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Microscopic and Molecular Identification of *Bombyx mori* Nuclear Polyhedrosis virus (*Bm*NPV) using *lef-8* and *polhe* Primers

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ABSTRACT: Silkworms infected with grasserie were collected at the 4th and 5th instars of their rearing. Sample was collected by a needle puncture limbs of thorax, white fluid containing virus was collected. Collected sample was subjected to centrifuge at 2500 rpm for 2 minutes for isolation of virus from debris and process was repeated till clear white color pallet was obtained. The pallet was examined under compound microscope and FESEM for the presence of *Bombyx mori* Nuclear Polyhedrosis Virus (*Bm*NPV). After positive microscopic results DNA was extracted and examined for the presence of two specific genes *viz.*, late expression factor or *lef-8* (conserved in NPV) and polyhedrin or *polhe* (conserved in *Bm*NPV). PCR products were resolved by electrophoresis on 0.8 % and clear bands was seen at 650 – 700bp for *lef-8* gene and at 160 – 170bp for *polhe* gene. Observed bands clearly confirm the presence of *Bm*NPV virus responsible for grasserie disease.

Keywords: Silkworm, *Bm* NPV, Polymerase chain reaction, *lef-8*, *polhe*, Virus etc.

INTRODUCTION

The silk industry faces severe setbacks in the past due to frequent disease outbreaks because the silkworm, *Bombyx mori* L. is highly susceptible to various microbial infectious diseases like grasserie, pebrine, flacherie and muscardine. These diseases contribute considerably to the cocoon crop which directly affects the farming community due to reduced returns (Govindan *et al.,* 1995). Despite extensive studies conducted and efforts made, the disease has been frequently causing concern to sericulture industry. Approximately 40 percent crop losses are attributed to different infectious diseases (Sheebarja *et al.,* 2007). Nuclear polyhedrosis is most deadly disease (Sengupta *et al.,* 1990; Dandrin *et al.,* 2000) and the loss caused by *Bm*NPV disease accounts for more than 60% of the total losses caused by all diseases (Chen *et al.,* 2014). All the silkworms irrespective of type and age are susceptible to the disease, however late age worms are more susceptible as compared to chawkie worms and is commonly called late age silkworm disease. During early stage of disease, infected silkworms do not exhibit any symptoms and/or any appetite change, however, the microscopic examination of haemolymph reveals the presence of polyhedral bodies. The viral multiplication is detected as early as 6 hours after polyhedral infections (Summers *et al.,* 1987) and expression of symptoms takes 72-96 hrs for larvae upto 3rd instar, 96-120hrs in 4th instar and beyond 120hrs in 5th instar. *Bm*NPV contains double standard circular DNA and a number of nuclear polyhedrosis viruses have been isolated from different lepidopteron insects (Federici *et al.,* 1997; Blissard *et al.,* 2000). There are 24 proteins in silkworm midgut which interact with BmNPV virus, these proteins help in viral transportation, propagation, metabolism and apoptosis (Feng *et al.,* 2020). Grasserie disease is very destructive leading to economic loss and improvement in the survival rate can help to minimize the loss. Advances in molecular techniques can help to create the breeding strategies for resistant strains of silkworm (Chen *et al.,* 2022). Any manipulation at the gene level or gene interference can help to enhance the resistance of the silkworm against the *Bm*NPV. Proteomics studies by Li *et al.* (2023) identified various genes resistant to *Bm*NPV, helps to understand the genetic basis of resistance and breed selecting programs. *Bm*STAT gene interference in silkworms can increase viral replication and reduces the survival rate (Li *et al.,* 2023). *Bm*SPP gene manipulation can affect virus proliferation and host survival, suggesting its potential as viral resistance in silkworms (Feng *et al.,* 2024). Diet improvement can also help silkworms to fight against the viral disease. Probiotic supplements can enhance the survival ratio from 56% to 92% of infected silkworms, indicates potential of probiotics in enhancing immunity of silkworms against *Bm*NPV (Zhou *et al.,* 2023). Various strategies can be adopted, which will be more relevant and economically feasible to control the viral infection. Before applying and

looking for good preventive measures or enhancing immunity diets, it is important to have fast, feasible and accurate method to identify the virus. In the present study we isolate purify and identify the virus (*Bm*NPV) causing grasserie disease in silkworms.

MATERIALS AND METHODS

A. Collection of infected silkworms

Infected silkworms were collected from the rearing site having the symptoms of grasserie disease like yellow color, sluggish movement, hanging upside down, loss of appetite etc. using the standard insect collection technique (Donald *et al.*, 1981). Insects were brought in the wide mouthed specimen bottles.

B. Collection of viral sample from infected insects.

A small prick with the help of sharp needle was made on the prolegs of silkworms and the white fluid containing polyhedral bodies were collected in the eppendorf tubes (Fig. 1A).

C. Isolation, purification of virus

Virus was isolated and purified when collected sample was subjected to centrifuge at 2500 rpm for 2 minutes for isolation of virus from debris. The centrifuge process was repeated for several times by adding distil water, keeping the lower pallet and discarding the supernatant each time till clear white color pallet was not obtained. The viral concentration was determined using Neubauerhaemocytometer as described by Cantwell *et al*. (1974). The highly purified viral particles were stored in refrigerator for further use (Fig. 1B).

D. Microscopic Examination of virus under compound microscope

The white fluid containing polyhedral bodies were examined under the microscope [Magnus (Model-CH20i-Bi)] at 600X magnifications for the presence/ absence of virus in the sample. The virus in the observed sample was photographed at 600X magnification (Fig. 2A).

E. Field Emission Scanning Electron Microscopy (FESEM) of virus.

Purified virus was subjected to Field Emission Scanning Electron Microscopy (FESEM). The facility was taken from National Institute of Technology (NIT) Srinagar by paying the required fee for the procedure using Gemini SEM 500 from Carl Zeiss (high-resolution field emission scanning electron microscope (FE-SEM)). The magnification power of the microscope is 50 times to 20,00,000 times and virus was observed at 10,000X magnification (Fig. 2B).

F. Extraction and Amplification of viral DNA

DNA was extracted and purified by following the standard method (Jose *et al.*, 2012). Polymerase chain reaction (PCR) was performed for the presence of the *lef-8* gene (conserved sequence for NPV) and polyhedrin or *polhe gene* (conserved sequence for *Bm*NPV). Briefly, one set of specific primer pair was employed to produce an amplicon of 650 – 700bp for *lef-8* gene, and another set of specific primer pair was employed to produce an amplicon of 160 – 170bp of *polhe*gene (Table 1).

The amplification was carried out in a 20.4µl reaction volume containing Milli-Q water, 1XGo Taq buffer (Promega), 1.5mM $MgCl_2$, 0.2mM dNTPs mixture, 0.2µM each of forward and reverse primer, 5 units of Taq DNA polymerase (Promega) and 30-60ng of template DNA. The samples were amplified in preheated thermal cycler (T100-thermal cycler, Bio-rad), with initial denaturation at 95°C for 3 min, followed by 35 cycles each consisting of 95°C for 40 sec, 54°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were resolved by electrophoresis on 0.8 % ethidium bromide-stained agarosegels at 100V for 20 minutes and visualized by Gel-Doc (Bio-Rad). DNA markers of 1kb and100bp were used as size standard.

Table 1: Selected Primers used in Polymerase Chain Reaction (PCR).

Target Strains	Primer Name	Sequence	Number of Nucleotide	Type of Disease
Lef-8	prL8-1	5´CAGGAAACAGCTATGACCCAYGGHGARATGAC3´	32	NPV
	prL8-2	5´CAGGAAACAGCTATGACCAYRTASGGRTCYTCSGC3´	35	
Polhe	Polhe *for*	5´CGTGTACGACAACAAGTACTACA3´	23	*Bm* NPV
	Polhe *rev*	5´AAAGTGAGTTTTTGGTTTTTGCC3´	23	

Source: *lef-8* [Jose *et al.*, 2012]; *Polhe* [Zubiaur *et al*., 2016].

RESULTS AND DISCUSSION

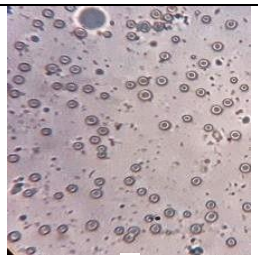
Identification of virus was done using microscopic technique and the molecular technique. The virus was purified and was imaged at 600x magnification and at 10,000x magnification through FESEM (Fig. 2A and B). The amplification of the viral DNA was carried and run in 0.8% agarose gel and observed under UV bio rad against *lef-8* and *polhe*. Dense bands were observed in the *Bm*NPV sample at 700bp and 170bp against *lef-8* and *polhe* respectively (Fig. 3A and B) and no bands were observed in control. Early description of microorganisms including viruses were solely based on the morphological features particularly shape and size which resulted in duplication of various viruses however molecular studies have drastically changed traditional taxonomy. In India, about 35 insect viruses have been recorded from the Baculovirus group, the most important being the NPV's of *Helicoverpa armigera, Spodoptera litura, Spilosoma oblique* (Walker), *Achaea janata* (L.) and *Amsacta albistriga*

(Walker) and the Granular viruses of *Achaea janata, Spodoptera litura, Helicoverpa armingera,* and *Chilo* spp.(Pawar *et al.*, 1992). The results of the present study shows the methods of isolation, purification and identification of the BmNPV. DNA sequencing is highly sensitive technique for amplification of target and is now widely used for detection and identification of insect viruses (Kool *et al.*, 1991; Williams *et al.*, 1993; De Moraes *et al.*, 1997). Jose *et al.* (2012); Zubiaur *et al.* (1996) reported *lef-8* and polyhedrin or *polhe* genes are conserved sequences for NPV and *Bm*NPV respectively. In the present investigation, viral DNA was extracted from the infected insects and both *lef-8* and *polhe* genes were utilized for the screening of the viral DNA (Fig. 4). Using the *lef-8* and *polhe* primer sets, a substantial amplification band was found for the *Bm*NPV DNA sample at 700bp and 170bp respectively. Our results coincide with that of Jose *et al.*, (2012); Zubiaur *et al.* (1996). This investigation gives complete set of procedure for the purification and identification of *Bm*NPV virus infecting silkworm and causing grasserie disease.

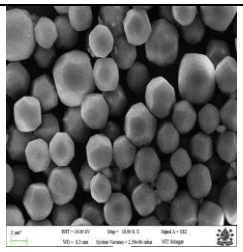
A

B

Fig. 1A. Collection of Silkworm Viral Sample **B-** Purified Viral Sample.

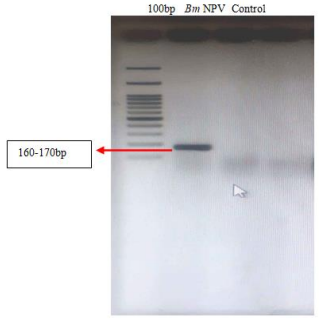


A

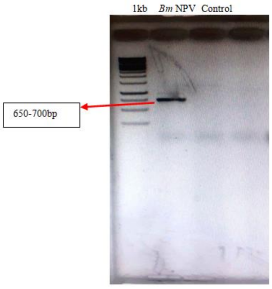


B

Fig. 2A. Microphotographs at 600 X magnification **B-**Microphotographs at 10.00KV magnification.



A



B

Fig. 3A. Band showing *polhe* gene at 160-170bp **B-** Band showing *lef-8* gene at 650-700bp.

CONCLUSIONS

Based on the result from present study, we can conclude that if we have to go for the fast and early detection grasserie disease. We need fast and effective fast method without any confusion to cope the virus and start precautions. Early detection can help to take necessary measures and help to save the silkworm cocoon crop from futher damage.

FUTURE SCOPE

Early detection of grasserie disease can help to manage the disease early, but we still need to do lot of research to learn the mechanism of host pathogen interaction. DNA extraction and sequencing of the virus may help to come up with some effective insecticides and help the farmers to save cocoon crop.

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Conflict of Interest. None.

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