Annexure-III

Reprints of Published Papers
Transovarial Transmission of Nucleopolyhedrovirus in Black-Inch Looper Caterpillar, *Hyposidra talaca* (Walk.) (Lepidoptera: Geometridae)

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**ABSTRACT**

*Hyposidra talaca* is one of the main pests of north-eastern tea plantation of India. *Hyposidra talaca* nucleopolyhedroviruses (HytaNPV) plays a key role to control the pest outbreak. Here the investigation of transovarial transmission of HytaNPV in the black-inch tea looper caterpillar, *H. talaca* was carried out. The egg masses were collected from a tea garden of Dooars region of India. The larvae reared from these eggs showed typical signs of nucleopolyhedrovirus infection. The phase contrast microscopic view indicated the HytaNPV caused mortality of larvae with an accumulation of large number of polyhedral occlusion bodies. Later the polyhedrin gene amplified from larva proved HytaNPV infection and presented the information of vertical transmission of virus from parent to offspring (transovarial transmission). HytaNPV can remain in larval population of *H. talaca* and be transmitted to the next generation. Hence, HytaNPV can be advantageous for longstanding control of *H. talaca*. This study reflects the important aspects of HytaNPV and proved its usefulness in biological control programme.

Introduction

Baculoviruses are well-known as the largest and most widely studied virus of insects. Till now, more than 700 baculoviruses have been isolated mainly from the insect species of the orders Diptera, Hymenoptera and Lepidoptera. In the search of biopesticides, baculoviruses have been analyzed and found prospective against field and forest pests (Moscardi, 1999). Nucleopolyhedroviruses (NPVs), which are a part of baculovirus, are pathogenic for invertebrates, particularly insects of the Lepidoptera. The virus is specified as having large double-stranded DNA genome within rod-shaped enveloped virion. The PCR technique has been shown to be suitable for virus identification (Mo-reaes and Maruniak, 2001; Galal, 2009; Hewson et al., 2011).

In *Hyposidra talaca*, a nucleopolyhedrovirus (HytaNPV) is an extremely infectious natural agent causing most destructive disease to the tea pest *Hyposidra talaca*. Mukhopadhyay et al. 2010, first reported the occurrence of HytaNPV in Terai and the foothills of Darjeeling.

The Indian tea has been one of the favorite beverages of the world for ages. But the tea lovers might be deprived of their favorite brew if the growth of the harmful pests like *Hyposidra talaca* cannot be checked. *H. talaca* (Lepidoptera: Geometridae) is a major defoliating tea pest, it creates a destructive disease to the tea plant *Hyposidra talaca*. Mukhopadhyay et al. 2010, first reported the occurrence of HytaNPV in Terai and the foothills of Darjeeling.

Antony et al. 2011, established the transovum transmission of HytaNPV by performing cloning and sequencing of a partial segment of HytaNPV polyhedrin gene. This virus has the ability to sustain in the host species at sub-lethal level and can be passed from one generation to the next (transovarial transmission). Successive generations of insect host are infected by polyhedra, thus the host serves as reservoir of inoculums. This characteristic helps in using NPV for biocontrol of the pest. The main aim is to produce a biopreparation of HytaNPV against *H. talaca*. Before that it is important to study the transovarial transmission of HytaNPV in its host. Due to the high potency of the virus as chemical alternative, finding out the transovarial transmission of the virus is a matter of great interest. This study may play an imperative role in the programs of integrated pest management (IPM). This study focuses on the transmissibility of HytaNPV from parents to progeny one of the most important parameters to consider when using NPV in a biological control programme. This study can be useful for the improvement of HytaNPV as biological control agent.

Materials and methods

**Insect rearing**

Gravid *H. talaca* females were collected manually using light trap from the Rajabhat tea estate (Eastern Dooars, 26°39’N, 89°29’E), Jalpaiguri, West Bengal, India. The collection site was located 87 km away from North Bengal Regional Research and Development Centre, Tea Research Association (TRA), India. Individual female was maintained in each open end glass vial covered with muslin cloth. The gravid females laid their eggs in the glass vials. The freshly laid eggs were brought to laboratory and 8 % formalin was used to sterilize the surface of the eggs for 15 min at room temperature. They were reared in an insect rearing room of Plant Protection Department of TRA. The eggs were kept to a sterile Petri dish just after several cleaning with distilled water and rinsed with 70% ethanol. Eggs collected from each female were kept separately and were maintained in a wooden cage of 30 cm × 30 cm × 35 cm size. They were reared at 28 ± 2 °C, 72 ± 3 % relative humidity and a 13L: 11D photoperiod. The newly hatched larvae, all from the
same parent were released onto fresh tender shoots of tea dipped in glass tubes. Each larva was reared in a separate cage, and fresh tea leaves were provided daily. Fresh tea leaves were supplied as food after sterilizing with 10% formalin for 5 min and rinsed with sterile double-distilled water. The larva that showed typical NPV signs was collected in a 1.5 ml eppendorf tube. Later the viral DNA was isolated and the degenerate primers were used for the PCR study of the isolated DNA. Later the amplified PCR products were cloned and sequenced.

**Disease Identification**
The larvae were monitored regularly to identify the symptoms of NPV infection (Sinu et al., 2011). The larvae were dissected after the recognition of signs of NPV infection. The tissues of dissected larvae were examined through eyes. Immediately after that the smears of the tissues were examined under light microscopy. After identification of polyhedral occlusion bodies (POB) of virus, these were dissolved in 1 N NaOH and were examined under a phase-contrast microscope (Thomas, 1974). The larval progeny that showed NPV symptoms were homogenized individually in distilled water. Then polyhedral occlusion bodies were isolated and the quantification of POBs was carried out by using Neubauer haemocytometer (Marienfeld, Germany) under phase contrast microscope (Olympus BX 51).

**Viral DNA extraction and PCR amplification for detection of NPV in H. talaca progeny**
Larvae showed signs of NPV infection were taken for PCR study. The PCR study was performed to confirm transovarial transmission. Viral DNA was extracted individually from each larval sample and purified with QIAamp DNA Mini Kit (Qiagen) according to manufacturer’s protocol. The final DNA was obtained after several cleaning with ethanol and diluted wash solution. Later DNA was eluted and re-suspended in 20 µl molecular grade water (Himedia). The isolation of DNA was confirmed by electrophoresis in 1% agarose gel and quantified with Biophotometer (Eppendorf).

A highly conserved region of polyhedrin gene from HytaNPV was amplified. The PCR was performed using the degenerate primer (F: 5’-GGACCSSGGYAARAAAYCAAA AAA-3’ and R: 5’-GCRTCWGGYCGAAAYTCTYT-3’) designed according to Antony et al. (2011). The PCR reaction was carried out taking 50-100 ng of viral DNA in a 25µl reaction solution containing 1X PCR buffer (Invitrogen, USA), 1.5 mM MgCl, (Invitrogen, USA), 0.5 mM dNTPs (Bangalore Genei, India), 1 U Platinum Taq DNA polymerase (Invitrogen, USA), 0.5 µM of each primer. PCR amplification was performed in a DNA thermal cycler (Veriti Thermal Cycler, Applied Biosystems, CA, USA). The conditions used were initially with denaturation for 1 cycle at 94°C for 5 min; followed by 35 repeated cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and the final extension cycle at 72°C for 7 min. The PCR products were separated by 1.5% agarose gel electrophoresis with a 100-bp DNA ladder as a size marker (Genei, Bangalore).

**Cloning and sequencing of the polyhedrin gene of NPV**
The band of the expected size (527 bp) was eluted from the gel using HiPura Agarose Gel DNA Purification Spin Kit (Himedia) following the manufacturer’s protocol. The eluent was ligated into pGEM-T vector (Promega, UK) in a 3:1 (insert: vector) molar ratio with T4 DNA ligase as described by the manufacturer. Ligated products were transformed into Escherichia coli DH10B competent cells (Invitrogen, USA). Following amplification of recombinant clone with M13 universal forward and reverse primers, the sequencing was performed using the BigDyeTerminator v1.0 cycle sequencing kit (Applied Biosystems) in ABI 3500 Genetic Analyzer (Applied Biosystems).

**Results**

**Disease Identification**
The larvae that were grown from the field collected H. talaca eggs showed typical signs of virus infection. Primary signs of infection in larval stage are loss of appetite, lethargic movement, and climb to the top of the twigs and hanging upside down by their abdominal legs. Also flaccidity and rupturing of the cuticle of larvae were noticed. These symptoms suggested nucleopolyhedrovirus (NPV) infection (Figure 1). The haemolymph contained a large number of polyhedral occlusion bodies. Later the presence of NPV was ascertained by phase contrast microscopic study (Figure 2). The number of polyhedral occlusion bodies was calculated by haemocytometer showing the presence of approx. 5.9 × 10^8 POBs per ml of cadaver.

![Figure 1. Clockwise representation of the transovarial transmission of Hyposidra talaca nucleopolyhedroviruses from parent to offspring.](image)

**Diagnosis of the disease in H. talaca progeny by PCR amplification and polyhedrin gene sequencing**
The targeted sequence was amplified from the extracted DNA of the purified polyhedra by using the degenerate primer set. The successful amplification of the specific polyhedrin gene region of NPV confirmed the viral infection in the larvae. The bands obtained from PCR products were identical (Figure 3) and contained the nucleotide sequence of 527 base pair (bp) which confirmed the presence of NPV in the larval samples. A Blast search confirmed 100% similarity with the previously published HytaNPV sequence (Antony et al., 2011). The PCR amplification of the NPV...
polyhedrin gene from larvae suggested the viral infection. These results conclude that the HytaNPV sequence amplified from the *H. talaca* progeny was derived from the parent through transovarial transmission.

**Figure 3.** Agarose gel electrophoresis of specific PCR amplified products from HytaNPV infected *H. talaca* larvae of F1 generation. Lane M- 100 bp Ladder DNA marker; Lane L1- L4: samples of positive PCR products. The band lies between 500-600 bp (base-pair) and the size of the band is 527 bp. This result confirms the vertical transmission of HytaNPV.

**Discussion**

Virus is the subject of importance for the development of biopesticides to maintain ecosystem in agricultural field. In this regard, baculoviruses like NPVs are considered as potential biocontrol agent and have been applied successfully against larvae of many insect pests in the world (Hu et al., 1993; Ma et al., 2007). NPV has the ability to transmit vertically from adults to their offspring through transovarial transmission (within the egg itself) and via transovum transmission (surface contamination of the eggs) (Jehle et al., 2006). In this study, the transovarial transmission of NPV in *H. talaca* was examined. The transovum transmission of HytaNPV in *H. talaca* has been reported previously by Antony et al., 2011. Here, field collected eggs were surface sterilized, and the neonates larvae were reared under laboratory conditions. The larvae showed symptoms typical of NPV infection, indicating the vertical transmission of NPV. This is the first report on the transovarial transmission of HytaNPV in *H. talaca*. Though previously published studies have been reported the vertical transmission of NPV in many lepidopteran pests (Burden et al., 2002; Fuxa et al., 2002; Petrik et al., 2003; Khurad et al., 2004; Vilaplana et al., 2009).

UV radiation is the main constrain in successful application of NPV as a biological control agent as it hinder the activity of NPV (Petrik et al., 2003). Though sustain of the NPV in the host species and vertical transmission of NPV resolve the problem. The larvae hatched from field-collected eggs showed NPV infection, and the PCR amplification of the isolated viral DNA from the infected larvae and the sequencing confirmed the presence of viral gene in the offspring. The larvae contain the HytaNPV at sub-lethal levels can survive and the moths emerge from such larvae can vertically transmit the virus to their offspring. The pest has short life cycle with multiple overlapping generations and presence in winter months (Das et al., 2010), so along with the pest HytaNPV can remain in the tea field throughout the year. Thus, the obtained result reflects a crucial prospect of HytaNPV that can be used in the biological control of *H. talaca*, specially the virus can persist in the larval population and can be transmitted from one generation to the next. These characteristics support the use of HytaNPV as a potent biological control agent and competent alternative to chemical insecticides in integrated pest management (IPM).

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**References**

Molecular detection and pathogenicity of a nucleopolyhedrovirus isolated from looper caterpillar (*Hyposidra talaca*), a tea pest

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**Abstract** *Hyposidra talaca* is a major defoliating pest of tea plants in north-eastern part of India. In this study, we look for variations (if any) in the attacking virus. Viral samples were collected from different regions of the northern part of West Bengal in India and were analyzed by PCR technique to study the variations across the region. The partial segment of the HytaNPV polyhedrin gene was cloned and sequenced. A 527 bp nucleotide sequence containing highly conserved region from polyhedrin gene of HytaNPV was observed. The blast homology search for studied polyhedrin gene showed 98% sequence identity with the sequence of previous reported NPV of *H. talaca*, *H. infixaria* and *Buzura suppressaria*. Pathogenicity study against second instar *H. talaca* indicated that the LC50 values ranged from 4.61 × 10^5 to 7.57 × 10^5 polyhedral occlusion bodies per ml (POBs/ml) and the LT50 values ranged from 4.2 to 6.66 days. Sequencing result reveals that the same HytaNPV strain dominates across this area and the pathogenicity indicates its potential as an alternative to chemical insecticides to control *H. talaca*.

**Keywords** *Hyposidra talaca* • Nucleopolyhedrovirus • Polyhedrin gene • PCR • Bioactivity

**Introduction**

Baculoviruses are renowned for most widely studied virus of insects. More than 700 baculoviruses have been isolated mainly from the insect species of the orders Diptera, Hymenoptera and Lepidoptera. Nucleopolyhedroviruses (NPVs), which are a part of baculovirus, are considered as potential biocontrol agents and have been applied successfully against larvae of many insect pests in the world (Hu et al. 1993; Ma et al. 2007). NPVs are mainly pathogenic for insects of Lepidoptera. Polyhedrin gene of NPV is considered as one of the most conserved baculovirus genes (Jehle 2004), and for the development of the genetic amplification technique, this gene proved its efficiency (Woo 2001). Polymerase chain reaction (PCR) technique can amplify target DNA sequence and it has been used in baculovirus screening since the last three decades (Burand et al. 1992; Ikuno et al. 2004; Galal 2009; Hewson et al. 2011; Arneodo et al. 2012). The PCR technique is suitable for diagnosis of viruses as identification,
The characteristics of NPVs that empower it to be useful as biopesticide are its high pathogenicity, host specificity, less chances of cross-inf ectivity and having the ability of rapid distribution. The virus has stable occlusion bodies and can effectively suppress a target pest from a crop field (Moscardi 1999). In this regard, *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) is an extremely infectious natural agent causing most destructive disease to the tea pest *Hyposidra talaca*. In India, HytaNPV is reported in Terai and Dooars part of North Bengal (Mukhopadhyay et al. 2010; Antony et al. 2011; Sinu et al. 2015). The development of such control agent starts with the isolation of naturally occurring entomopathogens and followed by their identification, characterization as well as the evaluation of their virulence.

The *H. talaca* looper caterpillar (Walk.) (Lepidoptera: Geometridae) is an economically significant defoliating pest of the tea belt in the north-eastern part of India. Previously, *Buzura suppressaria* (Guen.) (Lepidoptera: Geometridae), a major tea defoliator in India and China, was dominant in this region, but gradually it was suppressed by the current destructive pest *H. talaca* (Hazari ka et al. 2009). In the last decade, *H. talaca* was introduced into tea agro-ecosystem and caused severe damages in the tea industry. It spread all over the northern part of West Bengal (WB) and Assam in 2006 (Sinu et al. 2011). As the management of *H. talaca* is carried out with their population monitoring and respective damage control application of chemical pesticides, which are mainly organophosphates and synthetic pyrethroids (Sannigrahi and Talukdar 2003; Sarker and Mukhopadhyay 2006), there is an urgent need to find an alternative control method. Among few natural enemies of the pest, HytaNPV is found to be active and causes epizootics within a dense population of *H. talaca* (Sinu et al. 2015). Successive generations of insect host are infected by polyhedra; thus the host serves as a reservoir of inoculums.

Due to the high potency of the virus as chemical alternative, finding out the naturally improved isolates of the virus is a matter of great interest. PCR-based diagnosis was used for identification, establishment of relationship and also to find out the variation within the tested HytaNPVs. The primary aim of the study was to identify the most potential isolate of HytaNPV. Examination of the samples from four different geographic locations was done for the above-mentioned identification and simultaneously infectivity evaluation was conducted to determine their successful practical use, by laboratory bioassay. This study may play an important role in the programs of integrated pest management (IPM).

**Materials and methods**

**Larval rearing**

To investigate the sensitivity of *H. talaca*, the second instar larvae of this looper caterpillar were originally collected from Dooars region, West Bengal, India. The collection site was free of pesticides since 6 months and the larvae were carried with leaves to reduce their transportation disturbances. They were reared in the laboratory of the Plant Protection Department, North Bengal Regional Research and Development Centre, Tea Research Association (TRA), for three successive generations at 28 ± 2 °C, 72 ± 3% relative humidity and a 13L:11D photoperiod. Fresh tea leaves were provided as food after sterilizing with 10% formalin for 5 min and rinsed with sterile double-distilled water. These larvae were used for viral propagation and bioassays. The larvae were also examined daily to eliminate the secondary infection.

**Viruses**

The HytaNPV samples, used in the PCR study, were collected during 2012–2013 from geographically distinct localities covering eastern Dooars, western Dooars, central Dooars and Terai region of West Bengal, India. The average rainfall of Terai and Dooars are approximately 3500 and 3160 mm, respectively, situated between 26° 16′ and 27° 12′ north latitudes and 87° 59′ and 89° 53′ east longitudes. The virus samples were collected from ten tea estates located across these four distinct geographic regions (Table 1). From each collection site, 50–60 virus-infected larvae were sampled. The virus-infected larvae were collected as samples considering the primary signs of typical NPV infection in larval stage like flaccidity, rupturing of the cuticle and hanging upside down by their abdominal legs. Later, the presence of HytaNPV was ascertained by microscopic view and PCR study. The individual larva was collected in 1.5 ml centrifuge tube and the collected samples were kept at −20 °C for future use.

**Purification of HytaNPV**

For molecular analysis, each of aqueous homogenate of viral samples containing polyhedral occlusion bodies (POBs) was purified by two rounds of centrifugation. The putrefied samples were homogenized with pestle and mortar, and the concentrates were diluted with sterile double-distilled water. The crude filtrate was initially centrifuged for 5 min at 600 rpm to remove larger contaminants. The supernatant was collected in a new centrifuge tube for further centrifugation at 8000 rpm for...
20 min to pellet the virus and then washed with sterile double-distilled water for three times. The pellets were finally dissolved in 1 ml sterile double-distilled water and conserved at −20 °C. The quantification of POBs was carried out by using Neubauer hemocytometer (MARIENFELD, Germany) under phase-contrast microscope (OLYMPUS BX 51). For bioassays, the viral samples were purified by following the same method.

**Virus propagation**

Four HytaNPV isolates (SMB, RJB, WSB and TLP), belonging to four different geographic regions, were taken for bioassay tests and multiplied in second instar *H. talaca* larvae for mass propagation. In case of each isolate, the multiplication of HytaNPV was conducted by isolation of POBs from infected cadavers. For multiplication of each isolate, 150 larvae were fed on tea leaves dipped with virus suspension in a concentration of 10⁷ POBs/ml. After 2 h of starvation, they were exposed to virus-contaminated leaves for 24 h. Virus-induced larvae were kept as 30 larvae per group and reared in the laboratory at 28 ± 2 °C, 72 ± 3% relative humidity and a 13L:11D photoperiod. Microscopic view confirmed the presence of POBs in the cadavers of virus-induced larvae.

**Viral DNA extraction and PCR amplification**

After following the purification method, viral DNA was extracted from POBs of each of ten field collected HytaNPV samples and purified with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. The final DNA was obtained after cleaning several times with ethanol and diluted wash solution. Later, DNA was eluted and re-suspended in 20 μl molecular-grade water (Himedia). The isolation of DNA was confirmed by electrophoresis in 1% agarose gel and quantified with BioPhotometer (Eppendorf).

A highly conserved region of polyhedrin gene from HytaNPV isolates was amplified. The PCR was performed using the degenerate primer (F: 5’-GGACCCGYYGYAAC-3’ and R: 5’-GCACTGGGYYCGAYTCT-3’) designed according to Antony et al. (2011). The PCR reaction was carried out taking 50–100 ng of viral DNA in a 25 μl reaction solution containing 1X PCR buffer (Invitrogen, USA), 1.5 mM MgCl₂ (Invitrogen, USA), 0.5 mM dNTPs (Bangalore Genei, India), 1 U Platinum Taq DNA polymerase (Invitrogen, USA) and 0.5 μM of each primer. PCR amplification was performed in a DNA thermal cycler (Veriti Thermal Cycler, Applied Biosystems, CA, USA). The conditions used were initially denaturation for one cycle at 94 °C for 5 min, followed by 35 repeated cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and the final extension cycle at 72 °C for 7 min. The amplified products were resolved in 1.5% agarose gel stained with ethidium bromide.

**Cloning and sequencing of the polyhedrin gene**

The positive PCR product of the SMB isolate was eluted from the gel using HiPura Agarose Gel DNA Purification Spin Kit (Himedia, India) following the manufacturer’s protocol. The eluent was ligated into the pGEM-T vector (PROMEGA, UK) in a 3:1 (insert: vector) molar ratio with T₄ DNA ligase as described by the manufacturer. The ligated products were transformed into *Escherichia coli* DH10β competent cells (INVITROGEN, USA). Following amplification of recombinant clone with M13 universal forward and reverse primers, the sequencing was performed using the BigDye Terminator v1.0 cycle sequencing kit (Applied Biosystems) in ABI 3500 Genetic Analyzer (Applied Biosystems).
Sequence comparison and phylogenetic analysis

The sequence data were assembled into contig by DNA Dragon Version 1.5.6 (SequentiX, Germany) followed by the multiple-sequence alignments construction with the highly similar DNA and protein sequences using the Clustal W program (Thompson et al. 1994). The obtained nucleotide sequence was blasted to the GenBank database (blastx) to retrieve similar nucleotide and amino acid sequences, which were used for phylogenetic tree construction. The phylogenetic tree of aligned amino acid sequences was generated by the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) using Molecular Evolutionary Genetics Analysis (MEGA) v6.06 (Tamura et al. 2013). To estimate the confidence limits of nodes, 1000 bootstrap samples were generated. To reduce the impact of partial sequences of polyhedrin available in the GenBank database and to maximize the use of the available information, the pairwise comparison of amino acid sequences was performed by ClustalW with the default parameters.

Bioassays

The biological activity of four HytaNPV isolates, viz., SMB, RJB, WSB and TLP (belonging to four different geographic regions), was tested against early second instar H. talaca larvae. Bioassay tests were conducted using purified viral suspension by leaf-dip feeding technique. The virus concentrations were quantified with a phase-contrast microscope and a Neubauer hemocytometer. The concentrations of each tested isolates were prepared from the following stock concentrations in POBs/ml: $3.6 \times 10^9$, $5.7 \times 10^7$, $2.9 \times 10^6$ and $8.1 \times 10^5$ for SMB, RJB, WSB and TLP isolates, respectively. The experiments were performed using six concentrations of each virus isolate containing $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ POBs/ml. Larvae were taken from the virus-free rearing culture and starved for 2 h before feeding viruses. Leaves were dipped in viral suspension, air-dried and fed to larvae for 24 h prior to feeding on fresh foliages until death or pupation. In the control treatment, the virus suspension was replaced by double-distilled water. For each isolate, three replicates each of 30 larvae were used for each virus concentration and three replicates (30 larvae per replication) of double-distilled water-treated leaves served as control. Larvae were incubated at $27 \pm 1 \degree C$, $72 \pm 3\%$ RH and a 13L:11D photoperiod. Viruses-induced cumulative mortality was recorded daily till death and the mortality response data were analyzed on the basis of mortality on day 7 post-inoculation. The diseased cadavers were collected and kept in $-20 \degree C$ for further analysis. Later virus infections were confirmed by the presence of POBs in the cadavers, when viewed under phase-contrast microscopy.

Statistical analysis

The mortality of larvae was tabulated daily for all NPV isolates vs. dose combinations (4 × 6). Probit analysis was performed using IBM SPSS release 23.0.0.0 on the basis of mortality data obtained after 7 days of post-inoculation. The median lethal concentrations (LC50) for second instar were obtained from the SPSS probit model. Median lethal time (LT50) was also determined for each concentration using the equation (Biever and Hostetter 1971):

$$\text{LT}_{50} = a + e(c - b)/D$$

where ‘$a$’ is the number of hours from the initiation of the test until the reading made immediately before the 50% mortality was recorded; ‘$b$’ is the total number of larvae dead at the reading immediately before the 50% mortality was recorded; ‘$c$’ is the 50% of the total number tested (in our case, it is 45); ‘$D$’ is the number of larvae dying in 24 h during which the 50% mortality was reached; and ‘$e$’ is the number of hours between mortality counts (24 h in this case).

Results

Field survey

The virus-infected larvae were collected from different geographic locations (Dooars and Terai) by primary observation of typical NPV signs and subjected to further studies (Fig. 1). The virus was diagnosed by microscopic study, propagated in H. talaca laboratory colony, purified and kept at $-20 \degree C$ for future studies. The number of polyhedral occlusion bodies was calculated by a hemocytometer showing the presence of approximately $8.7 \times 10^9$ POBs/ml of cadaver.

PCR amplification and sequencing

The degenerate primer set was used to amplify the targeted sequence from the isolated DNA of the purified polyhedra and successful amplification of the specific polyhedrin gene region of ten isolates of HytaNPV was performed. Amplification was not obtained from the extract of control larvae. The bands obtained from the PCR products of all ten isolates were identical (Fig. 2). Hence, the sequencing was performed by taking only the SMB isolate of the Terai region; then it was compared and cross validated with previously reported HytaNPV (from Dooars region). Hereafter, the studied Terai isolate is denoted as HytaNPV-P. The amplified product of the concerned isolate showed a
The nucleotide sequence of 527 bp (GenBank accession no. KP027542). The deduced protein consists of 175 amino acids with the predicted molecular mass of 19.52 kDa.

The obtained nucleotide sequence was blasted in the NCBI GenBank database, the NPV polyhedrin gene was identified and the blastn algorithm indicated the significant homology with 29 NPV isolates. The blast results for the polyhedrin gene of HytaNPV-P (acc. no. KP027542) at the nucleotide level revealed the highest homology of 98% with the polyhedrin gene of HytaNPV (acc. no. JF510035), H. infixaria NPV (HyinNPV, acc. no. JF510036) and B. suppressaria NPV (BusuNPV, acc. no. JF510034) previously reported by Antony et al. (2011) and the lowest sequence identity of 79% was observed in Plusia orichalcea NPV (acc. no. AF019882). On comparing, the multiple-sequence alignment of nucleotide was generated with HytaNPV-P, HytaNPV, HyinNPV and BusuNPV of Indian and Chinese isolate (Fig. 3). In comparison with previously reported HytaNPV, it exhibited ten nucleotides changes (at 6, 12, 15, 36, 102, 174, 192, 513, 519 and 522 positions) throughout the DNA segment.

The deduced amino acid sequence was compared to all other reported polyhedrins in the GenBank database, and HytaNPV-P polyhedrin (acc. no. AJN00735) showed 87–100% homology with other lepidopteran NPVs. The results showed 100% sequence homology with HytaNPV (acc. no. AEK86285), HyinNPV(acc. no. AEK86286) and BusuNPV (acc. no. AEK86284). In spite of the above similarities, it created 98% sequence identity with the Chinese isolate of BusuNPV (acc. no. YP009001778), 97% sequence identity with Ectropis obliqua NPV (EcobNPV, acc. no. YP874194; also reported from China), and Hemileuca sp. NPV (acc. no. YP008378217), and 95% sequence identity with Helicoverpa armigera NPV (acc. no. ABW06597) and Mamestra configurata NPV (acc. no. NP613084). The lowest sequence identity of 88% was observed with Bombyx mori polyhedrin (acc. no. NP047414). The multiple-sequence alignment for amino acid was also performed taking the same NPV isolates as described in nucleotide alignment (Fig. 4). There was no difference found with previously reported HytaNPV, HyinNPV and BusuNPV (Indian Isolate). A difference of
three amino acids (at 87, 146 and 198 positions) was noticed with the BusuNPV of the Chinese isolate.

**Phylogenetic analysis**

Phylogenetic analysis of the polyhedrin gene was performed to find out the relationship between HytaNPV-P and other NPVs. The studied data were recognized in the NCBI taxonomy database as a HytaNPV which was referred here as HytaNPV-P. Phylogenetic analysis of the HytaNPV-P polyhedrin showed a high degree of relationship with a large number of published amino acid sequences. A neighbor-joining tree was generated from the amino acid sequences of 26 NPV polyhedrin genes. Phylogenetic analysis indicates that the NPV infecting the *H. talaca* species in the Terai region of India is closely related to HytaNPV, HyinNPV and BusuNPV of the Indian isolate and comes under the same clade (Fig. 5). This suggested that the collected virus-infected larvae from the Terai region were very likely infected by the same strain. It also appeared to have close relation with Chinese isolates of BusuNPV, supported by high bootstrap value. High protein sequence homology of the polyhedrin region of HytaNPV-P with BusuNPV (98%) of the Chinese isolate and phylogenetic analysis indicates that they are very closely related. Phylogenetic study clearly indicates that in India, a variant of Chinese isolate of BusuNPV infects the *H. talaca*.

**Bioassays**

LC50 and LT50 values were represented in Table 2 for the tested isolates. The mortality ranged from 84 to 22% and it is clear that *H. talaca* larvae were susceptible to the applied concentrations. The result showed that the second instar larvae were the most susceptible.
was most susceptible to SMB isolate with an LC$_{50}$ of 8.15 $\times$ 10$^9$ POBs/ml. Larvae were also susceptible to infection with LC$_{50}$ values of 3.87 $\times$ 10$^8$, 1.89 $\times$ 10$^5$ and 2.79 $\times$ 10$^5$ POBs/ml, for RJB, WSB and TLP isolates, respectively, within the same time frame. The results of the study reflected the dose-dependent mortality, with the rising dose of POBs, the larval mortality showed an increasing trend. In inoculated larvae started to die at second day post-infection. In untreated control, no mortality was found. The estimated LT$_{50}$ values varied from 3.25 to 6.67 days. An instance, for highest concentration (1 $\times$ 10$^8$ POBs/ml) of the LT$_{50}$ value of the WSB isolate was lowest at 3.25 days. When treated with the highest dose, the LT$_{50}$ values were 3.5 days for the SMB isolate, isolate, acc. no. AEK86284) and BusuNPV (Chinese isolate, acc. no. YP009001778). Positions containing different amino acids are shaded.

### Discussion

Virus is now an important subject for the development of biopesticides and to maintain the ecosystem in the agricultural field. Search of a new natural isolate of virus, which contains better biopesticidal characteristics, to control insect pest is still an important aspect in biological control. In this regard, baculoviruses like NPVs are well known for their variability, frequent genetic variation and 3.86 days for the RJB isolate and 3.89 days for the TLP isolate. On the other hand, the result also indicates a gradual increase in tolerance with lower concentration.

**Fig. 4** Deduced amino acid sequence alignment of HytaNPV-P poyhedrin gene (acc. no. AJN00735) with HytaNPV (acc. no. AEK86285), HyinNPV (acc. no. AEK86286), BusuNPV (Indian isolate, acc. no. AEK86284) and BusuNPV (Chinese isolate, acc. no. YP009001778). Positions containing different amino acids are shaded.
differences in their biology. These qualities remain intact even when the NPV isolates are sampled from the same species of different geographical locations (Cory et al. 2005).

*H. talaca* plays a leading role in reducing tea production throughout the north-eastern tea hub of India (Basu Majumdar and Ghosh 2004). *H. talaca* was reported as a tea defoliator in Java (Yunus and Ho 1980) and it was also reported as a major pest of mango, cacao and other forest trees (Entwistle 1972; Muniappan and Viraktamath 1986; Singh and Singh 2004). Some entomopathogenic bacteria and NPV, infecting the pest, have been reported from north-eastern part of India (Mukhopadhyay et al. 2010; Antony et al. 2011). However, commercial use of these microorganisms in this region is still far away. Unlike China and Japan, there is no considerable progress in using naturally occurring baculoviruses in integrated pest management of tea in India. In China, significant response of BusuNPV and EcobNPV against *B. suppressaria* and *E. oblique* made these NPVs commercially available (Hu et al. 1993; Ma et al. 2007). In India, HytaNPV can be an effective control measure against *H. talaca*. The result of the PCR study showed the presence of single NPV strain across the entire north-eastern tea hub of India. In lepidopteran NPVs, the full length of polyhedrin gene ranges from 483 to 747 bp (Jehle et al. 2006), and the 527 bp partial segment of the studied polyhedrin gene also fits within it. In the present study, the partial amplification of HytaNPV-P infecting *H. talaca*, showed 98% nucleotide sequence identity and 100% similarity in amino acids with previously published HytaNPV (Antony et al. 2011). HytaNPV-P also showed the same sequence identity with HyinNPV and BusuNPV (Indian isolate). This implies that HytaNPV-P is closely related to the aforesaid NPVs.
Interestingly, HytaNPV-P also showed high sequence identity with the Chinese isolate of NPV infecting *B. suppressaria*. In this case, HytaNPV-P showed 98% similarity in amino acids with BusuNPV (acc. no. YP009001778, Chinese isolate) and only three amino acid differences were observed in the polyhedrin gene of HytaNPV-P and BusuNPV (Chinese isolate). This similarity indicates that HytaNPV-P is also related to the isolate of BusuNPV from China.

Phylogenetic studies have shown that HytaNPV-P, HytaNPV, HyinNPV and BusuNPV (Indian isolate) belong to same clade and the derived tree also encourages the fact that a variant of the Chinese isolate of BusuNPV (acc. no. YP009001778) infects the HytaNPV-P. However, full genome sequencing is necessary to confirm whether the HytaNPV-P altogether is a different virus or a different isolate of BusuNPV (China).

After ingestion of polyhedral occlusion bodies by larvae, NPV virions reach the midgut and spread to cause infection (Petrik et al. 2003). The results showed the effectiveness of the application of isolated NPV through tea leaves. In the present study, the SMB isolate of the Terai region showed higher LC$_{50}$ value for second instar than the previously reported value of $2.8 \times 10^3$ POBs/ml (from Terai region) by Mukhopadhyay et al. (2011). On the other hand, we found the RJB isolate (from Eastern Dooars), WSB isolate (from Western Dooars) and TLP isolate (from Central Dooars), which were respectively 5 times, 2.3 times and 3.4 times less active than the SMB isolate. The reasons for the variability may be the different geographic locations.

### Table 2

Dose–mortality response of second instar *Hyposidra talaca* larvae on four geographic isolates of *H. talaca* nucleopolyhedrovirus (HytaNPV) within 7 days of inoculation

<table>
<thead>
<tr>
<th>HytaNPV isolate</th>
<th>No. of tested 2nd instar larvae ($n$)</th>
<th>Concentration (POBs/ml)</th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>LC$_{50}$ POBs/ml (95% confidence limits)</th>
<th>Chi square ($\chi^2$)</th>
<th>LT$_{50}$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMB</td>
<td>90</td>
<td>$1 \times 10^8$</td>
<td>84</td>
<td>0.332 ± 0.035</td>
<td>$8.15 \times 10^4$ (3.39 $\times 10^4$–$1.78 \times 10^5$)</td>
<td>0.097</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>$1 \times 10^7$</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
<td>4.27</td>
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<td>90</td>
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<td>63</td>
<td></td>
<td></td>
<td></td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>$1 \times 10^5$</td>
<td>51</td>
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<td>6.67</td>
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<td>38</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>Control</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>RJB</td>
<td>90</td>
<td>$1 \times 10^8$</td>
<td>77</td>
<td>0.297 ± 0.034</td>
<td>$3.87 \times 10^5$ (1.62 $\times 10^5$–9.44 $\times 10^5$)</td>
<td>0.108</td>
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<td>WSB</td>
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<td>83</td>
<td>0.350 ± 0.035</td>
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<td>Control</td>
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<tr>
<td>TLP</td>
<td>90</td>
<td>$1 \times 10^8$</td>
<td>78</td>
<td>0.300 ± 0.034</td>
<td>$2.79 \times 10^5$ (1.17 $\times 10^5$–6.65 $\times 10^5$)</td>
<td>0.081</td>
<td>3.89</td>
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<td>Control</td>
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</table>

Dose–mortality distribution with corresponding LC$_{50}$ and LT$_{50}$ values; Slope represents regression derivation. Data fit probit model by $\chi^2$ test at $\alpha = 0.05$, degree of freedom ($df$) = 5
(Terai and Dooars) and differences in larval age (Payne et al. 1981) and feeding habit (Lacey et al. 2002). The number of virions per occlusion body and the host susceptibility differences to NPV (El-Salamouny et al. 2003) can be the other possible causes. However, for cross reference, there is no other report of bioassay available at this point for this particular tea pest. Stiles and Himmerich (1998) also observed higher LC50 of AeMNPV against second instar Heliothis zea (i.e., 6.38 × 106 POBs/ml). Second instar larvae of Spodoptera littoralis were also highly susceptible to the SpliMNPV at the higher concentration of 2 × 106 POBs/ml, which caused a high rate of mortality than the lower concentration (Sutanto et al. 2014).

The virulence of baculoviruses is measured by the killing speed of the concerned virus to the insect host (Van-Beek and Hughes 1998) and is necessary to know the correct time for pest management programs. The highest inoculums of 108 POBs/ml of HytaNPV showed rapid killing speed with LT50 value of 3.25 to 3.89 days after treatment for four isolates. Lower concentrations take longer time to meet the same level of larval mortality.

The result shows decreasing trend in mortality with the decreasing dosage. Although this relation between the rate of change in mortality with the lessening dosage is not directly proportional, as the dose decreases ten times the mortality rate is decreased well less than ten times. Previous studies also support this observation (Jankevica and Zarins 1999; Sajap et al. 2000; Sethuraman and Narayanan 2010; Mukhopadhyay et al. 2011; Sutanto et al. 2014). However, the regression result in Table 2 shows that a positive correlation exists between mortality and dosage, as all the slope values are positive and infinitesimal Chi squares signify that there is a good linear fit. Both the studies of LC50 and LT50 satisfied the insecticidal characteristics of HytaNPV. HytaNPV isolated from H. talaca is being developed as a biopesticide for the control of H. talaca.

**Conclusion**

In this paper, we described the cloning and sequencing of a highly conserved region of polyhedrin gene and the insecticidal activity of HytaNPV. The PCR study revealed the presence of the same viral strain throughout the northeastern tea belt. The high degree of phylogenetic agreement between the studied and previously reported HytaNPV polyhedrin gene sequence also supports this inference. This is the first report of sequencing of HytaNPV polyhedrin gene from the Terai region. The availability of HytaNPV may help in biopesticide production. The obtained results of bioassays support the use of HytaNPV as a potent biological control agent and a competent alternative to chemical insecticides in integrated pest management (IPM). The aforementioned characterization of the newly isolated HytaNPV would provide better understanding of the molecular properties of this virus and be helpful in the development of HytaNPV as a biopesticide.

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**Compliance with ethical standards**

**Disclosure** The sequence reported in this paper has been deposited in NCBI under accession no. KP027542. No potential conflict of interest was reported by the authors.

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Biological activity of Hyposidra talaca nucleopolyhedrovirus against all instar stages of a destructive tea pest Hyposidra talaca (Lepidoptera: Geometridae) in India

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Biological activity of *Hyposidra talaca* nucleopolyhedrovirus against all instar stages of a destructive tea pest *Hyposidra talaca* (Lepidoptera: Geometridae) in India

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**ABSTRACT**

*Hyposidra talaca* (Walker) (Lepidoptera: Geometridae) is the most harmful pest of northeastern tea hub of India that devastates the tea production by feasting on the tea leaves. *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) is a natural enemy of the aforesaid pest, as it poses great obstruction in the multiplication of the pest by causing significant larval mortality. The study was undertaken to screen the virus activity against first to fifth instar *H. talaca* larvae. Early instar stages are found more susceptible than the late stages as they tend to reflect highest LC\(_{50}\) value for fifth instar as \(4.3 \times 10^7\) POB/ml and lowest LC\(_{50}\) value for first instar as \(7 \times 10^4\) POB/ml within seven days of inoculation. LT\(_{50}\) values vary between 2.47 and 8.45 days for neonates to fifth instar. The high record of virulence of HytaNPV indicates its bright prospect as a useful microbial biopesticide.

**1. Introduction**

The Indian tea has been one of the favourite beverages of the world for ages. But the tea lovers might be deprived of their favourite brew if the growth of the harmful pests like *Hyposidra talaca* cannot be checked. *Hyposidra talaca* (Lepidoptera: Geometridae) is a defoliating tea pest, it creates a periodical and regular problem in northeastern tea plantations of India. During the last decade, this moth has established a population and continued to disperse throughout the tea belt. Due to their alarming presence in extensive areas and intensity of causing damage, the larvae are responsible for huge economical losses and the pest has also suppressed the previous main species *Buzura suppressaria* (Quarterly advisory bulletin 2008).
The presence of *Hyposidra* was reported from cinchona, tea, coffee, cocoa and fruit trees in tropics (Entwistle 1972). In India, it was reported as checking the growth of the weed, *Chromolena odorata*, as a major control agent (Muniappan & Viraktamath 1986) whereas from Western Himalayas, it was recognised as a pest of forest tree species, *Quercus Incana* Roxb (Singh & Singh 2004). Among 26 species of *Hyposidra*, *H. talaca* was first reported as a tea pest from Indonesia and later was reported as a destructive tea pest from Dooars region of West Bengal (Basu Majumdar & Ghosh 2004). *H. talaca* has spread all over the northern parts of West Bengal and Assam within few years (Sinu, Antony, & Mallick 2011). It has a short life cycle with multiple overlapping generations. Apart from that, factors like faster multiplication, lack of efficient natural enemies and active presence in winter months (Das et al. 2010) cause high density of larvae. Current control measures focus on routine applications of chemical insecticides mainly organophosphates and synthetic pyrethroids to suppress the pest as there is a scarcity of effective insecticides (Sannigrahi & Talukdar 2003; Sarker & Mukhopadhyay 2006).

Massive increase in the use of pesticides poses threats to the ecosystem and human health particularly in developing countries. Among the common consequences of overusing the pesticides are pesticide resistance problem, secondary pest emergence, pest resurgence with variation in susceptibility, death of natural enemies and problem of the presence of pesticide residue in food (Antony et al. 2011). For safe food production, there is an urgent need for eco-friendly alternative such as baculovirus in plant protection. In this regard, nucleopolyhedroviruses (NPVs) have already proved their potential against many pests in the world (Wood & Granados 1991; El-Salamouny et al. 2003). In China, the large area of tea plantation was controlled by *B. suppressaria* NPV (Shi 1985). Hazarika et al. (1995) reported the presence of NPV in fifth instar tea bunch caterpillar (*Andraca bipunctata*). Although having natural presence, NPV has not been applied as biopesticide of tea pest except certain cases in Japan and China (Nakai & Kunimi 1997, 1998; Ishii et al. 2002) where biological control of pest population with NPV is one of major control programme (Chandel et al. 2004). In India, Mukhopadhyay et al. (2010) first reported the occurrence of *Hyposidra talaca* nucleopolyhedrovirus (HytaNPV) in Terai and the foothills of Darjeeling. HytaNPV is found active and causes epizootics within dense population of *H. talaca* larvae (Antony et al. 2011; Sinu et al. 2011; Sinu et al. 2014). The favourable characteristics of being highly pathogenic, host specific and having stable occlusion bodies empower it to be effectively used as biopesticide and alternative to chemical pesticides. Polyhedral occlusion bodies (POBs) protect virions to retain their infectivity for several years in the environment and help them to persist in soil for long (Millar 1997).

In spite of having high host specificity and intense virulence to harm the susceptible insects, still there is a lack of interest in India in utilising HytaNPV to control the pest in a large scale. It is necessary to know the lethal concentration and the lethal time of HytaNPV against different instars to make effective use of the virus to hinder the pest outburst and to eradicate the new colonies by taking
early stage control measures. This study focuses on pathogenicity of HytaNPV. PCR study was also carried out to confirm the presence of HytaNPV in the field collected samples. Keeping the above fact in mind, bioassays were performed with biopreparation of HytaNPV to determine difference in its biological activity against all larval stages of *H. talaca*. HytaNPV can be considered as one of the safe biological insecticides and has a great potential to be used in pest control in extensive areas of northeastern tea belt in India.

2. Materials and methods

2.1. Insect rearing

*H. talaca* moths were collected for study by light trap from experimental plot of North Bengal Regional R & D Centre, Tea Research Association (TRA), West Bengal, India. The colony of the moth was established and maintained at the rearing laboratory of Plant Protection Department, TRA. The moths were transferred pairwise to plastic cages, with piece of cotton wetted with sugar solution (10%) for egg laying. The eggs, obtained from the moths, were kept in plastic containers until hatching. The egg masses were surface-sterilised with 10% formalin for 10 min. and after emergence of larvae they were reared in wooden cages (30 × 30 × 35 cc). They were reared for three successive generations at 28 ± 2 °C, 72 ± 3% relative humidity and a 13L: 11D photoperiod. Fresh tea leaves were provided as food, after sterilising those leaves with 10% formalin for 5 min and also rinsing with sterile double-distilled water. The larvae were maintained under proper hygienic condition and were examined daily to eliminate the secondary infection. Later on these larvae were used for viral propagation and bioassay tests.

2.2. Viruses

The virus-infected *H. talaca* larvae were collected from Central Dooars (26°43′N, 89°03′E) region of West Bengal, India. The infected larvae were distinguished by primary signs of typical NPV symptoms in larval stages like flaccidity, rupturing of the cuticle and hanging upside down by their abdominal legs. Those infected larvae were then collected to be used as samples for the experiment. Later the presence of HytaNPV in the affected larvae samples was affirmed by phase contrast microscope (Olympus BX 51) and PCR study. The collected samples were kept in a freezer at −20 °C and were further used for the experiments.

2.3. Purification of HytaNPV

The viral polyhedral occlusion bodies (POBs) were purified from aqueous homogenate of cadavers by two rounds of centrifugation. The putrefied samples
were homogenised with pestle and mortar, and the concentrates were diluted with distilled water. The homogenates were filtered through three layers of sterile cheesecloth to remove debris. The crude filtrate was initially subjected to centrifugation for 5 min at 800 rpm to remove larger contaminants. The supernatant was collected for further centrifugation at 8000 rpm for 20 min at room temperature in 1 ml eppendorf tubes to pellet the virus and was washed with distilled water for three times. The pellets were finally dissolved in 1 ml sterile double-distilled water and conserved at −20 °C. The quantification of POBs was done using Neubauer haemocytometer (Marienfeld, Germany) under phase contrast microscope. In the above way, HytaNPV samples were purified for PCR study, viral propagation and bioassay tests.

2.4. Virus propagation

The propagation of HytaNPV was carried out in the third instar stage of *H. talaca*. The POBs were isolated from collected cadavers and virus concentration was quantified with Neubauer haemocytometer under microscope. The larvae were fed on tea leaves dipped with virus suspension in a concentration of $10^7$ occlusion bodies per ml. After 2 h of starvation, the larvae consumed the inoculated leaves for 24 h. The Larvae were kept in 30 per group and were supplied with fresh, sterilised, tea leaves as additional diet from next day. They were maintained in the laboratory at ±28 °C, 72 ± 3% relative humidity and a 13L:11D photoperiod.

2.5. Viral DNA extraction and PCR amplification

The Viral DNA was extracted from the field collected HytaNPV samples and purified with QIAamp DNA Mini Kit (Qiagen) according to manufacturer’s protocol. The final DNA was obtained after several cleaning with ethanol and diluted wash solution. Later DNA was eluted and resuspended in 20 μl molecular grade water (Himedia). The isolation of DNA was confirmed by electrophoresis in 1% agarose gel and quantified with Biophotometer (Eppendorf).

A highly conserved region of polyhedrin gene from HytaNPV was amplified. The PCR was performed using the degenerate primer (F: 5′-GGACCSGGYAARAAYCAA AAA-3′ and R: 5′-GCRTCWGGYGCAAAYTCYTT-3′) designed according to Antony et al. (2011). The PCR reaction was carried out taking 50–100 ng of viral DNA in a 25 μl reaction solution containing 1X PCR buffer (Invitrogen, USA), 1.5 mM MgCl₂ (Invitrogen, USA), 0.5 mM dNTPs (Bangalore Genei, India), 1 U Platinum *Taq* DNA polymerase (Invitrogen, USA), 0.5 μM of each primer. The amplification was done using gradient PCR (Veriti Thermal Cycler, Applied Biosystems, CA, USA). The conditions used were initially with denaturation for 1 cycle at 94 °C for 5 min; followed by 35 repeated cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and the final extension cycle at 72 °C for 7 min. The amplified products were resolved in 1.5% agarose gel stained with ethidium bromide. The
positive PCR products were eluted from the gel using HiPura Agarose Gel DNA Purification Spin Kit (Himedia, India) following the manufacturer’s protocol. The eluents were ligated into pGEM-T vector (Promega, UK) in a 3:1 (insert:vector) molar ratio with T₄ DNA ligase as described by the manufacturer. Ligated products were transformed into *Escherichia coli* DH10β competent cells (Invitrogen, USA). Following amplification of recombinant clones with M13 universal forward and reverse primers, the sequencing was performed using the BigDye Terminator v1.0 cycle sequencing kit (Applied Biosystems) in ABI 3500 Genetic Analyzer (Applied Biosystems).

### 2.6. Bioassay

The biological activity of HytaNPV was tested against all larval instar stages of *H. talaca*. The laboratory reared looper caterpillar larvae from the third generation were selected for the evaluation. The larvae, that had uniform size and age, were selected from each instar stage throughout the experiment. Bioassays were performed using five concentrations of purified pathogenic suspension of HytaNPV and the larvae were inoculated with leaf-dip feeding method. HytaNPV concentrations, containing $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ and $1 \times 10^9$ POBs/ml were prepared from original stock solution ($1 \times 10^{11}$ POBs/ml) by diluting it with sterile double-distilled water. The larvae after starvation for 2–3 h were released on the treated leaves. Leaves were dipped in viral suspension, air-dried and fed to larvae for 24 h. There were three replications for each concentration with 30 larvae per replication. Three replicates (30 larvae per replication) of sterile, double-distilled water-treated leaves were also kept as control. The insect culture was maintained at 28 ± 2°C, 72 ± 3% relative humidity and a 13L:11D photoperiod. After feeding them on the virus contaminated leaves, fresh sterilised foliage of tea were provided daily till the death of larvae. Virus induced cumulative mortality was recorded daily and diseased cadavers were collected and kept in a freezer at −20°C for further analysis. By following the above procedure, bioassays were performed for each instar stage. The LC₅₀ and LT₅₀ values were computed to find out the differences in virulence of HytaNPV against different instar by Probit analysis (Finney 1971).

### 2.7. Statistical analysis

Mortality was tabulated daily for all instar vs. dose combinations ($5 \times 5$). Probit analysis was performed using IBM SPSS release 23.0.0.0 on the basis of mortality data obtained after 7 days of post-infection. The median lethal concentrations (LC₅₀) for each instar were obtained from the SPSS probit model. The same mortality distribution for each doses were used to derive the medial lethal concentrations (LT₅₀) using probit model.
3. Results

3.1. Field survey

The virus-infected larvae were collected from Dooars region by primary observation of typical NPV symptoms and subjected for further studies. The virus was diagnosed by microscopic study, propagated in *H. talaca* laboratory colony, and was purified and kept at −20 °C for future studies. The number of polyhedral occlusion bodies was calculated by haemocytometer showing the presence of approx. $5.9 \times 10^9$ POBs per ml of cadaver.

3.2. PCR amplification

The degenerate primer set was used to amplify the targeted sequences from the extracted DNA of the purified polyhedra and to perform successful amplification of the specific polyhedrin gene region of HytaNPV. Amplification was not obtained from the extract of controlled larvae. The bands obtained from PCR products were identical (Figure 1) and contained the nucleotide sequence of 527 base pair (bp) which confirmed the presence of HytaNPV in the field collected samples. A Blast search confirmed 100% similarity with the previously published HytaNPV sequence (Antony et al. 2011).

3.3. Mortality response of *H. talaca* larvae to HytaNPV concentrations

The pathogenicity of HytaNPV was evaluated against all instar stages of *H. talaca* larvae. Tables 1–5 present the LC$_{50}$ and LT$_{50}$ values for the tested HytaNPV. Infected
Table 1. Susceptibility of neonate *H. talaca* larvae to the different concentration (POBs/ml) of nucleopolyhedrovirus of *H. talaca* (HytaNPV) within seven days of inoculation.

<table>
<thead>
<tr>
<th>Concentration (POBs/ml)</th>
<th>No. of tested larvae (n)</th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>Chi-square ($\chi^2$)</th>
<th>LC$_{50}$ (POBs/ml) (95% confidence limits)</th>
<th>LT$_{50}$ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^9$</td>
<td>90</td>
<td>96.7</td>
<td>0.452 ± 0.058</td>
<td>0.102</td>
<td>$7 \times 10^4$ (1.62 $\times$ 10$^4$−1.86 $\times$ 10$^5$)</td>
<td>2.47 (2.42−2.52)</td>
</tr>
<tr>
<td>$1 \times 10^8$</td>
<td>90</td>
<td>92.2</td>
<td>3.12</td>
<td>(3.06−3.17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>90</td>
<td>84.4</td>
<td>3.72</td>
<td>(3.66−3.77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>90</td>
<td>70</td>
<td>4.27</td>
<td>(4.22−4.32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>90</td>
<td>52.2</td>
<td>5.1</td>
<td>(5.05−5.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Distribution of mortality, LC$_{50}$ and LT$_{50}$ values with respect to various doses; Slope represents regression derivation. Data fit probit model by $\chi^2$ test at $\alpha = 0.05$, Degree of Freedom (df) = 3. Obtained $\chi^2$ value, confidence limits for LC$_{50}$ and LT$_{50}$ are tabulated accordingly.

Table 2. Susceptibility of second instar *H. talaca* larvae to the different concentration (POBs/ml) of nucleopolyhedrovirus of *H. talaca* (HytaNPV) within seven days of inoculation.

<table>
<thead>
<tr>
<th>Concentration (POBs/ml)</th>
<th>No. of tested larvae (n)</th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>Chi-square ($\chi^2$)</th>
<th>LC$_{50}$ (POBs/ml) (95% confidence limits)</th>
<th>LT$_{50}$ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^9$</td>
<td>90</td>
<td>92.2</td>
<td>0.374 ± 0.05</td>
<td>0.068</td>
<td>$1.67 \times 10^3$ (3.77 $\times$ 10$^3$−4.54 $\times$ 10$^3$)</td>
<td>3.75 (3.7−3.8)</td>
</tr>
<tr>
<td>$1 \times 10^8$</td>
<td>90</td>
<td>84.4</td>
<td>4.34</td>
<td>(4.29−4.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>90</td>
<td>75.6</td>
<td>4.96</td>
<td>(4.91−5.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>90</td>
<td>61.1</td>
<td>5.92</td>
<td>(5.87−5.97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>90</td>
<td>46.7</td>
<td>6.79</td>
<td>(6.74−6.84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Distribution of mortality, LC$_{50}$ and LT$_{50}$ values with respect to various doses; Slope represents regression derivation. Data fit probit model by $\chi^2$ test at $\alpha = 0.05$, Degree of Freedom (df) = 3. Obtained $\chi^2$ value, confidence limits for LC$_{50}$ and LT$_{50}$ are tabulated accordingly.
Table 3. Susceptibility of third instar *H. talaca* larvae to the different concentration (POBs/ml) of nucleopolyhedrovirus of *H. talaca* (HytaNPV) within seven days of inoculation.

<table>
<thead>
<tr>
<th>Concentration (POBs/ml)</th>
<th>No. of tested larvae (n)</th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>Chi-square (χ²)</th>
<th>LC₅₀ (POBs/ml) (95% confidence limits)</th>
<th>LT₅₀ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁹</td>
<td>90</td>
<td>68.9</td>
<td>0.180 ± 0.043</td>
<td>0.021</td>
<td>1.97 × 10⁶ (1.87 × 10⁵−8.87 × 10⁶)</td>
<td>4.31 (4.25−4.36)</td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>90</td>
<td>62.2</td>
<td>4.61 (4.56−4.66)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁷</td>
<td>90</td>
<td>54.4</td>
<td>5.4 (5.35−5.45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>90</td>
<td>47.8</td>
<td>6.53 (6.48−6.58)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁵</td>
<td>90</td>
<td>41.1</td>
<td>6.26 (7.21−7.31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90</td>
<td>0</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Distribution of mortality, LC₅₀ and LT₅₀ values with respect to various doses; Slope represents regression derivation. Data fit probit model by χ² test at α = 0.05, Degree of freedom (df) = 3. Obtained χ² value, confidence limits for LC₅₀ and LT₅₀ are tabulated accordingly.

Table 4. Susceptibility of fourth instar *H. talaca* larvae to the different concentration (POBs/ml) of nucleopolyhedrovirus of *H. talaca* (HytaNPV) within seven days of inoculation.

<table>
<thead>
<tr>
<th>Concentration (POBs/ml)</th>
<th>No. of tested larvae (n)</th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>Chi-square (χ²)</th>
<th>LC₅₀ (POBs/ml) (95% confidence limits)</th>
<th>LT₅₀ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁹</td>
<td>90</td>
<td>60</td>
<td>0.109 ± 0.042</td>
<td>0.119</td>
<td>4.9 × 10⁶ (3.86 × 10⁵−9.86 × 10⁶)</td>
<td>4.94 (4.89−5)</td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>90</td>
<td>55.6</td>
<td>5.43 (5.38−5.48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁷</td>
<td>90</td>
<td>52.2</td>
<td>6.05 (6−6.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>90</td>
<td>45.6</td>
<td>6.86 (6.8−6.91)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁵</td>
<td>90</td>
<td>43.3</td>
<td>7.64 (7.59−7.69)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90</td>
<td>4.4</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Distribution of mortality, LC₅₀ and LT₅₀ values with respect to various doses; Slope represents regression derivation. Data fit probit model by χ² test at α = 0.05, Degree of freedom (df) = 3. Obtained χ² value, confidence limits for LC₅₀ and LT₅₀ are tabulated accordingly. In control, mortality is suspected due to cross infectivity.
Table 5. Susceptibility of fifth instar *H. talaca* larvae to the different concentration (POBs/ml) of nucleopolyhedrovirus of *H. talaca* (HytaNPV) within seven days of inoculation.

<table>
<thead>
<tr>
<th>Concentration (POBs/ml)</th>
<th>No. of tested larvae <em>(n)</em></th>
<th>HytaNPV caused mortality (%)</th>
<th>Slope ± SE</th>
<th>Chi-square <em>(χ²)</em></th>
<th>LC₅₀ (POBs/ml) (95% confidence limits)</th>
<th>LT₅₀ (days) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁹</td>
<td>90</td>
<td>58.9</td>
<td>0.153 ± 0.042</td>
<td>0.070</td>
<td>4.3 × 10⁷ (7.33 × 10⁶−8.25 × 10⁸)</td>
<td>5.41 (5.36−5.47)</td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>90</td>
<td>51.1</td>
<td>5.97 (5.91−6.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁷</td>
<td>90</td>
<td>46.7</td>
<td>6.68 (6.63−6.73)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>90</td>
<td>40</td>
<td>7.67 (7.62−7.73)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 × 10⁵</td>
<td>90</td>
<td>34.4</td>
<td>8.45 (8.4−8.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: Distribution of mortality, LC₅₀ and LT₅₀ values with respect to various doses; Slope represents regression derivation. Data fit probit model by χ² test at α = 0.05, Degree of Freedom (df) = 3. Obtained χ² value, confidence limits for LC₅₀ and LT₅₀ are tabulated accordingly.
larvae almost invariably climbed to the top of the twigs and attached themselves to the twig prior to death (Figure 2). The cadavers were sampled from bioassay containing typical NPV symptoms and microscopic view indicated the presence of HytaNPV (Figure 3).

**Figure 2.** Cadavers of different instar stages of *H. talaca* showing symptoms of nucleopolyhedrovirus infection.
Note: Clockwise from left: neonate larvae (A); second instar (B); third instar (C); fourth instar (D); and fifth instar (E).

**Figure 3.** Polyhedra of HytaNPV in 40X under EVOS® FL Cell Imaging System (Invitrogen) containing embedded virions which are released in the alkaline pH of the insect midgut.
Note: Bar marker represents 100 μm.
The HytaNPV activity differed significantly from first to fifth instar. The LC$_{50}$ values for HytaNPV ranged from $7 \times 10^4$ POBs/ml to $4.3 \times 10^7$ POBs/ml across all instar stages. The virus showed an acute pathological effect on first larval instar. The mortality rate for neonates reached 96.7% for the tested dose of $1 \times 10^9$ POBs/ml within seven days of inoculation and inoculated larvae started to die at second day post-infection. While the mortality reached to 92.2, 68.9, 60 and 58.9% for second, third, fourth and fifth larval instar when infected with the same dose ($1 \times 10^9$ POBs/ml). LC$_{50}$ values determined were $7 \times 10^4$ and $1.67 \times 10^5$ POBs/ml for the larvae of neonates and second instar, respectively (Tables 1 and 2). However, third instar larvae appeared to be less susceptible to the virus than the first and second instar larvae with the LC$_{50}$ value of $1.97 \times 10^6$ POBs/ml, for the same viral concentrations (Table 3). The highest peak of mortality for first instar was observed on 3–4 days post-infection, on 4–5 days for second instar and on 5–6 days for third instar. LC$_{50}$ against fourth and fifth instar were raised to $4.9 \times 10^6$ and $4.3 \times 10^7$ POBs of HytaNPV per millilitre, respectively (Tables 4 and 5). The increasing LC$_{50}$ values for fourth and fifth instar inferred that they were significantly less susceptible than that of early stages. The results of the bioassays showed dose-dependent mortality (Figure 4). First instar was the most susceptible with an LC$_{50}$ of $7 \times 10^4$ POBs/ml, second and third instar stages were moderately susceptible to infection, while fourth and fifth instar were only semi-permissive to infection. In every instar experiment, almost all the controlled uninfected larvae survived and moulted to the next instar.

The trend analysis on progression of mortality with respect to the progression of time was carried out for each dose and for each instar followed by the estimation of respective LT values (Tables 1–5). The estimated LT$_{50}$ values varied from 2.47 to 8.45 days. Under the same dose, the LT$_{50}$ values gradually increased from

![Figure 4](image.png) Relative measurement on Log$_{10}$ of median lethal concentration for various instar stages of *H. talaca*. 

S. DASGUPTA ET AL.
neonates to fifth instar. As an instance, for higher concentration (1 × 10^9 POBs/ml) the LT50 of first instar calculated was 2.47 days, while in fifth instar it moved up to 5.41 days. LT50 values were 3.75, 4.31 and 4.94 days for the second, third and fourth instar, respectively, when treated with highest dose. On the other hand, the result also indicates a gradual increase in tolerance with lower concentration. Evidently, the LT50 values for lowest concentration (1 × 10^5 POBs/ml) for neonates to fifth instar were obtained as 5.1, 6.79, 7.26, 7.64 and 8.45 days.

4. Discussion

In the present day, the extended study of baculoviruses as biological agent opens a promising research line to overcome the late response of the virus towards pest. In parallel, another study is going on to find out potent baculovirus, containing better biopesticidal characteristics. In northeastern part of India, HytaNPV can be used as an effective control measure against the main destructive pest of tea plantation. The presence of some entomopathogenic bacteria and NPV virus has been reported from this region (Mukhopadhyay et al. 2010; Antony et al. 2011; Sinu et al. 2011). The commercial use of these micro-organisms in this region of India is far from being implemented, although the use of six baculoviruses as biopesticides against tea caterpillars in China was reported by Hu (1998). Baculoviruses like NPVs are the most promising, having the ability of rapid distribution, species-specific action; less chances of cross-infectivity and they also can effectively suppress a target pest from a crop field (Moscardi 1999).

*H. talaca* hinders the tea production in north-east India by causing severe defoliation of the tea bushes. After ingestion of polyhedral occlusion bodies by the larvae, NPV virions reach to the midgut and spread to cause infection (Petrik et al. 2003). From PCR study, 527 bp nucleotide sequences was obtained and indicated the presence of HytaNPV in field collected larval samples (Antony et al. 2011). The results of the bioassay experiment show the effectiveness of the application of isolated NPV through tea leaves against all the *H. talaca* instar (Sinu et al. 2014). This is the first report of HytaNPV bioassay on all *H. talaca* instar except the second.

The neonates were most susceptible to HytaNPV as the LC50 value was rationally the lowest. The bioassay of second instar caterpillar (from Dooars region) showed higher LC50 value with respect to previously reported value of 2.8 × 10^3 POBs/ml (from Terai region) by Mukhopadhyay et al. (2011). The reasons of variability may be different geographic locations (Terai and Dooars), differences in larval age (Payne et al. 1981) and feeding habit (Lacey et al. 2002). The number of virions per occlusion bodies and the host susceptibility differences to NPV (El-Salamouny et al. 2003) can be the other possible causes. Stiles and Himmerich (1998) also observed higher LC50 of AcMNPV against second instar *Heliothis zea* (i.e. 6.38 × 10^5 POB/ml). However, for cross reference, there is no other report of bioassay available at this point for this particular tea pest. The LC50 values for fourth and fifth instar, in the experiment, were significantly higher than neonates
due to difference in larval age. Related study reflected that the susceptibility of *Helicoverpha armigera* differed on the basis of the larval age when HearNPV was applied (Cherry et al. 2000). With the increase in dose of POBs, the percentage of larval mortality showed an increasing trend on the death of larvae. First and second instar larvae of *Spodoptera littoralis* were also highly susceptible to the SpliMNPV at the higher concentration of $2 \times 10^6$ POB/ml that higher concentration caused high rate of mortality than the lower concentration (Sutanto et al. 2014). Early instar *H. talaca* larvae were highly sensitive and susceptible to the HytaNPV than the late instar larvae to the same tested viral concentration. These results prove that it is better to control the pest in early stages.

The virulence of baculoviruses is measured by the killing speed of the concerned virus to the insect host (Van-Beek & Hughes 1998) and it is necessary to know the correct timing for pest management programmes. LT values presented in the tables indicated that for young larvae, highest inoculums of $10^9$ POB/ml of HytaNPV showed rapid killing speed with LT$_{50}$ value of 2.47 days of treatment where for second instar, it was recorded one day more than neonates and gradually fifth instar having maximum LT$_{50}$ value of 5.41 days. Similar kind of result was obtained in case of early instar larvae of codling moth (*Cydia pomonella*) when treated with AfMNPV and AcMNPV (Lacey et al. 2002). Also early instar of western spruce budworm required less time to be killed by CfMNPV than late instar (Duan & Otvos 2001). In case of *Chilo partellus*, to reach 100% mortality, there was gradual increase in time from second to fourth instar when CpNPV was applied (Sethuraman & Narayanan 2010) and higher dose killed the larvae at a faster rate. Lower concentrations ($10^5$–$10^8$) required greater time to meet the same level of larval mortality.

This study clearly indicates that *H. talaca* should be controlled in the early stage to prevent the destruction of the crop, to save the time for the pest management and to use small amount of biopesticide so that economical aspect can also be met. In the experiment, both the studies of LC$_{50}$ and LT$_{50}$ satisfied the insecticidal characteristics of HytaNPV. These characteristics stimulate the formulation of a biopesticide on HytaNPV by keeping larval stage in mind. To develop such kind of biological product is the point of interest in many pesticides companies.

5. Conclusion

In this experiment, we showed the insecticidal activity of HytaNPV and described the pathogenicity for all the larval stages of *H. talaca* to formulate a biopesticide. This is the first report of bioassay of HytaNPV. The results seem coherent and infer the fact that HytaNPV can be developed as an important tool to control *H. talaca* outbreak. This control system is economical and eco-favourable. As this study is lab-based, field application is required for acquiring the applied knowledge and to accept this assumption. These data can be used for evaluation and improvement in HytaNPV as biological control agent.
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Disclosure statement

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