Advances in Bioresearch
Adv. Biores., Vol 13 (1) January 2022: 09-13

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.13.1.913

Advances in Bioresearch

ORIGINAL ARTICLE

Characterization of *Bombyx mori* Nuclear polyhedrosis virus (*Bm*NPV) using microscopic techniques

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ABSTRACT
The mulberry silkworm Bombyx mori L. *is an economically important insect which is infected with a baculovirus, Bombyx mori Nuclear polyhedrosisvirus (Bm*NPV*) that causes grasserie disease to mulberry silkworms which produces serious economic loss to sericulture industry for more than 50 per cent. The skin of infected silkworm becomes fragile with swollen intersegmental region and the haemolymph appears turbid milky white. Hence it becomes essential to have disease diagnostic methods which permit the rapid identification of the initial inoculums. An attempt was made to isolate and identify the polyhedral bodies using microscopic techniques viz., Light microscope and Field emission scanning electron microscope (FE-SEM). The present results revealed the presence of polyhedral occlusion bodies (POBs) which indicate the presence of disease-causing virions in the silkworm haemolymph and diseased cadaver's tissues. The NPV detection technique is therefore needed to stop the outbreak and spread of the disease in silkworm rearing units for early prevention of the disease.*
Key words: *Bombyx mori, Bm*NPV, Polyhedra, Occlusion bodies (OBs)

Received 08.06.2021 Revised 16.09.2021 Accepted 15.11.2021

How to cite this article:
S. Ranjith kumar, K. Chozhan, K. A. Murugesh, R. Rajeswari and K. Kumaran. Characterization of *Bombyx mori* Nuclear polyhedrosis virus (*Bm*NPV) using microscopic techniques. Adv. Biores. Vol 13 [1] January 2022. 09-13

INTRODUCTION

The mulberry silkworm, *Bombyx mori.* L (Lepidoptera: Bombycidae) is an economically important insect domesticated for silk production. Sericulture is mainly practiced in five states namely, Karnataka, Andhra Pradesh, West Bengal, Tamil Nadu and Jammu & Kashmir jointly accounting for about more than 90 per cent of the total mulberry silk production in the country. Silkworms are susceptible to a number of diseases caused by different infectious agents. The *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) causing grasserie disease in silkworm is the most harmful viruses in the sericulture industry, often causing severe economic losses[13], [15].

*Bm*NPVis included in baculovirus group belonging to the Family Baculoviridae, Genus Alphabaculovirus. The name baculovirus is derived from the greek word, "baculum" meaning rod. The polyhedra are highly refractive and transparent and their size varies from 0.5 to 1.0 micron in diameter. Among viruses, nuclear polyhedrosis viruses (NPV) have caused the highest damage to silkworm, *B. mori* in tropical regions [1]. NPV infects all larval instars but more commonly in the 4th and 5th instars, during all seasons and cause 20-50 % cocoon crop losses in India [10].

As the disease matures, silkworm losses its appetite and the skin tension are lost usually five to seven days after infection of the disease, the inter-segmental membranes of the body become swollen and the skin become transparent. The diseased larva in the final stages of infection shows swelling in the intersegment membranes giving the larva the appearance of the bamboo cane with distinct nodes [8].*Bm*NPV affect the midgut epithelial cells, tracheal system, heamolymph cells, fat bodies, *etc*of the silkworms.

In this paper, we report the methodology to identify the presence and structure of *Bm*NPV viral polyhedra, through Light microscopic examination and Field emission scanning electron microscopy technique.

MATERIAL AND METHODS

Sample collection

Disease infected larvae were collected from farmers' rearing houses in Mettupalayam, Coimbatore district (Fig.1).The POBs **were extracted from individual diseased larvae.** The extracted polyhedra with dilution of 10^6 POBs/ml is dipped with disease free mulberry leaves and shade dried for some time. The POBs dipped leaves were given as first feed once during III instar double hybrid larvae. The symptoms of the NPV infected silkworm in the fourth and fifth instar larva, which are visible about a week after infection. The worms show symptoms viz*.,* translucent body, restlessness of the worms – crawling around the trays and hanging upside down, secretion of milky white ooze fluid which contains polyhedraand the intersegmental membrane swelling which is peculiar characteristics of grasserie infected silkworm larvae.

Isolation of NPV from haemolymph of infected silkworms

The grasserie infected fifth instar larvae were collected and the abdominal legs of larvae were cut open, and the haemolymph containing the polyhedral occlusion bodies (POBs) were collected in the presence of phenylthiourea to prevent melanization of haemolymph [14]. The POBs were then semi-purified by centrifugation. The suspension was centrifuged at 500 rpm for about a minute to sediment the floating lipids or crude tissue debris, if any. Then the supernatant was centrifuged at 3000 rpm for three minutes. The pellet obtained was washed three times in distilled water and finally suspended in distilled water(0.5 ml per insect equivalent).

The purity of the polyhedra was examined under microscope and quantified by counting in a Neubauer haemocytometer. For long term storage, required concentration of POBs was prepared by adding distilled water, polyhedra were freeze-dried and stored at 4^{o} C.

Isolation of NPV from infected silkworm cadavers

The viral suspension was prepared from 5^{th} instar silkworms died due to NPV. Infected silkworms were homogenized in 50 ml of distilled water using pestle and mortar. The homogenate was filtered through muslin cloth. The filtrate was then centrifuged at 500 rpm for 30 seconds and the supernatant was collected and again centriguged at 3000 rpm for three minutes. The supernatant was discarded and the pellets obtained were suspended in distilled water[16]. The resulting pellet containing the polyhedra, identified by the whitish coloration was re-suspended in distilled water and placed in sterile flasks and stored in refrigerator for further use.

Light microscope examination

A wet smear of the homogenized liquid using a drop of haemolymph or a small part of larval tissue was spread on a clean cavity slide. The slide was then dipped in 10% Giemsa's stain for 10 minutes. The excess stain was than washed with running tap water for 5-10 seconds. The prepared smear was examined using the oil immersion (1000x) magnification of light microscope LABOMED Lx500 and the magnified images were observed and captured in computer using image analysis software

Field Emission Scanning Electron Microscope (FE-SEM)

Field Emission Scanning Electron Microscope (MIRA-3 TESCAN) utilized at the Bharat Ratna Prof. CNR Rao research Centre at Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore was used to visualize the shape of the NPV.

NPV inoculum was fixed in 5% glutaraldehyde (aqueous solution) + 0.2% tannic acid for 2h, dehydrated in a standard aqueous acetone series (50%, 70%, 80%, 90% and twice in 100%) about five seconds each bath. Samples of that inoculum were then seeded onto stubs, previously covered with copper tape, and were allowed to air dry, and particles were viewed on FE-SEM [6].

RESULTS AND DISCUSSION

The grasserie disease caused by *Bm*NPV is one of the serious threats to sericulture industry that affects *B. mori* silkworms in different stages of its life cycle leading to significant economic losses at the farmers' level. Among various diseases of mulberry silkworm, NPV of *B. mori* is known to occur in all larval stages of development and largely manifested in the fifth instar of larvae in all the rearing seasons and causing 30–50% loss in cocoon production[11].

The symptoms of NPV from collected disease infected silkworms were observed showing clearly the swelling of intersegmental membranes (Fig.1), shining and translucent body which is the preliminary confirmation of the presence of *Bm*NPV occlusion bodies in the silkworms. The infected larva showed loss

of appetite and in addition the integument becomes very fragile and intersegmental region swells. Infected worms secrete milky white ooze fluid containing polyhedra which is peculiar symptom of grasserie infection and the worms die at later stages. Due to restlessness of the worms, the worms' crawls around the trays and behave differently, becoming easily agitated when disturbed, and are frequently found lying at the edge of the rearing beds and move upwards and hangs upside down. If infection occurs in the early instars, the worms fail to spin and die, whereas if the infection occurs at later stages, the worms could spin the cocoons but subsequently die inside producing melted cocoons, and the affected cocoons become unfit for reeling [12].

Light microscope examination of smears from individual maturely diseased larvae revealed the presence of virus particles and the images of polyhedra are observed and recorded using image analyzer (Fig.2). The smear test allows recognition of the occlusion bodies of nuclear polyhedrosis viruses (NPV) and the images clearly described the presence of Polyhedral bodies.The fluid inside the dead larvae is composed largely of virus particles and are produced inside of one larva. The NPV viewed with the Field Emission Scanning Electron microscope (FE-SEM) revealed typical baculovirus Occlusion Bodies (OBs) of type Nuclear polyhedrosis virus (NPV) with polyhedral/ hexagonal structures(Fig.3).The polyhedral had an average size ranging from 0.8 micro meter to 3.1 micro meter in diameter. In this case, the NPV polyhedra were clear, with the faces smooth and perfectly defined.The microscopic observation of a drop of haemolymph of the diseased larvae indicates the presenceof large number of hexagonal/pentagonal or tetragonal structures – the OBs/polyhedra.The polyhedral structure of different NPV of Lepidopterais formed basically by the same constituents: virions, protein matrix of poliedrin, and viral envelope. However, the NPV affects the useful insects such as silkworm *B. mori* and the virus was identified in studies of light and electron microscopy, and symptomatologic analyses[2]. Natural virus isolation, obtained from virus affected larvae of the beet armyworm was collected and occlusion bodies (OB's) were identified and purified[9].

Techniques for viral polyhedra processing were described in several publications [3],where the material is fixed in glutaraldehyde and post fixedin osmium tetroxide, in several concentrations. However, the images obtained do not differ fromthat obtained with the use of the techniques, describedin this work, where the faces of the polyhedron appeared smooth, facilitating the morphologic descriptions. Similar methodology is described by [4], [5], used to determine the morphology of SNPV and MNPV of *Thysanoplusiaorichalcea*(L.) (Lepidoptera: Noctuidae).The most advanced disease diagnosis technique is the use of antibody-based biosensors and lateral flow assays which exhibit high sensitivity and specificity and thus, can serve as good detectors of NPV with unprocessed or crude samples. The most effective solution is timely detection of NPV infection in silkworm rearing to stop further spread of the disease.

Fig.1. *Bm*NPV infected silkworm larvae showing intersegment swollen symptom

The NPV detection technique is needed to stop the outbreak and spread of the disease in rearing units, to take suitable preventive measures, to initiate appropriate control measures and for certification in National and International trade. The grasserie disease epidemics are driven by the amount of initial inoculum and the rate of transmission, it is essential to have diagnostic procedures which allow rapid detection of initial inoculum. The level at which the disease becomes apparent depends to a large extent on the ability of the observer to recognize the disease. The diagnostic kits for detection of NPV have been

developed but they are not widely used on a commercial scale and often they fail to provide the indispensable and timely advantages desired for early disease intervention[7].

Fig.2. Light microscopic examination of purified suspension of *B. mori* showing polyhedral occlusion bodies (1000x)

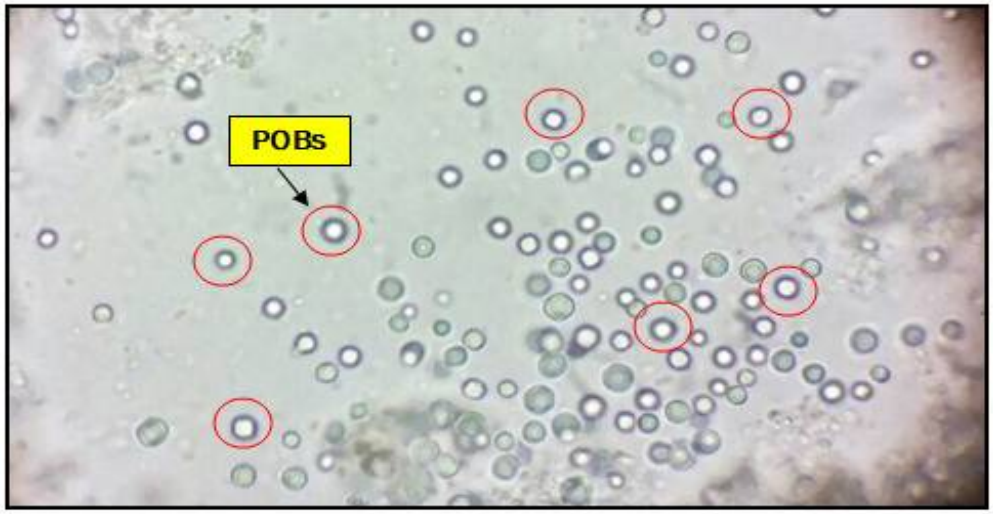
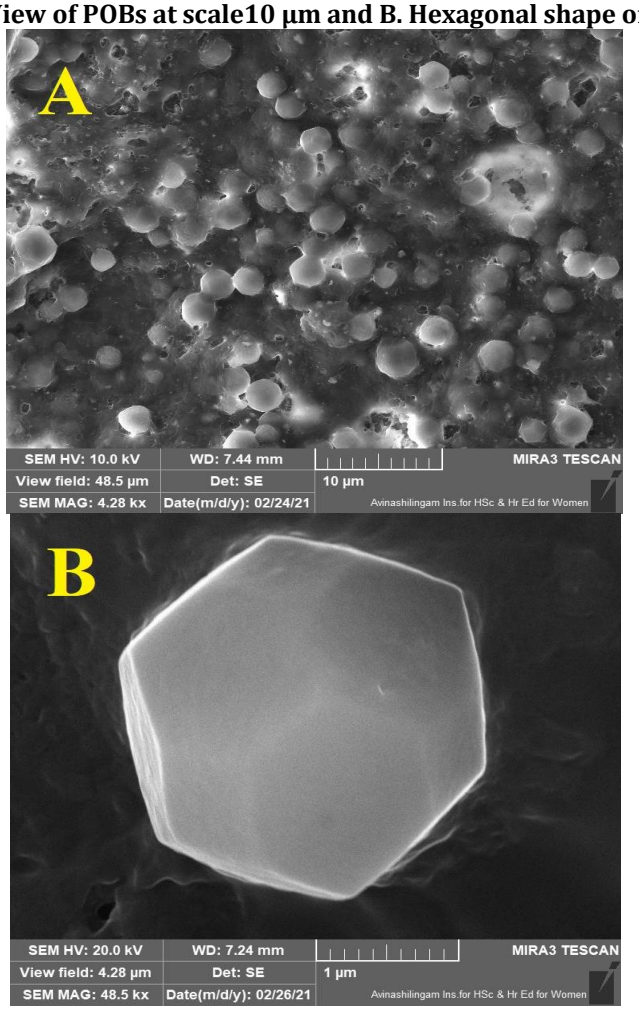


Fig.3. Field Emission Scanning Electron Micrograph of polyhedral OBs extracted from *Bm*NPV infected larvae. A. View of POBs at scale10 µm and B. Hexagonal shape of POBat scale 1 µm.



CONCLUSION

The current investigation reveals the symptom of *Bm*NPV larvae and the presence of polyhedral bodies from the filtrate and haemolymph of NPV affected silkworm larvae.Light microscope and Field emission scanning electron microscope were used to analyze and confirm the presence and structure of the polyhedral occlusion bodies (POBs) is shown clearly in the figures 2 and 3.

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