



## Comparative studies on malpighian tubule of *Bombyx mori* L, after grasserie disease caused by *Bombyx mori* Nuclearpolyherosis Virus (*BmNPV*)

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### Abstract

Larval malpighian tubule of multivoltine breed silk worm *Bombyx mori* L. (Nistari) was studied in both favorable and unfavorable rearing seasons after Grasserie caused by *Bombyx mori* Nuclearpolyherosis Virus (*BmNPV*) using histological analysis, scanning electron microscopy and transmission electronic microscopy for detailed study.

**Keywords:** *Bombyx mori* L, Nistari, Multivoltine breed, Malpighian Tubule, Nuclearpolyherosis Virus (*BmNPV*), Favorable rearing season

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## 1. Introduction

West Bengal, the major silk producing state in Eastern India occupies a unique geographical position (85.8° - 89.8° East, 21.5° - 27.3° North) and mulberry silkworm rearing is practiced mainly in the districts of Malda, Murshidabad and Birbhum. The climatic condition of West Bengal may broadly be divided into the hot dry period (from March - early June); the monsoon with high temperature and high humidity (from late June-October) and the winter (from November - February) (Anonymous, 2019). Silkworms suffer from various diseases like pebrine (microsporida), flacherie (bacteria), grasserie (virus) and muscardine (fungus). These pathogens have a great role in deterioration of sericulture industry. Further, most of the commercially silkworm races exploited in India are prone to infection and silkworm races are reared in India since decades without any major change. As a result, their tolerances against diseases are lost (Veeranna, 1999). The viral disease called 'Nuclearpolyhedrosis' is caused by Nuclearpolyhedrosis Virus (NPV) (Family: Baculoviridae) in *B. mori*. Literatures are available on the pathogenic effect of NPV on its host. Due to pathogenicity of NPV, unequal growth can be expected in comparison to the normal counterpart in the malpighian tubule affecting the excretion of the animal.

## 2. Materials and methods

### 2.1. Rearing Schedule

For three consecutive favorable rearing seasons, month wise February-March (2018-21) and November-December (2017-20), all the experimental procedure was conducted. All the recommended package of practices

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of rearing was followed. The rearing was followed after Krishnaswami (1978). The egg of *B. mori* L multivoltine breed of Nistari were taken from Silkworm Breeding and Genetics Laboratory of Central Sericultural Research and Training Institute, Berhampore, West Bengal, India and reared using traditional methods after feeding with tender Mulberry: *Morus alba* (S-1635 variety) leaves. At first the eggs were washed in 2% formalin solution for surface disinfection. Rearing house as well as rearing appliances was also disinfected by spraying with 5% normal bleaching powder solution. After proper room disinfection and taking proper measures for disinfection the rearing was commenced. Brushing of silkworm larvae is a process of separating the newly hatched larvae from the empty egg shells or egg sheets and transforming them to rearing bed. On the hatching day the egg sheets were exposed to light in the morning and at least one hour of exposure can result uniform hatching (Saha et al. 2008). Chopped tender mulberry leaves were provided (size: 0.5 cm<sup>2</sup>) by sprinkling as thin layer over the hatched larvae. The silkworm larvae were kept for 10-20 min as such. The larvae were then transferred to rearing trays providing optimum spacing. Wet pad of foam and paper greased with paraffin was provided in the tray to maintain the optimum humidity. Larval stages of silkworm could be divided into five instars and during this period molting occurs four times. The first three instars are called young age or *chawki* and the last two instars are called late age. The young age larvae were fed with tender leaves of mulberry plant and the fourth instar larvae were fed with mature leaves whereas the fifth instar larvae were given with cut twigs and mature leaves throughout the rearing and it was followed in all the rearing seasons.

## 2.2. Isolation and purification of virus

Grasserie infected pupae, obtained from silk growers of Birchandrapur village; Birbhum District of West Bengal, India was used in the present study. The polyhedra were isolated from the infected pupae and purified by centrifugation at 3000 rpm for 10 min using percoll cushions (PVP coated silica particles, Sigma chemicals Co., USA). After centrifugation, the polyhedra were stored at 4 °C. Third instar '0' hour larvae were perorally inoculated with *BmNPV* (1 × 10<sup>6</sup> Obs/ml). Polyhedra were counted using a Neubauer haemocytometer (German Fine Optic) under light microscope (×600) and determined the inoculum concentration after Undeen (1997). *BmNPV* isolates were propagated in *B. mori* silkworm larvae and purified from live moth using Percoll Cushions (PVP coated Silica particle, Sigma Chemicals Co., USA).

## 2.3. Inoculum concentration and experiments

Freshly ecdysed Vth instar 100 larvae were considered for the experiment. 1.5 × 10<sup>8</sup> Obs/ml were inoculated using conventional diet contamination method at '0' hour (Bhattacharya et al., 1992). Briefly the procedure involved dipping leaf dishes (28.27 sq. cm) in 1.0 ml of the polyhedral suspension, drying and then allowing the larvae to feed on the dishes for a period of 6 h. Normal and *BmNPV* infected samples were maintained. *BmNPV* inoculated Vth instar larvae were isolated from the population for different investigation.

## 2.4. Histopathological study

The method was followed after Baker (1958). In the laboratory animals were anaesthetized followed by sacrificed in anhydrous condition. Both normal and *BmNPV* infected tissues were dissected out and then fixed for histological studies. Alcoholic Bouin's fluid was used as fixative. After fixation, the samples were processed according to the general paraffin processing protocol involving dehydration of the tissue by gradation of alcohol, like 30% ethanol, 50% ethanol and 70% ethanol. Storage at 70% ethanol + lithium carbonate treatment for picric acid, 90% ethanol and finally 100% ethanol treatment was done. Later, clearing was done by using cedar wood oil. One to 2 h was used for clearing. Malpighian Tissues can be left in cedar wood oil indefinitely. It does not harden the tissue. After clearing, the samples were transferred to a mixture of xylene saturated with paraffin. It was kept in the paraffin at about 35 °C for 15 min. Hot paraplast was infiltrated in a 58 °C paraffin oven in an open container. Three consecutive changes following time sequences: 30 min, 30 min and 1 h respectively were done. A small amount melted paraplast was poured (fresh) into stainless steel container. The paraplast infiltrated tissues were transferred with forceps and oriented properly in the centre of the depression. The top surface of the paraplast was cooled by blowing gently on it. As soon as a scum of paraplast has formed on top, the cup was sunk gently into a cold water bath. The cup was cooled thoroughly in cold running tap water. Embedding was done following all the precautions and the blocks were prepared for sectioning with rotary microtome after trimming and mounting on a metal block holder. The thickness of sections was set at 5 μm using rotary microtome. Clean glass slides smeared with Myers' albumen and flooded

with distilled water were used for placing the tissue sections, stretched in hot oven. Sections were dried overnight before staining. The sections were covered carefully with a coverslip without drying. Photomicrographs were taken from tissue sections with oil immersion under light microscope ( $\times 1000$ ) (Leitz Diaplan Phase Contrast Microscope).

### **2.5. Scanning Electron Microscopic (SEM) Study**

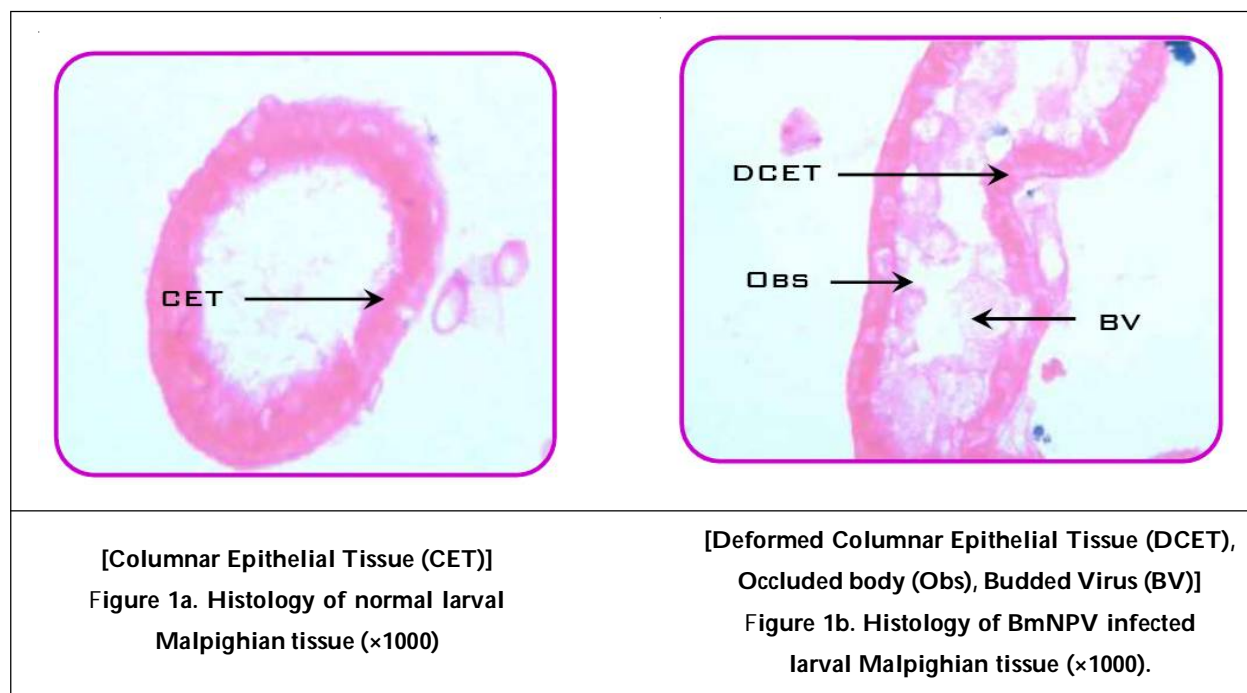
4g dry powder of Paraformaldehyde (EM grade) was dissolved in 100 ml of distilled water at 60 °C. Mouth of conical flask was covered with aluminum foil. A drop of 0.1N sodium hydroxide was used for dissolve properly. The solution was cooled and filtered. Equal volume of 0.2 M phosphate buffer (pH 7.4) to the paraformaldehyde solution to make it a 2% solution in 0.1 M phosphate buffer was added. Required quantity of glutaraldehyde (from the concentrated 25% stock solution, EM grade) was added in paraformaldehyde solution. In this case, 10 ml of glutaraldehyde (from stock solution) was added to 90 ml of 2% paraformaldehyde solution. The final concentration of the fixative was 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. Similar technique was followed after Inoue and Osatake (1988). Silkworms were perfused, first with 0.9% phosphate buffer saline of pH 7.2 for 10 min and then with 2% paraformaldehyde for 20 min. Larval Malpighian tussue at different larval stages of both healthy and infection of Nistari were collected for SEM study. Larval integument was fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 8-12 h at 4 °C. 5-7 mm in length and breadth of the integument sample was taken for the study followed by wash in buffer for 3 times, each for one-hour duration at 4 °C. The samples were kept inside a thermos flask using cool pack for transportation. *Critical Point Drying for SEM* was conducted with Hitachi (HCP-2). Gold coating was done by IB2 Ion Coater (Eiko Engineering, Japan). Samples were observed under SEM (S-530–Hitachi, Japan.) and photomicrograph was taken ( $\times 300 - 1500$ ). {Conducted at: **University Science Instrumentation Centre (USIC), The University of Burdwan, West Bengal**}

### **2.6. Transmission Electron Microscopic (TEM) Study**

Paraformaldehyde solution was made in 100 ml of distilled water by dissolving 4 g of dry power (EM Grade) at 60 °C. The mouth of the conical flask was covered with aluminum foil before the solution was cooled and filtered; later equal volume of 0.2 M Phosphate buffer (pH 7.4) was added to the paraformaldehyde solution to make it a 2% solution in 0.1 M Phosphate buffer. Then required quantity of glutaraldehyde (from the 25% concentrated stock solution, EM Grade) is added to the paraformaldehyde solution. To prepare 0.2M phosphate buffer (pH 7.4), 3.12 g of sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ : Molecular weight 156) was added to 100ml of distilled water and solution A was prepared. 2.84 g of disodium hydrogen orthophosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ : MW 142) was added to 100 ml of distilled water to make solution B. 19 ml of solution A was mixed with 81 ml of solution B and the final pH was adjusted with HCl or NaOH). Each sample was washed in buffer for three times each for one hour duration at 4°C. The samples were kept in thermos flask using cooling pack to the instrumentation centre. The technique was followed after Hall (1995). The samples were post fixed in 1% osmium tetroxide ( $\text{OsO}_4$ ) for 1 h at 4 °C. The samples were dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1  $\mu\text{m}$ ) were cut with an ultramicrotome, mounted on to glass slide, stained with aqueous toluidine blue and observed under light microscope for gross Observation of the area and quality of the tissue fixation. For electron microscope examination thin sections of gray silver color interference (70-80 nm) were cut and mounted in 300 mesh-copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D transmission electron microscope (FEI Company, The Netherlands) at an operating voltage 80 kV. Images were digitally acquired by using a CCD camera (Megaview III, FEI Company) attached to the microscope. *BmNVP* inoculated Vth instar larvae were isolated from the population for the electron micrograph study. Each larva was perfused in 0.9% phosphate buffer saline (pH 7.2) for 10 min followed by 2% paraformaldehyde solution for 20 min. Larval Malpighian tubule were dissected out and cut into pieces measuring 2  $\times$  2 mm size. The sample was fixed in a mixture of 2% paraformaldehyde solution and 2.5% glutaraldehyde in 0.1M phosphate buffer for 8 to 12 h at 4 °C. Samples were observed under TEM (268 D (10kV) of FEI, Company, The Netherlands and photomicrograph were taken ( $\times 1000 - 8000$ ). {Conducted at: All India Institute of Medical Sciences (AIIMS), New Delhi, India}.

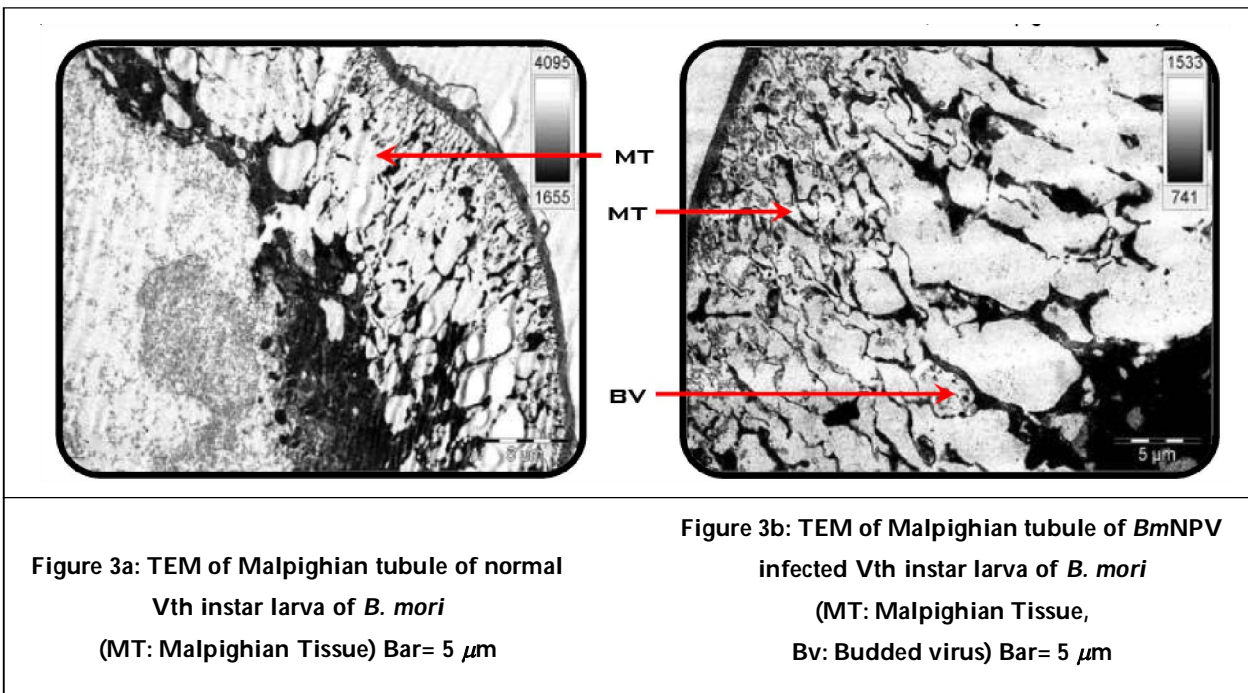
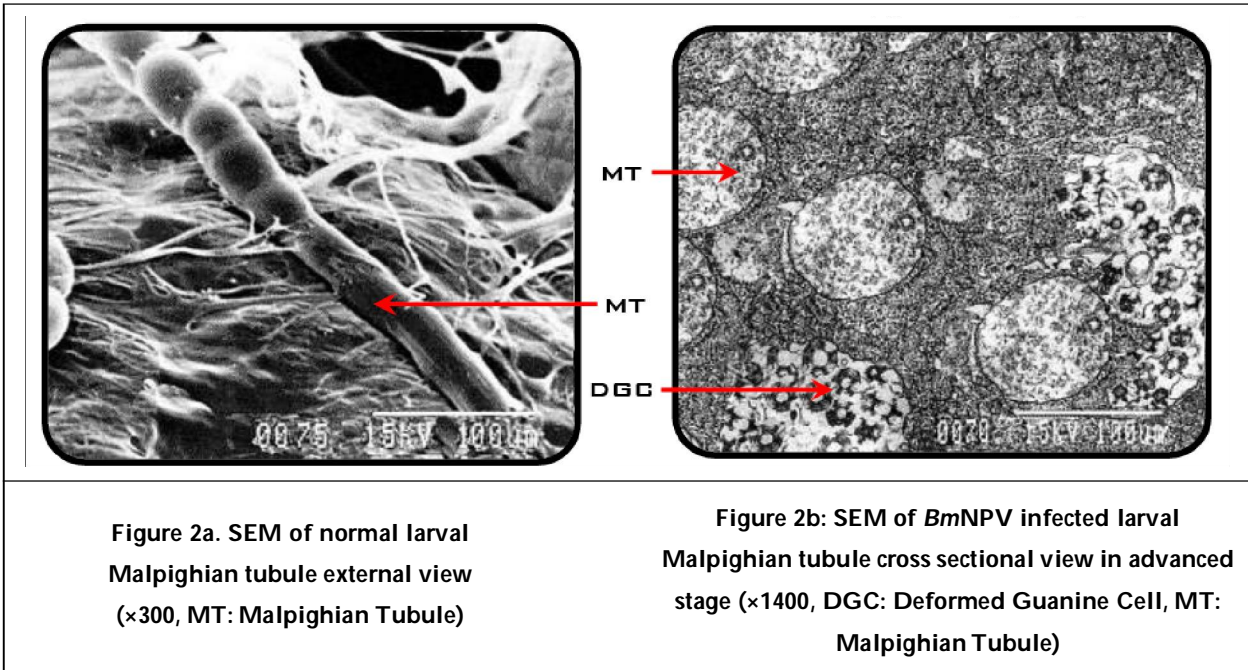
### 3. Results and discussion

Histology of Malpighian tubule reflects that the normal tissue section of Malpighian tubule in the silkworm shows inner hollow space surrounded by thick densely packed columnar epithelial tissue layer. The cells are provided with prominent nucleus (Figure 1a). The affected Malpighian tissue section of the silkworm shows presence of budded virus within the Malpighian tissue and they appear like oval hollow portions within the soft tissues and at late stage of infection they are released in the hollow space of the Malpighian tubule. The Occluded bodies (Obs) are also present in the inner lining of the Malpighian soft tissue and they are also released in the open space of the tubule before which they are arranged in the internal periphery of the tubule in the form of cluster (Figure 1b).



As per SEM observations, Malpighian tubules show layers of which bilayered laminae containing less permeable layer inside the outer more permeable one. Malpighian tubule, along with silk gland, heart and muscle tissues are not known to be involved with the synthesis of large haemolymph proteins (Brancalhao *et al.*, 2009). The Malpighian tubule under SEM study reveals that the tubule lies on the soft surface. The cross sectional view of the same indicates presence of guanine cells (Torquato *et al.*, 2006), and they are present on the periphery and there are alternate prominent dark and light striations observed in the cross section of the Malpighian tubule (Figure 2a). The grasserie infected larval Malpighian tubule under SEM lack alternate prominent dark and light striations as observed in the normal counterpart; rather they are diffused with deformed guanine cells. Infected Malpighian tubule is quite loose and the peripheral membrane is discontinuous in respect to the normal counterpart. Presence of occluded bodies (Suzuki, 2002) in between the striations of the Malpighian tissue layer is clearly observed (Figure 2b).

Larval Malpighian tubule under transmission electron microscopy revealed that healthy Malpighian tubule is compact and it is guarded by continuous outer membrane (Figure 3a) but the affected counterpart is quite loose and the peripheral membrane is discontinuous in respect to the normal counterpart (Khurad *et al.*, 2004). Presence of occluded bodies in between the striations of the Malpighian tissue layer is clearly observed. The Malpighian tubule of healthy silkworm larvae showed that the presence of inner soft Malpighian tissue guarded by the continuous peripheral membrane whereas the infected counterpart showed that the inner Malpighian tissue were loosened and broken, containing the occluded bodies in between the striations of the Malpighian cells. The second form of the virus, called budded virus (BV), never becomes occluded, spreads infection from cell to cell. The Malpighian tubule of healthy larvae exhibited the presence of inner soft tissues guarded by continuous peripheral membrane (Bravo-Patino and Ibarra, 2000). However, in infected larva the inner Malpighian tissue was loose and fragmented with Obs in between the striations of Malpighian cells. BV spreads infection from cell to cell (Figure 3b).



#### 4. Conclusion

Malpighian tubule of healthy silkworm larvae showed that the presence of inner soft Malpighian tissue is guarded by the continuous peripheral membrane whereas the infected counterpart showed that the inner Malpighian tissue are loosened and with full of Obs in between the striations of the malpighian cells. The outer membrane lost its basic round structure and became fragile (Haas-Stapleton et al., 2005). BVs were found more in groups and larger in size as compared to Obs.

Absence of alternative striations in grasserie infected larval Malpighian tubule under SEM is quite prominent and is diffused with deformed guanine cells (Zhou et al., 1998). In the Malpighian tubule *BmNPV* virions have approximate mean diameter of 95 nm and length of 315 nm. Therefore, BV is retained in Malpighian tubules and this is an interesting finding in the present study.

The TEM examination of different parts of the *BmNPV* infected *B. mori*, compared with the normal uninfected counterpart clearly revealed that the peripheral membrane of infected larval Malpighian tubule began to

diffuse to form a continuous larger structure to incorporate maximum number of Obs. The fully transformed structures gradually start to discharge the maximum number of Obs in adjacent matrix and the polyhedral structures were released for further multiplication of *BmNPV* in the form of budded virus.

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