in most years (Reed and Pawar, 1982).

### 2.1.2 *Spodoptera litura* (Fabricious) (Lepidoptera: Noctuidae):

*Spodoptera litura* (Fabricious) well known as tobacco caterpillar or tobacco armyworm is an important polyphagous crop pest of national status in India. It enjoys wide distribution and besides all over India it has been recorded in entire South and South-East Asia, Australia and Pacific islands (CAB 1967). The distribution of this pest in India was reported by Hampson (1919), Cotes and Swinhoe (1888). Earlier it is known to be a sporadic pest but in recent past it has emerged as major polyphagus pest. It is a major pest on tobacco, groundnut, chillies, cole crops,

sunflower, cotton and taro (*Colacasia*) etc (Mousa *et al.,* 1960). Ayyanna *et al.* (1982) has reported it as a major pest on groundnut. It is reported to feed on 112 species of plants belonging to 44 families' worldwide and 60 species of cultivated crops and wild plants are recorded as hosts in India (Thobbi, 1961).

The biology of *Spodoptera litura* varies with the host and climatic conditions. The outbreak of this pest generally followed by a good rainfall after a long dry spell (Chellaiah, 1985). Field studies conducted in Japan on the seasonal abundance of *S. litura* on crops like groundnut, sweet potato, and taro indicated that there were four generations between July and October (Miyahara *et al.,* 1971). In southern part of India this pest is known to complete 10-12 generations in a year (Rao *et al.,* 1991). The occurrence and abundance of *S. litura* on different crops in various geographical locations was studied by various workers world widely (Singh and Hoi, 1972; Islam *et al.,* 1983; Tiwari *et al.,* 1980; Saini and Verkya, 1985; Khuhro *et al.,* 1986; Rajagopal *et al.,* 1988). Its life cycle on several crops has been studied at several locations by many workers (Tiwari *et al.,* 1980; Bhalani and Talati, 1984). Various stages of the life cycle are outlined in Figure 6. The egg, larval and pupal period is 3, 12-26 and 6-16 days, respectively, with a total life span 49 to 53 days (female) and 50 to 56 days (male) at $20^0$C. Threshold temperatures and thermal requirements for development of *S. litura* were determined by Rao *et al.,* (1989). Garad, *et al.,* (1984) recorded greatest net reproductive rate on okra and least on groundnut at $26.8 \pm 2^0$C in the laboratory.

The eggs hatch in 3-4 days and the young larvae ($1^{st}$ to $3^{rd}$ instars) initially feed in groups on the surface cells of the leaves (scraping injury) leaving the opposite epidermis of the leaf intact. Generally the later instar ($4^{th}$ to $6^{th}$ instars) larvae disperse and spend the day in ground under the host plant, feeding at night and early in the morning. These larvae start feeding the whole leaf resulting in complete skeletinization of the plants. Under natural infestation in epidemic conditions, the entire foliage is consumed leaving only the mid ribs. It is an extremely serious pest on tobacco in India. It was estimated that two, four and eight larvae per plant reduced yield by 23-24%, 44.2% and 50.4%, respectively (Patel *et al.,* 1971). In controlled experiments on soyabeans in India, crops

chemically protected from *S. litura* and other pests yielded over 42% more than crops which were not sprayed (Srivastava *et al.,* 1972). On *Colocasia esculenta*, an average of 4.8 4$^{th}$-instar larvae per plant reduced yield by 10%, while 2.3 and 1.5 larvae reduced yield of brinjal and capsicum in glasshouses by 10% also (Nakasuji and Matsuzaki, 1977).

### 2.1.3 *Amsacta albistriga* (Walker) (Lepidoptera: Arctiidae):

*Amsacta albistriga* (Walker), commonly known as Red Hairy Caterpillar (RHC) is a pest of several rainy season crops in Asia especially on groundnut in South India (Nagarajan *et al.,* 1957; Nagarajan and Ramachandran, 1958; Narayana and Ranga Rao, 1959; Mukundan, 1964; Venkataraman *et al.,* 1970; Saroja *et al.,* 1971; Paramasivam *et al.,* 1973). *A. albistriga* is a common species in south India and *A. moorei* is a common species in north India. This pest frequently occurs in Tamil Nadu at Coimbatore, Madurai and Pollachi districts (Kuppuswamy *et al.,* 1966) and adjoining areas (Nagarajan and Ramachandran, 1958). Epidemics of this pest also occurred in Srikakulum, Visakhapatnam, Cuddapah, Kurnool, Anantapur, and Chittoor districts of Andhra Pradesh; an outbreak of this pest in Chittoor district occurred in 1975 (Siva Rao *et al.,* 1977). In Karnataka, the pest is of some importance in Raichur district.

It is a polyphagous insect but particularly destructive to groundnuts (Mukundan, 1964). Other plants recorded as hosts of this insect are finger millet, cowpea, castor, cholam, cotton, sorghum, pearl millet, maize, soybean, horsegram, greengram, blackgram, clusterbean, pigeonpea, sesame, jute, sunnhemp and several weeds (Nagarajan *et al.,* 1957). The RHC infestation is sporadic, but in certain years it can become serious and cause heavy losses (75%). In some areas in Tamil Nadu, Andhra Pradesh and Karnataka this pest occurs regularly in rainy season (Venkataraman *et al.,* 1970; Sandhu and Brar, 1977; Siva Rao *et al.,* 1977).

The field biology of RHC has been studied by many researchers (Nagarajan *et al.,* 1957; Nagarajan and Ramachandran, 1958; Mukundan, 1964; Ramaswamy and Kuppuswamy, 1973). The adult moths emerge from pupae present in the soil after the heavy rains at the onset of the south-west monsoon (usually in June). Various stages of the life cycle is depicted in Figure 6. They

copulate immediately and oviposit in the same night. The female moths lay 800-1000 eggs in masses on leaves, grasses and soil surface. After 3-4 days small larvae come out and feed gregariously by scraping the green portion of the leaves giving them papery appearance. Once the larvae become older ($3^{nd}$ to $5^{th}$ instar), they feed individually on leaf lamina leaving only the petiole, midrib and the main stem. In severe cases it feeds all the parts of plant leaving only main stem (Nagarajan *et al.,* 1957). After 20-30 days of active feeding grown up ($6^{th}$ instar) larva move towards barren areas, burrow into undisturbed soil and pupate at a depth of 10 to 20 cm. The pupae remain in the soil in diapause stage till the next monsoon. The pupation behaviour of *A. albistriga* was studied by Reddy *et al.* (2004). In most places this pest has one generation per year. However, a second generation was reported from Pollachi region of Tamil Nadu in the month of August (Ramaswamy *et al.,* 1968) and from Pavagada region of Karnataka after the northeast monsoon during October and November (Veenakumari *et al.,* 2007). This insect can adapt and synchronize with weather to its best advantage (Nagarajan and Ramachandran, 1958). When an outbreak of this pest occurs, a total yield loss over a large area is not uncommon (Nagarajan *et al.,* 1957). However, no clearcut relationship between larval numbers and yield loss has been worked out and most pest-control trials have only indicated the reduction in larval number 24 h after pesticides application without reporting any yield gains (Narayana and Ranga Rao, 1959; Kuppuswamy *et al.,* 1965; Ramaswamy *et al.,* 1969; Venkataraman *et al.,* 1970; Saroja *et al.,* 1971; Paramasivam *et al.,* 1973).

**Figure 2: Larva of tobacco caterpillar (*Spodoptera litura*) (A) and red hairy caterpillar (*Amsacta albistriga*) (B) in the act of defoliating groundnut crop.**

**A**

**B**

## 2.2 Need for the Alternative Pest Management Strategies for Chemicals:

Intensive and indiscriminate use of insecticides to control these three pests, in particular *H. armigera*, has resulted in development of resistance to synthetic insecticides such as organochlorine, organophosphate and carbamate groups of insecticides (Harries *et al.,* 1971). The first report on development of resistance in *H. armigera* to pyrethroids was published in late eighties (Dhingra *et al.,* 1988; McCaffery *et al.,* 1989). Subsequently, high levels of pyrethroid resistance were reported in cotton and pulse growing regions of India (Mehrotra and Phokela, 1992; Armes *et al.,* 1992, 1994 and 1996; Sekhar *et al.,* 1996). Kranthi *et al.* (2001) has been studied the pyrethroid resistance in 54 field strains of *H. armigera* collected between 1995 and 1999 from 23 districts in seven states of India and concluded that the enhanced cytochrome p450 and esterase enzyme activities were probably important mechanisms for pyrethroid resistance in some parts of the country where the use of pyrethroids was high. Kranthi *et al.* (2002) reported the high level of resistance in *H. armigera* to many of the commonly used insecticides. In China, extensive spraying with chemical insecticides has decimated natural enemies and has reduced natural control of cotton bollworm infestations (Liu *et al*., 2000; Yang *et al*., 2000).

In Andhra Pradesh, insecticide resistance to the populations of *S. litura* was first reported by Ramakrishnan *et al.* (1984). Issa *et al.* (1984a, b) surveyed the resistance to organophosphorous insecticides and pyrethroids in field strains of the *S. litura* during 1980-1984 cotton growing seasons in Egypt. Sawicki (1986) reported that the resistance to synthetic pyrethroids in *S. litura* and *S. litturalis* can be countered successfully. In Pakistan, the insecticide reisistance was evaluated in field populations of *S. litura* to endosulfan, organophosphates, carbamates and pyrethroids during 1997-

2005 (Mushtaq Ahmad *et al.,* 2007). The base line toxicity of diffeent insecticides against common cutworm *S. litura* was studied by diffeent monitoring methods, according to this data, the two field populations were shown to have high resistance to organophosphates and carbamates (Huang *et al.,* 2006). Armes *et al.* (1997) reported the status of insecticide resistance in *S. litura* in Andhra Pradesh. Murugesan and Dhingra (1995) reported the variability in resistance pattern of various groups of insecticides evaluated against *S. litur*a during a period spanning over three decades. The relative susceptibility of different larval instars of *S. litur*a to some synthetic pyrethroids has studied by Rao and Dhingra (1996).

The published data on resistance to synthetic insecticides in larvae of *A. albistriga* is very limited. Every year, several tons of pesticides are used to control this pest in Tamil Nadu, Andhra Pradesh and Karnataka. In spite of this, the pest recurs unabated each year causing considerable damage to sesamum*,* red gram, cotton, cowpea and castor in addition to groundnut (Veenakumari *et al.,* 2007).

In addition to the development of resistance to synthetic insecticides, it has also resulted in resurgence of secondary pests, destruction of natural enemies, environmental pollution and health hazards (Armes *et al.,* 1992, Kranthi *et al*., 2002). These problems reaffirmed the need to focus attention on alternate pest management strategies with special emphasis on these pests. Integrated Pest Management (IPM) methodology has been developed through the establishment of farmer field schools to improve the farmer's knowledge. Many farmers were trained to implement IPM with significant profits (Wang, 2000). Alternative pest management strategies include use of parasitoids; predators, plant products, pheromones and microbial biopesticides were emphasized. Currently, there are reports on more than 170 active ingredients in over 500 biocontrol products (Copping, 1998). Approximately 45 biopesticide products were registered by the US-EPA (United states environment protection agency) by 1995 and it was estimated that this number would increase as more products being developed / tested (Murphy *et al.,* 1995; Copping and Menn, 2000).

## 2.3 Biopesticides as Alternatives to Chemical Insecticides:

Biopesticides mainly include parasitoids, predators, fungi, bacteria, viruses, protozoa, nematodes, plant products and pheromones for the biological control of insect pests, among these entomopathogenic bacteria, fungi and viruses such as *Baculoviruses* have the potential to play an important role for the management of economically important insect pests (Dent and Jenkins, 2000; Moscardi, 1999). Insect-resistance based on a genetic modification through the introduction of toxic genes from *Bacillus thuringiensis* (e.g. Bt cotton), became available to farmers. There is renewed interest in classical biological control as a strategy in the management of severe pests like *Helicoverpa* by introducing natural enemies (King and Coleman, 1989). A more frequently tried method to achieve control with natural enemies has been used by augmentation of artificially reared parasites or predators (King and Coleman, 1989; Xia, 1997b). While the technical feasibility for controlling by this method has been demonstrated, the results in the field have not always been consistent (King and Coleman, 1989). Pathogens such as baculoviruses are attractive alternatives and have been used for more than 20 years with great success (Zhang, 1989). There are several advantages of using insect viruses in pest management: they are nonpathogenic to vertebrates and plants; They are well-studied systems from both pest management and molecular biology points of view; they leave no undesirable residues and can be used at the 'cottage scale' (FAO/WHO, 1973; Moscardi, 1999).

## 2.4 Entomopathogenic Viruses:

Insect viruses have been studied for many years due to an intrinsic interest in the general study of diseases of invertebrates and, more particularly, because of their potential as environmentally benign pest management agents (Evans, 1986). It is possible that association of viruses with invertebrates is ancient, possibly antedating the colonization of dry land by arthopods (Reik, 1970). The interest in insect diseases can be traced to the sixteenth century when a 'wilting disease' of

silkworms was first formally described (Benz, 1986). The study of ecology of insect viruses and their potential use for pest management agents began with the pioneering work of Steinhaus (1956).

Insect viruses are obligate, intracellular pathogenic entities. According to the International Commission on Taxonomy of Viruses (ICTV), those pathogenic to arthropods (insecta in particular) belong to at least 12 distinct families (Van Regenmortel *et al.,* 2000). In Table 1 classification of viruses that infect invertebrate (insect) hosts were listed. Early in the 20$^{th}$ century the disease in silkworms was attributed to a virus infection, and in 1947, visualization of rod-shaped virions, which are known to be characteristic of the virus family baculoviridae, was reported (Miller, 1996). Out of 12 families, baculoviruses have been well studied because of their potential as pest control agents (Black *et al.,* 1997; Van Beek and Hughes, 1998) and, more recently, for their prominence as expression vectors for a wide range of heterologous genes (Luckow, 1995; Miller, 1988; Choi *et al.,* 1999; Smith *et al.,* 1983). The advantages of baculoviruses for pest control include, their restricted host range (Grőner, 1986) and non-target effects on useful insects and lack of toxic residues, allowing growers to treat their crops even shortly before harvest, with low probability to develop stable resistance (Monobrullah, 2003). *In vivo* and *in vitro* tests with several vertebrate, invertebrate, and plant species have not demonstrated any pathogenic, toxic, carcinogenic, or teratogenetic effects after exposure to these viruses (Banowetz *et al.*, 1976; Huber and Krieg, 1978; Ignoffo, 1973; Ignoffo and Rafajko, 1972; Lautenschlager *et al.,* 1977; Roder and Punter, 1977). Baculoviruses are stable and can be stored as aqueous suspensions or dried powders for long periods without any loss of activity (David and Gardiner, 1967a). They are resistant to many chemicals and persist in the soil for many years (David and Gardiner, 1967b), and their activity is not altered significantly by relative humidity (David *et al.,* 1971), precipitation (David and Gardiner, 1966), or prolonged exposure to normal field temperatures (Yendol and Hamlen, 1973). In addition, they are highly compatible with other methods of pest control and are well suited for use in integrated pest management programs. They can be used concurrently with most

chemical insecticides, reducing effective doses of the latter to environmentally acceptable levels (Falcon, 1971; Hunter *et al.,* 1975; Jaques and Long, 1978). Baculoviruses differ significantly from chemical insecticides in that they are components of nature. Large quantities of virus are released into the environment during natural epizootics, which are common, widespread, and often important in regulating insect population levels (Federici, 1978; Injac, 1973). There is evidence that the amount of virus which is artificially placed into the environment for insecticidal purposes is minimal compared with the amount produced during such epizootics (Thomas, 1975).

## 2.5 Baculoviruses:

Baculoviruses are occluded, double stranded DNA (dsDNA) viruses, and characterized by the presence of occlusion or inclusion bodies (OBs). The nature and significance of these occlusion bodies remained a mystery for a long time until the electron microscope (EM) was available that the virus particle could be isolated and identified as the infectious viral agent. Based on the size, shape and occluded virion phenotype the baculoviruses are classified in to two genera, *Nucleopolyhedroviruses* (NPVs) and *Granuloviruses* (GVs*)* (Rohrmann, 1999; Winstanley and O.Reilly, 1999; Blissard *et al*., 2000; Fauquet *et al.,* 2004).

The EM observation of NPVs reveal polyhedral to irregular shaped occlusion bodies with size 0.15 to 15 µm in diameter composed of matrix protein (30-40 % of total viral protein) called polyhedrin, which crystallizes around many enveloped nucleocapsids (Hooft van Iddekinge *et al.,* 1983). Different NPVs are characterized by their occluded virions being present either as single (SNPV) or multiple (MNPV) nucleocapsids within the envelope (Fig 3). Both SNPVs and MNPVs may contain 20–200 virions depending upon species (Rohrmann, 1999). The GVs have small occlusion bodies (0.25 to 0.5 µm in cross- section) ellipsoidal in shape; normally contain a single nucleocapsid, which are enveloped and are composed of a major matrix protein called granulin (Funk *et al.,* 1997; Winstanely and O' Reilly, 1999; Crook, 1991).

NPVs are found moslty in the order Lepidoptera but they are also present in other insect species in the orders such as Hymenoptera (31 species), Diptera (27 species) and Coleoptera (5 species) as well as from the crustacean order Decapoda (shrimp), where as GVs are only found within the order Lepidoptera (Blissard *et al*., 2000; Federici, 1997; Martignoni and Iwai, 1986b; Adams and Bonami, 1991; Couch, 1974). In India, about 35 insect viruses have been recorded from

**Table 1: Classification of virus families containing insect pathogenic viruses.**

| **Family** | **Genera occuring in insects** | **Examples of virus** |
|---|---|---|
| **DNA viruses** | | |
| Ascoviridae | Ascovirus | *Trichoplusia ni* AV |
| Baculoviridae | Nucleopolyhedrovirus | *Helicoverpa armigera* NPV |
| | Granulosis virus | *Cydia pomonella* GV |
| Iridoviridae | Iridovirus | *Chilo* iridiscent virus |
| | Chloriridovirus | Mosquito iridescent virus |
| Parvoviridae | Densovirus | *Galleria mellonella* densovirus |
| Polydnaviridae | Ichnovirus | *Campoletis sonorensis* PV |
| | Bracovirus | *Cotesia melananoscela* PV |
| Poxviridae | Entomopoxvirus | *Amsacta moorei* EPV |
| Unclassified | - | *Oryctes* virus<br>Hz-1 virus<br>Bee filamentous virus<br>Tsetse virus<br>Narcissus bulb fly virus |
| **RNA viruses** | | |
| Birnaviridae | Birnavirus | *Drosophila* X virus |
| Caliciviridae | - | Chronic stunt virus |
| Nodaviridae | Nodavirus | Black beetle virus |
| Picornaviridae | - | Cricket paralysis virus |
| Reoviridae | Cytoplasmic polyhedrosis virus | *Bombyx mori* CPV |
| Rhabdoviridae | - | Sigma virus |
| Tetraviridae | Tetravirus | *Nudarelia β* virus |
| Unclassified | - | Various bee paralysis viruses |
| | - | Various *Drosophila* viruses |

Source: Fujitha *et al*. (1998)

the baculovirus group, the most important being the NPV of *H. armigera, S. litura, Spilosoma obliqua* (Walker)*, Achoea janata* (Linnaeus) and *A. albistriga* (Walker) and GVs of *A. janata*, *S. litura*, *H. armigera* and *Chilo* spp. (Pawar and Thombre, 1992).

The size and shape of occlusion bodies in NPVs varies considerably not only between the POBs from different insects, but often also within the same species. For example, majority of the polyhedral occlusion bodies of *H. armigera* NPV are spherical while some of them are irregular in shape and the size ranged from 0.6 to 2.3µm average to 1.35µm. The diameter of polyhedra ranges about 0.5 to 1.5µm, depending on the insect species. Fig. 3 shows the differentiation in the cross section of a typical baculovirus (NPV and GV) occlusion bodies (OBs). In the boundary of OB the protein envelope (PE) appears as an electron dense layer made up of PE protein or envelope protein and shown to be very sensitive to alkaline proteases (Russell and Rohrmann, 1990a; Van Lent *et al.,* 1990). The distance between envelope and crystalline matrix (polyhedrin or granulin) is not uniform around the occlusion body. The fine structure of occlusion body reveals crystalline lattice of the occlusion body protein molecules, which are arranged in cubic system. Although there is no true membrane covering the OB, difficulties in staining OB, the retention of their shape, and the presence of a membrane-like coat following chemical and physical treatment indicate that the exterior portion of OB is different for the interior portion. On the whole they are very stable and can persist indefinitely in the environment (Bergold*,* 1982).

The infectious, rod-shaped virions are randomly occluded in OBs without any apparent disruption of the lattice; an 8 nm layer separates virion from the protein matrix. The size of the virions

with dimensions in the range from 4.0 -140nm × 250 - 400nm. Alkaline-liberated virions readily lose their envelopes to reveal nucleocapsids each made up of a capsid surrounding a DNA core. The capsid, in turn, consists of protein subunits arranged along its long axis. The virions contain large circular, covalently closed, dsDNA genome with size in range of 80- 180 kbp packed in the nucleocapsid (Blissard and Rohrman, 1990, Volkman *et al.,* 1995). Among the baculoviruses, NPVs attracted the attention of pest control scientists interested in looking for an alternative to pesticides because they cause a highly infectious disease that kills in 5-7 days. These viruses attack some of the most important Lepidopteran crop pests including species of *Heliothis*, *Helicoverpa* and *Spodoptera.* Some of the related GV species are also highly infectious e.g. *Cydia pomonella* (apple codling moth) GV and *Plutella xylostella* (diamond back moth) GV. However not all GVs are as fast acting as NPV because morphologically they had single envelop with single nucleocapsid per occlusion body (Winstanely and O' Reilly, 1999) (Fig 3). In general, the host range of most NPV is restricted to one or a few species of the genus or family of the host where they were originally isolated. However, it also represents an important commercial draw back, restricting the use of these products to specific key pests or closely related pest complexes, such as *Heliothis* and *Helicoverpa* species (Chakraborthy *et al.* 1999). Some of the few exceptions having a broader host range are (i) *Autographa californica* MNPV infecting more than 30 species from about 10 insect families, all within the order Lepidoptera, (ii) *A. falcifera* NPV infecting more than 31 species of Lepidoptera from 10 insect families and (iii*) M. brassicae* MNPV which was found to infect 32 out of 66 tested Lepidopteran species from 4 different families (Groner, 1986; Doyle *et al.,* 1990; Hosteller and Puttler, 1991). In contrast to NPV, the host range of GV appears to be even narrower and mostly restricted to a single species. Over 20 species of baculoviruses have been developed or registered as commercially available insecticides and over 30 different products have been registered as commercial insecticides based upon NPV or GV. Some examples of commercially available virus-based pesticides registered for pest control in different countries were listed in Table 2.

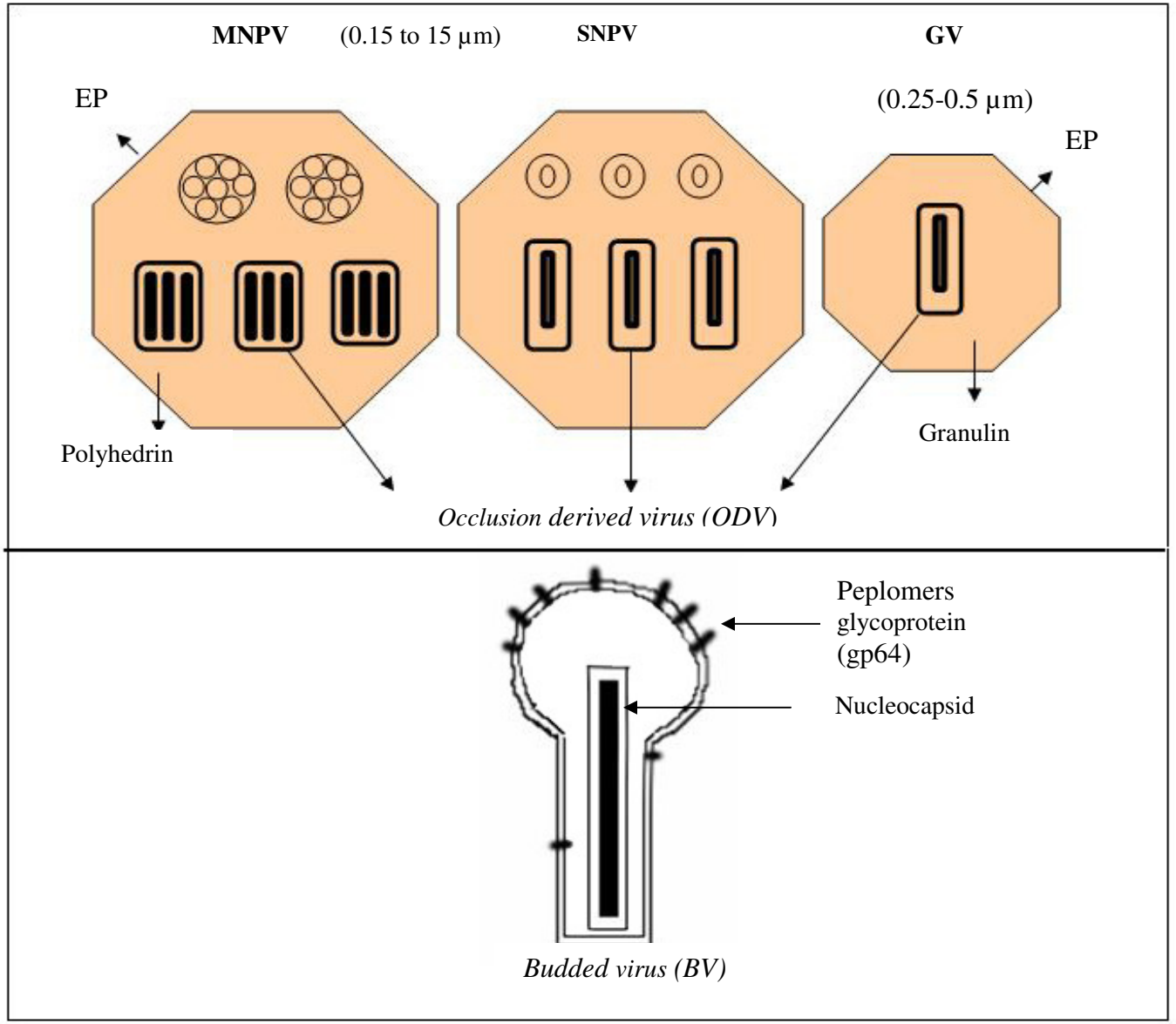
**Table 2: Examples of commercially available virus-based pesticides registered for pest control in different countries**

| S.No | Host insect | Crop | Commercial name | Country |
|---|---|---|---|---|
| 1 | *Anagrapha falcifera* (Kirby) | Cotton<br>Vegetables | | USA |
| 2 | *Anticarsia gemmmatalis* (Hubner) | Soybean | Baculoviron<br>Baculovirus<br>Nitral<br>Coopvirus | Brazil |
| 3 | *Autographa californica* (Speyer) | Cabbage<br>Cotton<br>Ornamentals | VPN 80 | Gautemala |
| 4 | *Buzurz suppresalis* (Guenee) | Oil tree, Tea | Tung | China |
| 5 | *Heliothis virescens* (Fab.) | Cotton | Elcar | USA |
| 6 | *Helicoverpa armigera* (Hubner) | Cotton,<br>Tomato | Virin-HS | Russia |
| 7 | *Helicoverpa zea* (Boddie) | Cotton | Elcar, Gemstar | USA |
| 8 | *Hyphantria cunea* (Drury) | Forest<br>mulberry | Virin-ABB | Russia |
| 9 | *Lymantria dispar* (Linnaeus) | Forests | Gypcheck<br>Disparvirus<br>virin-ENSH | USA<br>Canada<br>Russia, China |
| 10 | *Mamestra brassicae* (Linnaeus) | Cabbage | Mamestrin,Virin-EKS | France, Russia |
| 11 | *Neodiprion lecontei* (Fitch) | | Lecont virus | USA,Canada |
| 12 | *Neodiprion sertifer* (Geoffroy) | | Monisarmio<br>Virus<br>Virox | Finland<br>UK<br>UK |
| 13 | *Orgyia pseudotsugata* (McDunnough) | Forests | TM biocontrol<br>Virtuss | USA<br>Canada |

| | | | | |
|---|---|---|---|---|
| 14 | *Spodoptera exigua* (Hubner) | Ornamentals<br>Vegetables<br>Garden pea<br>Grapes | SSPOD-X | USA<br>Thailand |
| 15 | *Spodoptera frugiperda* (J.E.Smith) | Maize | | Brazil |
| 16 | *Spodoptera littoralis* (Boisduval) | Cotton | Spodopterin | Africa |
| 17 | *Spodoptera litura* (Fabricius) | Vegetables | | China |
| 18 | *Spodoptera sunia* (Guenee) | Vegetables | VPN 82 | Guatemala |

Source: Moscardi, 1999

**Figure 3: Morphological Characteristics of Nucleopolyhedroviruses (NPVs) and Granulovirues (GVs)**



## 2. 6 The Life Cycle of a Typical Baculovirus:

An understanding of the lifecycle of baculovirus and its mode of replication (Figs 4 and 5) is essential for an understanding of virus production dynamics. The replication of *Autographa californica* MNPV has been most extensively studied in larvae of *Trichoplusia ni* and in cultured cells of *S. frugiperda* and serves as a model for NPV and GV replication in Lepidoptera (Granados and Williams, 1986; Federici, 1997; Williams and Faulkner, 1997). The infection occurs when the susceptible larva feed on the virus-lased plant parts and it must be emphasised that these viruses have no contact effect and cannot infect an insect unless eaten (Blissard and Rohrmann, 1990). A unique feature of the baculovirus life cycle is the production of two virion phenotypes: Those virions found within polyhedra are termed polyhedra-derived virus (PDV) or occlusion derived virus (ODV) which is important for the horizontal spread of the virus in the environment and a second form which is involved in the spread of the viral infection and found in the haemocoel of the infected host insect is termed budded virus (BV) (Fig 3). These two phenotypes are structurally distinct and destined for two different functions, both of which are essential for virus survival in nature (Funk *et al.,* 1997). The differences between BV and ODV and various functions were detailed in Table 3.

Following consumption of occlusion bodies, in the alkaline environment of the midgut (pH >9.5), the protective crystal matrix protein (polyherin / granulin) dissolve rapidly and the infectious occlusion derived virions (ODVs) are released in to the gut (Blissard and Rohrmann, 1990). There is evidence that the dissolution of the occlusion body matrix might be facilitated by an insect derived alkaline protease, which is associated with the occlusion body matrix (Harrap *et al.,* 1977). The released virions pass through the peritrophic membrane (PM) (proteinaceous-Chitinaceous layer secreted by midgut cells to protect epithelium) and after the attachment to the microvilli of the midgut epithelium, the nucleocapsids enter the cell lumen either via fusion of the virion envelope with epithelial membrane or by viropexis (Patel *et al.,* 1967).

**Table 3: Differences between BV and PDV phenotypes (Fig. 3):**

| Character | BV | ODV |
|---|---|---|
| **Virions** | As single nucleocapsids in loose – fitting viral envelope | Possess one or many nucleocapsids in single tight – fitting envelopes. |
| **Time of production** | During early stages of infection | During terminal stages of viral infection |
| **Responsible for** | Secondary infection<br>(Spreads the infection from cell to cell within the host) | Primary infection |
| **Virion envelope** | Derived from plasma membrane | Acquired in the nucleus |
| **Mode of entry into host cells** | By adsorptive endocytosis | By fusion of viral envelope with microvilli of midgut epithelial cells |

The nucleocapsids are transported, most likely under involvement of the cellular microtubular structures, to the nucleus. Where they uncoat as early as 1 h post infection (PI), the DNA is exposed and the virus replication is initiated in the host cell (Miller and Lu, 1997). The newly produced nucleocapsids traverse the nuclear membrane, the cytosol and bud through the basal lamina of the mid gut cells into the hemolymph. These progeny virus particles are called extra cellular virus (ECV) or budded virus (BV) and consists of naked nucleocapsids (Miller and Lu, 1997). During exit from the cells, BVs acquire a new envelope and protein structures (peplomers), which consists of plasma membrane containing viral encoded glycoprotein, termed GP64 (Blissard and Rohrmann, 1989) (Fig 3). Gp64 is expressed both early and late in infection and is transported to and incorporated into the cell membrane. As nucleocapsids bud through the cell membrane and exit the cell, they become enveloped in the GP64-modified cell membrane. GP64 is required for the spread of the infection to other cells and for the virus to exit from an infected cell (Monsma *et al.,* 1996). GP64 appears to be pivotal for the interaction between the BV envelope and susceptible host cells through a possible interaction with a cell membrane receptor molecule and then a final fusion with the endosomal membrane (Blissard, 1996). BV primarily enters cells by endocytosis, a pathway in which the entire virion is endocytosed into an intracellular vesicle called an endosome. The acidification of the endosome is thought to initiate fusion of the virus envelope with endosomal membrane, releasing the viral nucleocapsid into the cytoplasm (Kingsley *et al.,* 1999; Markovic *et al.,* 1998). Some production of polyhedra may also occur during the first 48 h PI, but these OB are usually small and defective containing no virions (Miller and Lu, 1997). At this point the first cycle of viral replication completes and the second phase of infection in the other body tissues follows. Just as the OB is the form of the virus designed to carry the infection from insect to insect, the ECV or BV is the form in which the virus spreads from the initial site of infection in the midgut to the other tissues of the body of the insect (Monsma *et al.,* 1996; Oomens and Blissard, 1999). For most NPV and GV infecting lepidopteran host larvae, virus occlusion is not observed in midgut epithelial cells. These cells

release BV into the hemolymph which then systematically spread the virus infection among susceptible cells and tissues. Engellhard *et al.* (1994) studied the infection pathway in fourth instars of *Trichoplusia ni* larvae, based on the observations they postulated that in early infection of midgut, the tracheal system (tracheoblast and the tracheal matrix) might directly contribute to the systemic spread of BV. It is in the midgut tissue that the host insects are sometimes able to contain and halt the infection by destroying or shedding infected cells before the first cycle is completed (Engelhard and Volkman, 1995).

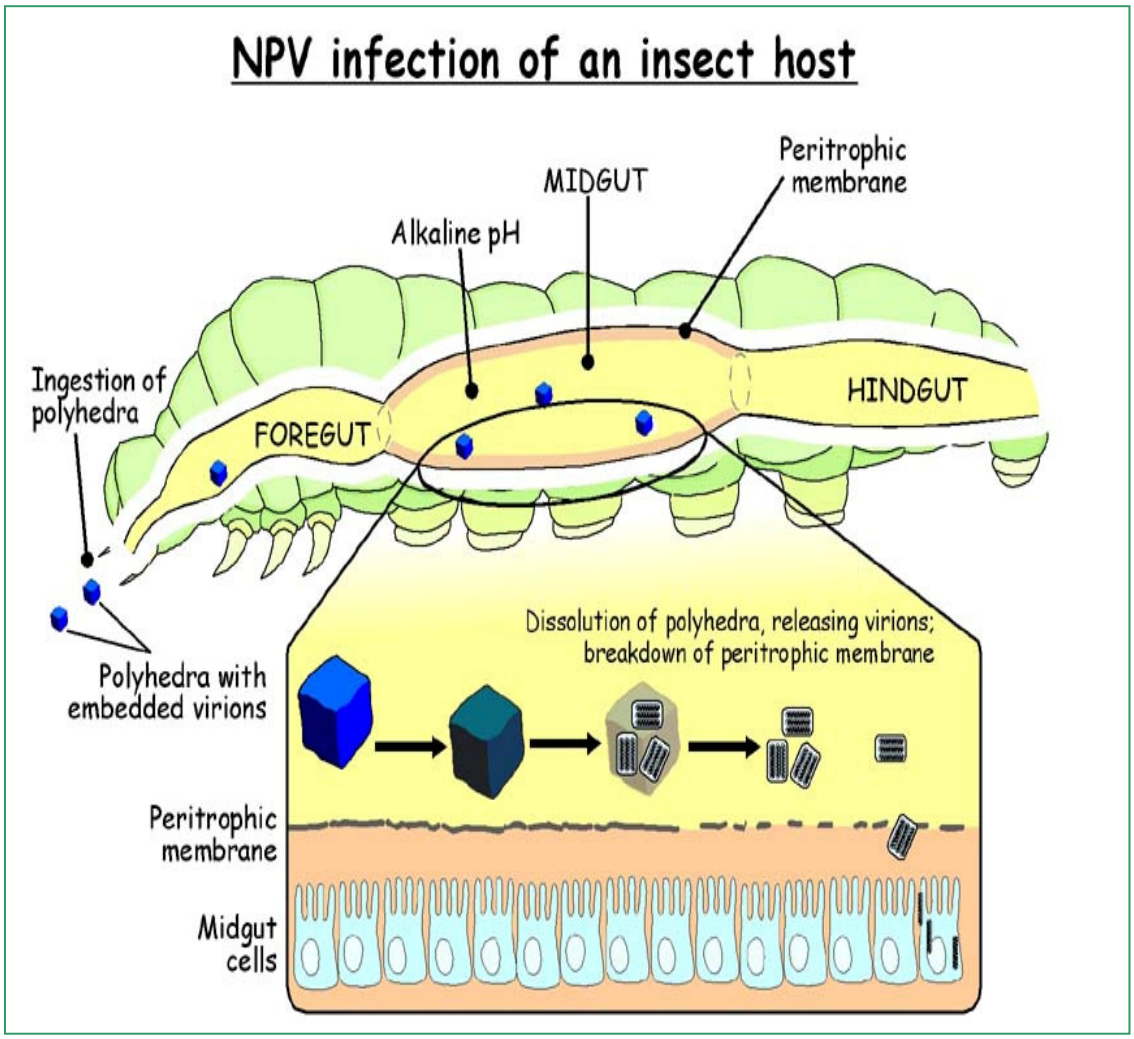
After the midgut cycle the BV particles spread throughout the body in the haemolymph and infect in turn the cells of haemocytes, tracheal cells, fat body, muscle cells, hypodermis, nerve cells, as well as reproductive and glandular tissues (Granados and Lawler, 1981; Federici, 1997). It is in these tissues that a second cycle of infection occurs and occlusion bodies (OBs) are produced. If the infection gets established and this second cycle of virus replication occurs it is often found that 90% or more of susceptible cells may become infected (Federici, 1997). In these the nuclei become swollen and nucleocapsids are produced, but unlike in the first cycle large amounts of polyhedrin or granulin are also synthesised and condense to form crystals in which the nucleocapsids become embedded to form new OBs and so complete the cycle (Hamblin *et al.,* 1990; Wood *et al.,* 1994). This OB production in a species such as *H. armigera* starts around 5 days after infection and peaks 7-8 days post infection (PI). The massive destruction of body tissue that accompanies the production of OB eventually kills the insect. The final stage of baculovirus infection is breakdown of the larval cuticle and the release of the occlusion bodies into the environment to spread and infect other insects. The cuticles of insect larvae consist mainly of chitin fibres embedded into a proteinacous matrix. Two baculovirus genes, encoding a chitinase (*ChiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitninase is a chitin-degrading enzyme with endo- and exo-molecular specificity, whereas cathepsin has cysteine protease activity (Slack *et al.,* 1995; Hawtin *et al.,* 1995; O'Reilly, 1997). In a typical nucleus 10-50 OBs are produced giving $10^9$-$10^{10}$ OB per

insect. On death these OB may comprise in total 10% of the insects weight. This astonishing high productivity of NPV or GV in larvae is another reason for their attraction as biopesticides and is unmatched by any other type of virus. Successfully infected insects secrete NPV or GV during the later stages of infection and move about host plants spreading virus extensively before death. OBs also remain active if eaten and passed through predators and, therefore, the activities of birds, mammals and other larval predators may be important in spreading baculovirus epidemics (Boucias *et al.,* 1987; Entwistle *et al.,* 1993; Vasconcelos *et al.,* 1996b).

The gross pathology of a typical baculovirus (NPV) infection in lepidopteran larvae was summarised by Granados and Williams, 1986; Federici, 1997; Williams and Faulkner, 1997 as follows:

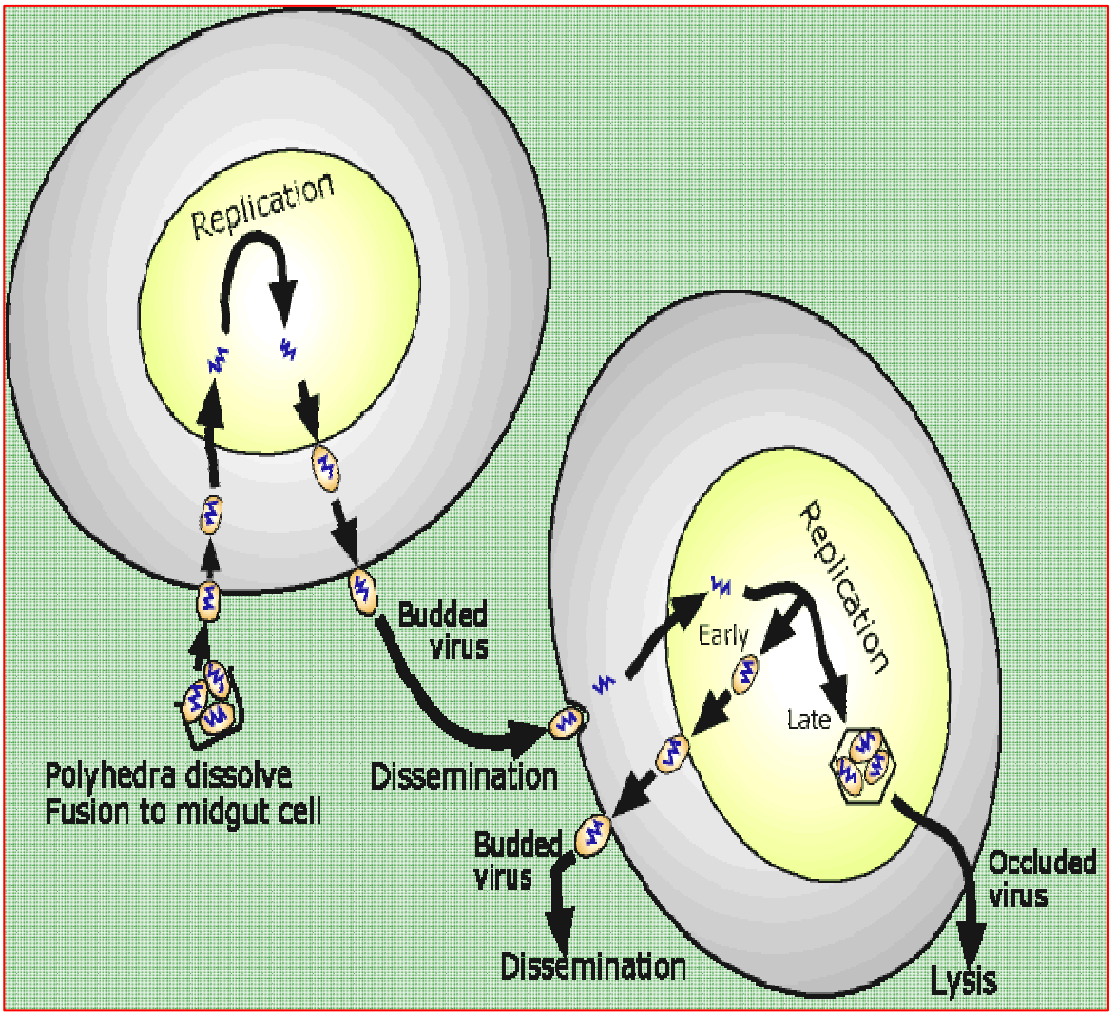
- Day 1-3 PI: Infected larvae normally do not show obvious signs of disease.
- Day 4-6 PI: Diseased larvae only react slowly to tactile stimuli. The larvae start to appear swollen, glossy and moribund.
- Day 6-7 PI: Diseased larvae stop feeding and begin to die. Diseased larvae of some species (e.g. *Lymantria, Heliothis, Amsacta* etc) crawl to the top of the twings (negative geotropism) on which they were feeding (Fig 10).
- Day 7-10 PI: Diseased larvae die and may liquefy, the cuticle ruptures and polyhedra are released.

**Figure 4: The mode of infection of a typical baculovirus in lepidopteran larvae**




Source: http://www.microbiologybytes.com/virology/kalmakoff/baculo/baculo.html

**Figure 5: An general overview of the replication cycle of NPVs**




Source: http://www.microbiologybytes.com/virology/kalmakoff/baculo/baculo.html

## 2.7. *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV):

NPVs are naturally occurring pathogens of *H. armigera* and have wide distribution in Asia, Africa and Australia. Strains of these viruses have been developed as commercial biopesticides in America, Australia, India, China and Thailand. Development of NPV-based pesticides to manage *Helicoverpa* species began in 1961. Progressed through various research and developmental phases, and attained technical realization as first commercial viral pesticide in 1973 (Ignoffo and Couch, 1981). The history of baculoviruses in India reveals that an impetus for the development of an NPV of *H. armigera* (HaNPV) was provided through its first discovery from the laboratory culture of its host in Gujarat (Patel *et al*., 1968). Jacob (1972) and Rabindra and Subramanian (1974) described the symptoms of disease, susceptibility of different instars of *H. armigera* and host-pathogenic relationship.

HaNPV has been shown to be highly effective in controlling *H. armigera* on a range of crops, including legumes (Rabindra *et al.,* 1992), oil seeds (Rabindra *et al.,* 1985), cotton (Jones, 1994) and vegetables (Jones *et al.,* 1998). Field trials on chickpea in India showed that HaNPV applications could control *H. armigera* more effectively than either chemical insecticides or commercially formulated *B. thuringiensis* (Cherry *et al.,* 2000). *H. armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV), has been used successfully in china to control this pest in an area of about 100,000 hectares since its first isolation in 1975 in Hubei province of China (Zang 1994), with minimal adverse effects on the biotic environment (Chen *et al.,* 2000b). For insect species such as cotton bollworm that have developed resistance to chemical and/or *Bt* insecticides, the application of HaNPV is one of the few options left for effective bollworm control (Trumble, 1998). Eight HaSNPV genotypes have been identified and partially purified from wild-type HaSNPV isolation by using an *in vivo* cloning method, among these one genotype (HaSNPV-G4) is present at the highest frequency and is the predominant genotype of this isolate (Sun *et al*., 1998). HaSNPV appears to have a host range restricted to members of the genus *Helicoverpa* including *H.*

*zea*, *H. virescens* and *H. assulta* (Herz *et al*., 2003; Ignoffo and Couch, 1981). *H. zea* single nucleocapsid NPV (HzSNPV) has almost the same host range (Ignoffo *et al*., 1983). There is no significant difference in the infectivity of HaSNPV and HzSNPV in *H. armigera* larvae (Sun and Zhang, 1994) or in *H. zea* (Hughes *et al*., 1983). HzSNPV was registered as one of the first commercial baculovirus pesticides (Viron-H, Biocontrol-VHZ, ElcarTM) in the 1970s and has been extensively used to control the cotton bollworm in the USA and other countries (Shieh, 1989; Cunningham, 1998). HaSNPV has been adopted for mass production as a viral pesticide and has been widely used to control the insect pests in China (Zhang *et al*., 1995) and in other countries (Jones, 1994) as well. HaSNPV insecticide is recommended to be applied to control early instars of the pest larvae through either a high volume spray or an ultra low volume spray method. Depending on the size and density of the crop and the age and density of the pest population, the dosage of virus application is suggested to be 1.2-2.4 $\times$ $10^{12}$ POBs/ ha, which is 1.2-2.4 kg of wettable powder, or 0.6-1.2 L of liquid concentrate. Normally 2-3 sprays are needed to control natural infestation with 4-5 days interval. The surviving number of cotton bollworm larvae was reduced by 83.7-91.7 % when cotton was sprayed with this spray regime (Zhang *et al*., 1995). However, like other baculoviruses HaSNPV have some limitations which restricted its widespread commercial use. The major disadvantage is slow speed of action when compared to chemical insecticides. During which the insects can still cause serious damage to the crop. To enhance the efficacy of HaSNPV as a pesticide, it has been genetically modified by deletion of the *egt* gene from its genome (recombinant HaCXW1) and insertion of an insect-selective scorpion toxin (AaIT) gene controlled by the HaSNPV *polyhedrin* promoter (recombinant HaCXW2) (Chen *et al*., 2000b). In cotton field plots, artificially released *H. armigera* larvae treated with either HaCXW1 or HaCXW2 were killed faster than larvae in plots treated with wild-type HaSNPV (Sun *et al*., 2002b, 2004).

## 2.8. *Spodoptera litura* nucleopolyhedrovirus (SlNPV):

Dhandapani *et al.* (1982) observed an epizootic in larvae of *S. litura* on daincha (*Sesbania bispinosa,* a green manure crop) in Tamil Nadu, which was traced to a NPV, not previously observed in India. It is suggested that virus is spread through soil-borne particles.

In field test in Japan, *Sl*NPV at the rate of $1 \times 10^{11}$ POB/0.1 ha gave good results against larvae in the $1^{st}$-$3^{rd}$ instar, but $3 \times 10^{11}$ POB/0.1 ha was necessary to control 4th stage instars. Active polyhedra were recovered in the soybean plots 1 month after the treatments (Okada *et al.,* 1977). In Tamil Nadu, application of *Sl*NPV against *S. litura* on knolkhol, beetroot, tobacco, cotton, cauliflower, cabbage and castor was studied in pot experiments. Maximum larval mortality was obtained on tobacco (96.6 %), cauliflower (96.6 %) and cabbage (93.3 %). Application of the virus to the lower leaf surface of tobacco or cotton caused higher mortality than on upper leaf surface (Santharam and Jayaraj, 1987). Sachithanandam *et al.* (1989) tested the efficacy of dust and wettable powder formulations of nuclear polyhedrosis virus (*Sl*NPV) under greenhouse conditions on groundnut. Wettable powder and dust formulations at the rate of $4\text{-}6 \times 10^7$ POBs/pot were as effective as chlorpyrifos 0.04% spray or carbaryl 5% dust against $3^{rd}$ instar larvae of *S. litura*. The virus formulations, however, were inferior to the unformulated virus. At higher doses, the wettable powder ($0.8 \times 10^8$ POB/pot) and dust ($1.2 \times 10^8$ POB/pot) were equivalent to unformulated virus ($0.8 \times 10^8$ POB/pot) causing 76.6, 70.0 and 80.0% mortality, respectively. Rao *et al.* (1987) added a number of adjuvants to SlNPV to observe its efficacy against *S. litura* on tobacco in nurseries and in the field in Andhra Pradesh. The addition of 0.25% boric acid enhanced the mortality of *S. litura* due to SlNPV. The virus application reduced leaf damage to a grater extent than endosulfan in the nursery.

In Japan, green house studies were carried out on the use of SlNPV @ $3 \times 10^8/m^2$ and Dipterex (trichlorfan) @ 0.05% a.i./200 $ml/m^2$ on strawberries against *S.litura.* The virus was slow in its action, but 20 days after spraying with SlNPV or trichlorfan the number of larvae per plant

was 1 and 2 respectively, compared to 17 in the untreated greenhouse plants. To examine the persistence, neonate larvae were released in plots 1, 11, and 19 days after treatment with virus, *B.t.* or trichlorfan. Larvae were controlled only in the SlNPV- treated plants (Nemoto and Okada, 1987). In soybean, field applications of SlNPV@ $3 \times 10^{12}$ and $1.5 \times 10^{12}$ POB/25 litres/ha increased the yield to 170% compared to that of untreated field and suppression continued for one month in comparison to 10 days when methomyl was applied @ 450 g a.i./ha (Asayama and Takimoto, 1987). In aerial application of the SlNPV @ $1.3 \times 10^{9}$ POB/ml was found to be ineffective while ground application @ $1.2 \times 10^{7}$ POB/ml was most effective. Larval mortality in aerially and ground treated plots was 54-83% and 87-93%, respectively (Okada, 1987).

In India, two field trials were conducted in Tamil Nadu, during the Kharif seasons of 1985 and 1986 with SlNPV @ 250 LE/ha (3 sprays), 0.08% chlorpyrifos (2 sprays), 0.08% chlorpyrifos + SlNPV @ 250 LE/ha (2 sprays) and initial spraying of SlNPV followed 3 days later with 2 sprays of chlorpyrifos against *S. litura* on cowpea. An initial treatment of SlNPV followed 3 days later by 2 sprays of chlorpyrifos gave the greatest reduction in pest numbers and highest grain yield in both years (705 and 467 kg/ha., respectively) (Sivaprakasam *et al.,* 1988). Dhandapani and Jayaraj (1989) observed that SlNPV @ 250 LE/ha at 10 day intervals was as effective as fenpropathrin @ 100 g a.i./ha + SlNPV (water dispersible powder) @ 125 LE/ha against *S.litura* on chillies.

In Vietnam, a new isolate of SlNPV was applied in local soybean fields, and the effect of its application was examined at high and low doses ($1.7 \times 10^{8}$ and $3.3 \times 10^{7}$ POB/$m^2$, respectively). The percentage of larvae infected with NPV increased from 22.2% on the day before NPV application (Day 0) to 50.8% (Day 6) in the high dose treatment plot, and from 7.9% (Day 0) to 35.7% (Day 6) in the low dose plot (Madoka Nakai and Nguyen Thi Thu Cuc, 2005).

## 2.9 *Amsacta albistriga* nucleopolyhedrovirus (AmalNPV):

The occurrence of NPV in groundnut red hairy caterpillar, *Amsacta albistriga* was first reported by Jacob and Subramaniam (1972). After that both field as well as laboratory studies were conducted on this virus in Tamil Nadu (Rabindra and Subramaniam, 1975; Jayaraj *et al.,* 1976; Narayanan *et al.,* 1978; Chandramohan and Kumaraswami, 1979; Baskaran *et al.,* 2001). Limited field application of this virus in 1979 resulted in large-scale outbreak of the disease in *A. albistriga* populations on groundnut during the succeeding year in Lakkepalayam village in Tamil Nadu (Rabindra and Balasubramanian, 1980). Muthukrishnan *et al.* (1998) conducted the *in vivo* studies on the susceptibility of *Amsacta albistriga*, to nuclear polyhedrosis virus. Similar studies were conducted at Pavagada in Karnataka; the results were quite encouraging as the virus effectively suppressed the pest, indicating that *A. albistriga* NPV (*Amal*NPV) has potential to be used as an effective microbial pesticide against this pest without posing any environmental hazard (Veenakumari *et al.,* 2005). The bio-safety studies of this NPV showed that it is non-hazardous to white mice and common carp *Cyprinus carpio* L. proving its safety to mammals and other non-target organisms (Narayan *et al.,* 1977a, b). The effect of host plants on the infectivity, incubation period and yield of NPV to groundnut red hairy caterpillar; *A. albistriga* was studied by Murali Baskaran *et al.* (1998). ICRISAT's research resulted in utilization of the NPV to control the RHC population on groundnut (Rao *et al.,* 2006). Currently, researches at the School of Biological Sciences, Madhurai Kamraj University, Tamil Nadu, India, has been working on the development of a recombinant baculovirus against *H. armegira* and *A. albistriga* (Personal communication).

## 2.10 Mass Multiplication of NPVs on Field Collected and Laboratory Reared Larvae:

Historically, several entomopathogenic viruses have been produced in susceptible host insects, because of the following reasons. i) The insect host is an efficient virus producer (Ignoffo and Couch, 1981). ii) Automation of *in vivo* rearing and *in vivo* production systems is feasible (Powell and Robertson, 1993; Bell and Hardee, 1995). Some baculovirus species may be produced in insect cell cultures, but the associated costs are relatively high (Hink, 1982). Therefore, all NPVs that have been developed as commercial products thus far have been produced in host larvae.

Like several other entomopathogenic viruses HaNPV, being an obligate pathogen, can be multiplied only on its host larvae (Ignoffo and Anderson, 1979). Rabindra and Jayaraj (1986) and Kennedy and Sathiah (2001) described mass multiplication techniques of HaNPV in detail. The *in vivo* virus production has several advantages like (1) Successful use of viruses produced in the host to control insect pests (Ignoffo and Couch, 1981; Bell, 1991). (2) Research is continuing in this area to produce more efficient systems, which makes this approach an economically viable one. (3) In many areas of the world, virus production in the host is the only approach feasible (Katagiri, 1981; Moscardi *et al*., 1981).

*In vivo* mass production systems have changed little over the past 30 years. The development of semi-synthetic artificial diets by Vanderzant *et al.* (1962) resulted in rearing and virus production systems for the cotton bollworm (*H. zea*), the tobacco bud worm (*H. virescens*) and the cabbage looper [*Trichoplusia ni* (Hubner)] by Ignoffo (1965). The initial rearing system was made more efficient by the introduction of disposable multicelled plastic trays (Ignoffo and Boeing, 1970), automation in rearing and automation in virus inoculation and harvesting. Optimal virus production is the result of interrelationships of host-pathogen-environment and each factor in this triad must be assessed for influence on quantity and quality of the product. Research in these areas has been summarized (Shapiro *et al*., 1986; Shapiro and Bell, 1981, 1982). Basic methods were described in

compact form by Klmakoff and Longworth (1980). A broader and more complete account of some aspects on virus production and role of virus in insect pest control has been given by Burges (1981). Falcon (1976) reviewed the problems associated with commercialization. Subsequent development and industrialization for mass rearing process, improvements in viral recovery procedures and formulation of the viral product made it possible for commercialization of HaNPV (Shieh, 1978). Further, Ignoffo and Couch, 1981 improved the method of mass production of baculovirus of *Helicoverpa* from the laboratory reared *Helicoverpa* larvae through which seven to nine times more active virus and two to five times more polyhedral occlusion bodies (POBs) were obtained from dead and diseased larvae.

Field collection of diseased larvae led to contamination with adventitious agents, which would pose a major problem in terms of safety and quality control, and as such it was not desirable for HaNPV production (Sherman, 1985). Because of the developments of semi-synthetic diet, containerization and automation, laboratory reared insect have been the hosts of choice. The advantages of these insects are i) Laboratory reared insects tend to be larger than field collected insects, because of the selection and adaptation to the laboratory environment (diet, temperature, humidity and photoperiod). ii) They are normally disease free, which should result in virus product that is free from other pathogens. iii) The growth and development of laboratory-reared insects tend to be faster than field insects, because of selection. iv) Virus yield among laboratory-reared insects tends to be greater than among field insects, since virus yield is dependent on host biomass (Hedlund and Yendol, 1974; Shapiro and Bell, 1981).

Although laboratory colonized insects provide several advantages over field insects as virus producers, field insects have also been used successfully to produce NPV from larvae of potato tuber moth (*Phthorimaea operculella* (Zeller)) in Australia (Matthiessen *et al*., 1978), the velvet bean caterpillar (*A. gemmatalis*) in Brazil (Moscardi *et al*., 1981), the European pine sawfly (*N. sertifer*) in

the United States (Rollinson *et al*., 1970) and a CPV from the pine caterpillar (*D. spectabilis*) in Japan (Katagiri, 1981) on natural foliage.

Different methods of mass production of baculoviruses, according to Pawar and Thombre (1992) are: i) large scale rearing of insects in the laboratory, ii) field collection of host larvae from infested crops and infecting them in the laboratory iii) field collection of diseased larvae from infested fields. Large collection of insect viruses at the rate of 20,000 host larvae have been reported from different crops *viz*., cotton, sunflower, pigeonpea, chickpea (Ignoffo, 1966a and 1966b; Anderson *et al*., 1972; Battu, 1992). Battu (1992) reported relatively lower levels of POBs obtained from field collected, diseased and dead insects. The number of larvae required to produce one LE ($6x10^9$ POBs) of virus from field collected larvae were higher (2.97) than laboratory reared ones (2.14) since field collected larvae were of different size unlike the uniform stages in the laboratory reared ones (Gopali and Lingappa, 2001a). At ICRISAT, for effective mass multiplication of *Amal*NPV, the field-collected larvae are released into an aluminum or polythene grid/enclosure (10 cm height) to confine the larvae inside the shaded enclosure and fed with plants already inoculated with the virus. The field technique for rearing larvae is advantageous, particularly in avoiding the handling of huge larval populations, rearing and inoculation. This would also facilitate farm level production and access to the biopesticide at the village level (Rao *et al.,* 2006). The laboratory level mass production technique for *Amal*NPV has been standardized by Veenakumari *et al.* (2006). In situ field level mass production of *Amal*NPV in a groundnut ecosystem was developed first time at Project Directorate Biological Control (PDBC), Bangalore, India (Veenakumari *et al.,* 2007).

## 2.11 Method of Virus Inoculation and POB Yield:

The virus used for the inoculation must confirm the quality control specifications of viral products as reported by Shieh and Bohmfalk, 1980. The inoculation dose is expressed in units of POB/ml, and the optimal dose varies with the virulent virus and age of the host (Ignoffo and Couch, 1981). Angelini and Labonne (1970) suggested that the best method to propagate the virus was to

spray a suspension on larval diet. They could get the larval mortality after seven to eight days. Shapiro and Bell (1981) reported that surface treatment is an efficient system that is easily automated and requires much less virus than diet incorporation. However, Odak *et al.* (1984) used soaked chickpea seeds treated with HaNPV to feed *Helicoverpa* larvae and found that the method was effective for mass production of virus. Bioassays were used to determine the activity of each batch of virus. Several modes of administration of virus were tried using different larval instars *viz*., surface treatment, diet incorporation and direct feeding (Ignoffo, 1966a). Earlier instars were highly susceptible to the virus (Rabindra and Subramanian, 1974) with $LT_{50}$ shorter than older ones. Narayanan (1979) reported that the early instars recorded 100% mortality. Whereas, late instars particularly from fifth instars pupated and gave rise to malformed adults with short and ruffled wings. The effect of NPV was directly related to the age of the larvae at the time of infection (Battu, 1990). Further, Battu (1992) reported that increasing dosages are required to kill the older larvae. The relative resistance of eight days old larvae was 2000 times more than that of one-day-old larvae. Further, he observed that fifth and sixth instar larvae could not be infected with the virus even at higher concentrations. Rabindra and Subramanian (1974) inoculated fourth instar larvae with a dose of $10^6$ POBs/ ml to harvest maximum yield. The $LC_{50}$ values for the first and third instar of *H. armigera* were $8.3\times10^3$ and $28.6\times10^5$ POBs per larva, respectively (Backwad, 1979). Narayanan (1979) found that the optimum dose of inoculum required for obtaining the maximum harvest of virus from the fourth instar larvae was $5\times10^4$POBs/cavity/larva by the diet surface contamination method. Where as, Shieh (1978) used $5\times10^5$ to $5\times10^6$ POBs/ml inoculum in each cavity and observed that there was significant interaction between the age of the larvae and dose of the virus with the recovery of POB.

Taun *et al.* (1989) described the pathogenicity of HaNPV to *H. armigera* using three different inoculation methods. The $LD_{50}$ values of fourth instar larvae that were fed on diet containing NPV or maize kernels soaked in virus suspension were $1.85\times10^6$ and $2.55\times10^5$ POBs per larva, respectively.

The inoculum imbibing method was more sensitive and convenient for inoculating the pest with virus. Whereas, Jayaraj and Sathiah (1993) described three methods of inoculation *viz*., head dipping, oral feeding and diet surface contamination and the latter method was the most economical and convenient for easy application. Ignoffo (1966b) estimated that at least $6 \times 10^9$ virus polyhedra were produced per larva in late instars of *H. zea* and he defined it as "one larval equivalent". The average yield of virus per larva infected after 5 to 7 days at 30°C was $1.5 \times 10^9$ polyhedra (Ignoffo, 1973). Teakle *et al.* (1985) observed that the least yield of $1.18 \times 10^7$ POBs/insect was from younger larvae of *H. armigera* compared to $3.6 \times 10^9$ POBs/insect from grownup larvae. Whereas, Shieh (1978) recovered $5 \times 10^9$ POBs/larva indicating that the yield of POBs was directly related to the age of the infected larvae. The host insect, insect diet, insect age and virus dosage, incubation, environment and preservation of virus infectivity were some of the major factors, which optimize the production of HzNPV (Carter, 1984). The virus yield increased exponentially with the age of larva at dosing in the range of zero to six days, the overall increase being approximately 100 fold (Teakle and Byrne, 1989). Battu (1990) reported an average yield of $1.81 \times 10^9$ POBs per larva of *H. armigera*. Similarly Pawar and Thombre (1992) reported that HaNPV yields per larva ranged from $0.95 \times 10^9$ to $3.5 \times 10^9$. Gopali and Lingappa (2001a) suggested $10^8$ POBs/ml as the optimum dose required for third and fourth instar larvae to achieve quicker and higher mortality of larvae for virus production and among different instars of *H. armigera*, the fourth instar larva was found ideal for virus production as it yielded higher quantity of virus per larva ($2.81 \times 10^9$ POBs).

*In vivo* mass production and control efficacy studies of *S. litura* NPV (SplNPV) were positively correlated with larval weight from 3rd instar to 5th instar larvae, maximum yield of $1.4 \times 10^9$ POBs/ml was obtained with early 5th instar larvae individually infected by diet–incorporation of inoculum of $3x10^6$ POBs/ml for 7 days of incubation at $30^0$C (Tuan *et al.,* 1998). Similarly, a maximum yield of $5.57 \times 10^9$ POBs / larva was obtained at the inoculum dose of 1966.2 POB/ $mm^2$ of *S. litura* NPV when exclusive harvest of cadaver was done (Senthil Kumar *et al.,* 2005). Jun *et al.* (2007) reported that the volume of POBs of SpltMNPV harvested at 5th day of post inoculation period was significantly lower

than that harvested on 7th day of post incubation period, which was significantly lower than that harvested after larval death and similar trend was observed in biological activity by dosing fifth instar larvae. To study the influence of virus inoculation method and host larval age on productivity of the NPV of the teak defoliator, *Hyblaea puera* (Cramer) was determined by different methods of inoculation (Biji *et al.,* 2006a).

## 2.12 Physico-Chemical Properties of the Occlusion bodies (OBs):

The physical and chemical properties of OBs from five NPVs and GVs were studied and compared by Summers and Smith (1976) and out lined as follows:

1) Occlusion bodies are insoluble in hot or cold water, alcohol, ether, chloroform, benzol or acetone.

2) They dissolve in aqueous solutions of NaOH, KOH, $NH_3$, $H_2So_4$ and $CH_3COOH$.

3) They are not digested by proteinases such as papain (pH 8.3), trypsin (pH 6.8) or pepsin (pH 3.3 to 4.0) but by pepsin at pH 2.0 to 2.9 and by trypsin and papain after alkali treatment.

4) They are heavier than water, a characteristic feature which helps to distinguish them from fat droplets which always float on top.

5) They are not destroyed by bacterial putrefaction.

6) The major component of the occlusion body is a single, viral encoded protein of molecular weight 25-33 kDa, called polyhedrin or granulin (Hooft van Iddekinge *et al*., 1983).

7) Occlusion bodies normally band at 54-56% sucrose on 40-65% w/w sucrose gradients at 100,000 g.

8) The buoyant density of ODVs in CsCl is 1.18-1.25 g/$cm^3$, which of BV in sucrose is 1.17-1.18 g/ $cm^3$.

## 2.13 Major Occlusion Body Protein or Matrix Protein (Polyhedrin / Granulin):

The most extensively characterized structural protein of baculoviruses is major occlusion body protein also called matrix protein or polyhedrin / granulin. The important characteristic feature of occluded insect viruses is their ability to produce virions sequesterd (occluded) within this crystalline matrix of occlusion body (Jacques, 1975; Rohrmann, 1986 and Rohrmann, 1992).

This occlusion body protein (Fig 3) stabilizes virions for long time survival and dispersal in the environment and, in the context of biological control, is convenient, safe and simply manipulated product (Blissard and Rohrmann, 1990). Occlusion provides such a selective advantage for insect viruses that it has apparently evolved independently at least three times (Rohrmann, 1992). In addition to baculoviruses, insect viruses from both the Reoviridae (cytoplasmic polyhedrosis viruses) (Payne and Mertens, 1983) and the Poxviridae [entomopox viruses (EPVs)] (Arif, 1984) also occlude their virions in protein matrix. Occlusion in both these virus groups is similar to that of baculoviruses in that the occlusion body protein gene is highly expressed during the very late phase of virus infection (Rohrmann, 1992; Funk *et al.,* 1997), protect the virions out side the host insect, and occluded virions are released by the high pH encountered in the midgut of a susceptible insect. Despite these similarities, there is no evidence of amino acid sequence functional identity between any of the occlusion body proteins from these different virus families has been detected (Arella *et al.,* 1988; Fossiez *et al.,* 1989; Yuen *et al.,* 1990).

For baculoviruses to occlude large numbers of virions efficiently, massive amounts of polyhedrin must be produced during the infectious cycle. This high level transcription of the polyhedrin gene is accomplished by an α-amanitin-resistant RNA polymerase which appears to have a different subunit composition to host RNA polymerase (Yang *et al.,* 1991). Although a hyper expressed gene product, polyhedrin is not necessary for growth of the virus in cell culture. This has been exploited in the development of the baculovirus expression system, in which the

polyhedrin gene is replaced by foreign genes under the control of the polyhedrin promoter (Smith *et al.,* 1983; Pennock *et al.,* 1984). The advantage of this system is that the recombinant baculoviruses express high levels of eukaryotic gene products that are usually folded and processed in a manner similar to the native proteins.

In addition to the current interest in baculoviruses owing to their widespread use as expression vectors, with the advent of genetic engineering, genetically modified baculoviruses have been shown to be capable infecting insects and expressing insect–specific toxin genes, insect hormones or insect enzymes which significantly accelerates the speed with which the virus kills a target insect (Tomalski and Miller, 1991; Stewart *et al.,* 1991; Maeda *et al.,* 1991b, Hammock *et al.,* 1990; Possee *et al.,* 1991; Bishop *et al.,* 1992; Bonning *et al.,* 1992; Bonning and Hammock, 1992; Hammock *et al.,* 1993; Hoover *et al.,* 1996; Harrison and Bonning, 2000; Tuan *et al.,* 2005). The importance of the occlusion body protein for stability and maintenance of infectivity of baculoviruses in the environment has been clearly demonstrated by field tests using polyhedrin deficient AcMNPV mutants (Hamblin *et al.,* 1990; Wood *et al.,* 1994).

## 2.14 Biochemical and Serological Characterization of Polyhedrin / Granulin:

The crystalline matrix of the occlusion body mainly consists of a single protein, called polyhedrin or granulin respectively. These proteins are about 245 amino acids (29 kDa) and hyper expressed during very late phase of virus infection and are not required for virus replication (Rohrmann, 1986; Rohrmann, 1992; Funk *et al.,* 1997) and constituting up to 18% or more of total alkali-soluble protein late in infection (Quant *et al.,* 1984). Summers and Smith (1976) compared the physical and chemical properties of five polyhedrins and granulins using polyacrylamide gel electrophoresis and two-dimensional high voltage electrophoresis of tryptic peptides. It was shown that each of the polyhedrins and granulins has a unique protein for a given virus with similar molecular weights of 28,000 $\pm$ 2000 Daltons. This protein crystal is extremely stable against solubilization by many solvents at neutral pH values and physiological conditions,

and highly resistant against the action of proteolytic enzymes (Bergold, 1947, 1948). The early reports on the proteins dissociated from this crystalline structures by weak alkali carbonate showed that this matrix consists of a heterogeneous mixture of peptides with sedimentation coefficients and estimated molecular weights (by gel electrophoresis) of 11.5S (275,000), 12.7S (336,000) and 12.8S (378,000) for NPV from three different insect species and 11.8S (300,000) for GV (Bergold, 1959; Summers and Egawa, 1973). The presence of multiple protein species also indicated by immunological and amino acid sequence analysis showed that the homogeneous protein in the crystalline structures of a NPV and GV contain at least two different antigenic structures (Longworth *et al.,* 1972; Scott and Young, 1973). These results suggested a complex composition and a possible subunit structure for the matrix. However, the antigenic specificity of a structural subunit or determinant thereof may change, dependent upon the state of aggregation of a protein (Yamaka and Ueta, 1964). Therefore, during the initial characterization of granulin and polyhderin, immunological studies must be correlated with physical and chemical techniques to properly characterize the protein. Early reports showed that the macromolecular structures of the larger protein subunits (approx 12S) could be dissociated by stronger alkaline treatment into subunits which are homogeneous with respect to molecular weights (Bergold, 1948).

Further studies of biochemical and biophysical properties of the solubilized matrix proteins were provided by the discovery that an alkaline protease was associated with the protein matrix of NPVs (Eppstein and Thoma, 1975; Summers and Smith, 1975; Payne, 1978). This protease was activated by the alkaline conditions used to solubilize the matrix and degraded matrix components to a mixture of lower-molecular-weight polypeptides. Several additional baculoviruses have been investigated, and similar protease activities have been detected (Crawford and Kalmakoff, 1977; Eppstein *et al.,* 1975; Kozlov *et al.,* 1975; McCarthy and Liu, 1976; Tweeten *et al.,* 1978). Inhibition of this activity by $HgC1_2$ or by heat treatment at 70°C for 30 min has allowed the matrix to be solubilized and recovered in a non degraded form (Eppstein *et al.,* 1975; Summers and Smith, 1975a; Tweeten *et al.,* 1978). After such treatment, the matrix

exhibits one major 12S component when it is analyzed by velocity sedimentation. These studies have indicated that the 12S molecule consists of eight subunits of granulin or polyhedrin. Comparative studies of granulin and polyhedrin revealed close similarities in molecular weights. All of the baculovirus matrix proteins examined to date have molecular weights in the range from 25,000 to 30,000 Daltons. Some of the previously determined molecular weights of polyhedrins and granulins were listed in Table 4. Electrophoretic and Immunological blotting analysis of polyhedrin proteins from three Japanese strains (K-3, G1-2 and G10-3) of *S. litura* multicapsid NPV (SlMNPV) indicated that the molecular weights of three strains were different (Ikeda *et al.,* 2004). Singh *et al.* (1979) compared the serological properties of polyhedrin protein and virions from four nuclear polyhedrosis viruses of *Plusiine* larvae (Lepidoptera: Noctuidae). Similarly the serological relationships of polyhedrin proteins from five different nuclear polyhedrosis viruses of lepidopterous insect species were compared (Pritchett *et al.,* 1979). Immunochemical studies with the polyhedrins from two *O. pseudotsugata* NPVs and *T. ni* NPV suggest that the 12S aggregate contains one of the major antigenic determinants observed in matrix preparations, the other antigenic site appears to be present in the monomeric polypeptide (Eppstein and Thoma, 1977; Rohrmann, 1977).

The solubilizing effects of various solvents on the proteinic crystalline structure of a granulosis virus of a *T. ni* was determined and further kinetic and morphological studies were conducted in an attempt to evaluate the nature of intermolecular binding forces which contribute to the construction and stability of the structure (Egawa and Summers, 1972; Kawanishi *et al.,* 1972). In addition to similarities in structure and size of matrix proteins from several baculoviruses are similar in amino acid composition. These proteins are characterized by high contents of aspartic and glutamic acid residues. Also prominent are the hydrophobic amino acids valine, isoleucine, and leucine. Comparative peptide mapping studies of granulins and polyhedrins have shown that many common peptides are present in the matrix proteins (Maruniak and Summers, 1978; Summers and Smith, 1975b). As suggested by Rohrmann (1977) and

Maruniak and Summers (1978) these peptides may represent regions of the protein that contribute to its aggregative properties and, thus, have been conserved through evolution. Other peptides are unique to individual matrix proteins and can be used to distinguish viral species.

The granulins from *T. ni* and *P. interpunctella* GV appear to be phosphorylated (Summers and Smith, 1975; Tweeten *et al.,* 1980). Whether this is typical of granulins and polyhedrins is not known. Yamamoto and Tanada (1978) have reported that a 126,000-molecular-weight component containing phospholipid and protein is associated with matrix preparations from the GV of *P. unipuncta*. This component enhanced infection of *P. unipuncta* by an NPV, and it has been suggested that this component is involved in the attachment of enveloped nucleocapsids to midgut cell membranes (Tanada and Watanabe, 1971).

**Table 4: Molecular weights (kDa) of occlusion body (OB) protein (polyhedrin / granulin) estimated in NPVs / GVs of some lepidopteran hosts.**

| *Host species* | **Molecular weight (kDa)** | **Reference** |
|---|---|---|
| **A. Genus Nucleopolyhedroviruses:** | | |
| *Autographa californica* | 30 | Summers and Smith, 1978 |
| *Anticarsia gemmatalis* | 29 | Summers and Smith, 1978 |
| *Epiphyas postvittana* | 28.8 | Hyink *et al.,* 1998 |
| *Helicoverpa armigera* | 28, 32 | Summers and Smith, 1978; Rivkin *et al.,* 1998 |
| *Helicoverpa zea* | 27 | Summers and Smith, 1978 |
| *Helicoverpa assulta* | 29 | Woo *et al.,* 2006 |
| *Lymatria dispar* | 30 | Stiles *et al.,* 1983 |
| *Mamestra configurata* | 31 | Li *et al.,* 1997 |
| *Rachiplusia ou* | 30 | Summers and Smith, 1978 |
| *Spodoptera litura* | 31 | Bansal *et al.,* 1997 |
| *Spodoptera frugiperda* | 32 | Escribano *et al.,* 1999 |
| *Trichoplusia ni* | 31 | Summers and Smith, 1978 |
| **B. Genus Granuloviruses:** | | |
| *Cirphis unipuncta* | 26.3 | Croizier and Croizier, 1977 |
| *L. pomonella* | 28 | Croizier and Croizier, 1977 |
| *Mamestra oleracea* | 26.4 | Croizier and Croizier, 1977 |
| *Pieris brassicae* | 28.2, 27.5 | Croizier and Croizier, 1977; Brown *et al.,* 1977 |
| *Plodia interpunctella* | 28 | Tweeten *et al.,* 1978 |
| *Pseudaletia unipuncta*<br>Hawaiian strain<br>Oregon strain | <br>28.7<br>29.1 | <br>Yamamoto and Tanada, 1978<br>Yamamoto and Tanada, 1978 |
| *Pygera anastomosis* | 26.9 | Croizier and Croizier, 1977 |
| *Spodoptera. frugiperda* | 28, 26 | Summers and Smith 1975a;1978 |
| *Trichoplusia ni* | 28 | Summers and Smith 1975b, 1978 |
| *Zeiraphera diniana* | 27.2 | Croizier and Croizier, 1977 |

## 2.15 Purification of Polyhedrin / Granulin:

Polyhedrin or Granulin is a highly stable protein it is insoluble in many solvents at neutral pH values and physiological conditions, and highly resistant against the action of proteolytic enzymes (Bergold, 1947, 1948). At the same time it is highly sensitive to alkali conditions. A standard protocol has been established by many workers for purification of polyhedrin or granulin, which involves the following common steps:

- Initial heat inactivation of alkaline proteases associated with larva-derived polyhedra.
- Alkali treatment (weak or strong Sodium carbonate buffer) of purified occlusion bodies to solubilize the polyhedrin or granulin.
- The alkali solubilized polyhedrin / granulin is further purified by either ultra centrifugation or isoelectric precipitation methods.

The quality of the polyhedrin preparation depends on the purity of the occlusion body suspensions. Therefore, before extraction of polyhedrin from occlusion bodies it is essential to purify the OBs by isopycnic banding in zonal rotors (Martignoni *et al.,* 1968). In this section, some of the protocols standardized previously for purification of polyhedrin or granulin were reviewed.

Purification of polyhedrin from *T. ni* NPV was standardized by initial heat treatment of gradient purified POBs, followed by incubation of POBs in 0.01M $HgCl_2$ in 0.01 M tris buffer (pH 7.8), dissolution of POBs in dilute alkaline saline (DAS) (0.1 M $Na_2 CO_3$, 0.15 M NaCl, pH 10.9) and then polyhedrin was collected as supernatant by ultra centrifugation of dissolved POBs at 100,000 × g for 30 min (Volkman and Falcon, 1982). Similarly, after initial heat treatment polyhedrin from two *Orgyia pseudotsugata* nucleopolyhedroviruses (OpSNPV and OpMNPV) was purified by dissolution of POBs in 0.1 volume of 1 M $Na_2CO_3$ 0.5 M NaCl buffer at $56^0$C for 10 min followed by centrifugation at 120,000 × g for 45 min (Quant *et al.,* 1984). Summers and

Egava (1973) purified the granulin from *Trichoplusia ni* granulovirus by dissolution of OBs in 0.07 M $Na_2$ $CO_3$ 0.05 M NaCl (pH10.7) at 5mg of OBs/ml for 1.5 to 2.0 h at room temperature then the granulin was clarified by the following two approaches. In first approach the dissolved OB suspension was subjected to centrifugation at 100,000 × g for 30 min. In second approach the dissolved OB suspension was layered on 10 to 40% (wt/vol) sucrose gradients and centrifuged at 25,000 rev/min by use of a SW41 rotor. Similarly, after heat treatment and alkali dissolution of gradient purified OBs of *A. californica, P. dispar, Trichoplusia ni,* and *Heliothis zea* and granulovirus from *T. ni* the polyhedrins or granulins were recovered from the top of sucrose gradients (density range of 1.15 to 1.27 g/ml) after centrifugation at 100,000 × g for 1 h (Smith and Summers, 1981). Brown *et al.* (1977) standardized the purification of granulin from gradient purified granules of *Pieris brassicae* granulovirus by alkaline disruption of granules using 0.1 M $Na_2$ $CO_3$ then virus particles were pelleted at 75,000 × g for 1 h and the supernatant contains mainly the granulin was subjected to iso- electric precipitation (pH 5.6) by slow addition of 0.1 M HCl then the precipitated polyhedrin was collected as sediment by centrifuging at 4000 × g for 20 min. Similarly polyhedrin from three nucleopolyhedroviruses from closely related hosts such as *Spodoptera littoralis, Spodoptera exempta* and *Spodoptera frugiperda* was purified by the alkali (0.1 M $Na_2$ $CO_3$) treatment of polyhedra followed by centrifuged at 70,000 × g for 1 h, supernatant was decanted and adjusted to pH 5.8 with 0.1 N HCl and the precipitated polyhedrin was pelleted at 4000 × g for 20 min (Harrap *et al.,* 1977). Instead of gradient purification, the POBs from *Autographa californica* nucleopolyhedrovirus were extensively washed with 0.1% SDS, virus particles were released by alkali (0.1M $Na_2$ $CO_3$) and centrifuged the dissolved POB suspension at 50,000 × g to pellet the virions then polyhedrin was precipitated from the supernatant by adjusting the pH to 5.8 (Roberts and Naser, 1982). Similarly, the polyhedrin from nucleopolyhedroviruses of *Autographa californica* and *Trichoplusia ni* (Hohmann and Faulkner, 1982) and granulin from *Epinotia aporema* granulovirus (EpapGV) (Parola *et al.,* 2003) were purified by isoelectric precipitation of alkali solubilized occlusion bodies. The putative

polyhedrin protein of monodon baculovirus (MBV) was isolated from infected post larvae by homogenization, differential centrifugation and density gradient centrifugation with verification by transmission electron microscopy (Attaphon *et al.,* 2005).

## 2.16 Production of Antibodies (Polyclonal / Monoclonal) Against Baculovirus Structural Components:

It has been well documented that repeated inoculations of a virus antigen into an animal will elicit a different antibody response than that obtained with a single or few injections of the same antigen (Casals, 1967). The rabbit is most frequently used animal for the preparation of polyclonal antibodies against baculoviruses and can be injected with whole OBs (Shamim *et al.,* 1994) or purified virions (Kelly *et al.,* 1978b and Smith and Summers, 1981), although the OBs are usually solubilized before injection (Crawford *et al.,* 1978). If guinea pigs are used, the OB must be solubilized before injection. To prepare antibodies against purified individual viral structural proteins, most commonly polyhedrin or granulin, purified protein preparations are electrophoresed through PAGE gels and the required bands are eluted individually in to PBS (Barta and Issel, 1978; Summers and Smith, 1975b and Sridhar Kumar *et al.,* 2007). Factors such as antigen purity, variability of antisera, and reaction of antisera with contaminating non viral antigens have led to several problems during standardization of serological assays during standardization of serological assays. To overcome this, monoclonal antibodies are obtained from cloned hybrids produced by the fusion of antigen-stimulated lymphocytes and myeloma cells (Chan and Mitchison, 1982). The purpose of this section is to review the polyclonal or monoclonal antibodies has been produced against the structural components of baculoviruses by researchers around the globe.

Polyclonal antibodies were prepared against the virus particles rather than polyhedrin protein of HaNPV to study the growth kinetics of virus in infected larvae (Kelly *et al.,* 1978b). Purified polyhedrin preparations (80µg) were used for production of monoclonal antibodies as

well as rabbit polyclonal antibodies to diagnose the NPV infection in infected larvae of *T. ni* (Volkman and Falcon, 1982) and in Lymantria dispar (Yu *et al.,* 1992). Quant *et al.* (1984) produced the monoclonal antibodies against purified polyhedrin preparations of two *Orgyia pseudotsugata* Baculoviruses (OpSNPV and OpMNPV). To diagnose the NPV infection, monoclonal antibodies were produced against 42K protein of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Naser and Miltenburger, 1982). The sequence of events in the infection of TN-368-10 and TN-368-13 cells by AcMNPV was investigated by polyclonal antibodies produced against purified AcMNPV larvae-derived occluded virions, highly purified polyhedrin and plasma membrane budded non-occluded virus (Summers *et al.,* 1978). To identify the baculovirus structural proteins that share interspecies antigenic determinants, polyclonal antibodies were produced in New Zealand white rabbits with a solution containing 75µg of intact virions of AcMNPV, *Porthetria dispar* MNPV, *T. ni* GV as well as highly purified polyhedrin of AcMNPV and HzSNPV (Smith and Summers, 1981). Knell *et al.* (1982) prepared six new antisera against SDS-disrupted viruses and additional GVs and studied their reactivity with structural polypeptides of 17 baculoviruses. To study the immunological relatedness of structural proteins of occluded and budded viruses of AcNPV, polyclonal antibodies were raised in New Zealand white rabbits against budded virus (BV), larvae-occluded virus, alkali liberated (LOVAL) and purified polyhedrin protein by injecting 80 µg of each antigen (Volkman, 1982). To detect the NPV infection in *Bombyx mori* larvae, monoclonal antibodies were produced to entire POBs in inbred BALB/c mice by injecting $10^7$/ml of gradient purified POBs (Shamim *et al.,* 1994). Crawford *et al.,* (1978) produced the polyclonal antibodies against *T. ni* SNPV, *T. ni* MNPV, *Euxoa messora* (*EM*) NPV, *Pieris rapae* (*Pr*) granulovirus and *Laspyresia pomonella* granulovirus and *Wiseana* spp. SNPV in New Zealand white rabbits as well as in Swiss white mice by injecting the purified polyhedra (3 ml) at concentration of 500 µg/ml after dissolving in 0.1 volume of 1 M $Na_2CO_3$ and then neutralized with 1M HCl. To identify the conserved epitopes on the polyhedrin protein of *Heliothis zea* nucleopolyhedrovirus, 12 anti-HzSNPV polyhedrin

monoclonal antibodies were produced (Huang *et al.,* 1985). To study the immunological relatedness of polyhedrin purified from nucleopolyhedroviruses of *H. armigera, S. litura* and *S. exigua,* polyclonal antibodies were produced against isolectric precipitates of polyhedrin and their cross- reactivity was evaluated (Tuan *et al.,* 1999). To study the serological relatedness of structural proteins among baculoviruses, monoclonal antibodies were produced against both non occluded virus (NOV) and polyhedrin preparations of *A. californica* (*Ac*) and *Choristoneura fumiferana* (*Cf*) nucleopolyhedroviruses (Hohmann and Faulkner, 1982). Similarly, Roberts and Naser (1982) has produced the monoclonal antibodies against a baculovirus, the AcNPV, and their ability to recognize other baculoviruses has also been tested. Harrap *et al.* (1977) has produced the polyclonal antibodies to purified polyhedra (5mg/ml), isolectric precipitated polyhedrin (5mg/ml), and purified virus particles (500µg /ml) of nucleopolyhedroviruses isolated from three closely related hosts such as *S. littoralis, S. exempta* and *S. frugiperda.*

To develop the diagnostic tools for detection and quantification of baculoviruses from infected larvae at field and laboratory level, to study the biosafety and environmental fate of recombinant and wild type baculoviruses, as well as quality control during mass production of baculovirus based bio pesticides, polyclonal antibodies were produced against isolectric precipitates of polyhedrin or granulin purified from *Epinota aporema* granulovirus (EpapGV) (Parola *et al .,* 2003), recombinant and wild type *Autographa californica* nucleopolyhedrovirus (AcAaIT and AcMNPV) (Ashour *et al.,* 2007), *Ha* NPV (Sridhar Kumar *et al.,* 2007). A synthetic peptide of 25 amino acid sequence (25Pmbv) generated from N-terminal sequence analysis of polyhedrin purified from monodon baculovirus (MBV) was conjugated with bovine serum albumin and used as an antigen for antiserum production in mice (Attaphon *et al.,* 2005).

## 2.17 Applications of Various Serological Tests in Identification, Characterization, Classification and Quantitative Detection of Baculoviruses:

There is clear evidence which suggests that intrinsic differences in baculovirus genomes and structural polypeptides are reflected in serological properties (Harrap *et al.,* 1977). Thus, another promising system for classification of baculoviruses is a system based on antigenicity (Huang *et al.,* 1985). Several immunological techniques in which antibodies formed against matrix proteins, enveloped nucleocapsids, and nucleocapsids are used are being investigated for their sensitivity and specificity in detection of baculovirus antigens. By comparative analyses of the antigenicities of polypeptides from these viral components, structural proteins that may determine virus strain-specific antigens can be identified (Harrap *et al.,* 1977). Of particular interest is the use of this information for developing procedures for reliable identification and monitoring of baculovirus levels in field insects and other environmental samples (Mazzone and Tignor, 1976). In this section the literature related to different immunochemical tools has been developed and evaluated for identification, characterization, classification and quantitative detection of baculoviruses has reviewed.

### 2.17.1 Tube precipitation and gel immunodiffusion:

When antibodies and macromolecular antigens react, they frequently form insoluble complexes (lattices) that visibly precipitate from solution. This phenomenon is the basis of many classic serologic tests. If the reaction occurs in liquid media in test tubes, it is called tube precipitation. If it is done by introducing antigen and antibody into different regions on an agar or agarose gel, allowing them to diffuse toward each other and form a band of precipitate at the junction of their diffusion fronts, it is called gel immunodiffusion.

The monoclonal antibodies produced against AcNPV specifically immunoprecipitated a polypeptide of MW 42 kDa from tissue culture derived extra cellular virus (Roberts and Naser,

1982). Similarly the radio immune precipitation assay was performed with monoclonal antibodies produced against *A. californica* and CfNPV, the antibodies AcV5, AcV6, AcV12 and AcV18 precipitated 12 or 13 [$^{35}$S] methionine-labeled polypeptides from non occluded viruses (NOV). Among those polypeptides, a single peptide of 64K was precipitated by AcV5, AcV6 and AcV18 while 42K polypeptide was specifically precipitated by Ac12 (Hohmann and Faulkner, 1982).

In insect virology, gel immunodiffusion tests have been used for characterization of structural proteins and identification of insect viruses of small, spherical DNA and RNA viruses because they move easily in the gel and antigen concentration usually is not a problem (Harrap and Payne, 1979). The properties of nucleocapsids and polyhedrin protein of a nucleopolyhedrovirus isolated from *Oryctes rhinoceros* and three closely related hosts such as *S. litturalis, S. exempta* and *S. frugperda* were determined by gel immunodiffustion in 1% (w/v) agarose in phosphate-buffered–saline (PBS), pH 7.4 in 60 min glass Petri dishes (Payne *et al.,* 1977 and Harrap *et al.,* 1977). Since precipitation is dependent upon antigen cross–linkage to form insoluble lattices, monoclonal antibodies cannot be used effectively in this procedure if they bind to only one determinant on a monomeric antigen (Yelton and Scharff, 1981). This can be overcome by mixing monoclonal antibodies that are reactive to different sites on an antigen. Huang *et al*. (1985) used this approach to determine whether monoclonal antibodies elicited to HzMNPV polyhedrin reacted with single or multiple epitopes of that molecule, and determined which pairs of monoclonal antibodies reacted with different epitopes, sufficiently separated to avoid antibody steric hindrance.

### 2.17.2 Hemagglutination inhibition:

Many intact viruses or viral–coded proteins have the capacity to agglutinate erythrocytes of certain species (hemagglutination). In some cases, the adsorption of viruses to host cells has been shown to involve the same receptor that mediates hemagglutinating activity (Howe and Lee, 1970). This hemagglutination (HA) reaction can be inhibited if specific antibodies attach to the effector proteins (the hemagglutinins) and prevent their attaching to receptors on the red blood

cells. The hemagglutination inhibition (HI) test has been used extensively for classification and identification of arthropod borne viruses (Clarke and Casals, 1958). With some viruses, the HI test is capable of identification to a subtype, group of strains, or even a strain level, while with other viruses it is useful for classification to the group level only (Casals, 1967). Similarly some insect-restricted viruses have demonstrated hemagglutinating activity (Cunningham *et al.,* 1966; Anderson *et al.,* 1981). Of these, the most extensive testing has been done with baculoviruses, but there is considerable disagreement as hemagglutinating activity. In some instances the polyhedrins have been implicated (Reichelderfer, 1974; Norton and Dicapua, 1975), while in others; enveloped nucleocapsids were found to be responsible (Anderson *et al.,* 1981).

### 2.17.3 Neutralization:

The neutralization test is based on the fact that many viruses are inactivated (neutralized) by antibodies that bind to critical sites, usually on the viral surface (Mandel, 1979). In the neutralization test, the remaining infectious activity is determined after virus exposure to antiserum. If the remaining activity is assessed *in vivo,* the antiserum concentration usually is held constant and the virus concentration is varied; if it is assessed *in vitro,* then the opposite is usually the case (Casals, 1967; Martignoni *et al.,* 1980).

The sensitivity (in terms of amount of antigen required to do the test) is closely associated with the infectious to physical particle ratio of the virus in the host system used. For example, it has been calculated that the physical to infectious particle ratio of the budded phenotype of AcMNPV infecting TN-368 cells *in vitro* is $1.28 \times 10^2$:1, while the ratio for the occluded phenotype (LOVAL) is $2.4 \times 10^5$:1 (Volkman *et al.,* 1976). To perform a neutralization test in this system, starting with 100 plaque forming units (PFU) in the absence of any inactivation, 1.4 ng of budded virus and 2.8 µg of LOVAL is required (Volkman *et al.,* 1976).

The specificity of the neutralization test is based on the “critical site” binding feature. In a sense, cross-reactivity is a measure of shared “critical sites”. Differences in the rate of binding of antibody to cross-reactive critical sites can be measured in kinetic neutralization experiments,

which is useful in discriminating among virus strains. Kelly *et al.* (1979) have used kinetic neutralization experiments to detect strain differences in insect-restricted viruses. It is thought that antibody usually neutralizes the virus by interfering with the initial virus-host cell interaction either directly or indirectly (Mandel, 1979). The neutralization assay can, therefore, be useful in detecting differences in specific interactions of viruses and host cell surfaces. This may be the case with the budded and occluded phenotypes of AcMNPV, which are neutralized by different populations of antibodies *in vitro* (Volkman *et al.,* 1976). It has been known for some time that the budded and occluded phenotypes of subgroup A and B baculoviruses (nuclear polyhedrosis viruses and granulosis viruses) are morphologically different from each other (Summers and Volkman, 1976; Adams *et al.,* 1977).

The neutralizing monoclonal antibodies were elicited to the budded phenotype of AcMNPV, four hybridoma clones produced antibody which neutralized the infectivity of AcMNPV NOV (Hohman and Faulkner, 1982). Subsequently, one neutralizing antibody was shown to bind specifically to envelop surface antigens of the budded virus. That same antibody did not neutralize or bind the occluded phenotype of AcMNPV (Volkman *et al.,* 1984).

### 2.17.4 Radioimmunoassay (RIA):

RIA is a simple theoretical model for detection of antigen-antibody-binding reaction of using radio labeled antibody ($^{125}$I) developed by Klmakoff *et al.* (1977). General predictions from the theory were confirmed by experimental results using a wide range of antigen-antibody systems. It was found that the greatest sensitivity of the RIA is achieved when the smallest amount of labeled antibody is used, and that whenever possible the antigen-antibody ratio should be greater than unity (Klmakoff *et al.,* 1977).

RIA provides a cheap and simple procedure that permits quantitation of low levels of baculovirus in infected larvae and in environment and to discriminate among distantly and closely related NPVs and GVs (Crawford *et al.,* 1978; Klmakoff *et al.,* 1977; Ohba *et al.,* 1977; Rohrmann, 1977). The sensitivity and cross-reaction of four solid-phase radioimmunoassays for

*T. ni* nuclear polyhedrosis virus containing singly enveloped virions were investigated (Crawford, *et al.,* 1978). Immunological comparisons were made to detect similar antigenic determinants among the structural polypeptides of NPVs and GVs using protein blot RIA (Summers and Hoops, 1980; Smith and Summers, 1981, Knell *et al.,* 1982).

### 2.17.5 Enzyme linked immunosorbent assay (ELISA):

In principle, ELISA and RIA are precisely the same, the ELISA makes use of an enzyme labeled (usually alkaline protease or penicillinase or peroxidase) antigen or antibody to signal the occurrence of an antigen-antibody reaction, further more the antigen does not have to be infectious for the assay to work as it does for neutralization (Weir, 1978 and Voller *et al.,* 1979, 1982). ELISA has been used more extensively than RIA in insect virology, probably because of expense and safety considerations involved in using $^{125}I$, its relatively short half life (60 days), and the cost of a gamma counter. Crook and Payne (1980) compared the three methods of ELISA for baculoviruses and agreed that the most sensitive assay for antigen detection in the absence of plentiful extraneous matter was the indirect test and in the presence of plentiful extraneous matter was the sandwich test.

### 2.17.5.1 The Indirect ELISA:

The applications of indirect ELISA in insect virology include host range studies, assessment of antigen purification techniques, and viral relatedness determinations. Some examples of these are given below. Indirect ELISA has been used as a sensitive test for viral antigen production in non permissive cells. Rubenstein *et al.* (1982) used it to detect *Estigmene acrea* granulosis virus antigen increase when fat body cells in culture were exposed to the virus. An increase in AcMNPV antigens in AcMNPV-exposed codling moth cells (cell line Cp 169) was detected using indirect ELISA (Langridge *et al.,* 1981a). Langridge *et al.* (1981b) also used the indirect ELISA when they repeated a published study to determine whether alkali liberated virus of the occluded phenotype of a NPV could be separated completely from polyhedrin by

sequential sucrose gradient and sepharose column purification techniques and concluded that even highly purified virions had elicited a considerable antibody titer to polyhedrin, indicating that they, in fact were not free of the protein (Bell and Orlob, 1977). Crook (1981) found he was able to discriminate between the granulosis virus of *Pieris brassicae* and *Pieris rapae*, using indirect ELISA. Brown *et al.,* (1982) reported using a variation of the indirect ELISA wherein enzyme-linked protein A was substituted for the second antibody in a study demonstrating antigenic relatedness of four baculoviruses from *Spodoptera* species. Roberts and Naser (1982) used monoclonal antibodies to probe for differences between *in vivo* and *in vitro* generated AcMNPV polyhedrin using direct and indirect ELISA. Hohmann and Faulkener (1982) used Indirect-ELISA to characterize the monoclonal antibodies produced against AcNPV and CfNPV were found to cross-react differently with polyhedrins and granulins from several species of baculoviruses. The relationship between three nucleopolyhedroviruses isolated from the larvae of *H. armigera, S. exigua* and *S. litura* in Taiwan was determined by assaying the polyhedrin in indirect ELISA with polyclonal antipolyhedrin antisera specific to each polyhedrin (Tuan *et al.,* 1999). A monoclonal antibody based indirect ELISA was developed and used for the differentiation of OpMNPV and OpSNPV and also for identification of their homologous polyhedrin in larval extracts (Quant, *et al.,* 1984). By using monoclonal antibodies in indirect ELISA, it was possible to detect virus antigens in NPV infected *H. armigera* and *Choristoneura fumiferana* larvae at about 6-9 hours after virus exposure, whereas disease symptoms of the larvae could only be observed after 5-6 days (Zang and Kaupp, 1988; Lu *et al.,* 1995). Similarly, the polyclonal antibody based indirect ELISA was developed for detection of HaNPV polyhedrin in infected larval extracts (Sridhar Kumar *et al.,* 2007). Alternatively, a monoclonal antibody against the 42K protein of AcMNPV was used in indirect ELISA for virus detection in dead larvae and for safety investigation (Naser and Miltenburger, 1982). An indirect competitive (IC) ELISA was developed and validated with various molecular methods to detect nucleopolyhedroviruses in larvae of the Douglas-Fir Tussock Moth *Orgyia pseudotsugata*

(Thorne *et al.,* 2007). Similarly, an IC-ELISA was standardized to evaluate the biosaftey of recombinant and wild type of nucleopolyhedroviruses of *A. californica* (Ashour *et al.,* 2007).

### 2.17.5.2 The Sandwich ELISA:

Sandwich ELISA is generally not as sensitive as the indirect ELISA, it can be used to detect antigen in a preparation which is highly contaminated with host tissue components. It is therefore, the method of choice for virus detection in crude virus extracts. The direct sandwich ELISA was very effectively used by Morris *et al.* (1981) to quantitatively monitor the degree of contamination of AcMNPV preparations with a small RNA virus, TRV. Kelly *et al.* (1978a) found that the direct sandwich ELISA was an effective tool for discriminating among purified preparations of five small iridescent viruses. In another study, Kelly *et al.* (1978b) used the same assay for detecting *Ha* NPV in *H. armiger* larval extracts. They reported a sensitivity of 1 ng virus per ml of extract. Langridge *et al.* (1981a) used the direct sandwich ELISA to monitor possible AcMNPV antigen increase in *L. dispar* larvae fed AcMNPV occlusion bodies but found no evidence for viral activity. An indirect sandwich ELISA assay has been established and used for monitoring the presence of *O. rhinocerous* baculovirus in field populations (Longworth and Carey, 1980). Payment *et al.*(1982) developed an indirect sandwich ELISA for *Euxoa scandens* cytoplasmic polyhedrosis virus that is sensitive enough to detect 10ng virus per ml of larval extract. Volkman and Falcon (1982) examined the possibility of using a monoclonal antibody as one component of an indirect sandwich ELISA for the detection of *T. ni* SNPV in infected *T. ni* larvae. They determined that the assay worked well if the monoclonal antibody was used as primary antibody, but not if it was used as the secondary antibody. With an antiserum against the polyhedrin component of the NPV of *Mamestra brassicae*, it was possible to detect polyhedra at a concentration of $2.44 \times 10^4$ polyhedra/ml by means of indirect sandwich ELISA (Riechenbacher and Schliephake, 1988). Similarly, polyhedrin specific monoclonal antibody based double antibody sandwich ELISA was developed for detection of *Lymantria dispar* MNPV and *Borrelina bombycis* NPV in infected host larvae or cultured insect cells (Ma *et al.,* 1984; Yu *et*

*al.,* 1992; Shamim *et al.,* 1994). As part of quality control of the production of a bioinsecticide based on *Epinotia aporema* granulovirus (EpapGV), a sensitive double antibody sandwich ELISA was developed for detection and quantification of the virus using polyclonal antibodies produced against granulin (Parola *et al.,* 2003).

### 2.17.6 Western blots:

Western blotting is a variation of immunoelectrophoresis that combines SDS-PAGE and RIA or ELISA can be used to determine which viral structural proteins are involved in serologic cross-reactions and it is a powerful tool for understanding the basis of serologic groupings of complex insect viruses (Towbin *et al.,* 1979). Smith and Summers (1981) demonstrated the power of western blotting by comparing the antigenic relatedness of 17 different species of baculoviruses from lepidopteran hosts. Knell *et al.* (1983) expanded these studies to find additional common antigenic determinants among different baculovirus sub groups. Smith and Summers (1981) also used the western blotting technique to compare the antigenic relatedness of the occluded and budded phenotypes of AcMNPV that they found to be considerably different. Volkman (1983) explored this further by doing reciprocal western blots of the two phenotypes. Roberts and Naser (1982) and (1983) used western blotting as a method of determining which of the AcMNPV structural proteins were reactive with monoclonal antibodies elicited to that virus. Furthermore, Naser and Miltenburger (1982) explored the possibility of using western blots in conjunction with a specific monoclonal antibody for the identification of AcMNPV. Similarly, western blotting was used to screen the monoclonal antibodies produced against the polyhedra of nuclear polyhedrosis virus infecting *Bombyx mori* larvae (Shamim *et al.,* 1994) and polyclonal antibodies produced against polyhedrin of HaNPV (Sridhar Kumar *et al.,* 2007).Western blotting was used to diagnose the NPV infection in the field larvae of *Orgyia pseudotsugata* by detecting the polyhedrin levels at various stages of infection in insects days post inoculation (Quant *et al.,* 1984).

### 2.17.7 Immunofluorescent and immunoperoxidase staining:

The first immunoassay developed by tagging an antibody with a substance to signal its reaction with an antigen was the fluorescent antibody (FA) technique. Coons *et al.* (1942) determined that fluorescein isothiocyanate (FITC) could be used to label antibodies without destroying their specificity. The FA technique has long been used for viral identification and for determining the location of viral antigens in infected cells during the course of replication (Casals, 1967; Schmidt and Lennette, 1973). Since the development of FA, immunocytochemical techniques have expanded to overcome some of the inherent limitations in using fluorescein as the signal, such as the requirement for a special microscope, signal fading, and incompatibility with histological staining operations (Volkman, 1982). One very successful method of overcoming these limitations, in additions to achieving much greater sensitivity and specificity, is the use of horseradish peroxidase-labeled antibodies. In addition to the usual direct and indirect strategies of staining used with FA, three and four-layered peroxidase anti-peroxidase (PAP) techniques have been developed which have enhanced sensitivity significantly (Naritoku and Taylor, 1982). Both immunofluorescent and immunoperoxidase staining procedures have been used to detect insect viruses. Krywienczyk (1963) and Shamim *et al.* (1994) used immunofluorescence to detect an NPV in *Bombyx mori*, and Kurstak and Kurstak, 1974 reported the use of immunoperoxidase to detect infections of *Tipula* iridescent virus and densonucleosis virus in *Galleria mellonella.* The indirect immunoperoxidase technique was used to monitor time course studies of AcMNPV in cell culture (Summers *et al.,* 1978). Peroxidase anti-peroxidase staining has been used to develop a quantitative assay for AcMNPV, for studying the kinetics of viral replication in single cells and to determine the location of viral antigens (Volkman and Goldsmith, 1981, 1982; Volkman, 1983). An extensive host range study of AcMNPV in vertebrate cell lines was conducted using the PAP technique to monitor intracellular increase in viral antigen (Volkman and Goldsmith, 1983). Recently, the indirect immunofluorescence was

used for evaluation of antigen-antibody reactivity on the surface of proteinaceous occlusion body towards application in reusable protein chip (Yoshikawa *et al.,* 2006).

### 2.17.8 Immunoprecipitation and immunoaffinity chromatography:

Immunoprecipitation is a widely used method for identifying the specificities of monoclonal antibodies (Yewdell and Gerhard, 1981). It can also be used for identification of viral antigens when specificity of the antisera is known. Immunoprecipitation has been used in insect virology to identify *in vitro* translated protein products of AcMNPV mRNA (Vlak *et al.,* 1981; Adang and Miller, 1982). In addition, it was used to identify the specificity of monoclonal antibodies generated to AvMNPV- (Roberts and Naser, 1982; Volkman *et al.,* 1984). Immunoaffinity chromatography is a technique that will become more and more popular as monoclonal antibodies gain wider usage. Roberts and Naser (1982) used to show specific binding of some of their monoclonal antibodies with AcMNPV polyhedrin. Similarly, Shamim *et al.,* (1994) used immunoaffinity chromatography to screen the monoclonal antibodies produced against polyhedra of *B. mori* NPV.

## 2.18 Need for the Molecular Level Identification and Characterization of Baculoviruses:

Molecular level identification, characterization and evaluation of phylogenetic status of a particular baculovirus are also important for establishment of purity of seed stock or master stock. Apart from the multiple or singly enveloped feature, NPV or GV cannot be identified visually from either light or electron microscopic studies. Microscopic and serological tools are unreliable for establishing the real identity of a given isolate and is not particularly helpful in providing clues about its host range and infectivity (Rovesti *et al.,* 2000). To identify viruses beyond the grouping in to GV or NPV we need to look at the DNA sequence using restriction endonuclease analysis or molecular probes, offers a relatively simple method for identification and differentiation of baculoviruses (Smith and Summers, 1978).

Examination of the DNA using these techniques has shown that many variants of a species may exist for example the MNPVs from *A. californica, T. ni, S. exempta, R. ou, Anagrpha falcifera* and *Galleria mellonella* can be considered to be variants of the same virus (Miller and Dawes, 1978; Smith and summers 1979; Summers *et al.,* 1980; Brown *et al.,* 1984, 1985; Harrison and Bonning, 1999). Many of the known baculoviruses could be grouped together depending on their degree of genetic relatedness, which does not reflect the taxonomic grouping of their host/hosts (Zanotto *et al.,* 1993). Among the NPVs with potential as pest control agents, the MNPVs isolated from *M. brassicae* (Lepidoptera: Noctuidae) and *H. armigera* (Lepidoptera: Noctuidae) were shown to be similar in terms of both biological activity and genomic homology (Smith and Summers, 1982; Figueiredo *et al.,* 1999; Rovesti *et al.,* 2000).

For identification of a particular NPV strain, the bioassay studies and restriction endonuclease profiles of different NPV species have been studied and compared by several investigators (Shapiro and Ignoffo, 1970; Odak and Rawat, 1982; Hughes *et al.,* 1983; William and Payne, 1984; Rabindra, 1992; Arora *et. al.,* 1997; Somasekhar *et.al.,* 1993; Geetha and Rabindra, 1999; Sudhakar and Madhavan 1999; Figueiredo *et. al.,* 1999; Rovesti *et.al.,* 2000).The existence of genetic variants with different biological activities may have important implications for development of biopesticides both in the possibility to select better naturally occurring strains and as a source material for genetic manipulation (Guo *et. al.,* 2006).

## 2.19 Identification and Molecular Characterization of Polyhedrin / Granulin Gene:

Polyhedrin / granulin is the major component of occlusion body (OB) and as often been studied. After the first report about localization of the polyhedron gene in AcNPV (Vlak and Smith, 1982; Hoofft van Iddekimge *et al.,* 1983) determined its nucleotide sequences. Polyhedrin / granulin is a protein of about 245 to 250 amino acids, and appear to be the most highly conserved baculovirus protein. These characteristics lead to the use of polyhedron or granulin sequences as the

base of baculovirus phylogenetic studies (Zanotto *et al.,* 1993). In this section, the literature has reviewed related to the baculovirus OB protein (polyhedron or granulin) gene has been identified and characterized previously by several workers.

A restriction digested fragment obtained from genome of the *Bombyx mori* nucleopolyhedrovirus contains the gene coding the viral occlusion body protein (polyhedrin) has been cloned, sequenced in its entirely together with some of its 5'and 3' flanking sequences and the primary structure of polyhedrin protein predicted from the nucleotide sequence of the gene was found to be somewhat different from the one previously reported (Latrou *et al.,* 1985). Polyhedrin gene of *Anticarsia gemmatalis* multiple nucleocapsid nucleopolyhedrovirus (AgMNPV) was identified in 2085 base pair fragment obtained by restriction digestion of its genome with *Sph*I-*Pst*I restriction endonucleases was cloned and sequenced, and the amino acid sequence obtained agreed with that deduced from the DNA coding region (Zanotto *et al.,* 1992). Similarly, the nucleotide sequence of granulin gene of the *Pieris brassicae* grnulovirus (Chakerian and Nesson, 1985) and polyhedrin gene of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus (Cowan *et al.,* 1994) was determined. The polyhedrin gene of SlMNPV was identified and characterized in the *Hind* III-F fragment of the viral DNA. The nucleotide sequence of the 1057 base pair (bp) region of this fragment contains an open reading frame without any intervening sequences for coding a polypeptide of 246 amino acids (Bansal *et al.,* 1997). The polyhedrin gene of a NPV isolated from bertha army worm, *Mamestra configurata* was identified from physical map constructed by digesting the genome with six restriction endonucleases, which has by convention been used as the zero point of REN maps of NPV, was determined by hybridizing the *A. californica* multicapsid nuclepolyhedrovirus HindIII-V fragment clone, which contains most of the polyhedrin gene, with genomic blots of MacoNPV (Li *et al.,* 1997). Similarly, the polyhedron gene region of a multinucleocapsid nuclear polyhedrosis virus (MNPV) isolated from the celery looper, *Anagrapha falcifera* was identified by hybridizing with the AcMNPV EcoRI-I fragment (Fedirici and Hice,

1997). The polyhedrin gene of an Israei *H. armigera* single nucleopolyhedrovirus was identified and characterized from restriction fragment; the nucleotide sequence encoded for a polypeptide of 246 amino acids and coincided with previously published HaNPV polyhedrin gene sequences (Rivkin *et al.,* 1998). Hyink *et al.* (1998) characterized the genome of a NPV isolated from the New Zealand light brown apple moth, *Epiphyas postvittana* using a strategy of single-stranded sequencing of the termini of restriction endonuclease fragment clones was employed to map the virus genome and the mapping was completed with Southern blotting and restriction analysis, polyhedrin gene has been fully sequenced and an ORF of 738 bp encodes a predicted protein of 28.8 kDa. As part of an effort to characterize the New Zealand's endemic Wiseana SNPV genome the polyhedrin gene was cloned and the nucleotide sequence was determined and the gene sequence was used, in conjunction with morphological and restriction endonuclease analysis to compare isolates from different sites and species of Wiseana (Sadler *et al.,* 1998). The 7.8 kb *Eco*RI-G fragment of *Rachiplusia ou* multicapsid nuclepolyhedrovirus (RoMNPV), containing the polyhedrin gene was cloned and sequenced, the nucleotide sequence and predicted amino acid sequence was used along with *Eco*RI and *Hind*III restriction profiles and bioassay studies to compare with other MNPVs (Harrison and Bonning, 1999).

A polymerase chain reaction (PCR) based detection system was developed for identification of polyhedrin gene in multiple nucleopolyhedroviruses by using a degenerate primers designed by comparing the polyhedrin amino acid sequences of twenty-six NPVs (Woo, 2001). Similarly, PCR based RFLP was developed for rapid identification and differentiation of *HaNPV* isolated from the environment by amplifying the 400 bp fragment from polyhedrin gene using degenerate primes (Christian *et al.,* 2001). A baculovirus was identified from *Lonomia obliqua* (Lepidoptera: saturniidae) is a pest of medical importance due to a potent toxin found in their spines, its molecular characterization was carried out by the identification of polyhedrin gene through constructing a partial genomic library with DNA fragments generated with EcoRI and its

DNA sequence was determined (Wolff *et al.,* 2002). The molecular characteristics of three Japanese strains (K-3, G1-2 and G10-3) of *S. litura* multicapsid nucleopolyhedrovirus was determined by restriction endonuclease analysis followed by cloning and sequencing of the polyhedrin gene fragment indicated that the three strains had different deduced amino acid sequences and molecular weights (Ikeda *et al.,* 2004). PCR with complementary primers to the polyhedrin gene region was used to diagnose *Bombyx mori* nucleopolyhedrovirus (BmNPV) infection, the PCR products were sequenced and the specificity of the amplification was confirmed by comparison with BmNPV polyhedrin sequences available in GenBank (Ikuno *et al.,* 2004). Woo *et al.* (2005) characterized the polyhedrin gene of Korean strain (SlNPV-K1) of *SlNPV* by the amplification of polyhedrin gene using a degenerate PCR primers, the sequencing results showed that the about 430 bp PCR product was a fragment of corresponding polyhedrin gene and further used it as a probe in Southern blot analysis of SlNPV-K1 restriction fragments.

The molecular characterization of a nucleopolyhedrovirus (SpliMNPV-Az) was isolated from diseased larvae of *S. littoralis*, at the island of *S. Miguel* in Azores, was carried out by restriction endonuclease analysis of the viral genome and further complete codons sequencing of the polyhedrin gene resulted in 750 bp fragment and was identical to the sequence of a SpliMNPV previously published (Martins *et al.,* 2005). Molecular characterization of a baculovirus (LyxyMNPV) isolated from infected larvae the casuarina moth, *Lymantria xylina Swinehoe* (Lepidoptera: Lymantriidae) through restriction endonuclease (*Bam*HI, *Eco*RI and *Eco*RV) analysis and the polyhedrin gene located in the *Bam*HI-D, *Eco*RI-C and *Eco*RV-K fragments was sequenced and furthermore, a rapid PCR-RFLP method was developed to distinguish LyxyMNPV from LdMNPV polyhedrin genes (Wu and Wang, 2005). The molecular cloning and characterization of *Antheraea mylitta* cytoplasmic polyhedrosis virus (AmCPV) polyhedrin gene was done by converting the segments 10 (S10) of the 11 double stranded RNA genomes to cDNA, cloned and sequenced (Uma *et al.,* 2005). Molecular characterization of a multicapsid nucleopolyhedrovirus

isolated from satin moth *Leucoma salicis* (Lepidoptera: Lymantriidae) was done by the amplification of conserved baculovirus conserved genes such as polyhedrin, lef-8 and pif-2 using degenerate primers and the resulting PCR products were cloned and sequenced (Jakubowska *et al.,* 2005). The polyhedrin gene sequence of a Korean strain of *H. assulta* nucleopolyhedrovirus was identified by the initial amplification of partial (430 bp) polyhedrin gene using degenerate primers and the resulting PCR product was cloned and sequenced, the sequenced polyhedrin (430 bp) fragment was used to probe the Southern blots to identify the location of the complete polyhedrin gene with in 6 kb *Eco*RI, 15 kb *Nco*I, 20 kb *Xho*I, 17 kb *Bgl*II and 3 kb *Cla*I fragments respectively and the fragment containing polyhedrin gene was cloned and sequenced. The open reading frame of HasNPV polyhedrin showed that 735 nucleotides which could encode 245 amino acids with predicted molecular mass of 29 kDa (Woo *et al.,* 2006).

The polyhedrin gene of a nucleopolyhedrovirus isolated from the diseased larvae of *Orgyia ericae* Germar was firstly analysed by cloning and sequencing of the restriction digested fragments of its genome (Yang *et al.,* 2006). First time the molecular characterization of a nucleopolyhedrovirus isolated from tea looper caterpillar, *Ectropis oblique* was done by sequencing the restriction digested fragments of *Eco*RI-L, *Eco*RI-N and *Hind*III-F and the location of potential ORF for polyhedrin gene was identified and compared the polyhedrin gene with previously published sequences (Ma *et al.,* 2006). The nucleopolyhedrovirus was firstly isolated from legume pod borer (LPB), *Maruca vitrata*, at Tainan in Taiwan and its complete polyhedrin gene was identified by initial amplification of partial polyhedrin gene (530 bp) using degenerate primers and the resulting PCR product was cloned and sequenced, then two internal primers within the partial sequence of polyhedrin gene were synthesized and used for extending the sequenced regions from the termini successively by using genomic DNA as a template then cloned and sequenced the complete polyhedrin gene (Lee *et al.,* 2007). The partial polyhedrin gene (405 bp) of an Egyptian

isolate of *Sl*NPV was successfully amplified by PCR using degenerate primes and subsequently, this DNA segment was cloned and sequenced (Seufi, 2008, unpublished).

## 2.20 Evaluation of Phylogenetic Status of Baculoviruses Based on Polyhedrin / Granulin Gene Sequences:

Investigation of phylogenetic relationships between baculoviruses and their hosts has provide potential clues about the understanding of biological adaptations such as virus- host specificity, but only broad aspects of virus-host associations have so far been identified (Brooks and McLennan, 1991; Zanotto *et al.,* 1993). Host range is of practical importance for pest control, since these characteristics are natural barriers that determine the use of these viruses and are relevant to safety assessment of genetically modified viruses. The methodology for phylogenetic estimation is undergoing refinement, but no one particular inference method has emerged which is superior for estimating phylogenies (Nei, 1996). Most baculovirus phylogenies consider estimations from tree construction algorithms of occlusion body protein (polyhedrin/granulin) sequences. The earlier polyhedrin / granulin based phylogenetic studies established that lepidopteran NPVs evolved from a common lepidopteran NPV ancestor rather than cross-infecting from different orders of arthropods (Rohrmann *et al.,* 1981). Studies by Zanotto *et al.* (1993) have revealed that prior to the divergence of the two major clades of lepidopteran NPVs (namely, Groups I and II) a clade comprising GVs diverged early in the evolution of the lepidopteran NPVs (Rohrmann, 1992; Zanotto *et al.,* 1993). Some of the representatives of Groups I and II are listed in Table 6 used for this study (Cowan *et al.,* 1994; Zanotto *et al.,* 1993). In this section, the phylogenetic status of some baculoviruses has been estimated previously based on polyhedrin /granulin gene sequences are reviewed as follows:

Comparison of primary structures of polyhedrin genes of nucleopolyhedroviruses of *B. mori* with that of *A. californica* suggest that considerable selective pressure has been exercised at the protein level during evolution and the nucleotide sequence comparisons of the two structural

genes reveal that the coding sequence have diverged significantly through the accumulation of silent and replacement substitutions. In contrast, a remarkable degree of sequence conservation was found to exist in the domains corresponding to the 5' and 3' non coding regions of the polyhedrin mRNAs (Latrou *et al.,* 1985). *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) and *Orgyia pseudotsugat*a MNPV (OpMNPV) are similar in terms of promoter structure and polyhedrin primary sequence, the polyhedrin gene of both viruses is transcribed in the anti-clockwise direction in relation to their physical maps and the region upstream from the polyhedrin gene of AgMNPV, OpMNPV, Bm NPV and AcMNPV was compared and this showed that the ORF common to all four viruses (Zanotto *et al.,* 1992). Analysis of the nucleotide sequence and deduced amino acid sequence of polyhedrin gene of a characteristically distinct SltMNPV indicate that this has more than 70% sequence identity to known polyhedrins, the coding region is preceded by an AT rich region containing the conserved late promoter motif TAAG and the upstream promoter and coding regions of this polyhedrin gene are more similar to polyhedrin of the NPVs of *S. frugiperda*, *S. exigua* and *Panolis flamea* (Bansal *et al.,* 1997). The sequence analysis of *M. configurata* nucleopolyhedrovirus polyhedrin gene showed that the ORF coding for a 246 amino acid polypeptide with 98.7% sequence identity with *Panolis flammea* nucleopolyhedrovirus (PaflNPV) polyhedrin protein and the putative polyhedrin gene sequence had 97.2% and 91.2% identity with the PaflNPV and *Mamestra brassicae* multicapsid nucleopolyhedrovirus (MabrMNPV) polyhedrin gene sequences respectively (Li *et al.,* 1997). Organization and molecular characterization of genes (p78, tyrosine phosphatase, protein kinase, lef-2 and ORF 327, 453 and 603) in polyhedrin gene region of *Anagrapha falcifera* multicapsid NPV (AfMNPV) showed that nucleotide sequence identity of 97% and amino acid sequence identity of greater than 98% with AcMNPV but the polyhedrin gene showed least relatedness between the two viruses, with a nucleotide sequence identity greater than 80% and deduced amino acid sequence identity of 90% , based on these results they concluded that the AfMPNV should be considered as a variant of the AcMNPV (Federici and Hice, 1997). Rivikin *et al.,*

(1998) found that the polyhedrin gene of an Israeli *H. armigera* single nucleopolyhedrovirus was 99.4% of homology to the *H. zea* NPV polyhedrin. Similarly, the phylogenetic relationship of *Epiphyas postvittana* nucleopolyhedrovirus with 23 other NPVs based on polyhedrin gene sequences shows EppoNPV to be a group I NPV and is very closely related to *Orgyia pesudotsugata* MNPV (Hyink *et al.,* 1998). The extent of divergence between the nucleotide sequence of polyhedrin gene of a New Zealand *Wiseana* SNPV isolate and other separate *Wiseana* species was small enough, however, to consider three SNPVs from *W. signata, W. cervinata* and *W. umbraculata* as different strains of a single SNPV species. In addition, the phylogenetic relatedness of this virus to 16 other NPVs from diverse insect genera suggests that the Wiseana SNPV was unique with in the baculoviridae, but was more closely related to the group II NPVs (Sadler *et al.,* 1998). The predicted amino acid sequence of RoMNPV polyhedrin shared more sequence identity with the polyhedrin of *Orgyia pesudotsugata* MNPV, in addition, the RoMNPV polyhedrin nucleotide sequence was almost completely identical (99.9%) to the previously published polyhedrin gene of *Anagrapha falcifera* MNPV (AfMNPV) (Harrison and Bonning, 1999). Phylogenetic analysis of the polyhedrin gene showed that the *Lonomia obliqua* MNPV (LoobMNPV) polyhedrin belongs to group I NPV and that it is closely related to the polyhedrin of the NPV of *Amsacta albistriga* (Wolff *et al.,* 2002). The nucleic acid sequence homology of the polyhedrin gene of Japanese SlMNPV is as high as 98.9% compared with Chinese strain, and 61.7% to 74.2% with other six NPVs compared (Ikeda *et al.,* 2004). The complete codon sequence of SpliMNPV-Az polyhedrin gene sequence was compared with other 38 polyhedrin genes from NPVs and with 6 granulin genes from GVs and resulted to be identical to the sequence of SpliMNPV previously published, thus indicating that natural host of SpliMNPV-Az must be *S. littoralis* (Martins *et al.,* 2005). Similarly, the phylogenetic analysis of polyhedrin gene of a nucleopolyhedrovirus isolated from *Lymantria xylina* multiple nucleopolyhedrovirus (LyxyMNPV) showed that LyxyMNPV is closely related to the *Lymantria dispar* MNPV (LdMNPV) (Wu and Wang, 2005). The nucleotide sequence comparison of

European *Leicoma Salicis* NPV (LesaNPV) polyhedrin gene with other published polyhedrin gene sequenced conformed the close relationship between LesaMNPV and OpMNPV (Jakubowska *et al.,* 2005). Similarly, the phylogenetic analysis of the polyhedrin gene sequenced from *Helicoverpa assulta* nucleopolyhedrovirus (HasNPV) showed that the polyhedrin gene of HasNPV shared 73.7% identity with the polyhedrin gene from AcNPV but were most closely related to *Helicoverpa* and *Heliothis* species NPVs with over 99% sequence identity (Woo *et al.,* 2006). The phylogenetic analysis of NPV isolated from *Orgyia ericae* Germer using polyhedrin gene sequence revealed that *O.ericae* NPV (OeNPV) was a member of the group II NPVs and was closely related to the *Buzura suppressaria* SNPV (BusuSNPV) and OpSNPV cluster (Li *et al.,* 2006). The phylogenetic analysis based on polyhedrin along with other conserved gene sequences of *Ectropis obliqua* single-nucleocapsid nucleopolyhedrovirus (EcobSNPV) indicated that this virus is closely related to the *Spodoptera exigua* multicapsid NPV (SeMNPV) and belongs to the previously described group II NPV (Ma *et al.,* 2006).

# CHAPTER - III

# *Materials & Methods*

**CHAPTER - III**

## MATERIALS AND METHODS

## Isolation and Propagation of NPVs from Major Lepidopteran Pests of Legume Crops in the Semi-Arid Tropics:

During natural epizootic conditions nucleopolyhedrovirus (NPV) symptoms were observed in the following lepidopteran pest populations on legume crops at ICRISAT farms.

1. *Helicoverpa armigera* on pigeonpea and chickpea crops,
2. *Spodoptera litura* on groundnut and
3. *Amsacta albistriga* on groundnut

The diseased larvae with fresh symptoms were carefully collected in to 2ml micro-centrifuge tubes and shipped to the laboratory for further studies.

### 3.1.1 Extraction of polyhedral occlusion bodies (POBs):

The viral occlusion bodies (OBs) were extracted from individual diseased larvae collected from fields with slight modifications to the method described by Christian *et al.* (2001). To each cadaver 1ml sterile distilled water was added. Then the cadaver was disrupted by vortexing for 60 - 120s and extract was filtered through glass wool. The glass wool was washed with 500µl of sterile distilled water and the filtrate centrifuged at 15000 × g for 5min. The supernatant was removed carefully; pellet was washed with 2ml of distilled water and centrifuged as described above. The pellet was resuspended in 1ml of sterile distilled water and stored at $4^0$C. The occlusion bodies were enumerated using Neubauer's haemocytometer mounted on a phase–contrast light microscope.

### 3.1.2 Mass multiplication:

Mass multiplication of HaNPV, SlNPV and AmalNPV were standardized at ICRISAT-NPV production laboratory. For mass multiplication, 4th instar larvae of *H. armigera* (Gopali and Lingappa, 2001a), late 4th instar or 5th instar larvae of *S. litura* (Jun *et al.,* 2007) and *A. albistriga* (Veenakumari *et al.,* 2006) were inoculated with virus. The method of virus inoculation and larval rearing procedures were detailed below:

### 3.1.2.1 *Ha*NPV mass multiplication:

*Ha*NPV was mass multiplied on laboratory reared as well as field collected healthy larvae as detailed below.

#### 3.1.2.1.1 Field collection of *H. armigera* larvae:

During 2006, third and fourth instar larvae of *H. armigera* were collected on various crops like pigeonpea, chickpea, pearl millet and occasionally on the weed, *Lagascea mollis* Cav. (during the off season). Collection was done in multicavity cell well trays (containing 50 cells) (size: 25x12 $cm^2$). Cell wells were kept ready one day before larval collection after sterilizing with 1% clorex solution, exposure to UV light.

Small pieces of semi-synthetic diet or soaked chickpea seeds were provided as diet in each cell well. From this collection uniform sized fourth instar larvae were selected and transferred to virus inoculation laboratory which is situated 2km away from rearing laboratory (insectary) for the multiplication of NPV on field collected *H. armigera* larvae.

#### 3.1.2.1.2 Establishment of laboratory culture of *H. armigera*:

Late fifth instar larvae were selected from the field collection and transferred on to semi-synthetic diet (Appendix I) in sterilized multicavity trays. The diet was supplemented regularly to ensure sufficient food and proper care was taken for sanitation. The pupating larvae were handled with care to minimize the disturbance. On pupation, pupae were surface sterilized in 1% clorex solution and washed with distilled water. Later they were transferred into plastic jars with vermiculate for adult emergence. After 7 to 8 days adults were separated based on sex and released into oviposition cages (45cm length: 30cm diameter) in 1:2 ratio of males to females. Adults were provided with 10% honey solution and vitamin stock solution as food using cotton swabs placed in a small plastic dish, which was placed at the bottom of plastic jar. Oviposition cages were provided with nappy liners for egg laying and they were regularly checked and replaced daily from day three to death of moths. Liners loaded with eggs were sterilized by dipping in 0.1% sodium hypochlorite solution. Liners were allowed to dry and then placed in clean sterilized plastic cups finely coated with a layer of artificial diet. The larvae emerged from eggs were reared together till second instar stage, and later they were transferred into individual sterilized cell wells provided with artificial diet to avoid cannibalism. Larvae were regularly monitored for their growth. Life cycle of *H. armigera* was shown in Fig 6**.** Once they attained the desired size i.e. late third instar they were used for the multiplication of virus. Before inoculation, larvae were weighed and average weight was calculated.

### 3.1.2.1.3 Virus multiplication:

Insects beginning to molt out of the third instar stage, determined by head-capsule slippage, were transferred to cell well trays with out diet for 16-24h. Artificial diet, which was prepared and poured in plastic trays prior to the inoculation was cut into pieces of 1 $cm^2$ and 0.5 cm thick were placed in virus inoculation cell well trays. Virus inoculation was done by the surface contamination method as given by Evans and Shapiro (1997). Fifty µl of virus suspension containing $10^8$ POBs/ml was dispensed over the surface of diet in the cell wells and spread

uniformly over the surface using blunt end of glass rod and allowed it to dry for 10-20min. Larvae were transferred to virus-infected diet and they were observed daily for mortality. Mortality of larvae started from 4$^{th}$ day and it continued up to 8-10 days. NPV was multiplied on 300 larvae in 6 batches with 50 4$^{th}$ instar larvae in each batch. After larval death, the cadavers were collected, in jars containing distilled water and stored at 4$^0$C for further processing.

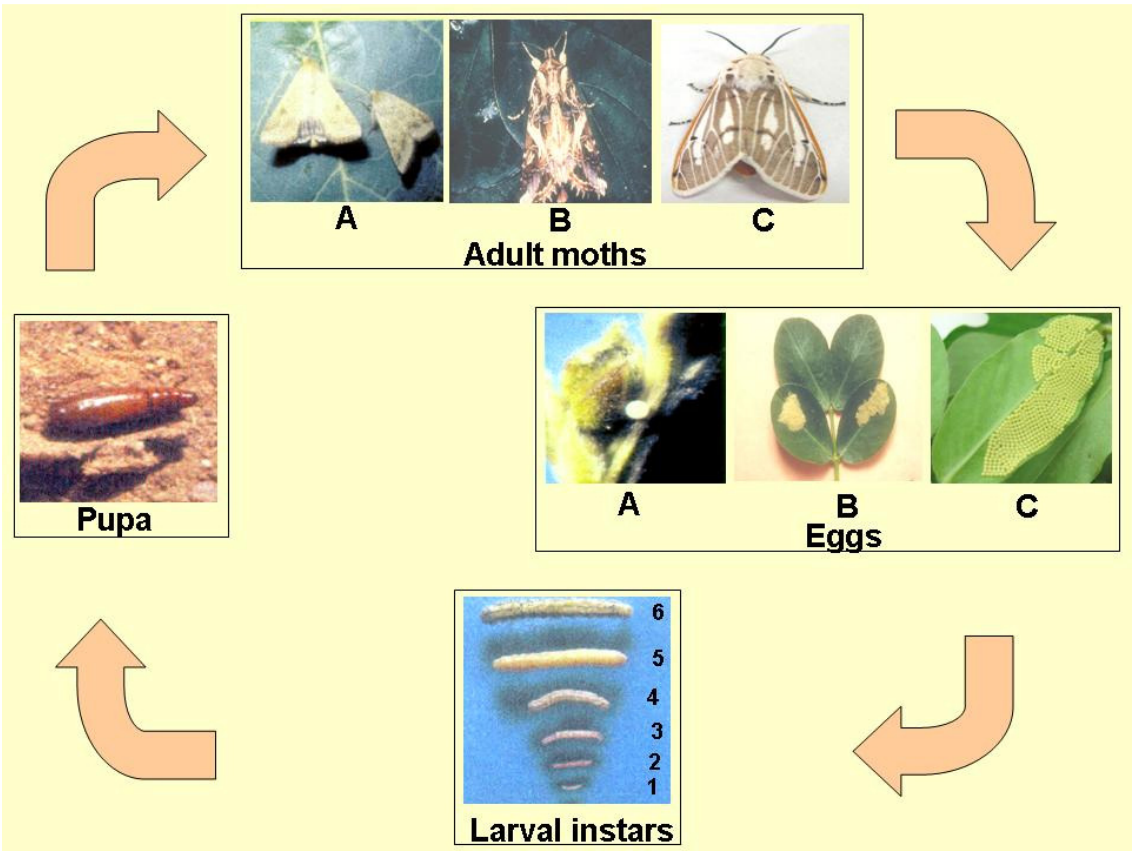
### 3.1.2.2 SlNPV mass multiplication:

SlNPV was mass multiplied on laboratory reared healthy larvae as detailed below.

#### 3.1.2.2.1 Establishment of laboratory culture of *S. litura:*

Initially, the *Spodoptera* culture was established from field collected egg masses at ICRISAT center. The egg masses collected from groundnut fields were disinfected with 1% clorax solution and pinned in jars containing artificial diet (Appendix II). After the 4$^{th}$ instar the culture was thinned and continued on fresh diet until pupation. On pupation, pupae were surface sterilized in 1% clorax solution and washed with distilled water. Later they were transferred into plastic jars with vermiculate for adult emergence. After 7 to 8 days adults were separated based on sex and released into ovipositional cages (45cm length: 30cm diameter) in 1:2 ratio of males to females. Adults were provided with 10% honey solution and vitamin stock solution as adult diet using cotton swabs placed in a small plastic dish, which was placed at the bottom of plastic jar. Ovipositional cages were provided with filter papers for egg laying and they were regularly checked and replaced daily from day three to death of moths. Filter papers loaded with egg masses were separated and pinned in jars containing artificial diet. In this process, the culture was continued under laboratory conditions. Various stages in the life cycle of *S. litura* were shown in Fig 6.

**Figure 6: Life cycle of *Helicoverpa armigera* (Hubner), *Spodoptera litura* (Fabricious) and *Amsacta albistriga* (Walker)**




Various stages in the life cycle of *H. armigera* (**A**) *S. litura* (**B**) and *A. albistriga* (**C**). The adult moths and their oviposition behaviour were clearly represented. The larval instars and pupal stages were commonly represented for three insects.

### 3.1.2.2.2 Virus multiplication:

For multiplication of virus, healthy late 4th instar larvae from the culture was shifted to plastic tubs (30 cm diameter, 10 cm height) with a thin layer of artificial diet. After shifting the larvae to virus production laboratory, the diet was treated with virus inoculum [previously preserved in the laboratory @ 0.25 LE per tub or $10^8$POBs/ml, this rate maximizes the POB yield (Okada, 1977)] and insect rearing was continued. Since, *Spodoptera* has very low cannibalistic behavior, 200-250 larvae were reared in each tub until the disease symptoms appeared (mostly from 7th day onwards). After the larval death, the cadavers were collected and processed for virus as explained in case of HaNPV.

### 3.1.2.3. AmalNPV mass multiplication:

AmalNPV was mass multiplied on laboratory reared as well as field collected healthy larvae as detailed below.

#### 3.1.2.3.1 Virus production from laboratory reared larvae:

Amsacta cultures were established in the laboratory from the field collected egg masses during early June from cowpea fields meant for green manure in ICRISAT farm. These egg masses were disinfected and kept for hatching. After egg hatch, the neonates were transferred on to groundnut leaves kept as bouquets in small wooden cages (50 × 30 × 30 cms). Enough foliage was maintained in the cages to provide sufficient food for the larvae particularly, as the larval stage advanced. Various stages in the life cycle of *A. albistriga* were shown in Fig 6. In order to produce NPV, some larvae were shifted to virus production unit, where the foliage was infected with previously collected virus. About 1 LE inoculum was used to cover 1000 larvae for virus production. The foliage was sprayed with virus for couple of times after larvae attained late 4th instar or early 5th instar. Larval infection was observed a week after inoculation and the dead

larvae were collected from the cage in distilled water and processed for virus as in case of HaNPV.

#### 3.1.2.3.2: Virus production from field collected larvae:

Since this species has one to two generations under field conditions, and they come as epidemic form in some locations, production of virus from field-collected larvae seems highly feasible, as laboratory rearing is not feasible due to its diapause behavior. Several thousands of above $4^{th}$ instar larvae were collected from groundnut fields at ICRISAT during the month of August, and were placed in big cages with ample plant material such as castor, sorghum as feed. The plant material in the insect rearing cages was treated with virus incoulum in order to infect and produce large quantities of virus from field-collected larvae. As the larvae start dying mostly a week after releasing in the cages, the dead larvae were collected in distilled water and processed for virus. Since *Amsacta* larvae has no cannibalism, about 1000 larvae were reared in 1 sq. m. cage area. Sowing of host plants 15 days before shifting the larvae can facilitate easy larval rearing and avoids replacing food with fresh plants. However, virus production from field-collected larvae cannot provide good proofing to prevent contamination from other viruses and bio-control agents.

## 3.1.3 Harvesting of POBs:

After collecting all dead larvae in distilled water, the larvae were ground in a blender. Suspension containing POBs was collected and filtered through the four layers of muslin cloth or plastic strainer to remove larval debris. Virus suspension centrifuged at 5000rpm for 10 to 15min (Remi R8C). POBs were collected as sediment at the bottom of the tube (Fig 7), which was dissolved in distilled water and stored at $4^0$C for further studies.

### 3.1.4 Enumeration, Dilution and Counting of POBs:

Enumeration of polyhedra in the viral suspension was done with the help of Neubauer's haemocytometer, which comprised a glass slide carrying calibrations in two replicates (Evans and Shapiro, 1997). One ml virus suspension was made up to 1000 ml by serial dilution, 1000 was taken as dilution factor. For counting of polyhedra, haemocytometer was used. Diluted virus suspension of 1 ml containing 0.1% teepol was drawn into pipette. Then the pipette was shaken vigorously closing the rubber tube tightly. The first three drops were discarded and the fourth drop was put in the groove of the haemocytometer and then standard cover slip was placed over the slide. Care was taken to see that the haemocytometer was clean and only required quantity of suspension was put to fill the calibrated area. After allowing polyhedra to settle down for two minutes, the polyhedra count was taken in 80 squares of the 1/400 sq. mm area at random with the help of stereomicroscope under 10 × 40 magnification. Proper care was taken to avoid duplication of counts of polyhedra on the lines of calibrations. This was done by counting polyhedra inside the squares as well as the polyhedra on the top and left sidelines only. Virus suspension of known concentration was prepared from the stock solution by suitable dilution with distilled water for various experiments. Concentration of stock solution was expressed as POB/ml which was calculated by using the following equation.

$$\text{Number of POBs/ml} = \frac{D \times X}{N \times K}$$

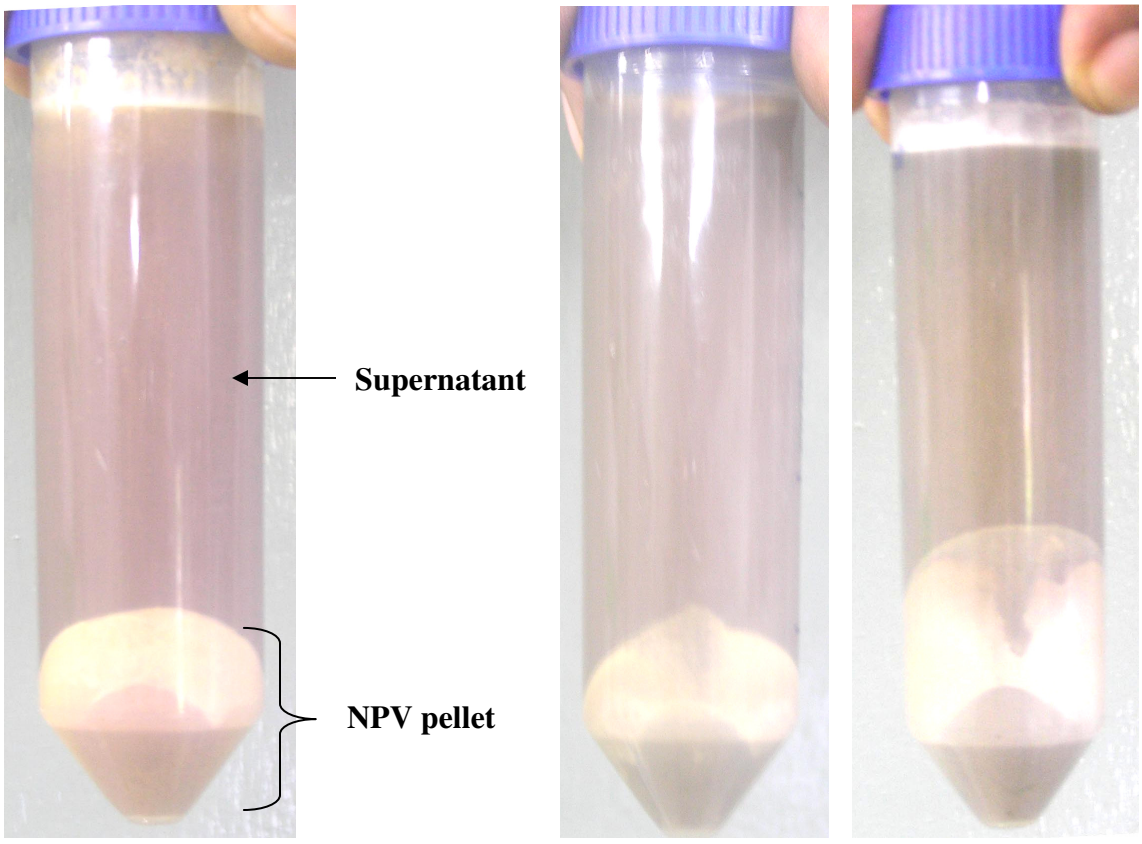
Where:

D= Dilution factor,

X= Total number of POBs counted,

N= Number of squares counted and K= Constant ($2.5 \times 10^{-7}$).

**Figure 7: Sedimented polyhedra at the bottom of tube after centrifugation in Remi R8C**




Centrifugation of virus-infected larval extracts at 5000 rpm in Remi-R8C resulted in sedimentation of POBs at the bottom of the tube. The Pellets of HaNPV (**A**) and SlNPV (**B**) were smaller than AmalNPN (**C**) subsequently the number of POBs also higher in C than A and B

**Working example:**

After centrifuge, 1 ml of the solution was diluted 1000 times and POBs counted in 100 squares was 421, then

D= 1000,

X= 421,

N= 100 and

K= $2.5 \times 10^{-7}$

$$\text{Thus POBs per ml} = \frac{1000 \times 421}{100 \times 2.5 \times 10^{-7}} = \frac{10 \times 421}{2.5 \times 10^{-7}} = \frac{4210}{2.5 \times 10^{-7}}$$

$$= \frac{1684}{10^{-7}}$$

$$= 1684 \times 10^{7} \text{ POBs/ml}$$

**Calculation of larval equivalent (LE):**

1 LE is equal to $6 \times 10^{9}$ POBs

$1684 \times 10^{7}$ POBs/ml is equal to:

$$= \frac{1684 \times 10^{7}}{6 \times 10^{9}}$$

$$= 2.806 \text{ LE/ml}$$

## 3.2 Electron Microscopic (EM) Studies:

To study the morphology (external and internal) of NPVs isolated from *H. armigera, S. litura* and *A. albistriga,* the purified POBs were studied by electron microscope (EM). The external morphology was studied by observing the POBs in scanning electron microscope (SEM) while the internal morphology was studied by the observation of cross sections of pellets of purified POBs in transmission electron microscope (TEM).

### 3.2.1 Scanning electron microscopic (SEM) studies:

The purified POB suspensions were transferred to vials and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 hr at $4^0$C and post fixed in 2% aqueous osmium tetroxide in the same buffer for 2 hr. After fixation, samples were dehydrated in a series of graded alcohol and dried to critical point drying. Dried samples were mounted over the stubs with double-sided conductivity tape. Finally, applied a thin layer of gold metal over the sample using an automated sputter coater (Model: JEOL-JFC 1600) for about 3 min. Then scanned the samples in scanning electron microscope (Model: JOEL-JSM 5600, JAPAN) at various magnifications. The sizes of the OBs were measured directly from the amplified photograph using a scale and dividing the value by the magnification of the photograph.

### 3.2.2 Transmission electron microscopic (TEM) studies:

Pellets of purified POBs were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 hr at $4^0$C and post fixed in 0.5% aqueous osmium tetroxide in the same buffer for 2 hr. After the post fixation samples were dehydrated in a series of graded alcohol, infiltrated and embedded in Araldite 6005 resin. Ultra thin sections (50-70 nm thickness) were cut with a glass knife on a Leica Ultra cut UCT-GA-D/E-1/00 ultramicrotome and mounted on grids. Then sections were stained with saturated aqueous uranyl acetate and counter stained with 4% lead citrate. Observed under TEM (Model: Hitachi, H-7500 from JAPAN) at various magnifications and at 80 KV current. The sizes of the OBs and nucleocapsids were measured directly from the amplified photographs using a precision ruler and dividing the value by the magnification of the photograph.

## 3.3 Purification of Polyhedral Protein (Polyhedrin) of NPVs:

Purification of polyhedrin from POB suspensions was standardized as per the protocols given by Quant *et al.* (1984), Harrap *et al.* (1977) and Summers and Egawa (1973) with slight modifications. The protocol steps were presented schematically in Fig 8.

### 3.3.1 Standardization of purification protocol:

- POB suspension was centrifuged at 5,000 rpm for 20 minutes in a Sorvall HB4 rotor. Supernatant was discarded and the pellets were resuspended in sterile distilled water and adjusted its concentration to $10^9$ POBs/ml.
- The aqueous POB suspension was subjected to heat-treatment at $70^0$C for 20-30min to inactivate the endogenous alkaline proteases.
- Then dissolved the POBs by adding 0.1 volume of dissolution buffer containing 1M $Na_2CO_3$-0.5M NaCl and incubated at $56^0$C for 15-20min in a water bath with occasional shaking.
- After dissolution of POBs, the suspension was cooled to $4^0$C and centrifuged at 120, 000 × g (26,000 rpm in a Beckman SW-28 rotor) for 60min to pellet virions and undissolved POBs.
- The supernatant containing predominantly the polyhedrin was further purified by two different approaches. The purity and yield of protein obtained in both the approaches was compared.

**First approach:**

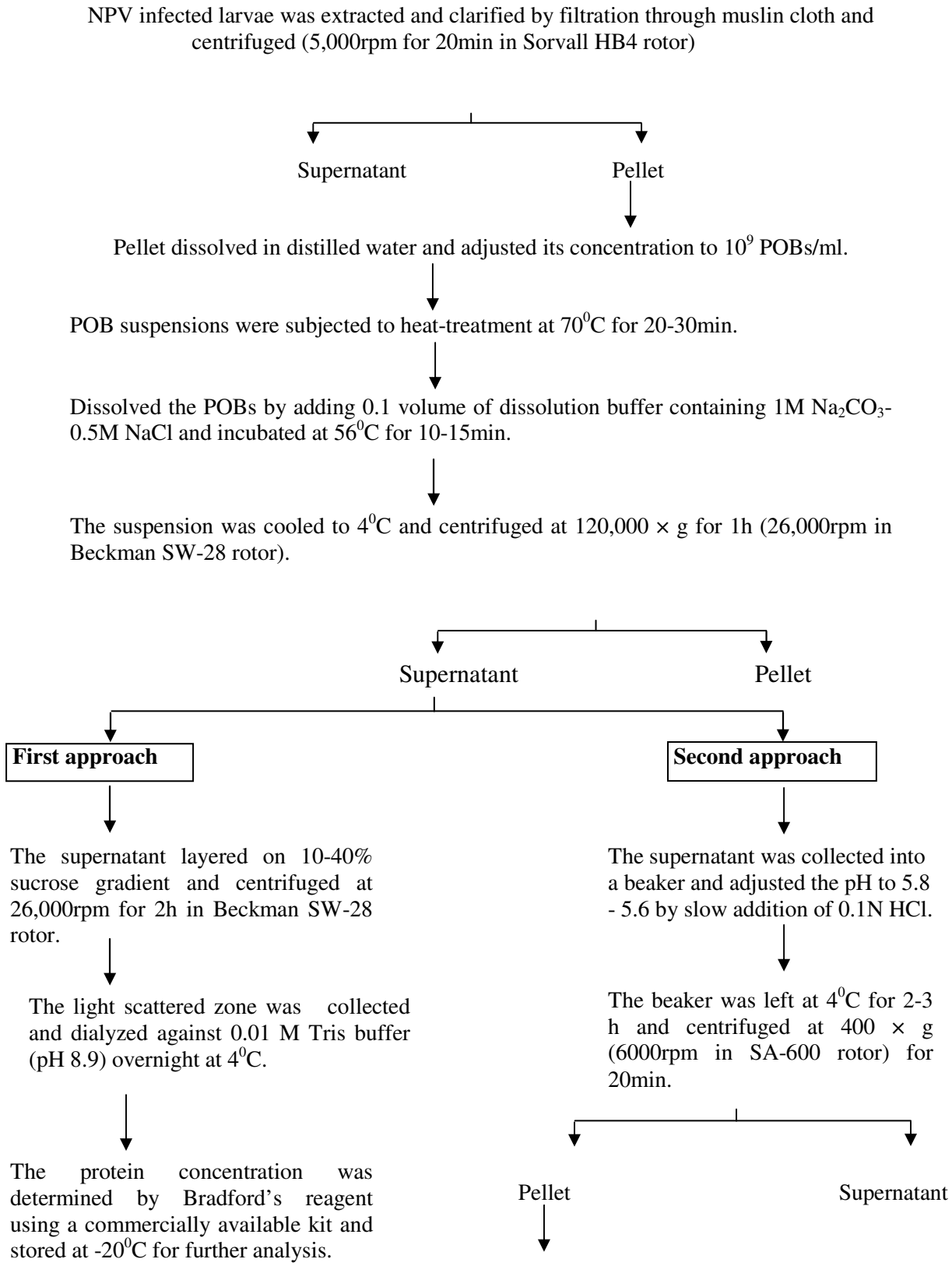
- The supernatant was collected and the remaining polyhedral remnants were cleared by layering on 10-40% sucrose gradient and centrifuged at 26,000 rpm for 2h in a Beckman SW-28 rotor.
- The light scattered zone was collected and dialyzed against 0.01M Tris buffer (pH 8.9) overnight at $4^0$C. The protein concentration was determined by Bradford's

reagent using a commercially available kit. The polyhedrin protein preparations were stored at -20$^0$C for further analysis.

**Second approach:**

- The supernatant was collected into a beaker and adjusted the pH to 5.8 - 5.6 by slow addition of 0.1N HCl. The polyhedrin is insoluble at this pH (isoelectric point of polyhedrin) and forms the precipitate.
- Then the beaker was left at 4$^0$C for 2-3h before centrifuging at 400 × g (6000 rpm in a SA-600 rotor) for 20 min and the polyhedrin pellet was resuspended in sterile distilled water.
- The pelleting step was repeated for 3-4 times and the final pellet was dissolved in same volume of sterile double distilled water and dialyzed against 0.01M Tris buffer (pH 8.9) overnight at 4$^0$C. The protein concentration was determined by Bradford's reagent using a commercially available kit. The polyhedrin preparations were stored at -20$^0$C for further analysis.

**Figure 8: Schematic representation of polyhedrin purification protocol**



The pellet was resuspended in sterile distilled water. The pelleting was repeated for four times and the final pellet was dissolved in same volume of sterile distilled water and dialyzed against 0.01M Tris buffer (pH 8.9) overnight at $4^0$C.

The protein concentration was determined by Bradford's reagent using a commercially available kit and stored at $-20^0$C for further analysis.

### 3.3.2 SDS–PAGE analysis of polyhedrin preparations:

The purity and integrity of polyhedrin preparations were analyzed in 12% SDS-PAGE gels as described in Kumar and Waliyar (2007). Purified polyhedrin samples at concentration about 100-200µg/10µl were assayed by separating them in polyacrylamide (PAGE) gels. The purified protein samples were mixed with equal volume of Laemmli buffer (Appendix III) and denatured by heat treatment in boiling water-bath for 3min. Samples (10µl) were loaded into wells of 12% SDS-PAGE (discontinuous gel, composition given in Appendix III) and electrophoresed at 100 volts for approximately 2 h in Broviga® apparatus. The gel was taken out from the apparatus and silver stained to visualize the proteins as detailed in Kumar *et al*. (2004) as given below:

- The gel was placed in fixative solution for 30min at room temperature with gentle shaking and solution was discarded.
- Then the gel was washed for three times (10min each wash) in distilled water.
- The gel was placed in DTT solution for 30min and then it was rinsed with distilled water.
- The gel was placed in silver nitrate solution for 30min with gentle shaking and then rinsed with distilled water.
- Then the gel was placed in developer solution till the bands appeared clearly.
- Reaction was stopped using stopper solution [1% (v/v) sodium acetate].

The molecular weights of the protein bands were estimated by comparing with the protein molecular weight standards (MBI Fermentas Cat# SM0441). Standard graph was prepared by plotting the distance migrated by protein standards on X-axis and molecular weights on Y-axis. The molecular weights of viral proteins were calculated from the standard graph by plotting the distance migrated by the viral proteins. Standard graph was prepared for each PAGE and average molecular weight calculated from three graphs was taken as molecular weight of the viral protein.

### 3.3.3 Electro-elution of polyhedrin:

In order to improve the quality of the polyhedrin protein preparation for immunization purpose to produce polyclonal antibodies, the polyhedrin protein (31 kDa molecular weight, Figs 21 and 23) was electro-eluted from the 10% SDS-PAGE gel as given in Harlow and Lane (1998) and the procedure was detailed below:

- The isoelectric precipitated polyhedrin sample (2ml) was mixed with equal volume of Laemmli buffer and denatured by heat treatment in boiling water-bath for 3min.
- Sample (4ml) was loaded into 10% SDS-PAGE maxi gel (discontinuous gel casted with 2mm thickness single welled comb (gel composition given in Appendix III) and electrophoresed at 100 volts for approximately 4-5h in Broviga ® apparatus.
- The gel was taken out from the apparatus and $1/4^{th}$ of the gel was separated longitudinally from one edge with the help of glass rod and blade. The remaining $3/4^{th}$ of the gel was placed in a glass tray having TE buffer (pH 7.8) and kept at $4^0$C until staining of the separated piece.
- The separated gel piece was stained for 1h with Comassie brilliant blue R250 at room temperature.

- After distaining, both stained and unstained parts of the gel were placed side by side on a glass plate. The exact portion corresponding to the polyhedrin protein band in unstained gel was separated.
- The separated gel (polyhedrin band) piece was made in to further small pieces and transferred in to a dialysis bag, which was already filled with TE buffer (pH 7.8). The dialysis bag was tagged properly and placed in SDS-PAGE tank buffer in horizontal electrophoresis unit and electrophoresed at 100 volts for 3-4h.
- The polyhedrin protein eluted in to dialysis bag was collected and protein concentration was determined specrophotometrically through Bradford method.
- The quality of the eluted polyhedrin protein was checked in 12% SDS-PAGE and stored as aliquots at -20$^0$C for further analysis.
- Before immunization the protein sample was concentrated up to required volume by evaporating the sample in speed vacuumed evaporator for 3-4h.

## 3.4 Production of Polyclonal Antibodies Against Polyhedrin:

The New Zealand White inbreed rabbits reared at the institute animal facility house were immunized intramuscularly (im) with 500 µg of electro-eluted polyhedrin per animal emulsified with complete Freund's adjuvant (CFA). Five injections were given at weekly intervals, and two weeks after the 5$^{th}$ injection, animals were bled for polyclonal antiserum (Harlow and Lane, 1998). Animals were boosted (im) after 5 weeks with the same dose of antigen emulsified in incomplete Freund's adjuvant (IFA). Animals were boosted at the time of decline in antibody titers. After the 5$^{th}$ injection, blood was collected from rabbits by making an incision in the marginal vein of the ear. The blood was allowed to clot at room temperature for 2-3 h or by exposed to 37$^0$C for 30min. After overnight refrigeration, the serum was collected with a Pasteur pipette and then centrifuged at 5,000rpm for 10min. The antisera were stored at 4$^0$C by adding 0.02% Sodium azide or in lyophilized form at -20$^0$C.

### 3.4.1 Monitoring antibody titers:

The titers of antisera produced against NPVs were determined by direct antigen coating (DAC) enzyme-linked immunosorbant assay (ELISA) as described in Kumar and Waliyar (2007) as detailed below:

#### 3.4.1.1 Direct antigen coating (DAC) enzyme-linked immunosorbant assay (ELISA):

Polyclonal antibodies were screened for the reactivity with polyhedrin protein of nucleopolyhedroviruses by DAC-ELISA. The reagents and buffers used for ELISA were given in Appendix IV. The polyhedrin (electro-eluted) purified from HaNPV, SlNPV and AmalNPV was used as coating antigen. Briefly, polyvinyl microtitration plates (Nunc MaxiSorb, Denmark) were coated with 100µl/well of polyhedrin (1µg/ml) diluted in coating buffer (50 mM Sodium carbonate/bicarbonate, pH 9.5) and incubated the plate in a humid chamber for 2h at $37^0$C or in a refrigerator ($4^0$C) overnight. After coating, the buffer was decanted gently and washed the plate with 3-4 changes of PBS-T, allowing 3min for each wash to remove unbound coating antigen, then serial dilutions of antiserum in PBS-T (1:500 to 1:128,000) were added at 100 µl/well, followed by incubation at $37^0$C for 1h or in a refrigerator ($4^0$C) overnight. Duplicates were maintained for each dilution of antiserum and assayed together 2 to 3 bleeds per plate. The plates were again washed 3-4 times with PBS-T. To detect the antigen-antibody complex goat anti-rabbit IgG-alkalinephosphatase (ALP)-labelled conjugate (Sigma, USA) diluted in PBS-T (1:4,000) was added at 100µl/well and incubated at $37^0$C for 1h. After another 3-4 times of washing phosphatase activity was measured by adding 100µl/well of Para-nitrophenyl phosphate substrate at 0.5mg/ml in 10% (v/v) diethanolamine buffer, pH 9.8 and incubated in dark place, at room temperature. The absorbance readings were recorded by an ELISA reader (Titertek Multiskan, Labsystems, Finland) at a single wavelength of 405nm after 1h of substrate reaction time. Maximum binding and background binding antibody concentrations were measured whether neither analyte nor antibody were added to the system.

## 3.5 Characterization of Polyhedrin-Polyclonal Antibodies:

Polyclonal antibodies produced against the polyhedrin of three NPVs were characterized by determining the specificity with their respective polyhedrins and cross-reactivity with heterologous polyhedrins from other NPVs.

### 3.5.1 Determination of Specificity of polyhedrin polyclonal antibodies by western immuno- blotting:

The specificity of polyhedrin polyclonal antibodies were determined by Western immunoblotting analysis of isoelectric precipitated polyhedrins (IPP), entire POB particle proteins (EPP), electro–eluted polyhedrin (EP) and healthy larval proteins (HLP) as described in Kumar and Waliyar (2007) as detailed below:

The reagents and buffers used for western immunoblotting were given in Appendex-V. Protein samples (10µl of each sample) were electrophoretically separated in 12% PAGE as described in section 3.3.2. Following SDS-PAGE, the separated proteins were electrophoretically transferred to nitrocellulose membrane. Transfer was performed at 200mA (constant current) using TE-22 mini transfer apparatus (Hoefer Scientific) and performed for 2-3h. After transfer, the nitrocellulose membrane was blocked with non-fat mild powder (eg. Nestle or Everyday) at 5% (w/v) in PBS-T by incubating the plate at room temperature with gentle shaking. Then the membrane was washed with PBS-T, thrice for 5 min each and incubated the membrane at room temperature for 1h in antibody buffer containing respective NPV- polyhedrin polyclonal antiserum at 1:5000 (v/v) dilution. After washing, the antigen-antibody reaction was visualized by colorimetric reaction by incubating the membrane at room temperature for 1 h in antibody buffer containing ALP-labelled anti-rabbit IgG at 1:4000 (v/v) dilution. After washing, the membrane was incubated with BCIP-NBT substrate solution in dark chamber at room temperature. The color development was recorded and stopped the reaction by washing the membrane in PBS-T for 5min and then placed it in distilled water. Then dried the membrane and photographed or digitized using a scanner.

### 3.5.2 Determination of cross-reactivity of polyhedrin-polyclonal antibodies:

The cross-reactivity of polyclonal antibodies was determined by subjecting the electro-eluted polyhedrin (EP) samples to DAC-ELISA and western immuno blotting as detailed below:

#### 3.5.2.1 DAC-ELISA:

DAC-ELISA was performed as described in section 3.4.1.1 in two different approaches as detailed below:

##### 3.5.2.1.1: Reciprocal test to determine the cross reactivity of the three polyclonal antisera:

In this approach variable concentrations (2000ng to 7.8ng /ml) of heterologous polyhedrins were coated in to ELISA plate in duplicate wells and fixed dilution (1:5000) of homologous antiserum (after booster dose bleed) was used to evaluate the cross-reactivity of the heterologous polyhedrins with that particular antiserum.

##### 3.5.2.1.2: Reciprocal DAC-ELISA with fixed heterologous polyhedrin concentration *vs.* variable homologous antiserum dilutions:

In this approach fixed concentration (1000ng/ml) of heterologous polyhedrins were coated into ELISA plate in duplicate wells and variable dilutions (1:1000 to 40,000) of homologous antiserum (after booster dose bleed) was used to evaluate the cross-reactivity of that particular antiserum with heterologous polyhedrins.

#### 3.5.2.2: Western immunoblotting:

To determine the cross-reactivity of homologous polyhedrin polyclonal antiserum with heterologous polyhedrins, the electro-eluted polyhedrins of three NPVs were separated in 12% SDS-PAGE as described in section 3.3.2 and subjected to western blotting as described in section 3.5.1 with respective antiserum at 1:5000 dilution.

## 3.6 Development and Evaluation Diagnostic Tools for NPVs:

To determine if the of polyhedrin specific polyclonal antibodies would be useful to monitor the various stages of NPV infection in larvae and to quantify the POBs in commercial NPV preparations, different immunochemical tools were standardized and evaluated their validation in routine application of diagnosis and quality control of NPVs.

## 3.6.1 Development of diagnostic tools:

To examine the ability of the polyclonal antibodies to diagnose the NPV infection, different age group larvae ($4^{th}$ and $5^{th}$ instars) of *H. armigera, S. litura* and *A. albistriga* were infected with their respective NPV in laboratory and evaluated the infection status by using various immunochemical tools. For example, for detection of POBs in larval homogenates, western immunoblotting and indirect immunofluorescence assay tools were developed, and for detection and quantification of polyhedrin content, DAC-ELISA and IC-ELISA were developed. The details of the standardization procedures were given below.

### 3.6.1.1 Infection of larvae and extraction of polyhedrin:

Healthy $4^{th}$ and $5^{th}$ instar larvae were transferred to cell well trays (*H. armigera* and *S. litura*) or plastic cages (*A. albistriga*) with out diet for 16-24h before released on to virus inoculated diet material. Larvae were infected with respective NPV by surface inoculation [as given by Evans and Shapiro (1997)] of their diet with greater than 50% lethal doses ($10^8$ POBs/ml). The larvae were reared under controlled conditions with 16:8h (L: D) photoperiod, 25 ± $2^0$C temperature and 70% relative humidity. Healthy controls for each instar were also maintained. Larvae were daily monitored for NPV infection until pupation. Larvae under live, dead, putrified and pupal stages were collected in to 2ml micro-centrifuge tubes, weighed and frozen at -$20^0$C. Polyhedrin was extracted from NPV infected larvae as described in Quant *et al.,* (1984) as detailed below:

Frozen larvae and pupae were homogenized at 0.1g of insect per ml in 0.01 M Tris (pH 8.2) - 0.15 M NaCl – 0.1 mM phenylmethylsulfonyl fluoride for 3-5min with a virtis blender at medium speed. This raw homogenate was directly used for Western immunoassay and indirect immunofluorescence assay for detection of POBs. For ELISA the total polyhedrin was extracted as follows: The larval homogenates were incubated with 0.1 volume of 1 M $Na_2CO_3$–0.5 M NaCl for 15-20min at $56^0$C to dissolve polyhedra. These preparations were centrifuged at 120,000 × g for 45min at $4^0$C (26,000 rpm in a Beckman SW-28 rotor) and the supernatants except those used in SDS-PAGE were heat treated for 20min at $70^0$C. Simultaneously healthy larvae were processed and prepared the extracts similarly. Total protein concentrations were determined by Bradford's reagent using a commercially available kit. Healthy and infected larval extracts were assayed in SDS-PAGE and then assayed in DAC and IC-ELISA to estimate the polyhedrin content.

### 3.6.1.2 SDS-PAGE analysis of healthy and infected larval extracts:

Extracts of infected and healthy larvae were assayed for detection of polyhedrin by separating them in 12% SDS-PAGE. Protein samples were mixed with equal volume of Laemmli buffer (Appendix III) and denatured by heat treatment in boiling water-bath for 3 min. Samples were loaded in to SDS-PAGE (discontinuous gel, composition given in Appendix III) at protein concentration of 200µg /10µl per well and electrophoresed at 100 volts for approximately 2h in Broviga ® apparatus. Gels were silver stained to visualize the proteins by the method given by Kumar *et al*. (2004). The molecular weights of the protein bands were estimated as described in section 3.3.2.

### 3.6.1.3 Western immuno assay for detection of POBs in larval extracts:

The raw insect homogenates (healthy and infected) were separated in 12% SDS-PAGE and subjected to western blotting as described in section 3.5.1 with respective antiserum at 1:5000 dilution.

### 3.6.1.4 Indirect immunofluorescence assay for detection of POBs in larval extracts:

The raw insect homogenates (healthy and infected) were heat-fixed on glass slides at 100 µl per slide for 2-3min. After fixing, the slides were washed for 3-4 times with PBS. Then slides were placed in a petri dish having a filter paper and incubated at $37^0$C for 1h with polyhedrin polyclonal antiserum (1:500) at 100µl per slide. After incubation slides were again washed with PBS, incubated with anti-rabbit Ig FITC conjugate (Sigma) at 1: 80 dilution for 1h at $37^0$C, followed by extensive washing with PBS. To confirm the antigen-antibody reactivity through fluorescence evaluation, slides were mounted in 80% glycerol and examined at various magnifications under fluorescence microscope (Olympus, Model: AX-70).

### 3.6.1.5 DAC-ELISA for detection and quantification of polyhedrin content in larval extracts:

All insect extracts (healthy and infected) were analyzed in DAC-ELISA as per the procedure given by Hobbs *et al.* (1987) as described in section 3.4.1.1. The total protein concentration for coating the ELISA plate was optimized by assaying the infected larval extracts at different concentrations (40, 20, 10, 5, 2.5 and 1.25µg/ml). ELISA readings were considered virus positive if the absorbance values of a sample differed by three-folds than those given by the healthy larval control.

To estimate the amount of poyhedrin present in infected larvae and to determine the effect of insect body proteins on the results of DAC-ELISA tests, extracts of uninfected larvae spiked with various amounts of polyhedrin. The polyhedrin was serially diluted from 1000 to 7.8 ng/ml in to fixed amount of healthy larval extract prepared in 50 mM carbonate buffer (pH 9.5). All homogenates (healthy and infected extracts) were adjusted to optimum coating concentration with coating buffer before being assayed. The results from DAC-ELISA were compared with a standard curve determined by serial dilution of spiked polyhedrin. The amount of polyhedrin present in larval extract was expressed as µg/mg of total protein concentration of larval extracts.

### 3.6.1.6 IC-ELISA for estimation of Polyhedrin content in larval extracts:

To eliminate the competition between insect and viral proteins for binding sites in the ELISA plate surface particularly when crude insect extracts were used in DAC-ELISA, an indirect competitive ELISA (IC-ELISA) was standardized and evaluated to estimate the polyhedrin content in insect extracts. The IC-ELISA was standardized as detailed below:

#### 3.6.1.6.1 Assay optimization:

Using a chequerboard system optimal concentrations for the coating antigen and antisera required for neutralization were determined by screening in a two dimensional titration analysis by DAC-ELISA and used in competitive inhibition studies. In order to optimize the assay protocols for estimation of heterologous polyhedrins, each homologous antiserum was screened with other two heterologous polyhedrins by two-dimensional (2D) titration analysis. The polyhedrin standards of HaNPV, SlNPV and AmalNPV were tested at concentrations ranging from 4 to 0.25µg/ml in 10-fold intervals. Each antiserum was tested at dilutions ranging from 1:1000 to 1:64,000. The combination of coating antigen and antibody dilution that resulted in the highest titer was selected for further development *i.e.* the antigen-antibody combination at which the lowest antigen concentration and highest antiserum dilution gives the maximum absorbance value after 60 min of substrate reaction time were considered as optimal concentration of antigen for coating ELISA plate and antibody dilution for ELISA. The polyhedrin standards were optimized from serial dilutions of 40 to 0.156, 20 to 0.078 and 10 to 0.039µg/ml. The dilution which gave the maximum regression was selected for further development. Before assay the total protein concentration of all insect extracts (healthy and infected) were adjusted to same optimum concentration.

#### 3.6.1.6.2 Assay procedure:

The procedure for IC-ELISA was essentially the same as that for the DAC-ELISA except that dilutions of standards and samples were incubated at $37^0$C for 1h with the antisera in PBS-T.

Samples (healthy and infected) were prepared as detailed in section 3.6.1.1. The details of the protocol steps were presented below:

The coating antigen (polyhedrin) was diluted in coating buffer (50 mM carbonate buffer, pH 9.5) to optimum concentration (1µg/ml) and added to the mocrotitre plate (150µl/well) and incubated the plate in a humid chamber for 2 hours at $37^0$C or in a refrigerator ($4^0$C) overnight. After coating, the buffer was decanted gently and washed the plate with 3-4 changes of PBS-T, allowing 3min for each wash to remove unbound coating antigen, then 100µl/well of serially diluted polyhedrin standards and samples were added to the wells then antiserum diluted in PBS-T at 1:4000 was added at 50µl/well, followed by incubation at $37^0$C for 1h or in a refrigerator ($4^0$C) overnight. The antibodies compete for bound antigen (to plate) and unbound antigen (in samples and standards) in the well. For each assay one row of buffer control (BC) was maintained by adding 100µl/well of PBST and one row of healthy control (HC) was maintained by adding of 100µl/well of healthy larval extract. The plates were again washed 3-4 times with PBS-T. To detect the antigen-antibody complex goat anti-rabbit IgG-alkalinephosphatase (ALP)-labelled conjugate (Sigma, USA) diluted in PBS-T (1:4,000) was added at 150µl/well and incubated at $37^0$C for 1h. After another 3-4 times of washing, phosphatase activity was measured by adding 150µl/well of Para-nitrophenyl phosphate substrate at 0.5mg/ml in 10% (v/v) diethanolamine buffer, pH 9.8 and incubated in dark place, at room temperature. The absorbance readings were recorded by an ELISA reader (Titertek Multiskan, Labsystems, Finland) at a single wavelength of $A_{405}$ nm after 1h of substrate reaction time. The results from IC-ELISA were compared with standard regression curves obtained by plotting $\log_{10}$ values of polyhedrin standards against optical density at $A_{405}$. Concentration of polyhedrin in the sample extract was determined from standard curves and expressed in µg/mg of total protein concentration of larval extracts.

### 3.6.1.6.3 Determination of percent competitive inhibition (CI):

To determine the affinity, specificity and sensitivity of polyhedrin polyclonal antiserum against homologous and heterologous polyhedrins, IC-ELISA was performed as described above. The percent of competitive inhibition (CI) was calculated by the following formula:

$$\% \text{ of competitive inhibition (CI)} = \frac{B}{B_0} \times 100$$

Where: B is the extinction of the well containing polyhedrin,

$B_0$ is the extinction of the well without polyhedrin (Buffer control).

From the above data standard competitive inhibiton curves were prepared by plotting the $\log_{10}$ values of polyhedrin standards against % of competive inhibiton of each standard concentration. The sensitivity of the assay was determined by calculating the concentration of polyhedrin required for 50% competitive inhibiton ($IC_{50}$) from the standard competitive inhibiton curve.

### 3.6.1.6.4 Determination of percent cross-reactivity (CR):

The percent of cross-reactivity of each polyclonal antiserum with heterologous polyhedrin was calculated as the $IC_{50}$ of the homologous polyhedrin divided by the $IC_{50}$ of the heterologous polyhedrin times 100.

$$\% \text{ of cross reactivity (CR)} = \frac{IC_{50} \text{ of homologous polyhedrin}}{IC_{50} \text{ of heterologous polyhedrin}} \times 100$$

### 3.6.1.6.5 Determination of percent recovery (PR):

To study the effect of insect body proteins on IC-ELISA results and to test the recovery of polyhedrin artificially spiked in to healthy insect extracts, polyhedrin standards at concentration of 20 - 0.078µg/ml were mixed into 25 and 50 µg/ml total protein concentrations of healthy larval extracts and both spiked and non-spiked samples were assayed in IC-ELISA. The recovery of polyhedrin in spiked healthy extracts were estimated by comparing the absorbance

values with the standard regression curve obtained from non-spiked samples and the % of polyhedrin recovered in spiked healthy extracts was calculated by using the following formula:

$$\% \text{ of recovery of polyhedrin (PR)} = \frac{\text{Amount of polyhderin estimated in spiked sample}}{\text{Amount of polyhedrin estimated in non-spiked sample}} \times 100$$

## 3.6.2 Development of quality control tools:

As part of the quality control during mass production of bio-insecticides based on HaNPV, SlNPV and AmalNPV, sensitive immunochemical tools such as DAC and IC-ELISA were developed and evaluated for the quantification of POBs in commercial NPV preparations. The protocols for the quality control tools were standardized as follows:

### 3.6.2.1 Preparation of standards and samples for ELISA:

A simple purification protocol was standardized for extraction of total polyhedrin from standard and sample NPV preparations as described below:

- Recently harvested NPV bottle was selected as stock standard POB suspension and the total POB concentration of the bottle was determined by counting the number of POBs as described in section 2.6 and its concentration was adjusted to 1 LE/ml ($6 \times 10^9$ POBs/ml) as working standard.
- 1 ml of known working standard POB suspension (1LE/ml) and unknown sample POB suspensions were taken in to 2ml micro centrifuge tubes and centrifuged at 10,000 rpm for 5min in a table top centrifuge. The supernatant was discarded and the pellet was dissolved in 1ml of resuspension buffer (0.01M Tris pH 8.2, 0.15 M NaCl and 0.1mM phenylmethylsulfonyl fluoride) and serially diluted in the same buffer from 1LE to 0.015 LE.

- To the standard and sample tubes 100 µl of POB dissolving buffer (1M $Na_2CO_3$ + 0.5 M NaCl) was added and incubated at $56^0$C for 15-20min in a water bath with occasional shaking.
- After dissolution of POBs, the suspension was cooled to $4^0$C on ice and centrifuged at 13000rpm for 60 min in a table top centrifuge. Supernatant was carefully collected and subjected to heat treatment at $70^0$C for 20 min and cooled to $4^0$C on ice. The total protein concentration was determined by Bradford's reagent using a commercially available kit.
- To check the purity, total polyhedrin extracted from standard and sample POB suspensions was assayed in 12% SDS-PAGE before being assayed in ELISA.

**Note:** To minimize the errors during extraction, 1LE/ml standard polyhedrin solution was prepared along with samples instead of all the standards at every time and serially diluted (1LE to 0.015LE) during ELISA experiment.

### 3.6.2.2 Assay optimization:

The extracts of standards and samples were evaluated in both DAC and IC-ELISA at 1:1000 dilution as described in sections 3.4.1.1 and 3.6.1.6. To calculate the number of POBs present in the unknown POB suspension, the absorbance values were compared with the standard regression curve obtained from the known standard POB suspension.

In order to determine the difference between microscope counting and ELISA results, some known and unknown samples were assayed in ELISA (DAC and IC-ELISA) and % of recovery was calculated by the following formula.

$$\% \text{ of recovery of POBs} = \frac{\text{Number of POBs estimated in ELISA}}{\text{No of POBs estimated in microscope counting}} \times 100$$

## 3.7 Application of immunochemical tools in optimization of conditions for productivity and quality of NPVs:

The immunochemical tools developed in this study were applied in optimization of conditions for the productivity and quality of NPVs during commercial production .The conditions optimized in this study were as follows:

- Identification of optimum age of the larvae for inoculation of virus to obtain maximum yield of virus.
- Identification of optimum time for harvesting of virus to obtain maximum yield of virus with low levels of bacterial contaminants.

### 3.7.1 Identification of optimum age of larvae for virus inoculation:

To study the effect of age of larvae on POB yield and to identify the optimum age of the larvae for inoculation of virus, NPVs were mass multiplied on different age group larvae. The total yield of NPV obtained was monitored by ELISA tools. The experiment was detailed as below:

Mass multiplication of NPVs was conducted on $2^{nd}$, $3^{rd}$, $4^{th}$, and $5^{th}$ instar larvae by diet surface contamination method (Fig 9) [as given by Evans and Shapiro (1997)] with greater than 50% lethal doses ($10^8$ POBs/ml). This rate maximizes the POB yield during mass multiplication (Okada, 1977). For each instar three replications were maintained each with twenty larvae**.** The larvae were reared under controlled conditions with 16:8 h (L: D) photoperiod, 25 ± $2^0$C temperature and 70% relative humidity. Larvae were observed daily for mortality. Mortality of larvae started from $4^{th}$ day and it continued up to 8-12 days depending up on the age of the larvae. At death, the cadavers were collected in jars containing approximately 10 times their own volume of distilled water. After collecting all dead larvae, the POBs were harvested as described in section 3.1.3. POBs harvested from all the age group larvae were dissolved in the same volume (10ml) of sterile distilled water. Then, total polyhedrin of POBs harvested from different age

group larvae was extracted as described in section 3.6.2.1 and assayed for the yield of NPV in ELISA (DAC as well as IC-ELISA) as described in section 3.6.2.2. From ELISA results the total POB yields among different age group larvae were determined and identified the optimum age of larvae for virus inoculation to obtain maximum virus yield.

### 3.7.2 Identification of optimum time for harvesting of virus:

To study the effect of period of harvest on POB yield during mass multiplication of NPVs and to establish the relationship between larval mortality and productivity and quality of NPV in larvae during mass multiplication, a bioassay experiment was conducted on optimum aged larvae of *H. armigera, S. litura* and *A. albistriga.* The productivity of virus in larvae at different intervals (post inoculation days) of experiment was monitored through ELISA (DAC and IC-ELISA) and western immunoassay tools. The experiment was detailed as below:

Healthy larvae of *H. armigera, S. litura* and *A. albistriga* were infected with respective NPV by surface inoculation [as given by Evans and Shapiro (1997)] of their diet with greater than 50% lethal doses ($10^8$ POBs/ml). This rate maximizes the POB yield during mass multiplication (Okada, 1977). For each virus three replications were maintained each with 20 larvae**.** As a control, the same number of larvae was fed the diet without POBs. For each virus three replications were maintained with twenty five larvae each. The larvae were reared under controlled conditions with 16:8 h (L: D) photoperiod, 25 ± $2^0$C temperature and 70% relative humidity. Two larvae from each replication were sampled on 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 days post exposure (dpe) to POBs, transferred to preweighed sterile micro-centrifuge tubes and weighed again in an electronic top pan balance. One larva was frozen at -$20^0$C for extraction of total polyhedrin and second larva was stored at $4^0$C for bacterial activity studies. Live larvae were sampled at 0 to 7 dpe, but both live and dead (virus killed) larvae were sampled at 8 and 9 dpe. At 10 to12 dpe, only dead larvae were sampled. Viral death generally caused liquefaction of the cadaver. Only cadavers that could be transferred whole from the cell wells or cages were used.

Insects were homogenized individually and assayed for detection of POBs by Western immunoblotting as described in section 3.6.1.3. Total polyhedrin was extracted from individual frozen larva as described in section 3.6.1.1 and all insect extracts were assayed for detection and quantification of polyhedrin levels by ELISA (DAC and IC-ELISA) as described in sections 3.6.1.5 and 3.6.1.6. For quantification of POBs the same extracts were again assayed in ELISA (DAC and IC-ELISA) as described in section 3.6.2.

**Figure 9: Bioassay studies**


**A** **B** **C**

Bioassay studies of NPVs against *Helicoverpa armigera* (**A**), *Spodoptera litura* (**B**) and *Amsacta albistriga* (**C**) by surface diet contamination method to optimize the age of larvae for virus inoculation and the time of virus harvest to obtain maximum virus yield with less bacterial contaminants.

### 3.7.3 Screening of bacterial activity in infected larvae days post exposure (dpe) to NPV:

The larvae collected on different days post exposure (dpe) to NPV were homogenized individually and screened for the bacterial load by plating samples on nutrient agar media (Miles and Misra, 1938). Sterilized molten agar medium (Himedia) was aseptically dispensed in volumes of 15ml into sterile petriplates and allowed to settle. One ml of larval homogenate was taken and ten fold serial dilutions ranging from $10^{-1}$ to $10^{-9}$ were prepared using sterile distilled water. Before plating, each petriplate was marked into six equal segments using a marker pen and each segment was plated with a different dilution. Six replicates for each dilution were maintained. Overcrowding has occurred at the higher concentrations (smallest dilutions) resulting in an underestimate of the numbers of viable bacteria present. Hence, in the present study observations were recorded at $10^{-3}$ dilution. Colony forming units per ml of solution was calculated using the following formula.

$$\text{CFU/ml} = \frac{\text{Number of colonies observed}}{\text{Volume plated}} \times \text{Dilution factor}$$

## 3.8. Evaluation of ELISA tools at field level efficacy studies of NPV:

ELISA tools developed in this study were also applied to monitor and evaluate the efficacy of NPV at field level at days post application (dpa). To study the infection status in individual larva and to estimate the % of infection in pest population after field application of NPV, field experiment was conducted during 2007 kharif season at ICRISAT forms with respect to *H. armigera* on pigeonpea crop. The experiment was detailed as below:

Field experiment was conducted with two treatments in three replications in randomized block design (RBD). Pigeonpea was sown with 60 × 15 cm spacing. The treatments used to study the effect on *H. armigera* were *Ha*NPV and control (no treatment). The field was sprayed with *Ha*NPV@ 250 LE / ha (Fig 10A) after the pest population was reached above ETL (Economic Threshold Level). From each plot (treatment and control) ten plants were randomly selected and

sampled 30-40 larvae per day (irrespective of the age), immediately shipped to the laboratory, weighed and frozen at -20$^0$C on 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 dpa of NPV. Live larvae were sampled at 0 to 4 dpa, both live and dead larvae were sampled at 5 to 10 dpa. Viral death generally caused liquefaction of the cadaver. Only cadavers that could be transferred whole from the plant parts were collected.

Total polyhedrin was extracted from individual frozen larva (Fig 10B) as described in section 3.6.1.1 and assayed for detection of polyhedrin by DAC-ELISA as described in sections 3.6.1.5 and for quantification of virus (POBs) by DAC and IC-ELISA as described in section 3.6.2. DAC-ELISA readings were considered as virus +ve if the absorbance values of a sample differed by three-folds than those given by the healthy insect control. Based on the ELISA results of individual larvae the % of NPV infection and gross virus concentration per day of field sampled larvae were estimated.

**Figure 10: Field application of NPV and extraction of polyhedrin from field sampled larvae to evaluate the efficacy of NPV by ELISA.**

**A** **B**

To evaluate the efficacy of NPV at field level by ELISA, field study was conducted by applying the NPV (250 LE/ ha) against *Helicoverpa armigera* on pigeonpea crop (**A**). Larvae were sampled from field at 0 to 10 days of post application (dpa), homogenized (**B**) and extracted the total polyhedrin to evaluate in ELISA.

## 3.9 Isolation and Characterization of Polyhedrin gene of NPVs:

In order to establish the purity of seed stock or master stock of NPV used for commercial viral insecticide preparations at ICRISAT, molecular level identification and evaluation of phylogenetic status of NPVs done by isolation, cloning, sequencing of polyhedrin gene and evaluation their phylogenetic status. The molecular studies were conducted as detailed below:

### 3.9.1 Optimization of PCR for amplification of complete polyhedrin gene of NPVs:

The complete polyhedrin gene of NPVs was isolated by optimizing the PCR using degenerate primers as detailed below:

#### 3.9.1.1 Design of degenerate primers:

The coding region of the polyhedrin gene, which is highly conserved among NPVs was targeted as template DNA. The sequence for this gene was previously determined for each of the NPVs infecting *Helicoverpa*, *Spodoptera* and *Amsacta* insect complexes (Table 5) were used to design the primers for this study. The polyhedrin gene sequences were analyzed by Multiple Sequence Alignment [CLUSTAL W (1.83)] in order to obtain a consensus sequence (Thompson *et al.,* 1994). Three degenerate oligomers were synthesized by solid phase chemistry and were generally obtained from commercial sources (Bioserve, Hyderabad, India).

**Table 5: List of full length polyhedrin gene sequences previously determined for NPVs of *Helicoverpa, Spodoptera* and *Amsacta* species used in this study to design degenerate oligomers.**

| S.No | Virus | Host | Public database accession number |
|---|---|---|---|
| **A. *Helicoverpa* complex**: | | | |
| 1 | HaNPV | ***Helicoverpa armigera*** | AF157012 |
| 2 | HaNPV | *Helicoverpa armigera* | AJ001917 |
| 3 | HaNPV | *Helicoverpa armigera* | NC003094 |
| 4 | HaNPV | *Helicoverpa armigera* | AF303045 |
| 5 | HaNPV | *Helicoverpa armigera* | A25670 |
| 6 | HaNPV | *Helicoverpa armigera* | U97657 |
| 7 | HaNPV | *Helicoverpa armigera* | NC002654 |
| 8 | HzNPV | ***Helicoverpa zea*** | NC003349 |
| 9 | HasNPV | ***Helicoverpa assulta*** | DQ157735 |
| **B.*Spodoptera* complex:** | | | |
| 10 | SlNPV | ***Spodoptera litura*** | NC003102 |
| 11 | SlNPV | *Spodoptera litura* | AF325155 |
| 12 | SlNPV | *Spodoptera litura* | AF037262 |
| 13 | SlNPV | *Spodoptera litura* | AY549963 |
| 14 | SlNPV | *Spodoptera litura* | AY549964 |
| 15 | SlNPV | *Spodoptera litura* | AY552474 |
| 16 | SlNPV | *Spodoptera litura* | DQ350142 |
| 17 | SlNPV | *Spodoptera litura* | DQ152923 |
| 18 | SlNPV | *Spodoptera litura* | X94437 |
| 19 | SliNPV | ***Spodoptera litturalis*** | D01017 |
| 20 | Sf NPV | ***Spodoptera frugiperda*** | J04333 |
| 21 | SeNPV | ***Spodoptera exigua*** | AF169823 |
| **C. *Amsacta* complex:** | | | |
| 22 | AmalNPV | ***Amsacta albistriga*** | AF118850 |

### 3.9.1.2 Extraction of viral DNA:

The genomic DNA of NPVs infecting *H. armigera, S. litura and A. albistriga* was extracted directly from POBs using the protocol given by Rabindra (2001) with slight modifications as detailed below:

- POB suspension (1ml) was taken in to 2ml micro-centrifuge tube and centrifuged at 10,000rpm for 2 min, supernatant was discarded carefully and the pellet was suspended in 1ml of 100mM Tris, 10 mM EDTA pH 7.8, 1.0 M NaCl and 5% PVP and centrifuged at 10,000rpm for 2 min.
- The supernatant was discarded and the pellet was dissolved in 0.5 ml of the same buffer. To this half the volume (250 µl) of 1M $Na_2CO_3$ was added and incubated at $37^0$C for 1hr followed by addition of 1% SDS and incubated at $37^0$C for 30min.
- Then centrifuged for 60sec at 6,500 rpm and the supernatant was taken in to a clean tube and to that equal volume of Tris saturated phenol was added and agitated gently for 5 min and centrifuged at 12,000 rpm for 2min in tabletop centrifuge.
- The upper aqueous layer was collected and to that equal volume Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and centrifuged at 12,000 rpm for 2min in tabletop centrifuge.
- The upper aqueous layer was collected and it was reclarified twice with equal volume of chloroform by spinning at 12,000 rpm for 2min.
- The upper aqueous phase was collected to which $1/10^{th}$ volume of 3M sodium acetate and 2.5% (v/v) cold ethanol were added and incubated at $-70^0$C for 1h.
- Then centrifuged at 12,000rpm for 15min, followed by washing the pellet with 70% cold ethanol and pellet was air dried at $37^0$C for 10min and dissolved in TE buffer and stored at $-20^0$C until use.

- The purity and concentration of the isolated genomic DNA sample was estimated by measuring the absorbance at 260 nm and 280 nm using UV-Visible spectrophotometer (Beckmann). The genomic DNA concentration was measured using the following formula:

  Genomic DNA concentration in µg/ml = absorbance at 260nm × 50µg/µl × dilution factor.

### 3.9.1.2.1 Electrophoresis:

The genomic DNA of NPVs was analysed by 0.8% agarose gel electrophoresis using horizontal gel electrophoresis system. A gel slab of required size containing 0.8% agarose and 6µl of 10mg/ml ethidium bromide was prepared in 1x TBE using gel mould. After setting time of 20 min the gel mould was placed in a tank containing sufficient quantity of 0.5 x TBE buffer (Appendix VI). The DNA along with DNA Marker (λ DNA Marker; Roche, Cat# 528 552) was loaded in wells of 0.8% agarose gel and electrophoresed at 100V for 1-2h. Removed the gel from the tray and visualized on a UV-transilluminator and photographed with a Polaroid$^{R}$ camera fitted with a UV filter.

### 3.9.1.3 Optimization of PCR conditions:

The PCR conditions were standardized as follows: each 25µl reaction mixture was set up as shown below:

| **Component** | **Volume** (µl) |
|---|---|
| 10 X reaction buffer | 3µl |
| $MgCl_2$ (25mM) | 3µl |
| Forward primer (10pm/µl) | 1µl |
| Reverse primer (10pm/µl) | 1µl |
| AmpliTaq Gold$^{TM}$ (1U) | 0.3µl |
| dNTPs mixture (100mM) | 1µl |
| Template DNA (50-60ng/µl) | 1µl |
| Double sterile distilled water | 14.7µl |
| **Total volume** | **25µl** |

PCR was carried out in Applied Biosystems thermocycler. The thermal cycles of the reaction were standardized by performing the PCR at various combinations of denaturation, annealing, and extension temperatures according to the Tm of the degenerate primers. PCR product was separated by 1% agarose gel electerophoresis as described in section 9.1.2.1. DNA Marker (λ DNA Marker; Roche, Cat# 528 552) was used to determine the size of PCR amplified product. Gel was stained with ethidium bromide (0.5µg/ml), viewed on a UV-transilluminator and photographed with a polaroid$^{R}$ camera fitted with a UV filter.

## 3.9.2 Cloning and sequencing of PCR product:

The PCR product was eluted from agarose gel, cloned and sequenced. The experimental details were given below.

### 3.9.2.1 Gel elution and purification of PCR product:

The PCR product of expected band with a size of approximately 750 bp was excised from the gel and eluted by using gel elution kit (Qiagen, MinElute Gel Extraction Kit) following manufacturer protocol as detailed below:

- The amplified DNA fragment was excised from the agarose gel with a clean, sharp scalpel and its size was minimized by removing extra agarose.
- Weighed the gel slice in a colorless tube and added 3 volumes of gel dissolving buffer (Buffer QG) to 1 volume of gel (300µl /100mg of gel).
- Incubated at $50^{0}$C until the gel slice has completely dissolved by vortexing the tube every 2-3min during the incubation.
- After the gel slice has dissolved completely, added 1 gel volume of isopropanol to the sample and mixed by inverting the tube several times.
- To bind DNA, the sample was transferred on to the MinElute column and centrifuged for 1min at 13000rpm on table top centrifuge.

- Added 500µl of Buffer QG to the MinElute column and centrifuged for 1 min at 13000rpm on table top centrifuge.
- To wash, added 750µl of Buffer PE to the MinElute column and centrifuged for 1 min at 13000rpm on table top centrifuge.
- Transferred the MinEute column to a clean 1.5ml microcentrifuge tube and centrifuged for 1min at 13000rpm on table top centrifuge to remove residual ethanol (Buffer PE).
- Placed the MinElute column in a clean 1.5 ml microcentrifuge tube.
- To elute DNA, added 10µl of Buffer EB (10mM Tris-HCl, 8.5) to the center of the membrane, allowed to stand for 1min, and then centrifuged for 1min at 13000rpm on table top centrifuge.

### 3.9.2.2 Ligation of purified PCR product in to cloning vector:

The purified PCR product was ligated in to pJET1/blunt cloning vector (Fig 11) (Fermentas, # K1221). The blunting reaction was set up as shown below:

| **Component** | **Volume** (µl) |
|---|---|
| 2 x reaction buffer | 10µl |
| PCR product (purified) | 5µl |
| DNA blunting enzyme (Fermentas, # K1221) | 1µl |
| Water, nuclease-free | 2µl |
| **Total volume** | **18µl** |

The reaction mixture was vortexed briefly and centrifuged for 3-5s. Then incubated at $70^0$C for 5 min and chilled on ice for several seconds. Then ligation reaction was set up by adding the following components to blunting reaction mixture:

| Component | Volume (µl) |
|---|---|
| pJET1/blunt cloning vector (50ng/µl) | 1µl |
| T4 DNA ligase (5 U/µl) | 1µl |
| **Total volume** | **20µl** |

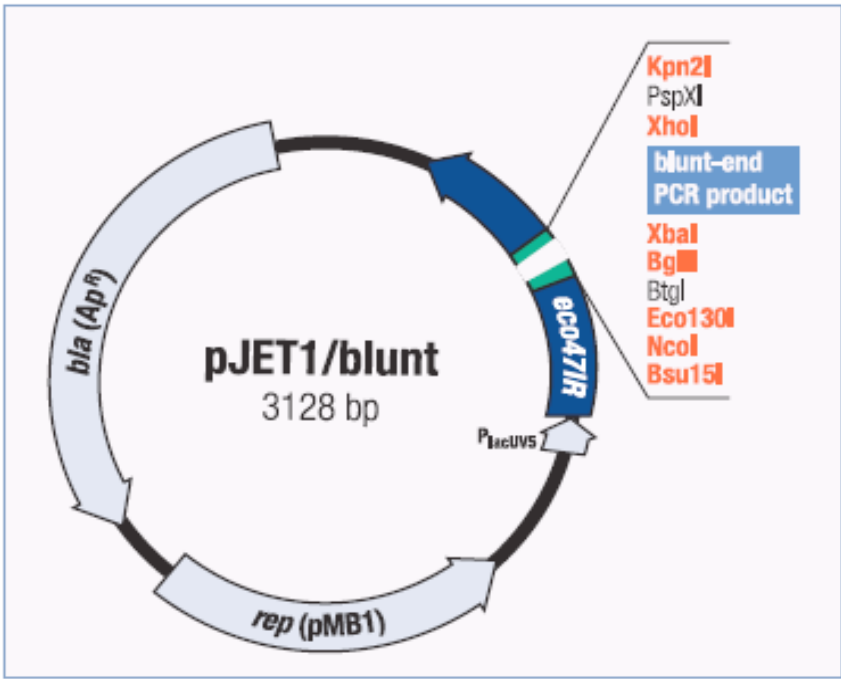
The reaction mixture was vortexed briefly and centrifuged for 3-4s. Then incubated at room temperature ($22^0$C) for 5min. The ligation mixture was used directly to transform competent *E.coli* cells.

### 3.9.2.3 Preparation of competent *E.coli* cells (DH5α strain):

- The log phase culture of *E.coli* (400µl) cells were inoculated in to 15ml of sterile LB broth and incubated at $37^0$C for 4h.

  **Note:** The optical density of the culture was measured during the incubation period and stopped the incubation period when the optical density reached to 0.5 OD at A 600nm.
- The culture was chilled on ice for 30min, transferred in to pre chilled 30ml glass tubes and the cells were collected as pellet by centrifugation at 5000rpm for10 min in SS34 rotor (Sorvall).
- The pellet was suspended in 1/10 volume (initial bacterial culture) of 100mM $CaCl_2$ solution and kept on ice for 15min.
- The cells were again pelleted and the final pellet was suspended in 1/20 volume of 100 mM $CaCl_2$ solution.
- To the cells, sterile glycerol was added to a final concentration of 15%, aliquoted (50µl/ tube) and stored at $-70^0$C for further use.

**Figure 11: pJET1/blunt end cloning vector map**



### 3.9.2.4 Transformation:

Competent cells were thawed rapidly by warming between hands and dispensed 100µl immediately into test tubes containing 10µl of ligation mixture. Gently swirled the tubes to mix and then immediately placed on ice for 10min. Then heat shocked the cells by placing tubes in to a $42^0$C water bath for exactly 2min and immediately placed on ice for 10min. Then added 1ml LB medium to each tube and placed the tubes on a roller drum at 250rpm for 1h at $37^0$C. Then plated aliquots of transformation culture on LB/ampicillin containing plates. When plates are dry, incubated for 12 to 16 hrs at $37^0$C.

### 3.9.2.5 Selection of clones for sequencing and BLAST search:

Single colonies were picked up with a sterile toothpick from overnight incubated plates and suspended in 50µl of LB broth and incubated at $37^0$C for 3-4h. After incubation, 4-6 colonies were screened for the conformation of polyhedrin gene insert in the putative recombinant clones.

### 3.9.2.5.1 Colony PCR for the conformation of inserted gene:

The colony PCR was performed using universal pJET1 forward and reverse sequencing primers [Bioserve Biotechnologies (India) Pvt.Ltd, Catalogue No # 51314 and 51315]. Each 25µl reaction mixture was set up as shown below:

| **Component** | **Volume (µl)** |
|---|---|
| 10 X reaction buffer | 3µl |
| $MgCl_2$ (25mM) | 3µl |
| pJET1 forward primer (10pm/µl) | 1µl |
| pJET1 reverse primer (10pm/µl) | 1µl |
| AmpliTaq Gold$^{TM}$ (1U) | 0.3µl |
| dNTPs mixture (100mM) | 1µl |
| Bacterial culture | 2µl |
| Double sterile distilled water | 13.7µl |
| **Total volume** | **25µl** |

PCR was carried out in Applied Biosystems thermocycler using the following parameters: initial denaturation step at $95^0$C for 10min followed by 35 thermal cycles of $95^0$C for 1min, $55^0$C for 1min and $72^0$C for 1min, with the final extension step increased to 10min. PCR product was verified by resolving the fragment in a 1% agarose gel electrophoresis along with DNA Marker (λ DNA Marker; Roche, Cat# 528 552) to check for the insert size.

### 3.9.2.6 Plasmid isolation:

Three clones were selected from the transformation event after colony PCR. The selected clones were inoculated in 5ml of LB medium with ampicillin (100µg/ml) in culture tubes and grown overnight at $37^0$C with vigorous agitation (220rpm). The plasmid DNA was isolated from over night culture by using QIA prep$^R$ Spin Miniprep Kit (250) (Qiagen) protocol according to the manufacturer recommendations. The protocol steps were presented below:

- The sub cultures (2ml) of each colony were centrifuged at 10,000rpm for 5min in a table top centrifuge to pellet the cells.
- Resuspended the bacterial pellet in 250µl of solution P1 (containing RNase A).
- Added 250µl of solution P2 and inverted the tube gently for 4-6 times.
- Added 350µl of buffer N3 and inverted immediately for 4-6 times and centrifuged at 12,000rpm for 10min in a table top centrifuge.
- The supernatant was applied to the QIA prep column, centrifuged at 12,000rpm for 30-60sec and discarded the flow through.
- Washed the QIA prep spin column by adding 0.75ml of buffer PE and centrifuge at 12,000 rpm for 30-60sec.
- Discarded the flow through and centrifuged for additional 1min to remove residual wash buffer.
- Then placed the QIA prep mini column in a clean 1.5ml micro centrifuge tube, added 50µl of buffer EB to the centre of the column and allowed to stand for 1min and centrifuged at 12,000 rpm for 1 min and collected the flow through.

- The elute (µl) was analyzed in 1% agarose gel electrophoresis.

### 3.9.2.7 Gene sequencing:

Plasmid confirmed that having inserted polyhedrin gene was subjected to sequencing. The sequencing PCR was carried out using Beckman Quick Master Mix, 100ng of plasmid DNA, and 5pm of pJET[1] forward and reverse primers. The PCR conditions are as follows: $94^0$C (30sec), $50^0$c (30sec), $60^0$C (4 min) for 30 cycles. The PCR product was precipitated using Sodium acetate and absolute alcohol to remove dye terminators. The sequencing reaction was performed using Beckman Coulter CEQ 8000 machine.

### 3.9.2.8 BLASTX search:

The nucleotide sequence of each clone was compared with sequences from various databases of National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by means of the basic local alignment search tool (BLAST) (Altschul *et al.,* 1997).

## 3.9.3 Phylogenetic Relation at Nucleotide Level of HaNPV-P Polyhedrin Gene with Known Polyhedrin and Granulin Genes:

Sequence data was analyzed using Lasergene software (DNASTAR, Madison, USA). A total 55 gene sequences of 41 NPV polyhedrin genes (including HaNPV-P strain) and 14 GV granulin genes (Table 6) were aligned by ClustalW (Thompson *et al.,* 1994) method using MgAlign tool of Lasergene software. The method parameters used for alignment are Gap penalty: 10.0; Gap length penalty: 0.20; Delay divergent seqs: 30%; DNA transition weight: 1.0; DNA weight matrix: IUB). Bootstrapping of phylogenetic tree was carried out using CLC workbench 3 software (CLC Bio).

### 3.9.4 Phylogenetic Relation at Amino acid Level of HaNPV-P Polyhedrin Protein with Known Polyhedrin and Granulin Proteins:

Nucleotide sequence of HaNPV-P polyhedrin gene clone was translated and the corresponding amino acid sequence along with known polyhedrins (40) and granulins (14) (Table 6) were aligned using MgAlign tool of Lasergene software. Bootstrapping of phylogenetic tree was carried out using CLC workbench 3 software (CLC Bio).

**Table 6: Accession numbers of known polyhedrin and granulin nucleic acid and amino acid sequences in public database used in this study for determination of phylogenetic status of HaNPV-P strain.**

| S.No | Virus | Group | Host | Public database accession numbers | |
|---|---|---|---|---|---|
| | | | | Nucleotide sequence | Amino acid sequence |
| **A. Genus: Nucleopolyhedroviruses (NPVs):** | | | | | |
| 1 | AcNPV | I | *Autographa californica* | K01149 | AAA46719 |
| 2 | AfNPV | N/D | *Anagrapha falcifera* | U64896 | AAB53357 |
| 3 | AhNPV | N/D | *Adoxophyes honmai* | NC_004690 | NP_818648 |
| 4 | AgMNPV | I | *Anticarsia gemmatalis* | Y17753 | CAA76844 |
| 5 | AmalNPV | I | *Amsacta albistriga* | AF118850 | AAD24463 |
| 6 | ApNPV | N/D | *Anthraea pernyi* | AB062454 | BAB58969 |
| 7 | ArceNPV | N/D | *Archips cerasivoranus* | U40834 | AAA93290 |
| 8 | AsNPV | N/D | *Agritis segetum* | DQ123841 | AAZ38167 |
| 9 | ArNPV | N/D | *Attacus ricini* | S68462 | AAP16625 |
| 10 | BmNPV | I | *Bombyx mori* | U75359 | AAB18336 |
| 11 | BsNPV | II | *Buzura suppressaria* | X70844 | CAA50194 |
| 12 | CfMNPV | I | *Choristoneura fumiferana* | U40833 | AAA93292 |
| 13 | CrNPV | N/D | *Choristoneura rosaceana* | U91940 | AAB51303 |
| 14 | EoNPV | II | *Ecotropis oblique* | DQ837165 | AAQ88174 |
| 15 | EpMNPV | N/D | *Epiphyas postvittana* | AF061578 | AAC72189 |
| 16 | HaSNPV | II | *Helicoverpa armigera* | NC_003094 | NP_203559 |
| 17 | HasNPV | II | *Helicoverpa assulta* | DQ157735 | AAZ83723 |

| | | | | | |
|---|---|---|---|---|---|
| 18 | HzSNPV | II | *Helicoverpa zea* | NC_003349 | NP_542624 |
| 19 | HcNPV | I | *Hyphantria cunea* | D14573 | BAA03427 |
| 20 | LdMNPV | II | *Lymantria dispar* | M23176 | AAA46742 |
| 21 | LoMNPV | I | *Lonomia obliqua* | AF232690 | AAF98122 |
| 22 | LsNPV | II | *Leucania seperata* | U30302 | AAA99736 |
| 23 | MbNPV | II | *Mamestra brassicae* | AB198073 | BAE06244 |
| 24 | McNPV | II | *Mamestra configurata* | AY126275 | AAM94988 |
| 25 | MdNPV | II | *Malacosoma disstria* | U61732 | AAD00095 |
| 26 | MnNPV | II | *Malacosoma neustria* | AJ277555 | CAB91643 |
| 27 | OpMNPV | I | *Orgyia pseudostugata* | M14885 | AAA64926 |
| 28 | OpSNPV | II | *Orgyia pseudostugata* | M32433 | AAA46739 |
| 29 | PfNPV | II | *Panolis flammea* | D00437 | BAA00338 |
| 30 | PnNPV | I | *Perina nuda* | U22824 | AAA64782 |
| 31 | PoNPV | N/D | *Plusia orichalcea* | AF019882 | AAC64234 |
| 32 | PxMNPV | N/D | *Plutella xylostella* | NC_008349 | YP_758474 |
| 33 | RoMNPV | N/D | *Rachiplusia ou* | NC_004323 | NP_702998 |
| 34 | SeMNPV | II | *Spodoptera exigua* | AF169823 | AAF33532 |
| 35 | SfMNPV | II | *Spodoptera frugiperda* | J04333 | AAA46737 |
| 36 | SlNPV | II | *Spodoptera litura* | X94437 | CAA64211 |
| 37 | SliNPV | II | *Spodoptera littoralis* | D01017 | BAA00824 |
| 38 | ToMNPV | N/D | *Thysanoplusia orichalcea* | AF169480 | AAD51629 |
| 39 | TnSNPV | N/D | *Trichoplusia ni* | AF093405 | AAC64160 |

| | | | | | |
|---|---|---|---|---|---|
| 40 | WsNPV | II | *Wiseana signata* | AF016916 | AAB97154 |
| **B. Genus: Granuloviruses (GVs):** | | | | | |
| 41 | AbGV | N/A | *Andraca bipunctata* | AY518318 | AAS86810 |
| 42 | AoGV | N/A | *Adoxophyes orana* | NC_005038 | NP_872455 |
| 43 | AsGV | N/A | *Agrotis segetum* | NC_005839 | YP_006343 |
| 44 | CfGV | N/A | *Choristoneura fumiferana* | AF439352 | AAC69544 |
| 45 | ClGV | N/A | *Cryptophlebia leucotreta* | AY229987 | AAQ21599 |
| 46 | CoGV | N/A | *Choristoneura occidentalis* | NC_008168 | YP_654422 |
| 47 | CpGV | N/A | *Cydia pomonella* | U53466 | AAK70668 |
| 48 | HbGV | N/A | *Harrisina brillians* | AF142425 | AAF66610 |
| 49 | PbGV | N/A | *Pieris brassicae* | X02498 | CAA26331 |
| 50 | PoGV | N/A | *Phthorimaea operculella* | AF499596 | AAM70199 |
| 51 | PxGV | N/A | *Plutella xylostella* | AF270937 | AAG27302 |
| 52 | SlGV | N/A | *Spodoptera litura* | NC_009503 | YP_001256952 |
| 53 | TnGV | N/A | *Trichoplusia ni* | K02910 | AAA43834 |
| 54 | XcnGV | N/A | *Xestia c-nigrum* | U70069 | AAB42059 |

*N/D-Not determined; N/A-Not applicable*

## 3.10. Development of PCR Based RFLP Marker for Identification and Differentiation of HaNPV-P (Patancheru) Strain:

In order to distinguish the HaNPV-PC strain with other NPVs based on its unique restriction sites present in the amplified portion of the polyhedrin gene a PCR-RFLP marker was developed. The experiment was as detailed below.

### 3.10.1 Restriction mapping analysis for identification of unique restriction sites:

To identify the unique restriction sites present in polyhedrin gene of HaNPV-P, the sequenced polyhedrin gene of HaNPV-P along with other known published polyhedrin gene sequences were subjected to restriction mapping analysis using BioEdit version 5.0.9. The unique restriction sites present at particular nucleotide positions in polyhedrin gene of HaNPV-P were identified and short listed the other NPVs which have same restriction sites at same positions.

### 3.10.2 PCR-RFLP analysis:

The unique restriction sites present in HaNPV-P strain polyhedrin gene was verified by PCR-RFLP study. The experiment was detailed as follows. Initially the polyhedrin gene of the HaNPV-P strain was amplified by using the degenerate primer set as described in section 9.1.3 then the PCR product was subjected to restriction endonuclease (REA) analysis with the selected restriction enzyme unique to HaNPV-P strain. The restriction digestion reaction was setup as shown below.

| DNA sample (PCR product) | 10x reaction buffer | BSA (2µg/ µl) | Restriction enzyme (10 U/ µl) | Milli Q water | Total volume |
|---|---|---|---|---|---|
| 12µl | 2µl | 1µl | 1µl | 4µl | 20µl |

The reaction mixture was incubated at $37^0$C for 3-4h and reaction was stopped by heat inactivation at 70 $^0$C for 10 min. To this mixture 5µl of DNA loading dye was added and separated in 12% native PAGE gel along with DNA Marker (λ DNA Marker; Roche, Cat# 528

552)**.** The gel was stained by soaking in ethidium bromide solution (0.5µg/ml of distilled water) for 20min, visualized on a UV-transilluminator and photographed with a polaroid$^{R}$ camera fitted with a UV filter.

The size of the restriction fragments was estimated by comparing with the marker DNA standards (λ DNA Marker; Roche, Cat# 528 552). Standard graph was prepared by plotting the distance migrated by DNA standards on X-axis and fragment lengths on Y-axis. The size of polyhedrin gene restriction fragments were calculated from the standard graph by plotting the distance migrated by the restriction fragmets. Finally the size of restriction fragmets was compared with the restriction map for verification of the unique restriction site present at particular nucleotide position.

# CHAPTER – IV

# *RESULTS*

# CHAPTER - IV
# RESULTS

Results of various experiments conducted in the present investigation are presented in this chapter.

## 4.1 Isolation and Propagation of NPVs from Major Lepidopteran Pests of Legume Crops:

During natural epizootic conditions NPV infections were observed in *H. armigera*; *S. litura* and *A. albistriga* pest populations at ICRISAT farms. Diseased larvae showed the following features:

- The diseased larvae were swollen, glossy and moribund.
- The larvae of *H. armigera* (Fig 12A) and *A. albistriga* (Fig 12B) were crawled to the top of the twings (negative geotropism) on which they were fed.
- The diseased larvae were died and the body tissues were liquefied, in some larvae the cuticle was ruptured and discharging of body fluid on to plant parts was observed.
- Observation of discharged body fluid under phase contrast microscope revealed that it consists of poly occlusion bodies (POBs) (Fig 13).
- Propagation of NPVs on respective larvae resulted in virus infection. The age of larvae used for multiplication of NPV, concentration of virus used for inoculation and yield of NPV obtained were presented in Table 7.

**Figure 12: NPV infected larvae of *H. armigera* and *A. albistriga***

**A**

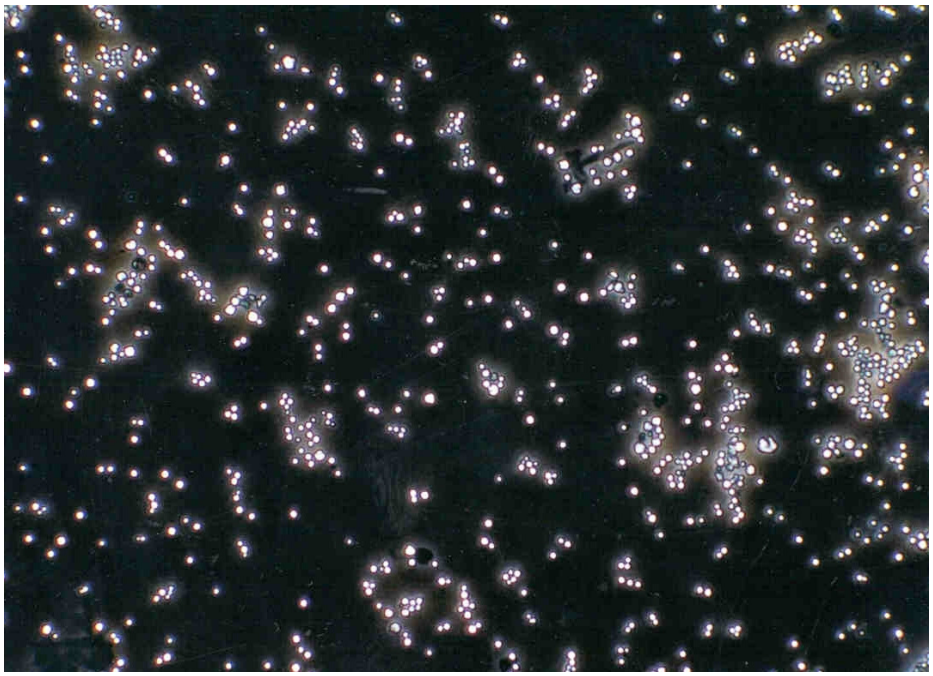
**B**

NPV infected larvae of *H. armigera* on pigeonpea (**A**) and *A. albistriga* on groundnut (**B**).

**Table 7: Yield of NPVs obtained during mass multiplication**

| NPV | Age of the larvae used for virus inoculation | Concentration of virus used for inoculation | Virus yield (±STDEV) | |
|---|---|---|---|---|
| | | | POBs/ml | POBs/larva |
| HaNPV | 4th instar (Field collected / lab reared) | $10^8$ POBs/ml | **Field collected larvae:** | |
| | | | $14.38 \pm 0.32 \times 10^9$ | $5.35 \pm 0.31 \times 10^9$ |
| | | | **Lab reared larvae:** | |
| | | | $12.47 \pm 0.7 \times 10^9$ | $5.18 \pm 0.45 \times 10^9$ |
| SlNPV | 5th instar (Lab reared) | $10^8$ POBs/ml | $14.45 \pm 0.28 \times 10^9$ | $5.73 \pm 0.17 \times 10^9$ |
| AmalNPV | 5th instar (Lab reared) | $10^8$ POBs/ml | $18.79 \pm 0.37 \times 10^9$ | $7.90 \pm 0.54 \times 10^9$ |

**Figure 13: Enumeration of poly occlusion bodies (POBs) under phase-contrast microscope (1000 X)**



POBs of NPVs were purified by differential centrifugation and enumerated under phase-contrast microscope at 1000 X magnification.

## 4.2 Electron Microscopic (EM) Studies:

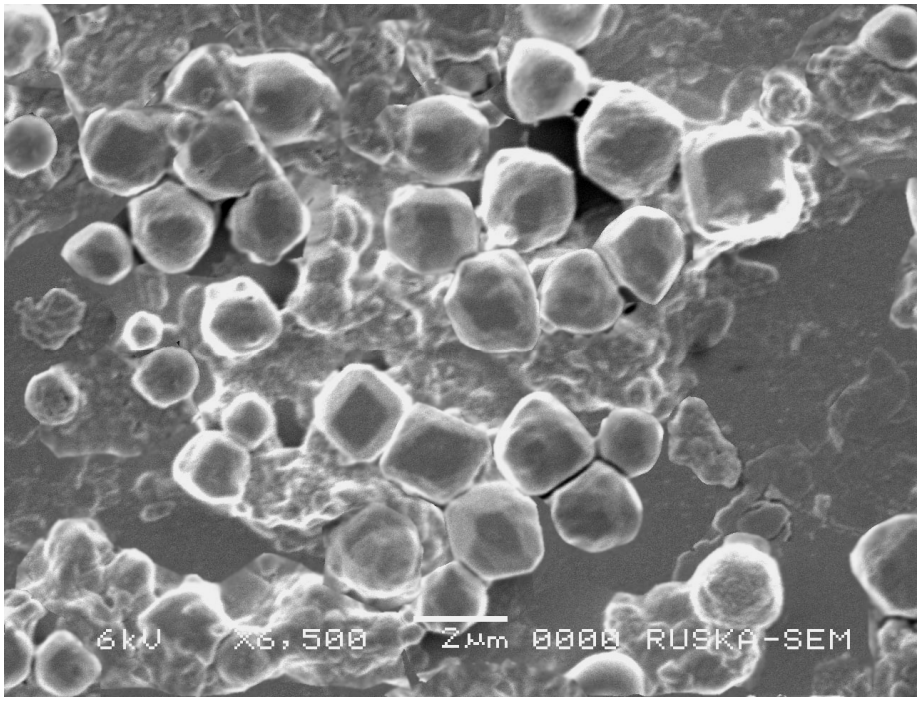
Electron microscopic (EM) studies of NPVs isolated from *H. armigera*; *S. litura* and *A. albistriga* revealed typical baculovirus occlusion bodies (OBs) with rod shaped nucleocapsids (NCs). The details of the EM study results were presented in Table 8. Under scanning electron microscope the POBs of HaNPV and SlNPV appeared as crystalline structures of variable shapes of size 0.5 to 2.5µm and 0.9 to 2.92µm in diameter (Figs 14 and 15); the POBs of AmalNPV were of 1.0 to 2.0µm in diameter (Fig 16). Under transmission electron microscope the cross-sectioned POBs revealed multiple nucleocapsids in each envelop, which were of bacilliform shaped structures of 277.7 × 41.6nm (HaNPV), 285.7 × 34.2nm (SlNPV) and 228.5 × 22.8nm (AmalNPV) in size. The POBs of HaNPV, and AmalNPV contained 2 to 6, (Figs 17 and 19) and SlNPV contained 5 to7 nucleocapsids per envelope (Fig 18).

**Table 8: Electron microscopic studies of NPVs**

| Virus | SEM | | TEM | | |
|---|---|---|---|---|---|
| | **Shape of OB** | **Size of OB** | **Type of virion** | **No of NCs/envelope** | **Dimensions of NC** |
| **HaNPV** | Irregular | 0.5 – 2.5µm | Multiple enveloped | 2 - 6 | 277.7 × 41.6nm |
| **SlNPV** | Irregular | 0.92 –2.92µm | Multiple enveloped | 5 - 7 | 285.7 × 34.2nm |
| **AmalNPV** | Irregular | 1.0 – 2.0µm | Multiple enveloped | 2 - 6 | 228.5 × 22.8nm |

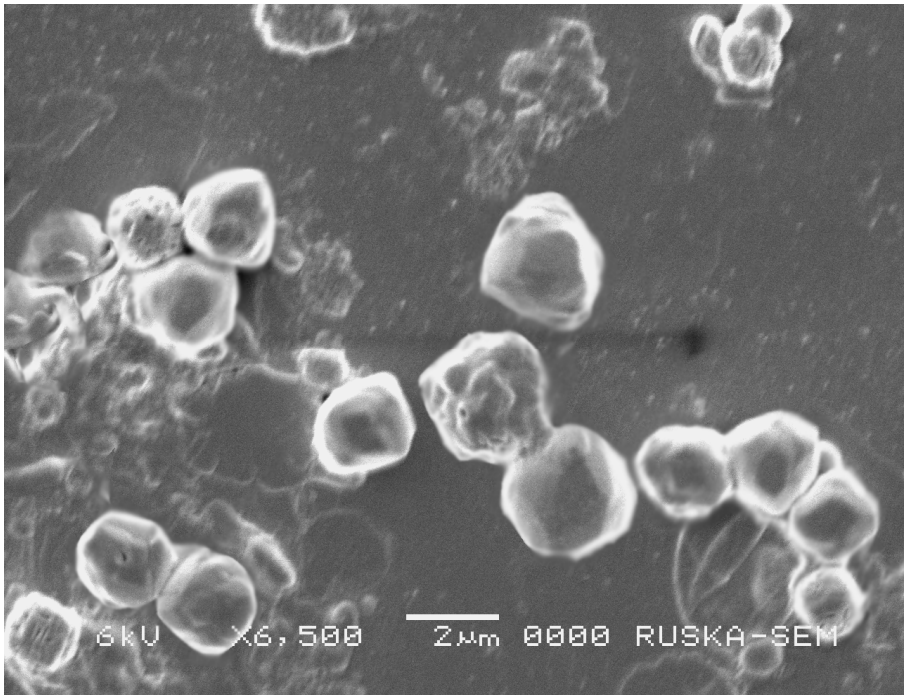
***SEM-****Scanning electron micrograph,* ***TEM****- Transmission electron micrograph;*
***OB****- Occlusion bodies;* ***NC****- Nucleocapsid.*

**Figure 14: Scanning electron micrograph (SEM) showing *H. armigera* nucleopolyhedrovirus (HaNPV) polyhedra purified by differential centrifugation (6, 500 X).**



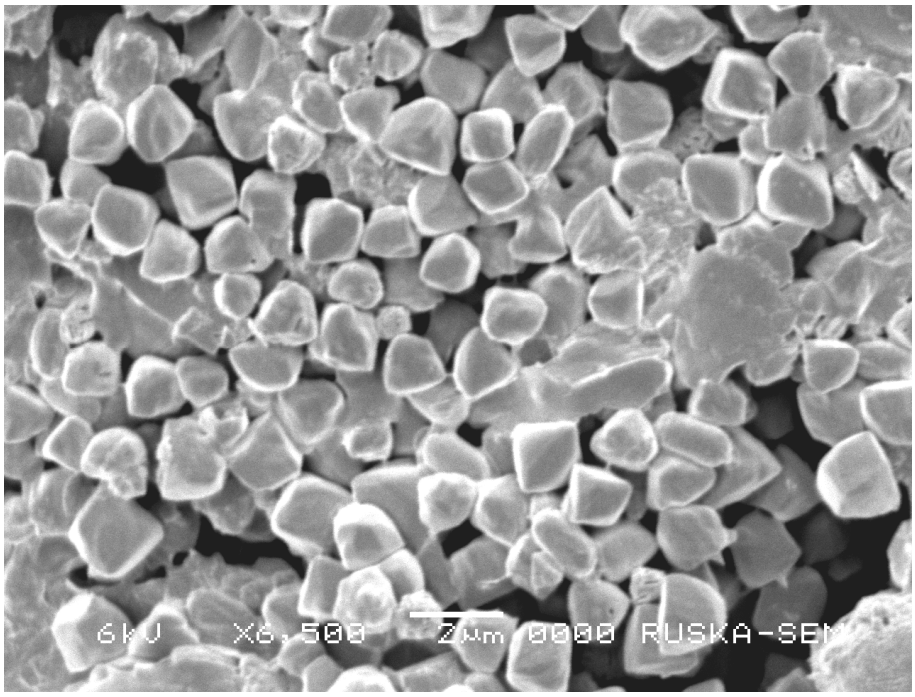

The purified aqueous POBs of HaNPV were dehydrated, mounted over the stubs, applied a thin layer of gold metal over the sample using sputter coater and then scanned under EM. Bar = 2µm.

**Figure 15: Scanning electron micrograph (SEM) showing *S. litura* nucleopolyhedrovirus (SlNPV) polyhedra purified by differential centrifugation (6, 500 X).**



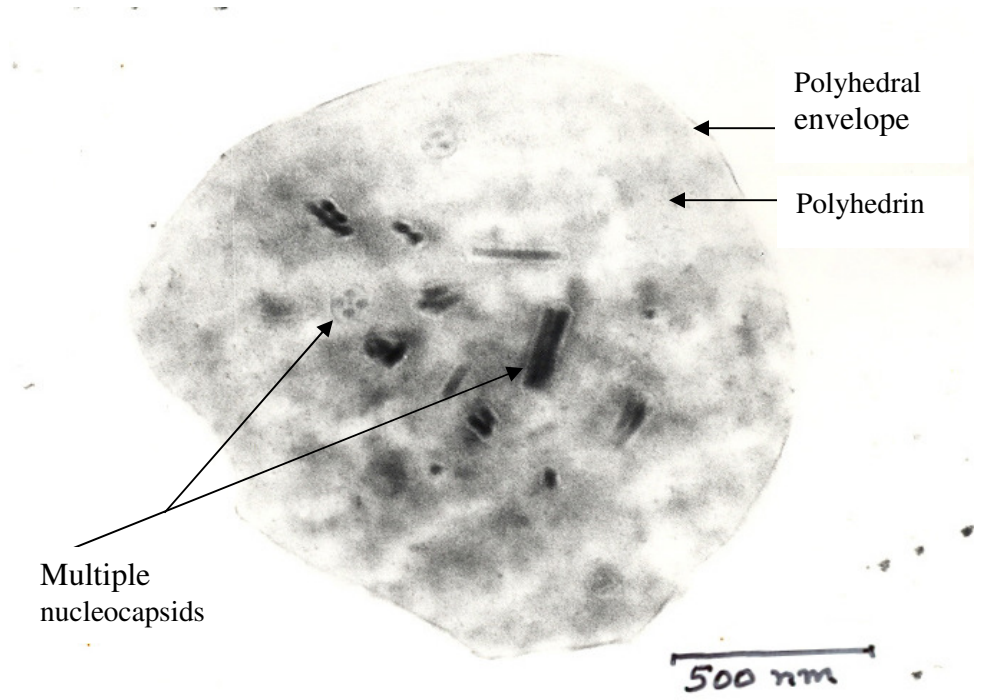

The purified aqueous POBs of SlNPV were dehydrated, mounted over the stubs, applied a thin layer of gold metal over the sample using sputter coater and then scanned under EM. Bar = 2µm.

**Figure 16: Scanning electron micrograph (SEM) showing *A. albistriga* nucleopolyhedrovirus (AmalNPV) polyhedra purified by differential centrifugation (6, 500 X).**




The purified aqueous POBs of AmalNPV were dehydrated, mounted over the stubs, applied a thin layer of gold metal over the sample using sputter coater and then scanned under EM. Bar = 2µm.

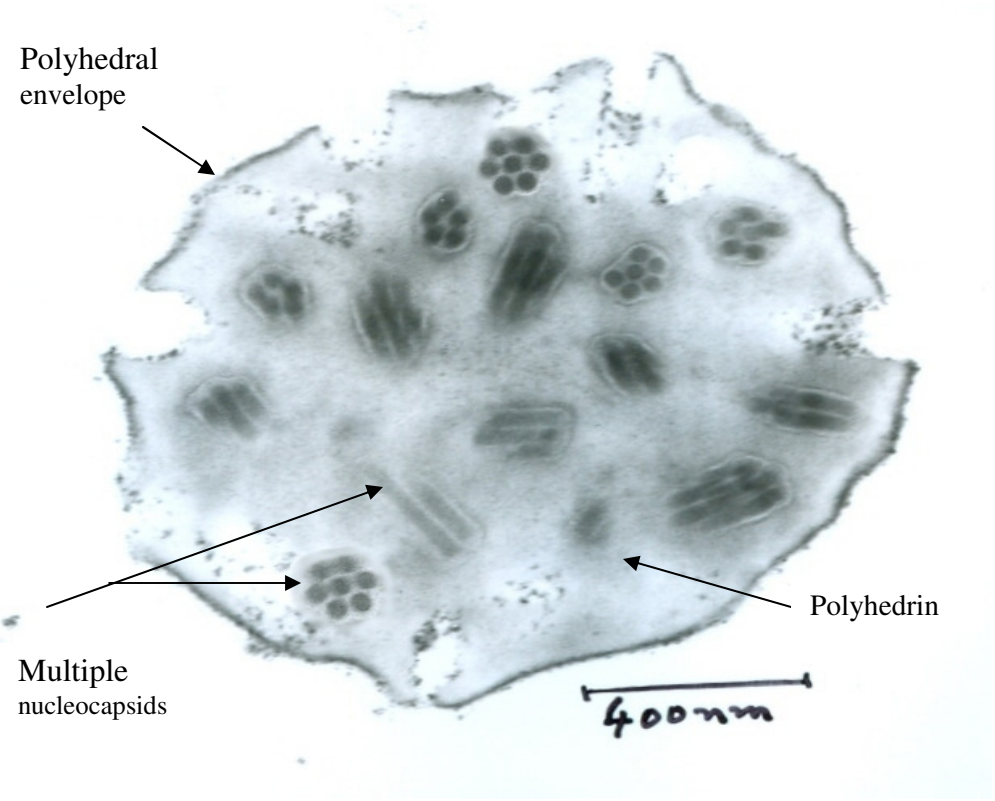
**Figure 17: Transmission electron micrograph (TEM) of cross section of polyhedra (POB) of *H. armigera* nucleopolyhedrovirus (HaNPV).**




Pellet of HaNPV POBs was subjected to ultra thin sections, mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with 4% Lead citrate and observed under TEM. Magnification = 20, 000 and Bar = 500 nm.

Details of polyhedra showing multiple nucleocapsids surrounded by a single membrane. The polyhedron envelope, the rod- like nucleocapsids and major occlusion body protein or matrix protein (polyhedrin) are indicated by arrow marks.

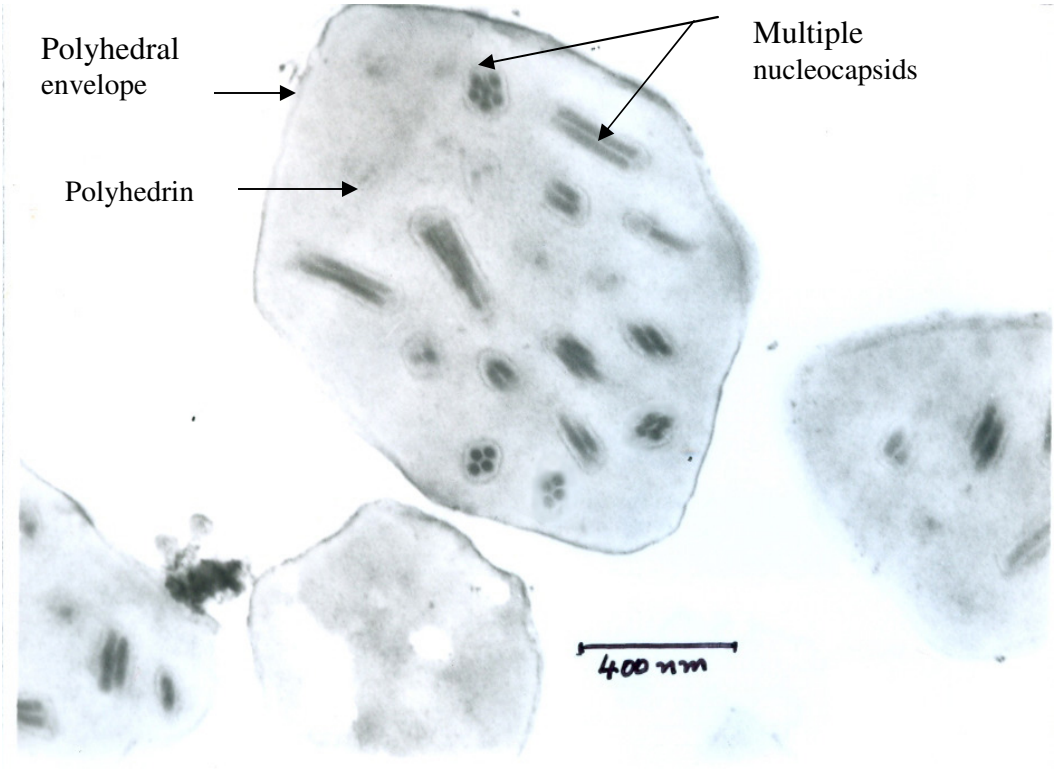
**Figure 18: Transmission electron micrograph (TEM) of cross section of polyhedra (POB) of *S. litura* nucleopolyhedrovirus (SlNPV).**




Pellet of SlNPV POBs was subjected to ultra thin sections, mounted on copper grids and stained with saturated aqueous Uranyl acetate and counter stained with 4% Lead citrate and observed under TEM. Magnification = 25,000 and Bar = 400 nm.

Details of polyhedra showing multiple nucleocapsids surrounded by a single membrane. The polyhedron envelope, the rod-like nucleocapsids and major occlusion body protein or matrix protein (polyhedrin) are indicated by arrow marks.

**Figure 19: Transmission electron micrograph (TEM) of cross section of polyhedra (POB) of *A. albistriga* nucleopolyhedrovirus (AmalNPV).**




Pellet of AmalNPV POBs was subjected to ultra thin sections, mounted on copper grids and stained with saturated aqueous Uranyl acetate and counter stained with 4% Lead citrate and observed under TEM. Magnification = 25,000 and Bar = 400 nm.

Details of polyhedra showing multiple nucleocapsids surrounded by a single membrane. The polyhedron envelope, the rod- like nucleocapsids and major occlusion body protein or matrix protein (polyhedrin) are indicated by arrow marks.

## 4.3 Purification of Polyhedral Protein (Polyhedrin) of NPVs:

Purification protocol for polyhedrin protein of NPVs was standardized by the following steps: initial heat inactivation of endogenous proteases; alkali disruption of POBs and release of virions, and ultracentrifugation to pellet virions. Further purification was achieved by either of the following approaches: (i) in this approach further purification of polyhedrin was achieved through centrifugation by layering on 10-40% liner sucrose gradient; and (ii) in this approach further purification of polyhedrin was achieved through precipitation of polyhedrin at isoelectric pH.

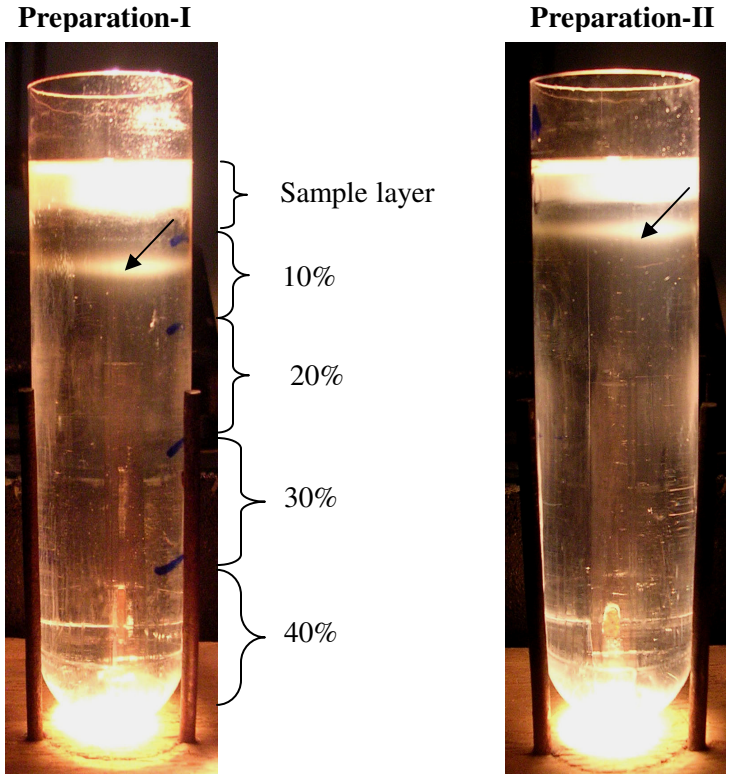
In 10-40% linear sucrose gradient centrifugation, the polyhedrin formed one diffused light scattered zone in 10% sucrose region (Fig 20). In isoelectric precipitation method the polyhedrin of all the three NPVs was precipitated at pH between 5.5 and 5.6. The precipitated polyhedrins of HaNPV, SlNPV and AmalNPV were settled as sediments at the bottom of the beakers were showed in Fig 22.

In 12% SDS-PAGE analysis, samples derived from light scattered zone as well as sample layers of sucrose gradients revealed that both samples were equally pure and proteins resolved as single band of estimated molecular weight ~31 kDa (HaNPV) (Fig 21). Similarly, polyhedrin purified by isoelectric precipitation method reveled that the molecular weight of major polyhedrin proteins of three NPVs were 31.65 kDa (± 0.00) (HaNPV), 31.29 kDa (± 0.00) (SlNPV) and 31.67 kDa (± 0.295) (AmalNPV), respectively. In addition, these preparations contained some minor molecular weight peptides of about 7-27 kDa and a high molecular weight peptide of about 60-70 kDa fragment (Fig 23). This has revealed that three NPVs have 6-8 minor polypeptides.

The yield of the polyhedrin obtained in sucrose gradient method was 1mg/ml, while the isoelectric precipitation method was about 15-20mg/ml from standard POB preparations ($10^9$ POB/ml). Due to lack of consistency in the purity and quality of the polyhedrin preparation in both the methods, the polyhedrin was electro-eluted from 10% SDS-PAGE for immunization purpose. In 12% SDS-PAGE the electro-eluted polyhedrin was appeared as single protein band

without any degraded peptides (Fig 24). Before immunization the polyhedrin was electro-eluted freshly. The purified polyhedrin preparations (of both methods) were aliquoted in to small tubes and stored at -20$^0$C for further use.

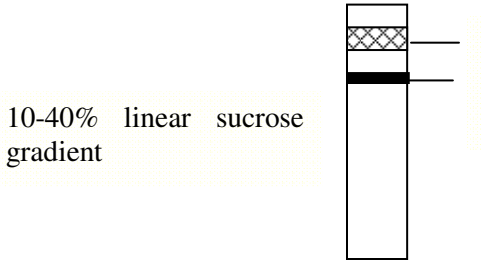
**Figure 20: Purification of polyhedrin protein through 10-40% linear sucrose gradients**

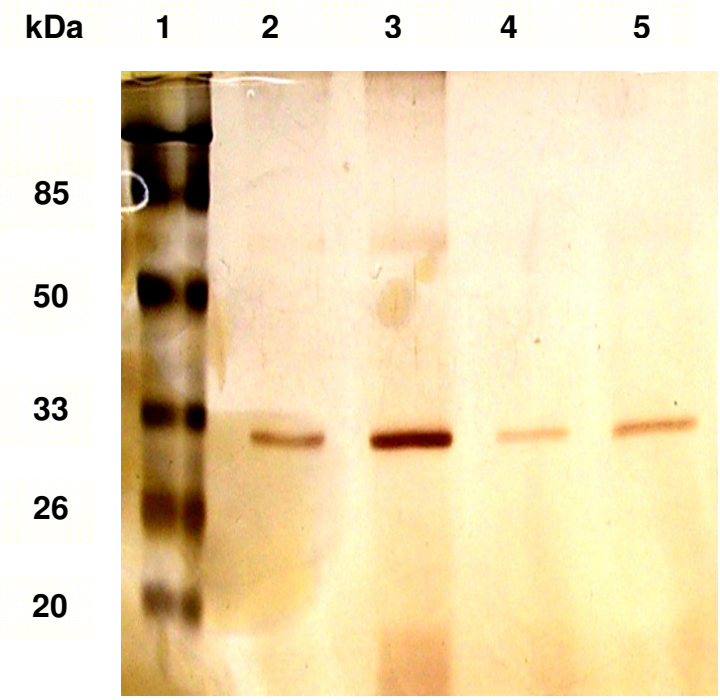



Purification of polyhedrin protein from NPVs in 10-40% linear sucrose gradients resulted in single light scattering zone in 10% sucrose region. Length of sucrose gradient and sample layered on it are indicated. Position of light scattering zones in both the preparations (I and II) are indicated with arrows.

**Figure 21: Separation of HaNPV polyhedrin collected from sucrose gradient in 12% SDS-PAGE**

Lane 1, Protein molecular weight marker
Lane 2, Light scattered zone (Preparation I)
Lane 3, Light scattered zone (Preparation II)
Lane 4, Sample layer (preparation I)
Lane 5, Sample layer (Preparation II)




**B) Silver stained 12% SDS -PAGE gel**

Light scatter zone and sample layer were collected and separated in 12%SDS- PAGE and the gel was silver stained (**B**). Sizes of protein molecular weight marker (kDa) are indicated. Pictorial representation of sample separation in sucrose gradient is shown in **A**.

**Figure 22: Isoelectric precipitation of NPV polyhedrin**


Purification of polyhedrin by isoelectric precipitation method. The polyhedrins of HaNPV (**A**), SlNPV (**B**) and AmalNPV(**C**) were precipitated at their isoelectric points and settled as sediment at the bottom of beaker.

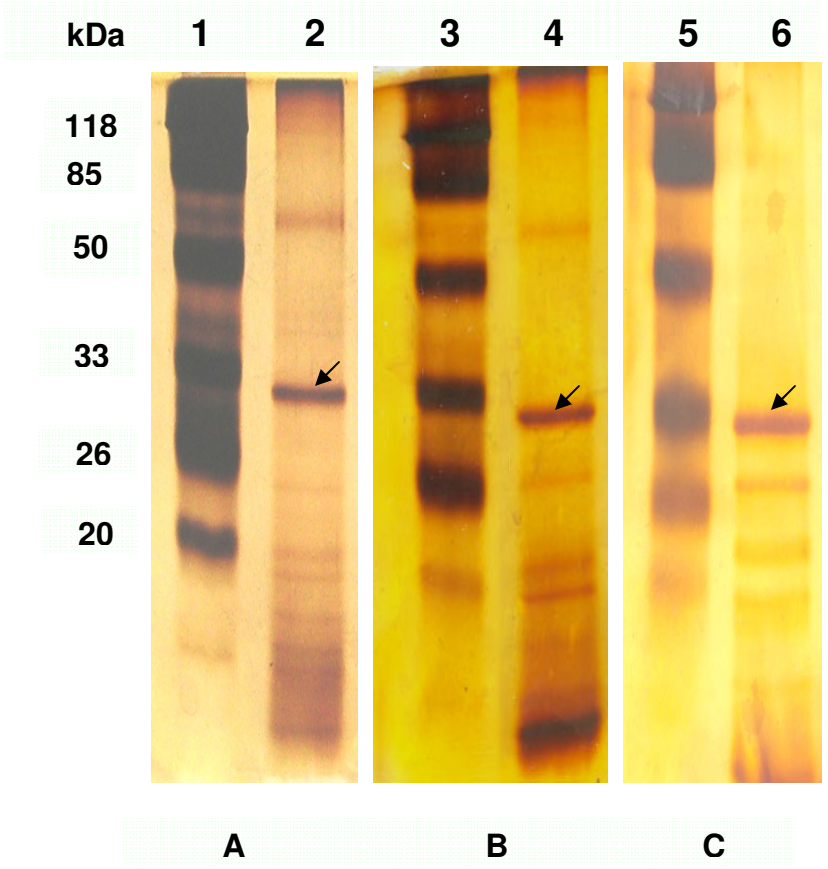
**Figure 23: 12% SDS-PAGE profiles of isoelectric precipitated polyhedrin protein preparations**

Lanes 1, 3 and 5 Protein molecular weight markers

Lane 2, HaNPV polyhedrin preparation

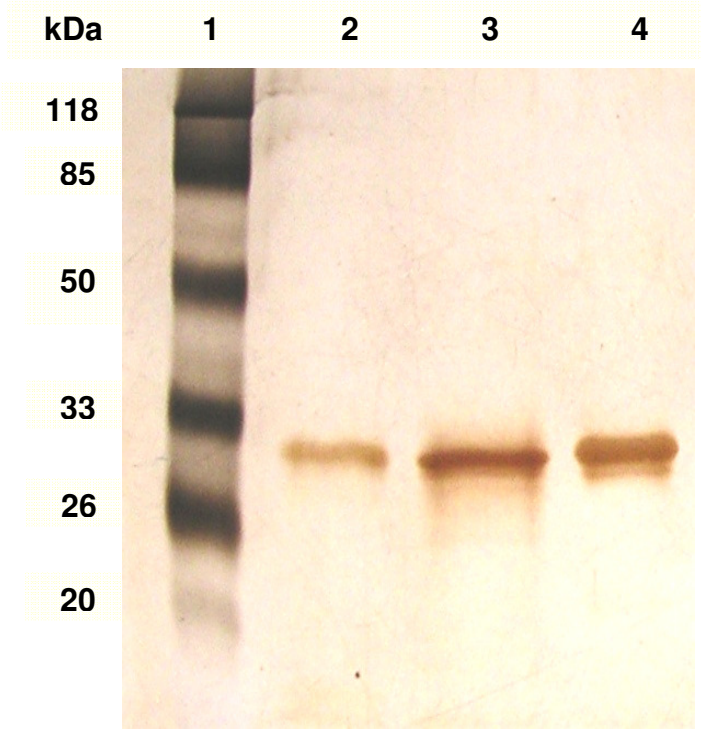
Lane 4, SlNPV polyhedrin preparation

Lane 6, AmalNPV polyhedrin preparation




Purification of polyhedral protein of HaNPV (**A**), SlNPV (**B**) and AmalNPV (**C**) by isoelectric precipitation method. The purity and integrity of the preparations were checked in 12% SDS-PAGE and the gels were silver stained. Sizes of protein molecular weight marker (kDa) are indicated. The major polyhedrin protein band at 31 kDa was indicated with arrow marks. Several minor polypeptides were also detected in three preparations.

**Figure 24: 12 % SDS-PAGE profile of electro-eluted polyhedrin**




Lane 1, Molecular weight marker,
Lane 2, HaNPV polyhedrin,
Lane 3, SlNPV polyhedrin,
Lane 4, AmalNPV polyhedrin

Polyhedrin band from a preparative 10% SDS-PAGE was excised and electro-eluted. The eluted polyhedrin (100-200µg) was seperated in 12% SDS-PAGE. The polyhedrin was appeared as single protein band in silver stained gel. Sizes of protein molecular weight marker (kDa) are indicated.

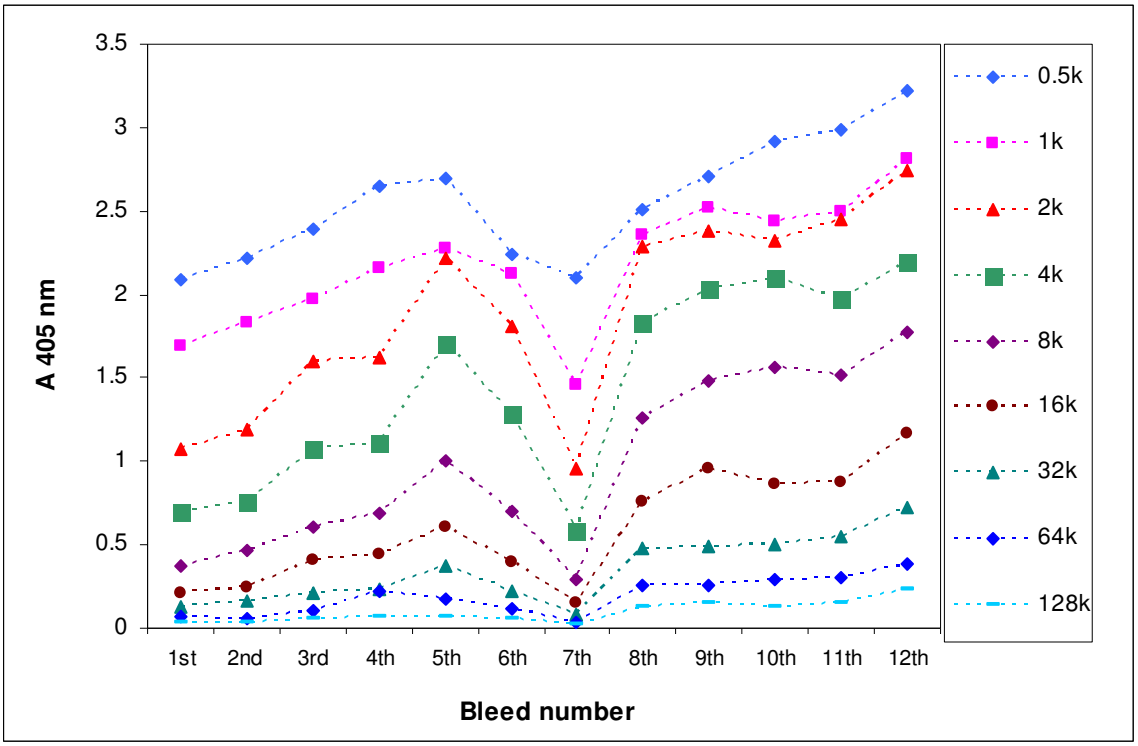
## 4.4 Production of Polyclonal Antibodies against Polyhedrin of NPVs:

To produce the polyclonal antibodies against the polyhedrins of HaNPV, SlNPV and AmalNPV, 500 µg of the electro-eluted polyhedrin was used as antigen to immunize the New Zealand White inbreed rabbits. The reactivity and the antibody titers of the bleeds were determined by DAC-ELISA reveled that the protocol used for immunization gave a good immune response in rabbits.

The DAC-ELISA results indicated that the polyclonal antibodies are highly reactive with the polyhedrin and the antibody titer in the bleeds was gradually increased up to 5 weeks (up to $5^{th}$ bleed) and then declined. After booster dose (from $8^{th}$ bleed onwards) antibody titer was increased. The absorbance readings of different bleeds of antiserum (against HaNPV-polyhedrin) at various dilutions were represented in the Fig 25.

The concentration (500µg) of polyhedrin of three NPVs used for immunization gave an antibody titer of 1:5000 dilution, 18 weeks after initiation of immunization. The antibody titer of each bleed was determined; the working dilution was optimized and labeled on each vial. The antisera vials were lyophilized and stored at $-30^{0}$C for further use.

**Figure 25: The antibody titer of polyhedrin polyclonal antiserum**



## 4.5 Characterization of Polyhedrin-Polyclonal Antibodies:

Polyclonal antibodies produced against the polyhedrin of HaNPV, SlNPV and AmalNPV were characterized by determining the specificity of antisera to detect their respective polyhedrins and investigated their ability to cross-react with other two heterologous polyhedrins.

The specificity was determined by Western immunoblotting analysis with three forms of polyhedrin [1. isoelectric precipitated polyhedrin (IPP) 2. electro-eluted polyhedrin (EP) and 3. Entire POB particle proteins (EPP)] and healthy larval proteins (HLP). The cross-reactivity of the antibodies was determined by DAC-ELISA and Western immunoblotting analysis of EP.

### 4.5.1 Specificity of antisera:

In western immunoblotting all three antibodies were specifically reacted with polyhedrin (31 kDa) and did not cross-reacted with HLP (Fig 26) indicating that the antibodies are highly specific to polyhedrin. The concentration of polyhedrin detected by polyhedrin polyclonal antibodies of three NPVs was 50-100µg of total protein in 10µl of isoelectric precipitations or electro-eluted polyhedrin preparations and 200µg of total protein concentration in 10µl of crude POB solutions.

The molecular weight of major polyhedrin protein belongs to three NPVs recognized by their respective antibodies was as follows: In HaNPV, 31.55 kDa in EPP, 31.27 kDa in IPP and 31.2 kDa in EP. In SlNPV and AmalNPV, the antibodies recognized the polyhedrin with similar molecular weights in three forms of polyhedrin (i.e. EPP, IPP and EP); they are 31.77 in SlNPV and 30.95 kDa in AmalNPV. Antibodies recognized some low molecular weight bands, which could be degraded polyhedrin peptides.

The low molecular weight proteins (molecular weights below polyhedrin protein) recognized by polyhedrin polyclonal antibodies were as follows: The antibodies of HaNPV polyhedrin detected the low molecular weight proteins with sizes of 26.64 kDa in IPP, 24.75 kDa in EPP, 20.47kDa in IPP, 16.49 in IPP, 15.14 in EPP and 11.43 in EP. Similarly, the antibodies of

SlNPV polyhedrin detected the low molecular weight proteins with size of 26.1 kDa, 24.57 kDa and 18.17 kDa in EPP and 16.91 kDa in IPP. Whereas the antibodies of AmalNPV polyhedrin detected the low molecular weight proteins with sizes of 26.54 kDa, 22.49 kDa and 18.74 kDa in IPP and 26.32 kDa, 18.86 kDa and 15.37 kDa in EP.

### 4.5.2 Cross-reactivity of antisera:

The cross-reactivity of the antisera was determined by DAC-ELISA and western blotting analysis of electro-eluted polyhedrin (EP). The antiserum with maximum antibody titer (i.e. after booster dose) was used for this study.

#### 4.5.2.1 DAC-ELISA:

The DAC-ELISA was performed essentially by two different ways to characterize the polyclonal antibodies as detailed below:

##### 4.5.2.1.1 Reciprocal test to determine the cross reactivity of the three polyclonal antisera:

DAC-ELISA performed to determine the cross reactivity of antiserum in detecting other NPVs, each antiserum showed strong cross reactivity with other two heterologous polyhedrins. The antibodies at 1:5000 dilutions were able to detect minimum 10-15ng/ml and maximum 1000ng/ml of their homologous polyhedrin and minimum 25-30ng/ml and maximum 1500-2000ng/ml of heterologous polyhedrins. Fig 27 showing the extent of cross-reactivity of fixed homologous antiserum dilution with variable concentrations of heterologous polyhedrins.

##### 4.5.2.1.2 Reciprocal DAC-ELISA with fixed heterologous polyhedrin concentration *vs.* variable homologous antiserum dilutions:

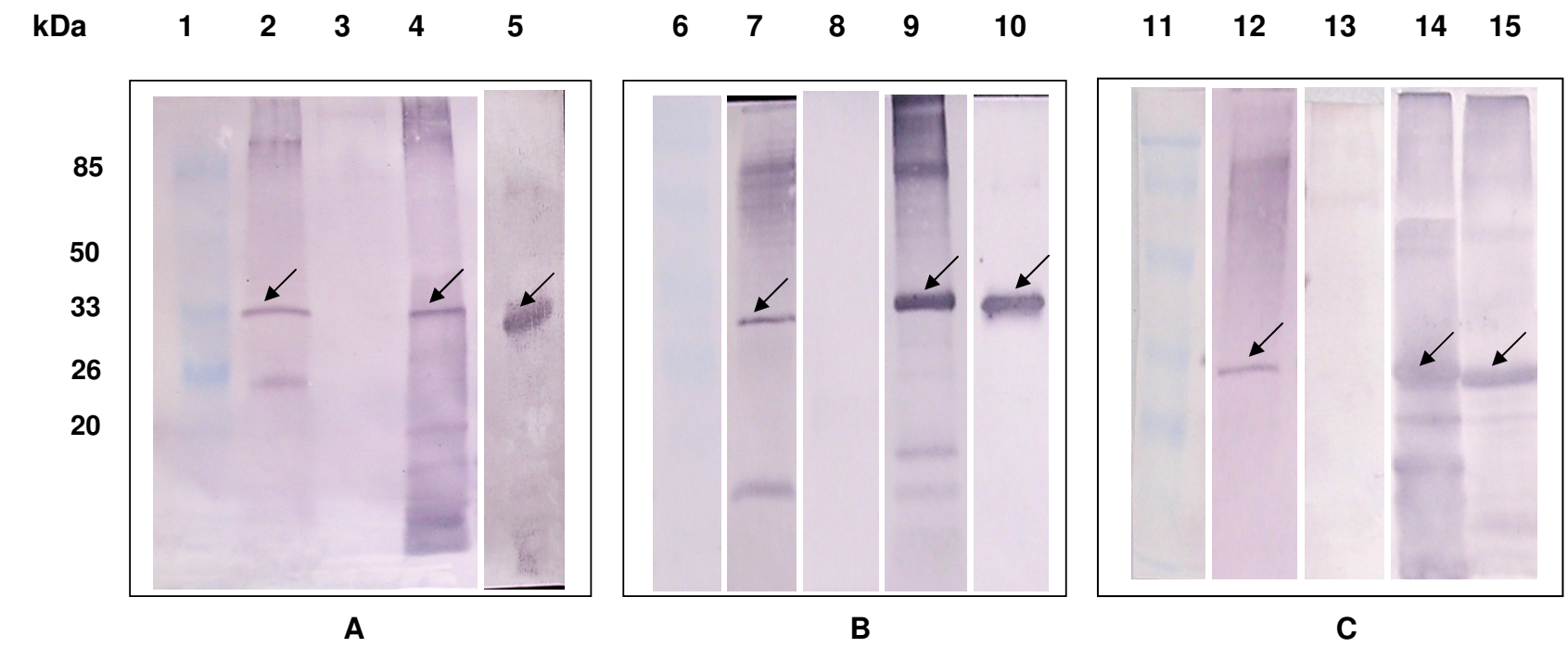
In reciprocal DAC-ELISA at fixed heterologous polyhedrin concentration (1000 ng/ml) and variable dilutions of homologous polyhedrin polyclonal antisera (1:1000 to 1: 40,000), the polyclonal antiserum of each NPV showed strong cross-reactivity with other two heterologous polyhedrins. The absorbance reading at particular dilution of the antiserum gave minimum of 1.0 OD or above within 1 h was considered as the maximum dilution of the antiserum to detect

polyhedrin. The HaNPV polyhedrin antiserum detected the homologous polyhedrin at 1: 25000 antiserum dilution and at the same the heterologous polyhedrins were detected at 1:15000 dilution. The SlNPV polyhedrin antiserum detected the homologous polyhedrin at 1:40000 dilution of antiserum and heterologous polyhedrins were detected at 1:25000 dilution. Similarly, the AmalNPV polyhedrin antiserum detected the homologous polyhedrins at 1:30000 dilution of antiserum and heterologous polyhedrins were detected at 1:15000 dilution. Fig 28 showing the extent cross-reactivity of the fixed concentration of heterologous polyhedrins at different dilutions of homologous antiserum. The combination of 1000ng/ml of polyhedrin concentration and 1:40,000 dilution of antiserum, the cross-reactive curves were diverged from each other, low level of cross-reactivity was observed and the antibodies were able to distinguish the homologous and heterologous polyhedrins. The combination at 1000ng/ml of polyhedrin concentration and 1:1000 dilution of antiserum, the cross-reactive curves were converged together, high level of cross-reactivity was observed and the antibodies were unable to distinguish the homologous and heterologous polyhedrins (Fig 28).

#### 4.5.2.2 Western immunoblotting:

In western immunoblotting the polyhedrin polyclonal antibodies were recognized both homologous and heterologous polyhedrins to a great extent indicates that the antibodies have strong cross-reactivity with heterologous polyhedrins (Fig 29).

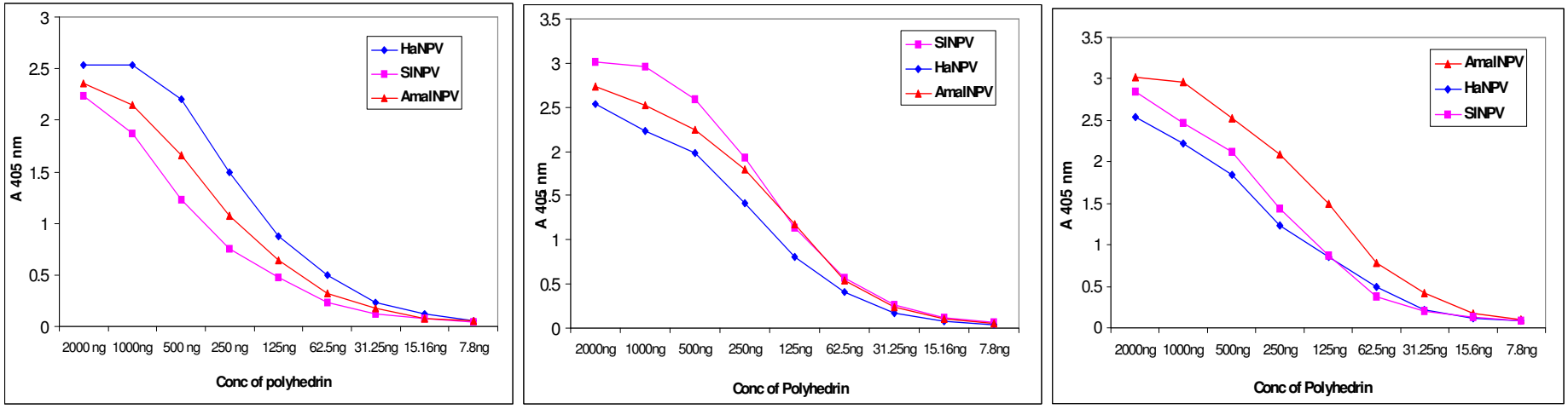
**Figure 26: Polyclonal antiserum showing the specific reactivity with polyhedrin protein.**




Lanes 1, 6 and 11, Protein molecular weight markers; Lanes 2, 7 and 12, Entire POB particles; Lanes 3, 8 and 13, healthy larval proteins; Lanes 4, 9 and 14, iso-electric precipitated polyhedrin; Lanes 5, 10 and 15, Electro-eluted polyhedrin.

In western immunoblotting, the polyclonal antibodies produced against polyhedrin protein of HaNPV (**A**), SlNPV (**B**) and AmalNPV(**C**) are specifically recognized the polyhedrin and are able to detect the polyhedrin in three forms such as polyhedrin in entire POB particle (EPP), iso-electric precipitated polyhedrin (IPP) and electro-eluted polyhedrin (EP) (rabbit injected polyhedrin). In addition to major polyhedrin, they also recognized some minor low and high molecular weight peptides. Sizes of the protein molecular weight marker was indicated. The major polyhedrin (31kDa) was indicated with arrow marks. Some minor low molecular weight bands recognized by antibodies could be degraded polyhedrin peptides.

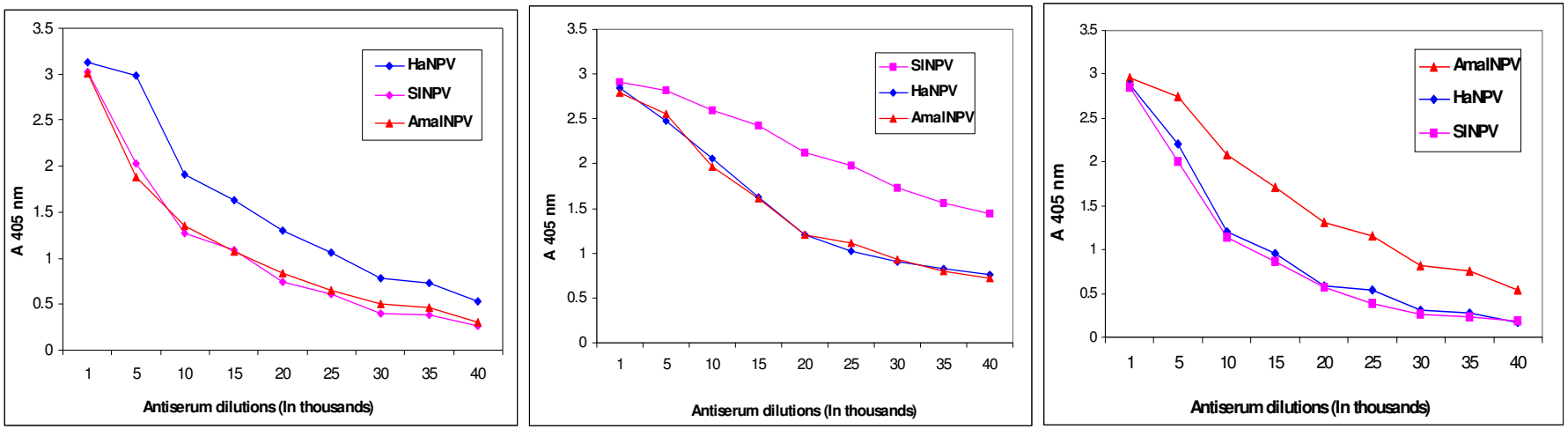
**Figure 27: Reciprocal DAC-ELISA to determine the extent of HaNPV, SlNPV and AmalNPV polyhedrin antibody cross-reaction**




**A** **B** **C**

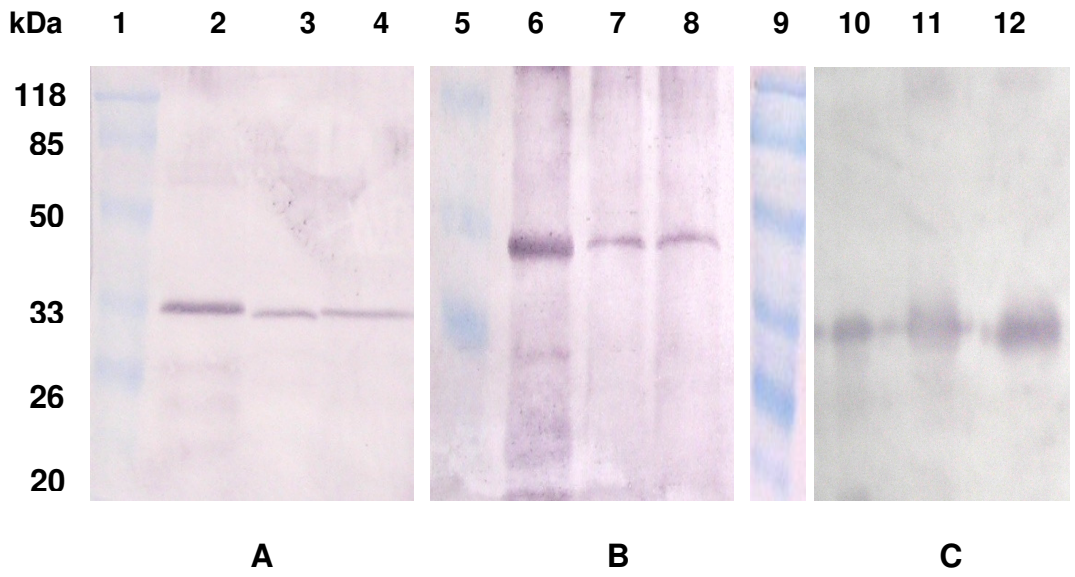
The homologous polyhedrin polyclonal antiserum of HaNPV (**A**), SlNPV (**B**) and AmalNPV (**C**) at fixed dilutions (1:5000) showing different degrees of cross reactivity with variable concentrations (2000 - 7.8ng) of heterologous polyhedrins.

**Figure 28: Reciprocal DAC-ELISA with fixed heterologous polyhedrin concentration *vs.* variable homologous antiserum dilutions**




The homologous polyhedrin polyclonal antiserum of HaNPV (**A**), SlNPV (**B**) and AmalNPV (**C**) at variable dilutions (1:1000 to 1:40,000) showing different degrees of cross-reactivity with fixed concentration (1000 ng/ml) of heterologous polyhedrins.

**Figure 29: Cross-reactivity of homologous polyhedrin polyclonal antiserum with heterologous polyhedrins in western immunoblotting.**




The homologous polyhedrin polyclonal antiserum of HaNPV (**A**), SlNPV (**B**) and AmalNPV(**C**) showed strong cross-reactivity with heterologous polyhedrins in Western immunoblotting analysis.

Lanes 1, 5 and 9, Protein molecular weight markers.

Lane 2, HaNPV polyhedrin
Lane 3, SlNPV polyhedrin
Lane 4, AmalNPV polyhedrin } A (HaNPV antiserum)

Lane 6, SlNPV polyhedrin
Lane 7, HaNPV polyhedrin
Lane 8, AmalNPV polyhedrin } B (SlNPV antiserum)

Lane 10, SlNPV polyhedrin
Lane 11, HaNPV polyhedrin
Lane 12, AmalNPV Polyhedrin } C (AmalNPV antiserum)

## 4.6 Development and Evaluation Diagnostic Tools for NPVs:

By using the polyhedrin polyclonal antibodies various immunochemical tools were developed and evaluated for the detection and quality control of NPVs.

## 4.6.1 Development of Diagnostic Tools:

The ability of the polyhedrin polyclonal antibodies to detect the polyhedrin in laboratory infected insect extracts was determined using artificially infected larvae, with uninfected larvae as controls.

### 4.6.1.1 Infection of larvae:

Healthy $4^{th}$ and $5^{th}$ instar larvae of *H. armigera, S. litura* and *A. albistriga* were infected with respective NPVs at >50% lethal doses ($10^8$ POBs/ml). The symptoms of NPV infection were observed from $4^{th}$ day after infection; the infected larvae reacted slowly to tactile stimuli and appear swollen, glossy and moribund. From $6^{th}$ day onwards the diseased larvae stopped feeding. On $7^{th}$ to $10^{th}$ days, most of the diseased larvae were dead and liquefied. Some larvae were survived after $10^{th}$ day and pupated. After $10^{th}$ day most of the dead larvae were putrefied and emitted the malodour. The infected larvae (live and dead), pupae and uninfected healthy larvae were sampled, homogenized and extracted the polyhedrin. Total protein concentrations of the larval extracts were listed in Table 12. The results of 12% SDS-PAGE assay of healthy and infected protein extracts, western immunoassay, indirect immunofluorescence assay of healthy and infected larvae and their evaluation in DAC and IC-ELISA were detailed below.

### 4.6.1.2 Separation of healthy and infected larval proteins in 12% SDS-PAGE:

Following separation of larval proteins in 12% SDS-PAGE there were some common proteins observed among healthy and infected larval extracts of these insect species. Some of them are present in both healthy and infected extracts but some are specific to healthy and infected larval extracts. In NPV infected larval extracts of three insect species, a highly expressed

protein (polyhedrin) with molecular weight of ~31 kDa was observed. The estimated molecular weights of polyhedrins are as follows: 31.0 kDa in *H armigera* and 31.3 kDa in *S. litura* and *A. albistriga* infected larval extracts.

#### 4.6.1.3 Western immunoassay for detection of POBs in larval extracts:

Analysis of healthy and infected larval homogenates showed that the polyhedrin polyclonal antibodies specifically detected a single polyhedron protein but not reacted with healthy larval homogenates, indicating antibody specificity to polyhedrin (Fig 30). Some times the antibodies recognized the minor polyhedrin fragments of sizes about 27kDa, which appears to be degraded polyhedron proteins (Fig 30C).

#### 4.6.1.4 Indirect immunofluorescence assay for the detection of POBs in larval extracts:

The efficacy of antibodies to react with POBs in infected larval homogenates was tested by indirect immunofluorescence assay. Fig 31 shows the stained POBs in infected larval homogenate of *H. armigera* that was probed with the *Ha*NPV polyclonal antibodies. Some times in infected homogenates, both POBs and dissolved polyhedrins were uniformly stained. There was no detectable staining (fluorescence) in the controls.

#### 4.6.1.5 Standardization of DAC-ELISA for quantitative detection of polyhedrin in larval extracts:

In DAC-ELISA the NPV infection was diagnosed successfully. The total protein concentration of 5µg/ml of larval extracts gave the maximum sensitivity to detect the NPV infection. The ELISA readings were considered virus positive if the absorbance values of a sample differed by three-folds than those observed in the healthy insect control. The samples tested in DAC-ELISA were infected larvae of either live, dead or putrefied conditions at $4^{th}$, $5^{th}$ instar and pupal stages. Simultaneously, healthy controls of each stage were also assayed. Based on the absorbance value the severity of the disease or virus titer in the larvae was determined (**Table 9**). To estimate the polyhedrin content in DAC-ELISA purified polyhedrin standards (1000 to 7.8ng/ml), healthy larval extracts (5µg/ml) spiked with purified polyhedrin standards

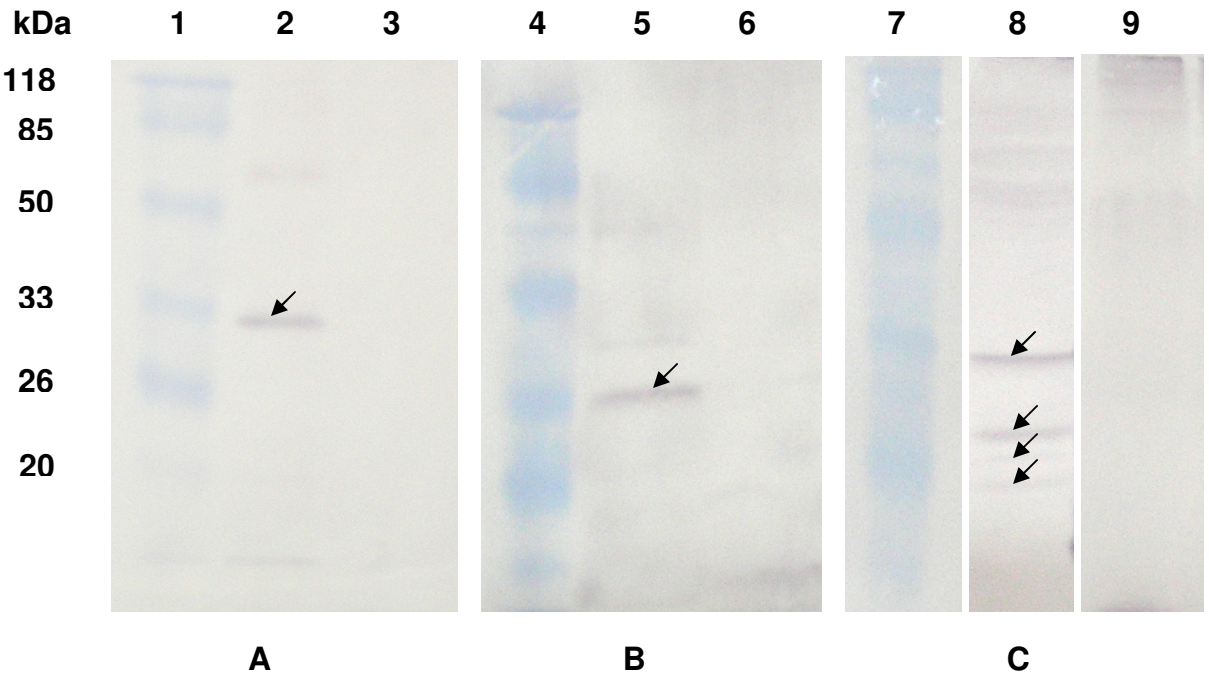
and healthy larval extracts were assayed. All homogenates (healthy and infected extracts) were adjusted to 5µg/ml with coating buffer before being assayed. The difference in the absorbance values of unspiked and spiked standards are indicated in the Fig 32. Antibodies detected the polyhedrin concentration as low as 15ng/ml of purified form and up to 30ng/ml in 5µg/ml of larval extracts. There was no cross-reaction between antibodies and healthy larval extracts (Fig 32). The results from DAC-ELISA were compared with a standard curve determined by serial dilution of polyhedrin spiked in healthy larval proteins.

**Figure 30: Diagnosis of NPV infection by western immunoblotting**

Lanes 1, 4 and 7, protein molecular weight markers,

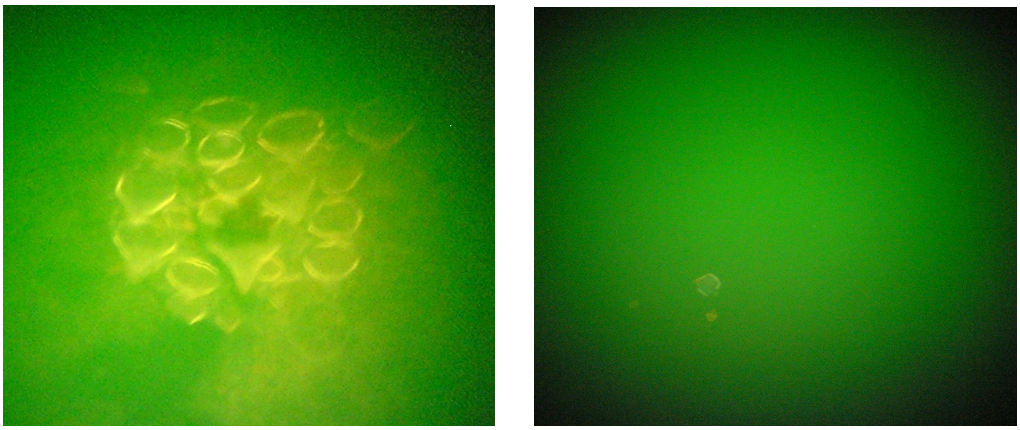
Lanes 2, 5 and 8, infected larval homogenates of *H. armigera, S. litura* and *A. albistriga,*

Lanes 3, 6 and 9, healthy controls of *H. armigera, S. litura* and *A. albisriga.*




Westernimmunoassay of NPV infected and healthy larval homogenates of *H. armigera* (**A**), *S. litura* (**B**) and *A. albistriga* (**C**) shows that the antibodies are specifically detected the POBs by recognizing the single polyhedrin protein of size 31 kDa. Some cases the antibodies recognized the polyhedrin degraded fragments of sizes 27-20 kDa (C).

**Figure 31: Diagnosis of NPV infection by indirect immunofluorescence assay**

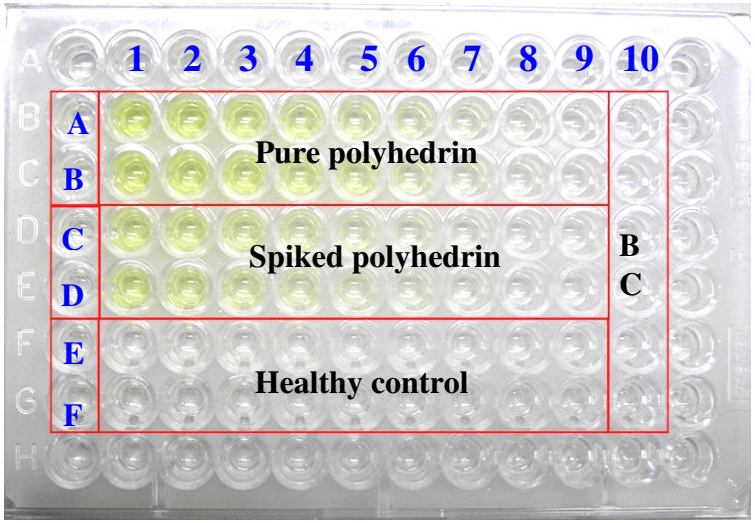


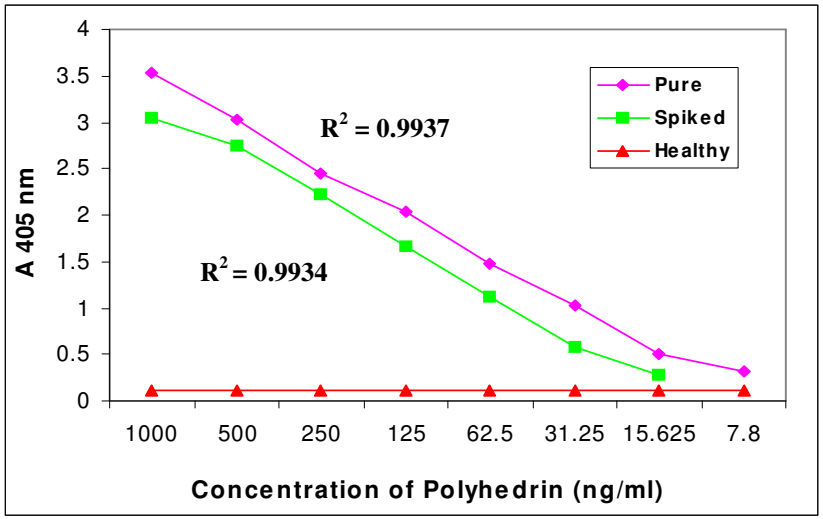
**A** **B**

Reactivity of polyhedrin polyclonal antiserum with POBs in infected larval extracts (**A**) by indirect immunofluorescence using anti-rabbit IgG FITC-conjugate. The specific fluorescence of HaNPV POBs and their disrupted empty polyhedral sacs with polyhedrin polyclonal antiserum observed under fluorescence microscope (Olympus, Model: AX-70) at magnification of (300 X). Same trarment did not resuled any detectable staining (fluorescence) in healthy controls (**B**)

**Figure 32: Quantitative detection of NPV polyhedrin using DAC-ELISA**

**Direct linear relationship between absorbance and polyhedrin concentration**




Lanes 1 to 9 (rows A, B and C, D): polyhedrin 1000 to 7.8ng /ml;
Lane 10 (rows A, B, C, D, E and F): Buffer control (BC)
Rows A and B: Purified polyhedrin;
Rows C and D: Pure polyhedrin artificially spiked into 5 µg/ml of healthy larval extract;
Rows E and F: Healthy control (5 µg/ml)
(A405nm values are directly proportionally to the polyhedrin concentration).

**Table 9: Diagnosis of NPV infection by DAC-ELISA**

| S.No | Age and condition of the larvae | Total protein concentration (mg/ml) | Protein concentration used for DAC-ELISA (µg/ml) | A 405 nm | Symptom severity |
|---|---|---|---|---|---|
| **A. *H. armigera:*** | | | | | |
| 1 | Healthy, 4th instar (live) | 5 | 5 | 0.082 | - |
| 2 | Infected, 4th instar (live) | 16.0 | 5 | 2.802 | ++++ |
| 3 | Infected,4th instar (dead) | 20.62 | 5 | 2.95 | ++++ |
| 4 | Infected, 4th instar (putrefied) | 22.1 | 5 | 3.05 | ++++ |
| 5 | Healthy, 5th instar (live) | 3.58 | 5 | 0.086 | - |
| 6 | Infected, 5th instar (live) | 7.74 | 5 | 0.682 | ++ |
| 7 | Infected, 5th instar (dead) | 21.66 | 5 | 2.664 | ++++ |
| 8 | Infected, 5th instar (putrefied) | 22.8 | 5 | 0.885 | +++ |
| 9 | Infected, pupa (live) | 4.82 | 5 | 0.315 | + |
| 10 | Infected, pupa (dead) | 24 | 5 | 0.2945 | + |
| **B. *S. litura:*** | | | | | |
| 11 | Healthy, 4th instar (live) | 5.21 | 5 | 0.036 | - |
| 12 | Infected, 4th instar (live) | 7.2 | 5 | 0.483 | + |
| 13 | Infected, 4th instar (dead) | 20.21 | 5 | 2.673 | ++++ |
| 14 | Infected, 4th instar (putrefied) | 20.5 | 5 | 0.832 | +++ |
| 15 | Healthy, 5th instar (live) | 5.3 | 5 | 0.04 | - |
| 16 | Infected, 5th instar (live) | 6.07 | 5 | 2.678 | ++++ |
| 17 | Infected, 5th instar (dead) | 22.11 | 5 | 3.03 | ++++ |
| 18 | Infected, 5th instar (putrefied) | 21.44 | 5 | 2.998 | ++++ |
| 19 | Infected, pupa (live) | 9.62 | 5 | 0.343 | + |
| 20 | Infected, pupa (dead) | 14.59 | 5 | 0.429 | + |
| **C. *A. albistriga:*** | | | | | |
| 21 | Healthy, 4th instar (live) | 10.6 | 5 | 0.045 | - |
| 22 | Infected, 4th instar (live) | 8.2 | 5 | 1.682 | ++++ |
| 23 | Infected, 4th instar (dead) | 21.5 | 5 | 1.891 | ++++ |
| 24 | Infected, 4th instar (putrefied) | 23.2 | 5 | 2.862 | ++++ |
| 25 | Healthy, 5th instar (live) | 12.3 | 5 | 0.041 | - |
| 26 | Infected, 5th instar (live) | 17.6 | 5 | 2.834 | ++++ |
| 27 | Infected, 5th instar (dead) | 22.4 | 5 | 3.12 | ++++ |
| 28 | Infected, 5th instar (putrefied) | 20.1 | 5 | 3.106 | ++++ |
| 29 | Infected, pupa (live) | 17.1 | 5 | 0.697 | ++ |
| 30 | Infected, pupa (dead) | 23.2 | 5 | 0.737 | ++ |

#### 4.6.1.6 Standardization of IC-ELISA for estimation of Polyhedrin content in larval extracts:

The optimal concentrations of coating antigen and respective antisera of HaNPV, SlNPV and AmalNPV were obtained by checkerboard titration assays. The combination of coating antigen and antibody dilution that resulted in the highest titer was selected for further development. The antigen concentration of 1µg/ml and the antibody dilution of 1:4000 were optimized to assay homologous antigens (Fig 33A) and the antigen concentration of 2µg/ml and the antibody dilution of 1:2000 were optimized to assay heterologous antigens (Fig 33B and C). The polyhedrin standards were spiked in to 25 or 50µg/ml of healthy larval proteins and optimized from serial dilutions of 40 to 0.156, 20 to 0.078 and 10 to 0.039 µg/ml. The concentrations of 20 to 0.078µg/ml was found to be the best regression curve (Fig 35). The sensitivity of IC-ELISA was 0.156µg/ml of homologous polyhedrins and 0.31 to 0.35µg/ml for detection of heterologous polyhedrins in 25 or 50µg/ml of insect total protein extract.

A competitive inhibition experiment was conducted in parallel to determine the sensitivity of the assay against homologous and heterologous polyhedrins. In Table 10 the selected competitive ELISA screening data of homologous polyhedrin polyclonal antisera against homologous and heterologous polyhedrins was presented. From this data the concentration of polyhedrin required for 50% competitive inhibition ($IC_{50}$) and % of cross-reactivity of each antiserum with heterologous polyhedrins were calculated.

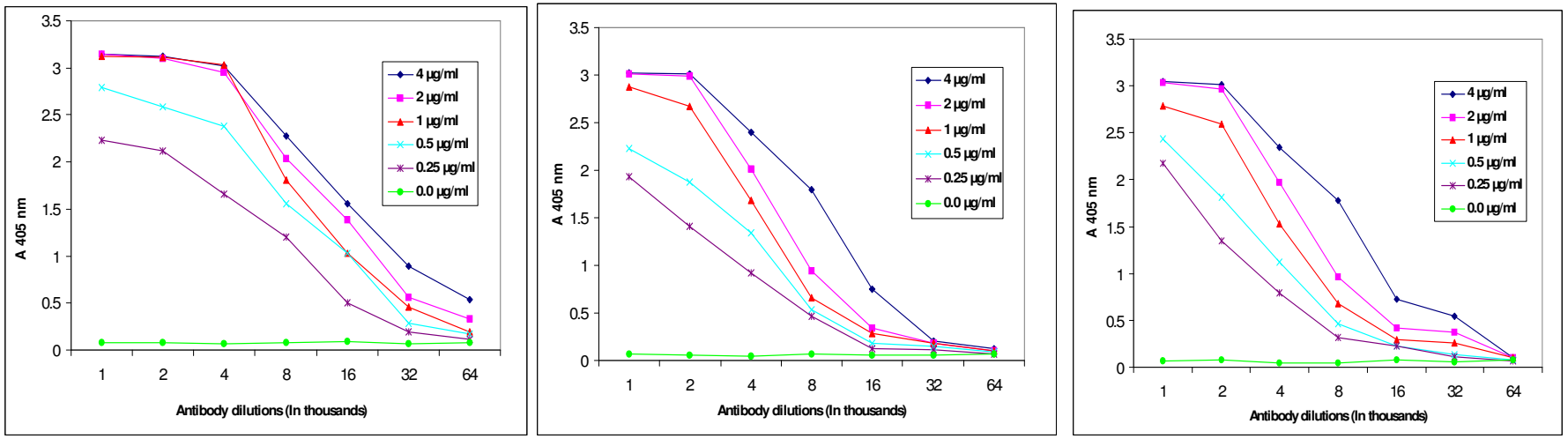
For HaNPV-polyhedrin polyclonal antiserum, the $IC_{50}$ (Fig 34A) was calculated to be 1.10µg/ml and heterologous polyhedrins were calculated to be 2.0µg/ml of SlNPV polyhedrin and 2.20µg/ml of AmalNPV polyhedrin. For SlNPV-polyhedrin polyclonal antiserum, $IC_{50}$ (Fig 34B) was calculated to be 1.26µg/ml and heterologous polyhedrins were calculated to be 2.25µg/ml of HaNPV polyhedrin and 2.85µg/ml of AmalNPV polyhedrin. For AmalNPV-polyhedrin polyclonal antiserum, $IC_{50}$ (Fig 34C) was calculated to be 1.19µg/ml and heterologous

polyhedrins were calculated to be 1.82 µg/ml of Ha NPV polyhedrin and 2.32µg/ml of SlNPV polyhedrin.

The percent cross-reactivity of each antiserum with their homologous polyhedrins was calculated to be 100% while with heterologous polyhedrins the antisera showed differential cross-reactivity (Table 11). The HaNPV- polyhedrin polyclonal antiserum has showed 54.72% and 50.0% of cross-reactivity with SlNPV and AmalNPV polyhedrins. The SlNPV- polyhedrin polyclonal antiserum showed 56.0% and 43.85% of cross-reactivity with HaNPV and AmalNPV polyhedrins. Similarly, AmalNPV-polyhedrin polyclonal antiserum showed 65.38% and 51.29% of cross-reactivity with HaNPV and SlNPV polyhedrins.

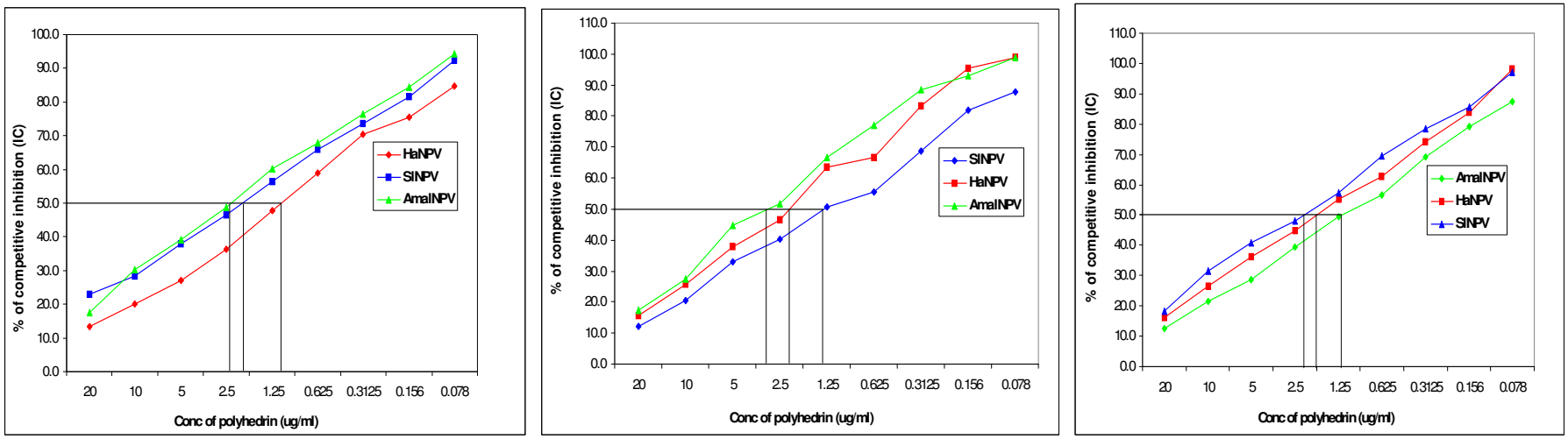
In order to study the effect of insect body proteins on IC-ELISA and test the % of recovery of artificially spiked polyhedrin to determine the effect of insect proteins, recovery experiments were conducted. In recovery experiments, 25 and 50µg/ml of insect body proteins did not show interference with artificially spiked polyhedrin (Fig 37). But when the concentration of insect body proteins increased above 50µg/ml the absorbance values were decreased slightly and false positive results were obtained. The percentage of recovery of artificially spiked polyhedrin was good from 25 or 50µg/ml of un-infected larval protein extracts. The details of the recovery experiment was presented in Table-12 and described as below. The % amount of polyhedrin (20 - 0.078µg/ml) recovered from 25µg/ml of un-infected larval protein extract was 95.7 ± 0.15 to 115.2 ± 6.4% for HaNPV polyhedrin, 82.1 ± 5.2 to 115.2 ± 6.4% for SlNPV polyhedrin and 89.7 ± 6.7 to 114.5 ± 0.4% for AmalNPV was recorded. Similarly, the % of amount of polyhedrin recovered from 50µg/ml of un-infected larval protein extract was 90.1 ± 1.3 to 116.8 ± 0.8 % for HaNPV polyhedrin, 89.8 ± 5.9 to 110.4 ± 4.8% for SlNPV polyhedrin and 88.95 ± 1.3 to 109.8 ± 2.6% for AmalNPV polyhedrin was recorded. Based on the recovery experiment the diagnosis of NPV infection by IC-ELISA was optimized for assaying the larval extracts by adjusting their total protein concentration to 25 or 50µg/ml.

**Figure 33: Titration analysis of various dilutions of polyhedrin polyclonal antiserum against homologous and heterologous polyhedrins with different concentrations as the coating antigens.**




Screening of homologous polyhedrin of HaNPV (**A**) and heterologous polyhedrins of SlNPV (**B**) and AmalNPV (**C**) at variable concentrations (4.0, 2.0, 1.0, 0.5, 0.25 and 0.0 µg/ml) against variable dilutions (1:1000 to 1:64,000) of homologous (HaNPV) polyhedrin polyclonal antiserum.

**Figure 34: Competitive inhibition curves for homologous and heterologous polyhedrins.**




The percent cross-reactivity of homologous polyhedrin polyclonal antiserum with heterologous polyhedrins was determined by competitive inhibition experiment carried out with homologous antiserum of HaNPV polyhedrin (**A**), SlNPV polyhedrin (**B**) and AmalNPV polyhedrin (**C**). The curves indicating that the amount of polyhedrin required to inhibit the reactivity of polyclonal antiserum in IC-ELISA with coated heterologous polyhedrins in the ELISA plate. By calculating the concentration of polyhedrin required for 50% competitive inhibition ($IC_{50}$) the sensitivity of the IC-ELISA was determined. The lines drawled on the figures were $IC_{50}$ values of homologous and heterologous polyhedrins with homologous antiserum.

**Table 10: Selected competitive ELISA screening against homologous and heterologous polyhedrins.**

| Polyhedrin | Coating antigen µg/ml | Antiserum dilution | IC50 µg/ml | Absorbance at 405 nm | | Slope | Intercept |
|---|---|---|---|---|---|---|---|
| | | | | min | max | | |
| **A. HaNPV polyhedrin polyclonal antiserum:** | | | | | | | |
| HaNPV | 1.0 | 1:4000 | 1.1 | 0.212 | 1.348 | 9.345 | 1.532 |
| SlNPV | 1.0 | 1:4000 | 2.01 | 0.185 | 0.854 | 8.795 | 12.149 |
| AmalNPV | 1.0 | 1:4000 | 2.2 | 0.192 | 0.876 | 9.392 | 10.672 |
| **B. SlNPV polyhedrin polyclonal antiserum:** | | | | | | | |
| SlNPV | 1.0 | 1:4000 | 1.26 | 0.2 | 1.46 | 9.55 | 2.286 |
| HaNPV | 1.0 | 1:4000 | 2.25 | 0.14 | 0.89 | 10.907 | 4.722 |
| AmalNPV | 1.0 | 1:4000 | 2.85 | 0.15 | 0.87 | 10.594 | 9.865 |
| **C. AmalNPV polyhedrin polyclonal antiserum:** | | | | | | | |
| AmalNPV | 1.0 | 1:4000 | 1.19 | 0.21 | 1.47 | 9.523 | 1.653 |
| HaNPV | 1.0 | 1:4000 | 1.82 | 0.17 | 1.03 | 9.888 | 5.899 |
| SlNPV | 1.0 | 1:4000 | 2.32 | 0.18 | 0.95 | 9.557 | 10.737 |

*Values given were averages of three separate experiments*

**Table 11: Cross-reactivity of homologous polyhedrin polyclonal antiserum with heterologous polyhedrins in IC-ELISA**

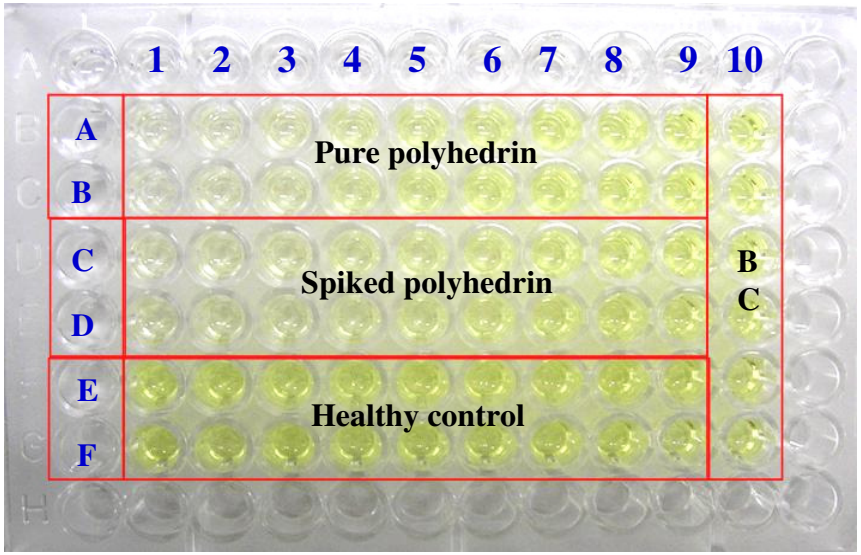
| S.No | Origin of polyhedrin protein | Cross reactivity* (%) |
|---|---|---|
| **A. HaNPV polyhedrin polyclonal antiserum:** | | |
| 1 | HaNPV | 100 |
| 2 | SlNPV | 54.72 |
| 3 | AmalNPV | 50 |
| **B. SlNPV polyhedrin polyclonal antiserum:** | | |
| 4 | SlNPV | 100 |
| 5 | HaNPV | 56 |
| 6 | AmalNPV | 43.85 |
| **C. AmalNPV polyhedrin polyclonal antiserum:** | | |
| 7 | AmalNPV | 100 |
| 8 | HaNPV | 65.38 |
| 9 | SlNPV | 51.29 |

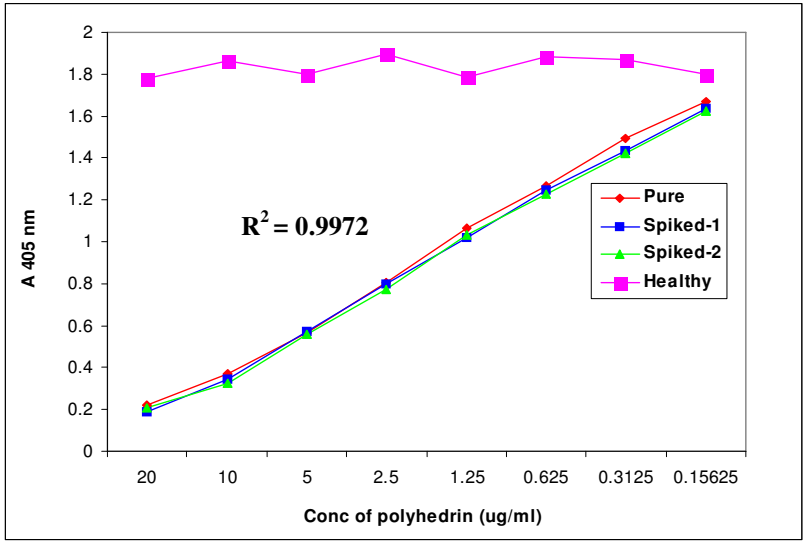
**Cross-reactivity was calculated as ($IC_{50}$ of homologous polyhedrin/ $IC_{50}$ of heterologous polyhedrin) × 100.*

*Heterologous polyhedrins were used as coating antigen.*

**Figure 35: Recovery of polyhedrin from artificially spiked healthy larval extracts in IC-ELISA**

**Indirect linear relationship between absorbance and polyhedrin concentration in IC-ELISA**




Lanes 1-9 (rows A, B and C, D): Polyhedrin 20 to 0.15625µg /ml;
Lane 10 (rows A, B, C, D, E and F): Buffer control [(BC) (0.0 µg /ml)];
Rows A and B: Pure polyhedrin;
Rows C and D: Pure polyhedrin artificially spiked in to 25 or 50 µg/ml of healthy larval extract;
Rows E and F: Healthy control (25 or 50 µg/ml).
(A 405nm values are indirectly proportionally to the polyhedrin concentration).

**Table 12: Recovery of polyhedrin from artificially spiked healthy larval extracts as determined by IC-ELISA.**

| S.No | Healthy extract used for spiking (µg/ml) | Polyhedrin spiked (µg/ml) | Polyhedrin estimated (µg/ml) | Recovery (%) |
|---|---|---|---|---|
| | | | Mean ± | |
| **A. HaNPV polyhedrin:** | | | | |
| 1 | 25 | 20 | 19.14 ± 0.03 | 95.7 ± 0.1 |
| 2 | 25 | 10 | 11.27 ± 0.38 | 112.7 ± 3.8 |
| 3 | 25 | 5 | 5.49 ± 0.08 | 109.8 ± 1.6 |
| 4 | 25 | 2.5 | 2.72 ± 0.02 | 108.8 ± 0.8 |
| 5 | 25 | 1.25 | 1.25 ± 0.09 | 100.0 ± 7.2 |
| 6 | 25 | 0.625 | 0.63 ± 0.02 | 101.1 ± 5.1 |
| 7 | 25 | 0.3125 | 0.32 ± 0.03 | 101.5 ± 8.7 |
| 8 | 25 | 0.15625 | 0.18 ± 0.01 | 115.2 ± 6.4 |
| 9 | 50 | 20 | 18.02 ± .02 | 90.1 ± 1.3 |
| 10 | 50 | 10 | 10.85 ± 0.36 | 108.5 ± 3.6 |
| 11 | 50 | 5 | 5.69 ± 0.02 | 113.8 ± 4.0 |
| 12 | 50 | 2.5 | 2.92 ± 0.02 | 116.8 ± 0.8 |
| 13 | 50 | 1.25 | 1.20 ± 0.08 | 96.0 ± 6.4 |
| 14 | 50 | 0.625 | 0.66 ± 0.03 | 105.6 ± 4.0 |
| 15 | 50 | 0.3125 | 0.34 ± 0.03 | 108.8 ± 9.6 |
| 16 | 50 | 0.15625 | 0.18 ± 0.01 | 115.2 ± 6.4 |
| **B. SlNPV polyhedrin:** | | | | |
| 17 | 25 | 20 | 17.02 ± 0.39 | 85.1 ± 1.9 |
| 18 | 25 | 10 | 8.21 ± 0.52 | 82.1 ± 5.2 |
| 19 | 25 | 5 | 5.22 ± 0.09 | 104.4 ± 1.8 |
| 20 | 25 | 2.5 | 2.85 ± 0.44 | 94.0 ± 2.4 |
| 21 | 25 | 1.25 | 1.32 ± 0.06 | 105.6 ± 4.8 |
| 22 | 25 | 0.625 | 0.72 ± 0.04 | 116.0 ± 7.2 |
| 23 | 25 | 0.3125 | 0.32 ± 0.01 | 101.7 ± 2.5 |
| 24 | 25 | 0.15625 | 0.17 ± 0.02 | 115.2 ± 6.4 |
| 25 | 50 | 20 | 19.48 ± 0.06 | 97.4 ± 0.3 |
| 26 | 50 | 10 | 8.98 ± 0.59 | 89.8 ± 5.9 |
| 27 | 50 | 5 | 5.02 ± 0.26 | 100.4 ± 5.2 |
| 28 | 50 | 2.5 | 2.74 ± 0.01 | 109.6 ± 0.4 |
| 29 | 50 | 1.25 | 1.28 ± 0.03 | 102.4 ± 2.4 |
| 30 | 50 | 0.625 | 0.69 ± 0.03 | 110.4 ± 4.8 |
| 31 | 50 | 0.3125 | 0.34 ± 0.01 | 109.1 ± 3.5 |
| 32 | 50 | 0.15625 | 0.17 ± 0.00 | 108.8 ± 0.0 |
| **C. AmalNPV polyhedrin:** | | | | |
| 33 | 25 | 20 | 17.94 ±1.34 | 89.7 ± 6.7 |
| 34 | 25 | 10 | 11.45 ± 0.04 | 114.5 ± 0.4 |
| 35 | 25 | 5 | 5.59 ± 0.35 | 111.8 ± 7.7 |
| 36 | 25 | 2.5 | 3.92 ± 0.39 | 109.6 ± 4.4 |
| 37 | 25 | 1.25 | 1.36 ± 0.04 | 108.8 ± 3.2 |
| 38 | 25 | 0.625 | 0.67 ± 0.05 | 107.2 ± 8.0 |
| 39 | 25 | 0.3125 | 0.33 ± 0.01 | 105.6 ± 3.2 |
| 40 | 25 | 0.15625 | 0.16 ± 0.01 | 105.6 ± 9.6 |
| 41 | 50 | 20 | 17.79 ± 0.26 | 88.9 ± 1.3 |
| 42 | 50 | 10 | 10.48 ± 0.23 | 104.8 ± 2.4 |
| 43 | 50 | 5 | 5.49 ± 0.13 | 109.8 ± 2.6 |
| 44 | 50 | 2.5 | 2.74 ± 0.11 | 109.6 ± 4.4 |
| 45 | 50 | 1.25 | 1.32 ± 0.01 | 106.0 ± 1.2 |
| 46 | 50 | 0.625 | 0.64 ± 0.02 | 101.6 ± 2.4 |
| 47 | 50 | 0.3125 | 0.31 ± 0.01 | 100.8 ± 4.8 |
| 48 | 50 | 0.15625 | 0.17 ± 0.05 | 109.4 ± 5.7 |

## 4.6.2 Development of Quality Control Tools:

As part of the quality control during mass production of bio-insecticides based on HaNPV, SlNPV and AmalNPV, sensitive immunochemical tools such as DAC and IC-ELISA were developed and evaluated for the quantification of POBs in commercial NPV preparations. The number of POBs present in sample was determined by extracting the total polyhedrin and compared with the standard regression graph of polyhedrin extracted from known number of POB standards such as $6 \times 10^9$ to $4.68 \times 10^7$ POBs /ml (1 LE to 0.0078 LE).

A simple purification protocol was standardized for extraction of total polyhedrin from 1 ml of standard and sample POB preparations. The protocol is similar to extraction of polyhedrin from larval homogenates with slight modifications to the method described in section 3.6.1.1. In 12% SDS- PAGE the purity of polyhedrin extracted from standards and samples was similar to polyhedrin purified by isoelectric precipitation method as detailed in Section 4.3. The intensity of the polyhedrin band was gradually decreased with decreasing in the number of POBs and at very low concentrations the band was not enough to visible. In addition to major polyhedrin, some minor low and high molecular weight polypeptides were also observed (Fig 36A and B). The extracts of standards and samples were evaluated in both DAC and IC-ELISA at 1:1000 dilution. The maximum regression ($R^2$) value for the standard curves of DAC and IC- ELISA were 0.9953 (Fig 37A) and 0.9977 (Fig 37B). The sensitivity of ELISA (DAC and IC-ELISA) was as low as $4.6875 \times 10^7$ POBs /ml (0.015 LE/ml). The ELISA results were comparable to the light microscope counting of POBs. The results of ELISA and microscope counting were incorporated in Table 13. The total number of POBs of NPV samples collected from ICRISAT-NPV production laboratory and some private samples collected from market were summarized in Table 14.

**Figure 36: 12% SDS-PAGE profiles of polyhedrin extracted from known number of POBs of standards and unknown number of POBs of samples.**

**A. Standards:**

Lane 1, Protein molecular weight marker

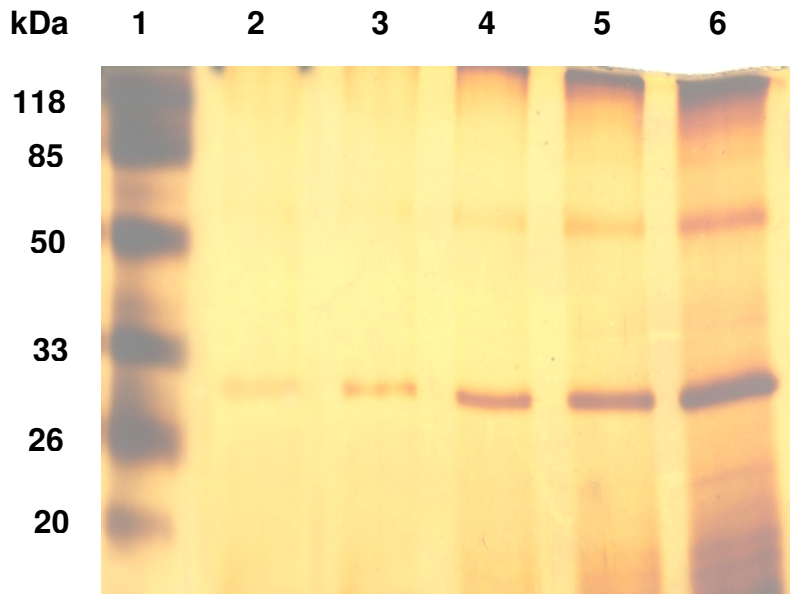
Lane 2, 0.0625 LE

Lane 3, 0.125 LE

Lane 4, 0.25 LE

Lane 5, 0.5 LE

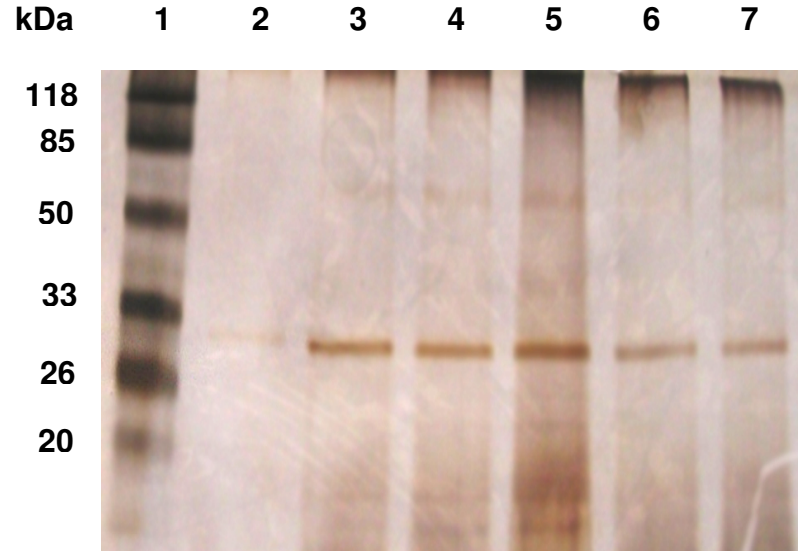
Lane 6, 1.0 LE




**B. Samples:**

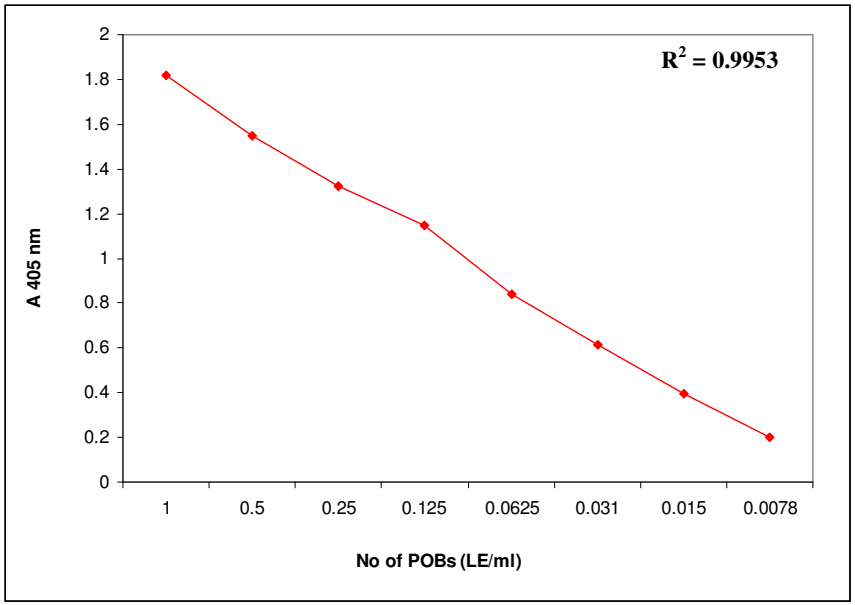
Lane 1, Protein molecular weight marker

Lanes 2-7, Samples with unknown number of POBs

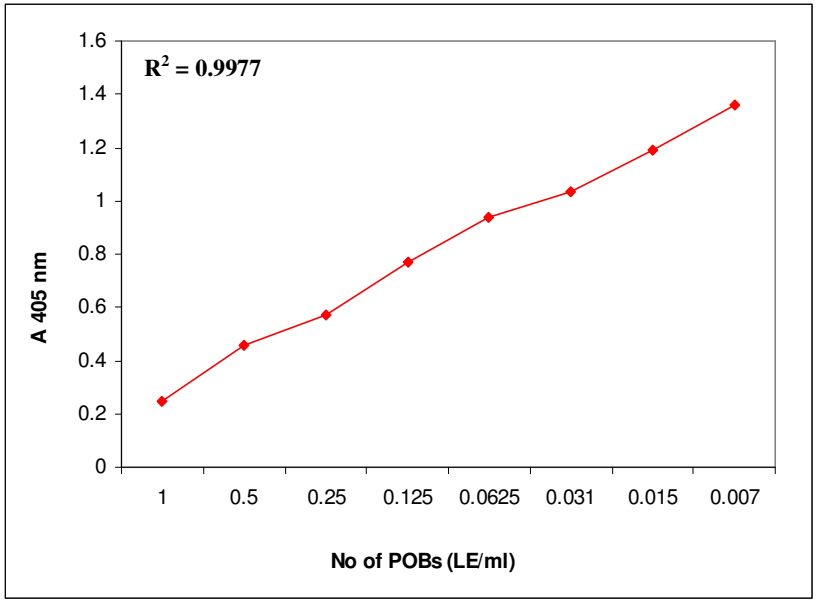



The purity of the polyhedrin extracted from POB standards (1LE to 0.0625 LE) (**A**) and from unknown number of POBs of samples (**B**) were checked in 12% SDS-PAGE and the gels were silver stained. Sizes of protein molecular weight marker (kDa) are indicated.
Note: 1 LE = $6 \times 10^9$ POBs/ml

**Figure 37: Linear relationship between absorbance and number of POBs in DAC and IC-ELISA**




A




B

Poly occlusion bodies (POBs) from standard NPV (1 LE/ml) bottle were serially diluted from $6 \times 10^9$ to $2.34 \times 10^7$ POBs/ml (1 to 0.0078 LE/ml), their total polyhedrin was extracted and analyzed in DAC (**A**) and IC-ELISA (**B**).
Note: 1 LE = $6 \times 10^9$ POBs/ml

**Table 13: Comparison of ELISA results with microscopic counting of POBs**

| Sample No. | Number of POBs (LE/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Microscope count (LE/ml) | DAC-ELISA (LE/ml) | IC-ELISA (LE/ml) | STDEV | Recovery (%) | |
| | | | | | DAC-ELISA | IC-ELISA |
| **1** | **1.0** | 0.972 | 1.053 | 0.0473 | 97.2 | 105.3 |
| **2** | **0.5** | 0.532 | 0.483 | 0.0289 | 106.5 | 96.6 |
| **3** | **0.25** | 0.235 | 0.233 | 0.0153 | 94.0 | 93.2 |
| **4** | **0.125** | 0.12 | 0.125 | 0.0025 | 96.0 | 100 |
| **5** | **0.0625** | 0.065 | 0.064 | 0.0013 | 104 | 102.7 |
| **6** | **0.031** | 0.030 | 0.035 | 0.0045 | 96.7 | 112.9 |
| **7** | **0.015** | 0.016 | 0.017 | 0.0008 | 108 | 113.3 |
| **8** | **0.4** | 0.43 | 0.38 | 0.05 | 107.5 | 95 |
| **9** | **0.246** | 0.22 | 0.23 | 0.013 | 89.4 | 93.4 |
| **10** | **0.14** | 0.123 | 0.134 | 0.009 | 87.8 | 95.7 |
| **11** | **0.086** | 0.08 | 0.092 | 0.012 | 93 | 106.9 |
| **12** | **0.05** | 0.043 | 0.048 | 0.005 | 86 | 96 |
| **13** | **0.026** | 0.026 | 0.03 | 0.002 | 100 | 115.3 |
| **14** | **1.09** | 0.973 | 1.02 | 0.047 | 89.2 | 93.57 |
| **15** | **0.5** | 0.532 | 0.55 | 0.025 | 106.5 | 110 |
| **16** | **0.7** | 0.77 | 0.80 | 0.03 | 110 | 114.2 |
| **17** | **1.0** | 0.972 | 1.06 | 0.0875 | 97.25 | 106 |
| **18** | **0.6** | 0.62 | 0.56 | 0.06 | 103.3 | 93.3 |
| **19** | **0.8** | 0.88 | 0.91 | 0.03 | 110 | 113.7 |
| **20** | **0.25** | 0.26 | 0.28 | 0.02 | 104 | 112 |

Note: 1 LE = $6 \times 10^9$ POBs/ml

**Table 14: Estimation of total POBs NPV based biopesticides collected from the ICRISAT-NPV production laboratory and commercial NPV samples collected from the market.**

| Sample No | Type of NPV | Place of collection | Total number of POBs (LE/ml) |
|---|---|---|---|
| **1** | **HaNPV** | ICRISAT | 1.06 |
| **2** | **SlNPV** | ICRISAT | 1.02 |
| **3** | **AmalNPV** | ICRISAT | 1.08 |
| **4** | **HaNPV** | Market | 0.05 |
| **5** | **HaNPV** | Market | 0.07 |
| **6** | **HaNPV** | Market | 0.01 |
| **7** | **HaNPV** | Market | 0.01 |
| **8** | **HaNPV** | Market | 0.1 |
| **9** | **SlNPV** | Market | 0.02 |
| **10** | **SlNPV** | Market | 0.2 |

Note: 1 LE = $6 \times 10^9$ POBs/ml

## 4.7 Application of Immunochemical Tools in Optimization of Conditions for Productivity and Quality of NPVs:

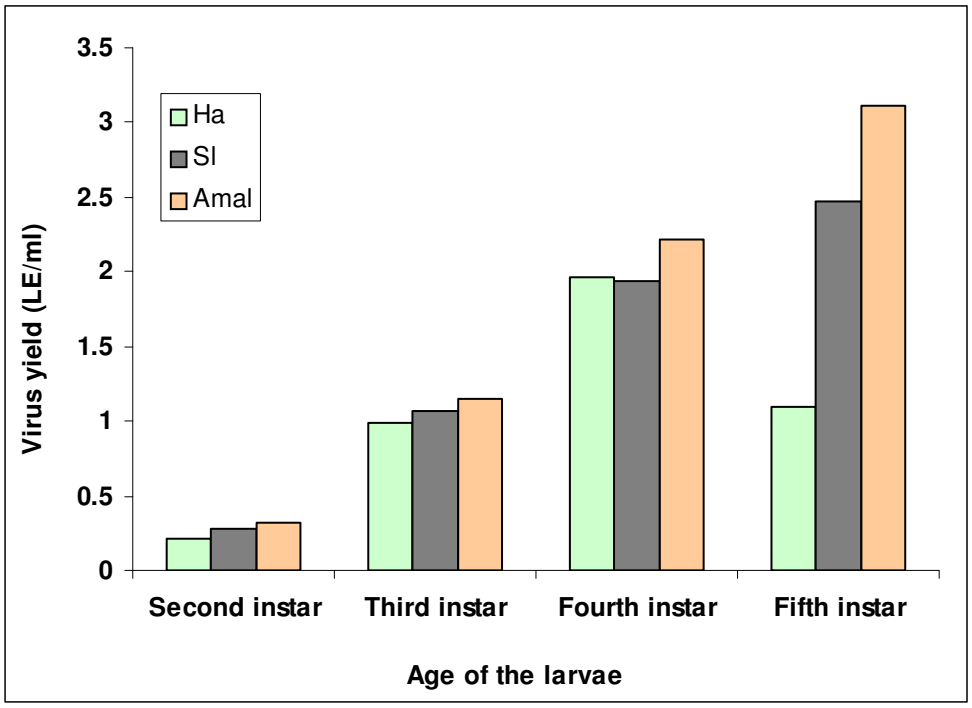
By applying the immunochemical tools the production process was optimized for impoved productivity and quality of NPVs during commercial production. Also evaluated the efficacy of NPV through ELISA by monitoring the infection status in pest populations after field application.

To optimize the conditions for productivity and quality of NPVs during commercial production, the optimum age of the larvae for virus inoculation and optimum time for virus harvesting were determined by applying the immunochemical tools in bioassay experiments. The results of the experiments were as follows.

### 4.7.1 Determining the optimum age of larvae for virus inoculation:

The effect of age of larvae on POB yield and optimum age of the larvae for inoculation of virus were determined by mass multiplying the NPVs on $2^{nd}$, $3^{rd}$, $4^{th}$, and $5^{th}$ instar larvae. The total yield of NPV obtained in each age group larvae determined by ELISA showed that the yield of NPV during mass multiplication was increased with increasing in age of larvae but it was not common with all the three insect species. For *H. armigera* the yield of NPV was increased gradually from $2^{nd}$ instar to $4^{th}$ instar stage and decreased in $5^{th}$ instar stage; whereas in *S. litura* and *A. albistriga* the yield was increased up to $5^{th}$ instar stage (Fig 38). The highest yield of NPV obtained for HaNPV when 4th instar larvae were infected (1.97 ± 0.035 LE/ml). The highest yield obtained for SlNPV when $5^{th}$ instar larvae were infectd (2.47 ± 0.097 LE/ml). The yield of AmalNPV was maximum when $5^{th}$ instar larvae were infected and was higher than HaNPV and SlNPV *i.e.* 3.11 ± 0.05. Based on these results, for *H. armigera* $4^{th}$ instar and for *Spodoptera litura* and *A. albistriga* $5^{th}$ instar larvae were identified as the optimum stages for inoculation of virus to obtain maximum yield. Different age group larvae and their POB yields determined by ELISA were furnished in Table 15.

**Figure 38: Effect of age of larvae on yield of NPV as determined by ELISA**




Application of ELISA in detemining the optimum age of larvae for inoculation to obtain maximum virus yield.
Note: 1 LE = $6 \times 10^9$ POBs/ml

***Ha**-Helicoverpa armigera; **Sl**-Spodoptera litura; **Amal**-Amsacta albistriga*

**Table 15: Effect of age of larvae on NPV yield as determined by ELISA**

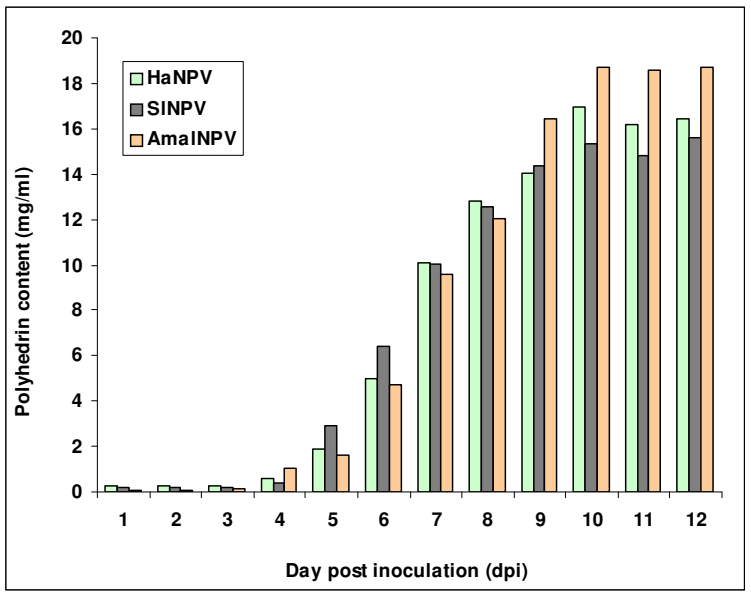
| Virus | Stage of infection / Yield (LE/ml) | | | | F.pr | SE± | LSD | CV |
|---|---|---|---|---|---|---|---|---|
| | 2nd instar | 3rd instar | 4th instar | 5th instar | | | | |
| **HaNPV** | 0.207 | 0.992 | 1.973 | 1.104 | < 0.001 | 0.03 | 0.12 | 0.60 |
| **SlNPV** | 0.283 | 1.066 | 1.997 | 2.47 | < 0.001 | 0.03 | 0.11 | 0.70 |
| **AmalNPV** | 0.32 | 1.153 | 2.217 | 3.113 | < 0.001 | 0.03 | 0.11 | 0.20 |

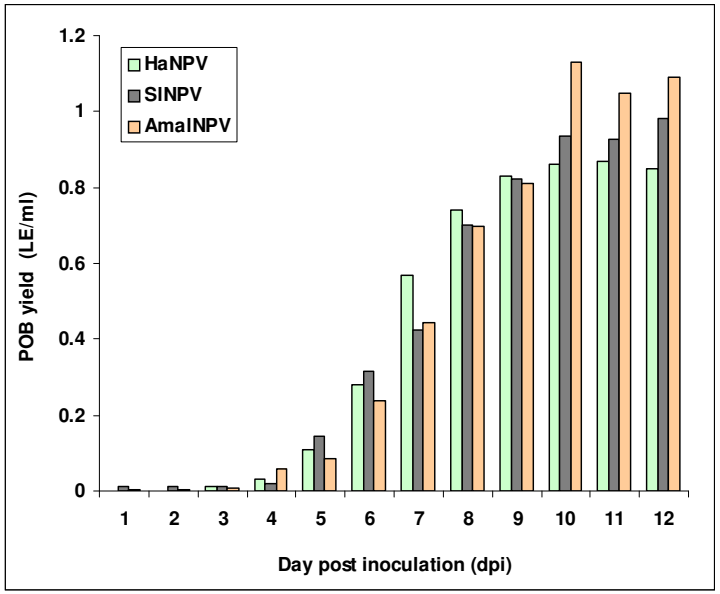
Note: 1 LE = $6 \times 10^9$ POBs/ml

### 4.7.2 Determining the optimum time for harvesting of virus:

The optimum period for harvesting POBs from the inoculated larvae were determined and established the relationship between larval mortality, productivity of virus and bacterial activity in larvae days post exposure to NPV by conducting bioassays (time course) experiment on optimum aged larvae. The productivity of virus in larvae at different intervals (post inoculation days) of the experiment was monitored through ELISA (DAC and IC-ELISA) and Western immunoassay tools. In ELISA the virus load was detected in infected larvae from 3 days after inoculation (dpi), but in western immunoblotting the virus load was detected from 4 dpi. In Fig 39A, the ELISA results were represented that the concentration of polyhedrin in sampled larvae was increased gradually from 3 to 10 dpi and subsequently no further increase was observed up to 12 dpi. Similar trend was observed with yield of virus on 3 to 12dpi which was presented in Fig 39B. The concentration of virus harvested from exclusively dead larvae on 10, 11 and 12 dpi were slightly higher than virus harvested on 9 dpi. However, in western immunoassay (Fig 40) the intensity of polyhedrin band was increased only from 4 to 9 dpi and subsequently no further increase was observed up to 12 dpi indicating that the virus harvested on 9 dpi was not greatly affect the total yield of NPV. The details of the polyhedrin content and POB yield estimated by ELISA on 1 to 12 dpi were presented in Table 16.

**Figure 39: Application of ELISA in bioassay (time course) experiment to study the effect of post inoculation period of harvest on NPV yield**




**A** **B**

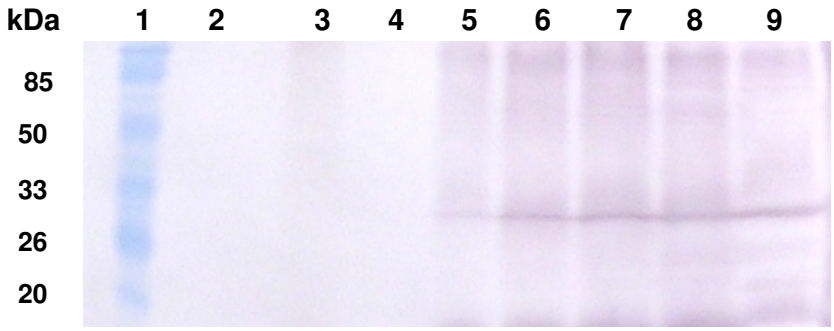
Application of ELISA tools in bio-assay (time course) experiments to estimate the virus titer in the larvae on different days post inoculation (dpi) by means of polyhedrin content (**A**) and number of POBs (**B**) in infected larvae collected at different intervals of the experiment (1 to 12 days post inoculation).
Note: 1 LE = $6 \times 10^9$ POBs/ml

***Ha**-Helicoverpa armigera; **Sl**-Spodoptera litura; **Amal**-Amsacta albistriga*

**Figure 40: Application of western immunoassay in bioassay (time course) experiment to study the effect of post inoculation period of harvest on NPV yield.**

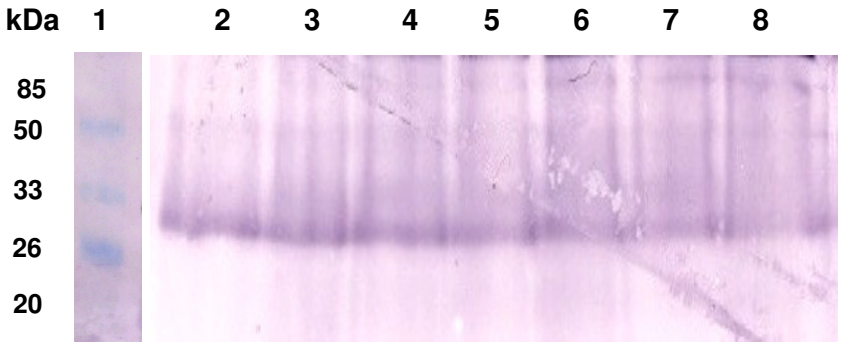
**A**

Lane 1, Protein marker
Lane 2, Healthy control
Lane 3, day 1
Lane 4, day 3
Lane 5, day 4
Lane 6, day 5
Lane 7, day 7
Lane 8, day 9
Lane 9, day 12



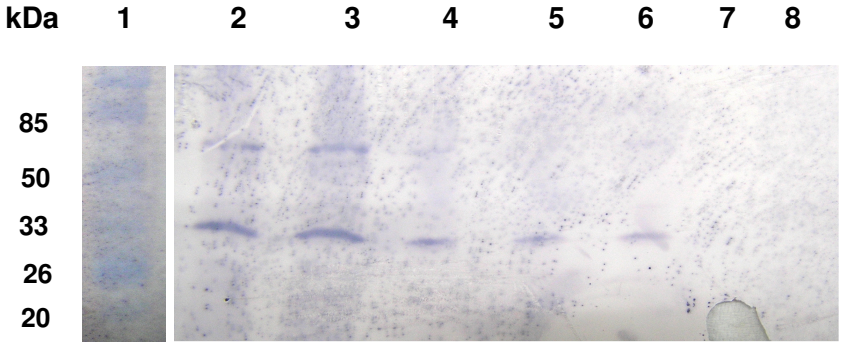

**B**

Lane 1, Protein marker
Lane 2, day 12
Lane 3, day 10
Lane 4, day 9
Lane 5, day 7
Lane 6, day 6
Lane 7, day 5
Lane 8, day 4




**C**

Lane 1, Protein marker
Lane 2, day 12
Lane 3, day 9
Lane 4, day 7
Lane 5, day 5
Lane 6, day 4
Lane 7, day 3
Lane 8, Healthy control




Application of Western immunoassay in bioassay (time course) experiment to identify the optimum time for harvesting of virus and to study the effect of post inoculation period of harvest on virus yield in larvae of *H. armigera* (**A**), *S. litura* (**B**) and *A. albistriga* (**C**). Polyclonal antibodies with respect to each NPV used at 1: 5000 dilution were specifically recognized the polyhedrin protein of size 31 kDa in larval extracts. Above figures indicates that the detectable virus load was observed from 4 days after inoculation (dpi) and virus concentration peaked by 9 dpi. Subsequently, no further increase in virus concentration was observed.

**Table 16: Effect of post inoculation period of harvest on NPV yield as determined by ELISA**

| Post inoculation day (dpi) | Virus titer | | | | | |
|---|---|---|---|---|---|---|
| | Polyhedrin content (mg/ml) | | | POB yield (LE/ml) | | |
| | *Ha* | *Sl* | *Amal* | *Ha* | *Sl* | *Amal* |
| | ± STDEV | | | | | |
| **1st day** | 0.26 ± 0.00 | 0.20 ± 0.01 | 0.046 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.002 ± 0.00 |
| **2nd day** | 0.26 ± 0.00 | 0.21 ± 0.00 | 0.077 ± 0.014 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.004 ± 0.00 |
| **3rd day** | 0.27 ± 0.00 | 0.21 ± 0.00 | 0.143 ± 0.045 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.007 ± 0.00 |
| **4th day** | 0.61 ± 0.04 | 0.42 ± 0.05 | 1.053 ± 0.08 | 0.03 ± 0.00 | 0.02 ± 0.00 | 0.053 ± 0.00 |
| **5th day** | 1.85 ± 0.22 | 2.40 ± 0.24 | 1.624 ± 0.05 | 0.11 ± 0.01 | 0.14 ± 0.02 | 0.083 ± 0.00 |
| **6th day** | 5.01 ± 0.33 | 6.39 ± 0.73 | 4.694 ± 0.57 | 0.28 ± 0.02 | 0.32 ± 0.05 | 0.235 ± 0.03 |
| **7th day** | 10.11 ± 0.90 | 10.01 ± 1.19 | 9.549 ± 0.36 | 0.57 ± 0.06 | 0.42 ± 0.05 | 0.468 ± 0.02 |
| **8th day** | 12.82 ± 0.74 | 12.72 ± 0.22 | 12.028 ± 1.64 | 0.74 ± 0.05 | 0.70 ± 0.10 | 0.601 ± 0.08 |
| **9th day** | 14.04 ± 1.25 | 14.22 ± 0.42 | 16.447 ± 0.30 | 0.83 ± 0.07 | 0.82 ± 0.02 | 0.823 ± 0.01 |
| **10th day** | 16.97 ± 1.44 | 16.07 ± 0.67 | 18.730 ± 1.25 | 0.86 ± 0.12 | 0.94 ± 0.06 | 1.001 ± 0.06 |
| **11th day** | 16.15 ± 1.03 | 16.26 ± 0.81 | 18.581 ± 0.45 | 0.87 ± 0.09 | 0.93 ± 0.02 | 0.961 ± 0.07 |
| **12th day** | 16.46 ± 0.67 | 16.50 ± 0.76 | 18.737 ± 0.34 | 0.85 ± 0.06 | 0.98 ± 0.06 | 1.014 ± 0.07 |

***Ha****-Helicoverpa armigera;* ***Sl****-Spodoptera litura;* ***Amal****-Amsacta albistriga*

Note: 1 LE = $6 \times 10^9$ POBs/ml

### 4.7.3 Screening of bacterial activity in larvae days post exposure to NPV:

Results clearly indicated that the bacterial population increased with the delay in harvest of NPV inoculated larvae. The highest number of bacterial colonies and colony forming units per ml (CFU/ml) were recorded in the larvae of *A. albistriga and* the lowest number of bacterial colonies and colony forming units per ml (CFU/ml) were recorded in the larvae of *H. armigera* (Table 17). The lowest number of bacterial colonies and CFU/ml of larval homogenate was observed on $5^{th}$ day sampled larvae and the highest number of bacterial colonies and colony forming units per ml was observed exclusively in dead larval homogenates after $9^{th}$ dpi onwards (10 to 12 dpi) (Table 17).

On $5^{th}$ dpi the number of bacterial colonies and CFU/ml were recorded as 23 and $1.15 \times 10^6$ for *H. armigera*, 28 and $1.4 \times 10^6$ for *S. litura* and 31 and $1.55 \times 10^6$ for *A. albistriga* larval homogenate. The number of bacterial colonies and CFU/ml recorded exclusively from dead larval homogenates were as follows: on $10^{th}$ dpi the number of bacterial colonies and CFU/ml were recorded as 151 and $7.55 \times 10^6$ for *H. armigera,* 162 and $8.1 \times 10^6$ for *S. litura* and 168 and $8.4 \times 10^6$ for *A. albistriga* larval homogenate. On $11^{th}$ dpi the bacterial population was recorded as 163 and $8.15 \times 10^6$ for *H. armigera,* 178 and $8.9 \times 10^6$ for *S. litura* and 181 and $9.05 \times 10^6$ for *A. albistriga* larvae. On $12^{th}$ day the bacterial population was recorded as 159 and $7.95 \times 10^6$ for *H. armigera,* 176 and $8.8 \times 10^6$ for *S. litura* and 187 and $9.35 \times 10^6$ in *A. albistriga* larval homogenate.

**Table 17: Effect of post inoculation period on bacterial activity in NPV infected larvae**

| Post inoculation day | Bacterial activity in NPV infected larvae | | | | | |
|---|---|---|---|---|---|---|
| | No. of bacterial colonies | | | CFU/ml | | |
| | *Ha* | *Sl* | *Amal* | *Ha* | *Sl* | *Amal* |
| **5th day** | 23 | 28 | 31 | 1,150,000 | 1,400,000 | 1,550,000 |
| **6th day** | 28 | 32 | 38 | 1,400,000 | 1,600,000 | 1,900,000 |
| **7th day** | 36 | 47 | 53 | 1,800,000 | 2,350,000 | 2,650,000 |
| **8th day** | 73 | 87 | 83 | 3,630,000 | 4,350,000 | 4,150,000 |
| **9th day** | 98 | 112 | 121 | 4,915,000 | 5,600,000 | 6,050,000 |
| **10th day** | 151 | 166 | 168 | 7,550,000 | 8,300,000 | 8,400,000 |
| **11th day** | 163 | 178 | 181 | 8,150,000 | 8,900,000 | 9,050,000 |
| **12th day** | 159 | 176 | 187 | 7,950,000 | 8,800,000 | 9,350,000 |

| Larva | F.Pr | SE± | LSD | CV |
|---|---|---|---|---|
| ***Ha*** | < 0.001 | 1.734 | 5.261 | 2.8 |
| ***Sl*** | < 0.001 | 2.624 | 7.958 | 5 |
| ***Amal*** | < 0.001 | 3.66 | 11.11 | 6.1 |

***Ha****-Helicoverpa armigera;* ***Sl****-Spodoptera litura;* ***Amal****-Amsacta albistriga*

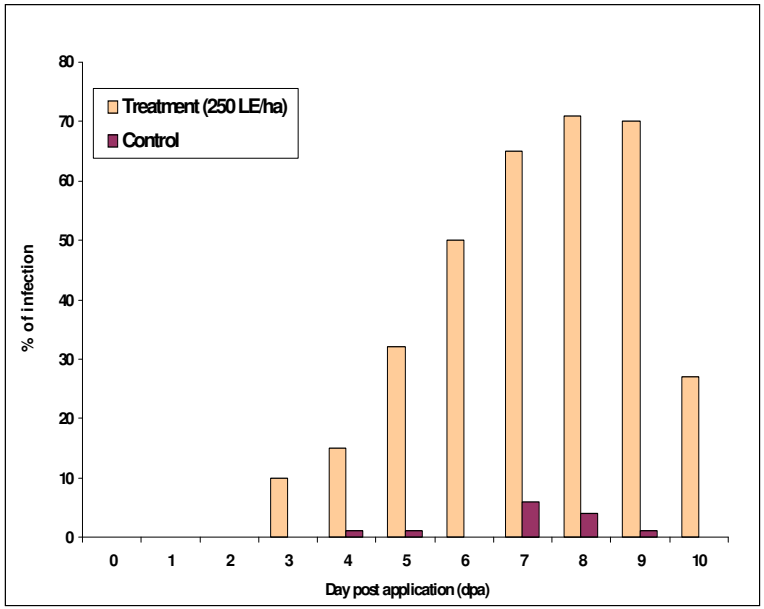
## 4.8 Evaluation of ELISA Tools at Field Level Efficacy Study of NPV:

The ELISA tools developed in the present study was applied to monitor the NPV infection status in field population of *H. armigera* on pigeonpea crop after field application of NPV. The DAC-ELISA results showed that the concentration of NPV used for field spray (250 LE/ha) successfully infected the field population. The details of total number of larvae sampled per dpa, number of NPV positive larvae observed by DAC-ELISA, percent of infection among sampled larvae per dpa and gross virus concentration (POBs) in infected larvae per dpa estimated by ELISA (DAC and IC) were represented in Table 18.

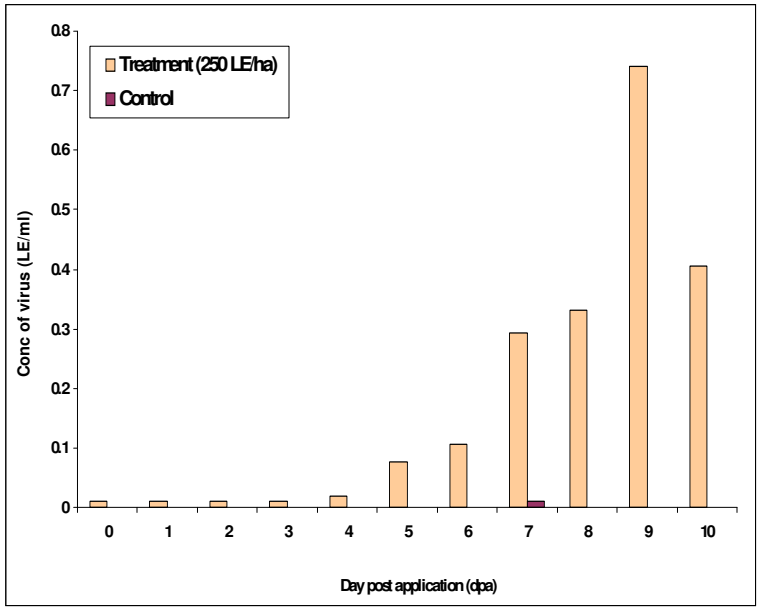
In Fig 41A**,** the percent infection in field sampled larvae per dpa determined by DAC-ELISA is presented. The curve indicates that 10 ± 1.7% of the field collected larvae were NPV positive on 3$^{rd}$ dpa, 15 ± 2.2% on 4$^{th}$ dpa, 32 ± 2.6% on 5$^{th}$ dpa, 50 ± 3.2% on 6$^{th}$ dpa , 65 ± 2.5% on 7$^{th}$ dpa, 71 ± 2.5%, on 8$^{th}$ dpa and 70 ± 5.9 on 9$^{th}$ dpa. But, on 10$^{th}$ dpa the percent infection was decreased to 27 ± 5.7%. In parallel, the DAC-ELISA results of the individual larvae collected from control (untreated) plot showed that most of the larvae were free of NPV and very few larvae were found to be NPV positive. The infection pattern observed in the larvae of control plot were as follows: The percent of NPV positive larvae were observed that 1 ± 1.9% on 4$^{th}$ and 5$^{th}$ dpa, 6 ± 1.9% on 7$^{th}$ dpa, 4 ± 5.1% on 8$^{th}$ dpa and 1 ± 1.9% on 9$^{th}$ dpa.

In Fig 41B the gross virus concentration (POBs) in infected larvae per dpa estimated by ELISA (DAC and IC) is represented. The ELISA results were detailed as follows: The concentration of virus during 0-4$^{th}$ dpa was negligible. Considerable level of virus tier was observed from 5$^{th}$ dpa to 10$^{th}$ dpa. The gross virus concentration in infected larvae was observed as 0.07 ± 0.01 LE /ml on 5$^{th}$ dpa, 0.10 ± 0.02 LE/ml on 6$^{th}$ dpa, 0.29 ± 0.067 LE/ml on 7$^{th}$ dpa, 0.33 ± 0.07 LE/ml on 8$^{th}$ dpa, 0.74 ± 0.07 LE/ml on 9$^{th}$ dpa and 0.41 ± 0.07 LE/ml on 10$^{th}$ day was observed.

**Figure 41: Monitoring of NPV infection at field level against *Helicoverpa armigera* on pigeonpea crop using ELISA**




**A**




**B**

Evaluation of ELISA tools at field level efficacy study of NPV against *H. armigera* on pigeonpea crop by estimating the percent infection in total field sampled larvae per day post application (**A**) using DAC-ELISA and estimation of virus concentration in infected larvae (**B**) using DAC and IC-ELISA.

Note: 1 LE = $6 \times 10^9$ POBs/ml

**Table 18: Evaluation of ELISA tools at field level efficacy study of NPV against *H. armigera* on Pigeonpea crop**

| Post application day (dpa) | Treatment (250 LE/ ha) plot | | | | Control plot | | |
|---|---|---|---|---|---|---|---|
| | Larvae sampled / dpa (No.) | NPV + ve larvae (No.) | Infection (%) | Virus conc. (LE/ml) | Larvae sampled / dpa (No.) | NPV + ve larvae (No.) | Infection (%) |
| | ± STDEV | | | | | | |
| **0** | 30 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 0.00 ± 0.0 | 30 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 |
| **1** | 34 ± 4.0 | 0 ± 0.0 | 0 ± 0.0 | 0.00 ± 0.0 | 32 ± 2.0 | 0 ± 0.0 | 0 ± 0.0 |
| **2** | 38 ± 2.1 | 0 ± 0.0 | 0 ± 0.0 | 0.00 ± 0.0 | 34 ± 1.0 | 0 ± 0.0 | 0 ± 0.0 |
| **3** | 34 ± 2.0 | 3 ± 0.6 | 10 ± 1.7 | 0.00 ± 0.0 | 34 ± 2.5 | 0 ± 0.0 | 0 ± 0.0 |
| **4** | 33 ± 3.1 | 5 ± 1.0 | 15 ± 2.2 | 0.02 ± 0.0 | 32 ± 1.5 | 0 ± 0.6 | 1 ± 1.9 |
| **5** | 36 ± 2.5 | 12 ± 2.5 | 32 ± 2.6 | 0.07 ± 0.01 | 31 ± 1.2 | 0 ± 0.6 | 1 ± 1.9 |
| **6** | 35 ± 5.0 | 17 ± 3.1 | 50 ± 3.2 | 0.10 ± 0.02 | 31 ± 1.0 | 0 ± 0.0 | 0 ± 0.0 |
| **7** | 35 ± 4.6 | 23 ± 3.8 | 65 ± 2.5 | 0.29 ± 0.06 | 31 ± 1.2 | 2 ± 0.6 | 6 ± 1.9 |
| **8** | 36 ± 5.3 | 25 ± 3.1 | 71 ± 2.5 | 0.33 ± 0.07 | 31 ± 1.0 | 2 ± 1.2 | 4 ± 5.1 |
| **9** | 35 ± 1.2 | 24 ± 2.1 | 70 ± 5.9 | 0.74 ± 0.07 | 30 ± 0.0 | 0 ± 0.6 | 1 ± 1.9 |
| **10** | 30 ± 0.0 | 8 ± 1.5 | 27 ± 5.7 | 0.41 ± 0.07 | 30 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 |

Note: 1 LE = $6 \times 10^9$ POBs/m

## 4.9 Isolation and Characterization of Polyhedrin Gene of NPVs:

A PCR protocol was standardized using degenerate primers to isolate the full length polyhedrin gene of NPVs isolated from *H. armigera, S. litura* and *A. albistriga* in the present study.

### 4.9.1 Optimization of PCR for amplification of full-length polyhedrin gene of NPVs:

#### 4.9.1.1 Design of degenerate primers:

Multiple sequence alignment of previously published polyhedrin gene sequences of NPVs infecting *Helicoverpa, Spodoptera* and *Amsacta* insect complexes available in GenBank showed that there is a great conservation among all the NPVs in these three insect complexes. Fig 42 showed the result of CLUSTAL-W multiple sequence alignment. Based on the output of multiple sequence alignment three degenerate primers were designed. The details of the primers are presented in Table 19. One degenerate primer (PG-C) was synthesized for 3'end region of polyhedrin gene for HaNPV, SlNPV and AmalNPV (polyhedrin gene C-terminal end primer). Another degenerate primer (PG-N) was synthesized for 5' end region of polyhedrin gene for HaNPV and SlNPV (polyhedrin gene N-terminal end primer). The third primer (AmalNPV PG-N) was synthesized for 5' end region of polyhedrin gene for AmalNPV (AmalNPV polyhedrin gene N-terminal end primer).

**Figure 42: (CLUSTAL W v1.83) multiple sequence alignment of previously published polyhedrin gene sequences available in NCBI GenBank of NPVs infecting *Helicoverpa, Spodoptera* and *Amsacta* insect complexes.**

1-100

```
gi|10.SlNPV NC_3102     ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|11.SlNPV AF325155    ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|13.SlNPV AY549963    ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|14.SlNPV AY549964    ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|12.SlNPV AF037262    ATGTATAGTCGTTATAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|15.SlNPV AY552474    ATGTATAGTCGTTATAGCGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATCTAGGTCACGTGATTAAAA
gi|16.SlNPV DQ350142    ATGTATAGTCGCTACAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATTTAGGTCACGTGATCAAAA
gi|20.SliNPV D01017     ATGTATAGTCGCTACAGTGCCTACAATTATAGTCCCCATCTGGGCAAAACCTATGTATACGATAACAAGTATTACAAAAATTTAGGTCACGTGATCAAAA
gi|17.SlNPV DQ152923    ATGTATAGTCGCTACAGTGCCTACAATTATAGTCCCCATCTTGGGCAAAACTATGTATACGATAACAAGTATTACAAAAATTTAGGTCACGTGATCCAAA
gi|1.HaNPV  AF157012    ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|4.HaNPV  AJ001917    ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|5.HaNPV NC_003094    ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|6.HaNPV AF303045     ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|3.HaNPV A25670       ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|8.HzNPV Nc_003349    ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|9.HasNPV DQ157735   ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|2.HaNPV  U97657      ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|7.HaNPV NC_002654    ATGTATACTCGTTACAG---------TTACAGCCCTACTTTGGGCAAAACCTATGTGTACGACAACAAATACTTTAAGAATTTAGGTGCTGTTATTAAAA
gi|18.SlNPV X94437      ATGTATACTCCGTACAG---------CTACAACCCGTCTCTGGGACGCACCTACGTGTACGACAACAAGTTCTACAAAAATCTAGGTTCGGTCATCAAGA
gi|21.SfNPV J04333      ATGTATACTCGTTACAG---------CTATAACCCATCTTTGGGTCGCACCTACGTGTACGACAACAAGTTCTACAAAAATCTAGGTTCGGTCATCAAGA
gi|19.SeNPV AF169823    ATGTATACTCGCTACAG---------CTATAACCCAGCCTTGGGTCGCACTTACGTGTACGACAACAAATTCTACAAGAATCTTGGTTCCGTCATCAAAA
gi|22.AmalNPV AF118850  ATGCCGGATTATTCGTACGC------GTACCGGCCCACCATTGGCCGCACATATGTGTATGACAATAAATATTACAAAAATCTAGGTTCAGTTATTAAAA
                        ***     *   *             **    **     * **    *  ** ** ** ** ** ** *     ** *** * ***    * **  * *
```

**Ha**-*Helicoverpa armigera;* **Has**-*Helicoverpa assulta;* **Hz**-*Helicoverpa zea;* **Sl**-*Spodoptera litura;* **Sli**-*Spodoptera litturalis;* **Se**-*Spodoptera exigua,* **Sf**-*Spodoptera frugiperda;* **Amal**- *Amsacta albistriga*

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gi|10.SlNPV NC_3102      TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACGGGTCCCGCGTATTAA
gi|11.SlNPV AF325155     TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACGGGTCCCGCGTATTAA
gi|13.SlNPV AY549963     TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACGGGTCCCGCGTATTAA
gi|14.SlNPV AY549964     TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACGGGTCCCGCGTATTAA
gi|12.SlNPV AF037262     TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACCGGTCCCGCGTATTAA
gi|15.SlNPV AY552474     TCAAAGAGTTTGCTCCCGACGCGCCTCTATACACGGGTCCCGCGTATTAA
gi|16.SlNPV DQ350142     TCAAAGAGTTTGCTCCCGACGCGCCACTCTACACCGGTCCCGCGTACTAA
gi|20.SliNPV D01017       TCAAAGAGTTTGCTCCCGACGCGCCACTCTACACCGGTCCCGCGTACTAA
gi|17.SlNPV DQ152923     TCAAAGAGTTTGCTCCCGACGCGCCACTCTACACCGGTCCCGCGTACTAA
gi|1.HaNPV   AF157012    TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|4.HaNPV   AJ001917    TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|5.HaNPV NC_003094     TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|6.HaNPV AF303045      TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|3.HaNPV A25670        TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|8.HzNPV Nc_003349     TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|9.HasNPV DQ157735     TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|2.HaNPV   U97657      TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|7.HaNPV NC_002654     TCAAAGAATTTGCACCTGACGCTCCGCTATACACTGGTCCTGCATATTAA
gi|18.SlNPV X94437       TCAAAGAGTTTGCGCCCGACGCCTCTCTATACAACGGACCCGCATATTAA
gi|21.SfNPV J04333       TCAAGGAGTTTGCTCCTGACGCACCCCTGTACAACGGACCCGCGTACTAA
gi|19.SeNPV AF169823     TCAAGGAATTCGCACCCGATGCGCCTCTTTACAACGGACCCGCCTATTAA
gi|22.AmalNPV AF118850   TGAAAGAGTTTGCGCCCGACGCACCTCTTTTCACAGGACCCGCATATTAA
                         * ** ** ** ** ** ** **  * ** * **  ** ** ** ** ***
```

**Table 19: Primers used for the amplification of polyhedrin gene region of NPVs isolated from *H. armigera, S. litura* and *A. albistriga***

| S.No | Primer | Target DNA | Primer size | Primer Sequence Text (5'-3') | Annealing Temp (Tm) |
|---|---|---|---|---|---|
| 1 | **PG-C (rp)** | HaNPV, SlNPV and AmalNPV polyhedrin genes | 24 bp | 5' TTA RTA BGC RGG WCC NKT GWA NAG 3' | $51^0$ C |
| 2 | **PG-N (fp)** | HaNPV and SlNPV polyhedrin genes | 17 bp | 5' ATG TAT CSB TAY AG 3' | $35^0$ C |
| 3 | **AmalNPV PG-N (fp)** | AmalNPV polyhedrin gene | 23 bp | 5' ATG CCG GAT TAT TCG TCG TAC GCB TA 3' | $53^0$ C |

Codes for mixed bases: M = A/C; R = A/G; W = A/T; S = C/G; Y = C/T; K = G/T; V = A/C/G; H = A/C/T; D = A/G/ T; B = C/G/T; N = A/C/G/T

### 4.9.1.2 Extraction of viral DNA:

Isolated viral DNA from three NPVs resolved as a single high molecular weight band in 0.8% agarose gels (Fig 43). Concentration of DNA samples was estimated in spectrophotometer and $A_{260:280}$ ratio was determined and presented in Table 20. The quantities of viral DNA obtained by the optimized protocol for extraction of DNA directly from POBs (infected larval extracts) was sufficient to carryout the PCR.

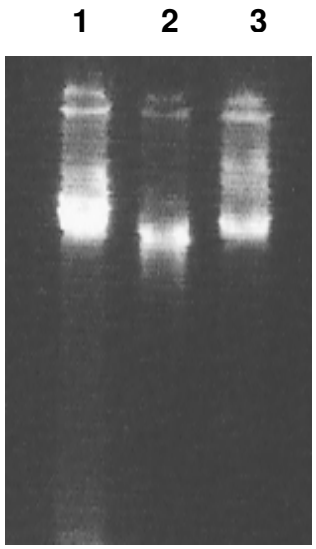
**Table 20: Spectrophotometric analysis of DNA of NPVs**

| Virus | 260 nm | 280 nm | 260/280 ratio | DNA concentration (µg/ml) |
|---|---|---|---|---|
| HaNPV | 0.026 | 0.016 | 1.626 | 65.287 |
| SlNPV | 0.033 | 0.031 | 1.061 | 65.005 |
| AmalNPV | 0.026 | 0.023 | 1.159 | 52.534 |

### 4.9.1.3 Optimization of PCR conditions:

The PCR parameters were standardized as given below: initial denaturation step at $95^0$C for 10 min, followed by 25 cycles at $95^0$C for 1 min, $37^0$C for 2 min, $72^0$C for 2 min followed by 10 cycles at $95^0$C for 1 min, $45^0$C for 3 min, $72^0$C for 3 min, and a final extension step at $72^0$C increased for 7 min. The thermal cycles optimized for amplification of polyhedrin gene of NPVs using degenerate primers were schematically represented in Fig 44.

**Figure 43: Resolution of DNA directly isolated from the NPV infected larval extracts (POBs)**



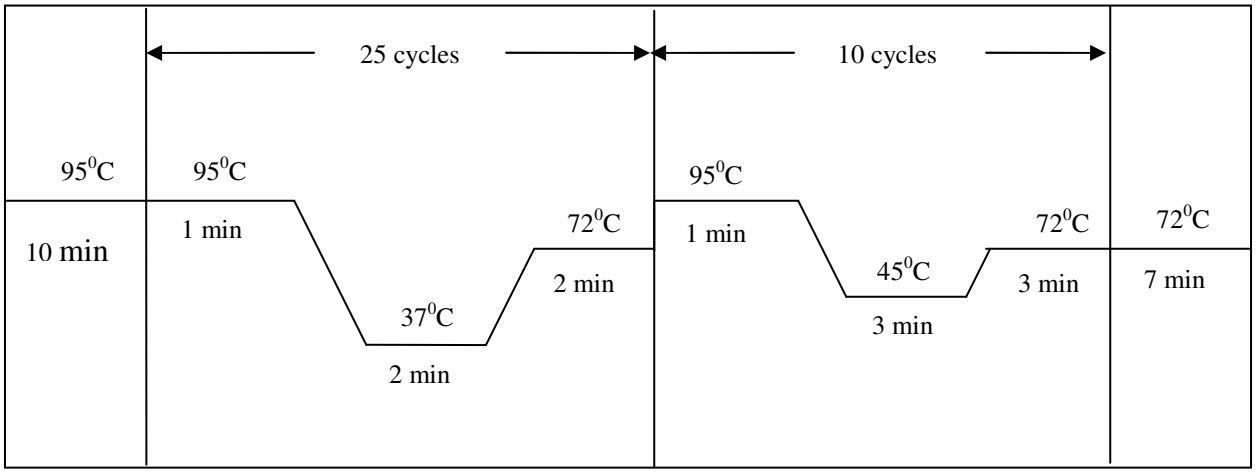

Lane 1, DNA isolated from POBs of HaNPV

Lane 2, DNA isolated from POBs of SlNPV

Lane 3, DNA isolated from POBs of AmalNPV

DNA isolated directly from the NPV infected larval extracts (POBs) separated in 0.8% agarose gel and visualized on UV-transilluminator.

**Figure 44: Optimization of thermal cycles in PCR for amplification of polyhedrin gene**



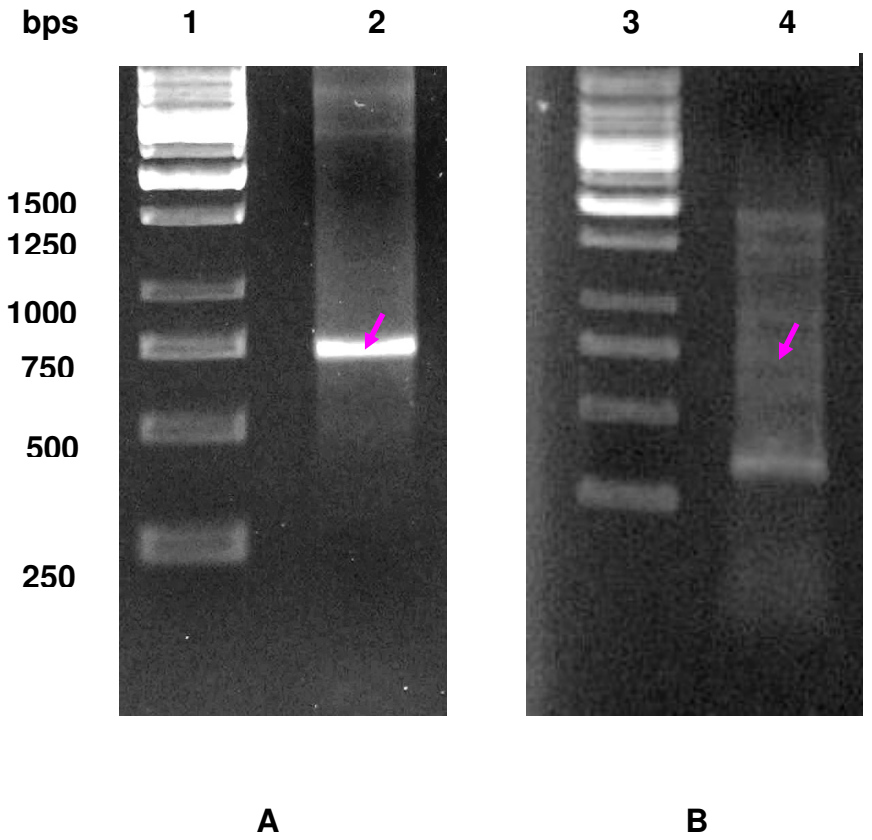

The primer combinations of PG-C and PG-N with HaNPV DNA template was successfully amplified the full-length polyhedrin gene with a major band of 744 bp (Fig 45A). Since the same primer combination with SlNPV DNA template did not result in any amplification products, at the same time the reverse primer from set one *i.e.* PG-C used in combination with Amal PG-N and Amal NPV DNA template resulted in non-specific amplicons with major band size of 350 bp and some minor bands with sizes of 592, 742, 929, 1188, 1391 bp products (Fig 45B).

**Figure 45: PCR amplified polyhedrin gene of NPVs using degenerate primers**

Lanes 1 and 3, DNA molecular weight markers

Lane 2, Amplified PCR product of HaNPV genomic DNA

Lane 4, Amplified PCR product of AmalNPV genomic DNA




Amplification of polyhedrin gene of HaNPV (**A**) and AmalNPV (**B**). The sizes of the DNA molecular weight markers were indicated and the DNA band size corresponding to the polyhedrin gene in two viruses was indicated with arrow marks.

## 4.9.2: Cloning and sequencing of PCR product:

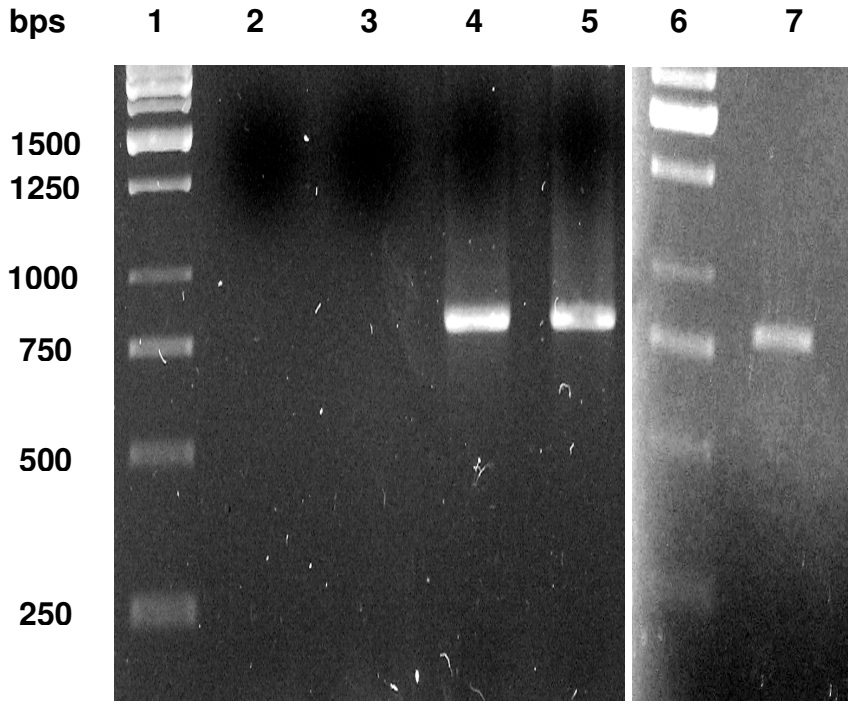
Cloning of agarose gel purified PCR product in case of HaNPV was worked well, while in case of AmalNPV, similar purification of the band with expected size (742 bp) from the agarose gel did not gave good results due to uncertainty in the amplification pattern *i.e.* multiple bands. Hence, the polyhedrin gene of AmalNPV could not be cloned and sequenced in this study. In the former case, the ligation and transformation event yielded over 50 colonies from which 10 colonies were sub-cultured and verified for the inserted gene by colony PCR. In the colony PCR, amplification of the clone using universal pJET1 forward and reverse sequencing primers [Bioserve Biotechnologies (India) Pvt.Ltd, Catalogue No # 51314 and 51315] gave the amplification of the expected size of about ~ 800 bp, that were taken into account for the plasmid sequence of 60 bp (Fig 11). Considering the sizes of previously published polyhedrin sequences, most amplification products were between 730-750 bp long.

Based on the colony PCR, three independent colonies with inserted polyhedrin gene were selected for sequencing (Fig 46). Gene sequencing analysis of selected clones resulted in 744 bp product. In BLASTX search the three sequences showed homology with baculovirus OB protein domain of known polyhedrin and granulin proteins from the GenBank data base. The sequence was deposited in GenBank with a public accession number of EU047914. In Fig 47**,** the nucleic acid and predicted amino acid sequence of the HaNPV-P (Patancheru strain) polyhedrin was represented.

**Figure 46: Colony PCR for the conformation of inserted gene in pJET1 cloning vector**

Lanes 1 and 6, DNA molecular weight markers

Lanes 2, 3, 4, 5 and 7, transformed colonies of recombinant clone generated from PCR amplified product of PG-C and PG-N primer set




Colony PCR for the conformation of insert size by using universal pJET1 forward and reverse sequencing primers. The sizes of the DNA molecular weight markers were indicated.

**Figure 47: Nucleic acid and predicted amino acid sequence of HaNPV-P (Patancheru strain) polyhedrin (Occlusion body protein)**

```
atgtatactcgttacagttacaaatccgtcgttgggaacgtccctacgtctacgacaaac
 M  Y  T  R  Y  S  Y  K  S  V  V  G  N  V  P  T  S  T  T  N
aagtactacaaaaatcttggatcagtcatcaaaaacgccaaccgcaaaaagcactatatc
 K  Y  Y  K  N  L  G  S  V  I  K  N  A  N  R  K  K  H  Y  I
gaacatgaactcgaggagaaaacactcgacccttagacagatatctggtggccgaagac
 E  H  E  L  E  E  K  T  L  D  P  L  D  R  Y  L  V  A  E  D
cccttcctgggaccgggcaaaaaccaaaaactaactttgtttaaagaaatcagaaatgtc
 P  F  L  G  P  G  K  N  Q  K  L  T  L  F  K  E  I  R  N  V
aagcccgacaccatgaagcttgtcgtaaactggagcggtaaagagtttctcagagaaact
 K  P  D  T  M  K  L  V  V  N  W  S  G  K  E  F  L  R  E  T
tggacccgtttcatggaagacagcttccctattgttaacgaccaagaagtcatggacgtt
 W  T  R  F  M  E  D  S  F  P  I  V  N  D  Q  E  V  M  D  V
ttccttgtaatcaacatgcgtcccactagacccaaccgttgtttcaaattcctggctcaa
 F  L  V  I  N  M  R  P  T  R  P  N  R  C  F  K  F  L  A  Q
catgctctgcgttgcgatcccgactatgtgccccacgaagtcatccgcatcgttgaaccg
 H  A  L  R  C  D  P  D  Y  V  P  H  E  V  I  R  I  V  E  P
tcctacgtgggcagcaacaacgaataccgcgtcagcttagccaagcgtggcggtggctgc
 S  Y  V  G  S  N  N  E  Y  R  V  S  L  A  K  R  G  G  G  C
cccgtgatgaatctgcactctgaatacaccaactctttcgaagagttcatcaaccgtgtc
 P  V  M  N  L  H  S  E  Y  T  N  S  F  E  E  F  I  N  R  V
atatgggagaacttctacaagccaattgtgtacgtaggcacagattcggctgaggaagag
 I  W  E  N  F  Y  K  P  I  V  Y  V  G  T  D  S  A  E  E  E
gaaattcttctcgaggtttctctggtgttcaaaatcaaagagtttgcgcctgatgcgcct
 E  I  L  L  E  V  S  L  V  F  K  I  K  E  F  A  P  D  A  P
ctatacatcggtcctgcttattaa
 L  Y  I  G  P  A  Y  *
```

### 4.9.3 Phylogenetic Relation at Nucleotide Level of HaNPV-P Polyhedrin Gene with Known Polyhedrin and Granulin Genes:

Polyhedrin gene sequence was aligned with previously published polyhedrin and granulin gene sequences by ClustalW method (Thompson *et al.,* 1994) using MgAlin tool of Lasergene software (DNASTAR, USA). Alignment of DNA sequences of 40 NPV polyhedrins and 14 sequences of GV granulins along with HaNPV-P polyhedrin gene and construction of phylogenetic tree (Fig 48) revealed two major branches that were considered as separate clusters of baculovirus occlusion body protein (polyhedrin or granulin), they are NPVs and GVs. NPVs were again divided into four major branches. They are group-I and group-II NPVs, LdMNPV and WsNPV were formed as separated branches. There are 16 NPVs in group-I, 23 NPVS in group-II (including HaNPV-P) were observed in separate clusters.

The maximum homology of 99.9% among group-I NPVs was noticed between AfNPV and RoMNPV and minimum homology of 77.4% among group-I NPVs was noticed between AmalNPV and ArNPV (Table 21). Due to less homology with remaining NPVs of Group-I the AmalNPV and LoMNPV were formed as separate branch within group-I NPVs. The AmalNPV was showing maximum homology of 80.9% with remaining group-I NPVs especially with EpMNPV and minimum homology of 77.4% was noticed with ArNPV. Similarly, the LoMNPV was showing maximum homology of 80.8% with remaining group-I NPVs especially with CrNPV and minimum homology of 77.6% was noticed with HcNPV. The maximum homology of 99.7% among group-II NPVs was noticed between MbNPV and McNPV and with minimum homology of 70.1% among group-II NPVs was noticed between LdMNPV and HzSNPV and WsNPV and HaNPV (Table 22). Due to less homology with remaining NPVs, the LdMNPV and WsNPV were formed as separate branches. The LdMNPV was showing maximum homology of 76% with remaining group-II NPVs especially with PfNPV and SfMNPV and minimum homology of 69.9% was noticed with HaNPV. At the same time maximum homology of 75.5%

with group-I NPVs especially with OpMNPV and minimum homology of 68.4% with LoMNPV was noticed. Similarly, the WsNPV was showing maximum homology of 74.6% with remaining group-II NPVs especially with SlNPV and minimum homology of 70.1% was noticed with HaNPV. At the same time maximum homology of 73.4% with group-I NPVs especially with ApNPV and minimum homology of 68.9% with AfNPV was noticed.

The polyhedrin gene sequence of HaNPV-P was more close to group-II NPVs. Among which, it was showing maximum homology of 98.2% with McNPV, 98% with MbNPV, 96.1% with LsNPV and 90.6% with PfNPV. At the same time with minimum homology of 72.4% was noticed with WsNPV. Bootstrapping analysis of phylogenetic tree with CLC work bench revealed that LdMNPV and WsNPV were separated as major branches and supported with 100% bootstrap values. The remaining branches are supported by high bootstrap values (Fig 49).

**Figure 48: Phylogenetic analysis at nucleotide level of HaNPV-P (←) polyhedrin gene with previously published polyhedrin and granulin genes collected from GenBank, using Mgalign tool of DNASTAR software.**

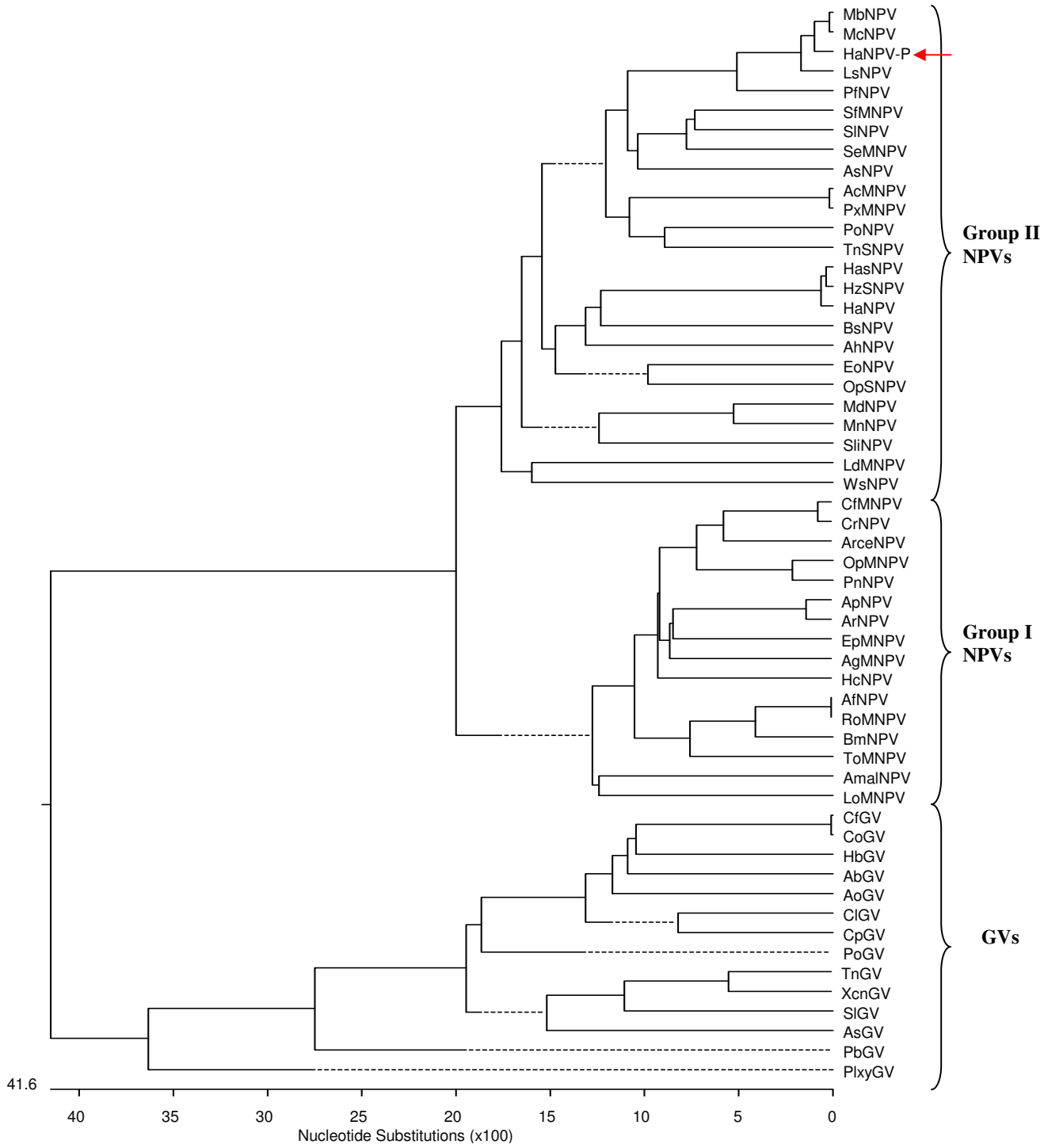


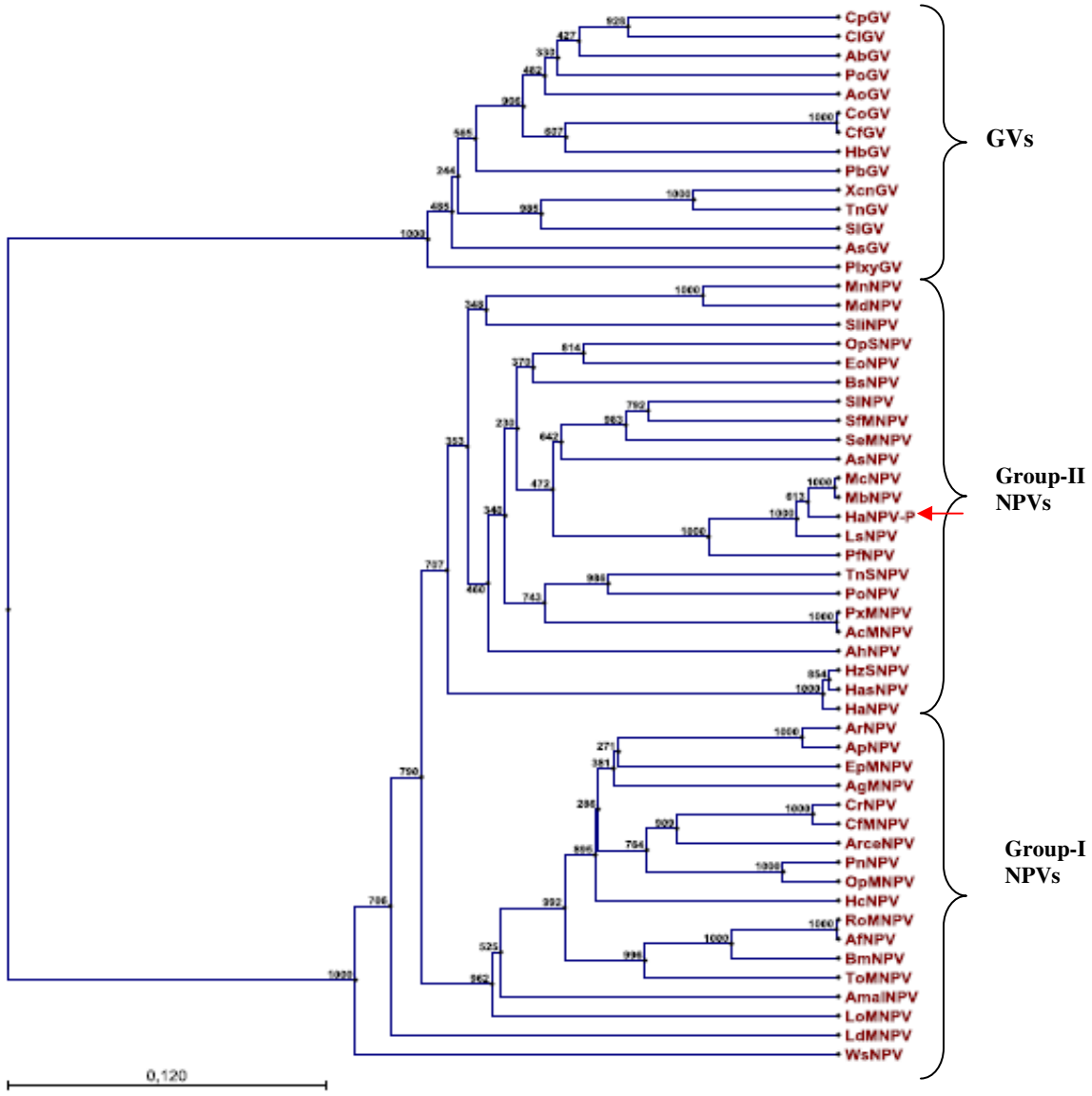
**Table 21: Pair Distances of nucleotide sequence of polyhedrin gene among group-I NPVs, aligned by ClustalW (Slow/Accurate, IUB) method (Percent Similarity in upper triangle Percent Divergence in lower triangle).**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | AfNPV | *** | 81.3 | 78.9 | 83.5 | 82 | 83.1 | 92.3 | 82.3 | 82.7 | 82.8 | 81.8 | 79.4 | 82 | 82.1 | 99.9 | 87 |
| **2** | AgMNPV | 21.8 | *** | 79.7 | 85.2 | 82.8 | 84.1 | 80.6 | 82.6 | 82.8 | 85.1 | 83.5 | 79.3 | 83.6 | 83.9 | 81.2 | 82.4 |
| **3** | AmalNPV | 25.1 | 24.1 | *** | 78.5 | 80.1 | 77.4 | 77.6 | 79.9 | 78.9 | 80.9 | 78.3 | 79.2 | 79.4 | 78.9 | 79.1 | 80.2 |
| **4** | ApNPV | 19.1 | 16.6 | 26.1 | *** | 85 | 97.3 | 82.1 | 84.4 | 85.2 | 86 | 83.9 | 80.4 | 85.2 | 84.4 | 83.3 | 82.1 |
| **5** | ArceNPV | 21.2 | 19.8 | 23.7 | 17.3 | *** | 82.9 | 80.6 | 89.5 | 88.9 | 84.6 | 82.5 | 77.7 | 86.9 | 86.6 | 81.8 | 81.8 |
| **6** | ArNPV | 19.6 | 18 | 27.7 | 2.8 | 19.6 | *** | 81.8 | 82.7 | 83.6 | 84.4 | 82.7 | 79.1 | 84.6 | 83.7 | 82.9 | 81.3 |
| **7** | BmNPV | 8.2 | 22.7 | 27.5 | 20.9 | 22.8 | 21.2 | *** | 80.8 | 80.9 | 81.7 | 80.8 | 78.7 | 81.6 | 81.3 | 92.4 | 86 |
| **8** | CfMNPV | 20.8 | 20.3 | 24.4 | 18 | 11.2 | 20.2 | 22.7 | *** | 98.4 | 84.6 | 82.7 | 80.3 | 87.1 | 86.7 | 82.3 | 81.5 |
| **9** | CrNPV | 20 | 19.8 | 24 | 16.7 | 12 | 18.9 | 22.3 | 1.5 | *** | 85.3 | 83.7 | 80.8 | 88.1 | 87.5 | 82.5 | 81.7 |
| **10** | EpMNPV | 20.2 | 17.3 | 22.9 | 16.1 | 17.5 | 17.8 | 21.4 | 17.4 | 16.6 | *** | 84.4 | 80.5 | 86.2 | 84.7 | 82.8 | 84 |
| **11** | HcNPV | 21.2 | 19.4 | 24.9 | 18.4 | 20 | 20.1 | 23 | 20 | 18.6 | 18.2 | *** | 77.6 | 86 | 85.2 | 82 | 82.4 |
| **12** | LoMNPV | 24.9 | 24.7 | 24.4 | 23.3 | 27 | 25.1 | 26.5 | 23 | 22.5 | 23.8 | 27.1 | *** | 78.2 | 79.8 | 79.7 | 79.7 |
| **13** | OpMNPV | 21.1 | 18.8 | 24.7 | 16.7 | 14.5 | 17.6 | 21.5 | 14.4 | 13.2 | 15.6 | 15.6 | 26.3 | *** | 95.9 | 81.8 | 83.5 |
| **14** | PnNPV | 20.9 | 18.3 | 25.5 | 17.7 | 14.9 | 18.6 | 21.8 | 14.9 | 13.9 | 17.5 | 16.6 | 23.9 | 4.2 | *** | 82 | 83.2 |
| **15** | RoMNPV | 0.1 | 22 | 24.9 | 19.3 | 21.2 | 19.8 | 8.1 | 20.8 | 20.2 | 20.2 | 21 | 24.7 | 21.3 | 21.1 | *** | 87.1 |
| **16** | ToMNPV | 14.6 | 20.3 | 23.2 | 20.9 | 20.9 | 22 | 15.7 | 22.1 | 21.3 | 18.7 | 20.3 | 24.3 | 18.9 | 19.3 | 14.4 | *** |

**Table 22: Pair Distances of nucleotide sequence of polyhedrin gene among group-II NPVs, aligned by ClustalW (Slow/Accurate, IUB) method (Percent Similarity in upper triangle Percent Divergence in lower triangle).**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | AcMNPV | *** | 74 | 76.7 | 74.8 | 75.7 | 78.2 | 72.9 | 72.9 | 72.6 | 74.4 | 79.1 | 80.1 | 79.9 | 75.7 | 75.5 | 75.6 | 81.7 | 80.8 | 99.9 | 75.5 | 78.7 | 76.3 | 76.3 | 79.4 | 73.4 |
| **2** | AhNPV | 30.3 | *** | 78 | 80.2 | 78.8 | 80.8 | 76.1 | 76.5 | 76.5 | 74.1 | 79.2 | 80.2 | 80.4 | 78.4 | 77.9 | 76.8 | 79.4 | 77.7 | 73.6 | 78.7 | 78.5 | 76.4 | 76.7 | 77.7 | 71.4 |
| **3** | AsNPV | 27.3 | 27.2 | *** | 77.5 | 77.5 | 79.8 | 74.5 | 74.6 | 74.9 | 73.8 | 80.4 | 80.4 | 80.3 | 76.8 | 76.5 | 78 | 82.2 | 78.1 | 76.8 | 82.5 | 82.2 | 78.3 | 81.6 | 78.9 | 72.4 |
| **4** | BsNPV | 30 | 23.3 | 27.6 | *** | 80.3 | 81.6 | 79.9 | 79.8 | 79.8 | 71.1 | 80.2 | 80.7 | 80.8 | 78.9 | 78.8 | 81.1 | 80.2 | 79.4 | 74.7 | 79.4 | 77.1 | 74.9 | 77.6 | 80 | 72.4 |
| **5** | EoNPV | 28.1 | 25.5 | 27.4 | 23.5 | *** | 82.2 | 76.5 | 76.8 | 76.8 | 75.5 | 80.8 | 82.5 | 82.3 | 78.7 | 78 | 83 | 81.5 | 78.9 | 75.3 | 80 | 78.9 | 78.1 | 78.7 | 78.7 | 74.3 |
| **6** | HaNPV-P | 23.3 | 22.6 | 24 | 21.2 | 20.6 | *** | 77.2 | 77.2 | 77.6 | 73.7 | 96.1 | 98 | 98.2 | 77.5 | 77.6 | 80 | 90.6 | 81.6 | 78 | 81.8 | 82.5 | 77.6 | 81.4 | 79.8 | 72.4 |
| **7** | HaNPV | 33.9 | 29.5 | 32.3 | 24.5 | 28.7 | 28.3 | *** | 98.9 | 98.8 | 69.9 | 75.4 | 76.1 | 76.1 | 76.9 | 77.9 | 79.1 | 75.4 | 76.7 | 73.2 | 76.4 | 75.3 | 74.8 | 74.9 | 77.6 | 70.1 |
| **8** | HasNPV | 33.9 | 28.9 | 32.3 | 24.9 | 28.3 | 28.3 | 1.1 | *** | 99.3 | 70.3 | 75.4 | 76.1 | 76.1 | 77.1 | 78.3 | 78.8 | 75.7 | 76.8 | 73.2 | 75.7 | 75.7 | 74.5 | 74.6 | 77.9 | 71 |
| **9** | HzSNPV | 34.4 | 28.9 | 31.9 | 24.9 | 28.3 | 27.7 | 1.2 | 0.7 | *** | 70.1 | 75.8 | 76.5 | 76.5 | 76.8 | 78.3 | 78.9 | 75.8 | 76.5 | 72.9 | 75.8 | 75.4 | 74.2 | 74.9 | 77.6 | 71 |
| **10** | LdMNPV | 30.9 | 32.1 | 32.1 | 39 | 30 | 31.9 | 39.5 | 38.7 | 39.3 | *** | 74.7 | 75.2 | 75.2 | 72.8 | 73.7 | 73.3 | 76 | 74.4 | 74.4 | 72.9 | 76 | 75.7 | 75.1 | 74.8 | 73.1 |
| **11** | LsNPV | 23.8 | 24.6 | 23 | 23.4 | 22.5 | 4 | 30.3 | 30.3 | 29.7 | 31 | *** | 97.6 | 97.6 | 76.7 | 77.3 | 79.2 | 90.6 | 81.2 | 79 | 81.4 | 82.2 | 77.5 | 81.6 | 79.5 | 71.3 |
| **12** | MbNPV | 22.4 | 23.4 | 23 | 22.7 | 20.3 | 2.1 | 29.3 | 29.3 | 28.7 | 30.9 | 2.5 | *** | 99.7 | 78 | 78.1 | 80 | 91.4 | 81.2 | 79.9 | 82.5 | 82.7 | 78.1 | 81.4 | 80 | 72.3 |
| **13** | McNPV | 22.7 | 23 | 23.2 | 22.5 | 20.5 | 1.8 | 29.3 | 29.3 | 28.7 | 30.9 | 2.5 | 0.3 | *** | 78 | 77.9 | 79.9 | 91.1 | 81.1 | 79.8 | 82.5 | 82.6 | 78.4 | 81.4 | 79.9 | 72.3 |
| **14** | MdNPV | 29 | 26 | 30.1 | 25.2 | 25.5 | 27 | 28.2 | 27.9 | 28.3 | 33.8 | 28.4 | 26.4 | 26.4 | *** | 90.4 | 77.1 | 77.1 | 77.7 | 75.6 | 77.5 | 77.2 | 78 | 76.9 | 77.9 | 74.5 |
| **15** | MnNPV | 28.8 | 27.4 | 30.1 | 25.4 | 26.7 | 27.1 | 26.7 | 26.1 | 26.1 | 32.4 | 27.5 | 26.3 | 26.7 | 10.5 | *** | 77.6 | 77.5 | 79.4 | 75.6 | 77.3 | 77.2 | 77.6 | 76.7 | 78.7 | 72.1 |
| **16** | OpSNPV | 28.7 | 28.8 | 27.8 | 22.1 | 19.6 | 24.4 | 25.5 | 25.9 | 25.7 | 34.7 | 25.4 | 24.4 | 24.6 | 28.4 | 27.6 | *** | 81.2 | 79.1 | 75.3 | 79.1 | 79.9 | 76.1 | 76.8 | 80 | 70.4 |
| **17** | PfNPV | 20.9 | 25.1 | 20.6 | 23.5 | 21.6 | 10.2 | 30.5 | 30.1 | 29.9 | 29.1 | 10.2 | 9.3 | 9.6 | 28 | 27.4 | 22.3 | *** | 81.5 | 81.6 | 81.5 | 82.6 | 77.1 | 81.6 | 79.8 | 72.4 |
| **18** | PoNPV | 21.7 | 27 | 26.4 | 24.7 | 25.2 | 22 | 28.6 | 28.4 | 28.8 | 30.9 | 21.9 | 21.9 | 22.1 | 26.9 | 24.5 | 25.1 | 21.5 | *** | 80.6 | 78.5 | 81.1 | 76.2 | 78.8 | 84.5 | 72 |
| **19** | PxMNPV | 0.1 | 30.5 | 27.5 | 30.2 | 28.3 | 23.5 | 34.1 | 34.1 | 34.6 | 31.1 | 23.9 | 22.6 | 22.8 | 29.2 | 29.1 | 28.9 | 21.1 | 21.9 | *** | 75.3 | 78.6 | 76.2 | 76.2 | 79.3 | 73.2 |
| **20** | SeMNPV | 29.3 | 25.5 | 20.3 | 24.4 | 23.5 | 21.6 | 28.9 | 30 | 29.8 | 33.6 | 21.8 | 20.7 | 20.7 | 27.1 | 27.3 | 24.8 | 21.5 | 26.3 | 29.5 | *** | 86.2 | 79.6 | 86.5 | 78.1 | 73.2 |
| **21** | SfMNPV | 24.2 | 26.7 | 20.7 | 27.9 | 25.2 | 20.7 | 30.7 | 30 | 30.5 | 28.5 | 21 | 20.3 | 20.5 | 27.4 | 27.5 | 24.1 | 20.3 | 22.1 | 24.4 | 15.4 | *** | 79.5 | 87.3 | 80.8 | 71.3 |
| **22** | SliNPV | 30 | 27.1 | 26.4 | 30.4 | 26.4 | 27 | 31.5 | 31.9 | 32.4 | 30 | 27.9 | 26.9 | 26.5 | 24.4 | 25 | 29.4 | 28.6 | 30.3 | 30.2 | 24.3 | 26.5 | *** | 78.7 | 76.2 | 72.1 |
| **23** | SlNPV | 26.8 | 28.3 | 21.3 | 27.1 | 25.5 | 21.5 | 32.1 | 32.6 | 32.2 | 30.4 | 21.2 | 21.6 | 21.6 | 27.8 | 28.2 | 28.5 | 22 | 25.1 | 27 | 15.6 | 14.5 | 26.4 | *** | 78.4 | 74.6 |
| **24** | TnSNPV | 22.7 | 27 | 25.5 | 23.7 | 25.8 | 24.2 | 27.2 | 26.8 | 27.2 | 31.7 | 24.4 | 23.7 | 23.9 | 26.9 | 25.7 | 23.7 | 24.1 | 17.8 | 22.9 | 27 | 22.4 | 28.4 | 26.3 | *** | 72.5 |
| **25** | WsNPV | 32.4 | 35 | 32.5 | 34.5 | 29.8 | 33.1 | 37.9 | 36.1 | 36.2 | 32.6 | 34.9 | 33.2 | 33.2 | 33.7 | 34.2 | 35.9 | 32.8 | 33.8 | 32.6 | 31.5 | 34.1 | 34.2 | 34.7 | 33.9 | *** |

**Figure 49: Phylogenetic analysis at nucleotide level, of HaNPV-P (←) polyhedrin gene with previously published polyhedrin and granulin genes collected from GenBank, Tree generated using CLC work bench (version 3.1), algorithm used was UPGMA with 1000 bootstrapping and values indicated on the branches of tree is the number of replicates taken in bootstrap analysis.**



### 4.9.4 Phylogenetic Relation at Amino acid Level of HaNPV-P Polyhedrin Protein with Known Polyhedrin and Granulin Proteins:

The nucleotide sequence of polyhedrin gene was translated in to protein (Fig 47) and the predicted amino acid sequence was aligned with previously published amino acid sequences of polyhedrins and granulins by ClustalW method (Thompson *et al.,* 1994) using MgAlin tool of Lasergene software (DNASTAR, USA). Alignment of amino acid sequences of 40 NPV polyhedrins and 14 GV granulins along with HaNPV-P polyhedrin and construction of phylogenetic tree (Fig 50) revealed two major branches that were considered as separate clusters of baculovirus occlusion body protein (polyhedrin or granulin), they are NPVs and GVs. NPVs were again divided into three major branches, they are group-I, group-II NPVs and LdMNPV alone formed a separate branch.

The maximum homology of 100% among group-I NPVs was noticed between the following NPVs: AfNPV and RoMNPV, PnNPV and CrNPV and ToMNPV and TnSNPV and minimum homology of 85.7% among group-I NPVs was noticed between the following NPVS: AcMNPV and AmalNPV and AcMNPV and BmNPV (Table 23 and Fig 52). Similarly, the maximum homology of 100% among group-II NPVs was noticed between MbNPV and McNPV and minimum homology of 79.4% was noticed between WsNPV and SfMNPV (Table 24 and Fig. 53).

The third branch NPV *i.e.* LdMNPV was showed 77.1% to 80.4% homology with group-I NPVs and 78.8 to 82.4% homology with group-II NPVs. Due to less homology with other gropu-II NPVs, WsNPV and SliNPV were formed as separate cluster within group-II NPVs. But, after bootstrapping WsNPV was completely separate from group-II NPVs and formed as a seperte branch of NPV. The amino acid sequence of HaNPV-P polyhedrin protein was more close to group-II NPVs with maximum homology of 95.5% with MbNPV and McNPV, 93.9% with

PfNPV and 93.5% with LsNPV and minimum homology of 79.4% with WsNPV and 81.8% with SliNPV. Bootstrapping analysis of phylogenetic tree with CLC work bench revealed that LdMNPV and WsNPV were separated as major branches among all NPVs supported by 100% bootstrap values. Remaining all branches were supported by high bootstrap values (Fig 51).

**Figure 50: Phylogenetic analysis at amino acid level of HaNPV-P (←) polyhedrin protein with previously published polyhedrin and granulin amino acid sequences collected from GenBank, using Mgalign tool of DNASTAR software.**

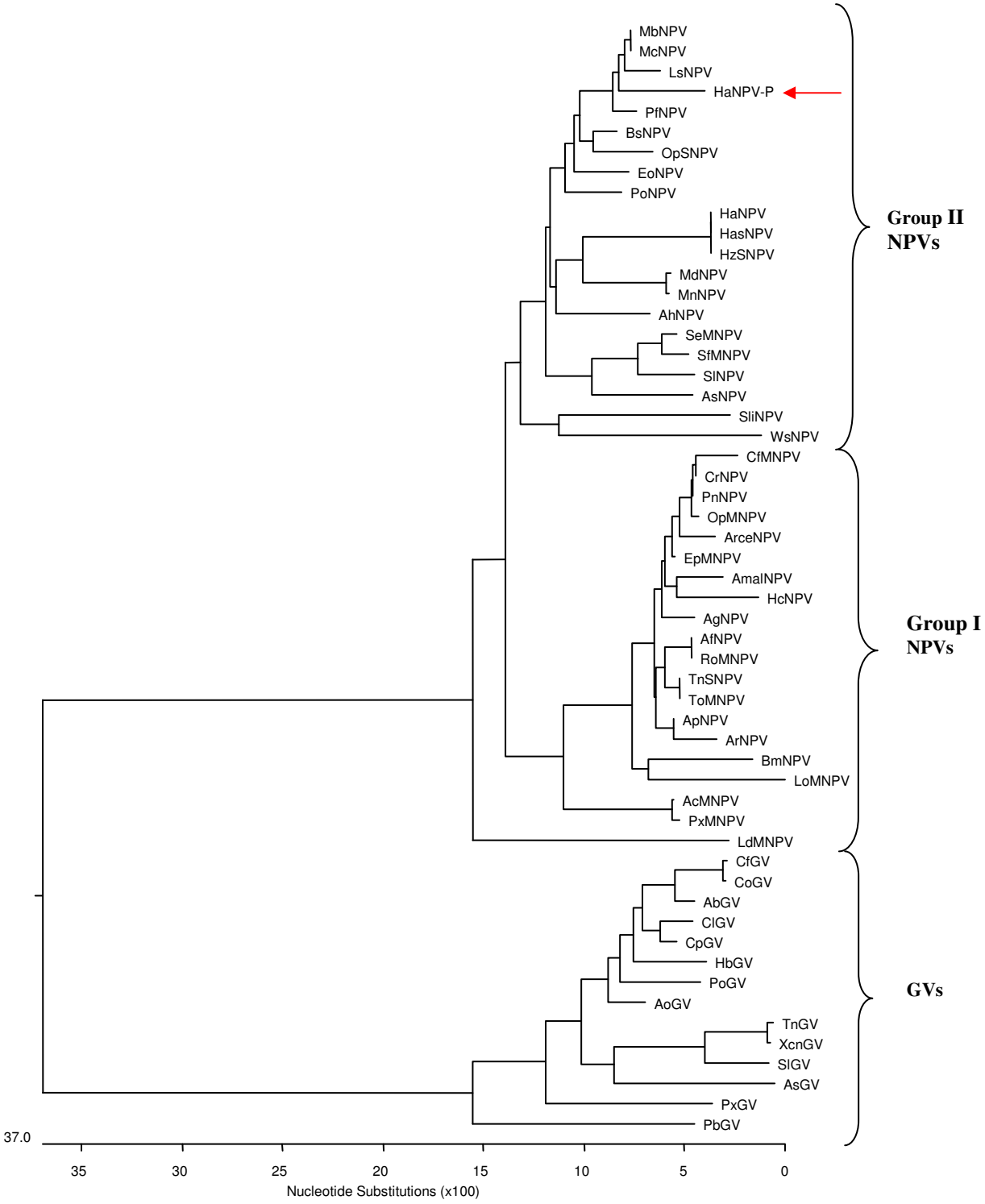


**Table 23: Pair Distances of amino acid sequence of polyhedrin protein among group-I NPVs, aligned by ClustalW (Slow/Accurate, IUB) method (Percent Similarity in upper triangle Percent Divergence in lower triangle).**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | AcMNPV | *** | 90.2 | 88.6 | 85.7 | 89.4 | 87.2 | 87.8 | 85.7 | 86.7 | 89.4 | 89 | 86.9 | 80.4 | 83.7 | 89.4 | 89.4 | 99.6 | 90.2 | 89.4 | 89.4 |
| **2** | AfNPV | 10.5 | *** | 95.9 | 93.5 | 97.6 | 95.1 | 95.5 | 92.2 | 94.7 | 96.7 | 97.1 | 93.5 | 79.2 | 89.4 | 96.3 | 96.7 | 90.2 | 100 | 98 | 98 |
| **3** | AgNPV | 12.4 | 4.2 | *** | 93.5 | 97.1 | 95.9 | 95.1 | 91.4 | 94.7 | 96.7 | 98 | 94.3 | 80 | 90.2 | 96.3 | 96.7 | 88.6 | 95.9 | 96.3 | 96.3 |
| **4** | AmalNPV | 13.9 | 5.1 | 5.1 | *** | 93.5 | 93 | 91.4 | 89 | 92.5 | 94.7 | 94.7 | 92.2 | 77.6 | 89.1 | 94.3 | 94.7 | 85.7 | 93.5 | 93.9 | 93.9 |
| **5** | ApNPV | 11.5 | 2.5 | 2.9 | 5.1 | *** | 96.3 | 98 | 93.5 | 95.1 | 97.1 | 98.4 | 94.3 | 79.6 | 91.4 | 96.7 | 97.1 | 89.4 | 97.6 | 98 | 98 |
| **6** | ArceNPV | 14 | 5.1 | 4.2 | 5.6 | 3.8 | *** | 93.4 | 89.3 | 95.6 | 96.7 | 97.1 | 92.6 | 77.8 | 87.7 | 96.3 | 96.7 | 86.4 | 94.2 | 95.9 | 95.9 |
| **7** | ArNPV | 13.4 | 4.6 | 5.1 | 7.3 | 2.1 | 6 | *** | 91.4 | 93.4 | 95.1 | 96.3 | 92.2 | 78 | 89.4 | 94.7 | 95.1 | 87.8 | 95.5 | 95.9 | 95.9 |
| **8** | BmNPV | 15.9 | 8.2 | 9.1 | 11 | 6.8 | 10.6 | 9.1 | *** | 88.9 | 91 | 91.8 | 89 | 79.2 | 88.6 | 91 | 91 | 85.3 | 92.2 | 91.8 | 91.8 |
| **9** | CfMNPV | 14.6 | 5.5 | 5.5 | 6 | 5 | 3.6 | 7 | 12 | *** | 98.2 | 96.9 | 91.6 | 76.5 | 87.2 | 97.8 | 98.2 | 86.7 | 94.7 | 96 | 96 |
| **10** | CrNPV | 11.5 | 3.3 | 3.3 | 3.8 | 2.9 | 2.5 | 5.1 | 9.6 | 1.8 | *** | 98.8 | 93.9 | 78.8 | 89.4 | 99.6 | 100 | 89.4 | 96.7 | 98 | 98 |
| **11** | EpMNPV | 11.9 | 2.9 | 2.1 | 3.8 | 1.7 | 2.1 | 3.8 | 8.7 | 3.2 | 1.2 | *** | 95.1 | 79.6 | 90.6 | 98.4 | 98.8 | 89 | 97.1 | 98.4 | 98.4 |
| **12** | HcNPV | 14.4 | 6.8 | 6 | 6.4 | 6 | 6.9 | 8.2 | 11.9 | 8.9 | 6.4 | 5.1 | *** | 78.4 | 87.8 | 93.5 | 93.9 | 86.9 | 93.5 | 93.5 | 93.5 |
| **13** | LdMNPV | 22.3 | 24 | 22.9 | 26.7 | 23.4 | 25.4 | 25.7 | 23.4 | 27.7 | 24.6 | 23.4 | 24 | *** | 77.1 | 78.8 | 78.8 | 79.6 | 79.2 | 78.8 | 78.8 |
| **14** | LoMNPV | 17.9 | 11 | 10.1 | 12.3 | 8.7 | 12.1 | 11 | 11.9 | 13.6 | 11 | 9.6 | 12.9 | 26.7 | *** | 89.4 | 89.4 | 83.3 | 89.4 | 89.8 | 89.8 |
| **15** | OpMNPV | 11.5 | 3.8 | 3.8 | 4.2 | 3.3 | 2.9 | 5.5 | 9.6 | 2.2 | 0.4 | 1.7 | 6.8 | 24.6 | 11 | *** | 99.6 | 89.4 | 96.3 | 97.6 | 97.6 |
| **16** | PnNPV | 11.5 | 3.3 | 3.3 | 3.8 | 2.9 | 2.5 | 5.1 | 9.6 | 1.8 | 0 | 1.2 | 6.4 | 24.6 | 11 | 0.4 | *** | 89.4 | 96.7 | 98 | 98 |
| **17** | PxMNPV | 0.4 | 10.5 | 12.4 | 13.9 | 11.5 | 14 | 13.4 | 16.4 | 14.6 | 11.5 | 11.9 | 14.4 | 22.9 | 18.5 | 11.5 | 11.5 | *** | 90.2 | 89.4 | 89.4 |
| **18** | RoMNPV | 10.5 | 0 | 4.2 | 5.1 | 2.5 | 5.1 | 4.6 | 8.2 | 5.5 | 3.3 | 2.9 | 6.8 | 24 | 11 | 3.8 | 3.3 | 10.5 | *** | 98 | 98 |
| **19** | TnSNPV | 11.5 | 2.1 | 3.8 | 4.6 | 2.1 | 3.4 | 4.2 | 8.7 | 4.1 | 2.1 | 1.7 | 6.8 | 24.6 | 10.5 | 2.5 | 2.1 | 11.5 | 2.1 | *** | 100 |
| **20** | ToMNPV | 11.5 | 2.1 | 3.8 | 4.6 | 2.1 | 3.4 | 4.2 | 8.7 | 4.1 | 2.1 | 1.7 | 6.8 | 24.6 | 10.5 | 2.5 | 2.1 | 11.5 | 2.1 | 0 | *** |

**Table 24: Pair Distances of amino acid sequence of polyhedrin protein among group-II NPVs, aligned by ClustalW (Slow/Accurate, IUB) method (Percent Similarity in upper triangle Percent Divergence in lower triangle).**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | LdMNPV | *** | 81.2 | 79.2 | 82.4 | 82.4 | 80 | 80 | 80 | 80 | 81.6 | 82.4 | 82.4 | 79.2 | 78.8 | 81.2 | 82.9 | 82.9 | 80.8 | 80 | 80 | 80 | 78.2 |
| **2** | AhNPV | 21.1 | *** | 88.6 | 93.5 | 92.3 | 88.6 | 89 | 89 | 89 | 90.7 | 91.9 | 91.9 | 89.8 | 90.2 | 92.3 | 91.5 | 91.9 | 89 | 88.2 | 85.8 | 88.2 | 84.4 |
| **3** | AsNPV | 24.4 | 12.4 | *** | 90.2 | 87.8 | 86.6 | 86.6 | 86.6 | 86.6 | 88.6 | 89.8 | 89.8 | 87.8 | 88.2 | 89.4 | 89.4 | 88.6 | 92.3 | 91.9 | 82.5 | 89.4 | 81.9 |
| **4** | BsNPV | 19.5 | 6.8 | 10.5 | *** | 94.3 | 91.5 | 90.7 | 90.7 | 90.7 | 93.1 | 94.7 | 94.7 | 91.9 | 91.9 | 95.9 | 95.1 | 94.7 | 91.5 | 91.5 | 86.2 | 91.5 | 84 |
| **5** | EoNPV | 19.5 | 8.2 | 13.3 | 5.9 | *** | 90.2 | 89 | 89 | 89 | 92.7 | 94.3 | 94.3 | 89.4 | 89.8 | 95.1 | 94.7 | 93.5 | 89.8 | 89.4 | 86.6 | 89.4 | 84 |
| **6** | HaNPV-P | 22.8 | 12.4 | 14.8 | 9.1 | 10.5 | *** | 84.6 | 84.6 | 84.6 | 93.5 | 95.5 | 95.5 | 87 | 87 | 90.7 | 93.9 | 91.5 | 87.8 | 87.4 | 81.8 | 86.6 | 79.4 |
| **7** | HaNPV | 22.8 | 11.9 | 14.8 | 10 | 11.9 | 17.3 | *** | 100 | 100 | 86.2 | 87.4 | 87.4 | 90.2 | 89.8 | 89.4 | 87.8 | 89 | 86.6 | 86.2 | 85.4 | 86.2 | 83.5 |
| **8** | HasNPV | 22.8 | 11.9 | 14.8 | 10 | 11.9 | 17.3 | 0 | *** | 100 | 86.2 | 87.4 | 87.4 | 90.2 | 89.8 | 89.4 | 87.8 | 89 | 86.6 | 86.2 | 85.4 | 86.2 | 83.5 |
| **9** | HzSNPV | 22.8 | 11.9 | 14.8 | 10 | 11.9 | 17.3 | 0 | 0 | *** | 86.2 | 87.4 | 87.4 | 90.2 | 89.8 | 89.4 | 87.8 | 89 | 86.6 | 86.2 | 85.4 | 86.2 | 83.5 |
| **10** | LsNPV | 20.6 | 10 | 12.4 | 7.3 | 7.7 | 6.8 | 15.3 | 15.3 | 15.3 | *** | 98 | 98 | 88.6 | 89 | 93.1 | 97.2 | 93.5 | 91.5 | 91.1 | 84.1 | 89.8 | 81.5 |
| **11** | MbNPV | 19.5 | 8.6 | 10.9 | 5.5 | 5.9 | 4.6 | 13.8 | 13.8 | 13.8 | 2.1 | *** | 100 | 90.2 | 90.7 | 94.7 | 98.4 | 95.1 | 91.9 | 91.9 | 85 | 91.1 | 82.7 |
| **12** | McNPV | 19.5 | 8.6 | 10.9 | 5.5 | 5.9 | 4.6 | 13.8 | 13.8 | 13.8 | 2.1 | 0 | *** | 90.2 | 90.7 | 94.7 | 98.4 | 95.1 | 91.9 | 91.9 | 85 | 91.1 | 82.7 |
| **13** | MdNPV | 23.9 | 10.9 | 13.3 | 8.6 | 11.4 | 14.3 | 10.5 | 10.5 | 10.5 | 12.4 | 10.5 | 10.5 | *** | 99.6 | 90.7 | 89.4 | 91.1 | 87.4 | 87.4 | 86.2 | 86.2 | 84.4 |
| **14** | MnNPV | 24.4 | 10.5 | 12.9 | 8.6 | 10.9 | 14.3 | 10.9 | 10.9 | 10.9 | 11.9 | 10 | 10 | 0.4 | *** | 91.1 | 89.8 | 90.7 | 87.8 | 87.8 | 85.8 | 86.6 | 84.4 |
| **15** | OpSNPV | 21.1 | 8.2 | 11.4 | 4.2 | 5.1 | 10 | 11.4 | 11.4 | 11.4 | 7.3 | 5.5 | 5.5 | 10 | 9.5 | *** | 95.1 | 94.7 | 91.1 | 91.1 | 87 | 90.2 | 83.5 |
| **16** | PfNPV | 19 | 9.1 | 11.4 | 5.1 | 5.5 | 6.4 | 13.3 | 13.3 | 13.3 | 2.9 | 1.6 | 1.6 | 11.4 | 10.9 | 5.1 | *** | 94.3 | 90.7 | 90.7 | 85 | 90.7 | 81.9 |
| **17** | PoNPV | 19 | 8.6 | 12.4 | 5.5 | 6.8 | 9.1 | 11.9 | 11.9 | 11.9 | 6.8 | 5.1 | 5.1 | 9.5 | 10 | 5.5 | 5.9 | *** | 91.1 | 91.5 | 85.4 | 90.2 | 83.1 |
| **18** | SeMNPV | 21.7 | 11.9 | 8.2 | 9.1 | 10.9 | 13.3 | 14.8 | 14.8 | 14.8 | 9.1 | 8.6 | 8.6 | 13.8 | 13.3 | 9.5 | 10 | 9.5 | *** | 98 | 84.1 | 94.7 | 81.5 |
| **19** | SfMNPV | 22.8 | 12.9 | 8.6 | 9.1 | 11.4 | 13.8 | 15.3 | 15.3 | 15.3 | 9.5 | 8.6 | 8.6 | 13.8 | 13.3 | 9.5 | 10 | 9.1 | 2.1 | *** | 83.3 | 95.5 | 79.4 |
| **20** | SliNPV | 22.8 | 15.8 | 20 | 15.3 | 14.8 | 20.5 | 16.3 | 16.3 | 16.3 | 17.9 | 16.8 | 16.8 | 15.3 | 15.8 | 14.3 | 16.8 | 16.3 | 17.9 | 18.9 | *** | 82.5 | 81.9 |
| **21** | SlNPV | 22.8 | 12.9 | 11.4 | 9.1 | 11.4 | 14.8 | 15.3 | 15.3 | 15.3 | 10.9 | 9.5 | 9.5 | 15.3 | 14.8 | 10.5 | 10 | 10.5 | 5.5 | 4.6 | 20 | *** | 80.2 |
| **22** | WsNPV | 24.7 | 17.6 | 21.3 | 16 | 16 | 20.2 | 18.6 | 18.6 | 18.6 | 19.2 | 17.6 | 17.6 | 17.6 | 17.6 | 16.6 | 18.6 | 17.1 | 21.3 | 21.9 | 18.6 | 23 | *** |

**Figure 51: Phylogenetic analysis at amino acid level, of HaNPV-P (←) polyhedrin protein with previously published polyhedrin and granulin amino acid sequences collected from GenBank, Tree generated using CLC work bench (version 3.1), algorithm used was UPGMA with 1000 bootstrapping and values indicated on the branches of tree is the number of replicates taken in bootstrap analysis.**

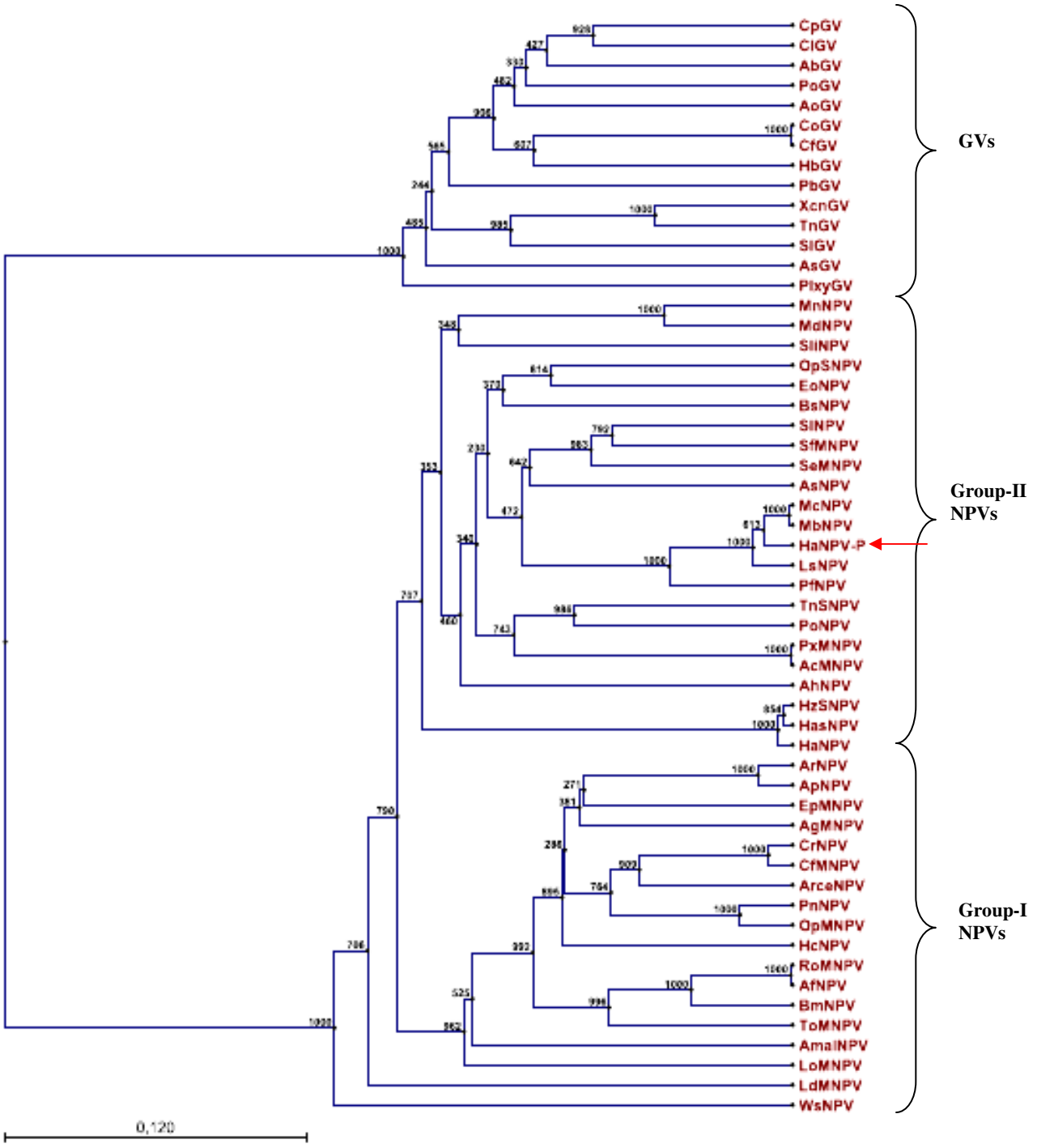
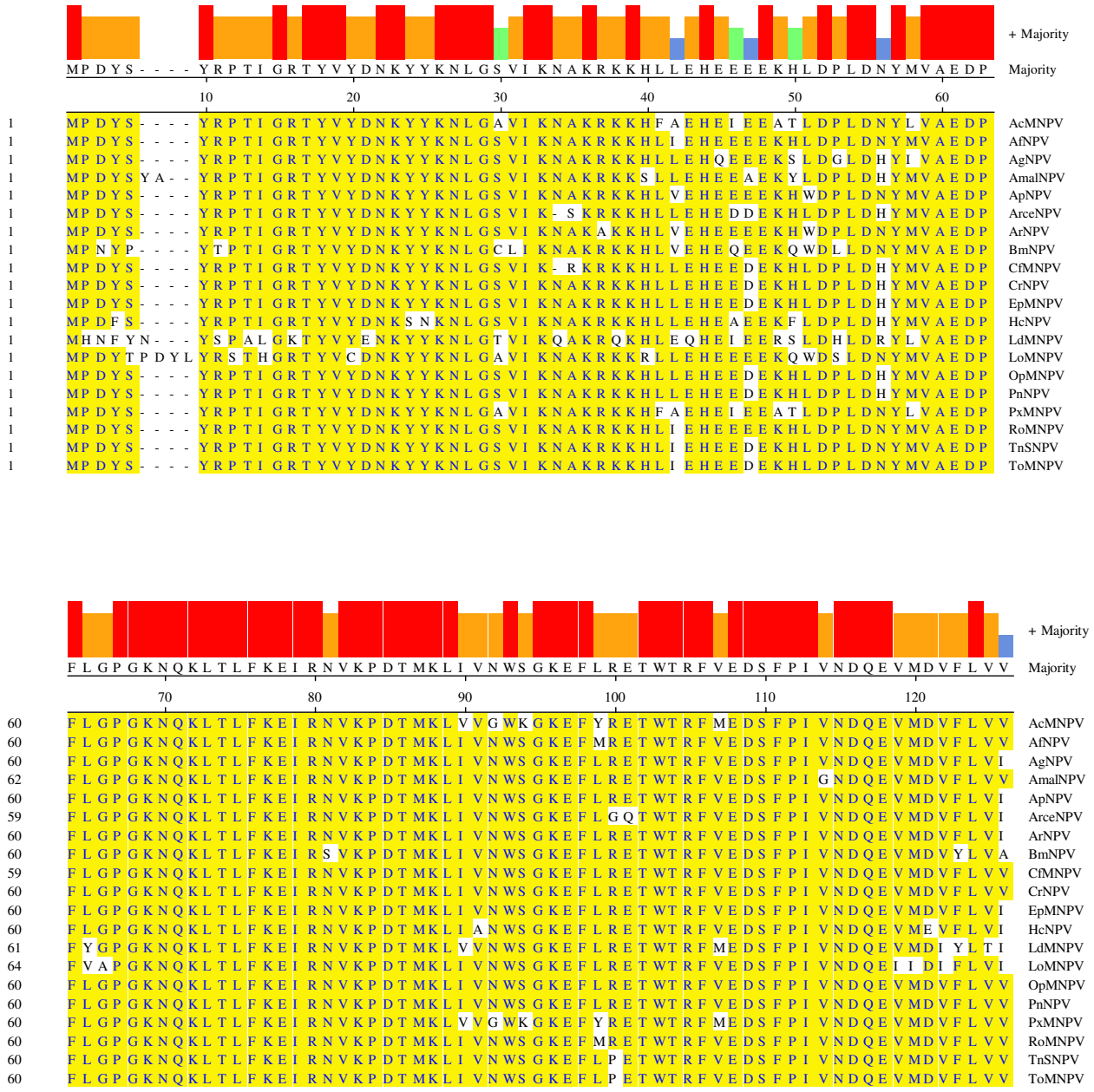
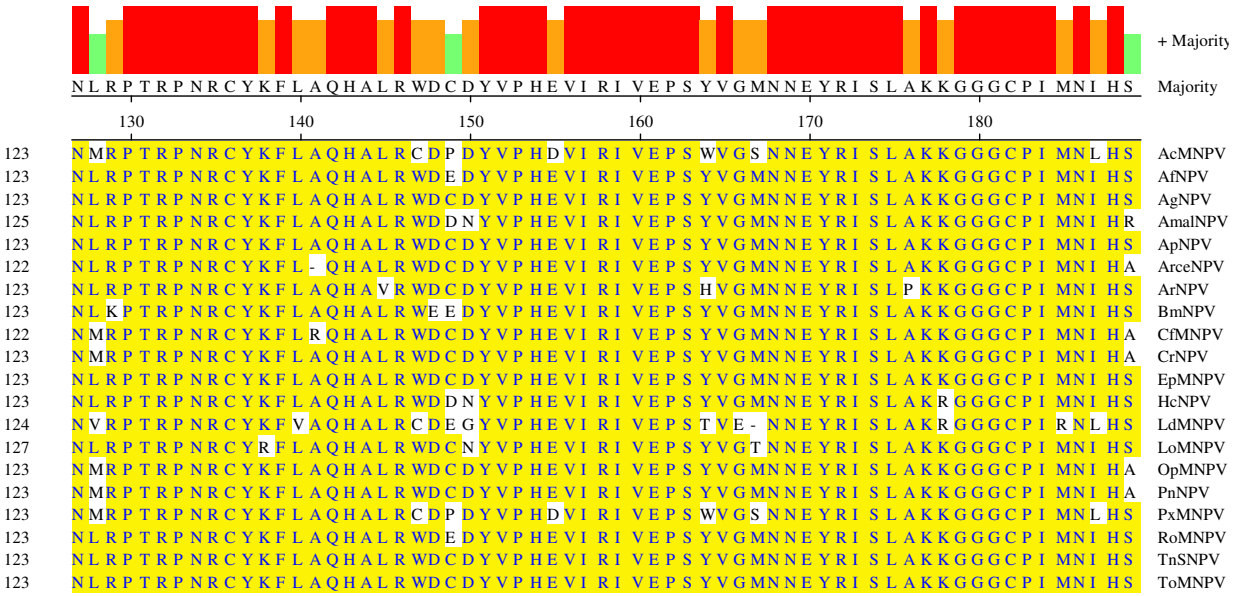


**Figure 52: Alignment of amino acid sequence of polyhedrin protein group-I NPVs, by Megalign using clastalW method.**

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Majority
130
140
150
160
170
180
123 N M R P T R P N R C Y K F L A Q H A L R C D P D Y V P H D V I R I V E P S W V G S N N E Y R I S L A K K G G G C P I M N L H S AcMNPV
123 N L R P T R P N R C Y K F L A Q H A L R W D E D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S AfNPV
123 N L R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S AgNPV
125 N L R P T R P N R C Y K F L A Q H A L R W D D N Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H R AmalNPV
123 N L R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S ApNPV
122 N L R P T R P N R C Y K F L - Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H A ArceNPV
123 N L R P T R P N R C Y K F L A Q H A V R W D C D Y V P H E V I R I V E P S H V G M N N E Y R I S L P K K G G G C P I M N I H S ArNPV
123 N L K P T R P N R C Y K F L A Q H A L R W E E D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S BmNPV
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123 N M R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H A CrNPV
123 N L R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S EpMNPV
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127 N L R P T R P N R C Y R F L A Q H A L R W D C N Y V P H E V I R I V E P S Y V G T N N E Y R I S L A K K G G G C P I M N I H S LoMNPV
123 N M R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H A OpMNPV
123 N M R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H A PnNPV
123 N M R P T R P N R C Y K F L A Q H A L R C D P D Y V P H D V I R I V E P S W V G S N N E Y R I S L A K K G G G C P I M N L H S PxMNPV
123 N L R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S RoMNPV
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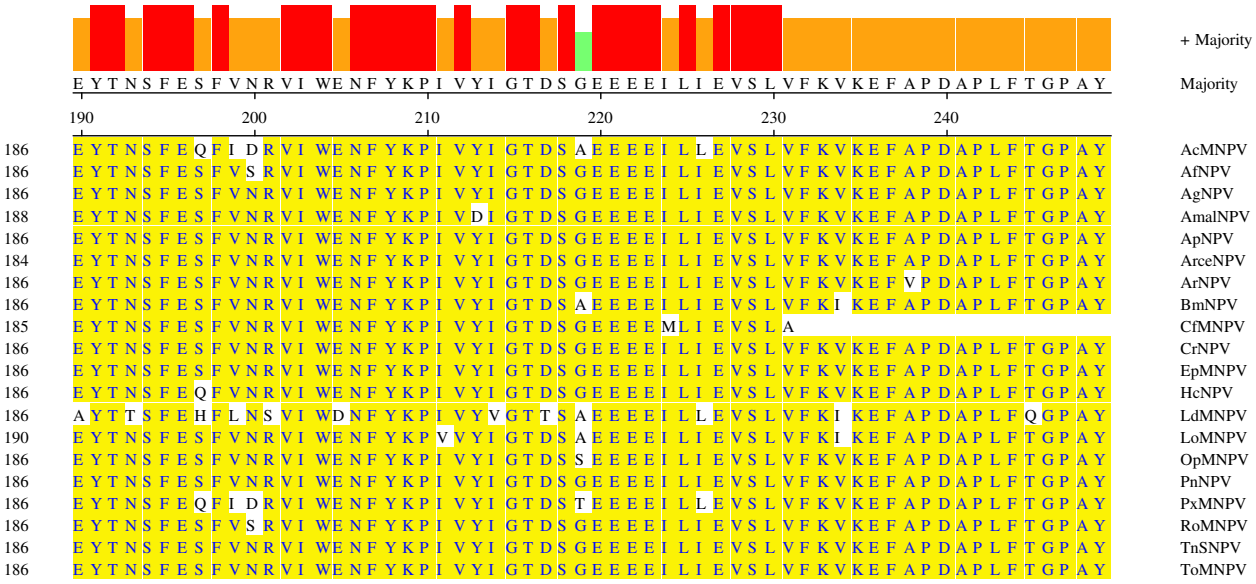
123 N L R P T R P N R C Y K F L A Q H A L R W D C D Y V P H E V I R I V E P S Y V G M N N E Y R I S L A K K G G G C P I M N I H S ToMNPV


+ Majority
E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y
Majority
190
200
210
220
230
240
186 E Y T N S F E Q F I D R V I W E N F Y K P I V Y I G T D S A E E E E I L L E V S L V F K V K E F A P D A P L F T G P A Y AcMNPV
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185 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E M L I E V S L A CfMNPV
186 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y CrNPV
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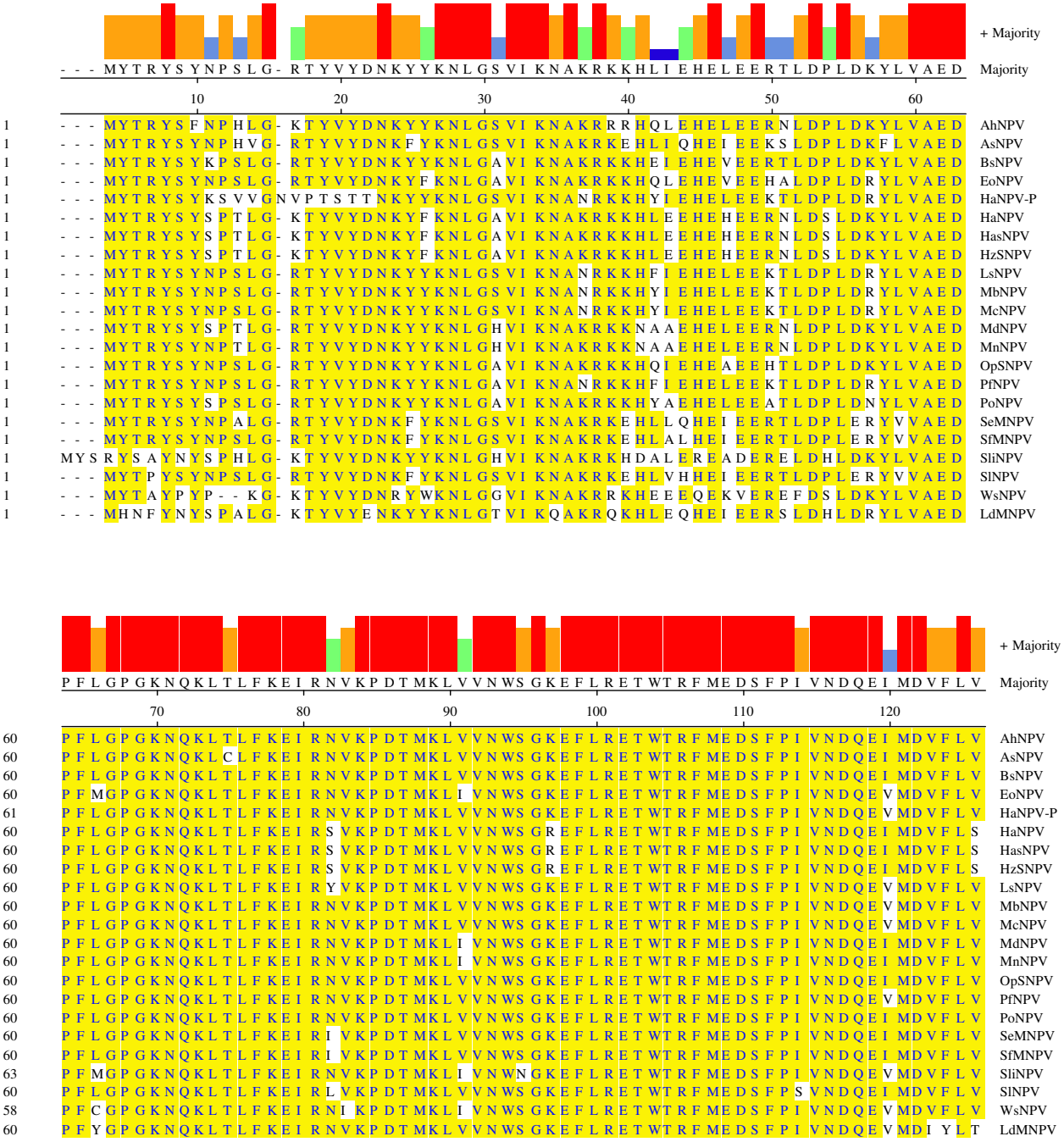
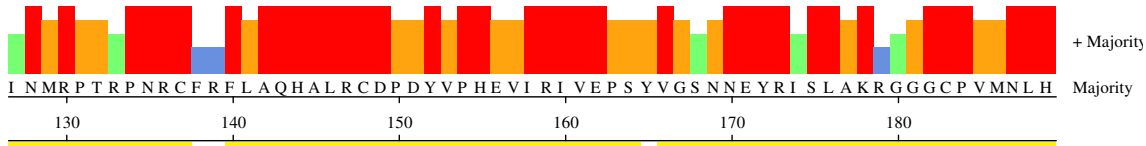
186 E Y T N S F E Q F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y HcNPV
186 A Y T T S F E H F L N S V I W D N F Y K P I V Y V G T T S A E E E E I L L E V S L V F K I K E F A P D A P L F Q G P A Y LdMNPV
190 E Y T N S F E S F V N R V I W E N F Y K P V V Y I G T D S A E E E E I L I E V S L V F K I K E F A P D A P L F T G P A Y LoMNPV
186 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S S E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y OpMNPV
186 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y PnNPV
186 E Y T N S F E Q F I D R V I W E N F Y K P I V Y I G T D S T E E E E I L L E V S L V F K V K E F A P D A P L F T G P A Y PxMNPV
186 E Y T N S F E S F V S R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y RoMNPV
186 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y TnSNPV
186 E Y T N S F E S F V N R V I W E N F Y K P I V Y I G T D S G E E E E I L I E V S L V F K V K E F A P D A P L F T G P A Y ToMNPV

**Figure 53: Alignment of amino acid sequence of polyhedrin protein group-II NPVs, by Megalign using clastalW method.**



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|---|---|---|
| 123 | I N M R P T R P N R C Y K F L A Q H A L R C D P D Y V P H E V I R I V E P S W V G S N N E Y R I S L A K R G G G C P V M N L H | AhNPV |
| 123 | V N M R P V K P N R C Y R F L A Q H A L R C D P D Y V P H E V I R I V E P V Y V G N H N E Y R I S L A K K G G G C P V M N L H | AsNPV |
| 123 | I N M R P T R P N R C Y R F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R I S L A K R G G G C P V M N L H | BsNPV |
| 123 | I N M R P T R P N R C Y K F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R I S L A K R G G G C P V M N L H | EoNPV |
| 124 | I N M R P T R P N R C F K F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R V S L A K R G G G C P V M N L H | HaNPV-P |
| 123 | V N M R P T K P N R C Y R F L A Q H A L R C D P D Y I P H E V I R I V E P S Y V G S N N E Y R I S L A K K Y G G C P V M N L H | HaNPV |
| 123 | V N M R P T K P N R C Y R F L A Q H A L R C D P D Y I P H E V I R I V E P S Y V G S N N E Y R I S L A K K Y G G C P V M N L H | HasNPV |
| 123 | V N M R P T K P N R C Y R F L A Q H A L R C D P D Y I P H E V I R I V E P S Y V G S N N E Y R I S L A K K Y G G C P V M N L H | HzSNPV |
| 123 | I N M R P T R P N R C F K F L A Q H A L R C D P D Y V P H E V I R I V E R S C V G T N N E Y R V S L A K R G G G C P V M N L H | LsNPV |
| 123 | I N M R P T R P N R C F K F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R V S L A K R G G G C P V M N L H | MbNPV |
| 123 | I N M R P T R P N R C F K F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R V S L A K R G G G C P V M N L H | McNPV |
| 123 | V N M R P T K P N R C F R F L A Q H A L R C D S D Y V P H E V I R I V E P S Y V G S N N E Y R I S L G K R Y N G C P V M N L H | MdNPV |
| 123 | V N M R P T K P N R C F R F L A Q H A L R C D S D Y V P H E V I R I V E P S Y V G S N N E Y R I S L G K R Y N G C P V M N L H | MnNPV |
| 123 | I N M R P T R P N R C F R F L A Q H A L R C D P E Y V P H E V I R I V E P S Y V G S N N E Y R I S L A K R G G G C P V M N L H | OpSNPV |
| 123 | I N M R P T R P N R C Y K F L A Q H A L R C D P E Y V P H E V I R I V E P S Y V G S N N E Y R V S L A K R G G G C P V M N L H | PfNPV |
| 123 | I N M R P T R P N R C F K F L A Q H A L R C D P D Y V P H E V I R I V E P S W V G S N N E Y R I S L A K K G G G C P I M N L H | PoNPV |
| 123 | I N M R P T R P N R C F R F L A Q H A L R C D P D Y V P H E V I R I V E P V Y V G T N N E Y R I S L A K K G G G C P V M N L H | SeMNPV |
| 123 | I N M R P T R P N R C F R F L A Q H A L R C D P D Y V P H E V I R I V E P V Y V G N N N E Y R I S L A K K G G G C P V M N L H | SfMNPV |
| 126 | V N M R P T R P N R C F R F L A Q H A L R C D P E Y V P H D V I R I V E P S Y V G T N N E Y R I S L A K K G G G C P V M N L H | SliNPV |
| 123 | I N M R P T R P N R C Y R F L A Q H A L R C D P D Y V P H E V I R I V E P S Y V G S N N E Y R I S L A K K G G G C P V M N L H | SlNPV |
| 121 | I N M R S T K P N R C F R F L A Q H A L R C D P D Y V P H E I I R I V E P S Y V G - N N E Y R I S L A K R G G G C P I M N L H | WsNPV |
| 123 | I N V R P T R P N R C Y K F V A Q H A L R C D E G Y V P H E V I R I V E P S T V E - N N E Y R I S L A K R G G G C P I R N L H | LdMNPV |


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|---|---|---|
| 186 | A E Y T N S F E E F I S R V I W E N F Y K P I V Y I G T D S A E E E E I L L E V S L V F K V K E F A P D A P L Y T G P A Y | AhNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y I G T D S A E E E E I L L E L S L V F K I K E F A P D A P L Y N G P A Y | AsNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K V K E F A P D A P L Y T G P A Y | BsNPV |
| 186 | A E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y S G P A Y | EoNPV |
| 187 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y I G P A Y | HaNPV-P |
| 186 | A E Y T N S F E D F I T N V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L I F K I K E F A P D A P L Y T G P A Y | HaNPV |
| 186 | A E Y T N S F E D F I T N V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L I F K I K E F A P D A P L Y T G P A Y | HasNPV |
| 186 | A E Y T N S F E D F I T N V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L I F K I K E F A P D A P L Y T G P A Y | HzSNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y N G P A Y | LsNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y N G P A Y | MbNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y N G P A Y | McNPV |
| 186 | S E Y T N S F E D F I N R V I W E N F Y K P L V Y I G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y T G P A Y | MdNPV |
| 186 | S E Y T N S F E D F I N R V I W E N F Y K P L V Y I G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y T G P A Y | MnNPV |
| 186 | A E Y T N S F E E F I N R V H W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y S G P A Y | OpSNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y N G P A Y | PfNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y S G P A Y | PoNPV |
| 186 | S E Y T N S F E E F I N R V I W E N F Y K P I V Y V G T D S G E E E E I L L E L S L V F K I K E F A P D A P L Y N G P A Y | SeMNPV |
| 186 | S E Y T H S F E E F I N R V I W E N F Y K P I V Y V G T D S G E E E E I L L E L S L V F K I K E F A P D A P L Y N G P A Y | SfMNPV |
| 189 | A E Y T T S F E S F I D K V I W Y N F Y K P I V Y V G T D S A E E E E I L L E V S L V F K I K E F A P D A P L Y T G P A Y | SliNPV |
| 186 | S E Y T H S F E E F I N R V I W E N F Y K P I V Y V G T D S G E E E E I L L E V A L V F K I K E F A P D A S L Y N G P A Y | SlNPV |
| 183 | L E Y T N S F E H F I N K V I W E N F Y K P I V Y I G T D S A E D E E V L F E V S L V F K I K E F A P D A P L F S G P A Y | WsNPV |
| 185 | S A Y T T S F E H F L N S V I W D N F Y K P I V Y V G T T S A E E E E I L L E V S L V F K I K E F A P D A P L F Q G P A Y | LdMNPV |

## 4.10. Development of PCR Based RFLP Marker for Identification and Differentiation of HaNPV-P (Patancheru) Strain:

In order to distinguish the HaNPV-P strain with other NPVs based on its unique restriction sites present in the amplified portion of the polyhedrin gene a PCR-RFLP marker was developed.

### 4.10.1 Restriction mapping analysis:

Restriction mapping analysis of HaNPV-P polyhedrin gene along with other known published polyhedrin sequences showed that one unique restriction site, *Xho*-I at nucleotide position 131 was found in NPV from *M. brassicae* and *M. configurata,* and at position 671 in NPV of *L. seperata.* Whereas in HaNPV-P, the *Xho*-I site was found at both 131 and 671 base pairs (Fig 54). But, the *Xho*-I site was not found in any of the HaNPV polyhedrin gene sequences deposited in the GenBank.

### 4.10.2 PCR-RFLP analysis:

The *Xho*-I restriction sites present in HaNPV-P polyhedrin gene was verified by PCR-RFLP study. In 12% native PAGE the digested product was separated as three different sized fragments which are corresponding to the restriction map. The sizes of the restriction fragments were estimated to be 540, 131 and 73 bps (Fig 55).

**Figure 54: Restriction mapping analysis of HaNPV-P polyhedrin gene using BioEdit version 5.0.9**

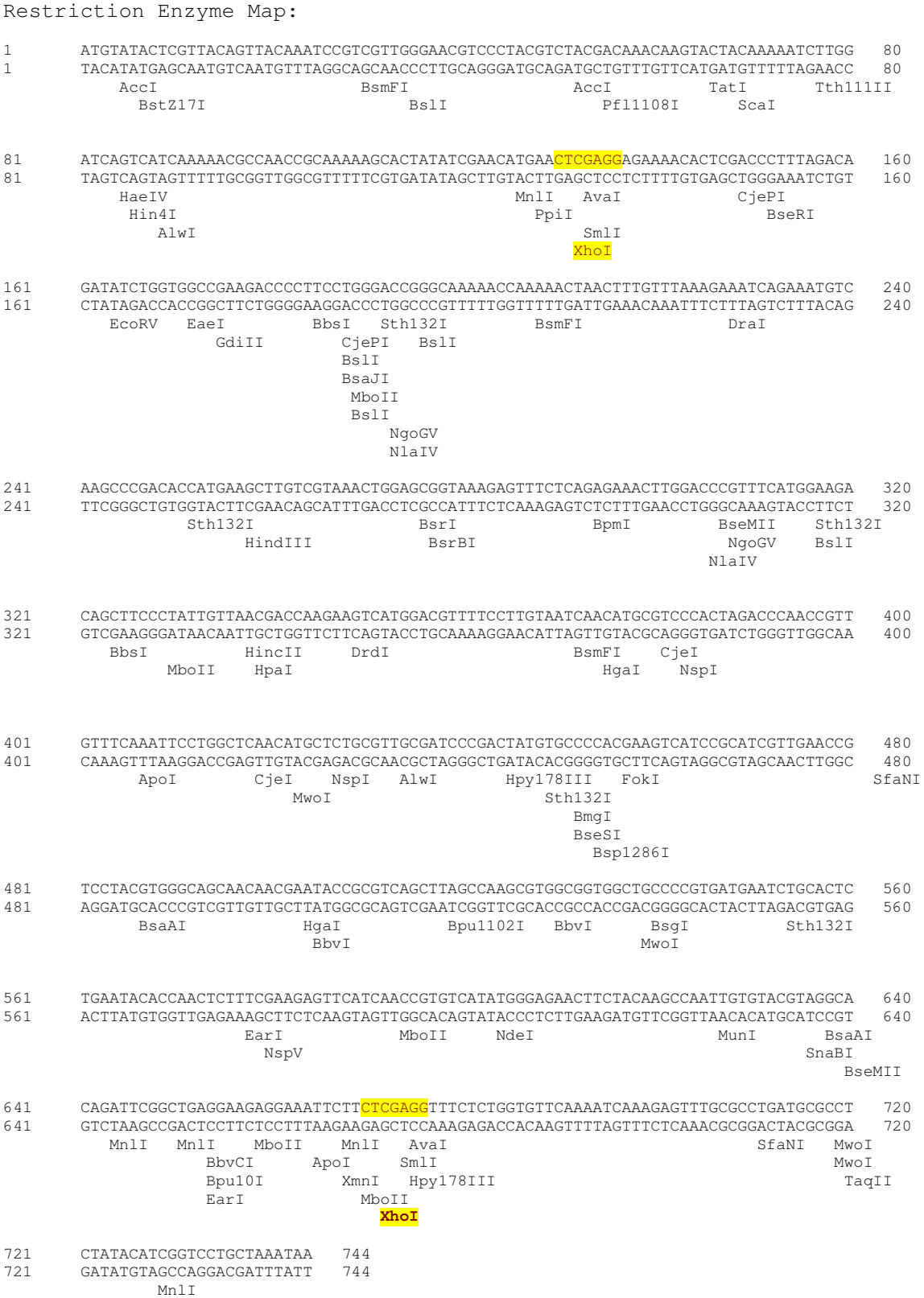
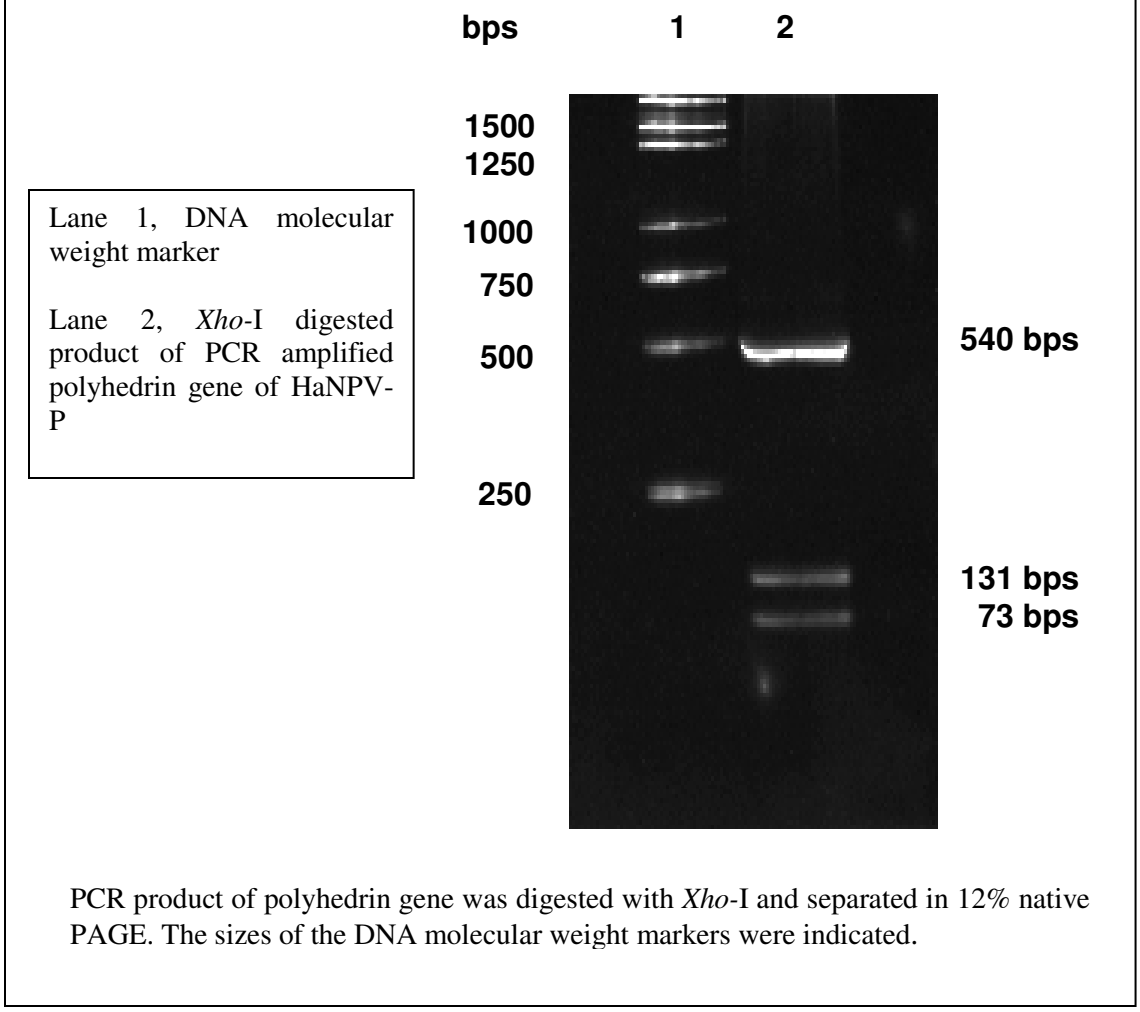


**Figure 55: PCR-RFLP analysis of HaNPV-P polyhedrin gene**




PCR product of polyhedrin gene was digested with *Xho*-I and separated in 12% native PAGE. The sizes of the DNA molecular weight markers were indicated.

# CHAPTER – V

# *DISCUSSION*

# CHAPTER-V
# DISCUSSION

Sole reliance on synthetic chemical pesticides to manage noxious pests like *H. armigera, S. litura* and *A. albistriga* has led to the development of several ill effects on the environment leading to ecological hostility and insect resistance to pesticides. To minimize these ill effects of synthetic chemicals, and at the same time to reduce the pest population, biological control methods through use of entomopathogenic microorganisms form as a potential pest control component of IPM. Currently a major focus in the management of these major pests has been through the use of NPV. Though NPV has distinct advantages such as host specificity, high virulence, economic feasibility, environmental safety and compatibility with other methods of pest control, it is underexploited due to its non-availability to farmers, and there is wide gap between demand and production due to non-availability of methods to scale up the virus production process. The reliability of the product is also a crucial issue in ensuring acceptance and sustained use by the farmers. The issue of erratic performance of viral biocontrol agents has been recognized as a significant factor in the limited successful commercialization (Lisansky, 1997). Many of the viral products available in the market were characterized as 'weak' with poor efficacy and questionable 'quality' (Harris, 1997). Many of the viral products produced in developing countries are failing to meet the acceptable standards (Kern and Vaagt, 1996). Without quality control aspects incorporating into NPV production, poor quality products will erode the confidence of farmers in microbial control products like NPV and significantly retard the promotion of this potential technology. Therefore it is necessary to have an effective diagnostic and quality control system for the development of a successful microbial control product (Shieh, 1989).

Mass production of NPV insecticide is simple and widely produced even at farmer level. Healthy larvae reared in the laboratory or collected from the fields are fed with low dose of NPV

and the virus produced in the insect is harvested and its concentration is estimated by counting POBs using a light microscope fitted with hemocytometer. Recently local production and utilization of NPV gained momentum in India through participation of scientists, farmers, NGOs and state agricultural and agriculture extension departments. Although, commercial production, quality and storage were still contentious issues, NPV is multiplied on field collected larvae and being applied on crops. Multiplying NPV on field-collected larvae was found to be easier and cost effective compared to laboratory-reared larvae, but efficacy and quality of which may be affected due to contaminants such as bacteria and fungi. The effectiveness of the viral insecticide is critically dependent on concentration of POB, which is expressed as LE (Larval Equivalent). Generally, a standard stock preparation consists of 1LE, *i.e.* $6 \times 10^9$ POBs/ml. While NPV insecticide production methods have been well established in many developing countries, the microscopic counting procedure used to screen larvae for NPV infection and quality control of the viral insecticide lots has low-detection efficiency, unknown specificity and is laborious and requires considerable skill. Because of this many NPV products produced have poor efficacy and found to be ineffective under field conditions. To over come these problems and for effective production of viral insecticides, it is necessary to have an efficient strategy for virus production, combined with rapid and specific diagnostic and quality control tools. At present appropriate, sensitive and reliable serological tools are not available and the development will go a long way in the quality control of insect viruses in developing countries. Once developed, the tools would be of immense value to public and private entrepreneurs, such as state biopesticide production laboratories and regulatory agencies. Furthermore, highly standardized, accurate and sensitive diagnostic tools for NPV detection in field-collected larvae would be beneficial to pest management personnel, because early detection of NPV disease could make it possible to predict the occurrence of an imminent epizootic and thus alter the pest control tactics to be employed. In addition, accurate identification of NPV species using molecular approaches is also important for establishing the identity of seed stock or master stock. The information is very limited in India on

molecular level identification and evaluation of phylogenetic status of commercial baculovirus preparations against major insect pests. Hence, the present study was undertaken to develop and evaluate the immunochemical tools for quantitative estimation of NPVs in commercial lots; to apply these tools in diagnosis of NPV infection at field level and to characterize and determine the phylogenetic status of NPV used for commercial viral insecticide preparations at ICRISAT, Patancheru, India.

## 5.1 Isolation and Propagation of NPVs from Major Lepidopteran Pests of Legume Crops:

During natural epizootic conditions NPV infections were observed in *H. armigera*; *S. litura* and *A. albistriga* pest populations at ICRISAT forms. The diseased larvae showed the typical baculovirus infection symptoms. The infected larvae showed pale swollen bodies and moribund. The larvae of *H. armigera* and *A. albistriga* were crawled to the top of the twigs (negative geotropism) on which they were fed (Fig 12). But the larva of *S. litura* was not showed this feature due to its burrowing and nocturnal habitat. Hence, the diseased larvae of *S.litura* were collected at the base of the plant or at peripheral layers of the soil near by root system. The initial signs of baculoviral infection are gradual changes in the color and luster of the integument. Infection of the epidermis caused the host to appear soft and in some larvae the cuticle was ruptured and discharging of body fluid on to plant parts was observed. Earlier these symptoms were also reported by others (Tanada *et al.,* 1993; Federici 1997; Aizawa 1963). Observation of discharged body fluid under phase contrast microscope revealed that it consists of POBs. The infectious virions are occluded in proteinaceous POBs also called polyhedra and are protected against environmental conditions for several years until the availability of susceptible host at particular life–stage from a given locality for significant period of time to maintain a continuous cycle of infection (Jacques, 1975; Rohrmann, 1986). Hence, baculoviruses cause lethal epizootic diseases in their host-insect populations (Gelertner and Federici, 1990) and have great potential to

be used as biological insecticides (De Moraes *et al.,* 1997). The role of NPVs in natural epizootics and their insect population dynamics were studied by many workers (Blissard *et al* 2000, Gelernter and Federici, 1990; Caballero *et al.,* 1992a; Weiser, 1987).

In the present investigation HaNPV was multiplied both on field collected and laboratory reared fourth instar larvae and the yield obtained was compared. There was a significant difference in parameters like POBs/ml and POBs/larvae which showed that NPV multiplied on field collected larvae recorded significantly higher yield compared to laboratory reared larvae (Table 7). Similarly, the field collected larvae of *A. albistriga* were yielded 5.05 times more virus/larva (Veenakumari *et al.,* 2007), the NPV of *Hyblaea puera* (Cramer) (Lepidoptera: Hyblaeidae), the teak defoliator, when mass produced *in situ* reportedly yielded 2.56 times more HpNPV than when mass produced in the laboratory (Sudheendrakumar *et al.,* 2004). By multiplying the HaNPV on third instar *H. armigera* larvae Pawar and Thombre (1992) recorded $0.95 \times 10^9$ to $3.5 \times 10^9$ POBs/larva. Gopali and Lingappa (2001a) inoculated fourth instar *H. armigera* larvae @$10^8$ POBs/ml and recorded higher larval mortality and higher quantity of viral yield per larva ($2.81 \times 10^8$ POBs) and suggested $10^8$ POBs/ml as optimum viral dose required for mass production of virus both on third and fourth instar *H. armigera* larvae. In the present study the POB yield obtained per larva was slightly higher than the earlier studies conducted by Gopali and Lingappa (2001a) and Pawar and Thombre (1992), which may be due to variation in the size of the larvae inoculated with the virus and the diet provided to the larvae. However, Shieh (1978) recovered $5 \times 10^9$ POBs/larva by inoculating grown up larvae of *H. armigera,* which is very close to the present value. The host insect, insect diet, insect age and virus dosage, incubation, environment and preservation of virus infectivity are some of the major factors, which optimize the production of *Hz*NPV (Carter, 1984). The optimum dose of viral inoculum also varies with the virulent strain and age of the host (Shapiro, 1992; Battu, 1987, 1990). Rabindra and Subramanian (1974) harvested maximum virus yield by inoculating $10^6$ POBs/ fourth instar larva. There was no much difference in number of larvae required for the production of one LE of virus

from the field collected and laboratory reared larvae. Where as earlier Gopali and Lingappa (2001a) reported that the number of field collected larvae required to produce one LE of virus was higher (2.97) than laboratory reared ones. This variation was due to assorted sizes or stages in the field collected larvae where as uniformity of larval age in laboratory reared larvae account for discrepancy in the viral productivity. However mass production of HaNPV on field collected larvae is more feasible when large scale production is aimed in short period. Pawar and Thombre (1992) and Thombre (1996) recorded lower productivity per larva than in the current study. This could possibly be due to the utilization of larger proportion of early instar larvae. Where as for mass multiplication of SlNPV and AmalNPV laboratory reared $5^{th}$ instar larvae emerged from field collected egg masses was used for virus inoculation due to long gestation period during $5^{th}$ instar stage for these insects. Subsequently, due to heavy body weight of these insects the yield of NPV was also recorded as higher than HaNPV. In the present investigation the POB yield of SlNPV obtained per larva ($5.73 \pm 0.17 \times 10^9$) was higher than the earlier studies ($1.4 \times 10^9$) reported by Tuan *et al.* (1998) and lower than the earlier studies ($9.7 \times 10^9$) reported by Jun *et al.* (2007) and close to earlier study ($5.572 \times 10^9$) reported by Senthil kumar *et al.,* 2005. Whereas, the yield of AmalNPV ($7.90 \pm 0.54 \times 10^9$ POBs/larva) was noticed to be higher than HaNPV and SlNPV. This is due to higher body weight of *A. albistriga* larvae and was naturally is always larger and heavier than that of other two insects. *In situ* field level mass production of AmalNPV in groundnut ecosystem was studied by Veenakumari *et al.* (2007) and recorded the yield of $1.052 \times 10^{10}$ POBs/larva this is close to our present study. At ICRISAT, for effective mass multiplication of *Amal*NPV, the field-collected larvae were released into an aluminum or polythene grid/enclosure (10 cm height) to confine the larvae inside the shaded enclosure and fed with plants already inoculated with the virus. The field technique for rearing larvae was found to be advantageous, particularly in avoiding the handling of huge larval populations, rearing and inoculation. This would also facilitate farm level production and access to the biopesticide at the village level (Rao *et al.,* 2006).

## 5.2 Electron Microscopic (EM) Studies:

The NPVs isolated from the larvae of *H. armigera, S. litura* and *A. albistriga* at ICRISAT forms were morphologically compared with each other by conducting electron microscopic studies this revealed typical baculovirus occlusion bodies. Scanning electron micrographs (SEM) showed that the OBs of HaNPV, SlNPV and AmalNPV were appeared as irregular shape structures with sizes ranged from 0.5 to 2.5µm, 0.9 to 2.92µm and 1.0 to 2.0µm in diameter (Figs 14, 15 and 16). This indicated that viruses isolated in this present investigation were NPVs rather than GVs. Generally NPVs are larger size and are irregular to polyhedral shape where as GVs are comparatively smaller than NPVs and their shape is round. Before characterization of any baculovirus from an insect host, initially it is necessary to conduct electron microscopic study (SEM and TEM) to determine whether it is NPV or GV or SNPV or MNPV. Transmission electron microscope (TEM) studies on cross-sections of purified POBs of these viruses showed that each occlusion body contains 2 to 7 (multiple) nucleocapsids packaged within a single viral envelope (Figs 17, 18 and 19). The nucleocapsids are elongated with parallel sides and two straight ends, measuring the sizes of 277.7 × 41.6 nm (HaNPV), 285.7 × 34.2 nm (SlNPV) and 228.5 × 22.8 nm (AmalNPV) (Table 8). Tuan *et al.* (1999) reported that the occlusion bodies of HaNPV and SlNPV isolated in Taiwan were irregular shape with sizes raged from 0.79 ± 0.22 µm (HaNPV) and 1.61 ± 0.32 µm (SlNPV), both the viruses were MNPVs and the nucleocapsids were bacilliform to cylindrical tubular shaped structures with dimensions of 319.80 ± 7.80 × 44.45 ± 4.54 nm (HaNPV) and 332.26 ± 13.55 × 47.16 ± 1.42 nm (SlNPV). In another study the polyhedra of the *Lymantria dispar*MNPV-NM isolate were observed as irregularly shaped, the average diameter of the polyhedra was 1.62 ± 0.33 µm, TEM revealed that LdMNPV-NM had bundles of virions in the nucleocapsid, which belonged to MNPV (Shim *et al.,* 2003). Similarly, Wolf *et al.* (2002), reported the morphology of a MNPV isolated from *Lonomia obliqua* (Lepidoptera: Saturniidae) with size ranged from 1 to 1.4 µm and the nucleocapsid dimensions of

270 × 36nm. Ma *et al.* (2006), observed the occlusion bodies in the mid gut tissues of the tea looper (*Ectrophis obliqua*) under TEM, the micrograh showed that the EcobSNPV were irregular shape and ranged in size from 0.7 to 1.7 µm in diameter and multiple rod-shaped virions measuring about 250 nm in length and 40 nm in width, were embedded in each OB with a single nucleocapsid packaged within the envelope of the virion. Woo *et al.* (1998) isolated the host range-expanded recombinant baculovirus, RecB-8 from BmN-4 cells, coinfected with *A. californica* and *B. mori* nuclear polyhedrosis viruses and morphology of their polyhedra compared in an electron microscope. Interestingly, the polyhedra of RecB-8 were tetrahedral although the polyhedrin gene was the same as that of the BmNPV parent which has icosahedral polyhedra. Thus the morphology of the RecB-8 polyhedra resulted from host cell factors and/or another viral genome in the host cells. Recently, Grasela *et al.* (2008) demonstrated the MNPV nature of a NPV infecting *T. ni* during isolation and characterization of a baculovirus associated with that insect parasitoid wasp, *Cotesia marginiventris,* or its host. Gales *et al.*(2007), studied the use of immunoglobulin heavy chain binding protein (BiP) as a signal sequence to help guide recombinant protein to the rough endoplasmic reticulum (rER), to characterize the distribution of recombinant proteins in infected SF9 cells 2 days post-infection using confocal laser scanning microscopy (CLSM) and TEM.

## 5.3 Purification of Polyhedral Protein (Polyhedrin):

The crystalline matrix of the occlusion body mainly consists of a single protein, called polyhedrin or granulin. These proteins are about 245 amino acids (29 kDa) and hyper expressed during very late phase of virus infection and are not required for virus replication (Rohrmann, 1986, 1992; Funk *et al.,* 1997) and constituting up to 18% or more of total alkali-soluble protein late in infection (Quant *et al.,* 1984). It is a highly stable protein, insoluble in many solvents at neutral pH values and physiological conditions, highly resistant against the action of proteolytic enzymes and at the same time it is highly sensitive to alkali conditions (Bergold, 1947,1948). In

the present study with an aim of production of polyclonal antibodies against poly occlusion body protein (polyhedrin) for the development of diagnostic and quality control tools during mass production of NPVs, the purification protocol for polyhedrin protein was standardized (Fig 8) with slight modifications to the methods given by Quant *et al.* (1984); Harrap *et al.* (1977) and Summers and Egawa (1973). The protocol steps involved initial heat inactivation of endogenous proteases; alkali disruption of POBs, ultracentrifugation of dissolved POBs to pellet the virions and undissolved POBs. Further purification was achieved by either of the following approaches, in one approach the supernatant of ultra-centrifuged dissolved POBs was layered on 10-40% liner sucrose gradient and in another approach the pH of the supernatant was adjusted to the isoelectric point of polyhedrin. Studies on biochemical and biophysical properties of the solubilized matrix proteins were provided by the discovery of an alkaline protease was associated with the protein matrix of NPVs (Eppstein and Thoma, 1975; Summers and Smith, 1975a; Payne 1978). This protease was activated by the alkaline conditions used to solubilize the matrix and degraded matrix components to a mixture of lower-molecular-weight polypeptides. Inhibition of this activity by $HgC1_2$ or by heat treatment at 70°C for 30 min has allowed the matrix to be solubilized and recovered in a non degraded form (Eppstein *et al.,* 1975; Summers and Smith, 1975; Tweeten *et al.,* 1978). Volkman and Falcon (1982) standardized the purification of polyhedrin from *T. ni* NPV by initial heat treatment of gradient purified POBs, followed by incubation of POBs in 0.01M $HgCl_2$ in 0.01 M tris buffer (pH 7.8), dissolution of POBs in dilute alkaline saline (DAS) (0.1 M $Na_2 CO_3$, 0.15 M NaCl, pH 10.9) and then polyhedrin was collected as supernatant by ultra centrifugation of dissolved POBs at 100,000 × g for 30 min. Similarly, after initial heat treatment polyhedrin from two *O. pseudotsugata* nucleopolyhedroviruses (OpSNPV and OpMNPV) was purified by dissolution of POBs in 0.1 volume of 1 M $Na_2CO_3$ 0.5 M NaCl buffer at $56^0$C for 10 min followed by centrifugation at 120,000 × g for 45 min (Quant *et al.,* 1984). In the present study of 10-40% linear sucrose gradient centrifugation, the polyhedrin formed one diffused light scattered zone in 10% sucrose region (Fig 20) due to its very low

density in solutions. Similarly, Summers and Egava (1973) purified the granulin from *T. ni* GV by dissolution of OBs in 0.07 M $Na_2 CO_3$ 0.05 M NaCl (pH10.7) at 5mg of OBs/ml for 1.5 to 2.0 h at room temperature then the granulin was clarified by the following two approaches. In first approach the dissolved OB suspension was subjected to centrifugation at 100,000 × g for 30 min. In second approach the dissolved OB suspension was layered on 10-40% (wt/vol) sucrose gradients and centrifuged at 25,000 rev/min by use of a SW41 rotor. Similarly, after heat treatment and alkali dissolution of gradient purified OBs of *A. californica, P. dispar, T. ni,* and *H. zea* and granulovirus from *T. ni* the polyhedrins or granulins were recovered from the top of sucrose gradients (density range of 1.15 to 1.27 g/ml) after centrifugation at 100,000 × g for 1 h (Smith and Summers, 1981). In isoelectric precipitation method, the polyhedrin of all the three NPVs was precipitated at pH between 5.5 and 5.6 (Fig 21). Most of the polyhedrins or granulins of baculoviruses are insoluble and precipitated at this pH due to their isoelectric property. Similarly, Brown *et al.* (1977) standardized the purification of granulin from gradient purified granules of *Pieris brassicae* GV by alkaline disruption of granules using 0.1 M $Na_2 CO_3$ then virus particles were pelleted at 75,000 × g for 1 h and the supernatant contains mainly the granulin was subjected to iso-electric precipitation (pH 5.6) by slow addition of 0.1 M HCl then the precipitated polyhedrin was collected as sediment by centrifuging at 4000 × g for 20 min. Similar approaches were followed by Harrap *et al.* (1977) for purification of polyhedrin from three nucleopolyhedroviruses from closely related hosts such as *S. littoralis, S. exempta* and *S. frugiperda.* Similarly, the polyhedrin from nucleopolyhedroviruses of *A. californica* and *T. ni* (Hohmann and Faulkner, 1982) and granulin from *Epinotia aporema* granulovirus (EpapGV) (Parola *et al.,* 2003) were purified by isoelectric precipitation of alkali solubilized occlusion bodies. Instead of gradient purification, the POBs from *A. californica* nucleopolyhedrovirus were extensively washed with 0.1% SDS, virus particles were released by alkali (0.1M $Na_2 CO_{3)}$ and centrifuged the dissolved POB suspension at 50,000 × g to pellet the virions then polyhedrin was precipitated from the supernatant by adjusting the pH to 5.8 (Roberts and Naser, 1982). Recently,

the putative polyhedrin protein of monodon baculovirus (MBV) was isolated from infected post larvae by homogenization, differential centrifugation and density gradient centrifugation with verification by transmission electron microscopy (Attaphon *et al.,* 2005). In 12% SDS-PAGE analysis, the light scattered zone as well as sample layers of sucrose gradients revealed that both samples were almost same, highly pure and appeared as single protein band with estimated molecular weight of approximately 31 kDa (HaNPV) (Fig 22). The same purification protocol in other preparations resulted in some minor low molecular weight as well as high molecular weight polypeptide contaminants. Similarly, the 12% SDS-PAGE analysis of the polyhedrin purified by isoelectric precipitation method reveled that the molecular weight of major polyhedrin proteins of three NPVs were 31.65 kDa (± 0.00), 31.29 kDa (± 0.00) and 31.67 kDa (± 0.295) *Helicoverpa, Spodoptera* and *Amsacta* respectively (Fig 23). This report is similar to that reported by Tuan *et al.* (1999) for three lepidopteran NPVs such as HaNPV, SlNPV and SeNPV. Recently, Ashour *et al.* (2007) reported the molecular weight of 32 kDa for recombinant and wild type *A. californica* nucleopolyhedrovirus (AcAaIT and AcMNPV). In addition to the major polyhedrin they are contaminated with some minor low molecular weight peptides of about 7-27 kDa and a high molecular weight peptide of about 60-70 kDa fragments (Fig 23), which could be the degraded peptides or dimmers of the 31 kDa polyhedrin protein. This has revealed that these three NPVs have 6-8 minor polypeptides. Finally, the yield of the polyhedrin obtained in sucrose gradient method was 1mg/ml, while the isoelectric precipitation method was about 15-20 mg/ml from standard POB preparations ($10^9$ POB/ml). Due to lack of consistency in the purity and quality of the polyhedrin preparation in both the methods, the polyhedrin was electro-eluted from 10% SDS-PAGE for immunization purpose.

The early reports on the proteins dissociated from this crystalline structures by weak alkali carbonate showed that this matrix consists of a heterogeneous mixture of peptides with sedimentation coefficients and estimated molecular weights (by gel electrophoresis) of 11.5S

(275,000), 12.7S (336,000) and 12.8S (378,000) for NPV from three different insect species and 11.8S (300,000) for GV (Bergold, 1959; Summers and Egawa, 1973). The presence of multiple protein species also indicated by immunological and amino acid sequence analysis showed that the homogeneous protein in the crystalline structures of a NPV and GV contain at least two different antigenic structures (Longworth *et al.,* 1972; Scott and Young, 1973). Summers and Smith (1975b) compared the physical and chemical properties of five polyhedrins and granulins using PAGE and two-dimensional high voltage electrophoresis of tryptic peptides and reported that each of the polyhedrins and granulins has a unique protein for a given virus with similar molecular weights of 28,000 $\pm$ 2000 Daltons. It is likely that lower molecular weight polypeptides detected in polyhedrin protein preparation after alkaline dissolution (Summers and Egava, 1973; Padhi *et al.,* 1975; McCarthy and Liu, 1976) are attributable to the activity of alkaline proteolytic enzymes (Eppstein and Thoma, 1975; Eppstein *et al.,* 1975). Which, in the NPVs of *Spodoptera* species may cleave the protein at a specific point to generate a polypeptide in the range 22,000-25,000 Daltons. This protease was activated by the alkaline conditions used to solubilize the matrix and degraded matrix components to a mixture of lower-molecular-weight polypeptides. Several additional baculoviruses have been investigated, and similar protease activities have been detected (Crawford and Kalmakoff, 1977; Eppstein *et al.,* 1975; Kozlov *et al.,* 1975; Mc Carthy and Liu, 1976; Tweeten *et al.,* 1978). These studies have indicated that the 12S molecule consists of eight subunits of granulin or polyhedrin and revealed that polyhedrins and granulins have close similarities in molecular weights. All of the baculovirus matrix proteins examined to date have molecular weights in the range from 25,000 to 30,000 Daltons. The solubilizing effects of various solvents on the proteinic crystalline structure of a granulosis virus of a *T. ni* was determined and further kinetic and morphological studies were conducted in an attempt to evaluate the nature of intermolecular binding forces which contribute to the construction and stability of the structure (Egawa and Summers, 1972; Kawanishi *et al.,* 1972).

## 5.4 Production of Polyhedrin Polyclonal Antibodies:

Polyhedrin, the major component of NPV polyhedra, is coded for by the virus, and its presence in larvae indicates the presence of NPV or an NPV infection. Hence, in the present study polyhedrin was used as antigen in production of polyclonal antibodies. Antibodies were raised in New Zealand White inbreed rabbits and were used for the development of diagnostic and quality control tools. It has been well documented that repeated inoculations of a virus antigen into an animal will elicit a different antibody response than that obtained with a single or few injections of the same antigen (Casals, 1967). In the present study the polyhedrin used for immunization was electro-eluted from a preparative 10% SDS-PAGE gel to avoid the unwanted minor protein contaminants in polyhedrin preparations such as some low molecular weight degraded peptides and some high molecular weight dimers (Fig 24). The rabbit is most frequently used animal for the preparation of polyclonal antibodies against baculoviruses and can be injected with whole OBs (Shamim *et al.,* 1994) or purified virions (Kelly *et al.,* 1978b and Smith and Summers, 1981), although the OBs are usually solubilized before injection especially if guinea pigs are used, (Crawford *et al.,* 1978). Factors such as antigen purity, variability of antisera, and reaction of antisera with contaminating non viral antigens have led to several problems during standardization of serological assays. To overcome this, individual viral structural proteins, most commonly polyhedrin or granulin, purified protein preparations are electrophoresed through PAGE gels and the required bands are eluted individually in to PBS (Barta and Issel, 1978; Summers and Smith, 1975 and Sridhar kumar *et al.,* 2007). In the present study 500µg of electro-eluted polyhedrin was used as antigen to immunize the animals, which gave an antibody titer of 1:5000 dilution (Fig 25) 18 weeks after initiation of immunization. Similarly, Crawford *et al.,* (1978) produced the polyclonal antibodies against *T. ni* SNPV, *T. ni* MNPV, *Euxoa messora* (*EM*) NPV, *Pieris rapae* (*Pr*) granulovirus and *Laspyresia pomonella* granulovirus and *Wiseana* spp. SNPV in New Zealand white rabbits as well as in Swiss white mice by injecting the purified

polyhedra (3ml) at concentration of 500µg/ml after dissolving in 0.1 volume of 1 M $Na_2CO_3$ and then neutralized with 1M HCl. At the same time purified polyhedrin preparations (80µg) were used for production of monoclonal antibodies as well as rabbit polyclonal antibodies to diagnose the NPV infection in infected larvae of *T. ni* (Volkman and Falcon, 1982; Volkman, 1982) and in Lymantria dispar (Yu *et al.,* 1992). Similarly, Quant *et al.,* (1984) produced the monoclonal antibodies against purified polyhedrin preparations of two *Orgyia pseudotsugata* Baculoviruses (OpSNPV and OpMNPV). To identify the conserved epitopes on the polyhedrin protein of *Heliothis zea* nucleopolyhedrovirus, 12 anti-HzSNPV polyhedrin monoclonal antibodies were produced (Huang *et al.,* 1985). To study the immunological relatedness of polyhedrin purified from nucleopolyhedroviruses of *H. armigera, S. litura* and *S. exigua,* polyclonal antibodies were produced against isolectric precipitates of polyhedrin and their cross- reactivity was evaluated (Tuan *et al.,* 1999). Harrap *et al.* (1977) has produced the polyclonal antibodies to purified polyhedra (5mg/ml), isolectric precipitated polyhedrin (5mg/ml), and purified virus particles (500µg /ml) of nucleopolyhedroviruses isolated from three closely related hosts such as *S. littoralis, S. exempta* and *S. frugiperda.* To develop the diagnostic tools for detection and quantification of baculoviruses from infected larvae at field and laboratory level, to study the biosafety and environmental fate of recombinant and wild type baculoviruses, as well as quality control during mass production of baculovirus based bio pesticides, polyclonal antibodies were produced against isolectric precipitates of polyhedrin or granulin purified from *Epinota aporema* granulovirus (EpapGV) (Parola *et al.,* 2003), recombinant and wild type *A. californica* nucleopolyhedrovirus (AcAaIT and AcMNPV) (Ashour *et al.,* 2007), and Ha NPV (Sridhar Kumar *et al.,* 2007).

## 5.5 Characterization of Polyhedrin-Polyclonal Antibodies:

Polyclonal antibodies produced against the polyhedrin of HaNPV, SlNPV and AmalNPV were characterized by determining the specificity of antisera to detect their respective polyhedrins

and investigated their ability to cross-react with other two heterologous polyhedrins. The specificity was determined by western immunoblotting analysis and the cross-reactivity of the antibodies was determined by DAC-ELISA as well as western immunoblotting analysis. In western immunoblotting all three antibodies were specifically reacted with polyhedrin (31 kDa) and did not cross-reacted with HLP indicated that the antibodies are highly specific to polyhedrin (Fig 26). In addition to the major polyhedrin (31 kDa), the polyclonal antibodies recognized some minor low molecular weight polypeptides of about 11-27 kDa and a high molecular weight peptides of about 43.6-99.14 kDa proteins (Fig 26). Some of these proteins could not be aligned with those polypeptides in silver stained gels of isoelectric precipitated polyhedrin preparations examined previously. Thus, there is general agreement that the molecular weight of the major polypeptide of polyhedra (variously referred to as polyhedrin, polyhedral protein, inclusion body protein, polyhedrin or granulin) is in the range of 28-33 kDa (Kozlov *et al.,* 1975; Summers and Smith, 1975; Padhi *et al.,* 1975; Mc Carthy and Liu, 1976). It is likely that lower molecular weight polypeptides detected in polyhedrin protein preparation after alkaline dissolution (Summers and Egava, 1973; Padhi *et al.,* 1975; Mc Carthy and Liu, 1976) are attributable to the activity of alkaline proteolytic enzymes (Eppstein and Thoma, 1975; Eppstein *et al.,* 1975). Which, in the NPVs of *Spodoptera* species may cleave the protein at a specific point to generate a polypeptide in the range 22-25 kDa. The presence of a protease raises some doubts concerning the effects of the enzyme on the polypeptides and antigenic properties of virus particles as well as polyhedrin. However, other studies have suggested that the proteolytic activity does not significantly affect the antigenicity of polyhedrin (Crawford and Kalmakoff, personal communication). In another proposal the occurrence and distribution of minor polypeptides of polyhedrin have been considered as a means of identifying field collected baculoviruses (Maskos and Milenburger, 1981). Their, presence in OB derived from larvae was attributed to proteolytic cleavage by an insect-host alkaline protease but their detection in OB harvested in vitro cannot be due to the same mechanism since tissue-culture derived OB lack the enzyme (Zummer and

Faulkner, 1979). Using hybridoma antibodies AcP1 and AcP2, it was apparent that many of the minor polypeptides possessed the same epitopes as 31 kDa protein and the reaction of AcP1 with polypeptides not detected by AcP2 confirmed that the two monoclonal antibodies recognized different polyhedrin epitopes (Hohmann and Faulkner, 1982). The monoclonal antibodies produced against nuclear polyhedra of BmNPV reacted with antigens of high molecular weight proteins (80-125 kDa); also react with a low-molecular weight protein of about 14 kDa (Shamim *et al.,* 1994). However it was not clear about the nature of 67 kDa protein, but most likely it may be aggregate polyhedrin protein and the low molecular weight protein (14 kDa) may be virion protein as described previously by using monoclonal antibodies against MNPV of *Orgyia pseudotsugata* (Quant *et al.,* 1984).

In the present investigation the cross-reactivity of homologous polyhedrin antisera with heterologous polyhedrins was determined by DAC-ELISA and western blotting analysis of electro-eluted polyhedrin (EP). The antiserum with maximum antibody titer (*i.e.* after booster dose) was used for this study. In DAC-ELISA at variable heterologous polyhedrin concentrations (1000 to 7.8 ng/ml) *vs*., fixed homologous antiserum dilution (1:5000), the polyhedrin polyclonal antiserum of each NPV was showed strong cross reactivity with other two heterologous polyhedrins (Fig 27). The antibodies at 1:5000 dilutions were able to detect minimum 10-15 ng/ml and maximum 1000 ng/ml of their homologous polyhedrin and minimum 25-30 ng/ml and maximum 1500-2000 ng/ml of heterologous polyhedrins. At fixed heterologous polyhedrin concentration (1000 ng/ml) and variable dilutions of homologous polyhedrin polyclonal antisera (1:1000 to 1: 40,000), the polyclonal antiserum of each NPV showed strong cross-reactivity with other two heterologous polyhedrins (Fig 28). The HaNPV polyhedrin antiserum detected the homologous polyhedrin at 1: 25000 times of antiserum dilution and at the same the heterologous polyhedrins were detected at 1:15000 dilution. The SlNPV polyhedrin antiserum detected the homologous polyhedrin at 1:40000 dilution of antiserum and heterologous polyhedrins were detected at 1:25000 dilution. Similarly, the AmalNPV polyhedrin antiserum detected the

homologous polyhedrins at 1:30000 dilution of antiserum and heterologous polyhedrins were detected at 1:15000 dilution. ELISA analysis rapidly generated useful information about the cross-reactivity of polyhedrins from several baculoviruses. Experiences with cross-reactivity studies by Mazzone and Tignor (1976); Crawford *et al.* (1978); Harrap and Payne (1979); Hohmann and Faulkner (1983) shown that conventionally prepared antisera discriminate only slightly between the polyhedrins and granulins. Even monoclonal antibodies prepared against a single NPV polyhedrin of BmNPV have shown cross-reactivity to a variable extent with four different strains of NPVs, *i.e.* BmNPV, AmalNPV, HaNPV and SlNPV, indicates that they recognize a common epitope shared with polyhedrin proteins in these organisms (Shamim *et al.,* 1994). Similarly, Tuan *et al.* (1999) assayed the polyhedrins of HaNPV, SlNPV and SeNPV by ELISA with polyclonal antiserum specific to each polyhedrin and demonstrated that there is a close relationship among polyhedrins of these viruses. Hohmann and Faulkner (1983) found that each monoclonal antibody that reacted with polyhedrin was either slightly or markedly different in its specificity. In the present study of western immunoblotting analysis, the polyclonal antibodies were recognized both homologous and heterologous polyhedrins to a great extent indicates that the antibodies have strong cross-reactivity with heterologous polyhedrins (Fig 29). Western blotting is a variation of immunoelectrophoresis that combines SDS-PAGE and RIA or ELISA can be used to determine which viral structural proteins are involved in serologic cross-reactions and it is a powerful tool for understanding the basis of serologic groupings of complex insect viruses (Towbin *et al.,* 1979). Smith and Summers (1981) demonstrated the power of western blotting by comparing the antigenic relatedness of 17 different species of baculoviruses from lepidopteran hosts. Knell *et al.,* (1983) expanded these studies to find additional common antigenic determinants among different baculovirus sub groups. Volkman (1983) explored this further by doing reciprocal western blots of the two phenotypes. Roberts and Naser (1982) and Hohman and Faulkner (1983) used the western blotting as a method of determining which of the AcMNPV structural proteins were reactive with monoclonal antibodies elicited to that virus.

Similarly, western blotting was used to screen the monoclonal antibodies produced against the polyhedra of nuclear polyhedrosis virus infecting *Bombyx mori* larvae (Shamim *et al.,* 1994) and polyclonal antibodies produced against polyhedrin of HaNPV (Sridhar Kumar *et al.,* 2007). Hohman and Faulkner (1983) studied the western blot analysis of AcNPV polyhedrin and of fragments produced by proteolysis and highlighted the specificity of monoclonal antibodies. In his study the AcP1 and AcP2 reacted with major polypeptide (p31), but only AcP2 reacted with fragments produced by digestion with Staphylococcal V8 protease. This demonstrated the presence of atlast two epitopes on polyhedrin subunit. The more universal reaction of AcP2 antibody with polyhedrins from other viruses indicated that there is a common antigen present in AcNPV, TnNPV, EmNPV and CfNPV polyhedrins resided on some of the V8-produced fragments of AcNPV. Smith and Summers (1981) proved that the differential cross-reactivity of AcMNPV and HzMNPV polyhedrin antisera to heterologous proteolytic peptides suggest that interspecies antigenic determinants are not identical on polyhedrins and granulins but are composed of a spectrum of related determinants.

## 5.6 Development and Evaluation of Diagnostic Tools for NPVs:

The objective of this study was to develop and evaluate the pathogen (NPV) detection methods for the IPM program of the *H. armigera, S. litura* and *A. albistriga*. Polyhedrin specific polyclonal antibodies were used to monitor the various stages of NPV infection in larvae and to quantify the POBs in commercial NPV preparations. For this different immunochemical tools were standardized and evaluated their validation in routine application of diagnosis and quality control of NPVs. Our results indicate that the immunochemical methods developed in the present study are appropriate for identifying HaNPV, SlNPV and AmalNPV infection in the larvae and for the quantification of NPVs in commercial preparations.

## 5.6.1 Development of Diagnostic Tools:

For qualitative detection of NPVs in larval homogenates, western immunoblotting and indirect immunofluorescence assay, and for quantitative detection DAC and IC-ELISA were developed and evaluated. Since, polyhedrin is the major component of NPV polyhedra, is coded for by the virus, and its presence in larvae indicates the presence of NPV or an NPV infection. Separation of larval proteins in 12% SDS-PAGE clearly differentiated the healthy and infected larvae. In NPV infected larval extracts a highly expressed protein (polyhedrin) with molecular weight of approximately 31 kDa was observed. At the same time in healthy larval extracts, a protein with size similar to polyhedrin was observed with less intensity than polyhedrin. The molecular weights of polyhedrins are as follows: 31.0 kDa in *H. armigera* and 31.3 kDa in *S. litura* and *A. albistriga* infected larval extracts. The same sized protein observed in healthy larval extracts with molecular weight of 31.72 kDa in *H. armigera*, 31.3 kDa in *S. litura* and 31.23 kDa in *A. albistriga* healthy larval extracts. In addition, there are some common proteins observed among healthy and infected larval extracts of these insect species. Some of them are present in both healthy and infected extracts but some are specific to healthy and infected larval extracts. Similar studies were conducted by Tuan *et al.* (1999), for the detection of NPV polyhedrin in the larvae of *H. armigera, S. litura* and *S. exigua* and found that the molecular weight of polyhedrins were all approximately 31 kDa. Quant *et al.* (1984), separated the extracts of OpSNPV- and OpMNPV infected and healthy tussock moth (*Orgyia pseudotsugata*) in SDS-PAGE with a 3% stacking gel and a 10% separating gel and the separated proteins were silver stained and differentiated the healthy and infected larval extracts based on the hyper expressed polyhedrin protein in infected larval extracts. Even though both healthy and infected larval extracts showed the similar size protein corresponding to polyhedrin in silver stained gels, western blot analysis of healthy and infected larval homogenates showed that the polyclonal antibodies specifically detected a single protein of polyhedrin with size of 31 kDa but not reacted with any other viral

proteins in infected homogenates (Fig 30). In healthy larval homogenates, the antibodies did not cross-reacted with any protein indicates that the antibodies are highly pure and specific to polyhedrin. Some times the antibodies recognized the minor fragments of sizes about 27 kDa and below in infected larval homogenates which could be degraded peptides of polyhedrin but not other viral proteins because they are found only in few cases (Fig 30C). Similarly, western blotting was used to diagnose the NPV infection in the larvae of *Bombyx mori* (Shamim *et al.,* 1994) and in the larvae of *Orgyia pseudotsugata* (Quant *et al.,* 1984). Similarly, the efficacy of antibodies to react with POBs in infected larval homogenates was tested by indirect immuno-fluorescence. The POBs were probed with polyhedrin polyclonal antibodies (1: 5000 dilution) and then the antigen-antibody complex was visualized by staining the slides with anti-rabbit Ig-FITC conjugate (Sigma) at 1: 80 dilution. The specific fluorescence of POBs and some times both POBs and dissolved polyhedrin were uniformly stained and no detectable staining was observed in the controls (Fig 31). The indirect immuno fluorescence technique has long been used for viral identification and for determining the location of viral antigens in infected cells during the course of replication (Casals, 1967; Schmidt and Lennette, 1973). Krywienczyk (1963) and Shamim *et al.* (1994) used immunofluorescence to detect an NPV in *Bombyx mori*. Kurstak and Kurstak (1974) reported the use of immunoperoxidase to detect infections of *Tipula* iridescent virus and densonucleosis virus in *Galleria mellonella* and by Summers *et al.* (1978) in the time course studies of AcMNPV in cell culture. Recently, the indirect immunofluorescence technique was developed for evaluation of antigen-antibody reactivity on the surface of proteinaceous occlusion body towards the application in development of reusable protein chip (Yoshikawa *et al.,* 2006). The ELISA has been shown to be a specific and sensitive method to detect NPVs. In the present study two methods of ELISA (DAC and IC-ELISA) were developed, compared and evaluated for diagnosis and quality control of NPVs. In DAC-ELISA total protein concentration of 5µg/ml of larval extracts gave the maximum sensitivity to detect the NPV infection. The samples tested in DAC-ELISA were healthy, infected larvae at live, dead and putrefied conditions of $4^{th}$, $5^{th}$ instar

and pupal stages. The samples showed the variation in total protein concentration and ELISA absorbance values irrespective of the larval condition (either live or dead or putrefied or pupal stages) which indicates that there is no relationship between the total protein concentration and age and condition (healthy, infected-live, infected- dead, putrefied and pupal conditions) of the larvae (Table 9). In qualitative analysis by DAC-ELISA there is no much variation in absorbance values between infected-live and dead larvae but in quantitative estimation by DAC and IC-ELISA, we found that there is a considerable level of difference in virus titer between live and dead larvae. Overall we found that the dead larvae have more virus titer than live larvae except in few cases where live larvae have more virus titer. The reason for this variation in virus titer with in the same age group larvae is due to variation in the size of the larvae inoculated with the virus. Another subject to be considered here is the variation in the virus titer in different age group larvae and this subject was discussed in detail in later section. In case, *H. armigera* the virus titer was higher in $4^{th}$ instar stage than in $5^{th}$ instar stage. This could be due to the less gestation period from $5^{th}$ to $6^{th}$ instar stage where the insects might have developed resistance to virus. But, in case of S. *litura* and *A. albistriga* the virus titer was higher in $5^{th}$ instar stage than $4^{th}$ instar stage larvae. This is due to somewhat prolonged time during molting of larvae from $5^{th}$ to $6^{th}$ instar stages during that period the insect immune system might have compromised to virus multiplication. The polyhedrin content of larval extracts was estimated in DAC-ELISA, by assaying the pure polyhedrin standards (1000 to 7.8ng/ml), healthy larval extracts (5µg/ml) spiked with pure polyhedrin standards and healthy larval extracts were assayed. Hence, all homogenates (healthy and infected extracts) were adjusted to 5µg/ml with coating buffer before being assayed. We determined empirically, however, that the assay was sensitive enough to be useful in detecting polyhedrin in larvae exposed to realistic field dosages of polyhedra. The interference observed by host tissue extract may have been at least partially due to the highly restricted binding of polyhedrin to the polyclonal antibody. The antigenic determinants on the polyhedrin molecule, recognizable by the polyclonal antibody, could have been vulnerable to

proteases in the host extract or coated or masked in some way either by extract components or by aggregation of the polyhedrin molecules due to higher dilutions of coating antigen. In DAC-ELISA antibodies detected the polyhedrin concentration as low as 15ng/ml of purified form and up to 30ng/ml in 5µg/ml of larval extracts and there was no cross-reaction between antibodies and healthy larval extracts (Fig 32). The results from DAC-ELISA were compared with a standard curve ($R^2$ = 0.9934) determined by serial dilution of polyhedrin spiked in to healthy larval proteins (Fig 32). The ELISA has proved to be a simple and sensitive method suitable for screening large numbers of samples for virus infection (Kelly *et al.* I978a; Zhang and Kaupp, 1988; Lu *et al.,* 1995) and for discriminating between related viruses (Kelly *et al.* 1978 b; Koenig, 1978). These workers have used the double antibody sandwich method for their assays but other methods which have potential advantages are possible. The direct method simplifies the procedure by coating the plate directly with virus and then adding antibody enzyme conjugate. The indirect method in which unlabelled antibody is added to virus coated plates uses a commercially available anti-IgG-enzyme conjugate which avoids the need for a different conjugate for each virus or virus component being assayed. Similarly, the relationship between three nucleopolyhedroviruses isolated from the larvae of *H. armigera, S. exigua* and *S. litura* in Taiwan was determined by assaying the polyhedrin in DAC-ELISA with polyhedrin polyclonal antisera specific to each polyhedrin (Tuan *et al.,* 1999) and similarly a monoclonal antibody based DAC-ELISA was developed for the identification and differentiation of OpMNPV and OpSNPV and also for detection of their homologous polyhedrin in larval extracts with sensitivity of 100ng/ml in the presence of host tissue extract, rather than 5ng /ml in its absence (Quant, *et al.,* 1984). Recently, a polyclonal antibody based DAC-ELISA was developed for detection of HaNPV polyhedrin with a sensitivity of 30ng/ml in the presence of host tissue extract rather than 15ng/ml in its absence (Sridhar Kumar *et al.,* 2007). However, due to competition between insect and viral proteins for binding to ELISA plate surface has reduced the detection sensitivity of the DAC-ELISA, particularly when crude insect extracts were used. Since it was found that host

tissue extract interfered with the assay, still we were able to determine its absolute sensitivity in the presence of unknown quantities of host tissue extract. To eliminate the competition between insect and viral proteins for binding sites in the ELISA plate surface in DAC-ELISA, We attempted to avoid the interference phenomenon by standardizing an IC-ELISA to estimate the polyhedrin content in insect extracts. Using a chequerboard system the combination of coating antigen concentration of 1µg/ml and the antibody dilution of 1:4000 were optimized to assay homologous antigens and the antigen concentration of 2µg/ml and the antibody dilution of 1:2000 were optimized to assay heterologous antigens (Fig 33). To estimate the amount of polyhedrin in larval extracts polyhedrin standards of 20 to 0.078µg/ml were spiked in to 25 or 50µg/ml of healthy larval extracts resulted in the regression ($R^2$) value of 0.9972 (Fig 35). The sensitivity of IC-ELISA was 0.156µg/ml of homologous polyhedrins and 0.35 to 0.31µg/ml of heterologous polyhedrins in 25 or 50µg/ml of insect total protein extract. Similarly, an IC-ELISA was standardized to evaluate the biosaftey of recombinant and wild type NPV of *A. californica* (Ashour *et al.,* 2007) with 2.5µg/ml of coating antigen concentration and antibody dilution was 1:4000. Based on the close $IC_{50}$ values between homologus and heterologus polyhedrins (Fig 34, Table 10) each antiseum showed the high percent of cross-reactivity with heterologous polyhedrins (Table 11). Similar results were obtained by Ashour *et al.* (2007) with his optimized IC-ELISA system was low for heterologous polyhedrins from wild type AcMNV, AcAaIT-field, and wild type SlNPV. In order to study the effect of insect body proteins on IC-ELISA and to test the % of recovery of artificially spiked polyhedrin in to 25 and 50µg/ml of un-infected insect body proteins. The results obtained with these mixtures were compared with pure virus at the same concentrations. The % of amount of polyhedrin (20 - 0.078µg/ml) recovered from 25 or 50µg/ml of larval protein extract was 82.1 to 116.8% (Table 12). Based on the recovery experiment the diagnosis of NPV infection by IC-ELISA was optimized for assaying the larval extracts by adjusting their total protein concentration to 25 or 50µg/ml. Among both ELISA methods, only IC-ELISA method was capable of detecting polyhedrin with the highest ratio of

host material through which no increase in colour intensity compared with pure virus (A 405nm values are indirectly proportionally to the polyhedrin concentration). Similarly, Crook and Payne (1980) examined the direct, indirect and double antibody sandwich methods of ELISA for their ability to detect and discriminate between granulosis viruses from *Pieris brassicae, Agrotis segetum* and *Cydia pomonella* and for their specificity in the presence of host material and they concluded that the indirect method was the most sensitive and capable of detecting down to about 1ng of dissolved capsules/ml compared with 10ng/ml for the double antibody sandwich method and 25ng/ml for the direct method and the double antibody sandwich method was more specific, showed greatest discrimination between different granulosis viruses.

## 5.6.2 Development of Quality Control Tools:

The effectiveness of the viral insecticide is critically dependent on concentration of POB, which is expressed as LE (Larval Equivalent). Generally, a standard stock preparation consists of 1LE, *i.e.* $6 \times 10^9$ POBs/ml. While NPV insecticide production methods have been well established in many developing countries, the microscopic counting procedure used to screen the larvae for NPV infection and quality control of the viral insecticide lots has low-detection efficiency, unknown specificity and is laborious and requires considerable skill (Wigley, 1976). Because of this many NPV products produced have poor efficacy and found to be ineffective under field conditions (Kern and Vaagt, 1996; Harris, 1997). Therefore it is necessary to have an effective diagnostic and quality control system for the development of a successful microbial control product (Shieh, 1989). Hence, in the present study as part of the quality control during mass production of NPVs used for commercial viral insecticide preparations at ICRISAT, Patancheru, India, we developed some sensitive and reliable immunochemical methods such as DAC and IC-ELISA and evaluated their performance in quantification of POBs in commercial NPV preparations. The number of POBs present in sample bottle was determined by extracting the total polyhedrin and compared with the standard regression graph of polyhedrin extracted

from known number (estimated by microscopic counting) of POB standards such as $6 \times 10^9$ to $2.34 \times 10^7$ POBs /ml (1 LE to 0.0078 LE) (Fig 37). A simple purification protocol was standardized for extraction of total polyhedrin from 1 ml of standard and sample POB preparations and checked their purity in 12% SDS-PAGE (Fig 36). The extracts of standards and samples were evaluated in both DAC and IC-ELISA at 1:1000 dilution. These ELISA methods are sensitive to a minimum of approximately $4.6875 \times 10^7$ POBs /ml (0.015 LE/ml), which is little bit higher to the range (100 to 2,000) of previous reports (Crook and Payne, 1980; Kelly *et al.,* 1978b; Longworth and Carey; 1980; Shamim *et al.,* 1994; Stark *et al.,* 1999). The ELISA methods can be used to quantify infection, unlike other methods and this feature may be applied to predict the potential inoculum required for future populations. Previously, it has been shown that the ELISA method can be used to quantify baculoviruses (Clark and Barbara, 1987). Tuan *et al.,* 1998 compared the visual counting of POBs by microscope, bioassay, SDS-PAGE, and ELISA for quality control of SlNPV products, and ELISA has proved to be better than SDS-PAGE. We found that there was a significant linear relationship between semi-purified baculovirus POBs and absorbnce at 405 nm with a maximum regression ($R^2$) value of 0.9953 for DAC-ELISA and 0.9977 for IC-ELISA (Fig 37). The ELISA results were comparable to light microscope counting of POBs (Table 13). The absorbance values suggesting that the ELISA method can be used to accurately quantify bit virus POBs and virus infections from tissue homogenates. Recently, Thorne *et al.* (2007) reported that the alkali treated POB lysates were assayed in IC-ELISA for estimation of total POBs in semi-purified commercial NPV samples or in insect tissue extracts with a sensitivity of minimum of approximately 850 POBs. Similarly, Parola *et al.* (2003) reported that the estimation of granulin in commercial GV suspensions of *Epinotia aporema* GV by DAS-ELISA with a sensitivity of 0.53ng/ml of purified OB suspensions, this represented $2.0 \times 10^4$ OBs/ml.

## 5.7 Application of Immunochemical Tools in Optimization of Conditions for Productivity and Quality of NPVs:

The immunochemical tools developed in the present study were applied in optimization of conditions for the productivity and quality of NPVs during commercial production by applying them in bioassay experiments. The conditions optimized were as follows:

1. Age of the larvae for virus inoculation to obtain maximum virus yield and
2. Time of virus harvest to obtain maximum virus yield with low levels of bacterial contaminants.

### 5.7.1 Identification of optimum age of larvae for virus inoculation:

The effect of age of larvae on POB yield or optimum age of the larvae for inoculation of virus played critical role in determining the productivity of virus with good yields. Hence, in the present study the optimum age of larvae for inoculation of virus was determined by mass multiplying the NPVs on $2^{nd}$, $3^{rd}$, $4^{th}$, and $5^{th}$ instar larvae and the total yield of NPV obtained in each age group larvae was determined by ELISA. The ELISA results showed that the yield of NPV during mass multiplication was increased with increasing in age of larvae (Fig 38, Table 15). In case, *H. armigera* the yield of NPV was increased gradually from $2^{nd}$ instar to $4^{th}$ instar stage and decreased in $5^{th}$ instar stage. This could be the less gestation period from $5^{th}$ instar to $6^{th}$ instar stages where the insects might have developed resistance to virus. But, in case of *S. litura* and *A. albistriga* the yield was increased up to $5^{th}$ instar stage due to somewhat prolonged gestation period than *H. armigera* during $5^{th}$ instar stage for these insects. Subsequently, due to heavy body weight of these insects the yield (2.47 ± 0.097 LE/ml for SlNPV and 3.11 ± 0.05 for AmalNPV) of NPV was also recorded as higher than HaNPV (1.97 ± 0.035 LE/ml). By multiplying the HaNPV on third instar *H. armigera* larvae Pawar and Thombre (1992) recorded $0.95 \times 10^9$ to $3.5 \times 10^9$ POBs/larva. Gopali and Lingappa (2001a) inoculated fourth instar *H. armigera* larvae @$10^8$ POBs/ml and recorded higher larval mortality and higher quantity of viral yield per larva ($2.81 \times 10^8$ POBs). In the present study the POB yield obtained per larva was

slightly higher than the earlier studies conducted by Gopali and Lingappa (2001a) and Pawar and Thombre (1992) which may be due to variation in the size of the larvae inoculated with the virus and the diet provided to the larvae. However, Shieh (1978) recovered $5 \times 10^9$ POBs/larva by inoculating grown up larvae of *H. armigera,* which is very close to the present value. Similarly, the fourth instar larvae for NPV production was selected by Biji et *al.* (2006a) for HpNPV and Rabindra and Subramanian (1974) for HaNPV. In the present investigation the POB yield of SlNPV obtained per larva ($5.73 \pm 0.17 \times 10^9$) was higher than the earlier studies ($1.4 \times 10^9$) reported by Tuan *et al.* (1998) and lower than the earlier studies ($9.7 \times 10^9$) reported by Jun *et al.* (2007) and close to earlier study ($5.572 \times 10^9$) reported by Senthil kumar *et al.* (2005). The yield of AmalNPV multiplied on 5$^{th}$ instar larvae was greater than 6$^{th}$ instar larvae due to its heavy weight and prolonged guestation period to enter in to 6$^{th}$ instar stage at which could be develop resistance, it enhance the multiplication of virus in the body tissues. This is supported by Narayanan *et al.* (1978); Moscardi (1999); Carter (1984) by stating that the yield of virus is known to be directly proportional to host larval weight, insect diet, insect age and virus dosage, incubation, environment and preservation of virus infectivity are some of the major factors, which optimize the production of NPV.

### 5.7.2 Identification of optimum time for harvesting of virus:

Selection of harvesting time is crucial in maximizing the yield, both to achieve peak NPV production in individual larvae and to avoid losses. Attempts made to identify the appropriate time of harvest after inoculation of the larvae with the virus by conducting the bioassay (time course) experiment. The productivity of virus in larvae at different intervals (post inoculation days) of the experiment was monitored through ELISA (DAC and IC-ELISA) and western immunoassay. This revealed that the POB yield was higher in cadavers (dead larvae) than live larvae. In ELISA the virus load was detected in infected larvae from 3 days after inoculation (dpi) but, in western immunoblotting the virus load was detected from 4 dpi. The ELISA results showed that the concentration of polyhedrin in sampled larvae was increased gradually from 3 to

10 dpi and subsequently no further increase was observed up to 12 dpi (Fig 39, Table 16). However, in western immunoassay (Fig 40) the intensity of polyhedrin band was increased only from 4 to 9 dpi and subsequently no further increase was observed up to 12 dpi indicating that the virus harvested on 9 dpi was not greatly affect the total yield of NPV. Quant *et al.* (1984) reported that the polyhedrin of OpNPVs was not detected in larvae by DAC-ELISA during 1 to 4 dpi of time course experiment but, he noticed that the polyhedrin from insects infected with OpMNPV could be detected by a visible color reaction as well as spectrophotometrically on 5 dpi, and the amount of polyhedrin was peaked by 8 dpi and subsequently no further increase up to 11 dpi. Similarly, Volkman and Falcon (1982) and Shamim *et al.* (1994) reported that the polyhedrin of *T. ni* NPV and *B. mori* NPV were detected on 4dpi during the time course experiment by using DAS-ELISA. Recently, Parola *et al.* (2003) reported that the granulin of *Epnotia aporema* GV (EpapGV) was detected as early as 1 dpi using DAS-ELISA. Curiously, in the present study the high-dose-exposed larvae showed no such evidence of polyhedrin production by day 1, but rather the polyhedrin from the ingested (or external) polyhedra apparently was degraded, or antigenically altered, because no polyhedrin could be detected in any of the larvae tested. It is possible that the delay in polyhedrin production observed in the larvae was due to secondary disease caused by microorganisms either ingested or already in the gut, the host being made more susceptible by the primary virus infection. The occurrence of secondary disease in virus infected larvae is not uncommon (Vago, 1963), and it is highly likely that secondary disease would interfere with the 'normal' timing of viral replication and production of polyhedra. The concentration of virus harvested from exclusively dead larvae on 10, 11 and 12 dpi were slightly higher than virus harvested on 9 dpi. Therefore, it may be best to harvest POBs after larval death. However, this involves the practical difficulty of harvesting ruptured and disintegrating larvae. Frequent observation and collection of larvae just after death over a period of several days should maximize the number of POBs to be harvested, but is quite laborious. Hence, the immunochemical tools developed in the present study were successfully applied to monitor the

POB production in NPV exposed larvae with less pain to technician to screen the large number of larvae in the production batches. Okada (1977) developed an apparatus that could be used to aspirate the collect ruptured and disintegrating larvae. Collection of larvae using this apparatus diminished the labor and loss of POBs associated with difficulties in harvesting liquefying larvae. Smitt and Vlak (1988) suggested a premature harvest of *S. exigua* larvae between four and seven days of post inoculation. It was also observed that production of polyhedra did not increase after the seventh day of inoculation. However, in the present study harvesting of virus exclusively from dead insects recorded maximum yield, as harvesting is delayed there was decrease in yield due to wastage of liquefied tissues. However, there was increase in number of bacterial and fungal contaminants in NPV-infected larvae during the incubation period, reaching high numbers after larval death (Ignoffo and Shapiro, 1978; Grzywacz *et al.,* 1998).

### 5.7.3 Screening of bacterial activity in larvae days post exposure to NPV:

This study is related to address the bacterial contamination levels in the NPV infected larvae and it showed significantly the highest bacterial population was recorded exclusively from the dead larvae. The highest number of bacterial colonies and colony forming units per ml (CFU/ml) were recorded in the larvae of *A. albistriga* and the lowest number of bacterial colonies and CFU/ml were recorded in the larvae of *H. armigera* and at the same time in each insect species, the lowest number of bacterial colonies and CFU/ml were recorded on $5^{th}$ day sampled larvae and the highest number of bacterial colonies and CFU/ml were recorded exclusively in dead larval homogenates after $9^{th}$ dpi onwards (10 to 12 dpi) (Table 17). This is due to secondary disease caused by microorganisms either ingested or already in the gut, the host being made more susceptible by the primary virus infection. The occurrence of secondary disease in virus infected larvae is not uncommon (Vago, 1963). In this experiment bacterial contamination levels on $5^{th}$ dpi the number of bacterial colonies and CFU/ml of raw insect homognetes were recorded as 23 and $1.15 \times 10^6$ for *H. armigera*, 28 and $1.4 \times 10^6$ for *S. litura* and 31 and $1.55 \times 10^6$ for *A. albistriga* larval homogenate. On $12^{th}$ day the bacterial population was recorded as 159 and $7.95 \times 10^6$ for

*H. armigera,* 176 and 8.8 × $10^6$ for *S. litura* and 187 and 9.35 × $10^6$ in *A. albistriga* larval homogenate. Martignoni *et al.* (1968) recorded 9.26 ×$10^8$ CFU per $10^9$ POB/ml of a raw NPV sample and they reported that the bacterial species associated in the viral production systems are generally harmless which belong to normal gut flora of the larvae, and arresting of their further growth is preferable as the presence of any human and vertebrate pathogen is not permissible (Podgwaite *et al.,* 1983, Cherry *et al.,* 1997). Recently, Podgwaite *et al.* (2006) reported that the gypsy moth nucleopolyhedrosis virus product, Gypchek®, during a 100-day production run consists of bacterial population of 5.97±1.51×$10^8$ CFU/ml. They did not detect obligate anaerobic or fecal coliform bacteria in any of the sample. *Bacillus cereus, Staphylococcus epidermidis, B. licheniformis, Streptococcus faecalis, Serratia liquefaciens*, and *Aspergillus niger* but the presence of opportunistic pathogens indicated that assiduous monitoring of the virus production facility and rigorous quality control of production batches are necessary. Purification methods based on centrifugation were found to be ineffective in removing bacteria hence improved methods of hygiene and harvesting appeared to be more valued in reducing contamination (Grzywacz *et al.,* 1997). McKinley *et al.* (1989) also suggested harvesting of live infected larvae to reduce the bacterial contamination. By harvesting the NPV infected larvae prior to death, it is possible to remove bacterial contaminants but the process involved is costly (Podgwaite, 1981) and often cause heavy losses which make the option uneconomic (Kelly and Entwistle, 1988; Kelly *et al.,* 1989). Recently, Rao and Meher (2004) suggested 6$^{th}$ day after post inoculation as ideal period of harvest when the mortality and NPV yield were in accord for optimal viral recovery to minimize the constraint of malodor associated with the *H. armigera* NPV production. Grzywacz *et al.* (1998) described that the virus product from live larvae was only contained with acceptable level of microbes. In this sense, harvesting virus from live larvae is probably advantageous over doing from NPV-killed larvae. But, in our study, mortality was not synchronized, instead occurred from 7 to 12 dpi, during which both infected but yet to be vital and live larvae were present together. However, our western immunoassay studies (Fig 40)

showed that the intensity of polyhedrin band was increased only from 4 to 9 dpi and subsequently no further increase up to 12 dpi and the ELISA results showed that the concentration of virus estimated in the larvae sampled on 9 dpi was slightly lower than virus estimated in exclusively dead larvae sampled on 10, 11 and 12 dpi. Even though there is a considerable difference in bacterial contamination levels between 7 and 9 dpi sampled larvae, but their difference in yield of virus is not ignorable. This indicates that there is no much difference between the yield of virus in the larvae screened on 9 and 12 dpi and at the same time the bacterial contamination is also low in 9 dpi larvae compared to 10, 11 and 12 dpi. Hence, the virus harvest from both live and dead larvae on 9 dpi is feasible and not greatly affects the total yield and quality.

## 5.8 Evaluation of ELISA Tools at Field Level Efficacy Study of NPV:

To integrate the ELISA tools into IPM program, we examined the ability of the antibodies to detect the NPV at various stages of infection in insect population at field level. For this field experiment was conducted during 2007 kharif season at ICRISAT forms with respect to *H. armigera* on pigeonpea crop by spraying the field with 250 LE/h after the pest population was reached above ETL (Economic Threshold Level). The larvae were sampled on 0 to 10 dpa and assayed for NPV in DAC-ELISA. After 4 days of treatment, the *H. armigera* larvae picked up the infection and crawled listlessly about the field. They climbed to near by top portions of the plants and dead larvae were seen hanging from the plants and subsequently dropped to the ground at the base of the plant. Apart from this observation, the ELISA results also showed that the concentration of NPV used for field spray was successfully infected the field population. The infection was initiated in field population on 3 dpa and the disease was peaked up to 8 and 9 dpa and then declined on 10 dpa (Fig 41). The DAC-ELISA results (Table 18) showed that 10 ± 1.7% of the field collected larvae were NPV + ve on 3 dpa, 15 ± 2.2% on 4 dpa, 32 ± 2.6% on 5 dpa, 50 ± 3.2% on 6 dpa , 65 ± 2.5% on 7 dpa, 71 ± 2.5%, on 8 dpa and 70 ± 5.9 on 9 dpa. But, on 10 dpa the % of infection was suddenly decreased to 27 ± 5.7%. On 10 dpa almost field larvae were

died only few larvae (live and dead) were recorded on plants. The very low % of infection in the larvae sampled on 10 dpa was due to the reasons we can predict are either lack of sufficient virus concentration to cause the infection or may be the virus lost its viability after certain period of field application or the larvae may not exposed to virus or they may be migrated from neighbor fields. If less number of larvae are infected, supplementary virus is inoculated to initiate the epizootics to prevent the insect damage (Stelzer *et al.,* 1977, 1979). Moore *et al.* (2004) conducted the field evaluation of HaNPV on Citrus in South Africa, he reported that, a concentration of $1.15\times10^7$ POBs/ml of HaNPV spray resulted in a 100% reduction in *H. armigera* larval infestation within 7 days on tomato plants in a hot house environment and a 10-fold lower concentration, $1.15\times10^6$ POBs/ml, resulted in a 100% reduction within 16 days. In two field trials on navel oranges, the lower concentration and an additional even lower concentration of $7.26\times10^5$ POBs/ml, resulted in a 100% reduction in *H. armigera* infestation within 14 days. Recently, Thorne *et al.*(2007) started the evaluation of OpNPVs in larvae that have emerged from field–collected egg masses by indirect ELSA method. The DAC-ELISA results of individual larvae screened from control (un-treated) plot showed that most of the larvae were NPV –ve and very few larvae were found to be NPV +ve (1 ± 1.9% on to 6 ± 1.9%). This is due to the migration of larvae from treatment plot to control plot or may be natural NPV infection occurred in field population which is frequently observe at ICRISAT forms. The virus strain [HaNPV-P (Patancheru strain)] used for this field study was isolated during such an out break of natural epizootic condition. The gross virus concentration (POBs) in infected larvae per dpa estimated by ELISA method during 0-4 dpa was negligible and the virus tier was gradually increased between 5 to 10 dpa (Table 18). The virus concentration in infected larvae was 0.07 ± 0.01 LE /ml on 5 dpa, 0.10 ± 0.02 LE/ml on 6 dpa, 0.29 ± 0.067 LE/ml on 7 dpa, 0.33 ± 0.07 LE/ml on 8 dpa, 0.74 ± 0.07 LE/ml on 9 dpa and 0.41 ± 0.07 LE/ml on 10 dpa. Even though the % of infection in larvae sampled on 10 dpa was very low, considerable level of virus titer was estimated from the infected larvae sampled on 10 dpa (Table 18). Because, laboratory bioassay study proved that the

rate of conversion of insect to virus increased from 5 to 10 dpa. Based on the ELISA results the pest management decisions may be guided by pathogen incidence, because increases are correlated with host declines (Otvos *et al.,* 1999; Stelzer 1979). The described ELISA methods in the present study may be effective and applicable to IPM programs.

## 5.9 Isolation and Characterization of Polyhedrin Gene of NPVs:

In order to establish the purity of seed stock or master stock of NPV used for commercial viral insecticide preparations at ICRISAT, an attempt was made at molecular level identification and evaluation of phylogenetic status of NPVs done by isolation, cloning, sequencing of polyhedrin gene and evaluation their phylogenetic status.

### 5.9.1 Isolation of Polyhedrin Gene:

For this a double round PCR protocol was standardized using degenerate primer set (Table 19) to isolate the full length polyhedrin gene of NPVs isolated from *H. armigera, S. litura* and *A. albistriga*. Due to variation in the annealing temperatures of the primer combinations the PCR protocol was standardized with dual rounds of annealing temperatures (Fig 44). Unfortunately, the degenerate primers were not successfully amplified the polyhedrin genes of SlNPV and AmalNPV. The primer combination with SlNPV DNA did not resulted in any amplification and the AmalNPV DNA template resulted in non specific amplifications. We could explain this fact either because of the use of degenerated primers with variation in the annealing temperatures or the nucleotides of N-terminal and C-terminal ends of the polyhedrin genes of these two viruses may be different from the previously published sequences from which the primers were designed or the influence of contaminants in the DNA extracted directly from POB samples, which are common inhibitors of the PCR (Wilson, 1997). Considering that the DNA extraction procedure from POBs could be cause of inconsistent results, we used a reliable protocol for extraction of DNA directly from POBs (infected larval extracts) and the quality of DNA was more than sufficient to carryout the PCR (Fig 43). In this way the same primer

combination with HaNPV DNA template was successfully amplified the full-length polyhedrin gene (~750 bps) (Fig 46). Similarly, PCR assays using degenerate / specific primers were described by several authors for detection and differentiation of NPVs by amplifying the polyhedrin gene (Seufi, 2008, unpublished, Lee *et al.,* 2007, Woo *et al.,* 2006, Jakubowska *et al.,* 2005, Wu and Wang, 2005, Woo *et al.,* 2005, Ikuno *et al.,* 2004, Christian *et al.,* 2001, De Moraes and Maruniak, 1997; Burand *et al.,* 1986). In the present investigation, lack of proper PCR products of SlNPV and AmalNPV polyhedrin genes, we confined only to the HaNPV-P (Patancheru strain) polyhedrin gene for its cloning, sequencing and phylogenetic analysis.

## 5.9.2 Cloning and sequencing of HaNPV-P polyhedrin gene:

Although the isolation of NPV from *H. armigera* was previously reported, the characterization of their polyhedrin gene was not reported yet from Indian subcontinent. Cloning of HaNPV-P polyhedrin gene in to pJET1 cloning vector was worked well, in the colony PCR, amplification of the clone using universal pJET1 forward and reverse sequencing primers gave the amplification of the expected size of about ~ 800 bp, that were taken into account for the plasmid sequence of 60 bp (Fig 46). Considering the sizes of previously published polyhedrin sequences, most amplification products were between 730-750 bp long. Gene sequencing analysis of selected clones resulted in 744 nucleotide long ORF with a predicted coding capacity for a polypeptide of 247 amino acids (Fig 47) as in the AmalNPV (AF118850). Rivkin *et al.* (1998) reported that 246 amino acid polypeptide in a local strain of Israeli HaNPV polyhedrin. Similarly, Bansal *et al.* (1997) reported that 246 amino acid polypeptide in SlNPV polyhedrin. This is the 8$^{th}$ report world wide and 1$^{st}$ report from Indian sub continent to be described the full length polyhedrin gene sequence of a NPV isolated from *H. armigera*. In BLASTX search the three sequences showed homology with baculovirus OB protein domain of known polyhedrin and granulin proteins from the GenBank data base.

### 5.9.3 Phylogenetic Analysis at Nucleotide and Amino acid Levels of HaNPV-P Polyhedrin with Known Polyhedrins / Granulins:

The polyhedrin is the major protein of the virus occlusion body and is the most conserved protein of NPVs (Rohrmann, 1992). After the first report about localization of the polyhedrin gene in AcNPV (Vlak and Smith, 1982; Hooft van Iddekimge *et al,* 1983) determined its nucleotide sequences. Polyhedrin/granulin is a protein of about 245 to 250 amino acids, and appears to be the most highly conserved baculovirus protein. These characteristics lead to the use of polyhedrin or granulin sequences as the base of baculovirus phylogenetic studies, since this is the gene from which the larger number of different examples is available for comparison (Zanotto *et al.,* 1993). To investigate the relatedness of HaNPV-P to other baculoviruses, the study compared the polyhedrin gene of HaNPV-P with fully-sequenced polyhedrin genes of lepidopteran NPVs and GVs available in GenBank.

Comparison of the nucleotide sequence of the coding region of the polyhedrin with DNA sequences of 40 NPV polyhedrins and 14 GV granulins by using MgAlin tool of Lasergene software (DNASTAR, USA) and onstruction of phylogenetic tree revealed two major branches that were considered as separate clusters of baculovirus occlusion body protein (polyhedrin or granulin), they are NPVs and GVs. NPVs were again divided into two major branches, they are group-I and group-II NPVs. There are 16 NPVs in group-I, 25 NPVS in group-II (including HaNPV-P) were observed in separate clusters (Fig 48).

The data was also analyzed by bootstrapping at 1000 replicates using CLC workbench 3 software (CLC Bio) and the obtained topology of the later phylogenetic tree agree with that of clastalW analysis (Fig 49). In both trees the HaNPV-P falls within the group-II of NPVs and is closely related to the NPVs isolated from *M. configurata* (98% homology), *M. brassicae* (96.1% homology), *L. separata* (90.6% homology) and *Panolis flammea* (90.6%) (Table 22). At the same time, minimum homology (72.4%) with NPV isolated from *Wiseana signata*. But,

at the same time the HaMNPV-P polyhedrin gene showed the 77.2 % homology with HaNPV. Similarly, in group-I NPVs, 99.9% homology was noticed between AfNPV and RoMNPV (Table 21). The high degree of homology between viruses isolated from different hosts represents that those viruses are isolates of the same virus.

Although, polyhedrin gene is still considered a reasonable marker for identification of a NPV and its neighbors, Herniou *et al.* (2003) and Lange *et al.* (2004) argued that it might not be the best baculovirus gene for phylogenetic studies because polyhedrin phylogenies often disagree with other gene phylogenies. While other phylogenetic analyses consistently group AcMNPV and BmNPV together, but phylogenies based on polyhedrin have AcMNPV as a sister group to the rest of the group-I NPVs (Herniou *et al.,* 2003). Phylogenies based on combined sequences of shared genes have been found to be more robust than those based on the sequences of individual genes (Herniou *et al.,* 2001, 2003). The present investigation is strongly supporting the earlier studies by Harrison and Bonning (1999), they reported that the NPVs of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus and studies by Smith and Summers (1982); Figueiredo *et al.* (1999); Rovesti *et al.* (2000), they stated that among the NPVs with potential as pest control agents, the MNPVs isolated from *M. brassicae* (Lepidoptera: Noctuidae) and *H. armigera* (Lepidoptera: Noctuidae) were shown to be similar in terms of both biological activity and genomic homology. Other studies have also shown that another virus, *Panolis flammea* MNPV (PfMNPV), is closely related to MbMNPV. These viruses shared a high degree of homology and both replicated in either *M. brassicae* or *P. flammea* causing similar mortality rates (Possee and Kelly, 1988). Thus, MbMNPV, HaMNPV and PfMNPV are all closely related and should probably considered variants of a single virus species because of their biological activity (Rovesti *et al.,* 2000). Due to the high degree of homology between the polyhedrin sequences of HaMNPV-P, McMNPV, and MbMNPV would be classified in the group-II NPVs as established by Zanotto *et al.* (1993). The HaNPV-P, McMNPV and MbMNPV sequences are also identical for 5' sequences between the TAAG consensus sequence and the ATG start site of polyhedrin and are typical of group-II as opposed to

Group-I polyhedrin untranslated sequences (Zanotto *et al.,* 1993). It is likely that the predominant genotype selected from viral isolate such as HaMNPV is dependent on prevalence of a particular host, e.g., *H. armigera* or *M. brassicae,* as well as other factors, such as geographical location. Clearly, there are problems in naming a baculovirus simply on the basis of the host from which it was originally isolated and therefore greater vigilance should be taken in naming new (and old) viruses. For example, studies on viruses from members of the same or different host species show similarity or variability in morphology, virulence and biological characteristics (Shim *et al.,* 2003). Many of the known baculoviruses could be grouped together depending on their degree of genetic relatedness, which does not reflect the taxonomic grouping of their host/hosts (Zanotto *et al.,* 1993). Based on the above reports one can comment like "variants of baculoviruses with heritable similarities in virulence and variations in host range arise spontaneously in nature".

In spite of the fact that the HaNPV-P position in the tree was consistent, the position of the same lineages changed depending on the method used. Several reasons, such as the significant variation in DNA composition of some lineages and the small size of the polyhedrin gene, may help in explaining the lack of resolution of some taxa. As the number of distinct polyhedrin sequences available increases, its shortcomings as a phylogenetic marker become worth considering. This small gene has both, considerable conservation at the amino acid level and variation at the DNA composition level due to unique codon usage preferences observed in the different viruses. This entails two problems. First, the small size of the dataset reduces the amount of available information to reconstruct the phylogeny. Second, the informative sites had significant variations in the DNA composition. For example, the polyhedrins of the LdMNPV, BusuNPV, OpMNPV, AmalNPV, and LoobMNPV were found to have biases nucleotide composition. In the presnt study, bootstrap analysis using 1000 replicates of phylogenetic tree with CLC work bench revealed that LdMNPV and WsNPV were separated as major branches and supported with 100% bootstrap values. The remaining branches are supported by high bootstrap values. Composition biases make it difficult to estimate the proper values for nucleotide transition matrices, which can induce

systematic errors in maximum likelihood inferences. This causes likelihood models to provide unstable position for several taxa and their association to Group-II NPV. Of course, the concatenation of more genes in common between the genomes of interest may provide more reliable information for the phylogenesis after the genome of HaNPV-P is fully sequenced.

Similarly, the phylogenetic analysis based on predicted amino acid of HaNPV-P polyhedrin with previously published amino acid sequences of polyhedrins and granulins by ClustalW method (Thompson *et al.,* 1994) using MgAlin tool of Lasergene software (DNASTAR, USA) revealed that the phylogenetic tree (Fig 50) showed two major branches that were considered as separate clusters of baculovirus occlusion body protein (polyhedrin or granulin), they are NPVs and GVs. NPVs were again divided into three major branches, they are group-I, group-II NPVs and LdMNPV alone formed a separate branch. Due to less homology with other group-II NPVs, WsNPV and SliNPV were formed as separate cluster within group-II NPVs. But, after bootstrapping WsNPV was completely separated from group-II NPVs and formed as a seperte branch of NPV.

When the predicted amino acid sequence deduced from the putative HaNPV-P polyhedrin gene was compared with the amino acid sequence of McMNPV, MbMNV, PfNPV and LsMNPV polyhedrins, it was shown to be nearly identical, being 95.5% with MbNPV and McNPV, 93.9% with PfNPV and 93.5% with LsNPV polyheddrins respectively (Table 24). The deduced amino acid sequence of HaNPV-P polyhedrin differs by only 11 amino acids with McMNPV and MbMNPV. Similalry, it differes by 15 amino acids with LsMNPV and PfMNPV polyherins (Fig 53). Although these amino acid changes do not occur in highly variable regions of polyhedrin (Zanotto *et al.,* 1993). The high degree of homology between the polyhedrin sequences of HaNPV-P, McMNPV, MbMNPV, PfMNPV and LsMNPV indicats that HaMNPV-P would be classified in the group-II NPV as established by Zanotto *et al.* (1993). Similarly, Li *et al.* (1997) proved that the polyhedrin amino acid sequence of McMNPV was close (97.6%) to MbMNPV and PfNPV polyhedrins and classified that McMNPV was a group-II NPV. Generally group-II NPVs have polyhedrins with 246 amino acids instead of 245 found in group-I. But, Wolff *et al.* (2002) observed a unique

characteristic feature in McMNPV with respect to polyhedrin is the size of predicted protein is 249 amino acids as in the SliNPV and the aminoacid sequence also more close to AmalNPV polyhedrin, which is having 247 amino acids. Most of the size differences among polyhedrins take place in clusters in the N-terminus of the proteins that are tyrosine-rich and where short motifs appear to be duplicated in the longer sequences or lost in the shorter ones. For example, the HaNPV-P has a duplication of P-L/A-Y tripeptides (Fig 47); LobMNPV has a duplication of a P-D-Y tripeptide and the SliNPV of a Y-S-R/A tripeptide (Wolff *et al.,* 2002).

## 5.10. Development of PCR Based RFLP Marker for Identification and Differentiation of HaNPV-P (Patancheru) Strain:

Phylogenetic analysis based on polyhedrin gene showed that, the HaNPV-P strain was more close to McMNPV, MbMNPV and LsMNPV than HaNPV. In the present study an attempt has been made to distinguish HaNPV-P strain from other NPVs through PCR-RFLP marker analysis based on its unique restriction sites present in the amplified portion of the polyhedrin gene. To identify the unique restriction sites, restriction mapping analysis of HaNPV-P polyhedrin gene along with other known published polyhedrin sequences was performed using BioEdit version 5.0.9. The unique restriction sites present at particular nucleotide positions in polyhedrin gene of HaNPV-P were identified and short listed the other NPVs which have same restriction sites at same positions. This has showed that one unique restriction site, *Xho-I* at nucleotide position 131 was found in NPV from *M. brassicae* and *M. configurata*, and at position 671 in NPV of *L. seperata*. Whereas in HaNPV-P, the *Xho*-I site was found at both 131 and 671 base pairs (Fig 54). Further verification PCR-RFLP analysis of HaNPV-P polyhedrin gene showed that, in 12% native PAGE the digested product was separated as three different sized fragments which are corresponding to the restriction map. The sizes of the restriction fragments were estimated to be 540, 131 and 73 bps (Fig 55). But, the *Xho*-I site was not found in any of the HaNPV polyhedrin gene sequences deposited in the GenBank. This indicated that the HaNPV-P strain was shown to be a mixture of closely related genotypes. The PCR, when combined the use

of REN analysis, can provide considerable resolution for use in diagnostic screens, it is relatively simple to use and can yield results very quickly. Not surprisingly, this approach is now starting for detection and identification of a range of insect viruses (Kool *et al.,* 1991; Williams, 1993, De Moraes and Maruniak, 1997; Bulach *et al.,* 1999). Similarly, Christian *et al.* (2001) developed a rapid method based on PCR-RFLP analysis for identification and differentiation of HaSNPV and AcMNPV groups by using a set of redundant primers to highly conserved region of polyhedrin gene. Based on REN analysis, Rovesti *et al.* (2000) reported that the HaNPV isolate was shown to be a mixture of many closely related genotypes but individual genotypes remained unchanged on passage in either *H. armigera* or *M. brassicae*. Doyle *et al.* (1990) noted that when MbMNPV$^{D}$ was passed in *Othosia cruda* there were minor changes in the restriction enzyme profile, which was attributed to the selection of a different variant. However, bioassay studies of Rovesti *et al.* (2000) showed that the two viruses HaMNPV and MbMNPV were successfully replicated in *H. armigera, M. brassicae* and *H. zea,* resulting in each case, in progeny virus which was essentially similar to the inoculum. Therefore, a viral insecticide based on these NPVS from *H. armigera* and *M. brassicae* would be more appropriately targeted against both insects. Similarly, Murillo *et al.* (2001) has reported that REN profiles of two SeNPV isolates (SeUZB and SeSP3) in Uzbekistan and Spain and MbNPV (Mb-PL) in Poland were closely related to previously described Spanish isolates of SeNPVs. At the same time the *Pst-I* and *Bgl-II* profiles of SeUZB and Mb-L were identical and very similar to the REN profiles of MbMNPV strain which is the active component of Mamestrin$^{R}$, a commercial bioinsecticide in France (NPP, Nagueres, France). In addition to SeMNPV, *S.exigua* is susceptible to other NPVS such as *A. californica* MNPV (Smits and Vlak, 1987), *S. liitoralis* MNPV (Munoz and Caballero, 1999) and MbMNPV (Wiegers and Vlak, 1984). In another case AcMNPV and BmNPV also show a high degree of genomic homology and different REN fragment profiles but do not share an overlapping host range and can be regarded as two different species. It is interesting to note that only minor changes in the virus genome, namely one or two amino acid substitutions in the

AcMNPV helicase P143, are sufficient to expand the host range of AcMNPV to *B. mori* larvae (Arguad *et al.,* 1998; Kamita and Maeda, 1997).

In the earlier days of baculovirology, it was believed that baculoviruses could only infect a single host species, and that no cross- infection occurred. This generalized the use of binomial Latin names of the insect species hosts to describe the new viral isolates. However, this practice can affect our understanding of baculovirus biology, and can also lead to confusion upon studying the classification and taxonomy of baculoviruses (Federici and Hice, 1997), and should be changed by more reliable methods based, for instance, in the genotypic characteristics of the viruses. A useful means for identification or description of baculoviruses is REN analysis of viral DNA, as firstly demonstrated by Lee and Miller (1978). This method has proved to be very useful not only for distinguishing distinct NPV species, but also different strains of one virus or even different genotypes within the same virus isolate (Smith and Crook, 1988; Munoz *et al,* 1998, 1999).

In general, baculovirus wild-type populations, from both different geographical isolates of the same virus and within a single isolate, where several genotypic variants of frequently coexist, show a considerable genetic heterogeneity. This heterogeneity is due to the enormous plasticity displayed by baculoviruses genomes which can undergo deletions (Munoz *et al.,* 1998), insertions (Jehle *et al.,* 1995), point mutations, recombinations (Croizier and Ribeiro, 1992), *etc.* This plasticity suggests that field isolates may be adapting to host and environmental conditions and that those isolates containing heterogeneous populations may be more valid for viral survival in the field (Possee and Rohrmann, 1997).

Investigations of geographic variability and the role of genotypic differences in the biology of baculoviruses are an important area of current research. Such studies may provide insight into the evolution of baculoviruses and their hosts and may also aid in the development more effective virus strains for biological control of insects. The improvement of HaNPV-P strain for a successful introduction into biological control for legume pod borer still requires detailed

knowledge of the molecular biology of this virus. This study sets the foundation for this and will serve in genetic engineering of the virus to enhance its potential as biological control agent.

# CHAPTER – VI

# *CONCLUSIONS*

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The important conclusions drawn from the present investigation are

- Nucleopolyhedroviruses (NPVs) isolated from *H. armigera, S. litura* and *A. albistriga* are belongs to multiple nucleocapsid NPVs (MNPVs). This is the 1st report from Indian sub-continent to be described the multiple nucleocapsid nature of NPVs isolated from these insect species.

- There was a significant difference in parameters like POBs/ml and POBs/larvae which showed that HaNPV multiplied on field collected larvae recorded significantly higher yield compared to laboratory reared larvae and the yield of SlNPV and AmalNPV was higher than HaNPV.

- Isoelectric precipitation method is simple and rapid protocol than sucrose gradient centrifugation method for purification polyhedrin from complex POB suspensions.

- Molecular weight of polyhedrins estimated in 12% SDS-PAGE are 31.65 kDa (± 0.00), 31.29 kDa (± 0.00) and 31.67 kDa (± 0.295) of HaNPV, SlNPV and AmalNPV respectively.

- The polyhedrin purified in both the methods (iso-electric and sucrose gradient) were contaminated with some minor molecular weight peptides of about 7-27 kDa and a high molecular weight peptide of about 60-70 kDa fragment. This has revealed that three NPVs have 6-8 minor polypeptide contaminants.

- In sucrose gradient purified polyhedrin preparations the minor peptide contaminants were relatively lower than isoelectric precipitated polyhedrin preparations but the yield of polyhedrin obtained was very less.

- Electro-elution of polyhedrin from PAGE gels is a best option to avoid the minor peptide contaminants in polyhedrin preparations during polyclonal antibody production against polyhedrin of NPVs.

- Polyclonal antibodies against polyhedrin of HaNPV, SlNPV and AmalNPV were produced by using 500µg of polyhedrin for immunization with an antibody titer of 1:5000 dilution, 18 weeks after immunization.

- The antibodies are highly specific to the polyhedrin and have no cross-reaction with insect body proteins. In addition to the major polyhedrin (31 kDa), the polyclonal antibodies recognized some minor low molecular weight polypeptides which could be degraded peptides of major polyhedrin. However, each antiserum has different degrees of cross-reactivity with heterologous polyhedrins in DAC-ELISA and western immunoblotting.

- Among various immunochemical tools developed using the polyhedrin polyclonal antibodies, indirect immunofluorescence assay, western immunoblot assay and DAC-ELISA were more convenient for detection of POBs in homogenates of NPV-infected larvae and viral insecticide preparations.

- Both DAC-ELISA and IC-ELISA were more conveninet for detection and quantification of polyhedrin protein in insect extracts and viral insecticide preparations but IC-ELISA is an appropriate test and allows quantification directly from alkali treated crude insect extracts / viral insecticide preparations.

- These tests are equally effective in detecting heterologous polyhedrins of closely related NPVs.

- These diagnostic tools are convenient, rapid and inexpensive for routine detection and quantification of NPVs and this technology will also be transferred to the bioproducts agribusiness units for commercialization of NPV production.

- Application of ELISA and western immunoblot assay in bioassay experiments during optimization of conditions for the productivity and quality of NPVs suggested that $4^{th}$ instar larvae is suitable for *H. armigera* and $5^{th}$ instar larvae are suitable for *S. litura* and *A. albistriga* for virus inoculation, and virus harvesting 9 days after inoculation from both live and dead larvae was better to get the maximum virus yield as well as to reduce the development of bacterial contamination.

- Application of ELISA tools at field level evaluation of efficacy of NPV against *H. armigera* on pigeon pea crop have proved that the useful ness of these tools in ecological and epidemiological studies of NPVs during IPM programs and also during the surveys of their persistence and out breaks of natural epizootics in the environment.

- Dual round PCR protocol using degenerate primers was a convenient method for isolation of full length polyhedrin gene of HaNPV-P strain.

- Gene sequencing analysis of HaNPV-P polyhedrin gene resulted in 744 nucleotide long ORF with a predicted coding capacity for a polypeptide of 247 amino acids. This is the 1$^{st}$ report from Indian sub continent and 8$^{th}$ report world wide to be described the full length polyhedrin gene sequence of a NPV isolated from *H. armigera*.

- Phylogenetic analysis at nucleotide as well as amino acid levels of polyhedrin gene showed that the virus belongs to group-II NPVs and is more closely related to the polyhedrins of NPVs isolated from *M. brassicae*, *M. configurata* and *L. seperata*. The virus was named as *Helicoverpa armigera nucleopolyhedrovirus,* Patancheru strain (HaNPV-P).

- PCR-RFLP analysis clearly differentiated the HaNPV-P polyhedrin with other published HaNPV polyhedrins by having one unique (*Xho-I*) restriction site at nucleotide positions 131 and 671 as in *M. brassicae* and *M. configurata* (131) and *L. seperata* (671) indicates that the HaNPV-P is a unique strain with in HaNPV species.

- The present investigation provides the insight into the evolution of baculoviruses and their hosts and may also aid in the development more effective virus strains for biological control of insects. The improvement of HaNPV-P strain for a successful introduction into biological control for legume pod borer still requires detailed knowledge of the molecular biology of this virus. This study sets the foundation for this and will serve in genetic engineering of the virus to enhance its potential as bio-control agent.

# *LITERATURE CITED*

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# *APPENDIX*

## APPENDIX -I

### *Helicoverpa armigera* (Hubner) larval diet:

**Diet ingredients:**

| S. No. | Ingredients | Quantity* |
|---|---|---|
| 1. | Chickpea flour | 300.0 g |
| 2. | Ascorbic acid | 4.7 g |
| 3. | Methyl-p-hydroxybenzoat | 5.0 g |
| 4. | Sorbic acid | 3.0 g |
| 5. | Aureomycin powder | 11.5 g |
| 6. | Vitamin stock solution | 10.0 g |
| 7. | Water | 450.0 g |
| 8. | Yeast | 48.0 g |
| 9. | Agar | 17.3 g |
| 10. | Water (for yeast/agar) | 800.0 ml |

* Diet sufficient for about 2000 larvae.

**Method of diet preparation:**

- Weigh all dry ingredients and have all wet ingredients in appropriate measuring cylinders.
- Add ingredients (S.No. 1-6) in a large bowl. Add water (S.No.7) and mix thoroughly using a blender
- Mean while heat water in a saucepan. When the water starts boiling add agar and mix thoroughly by stirring.
- Sprinkle yeast and mix thoroughly until an even consistency is obtained
- Add agar mixture to the ingredients (S.No.1-6) in the blender and mix thoroughly.
- Pour diet into trays and cell wells
- Leave to cool and transfer insects on to the diet.

## APPENDIX -II

### *Spodoptera litura* (Fabricious) larval diet:

### Diet ingredients:

| S.No | Ingredient | Quantity[1] |
|---|---|---|
| **Fraction-A:** | | |
| 1 | Water | 2000.0 ml |
| 2 | Kabuli gram[2] flour | 438.4 g |
| 3 | Brewer's yeast | 32.0 g |
| 4 | Sorbic acid | 4.0 g |
| 5 | Vitamin E (Viteolin capsules) | 4.6 g |
| 6 | Methyl parahydroxy benzoate | 6.4 g |
| 7 | Ascorbic acid | 10.4 g |
| 8 | Sorghum leaf powder | 160.0 g |
| **Fraction-B:** | | |
| 9 | Agar-Agar | 40.8 g |
| 10 | Water | 1600.0 ml |
| 11 | Formaldehyde (40%) | 3.2 g |

1: Amount used to prepare 15 jars of 300 g diet each

2: A cultivar of Chickpea (Cicer aiertnum)

### Method of diet preparation:

- All the ingredients of fraction-A except the Sorghum leaf powder are blended for 1 min.
- Sorghum leaf powder is soaked in 2 liters of warm water ($70^0$C) and blended with fraction-A ingredients for 3 min.
- Agar-Agar is boiled in 1.6 liters of water (fraction-B) and cooled to $40^0$C before adding to the blender containing fraction-A ingredients.
- Formaldehyde is finally added and all the constituents are blended for 3 min. Then the diet poured in to plastic jars and cooled.
- Each jar contains about 300g diet, which is sufficient for 100 larvae to develop successfully to IV instar.

# APPENDIX -III

## Polyacrylamide Gel Electrophoresis Reagents and Buffers:

**Stack gel buffer (1 M Tris-HCl, pH 6.8)**

Tris base 12.1 g

Dissolve in 70 ml distilled water, adjust pH to 6.8 with 1 N HCl and make up to 100 ml with distilled water.

**Resolving gel buffer (1 M Tris-HCl, pH 8.8)**

Tris base 12.1 g

Dissolve in 70 ml distilled water, adjust pH to 8.8 with 1 N HCl and make up to 100 ml with distilled water.

**Acrylamide/Bis (30:0.8 w/w) mixture**

Acrylamide 30 g

Bis acrylamide 0.8 g

Distilled water to 100 ml

Store this solution at 4ºC in amber coloured bottle or wrap the bottle with aluminium foil to avoid exposure to light.

**Precaution:** Acrylamide is a neurotoxin. Direct contact with skin or inhalation of acrylamide should be avoided. Prepare this solution in fume hood and always wear gloves.

**10% ammonium persulphate (APS)**

APS 100 mg

Distilled water 1 ml

**Note:** Always prepare fresh solution before use.

**Electrode (running or tank or TG) buffer, pH 8.3**

Tris base (25mM) 3 g

Glycine (250mM) 14.4 g

SDS 1 g

Distilled water 1 litre. No need to adjust pH. Store at room temperature.

| **Plug gel composition (Optional)** | |
|---|---|
| Acrylamide: Bis mixture | 1.75 ml |
| Resolving gel buffer | 1.0 ml |
| Distilled water | 1.0 ml |
| TEMED | 20 µl |
| 10% APS | 40 µl |

| **Stacking gel composition (4%)** | |
|---|---|
| Acrylamide:Bis mixture | 1.75 ml |
| Stack gel buffer | 1.25 ml |
| Distilled water | 7 ml |
| TEMED | 15 µl |
| 10% APS | 200 µl |
| 10% SDS | 100 µl |

**Resolving gel composition**

| Component | 10% | 12% | 14% | 16% |
|---|---|---|---|---|
| Acrylamide: Bis mixture | 10 ml | 12 ml | 14 ml | 16 ml |
| Resolving gel buffer | 11.25 ml | 11.25 ml | 11.25 ml | 11.25 ml |
| Distilled water | 9 ml | 7 ml | 5 ml | 3 ml |
| TEMED | 20 µl | 20 µl | 20 µl | 20 µl |
| 10% APS | 100 µl | 100 µl | 100 µl | 100 µl |
| 10% SDS | 300 µl | 300 µl | 300 µl | 300 µl |

**Note:** Mix acrylamide: bis solution, gel buffer, distilled water and TEMED mix well, then add APS, swirl the flask and immediately pour into the gel mould

**Precaution:** Unpolymerised acrylamide is a neurotoxin. Gloves should be worn when handling this solution.

*For denaturing gel only

**Laemmli buffer**

0.5 M Tris-HCl, pH 6.8

10% SDS

5% 2-amino-thioglycerpl

10% glycerol

0.05% bromophenol blue

## Silver Staining Reagents and Buffers

**Fixing solution :( Prepare freshly before use)**

Glacial acetic acid: 3 ml

Methanol: 50 ml

$dH_2O$ : 147 ml

**DTT wash solution {0.05% Dithiothreitol (DTT)}**

DTT: 5 mg

$DH_2O$: 10 ml

This can be stored in aliquots at $-20^oC$ dilute this to 1:100 freshly before use.

**Silver nitrate solution (Prepare freshly before use)**

Silver nitrate: 400 mg

$DH_2O$: 200 ml

**Developer solution (Prepare freshly before use)**

$Na_2Co_3$: 6 g

Formaldehyde: 100 µl

$dH_2O$: 200 ml

**Stop solution:**

Glacial acetic acid: 1 ml

$dH_2O$: 99 ml

## APPENDIX-IV

## ELISA- Reagents and Buffers:

**Carbonate buffer or coating buffer, pH 9.6**
$Na_2CO_3$ 1.59 g
$NaHCO_3$ 2.93 g
Distilled water to 1 l [No need to adjust pH]

## Phosphate buffer saline (PBS), pH 7.4

$Na_2HPO_4$ 2.38 g
$KH_2\ PO_4$ 0.4 g
KCl 0.4 g
NaCl 16.0 g
Distilled water to 2 l
No need to adjust the pH

**Phosphate buffered saline Tween (PBS-T)**
PBS 1 l
Tween-20 0.5 ml

**Antibody buffer (PBS-TPO)**
PBS-T 100 ml
Polyvinyl Pyrrolidone (PVP) 40,000 MW 2.0 gm
Ovalbumin (Sigma Cat. No. A5253) 0.2 gm

**Distilled water - Tween ($dH_2O$-T)**
Distilled water 2 l
Tween 20 (0.05% v/v) 1 ml

**Substrate buffer (diethanolamine buffer) for ALP system**
Prepare 10% diethanolamine in distilled water and store at 4 0C. Adjust pH to 9.8 with con.HCl. Prepare 0.5 mg/ml p-nitro phenyl phosphate (PNPP) in 10% diethanolamine, pH 9.8 (for each 15 mg table 30 ml substrate buffer is required). This solution should be prepared fresh. Don't store left over buffer.
**Note:** Diethanolamine is toxic and harmful to eyes. Take necessary care to avoid contact with skin. PNPP convert to p-nitrophenol after reacting with APL. Plates after adding substrate must be handled extremely carefully.

# APPENDIX-V

## Western Immunoblotting Reagents and Buffers:

### Transfer buffer

Tris (0.025 M) 9.1 g
Glycine 43.2 g (0.192 M)
Methanol 600 ml
$dH_2O$ to 3 l

**Tris-buffered saline (TBS)**
Tris 4.84 g (0.02 M)
NaCl 58.48 g (0.5 M)
Adjust pH to 7.5 with 1N HCl, then makeup to 2 l in $dH_2O$
**Note:** TBS can be replaced with PBS.

**TBS-Tween (TBS-T)**
Add 500 ml of Tween – 20 to TBS-T [Final concentration of Tween 0.05% (v/v)]

**Blocking solution (antibody buffer)**
TBS-T with 5% (w/v) non-fat mild powder (eg. Nestle or Everyday)

**Enzyme conjugate**
Goat anti-rabbit IgG conjugated to ALP or HRP to detect rabbit antibodies
Prepare appropriate dilution of the conjugate in antibody buffer. [**Note:** Usually 1:5000 for conjugate purchased from Sigma]

**Substrate:**

**Fast Red Substrate**

**Solution A:**
Napthol AS-BI phosphate 50 mg
Dimethyl formamide 20 ml
Distilled water 20 ml
Adjust pH to 8.0 with 0.1 M $Na_2CO_3$

**Solution B:**
Fast red/Fast blue RR salt 50 mg
0.2 M Tris-HCl buffer pH8.3 18 ml
Add 2 ml of solution A to solution B mix well, filter through glass wool or Whatman filter paper and use.

**2X Protein sample buffer (Lamelli buffer)**
(Please see section Appendex III)

**Electrode (running or tank or TG) buffer, pH 8.3**

(Please see section Appendex III)

# APPENDIX-VI

## Agarose Gel Electrophoresis Reagents and Buffers

**10 X electrophoresis buffer (TBE buffer, $P^H$ 8.3)**

Tris base (0.45 M) - 54 g

Boric acid (0.45 M)-27.5 g

0.5 M EDTA $P^H$ 8 (0.01 M)-20 ml

$dH_2O$ -1.0 liter

Sterilize by autoclaving and store at room temperature

**Working solution (0.5x) TBE**

5 ml of 10x TBE + 95 ml $dH_2O$-0.5x TBE

Final concentration of Tris base, Boric acid and EDTA in working solution is 0.045 M, 0.045 M and 0.001 M, respectively

**5 X sample buffer (gel loading buffer)**

Bromophenol blue 0.25%-5 mg

Xylenecyanol FF 0.25%-5 mg

Glycerol 30%-3 ml

Sterile $dH_2O$ -10 ml

**1% Ethidium bromide solution**

Ethidium bromide- 100 mg

$dH_2O$ -10 ml

Store in dark colour bottle at $4^0$C