Molecular Identification and Pathological characteristics of native isolated NPV against *Spodoptera litura* (Fabricius) in Pakistan

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Abstract

The cotton army worm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) is a destructive pest of various field crops and vegetables in Pakistan. Development of bio pesticide is an attractive strategy to minimize the problems of pest resistance, environmental pollution and human health concerns. The isolates of *S. litura* Nucleopolyhedroviruses (SltNPV) were collected from infected larvae fed on natural cotton crops. The NPV was isolated from the larvae and viral occlusion bodies (VOBs) were detected using a light microscope. The toxicity of native isolates against *S. litura* also studied by testing different concentrations (1 x 10^2 POB (Polyhedral occlusion bodies) mL^{-1}- 1 x 10^{10} POB mL^{-1}) from the occlusion bodies produced from NPV isolates against 2^{nd}, 3^{rd} , 4^{th} and 5^{th} instar larvae of *S. litura.* The rapid and sensitive polymerase chain reaction (PCR) technique was used for the molecular detection of NPV gene from native NPV diseased insect. Multiple sequence alignment and phylogenetic analysis were performed to compare *Sl*NPV- FSD15 based on *Lef-8* with other Lef-8 genes sequences clearly showed that our *Sl*NPV-FSD15 isolate belongs to *Spodoptera litura* associated NPVs. The biological activities of this NPV isolates were investigated under laboratory condition. The highest mortality of *S. litura* was observed at early instars. Against second instars of *S. litura,* LC_{50} values of NPV isolate ranged from 1.92×10^3 to 3.64×10^3OB/ml with LT50 values of 69.30 hrs. to 72.80 hrs. respectively. This study showed highly effectiveness and provides an opportunity to cut down the use of synthetic approaches and develop safe biological/microbial insecticides from NPV isolates, which in future may effectively control *S. litura.*

Keywords: *Spodoptera litura*, PCR, NPV, Bio pesticide, DNA barcoding

INTRODUCTION

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) is a cosmopolitan and polyphagous pest which cause severe losses in southern and northern districts of Pakistan (Ahmed *et al.,* 2016; Saleem *et al*., 2016). Itt is widely distributed throughout Asia, Africa and Europe (Nathan *et al*., 2005; El-Helaly, 2013) causing damageto numerous economically important cash crops such as cotton, groundnut, soybean, tomato, sweet potato, onion, clover chili, cauliflower and cabbage (El-bendary and El- Helaly, 2013; Saleem *et al.,* 2016).. Current methods to control this pest are based on the use of conventional insecticides which causes the development of resistance in the pest and further has a negative environmental and human impact. Extensive use of synthetic insecticides has led to outbreaks of insecticide resistance in *S. litura* (Bhatti *et al.,* 2013; Ahmed *et al*., 2015). The development of resistance in *S. litura* against organochlorine, organophosphates and synthetic pyrethroids has been reported in the areas of cotton belt of south Punjab, Pakistan (Ahmed *et al.,* 2015).. An attractive and alternate tool for *S. litura* control is the use of entomopathogens. Several species of insect viruses, bacteria, fungi and protozoa were isolated and investigated for control of *S. litura* (Çakici *et al.,* 2014). In recent years, more than 600 insect species belonging to order Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanura, and Trichoptera infected with baculovirus have been isolated (Haase *et al.,* 2015). Baculoviruses are insect-specific, diverse group of double-stranded DNA circular viruses. Baculoviruses belong to the family Baculoviridae which is further divided into two genera, the Nucleopolyhedroviruses (NPVs) and Granuloviruses (GVs) (Hu *et al*., 2003; Jehle *et al.*, 2006; Barreto *et al*., 2005). The NPVs are cuboidal shaped having occlusion body of 0.4 to 2.5 μm in size visible under electron microscope (Moser *et al*., 2001; Shapiro *et al.,* 2004). Lepidopteran pests, *Spodoptera exigua* (Murillo *et al*., 2001; Wu *et al*., 2012; khattab *et al*., 2013). *S. litura* (Lavina *et al.,* 2001; Martins *et al.,* 2005; Laarif *et al.,* 2011), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Mehrvar 2007, Kumar *et al*., 2012; Noune and Hauxwell, 2015) and *Malacosoma americanum* (Demir, 2014) have shown susceptibility to several isolates of NPV. Horizontal transmission of baculoviruses from one host to another occurs when a susceptible insect ingests food contaminated with occlusion bodies (OBs) (Takahashi *et al*, 2015). Several studies have reported the isolation of NPVs from *S. litura* (Lavina *et al*., 2001;

Martins *et al*., 2005; Lucien *et al.,* 2009, Laarif *et al*., 2011; Kumar et al., 2011), *H. armigera* (Figueiredo, 1999; Ogembo *et al*., 2007; Mehrvar ,2008 Kumar *et al*., 2012; Noune and Hauxwell, 2015), *S. exigua* (Murillo *et al*., 2001; Wu *et al*., 2012; khattab *et al*., 2013), *Trichoplusia ni* (Erlandson *et al*., 2007) *Diaphania pulverulentalis* (Pachippan *et al*., 2012), turnip moth (Jakubowska *et al*., 2005) and *Chrysodeixis includens* (Alexandre *et al*., 2010).

All NPVs have molecularly been characterized by restriction endonuclease mapping of viral DNA, showing that they can be distinguished from each other by one or more DNA restriction enzyme fragments. At biological level, some of these strains have presented better insecticidal activities, which make them more suitable to *S. litura* (Martins *et al*., 2005; Laarif *et al*., 2011), *H. armigera* (Ogembo *et al*., 2007; Mehrvar, 2008) and *S. exigua* control (khattab *et al.*, 2013). The present study was planned to identify NPVs as a biological control agent for *S. litura* from local Spodoptera NPV infected population. Therefore, a simple molecular procedure suitable for diagnosis of viruses in their natural hosts was adopted and report the evaluation of its biological activity against different instars is reported.

MATERIALS AND METHODS

NPV diseased insect collection

The original virus isolates were obtained from diseased *S. litura* larvae collected from cotton field of PARS-UAF. The larvae that showed baculovirus infection symptoms were brought to laboratory (Fig. 1A) and examined to confirm the presence of virus by light microscope with Giemsa staining according to Mustafa *et al*. (2001)., A thin smear of infected worm tissue was mounted and air dried. The smear was immersed for 1-2 min in Giemsa, rinsed under running tap water for 5-10 sec then the smear was stained for two hours in 10% Giemsa stain (10g of Giemsa dissolved in 100ml distilled water). The dye was rinsed off in running tap water for 5-10 sec and allowed to air dry, then examined under a light microscope to detect the occlusion bodies (OBs). After the examination, the diseased larvae were kept at -20°C until the purification of OBs (polyhedra)

Light microscope examination

Moribund larvae showing specific disease symptoms were individually examined for the presence of polyhedral inclusion bodies using light microscope. A wet smear of the homogenized liquid using a drop of haemolymph or a small part of larval tissue was spread on a glass slide and stained in 10% Giemsa's stain for 10 min. The excess stain was then washed with running water

for 5-10 second (Wigley, 1976). The prepared smear was examined using the oil immersion of phase contrast microscope. The smear test would allow recognition of the occlusion bodies of nuclear polyhedrosis viruses NPV.

NPV production and Purification

The diseased larvae of 3rd to 5th instar of *Spodoptera litura* were collected from cotton fields in Faisalabad, Pakistan, in 2013. The infected larvae showing baculovirus presence symptoms were brought to IGCDB laboratory and stored at -20 °C until the isolation of OBs (polyhedra). Isolation and purification of the NPV was performed in 4th instar of *S. litura*. Using haemocytometer (Hausser Scientific), NPV viral concentrations were quantified under a light microscope with six counts per hemocytometer. A stock suspension of NPV was prepared from *S. litura* which was diluted to various concentrations 1 x 10^2- 1 x 10^9 OBs/ml. Twenty-five larvae were placed in 50 ml tube containing 25 ml 0.5% SDS and homogenized in conical tubes using T-25 basic ULTRA-TURRAX set. Homogenates were flitered through 5 layers of cheesecloth set in a funnel fitted with a wire mesh. Then, filtrate was transferred to 50 ml round bottom polybicarbonate tubes.. POBs were settled at bottom of tube by centrifugation at 2700 rpm for 10 min at room temperature in 50 ml round bottom centrifuge tubes. Before final isolation, the pellets were re-suspended in 0.1% SDS and re-suspension was repeated with 0.5 M NaCl. Then the OBs were finally re-suspended in distilled water containing 0.02% sodium azide. Following the protocol of Cheng et al. (1990), POBs were further purified according to sucrose gradient centrifugation method.

DNA extraction and PCR

DNA was extracted from NPV infected larvae of *S. litura* with DNeasy Tissue kit (Qiagen, Hilden, Germany) according to manufacturer protocols. Quantity and quality of extracted virus DNA was estimated using UV spectrophotometer (Pico200) and by the ethidium bromide 1.5% agarose gel electrophoresis respectively. The DNAs obtained were stored at -20°C until used. The primer nucleotide sequences based on Nucleopolyhydroviruses (NPV) conserved gene late expression factor (Lef-8) was carried out for PCR in a reaction volume of 48 µl. The reaction volume contains ddH2O (33 µl), 10Xreaction buffer (5 µl), 1 mM dNTPs (5 µl), 200 µM of each forward and reverse primers (1 µl), and 1 µl of purified DNA (~ 50 ng). The lef-8 specific degenerate primers (prL8-1 and prL8-2) developed by Lange et al. (2004) was used in PCR. The amplification of DNA was accomplished with a PCR thermal cycler (PeqStar, Germany) under

following PCR cycling conditions. DNA samples were preheated to 95 °C for 4 min, followed by 35 cycles with denaturation temperature of 95 °C for 2 min, annealing temperature of 46 °C for 2 min, and extension temperature at 72 °C for 1 min. Then, final extension at 72 °C for 2 min was included for final amplification. In the first step of PCR, 0.2 µl of Taq polymerase and 1.8µl its dilution buffer was added in each tube making final reaction volume up to 50 µl.. The PCR product was analyzed by 1% agarose gel electrophoresis at 80 V for 1 h. After the PCR, gels were stained in solution of ethidium bromide (2µg/mL) and were visualized under UV light using fisher scientific gel documentation system (Syngene™ IG3) for the confirmation of virus presence.

Nucleotide Sequencing and Phylogenetic Analysis

Polymerase chain reaction product (745 bp) was purified using commercial kit and sequencing was performed and analyzed on AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA). The resulting Lef-8 sequence has been deposited in international database (NCBI). NPV Lef-8 sequences identified by Blast sequence homology were downloaded from Genbank database (http://www.ncbi.nlm.nih.gov). All Lef-8 sequences were aligned with ClustalW and phylogenetic analysis was performed under MEGA6 software (Tamura et al. 2013)

Insect Rearing and Bioassay study

The *S. litura* larvae used in this experiment derived from cotton fields of Punjab and reared on artificial diet consisting of chickpea flour 150g, sorbic acid 0.75 g, yeast powder 24g, linseed oil 6 ml, agar 8.4 g, vitamin mixture 0.02 g, ascorbic acid 2.35 g, methl-4-hydroxy benzoate 1.5 g, d H2O 550 ml and streptomycin 0.75 g. The rearing was done under controlled laboratory condition (25± 2^{O}C, 70± 5 RH, 14:10 h light: dark photoperiod). The diet was stored at 4ºC until use. About fifty 2^{nd} and 3^{rd} instar *S. litura* larvae were transferred in individual plastic vials containing 0.5 -1.5 g thin layer of artificial diet until they reached at fourth larval stage. Then, they were transferred to boxes containing 3 cm thick layer of vermiculite (<0.5 mm grain size) for pupation. Additional artificial diet was provided shortly before pupation. Pupae were collected and incubated at 25 ^{0}C. All the larvae were maintained at 25±2°C, 75% r.h and 16:10 (D:L) photoperiod (Jehle et al., 2013).

All the bioassays were performed according to Lucien et al. (2009) with slight modification. Briefly, Virulence of NPV was tested against 2^{nd}, 3^{rd} 4^{th} and 5^{th} instar larvae of *S. litura.* Molted larvae were distinguished by their head capsule slippage and transferred to

individual vials for 12 h. Different NPV suspensions (1 x 10^2; to 1x 10^9 POB mL^{-1}) were prepared and 5-10 µl of each viral concentration was socked with fresh piece of artificial ($2mm^2$) in vials. Molted Larvae were allowed to feed on the treated artificial diets until complete consumption. After consuming artificial diet, 25 larvae were individually shifted on diet without virus suspension. Larvae served with virus free diet constituted the healthy controls. LC50 and LT50 values were determined from data observed every day. Concentrations of OBs were determined with haemoctometer under phase contrast microscopy at 400X magnification. All the treatments were replicated thrice and maintained at 25±2°C, 75% r.h. and L16: D8 h photoperiod.

Statistical analysis

The data were analyzed using Probit analysis software to arrive at lethal concentration of virus required to cause 50% mortality (LC_{50}) and lethal time required to cause 50% mortality (LT50)

RESULTS

Symptoms of viral infection

The native *Spodoptera litura* nucleopolyhydrovirus (NPV) isolate was obtained from infected larvae collected from cotton field of Post graduate Agriculture Research Station (PRAS) of University of Agriculture Faisalabad. The symptoms of viral infection on *S. litura* collected from the cotton field were liquefied ruptured larval body (Fig. 1-A), and slow motion swollen larvae with red color cuticle (Fig. 1-B). The field collected virus isolate was cultured in a *S. litura* laboratory colony (Fig. 1-C-F). Then infected propagated colony was then purified and kept at -80°C for further studies.

Microscopic Examination

The viral occlusion bodies (VOBs) from diseased larvae were observed under a light as well as inverted microscope. A thin smear of infected larvae fluid was stained with drop of Giemsa, VOBs appearing as polyhedral and negatively stained particles. Under light and inverted microscope, NPV associated occlusion bodies were observed (Fig.2)

PCR of NPV specific gene

The infected samples collected from Faisalabad district were analyzed for PCR detection. The partial codons sequence of the late expression factor-8 (Lef-8) gene was amplified using

specific PCR primers. Gel electrophoresis analysis of the PCR product showed an amplification of single fragment at correct length (~745 bp)(Fig. 3).

DNA sequencing and Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Phylogenetic analyses were conducted in MEGA6 (Tamura et al., 2013) for the comparison of the SlNPV-FSD15-lef-8 gene sequences with the sequences of other lef-8 genes of various nucleopolyhydroviruses (NPVs) available in GenBank. The nucleotide sequence of the native NPV isolate (Slt NPV-FSD15) is composed of 745bp, submitted at GenBankNCBI. This sequence was aligned using ClustalW with the NCBI available nucleotide sequences of NPVs associated with different insects. The molecular phylogeny of nucleotide sequence of the SlNPV-FSD15-lef-8 showed maximum homology (99-100%) with nucleotide sequence of SpliNPV from *Spodoptera litura* of Germany, (AY706581.1), Japan (AB326103.1, AB583682.1, AB581187.1)), and Indian origin, (JF2760358.1),. The *Helicoverpa armigera* associated NPV formed separate clade clearly distinguished from *Spodoptera litura* NPV isolates with lowest similarity percentage index (Fig. 4) which was observed between 82-90%.

Biological activity of native SplNPV-FSD15 isolate

Bioassay ofisolated strain ofSpltNPV against 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instars larvae of *S. litura* under laboratory condition show a wide range of variation in its biological activity. The LC50 values in three experiments of SpltNPV were inversely correlated with the age of the larvae, LC50 values being the highest for fifth instars. Similarly, LT50 was as low as 69-72 hours for second instar larvae and regularly increased to reach at 144 to 146 hours for fifth larvae instars. LT50 values were not significantly different between the different independent experiments. Bioassay result revealed that the SpltNPV strain isolated from *S. litura* was highly effective especially against 2^{nd}, 3^{rd} instar larvae. In first experiment, LC50 values for 2^{nd} , 3^{rd} , 4^{th} and 5^{th} instar larvae were 2.64×10^{3}, $2.92\times10^{4,}$ $2.94\times10^{5,}$ and 2.15×10^{6} OBs/ml respectively. Briefly, in the third experiment, for 2^{nd} instars, the most virulent LC_{50} value (1.92×10^{3} POB mL^{-1}) was observed, followed by 2^{nd} and 1^{st} experiment (Table 1). The order of LC_{50} value in the case of three experiments of SpltNPV was second<third <forth <fifth instar. Similarly, LT_{50} value of 3^{rd} spltNPV experiment was the lowest (59 hours) followed by 2^{nd} and 1^{st} experiment (Table 2).

DISCUSSION

S. litura (Lepidoptera: Noctuidae) also called armyworm is one of the most destructive and notorious phytophagous insect pests on cotton, vegetables and other field crops. Microbial bio-pesticides based on native baculoviruses have great potential in agriculture with better insecticidal characteristics and higher safety' for environment. The NPV virus isolates was obtained from diseased *S. litura* collected cotton. The diseased larvae exhibited viral-like symptoms such as swollen bodies, moribund appearance when field collected and also when laboratory NPV treated. . Similarly, because of deposition of occluded viruses (OVs), infected larval bodies become pale and their cuticle ruptured discharging body fluid and ultimately killing the insect. Similar results were reported by Toprak *et al.* (2005). The viral occlusion bodies (VOBs) of the Pakistani nucleopolyhedrovirus isolates (*Sl*NPV-FSD15) were detected using a light microscope by staining thin smear of infected larvae and drop of VOBs with Giemsa stain which appeared polyhedral and negatively stained particles. The developed bio informative tools and application of latest genomic data facilitated the identification of viruses because of good primer designing. Here, we also used molecular techniques to characterize native *S. litura* associated NPVs that were found identical to SpltNPVs from other countries and clearly different from *Helicoverpa armigera* and *Mythimna separata* NPVs (Kouassai et al., 2009). Further, LC50 values against 2^{nd} -5^{th} instars with LT50 values of this NPV isolate (SltNPV-FSD15) indicated a significant effective control against *S. litura*. Result obtained by Laarif *et al*. (2011) indicated that amino acid and nucleotide sequences of *S. littoralis* (Tun-SlNPV) nucleopolyhedrovirus strain was almost identical with different GenBank deposited sequences of NPVs (Clem *et al*., 2013; Ikeda *et al*., 2013).

In bioassay, increasing value of LC50 and LT50 with the age of the host showed the susceptibility of the different larval stages; decline in *S. litura* susceptibility to NPV is due to dilution effect because larval weight increased as the insect grows (Briese *et al*., 1986). Lucein *et al*. (2009) and Bhutia *et al*. (2012) also found that LC50 and LT50 values were increased as larval age increased showing - against SpltNPVN. However, Trang *et al*. (2002) reported that older larvae of *S .litura* were not affected through ingestion, but intra haemocoelic infection of NPV in mature insect resulted in insect death (Rao *et al*., 2015). SpltNPV isolate from Biocontrol research laboratory (BCRL) was found to be virulent against 3^{rd} instar larvae of *S*

litura larvae with LT_{50} value (122.16 d) at 1×10^6 OB's/ ml (Bhutia *et al*., 2012). Subramanien *et al*. (2005) described that LT_{50} values for larvae of *S. litura* was dose-dependent. The LC_{50} value for the larvae of *S. litura* increases 15,000 times in 2 day-old larvae as compared to 8th day larvae (Trang *et al*., 2002). Similar trend for LC50 of SpltNPV isolate against 2nd and 3rd were (3.5 9 $\times10^4$ and 2.4 9$\times$ 10^5 OBs/ml) observed (Kumar *et al*., 2011). In summary, increasing dose of NPV caused significant reduction in the survival times of Lepidoptera larvae. This pattern was also observed in instar of *Mythima separata* and third instars of *S. litura* larvae (Koussoi *et al*., 2009). Minimum lethal time (LT50) (96-216 hrs) of different instars of *S. litura* suggests that application of SpltNPV isolate as bio insecticide would be economic and attractive strategy for the control of *S. litura*. (Lucein *et al*., 2009) .Contradictory results were found by Koussoi *et al*. (2009) who observed that high LC50 of MSNPV (180 hrs) and Indian NPV isolates (240 hrs) against *M. separata* made clear that their sole application would not be an efficient strategy for insect control, but should become effective bio insecticides when combined with enhancers. In our case, the very good results of LC50 and LT50 showed highly effectiveness of Slt NPV-FSD15 isolate against all tested instars of *S. litura* as compared to other reported SltNPV isolates. The biological activity of this isolate is under progress against other important lepidopterous pest of major agriculture crops.

CONCLUSION

The indigenous NPV strain associated to *S. litura* (SltNPV-FSD15) collected from Faisalabd cotton fields was successfully isolated from NPV infected larvae of *S. litura* and characterized and evaluated against this important insect pest. The results confirmed that this native isolate (SltNPV FSD-15) was highly effective against *S. litura* showing high mortality with reduced LT50 values as compared to other SltNPVs reported from other countries. We have also developed NPV based microbial insecticide using this native NPV isolate. The efficacy evaluation of some other native SltNPV isolates collected from various regions of Punjab is under process,

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Conflict of interest statement

The authors have no conflict of interest.

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Fig.1. NPV infected *S. litura* (A) Typical NPV symptom of NPV ruptured infected larvae, usually die 4–9 days after infection, disintegrate and release a virus-laden fluid (B) Slow move NPV infected swollen malformed pale brown larva; C- Slow move NPV infected swollen malformed dark larva from 5^{th} Instar (D-E) Malformed pre-pupae (F) Laboratory propagative NPV infected larva (G) Healthy larva

Fig.2. (A) NPV infected *S. litura* viral occlusion body's polyhedra under light microscopy (B) degenerating (ruptured) infected *S. litura* cell.

Fig.3. PCR detection of NPV from *S. litura* infected samples from Pakistan by using NPV LEF8-specific primers: Lane1-3 Non infected *S. litura* Larva, Lane 4-8 NPV infected *S. litura* larva (Pak-15 Faisalabad strain), M- 1 kb DNA Marker (Invitrogen).

Fig.4. Molecular Phylogenetic analysis by Maximum Likelihood method of Pakistani isolate SltNPV-FSD15 strain based on nucleotide sequences with the corresponding partial late expression factor-8 (Lef-8) gene of 12 nucleopolyhydroviruses associated with *S. litura* (NPVs). *S. litura* associated NPV isolates origin and GenBank accession numbers used in the sequence analysis and phylogenetic tree construction; AY706581.1 S37 (Germany), JF276035.1 (Banglore, India), AB451187.1 (AB326103.1, AB451187.1, AB583682.1 (Japan). The numbers represent bootstrap percentage values based on 1,000

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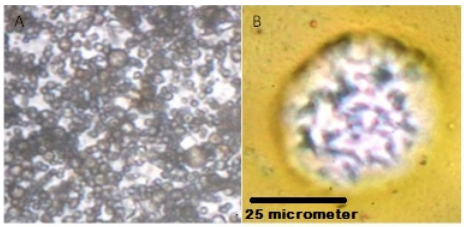

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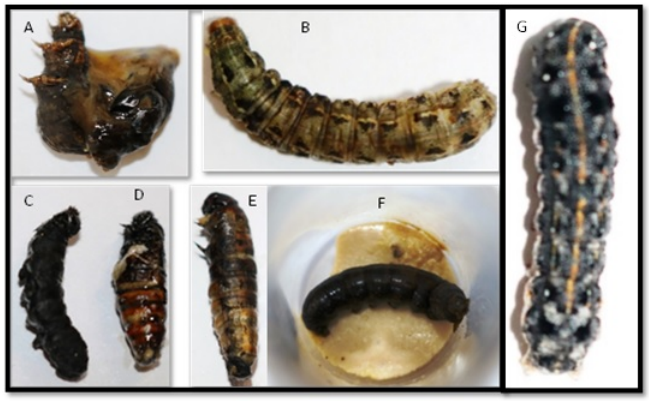

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Table 1.- LC50 (95% CL) values of SPLtNPV-FSD15 Isolate against 2nd, 3rd and 4th instar of *Spodoptera litura.*

Instars	SPLtNPV-1					SPLtNPV-2					SPLtNPV-3				
	LC50	Slope	X^2	Df	P	LC50	Slope	X^2	Df	P	LC50	Slope	X^2	Df	P
Second	2.64×10^3	10298210	3.02009	1	0.082	1.94×10^3	10672645	3.07360	1	0.080	1.92×10^3	10672645	3.05460	1	0.081
Third	2.92×10^4	9727687	3.87286	1	0.049	1.66×10^4	10301158	4.45550	1	0.874	1.91×10^4	9898068	5.46667	1	0.019
Fourth	2.94×10^5	248157064	0.173039	1	0.677	2.68×10^5	69371413	0.0973090	1	0.755	3.9×10^5	55947328	0.09-3141	1	0.764
Fifth	2.15×10^6	65593222	0.449124	1	0.503	2.34×10^6	56888296	0.294854	1	0.587	3.96×10^6	54577160	0.295128	1	0.587

Table 2.- LT50 (95% CL) value of SPLtNPV-FSD15 isolate against 2nd, 3rd, 4th and 5th instar of *Spodoptera litura.*

Instars	SPLtNPV-1					SPLtNPV-2					SPLtNPV-3				
	Lt50 (hr)	Slope	X^2	Df	P	Lt50(hr)	Slope	X^2	Df	P	Lt50(hr)	Slope	X^2	Df	P
Second	72.50	0.917231	0.304227	1	0.581	70.4	0.948467	0.587796	1	0.443	69.30	0.953284	0.657373	1	0.417
Third	95.76	0.802226	0.117478	1	0.732	98.4	0.786250	1.49456	1	0.222	93.6	0.839378	1.10078	1	0.294
Fourth	121.6	0.713918	0.0030889	1	0.956	125.2	0.700997	0.0038591	1	0.950	123.5	0.693892	0.118227	1	0.731
Fifth	144.64	0.654180	0.0038249	1	0.951	146.8	0.660854	0.0632983	1	0.801	145.8	0.676337	0.0005908	1	0.981