

## The Spreading Of *Spodoptera litura* Multiple Nucleopolyhedrosis Virus in Midgut of Third Instar of *Spodoptera Litura* Larvae

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**Abstract:** Analyses on the spreading of *SpltMNPV* (*Spodoptera litura* Multiple Nucleopolyhedrosis Virus) multiplied in midgut epithelium cell of in third instar of *S. litura* larvae has been done through histological studies. The aim of this study was to examine the distribution of *SpltMNPV* in *S. litura* larvae. The third instars of *S. litura* larvae that had been infected with *SpltMNPV* were incubated for 0, 24, 48, and 72 hours. Afterwards, the infected *S. litura* larvae were cut crosswise in the anterior, middle, and posterior of the midguts. The crosswise cuts at the midguts were fixed using Gilson solution, executed under the paraffin method, and cut as thick as of 4  $\mu\text{m}$ , and then stained with Hematoxylin and eosin. The results of this study indicate that *SpltMNPV* (PIB, Polyhedral inclusion bodies) have infected various organs of the *S. litura* larvae, i.e. lumen, peritrophic membrane, midgut epithelial layers, trachea, blood vessels, malpighian tubules, muscle cells and adipocyte. The only organ which is not infected by *SpltMNPV* is the cuticle.

**Keyword:** *SpltMNPV*, midgut epithelial cells, and *Spodoptera litura*

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### I. Introduction

*Spodopteralitura* is often known as Tropical armyworm. This worm is an agricultural pest which is very harmful because its attack is simultaneous and in bulk (Kartohardjono and Arifin, 2000). *S. litura* attacks cause 12.5% to over 20% destruction in soybean plantation of over 20 days of age (Adisarwanto and Widiyanto, 1999). The damage can cause the loss of 85% of the yield, or even crop failure (Marwoto and Suharsono, 2008). One of the viruses being developed to control *Spodoptera litura* is *SpltMNPV* (*Spodopteralitura* Multiple Nucleo polyhdrosis Virus). According to Asri & Isnawati (2005), *SpltMNPV* in  $10^6$  PIBs/ml (*Polyhedra inclusion bodies/ml*) concentrations is effective to control *S. litura* as to increase mortality rate into 80-90% in a laboratory-based experiment and  $10^7$  PIBs/ml in a greenhouse-based experiment (Asri, 2004).

The propagation of *SpltMNPV* is usually done using the *S. litura* worm. This method, however, faces many obstacles, i.e. the high rates of deaths of the larvae in the culturing process in laboratory. *SpltMNPV* cultured in an *in vitro* condition at the midgut epithelial cells of *S. litura* larvae is feasible (Asri and Nur, 2008). Usually *SpltMNPV* proliferates in epithelial cells or in their derivatives. Accordingly, this study examined the distribution of *SpltMNPV* in midgut of *S. litura* larvae.

### II. Materials and Methods

#### Multiplication of *SpltMNPV* in epithelial cell culture

Multiplication of *SpltMNPV* in the epithelial cells of *S. litura* was done through an *in vitro* method. The *SpltMNPV* used in this study was isolated from *S. litura* larvae died infected by viruses in areas in Central Java, Indonesia (Wahyuni, 2002). The *SpltMNPV* was purified through a centrifugation method (Arifin, 1994), and the concentration was calculated using a Haemocytometer. Polyhedra of *SpltMNPV* ( $1.1 \times 10^6$  PIBs /ml) was broken using  $\text{Na}_2\text{CO}_3$  0.5 M. The *SpltMNPV* without polyhedra was inoculated in midgut epithelial cell with a cell concentration of  $7.6 \times 10^7$  cells/ml. The infected epithelial cells were grown in Grace's medium enriched with fetal bovine serum 2.5% and incubated at the temperature of 28 – 30°C in three days. The *SpltMNPV* in Grace's medium was harvested through a centrifugation method of 3500 rpm for about 15 minutes. The number of *SpltMNPV* in Supernatant was counted using a Haemocytometer.

#### Infection of *SpltMNPV* in *S. litura* larvae

The amounts of *SpltMNPV* ( $7.8 \times 10^7$  PIBs/ml) were used to infect 30 of *S. litura* larvae through a feeding contamination method (Asri, 2009). These 30 infected larvae were grouped into three group, i.e. 10 larvae were incubated for 24 hours, another 10 larvae were incubated for 48 hours, and the rest 10 larvae were

incubated for 72 hours. In addition, 10 larvae were used as control group (given no treatment of *SpltMNPV*). During the incubation, the infected *S. litura* larvae were fed with artificial food and incubated at room temperature (28-30°C). The infected larvae were examined on morphological changes during the incubation.

### **Histology preparation**

The histological preparation was done using a modified paraffin method (Thamrin, *et.al*, 2012). The infected larvae incubated for 0, 24, 48, and 72 hours were fixed with Gilson solution. The larvae were cut crosswise at the anterior, middle, and posterior midgut. The midgut pieces were immersed in ethanol 70% for at least 24 hours. Dehydration was done in alcohol of 70% titration (4 x 20 minutes), 80% (2 x 20 minutes), 96% (1 x 20 minutes) and absolute alcohol (1 x 20 minutes). Clearing process was done in eugenol for 24 hours. Then, the samples were soaked in paraffin until they were frozen and were through the processes of Embedding, Trimming, and Cutting to 4 micron in size. The pieces were placed on glass objects which had been given Meyer albumin adhesive. The next step was HE coloring which was done by immersing in Xylol + Kl 1%, 15 minutes, absolute Xylol, alcohol series (absolute alcohol, 96%, 80%, 70%, each for 5 minutes); staining with Haematoxylin for 10 minutes, running water for 5 minutes, 100 ml of ethanol 70% + 5 drops of HCL for 10 seconds; distilling water for 5 minutes; staining with eosin, washed with distilled water and immersed in alcohol series (ethanol 70%, 80%, 96%, and absolute alcohol), xylol 1 each for 5 minutes and xylol 2 for at least 20 – 30 minutes. Then, they were covered with a cover glass and glued with *entellan*.

### **Observation of *SpltMNPV* infection on *S. litura* larvae's organs**

In this study, observation data were analyzed descriptively. The pieces of *S. litura* larvae were observed under a light microscope. The observation of the infected organs was done on the anterior, middle, and posterior midguts within 0, 24, 48, and 72 hours incubations. Determining the infected organs was based upon the discovery of polyhedral inclusion bodies of *SpltMNPV* at each organ.

## **III. Results and Discussion**

Table 1 shows the spreading of *SpltMNPV* in *S. litura* larvae's bodies which had been incubated for 0, 24, 48, and 72 hours. In the 0-hour incubation (control), all the organs (the lumen and the midgut epithelial cells) were still free from viruses. This shows that larvae used in this incubation were uninfected with *SpltMNPV*. In the 24-hour incubation, the organs infected by *SpltMNPV* were the lumen, peritrophic membranes and midgut epithelia, fat body/fat cells (at the lumen midgut), trachea and blood vessels (only at the posterior midgut). These findings are correspond with the results of the study by Engelhard, et al. (1994) stating that after 24-hour incubation, *AcMNPV* (*Autografa californica* Multiple Nucleopolyhedrosis Virus) has shown some infection signs on the epithelial midgut and tracheoblast. In this present study, there were some infection of PIB *SpltMNPV* on the posterior midgut of blood vessels. The findings in this study are rather different from Engelhard's study (1994), in which *AcMNPV* infected hemocyte in blood vessels only after 36 hours of incubation. This means that the spread of *SpltMNPV* is faster than the spread of *AcMNPV*. In the 48-hour incubation of *SpltMNPV*, it can be seen in the larvae's bodies that the virus had infected most of the organs (lumen, peritrophic membranes and midgut epithelia, fat body, trachea and blood vessels) except some muscle cells and the cuticles. This is in accordance with Prasad & Yogita (2006) which confirms that *S/NPV* attacks almost all organs (midgut, fat body, muscular layer, and basement membrane) at 48 hours post-infection. After 72-hour incubation, the worms had lost their appetite, their bodies started to change color and getting pale and slow in their movement. In this incubation, almost all organs in the midgut, anterior, middle, or posterior, were already infected, except the cuticles. In Engelhard's study (1994), all of the organs (midgut, trachea, blood vessels, muscle cells, and epidermis in cuticles) have been infected by *AcMPV* after 70 hours of incubation. In this study, the epidermis of the cuticles was infected, but the cuticle layers (chitin) were free from *SpltMNPV* infection. The cuticles were the last layers which were not infected by the virus. The virus still needs the cuticles to collect the virus from reproduction (Rohrmann, 2008).

Based on Figure 1, the spread of *SpltMNPV* in various organs of *S. litura* larvae is noticeable. The organs of *S. litura* infected by *SpltMNPV* are midgut, trachea, blood vessels, malpighian tubules, fat cells/fat body, and muscle cells. The only organ uninfected by *SpltMNPV* was the cuticle. The organs infected by *SpltMNPV* could be indicated by the *SpltMNPV* PIB existence in the organs (Figure 1).

The virus began to act only after being ingested through the food and getting activated in the alkaline pH of the midgut lumen. Midgut is the first organ targeted by the virus (Prasad & Yogita, 2006). Inside the lumen, *SpltMNPV* polyhedral will be split by alkaline pH, then the multiple nucleocapsid (MNPV) will break out and damage the peritrophic membranes by endopeptidase / metalloproteinase / *enhancins* enzyme. This causes much damage in the peritrophic membranes, so the peritrophic membranes are destroyed (Rohrmann, 2008). The next target cell is the midgut epithelial cells. The infection mechanism of *SpltMNPV* on midgut epithelial cell will begin by some multiple nucleocapsid of *SpltMNPV*, as to recognize the host cell membrane

by attaching to a specific place (receptor site) and release a particular compound that can change the cell membrane, so that the cell membrane invaginate and form a tunnel. According to Rohrmann (2008), multiple nucleocapsid is attached to larva midgut cells through the interaction between vp91 (virus protein 91) and midgut cells receptor. MNPV has sensitive receptor sites so that it can bind protein and change the conformation of the compounds in the epithelial cell membrane.

Table 1 The Spread of *SpltMNPV* in *S. litura* larvae

Hour	Histology	LM	PM	ME	Tr	BV	MT	FB	CE	MC
0 h (Control)	AM	-	-	-	-	-	-	-	-	-
	MM	-	-	-	-	-	-	-	-	-
	PM	-	-	-	-	-	-	-	-	-
24 h	AM	+	+	+	+	-	No	-	-	-
	MM	+	+	+	+	-	-	+	-	-
	PM	+	+	+	+	+	-	-	-	-
48 h	AM	+	+	+	+	+	No	+	-	-
	MM	+	+	+	+	+	+	+	-	-
	PM	+	+	+	+	+	+	+	-	+
72 h	AM	+	+	+	+	+	No	+	-	+
	MM	+	+	+	+	+	+	+	-	+
	PM	+	+	+	+	+	+	+	-	+

Note: Lm = Lumen, PM = Peritrophic Membrane, ME = Midgut Epithel, Tr = Trachea, BV = Blood Vessels, MT = Malpighian Tubules, FB = fat body/fat cells, CE = Cuticle Epithelia, MC = Muscle Cell. + = infected by virus, - = not infected by virus, NO = no organ, AM = Anterior Midgut, MM = Middle Midgut, PM = Posterior Midgut.

The next phase after the attachment of virus in a specific place on the host cell was penetration and releasing nucleocapsid in the envelope to the cytoplasm and, followed by biosynthesis of the virus components by forming virogenic stroma containing genetic materials and capsid protein (nucleocapsid). Virogenic stroma formation is regulated by a virus protein called PP31 (Rohrmann, 2008). The assembling of the virus components and the releasing of Polyhedra inclusion bodies (PIBs) of *SpltMNPV* became the last phase. The epithelial cells were destroyed and *SpltMNPV* spread to the nearby organs such as trachea, blood vessels, and malpighian tubules. This is in accordance with the histo-micrograph figure obtained by Prasad & Yogita (2006), in which *SpltMNPV* first enters the midgut lumen then damaging the peritrophic, epithelial tissues, fat body, blood circulatory system/hemocoel, trachea, muscle cells, and integument.

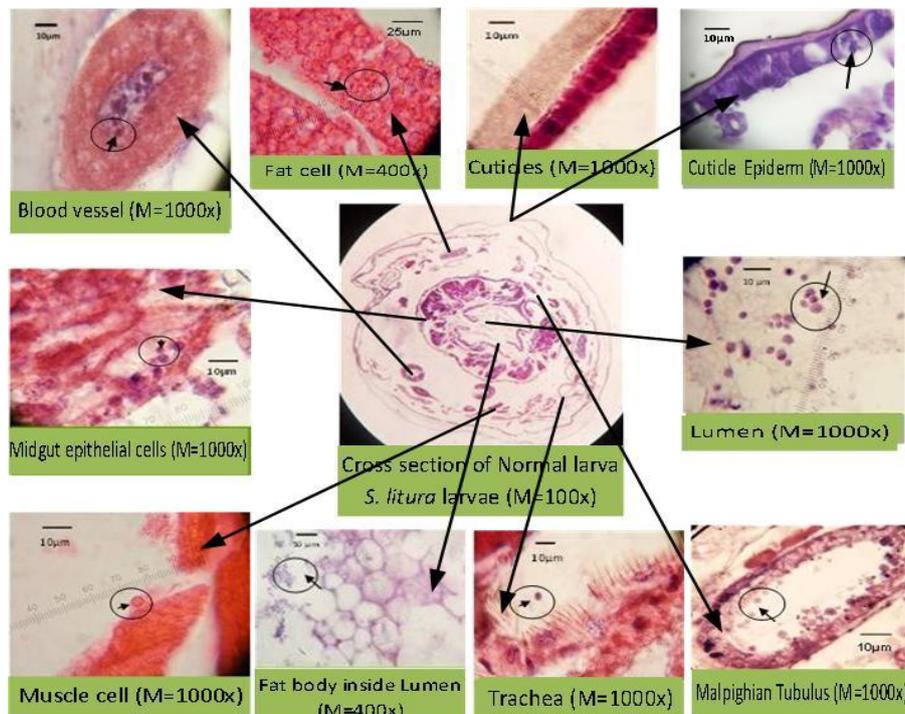


Figure 1. Organ potential to be infected by *SpltMNPV* at the cross section of midgut of *S. Litura* larvae (the short black arrow in circle points at the *Polihedra* inclusion bodies of *SpltMNPV*). The organs infected by *SpltMNPV* included lumen, peritrophic membrane, midgut epithelial layers, trachea, blood vessels, malpighian tubules, muscle cells and adipocyte. The only organ which is not infected by *SpltMNPV* was the cuticle.

Trachea is the second target of the virus infection (Engelhard, *et al.*, 1994). The center of the trachea polyhedral inclusion bodies of *SpltMNPV* are already visible. According to Anonymous (2006), trachea is one of the organs in charge of supplying oxygen and excreting CO<sub>2</sub>. This organ can be infected by *BmNPV* (*Bombyxmori* Nuclear Polyhedrosis Virus) and is responsible for spreading *BmNPV* to other organs (Torquato, *et al.*, 2006) by tracheal epidermal cell and cytoplasmic extension (Engelhard, *et al.*, 1994). On the other hand, blood vessels in lumen also are infected by *SpltMNPV*, and polyhedron of *SpltMNPV* is shown to be oval in shape. By entering the blood vessels, it is easy for *SpltMNPV* to spread to the whole body. According to Keddie *et al.*, (1989), systemic infection is mediated by free virus for some tissues, whereas infected hemocytes in blood vessels appears to spread virus to some other tissues.

In this study, malpighian tubules lumen were infected by *SpltMNPV* which caused the destruction of the malpighian tubules which caused the metabolism waste not be excreted from the *S. litura* larvae's bodies. This could cause poisoning in the *S. litura* larvae's bodies. The skin in insects comprises of cuticles which are composed of fibrils and chitin planted in the protein matrix. Cuticles help prevent pathogens from entering the body, but if the cuticles crack due to injury or degradation, it may cause infection (Kavanagh & Reeves, 2004). In this study the cuticles were not infected by *SpltMNPV*. *SpltMNPV* could not infect cuticles since cuticles do not have site receptor (vp91) for *SpltMNPV* (Rohrmann, 2008). Hence, the data showed that the *S. litura* larvae's muscle cells have polyhedra of *SpltMNPV*.

#### IV. Conclusion

*S. litura* larvae were infected by *SpltMNPV* in several organs, there are lumen, peritrophic membranes, trachea, blood vessels, malpighian tubules, muscle cells and fat cells. The only organ uninfected by the virus was the cuticle.

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