Histopathology of Spodoptera litura Larva Infected by Multiple Nuclear Polyhedrons Virus (SpltMNPV) in Photo-Protectant Formulation

by Mahanani Asri

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Maaf Pak didik, saya pembetulannya langsung disini ya pak. Pake bahasa Indonesia ya pak?

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Abstract

Spodoptera litura Multiple Nucleopolyhedrosis Virus (*Splt*MNPV) is a viral pathogen with the potential to control pests *Spodoptera litura*. The virus is effective for controlling *S. litura* with the mortality of 80-90%. Histopathologic preparations were made in two ways using paraffin with hematoxylin-eosin staining and IHC methods. *Splt*MNPV was detected in *S. litura* and its histopathologic characterized by the existence of polyhedral inclusion bodies distinctive color (dark brown) spread in the midgut lumen and epithelium. Tissues and organs that have been infected by *Splt*MNPV in vitro in a photo-protectant formula on the incubation time of 0, 1, 2 and 3 days using paraffin method is lumen, the midgut epithelium, epithelial skin, trachea, blood vessels, muscle cells, and Malpighi tubules while the uninfected parts were the cuticle and nerve cells) dengan menggunakan IHC,PIB *Splt*MNPV tidak ditemukan di otot.

Keywords; Immunohistochemistry, *Spodoptera litura* multiple nuclear polyhedrosis virus (*Splt*MNPV), Photo-protectant

Comment [A1]: Maaf pak penulisan nam spesies biasanya miring.

Comment [A2]: Yang sel otot yang menggunakan imunohistokimia pak. Kalau yang paraffin kutikula dan sel syaraf.. hasil iniada di table. Bagaimana pak?

Comment [A3]: Kontradiksi, di dalam hasi dan pembahasan yang organ yang tidak trinfeksi yang disebut hanya sel otot.

1. Introduction

Spodoptera litura is one of the harmful agricultural pests since its attacks are simultaneous in large quantities. Major crop losses of soybean by *S. litura* could reach 85% (Bedjo, 2007). One virus that is being developed as a bio-pesticide is *Spodoptera litura* multiple nucleopolyhedrovirus virus (*Splt*MNPV). *Splt*MNPV effectively controlled *S. litura* with mortality 80-90% in laboratory Asri (2005) and at a concentration of 10^6 PIBs/ml (polyhedral inclusion bodies/ml) and a concentration of 10^7 PIBs / ml in the greenhouse (Asri, et al 2004). In vitro breeding can be performed on epithelial cells of *S. litura* larval midgut instar 4, 5 and 6 in Grace's medium (Asri and Nur, 2007). Its application in the field, virus' pathogenicity has decreased caused by susceptible NPV even become inactive when exposed to sunlight, especially UV (Young, 2000). *Splt*MNPV pathogenicity can be protected from solar radiation by adding Kaolin and Ethyl-P-metoksinamat (Asri, 2013).

The level of virulence of the virus in killing its host can be seen through the visualization of the current presence of the virus to infect host tissue through paraffin and immunohistochemistry (IHC) methods. This method emphasizes the specific reaction between virus' polyhedrin and primary antibody produced by the infected host and visualized by adding a secondary antibody labeled streptavidin-biotin with a brown chromogen (Hayat, 2001).

2. Materials and Methods

2.1 SpltMNV Multiplication in Larval Midgut Epithelial Cell

*Splt*MNPV isolated from *S. litura* dead larvae infected with the virus from Central Java Indonesia (Wahyuni, 2002). *Splt*MNPV was purified by centrifugation and counted using hemocytometer initial concentration of 1.1 x 10^6 PIBs/ml. Its polyhedral was cracked using 0.5 M Na_2CO_3. *Splt*MNPV without polyhedral was inoculated into *S. litura* larvae epithelial cell cultures as 7.6 x 10^7 cells/ml in Grace's medium enriched with 2.5% fetal bovine serum. Cells were incubated at 28 - 30°C for 3 days. *Splt*MNPV was purified by centrifugation 3500 rpm 15 minutes and calculated using hemocytometer.

2.2 SpltMNPV Infection to the Third Instar Larvae

As many 7.8 x 10^7 PIBs/ml *Splt*MNPV were infected to 30 *S. litura* third instar larvae by contamination of feed (Asri, 2009). As 10 larvae were incubated at room temperature (28-30°C) for 24 hours, 10 larvae for 48 hours and 10 for 72 hours while the other 10 larvae as a negative control. During incubation, the infected larvae were fed using artificial feed. *Splt*MNPV was irradiated with sunlight for 12 hours and infected by larvae of *S. litura* incubated for 0, 24, 48 and 72 hours (for paraffin) and 0, 2, 4 and 6 days (for IHC).

2.3 Histological Preparation using Paraffin Method

Comment [IA4]: Metode pewarnaan hematoksilin eosin belum dituliskan.

One of 10 larvae were infected with the virus in each incubation was used as a sample. Infected larvae in the incubation 0, 1, 2 and 3 days were cut transversely in the middle of midgut. Each midgut made preparations for the series as many as 2 slides.

Histological preparations were made from a cross-section of *S. litura* larval midgut infected SpltMNPV propagated in vitro using modified paraffin mehode (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 front, middle and back of 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 + KI 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 705, 80%, 96%, and absolute alcohol), xylol 1 each for 5 minutes and xylol 2 for at least 20 – 30 minutes. Then, they were covered

Comment [A5]: Saya tambahkan metode paraffin menurut thamrin, et al 2012)

with a cover glass and glued with *entellan*.

2.4 Histological Preparation using IHC

Fixation with formalin buffer solution for 24 hours, then soaked in 70% ethanol least 24 hours. Dehydration is performed on an aspirator using alcohol solution series of 70%, 80%, 96% and absolute alcohol for ~~1~~ 3 x 30 minutes. The clearing process was done in aspirator by using Xylol 1 (15 minutes) and Xylol 2 (12 hours). The samples were inserted in the liquid paraffin-xylol (30 minutes) and liquid paraffin I, II and III respectively for 1 hour until frozen and embedded, trimmed and cut-sized 4 microns. Results pieces affixed to glass objects (Poly-L-Lysine) and put into the oven overnight. Deparaffinization was conducted by soaking in xylol (2x3 min) and a mixture of xylol-ethanol 100% (3 minutes) followed by ethanol series 96%, 80%, 70% and 50% for 3 minutes respectively, and rinsed with running water, phase antigen retrieval was done by using Proteinase-K 50µL and put in refrigerator overnight. Peroxidase blocking for 5 minutes, rinsed with distilled water and placed in PBS for 5 minutes and the rest of PBS minimized with a tissue. 40 µL of primary antibody (anti-polyhedrin from Sigma-Aldrich) was dripped on a specimen with a 1: 100 dilution (anti-polyhedrin in PBS) for 30 minutes. Rinsed with PBS (3x5 min) and then the specimen was split with the biotinylated antibody (Biotin-labeled Goat anti-Chicken IgY from Aves. LABB, INC) with a dilution of 1: 200 in PBS and left to stand for 30 minutes covered with a black cover and rinsed with Tris-buffer saline 3 x 5 minutes. The next stage is to shed Streptavidin peroxidase (30 minutes) rinsed with PBS (3x5 min) and entered into a staining with the chromogen substrate for 10 minutes, rinsed with sterile distilled water and given dye opponents are Mayer hematoxylin (2 -5 minutes) then rinsed with distilled water and dipped in water Ammonia 10x, rinsed with distilled water (2min), ethanol-rise (50%, 70%, 80%, 96% 3 minutes respectively and 100 % as much as 2 x 3 minutes) rinsed in Xylol 1 and 2 for 3 minutes, then covered with a cover glass and mounted with mounting medium.

2.4 Observation

Data analysis was done by the descriptive method. Results of a cross-section of *S. litura* larval midgut was observed using a light microscope. Observations made on the midgut infected organ front, middle and back, on incubation of 0, 24, 48 and 72 hours. The determination is based on the discovery of an infected organ polyhedral inclusion *Splt*MNPV bodies of each organ. While the determination of the cell, tissue or organ that indicate polyhedrin protein expression to see the emergence of a dark brown colour. Cell or tissue with blue-purple indicated that it is not infected by *Splt*MNPV because not detected polyhedrin protein.

3. Result and Discussion

3.1 Infected tissue and organ in photo-protectant formula on the body of larvae of S. litura were prepared by paraffin and IHC

Deployment SpltMNPV PIB by propagated in vitro protected by Kaolin and EPMS 20% in some tissues and organs, which were prepared by paraffin method can be seen in Table 1.

Comment [IA6]: Which one exactly did yo use?

Comment [A7]: Hanya buffer formalin. Pa tanpa Gilson. Di Gilson ada merkurinya tidal bisa untuk IHC.

Comment [IA8]: How many times the exa methods that u used 1 or 3 x 30 mins?

Comment [A9]: Pake yang 3 kali saja pak

Table 1. Tissue and organs of infected larvae of *S. litura* SpltMNPV with Photo-protectant in the form of kaolin and 20% irradiated EPMS 12 hours were prepared by the method of paraffin dengan fiksatif larutan Gilson

No	Lokasi midgut	Inkubasi (jam)	Ulangan	Infected organs									
				Lm	PM	EM	Tr	BV	TM	N	BF	Ct	M
1	Negative Control	24	1	-	-	-	-	-	-	-	-	-	-
2	Depan	24	1	+	+	+	+	-	Null	Inv	-	-	-
			2	+	+	+	+	-	Null	Inv	-	-	-
	Tengah	24	1	+	+	+	+	-	+	Inv	+	-	-
			2	+	+	+	+	-	+	Inv	+	-	-
	Belakang	24	1	+	+	+	+	+	+	Inv	-	-	+
			2	+	+	+	+	+	+	Inv	-	-	+
3.	Depan	48	1	+	+	+	+	+	Null	Inv	+	-	-
			2	+	+	+	+	+	Null	Inv	+	-	+
	Tengah	48	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Belakang	48	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	Null	Inv	+	-	+
4.	Depan	72	1	+	+	+	+	+	Null	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Tengah	72	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Belakang	72	1	+	+	+	+	+	+	Inv	+	-	V +
			2	+	+	+	+	+	+	Inv	+	-	+

Note: Lm = Lumen, PM = Peritrophic membrane, EM = Epithelia Midgut, Tr = Trachea, CE = cuticle epithelia, BV = blood vessel, TM = Tubules Malpighi, N = Neuron, BF = body fat, Ct = Cuticle, M = muscle, U = uninfected, I = infected, Inv = invisible organ, Null = no organ, G = Gilson, D= front midgut, T = middle midgut, B = back midgut (1) and (2) = repetition, GFE = Gilson fixative formulation *Splt*MNPV front midgut with Kaolin and EPMS, GME = Gilson fixative formulation *Splt*MNPV middle midgut with Kaolin and EPMS, GBE = Gilson fixative formulation *Splt*MNPV Back midgut with Kaolin and EPMS (dihilangkan karena sudah dimasukkan di judul table).

PIB SpltMNPV at 24 h incubation is already visible in the lumen of the midgut, peritrophic membrane midgut, epithelial midgut and trachea, while according Lucarotti (2012), based on data obtained from the image electron microscopy obtained PIB / OB

Comment [IA11]: Metode penyajian data kurang komunikatif.

Comment [A10]: Apa yang ini perlu dihilangkan?

Comment [A12]: Centang diganti positif c silang diganti negative ta pak? Tanda centa menunjukkan adanya PIBs pada organ tersebut.

mature from Balsam fir sawfly (*Neodiprion abietis*) *Gammabaculovirus* (Baculoviridae: NeabNPV) at 72 hpi seen in midgut epithelial cells of its larvae.

At an incubation period of 48 hours, PIB has spread to blood vessels, body fat and some muscle. At 72 hours of incubation, PIB has spread throughout the organ except the cuticle. PIB in the nervous system cannot be found because the cells are too difficult to be observed under light microscope magnification (400X). While Malpighi tubules in front of the midgut are not there so that PIB Malpighi tubule is only visible in the middle and back only.

This is in accordance with Prasad & Yogita (2006) which confirms that *S*lNPV 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 incubation. In this study, the epidermis of the cuticles was infected, but the cuticle layer, chitin, was free. The cuticles were the last layer which was not infected by the virus. The virus still needs the cuticles to collect the virus from reproduction (Rohrmann, 2008). Asri (2013), said that PIB SpltMNPV saw in all organs except the epithelium of the skin and nerve cells at 72 h incubation.

SpltMNPV PIBs can be seen after 2 days incubation on the lumen, peritrophic membrane, some of the midgut epithelium, and 4 days in the trachea, and Malpighi tubules, and blood vessels, PIBs are not visible in the muscle (Table 2). At 6 days, it extended into body fat.

Table 2. Judul table kok tidak ada pak?
Seharusnya (**The tissues and organs of S.litura larvae infected by *Splt*MNPV with photo-protectant such as kaolin and EPMS of 15% radiated for 12 hours, prepared by the method of imunohistochemistry, observable by the presence of PIB (polyhedral inclution bodies**

No	Sample	Inkubasi (hari)	Midgut dipotong secara	Infected organs							
				Lm	PM	EM	Tr	BV	TM	M	BF
1	Negative Control		1	-	-	-	-	-	-	-	-
2	In Vivo	2	CS	+	+	±	-	-	+	-	+
			LS	+	+	±	-	-	+	-	+
		4	CS	+	+	±	+	±	+	-	±
			LS	+	+	±	+	±	+	-	±
		6	CS	+	+	±	+	±	±	-	±
			1	+	+	±	+	±	±	-	±
3.	In vitro	2	CS	+	+	±	+	-	+	-	+
			LS	+	+	±	+	-	+	-	+
		4	CS	+	+	±	+	+	+	-	±

			LS	+	+	±	+	+	+	-	±
		6	CS	Prepupa (organs were purple and reddish brown)							
			LS	Prepupa (organs were purple and reddish brown)							
4.	EPMS 15%	2	CS	+	+	+	+	-	-	-	+
			LS	+	+	+	+	-	-	-	+
		4	CS	+	+	+	+	-	+	-	+
			LS	+	+	+	+	-	+	-	+
		6	CS	+	+	+	+	+	+	+	±
			LS	+	+	+	+	+	+	+	±

Note: CS = cross section, LS = longitudinal section, EPMS 15% = Ethyl-P Metoksinamat 15%, Lm = Lumen, TM = Tubulus Malpighi, PM = Peritrophic membrane, EM = Epithel Midgut, BF = body fat, Tr = Trachea, BV = Blodd vessel, U = uninfected, I = infected, Inv = invisible organ, Null = No organ, M = Muscle, + = ada PIB *Splt*MNPV, - = tidak ditemukan PIB *Splt*MNPV dan ± = ada dan tidak ada PIB *Splt*MPNV

*Splt*MNPV propagation mechanism beginning by oral infection, the virus get into the midgut lumen, penetrate into membrane peritrophic and basal lamina, infect cells in epithelial midgut and out towards hemolymph through tracheoblast of the trachea and spread to the tubular Malpighi, muscle, and fat cells as well some blood vessels (Passarelli, 2011).

S. litura which was on the second-day treatment of 6 days incubation entered prepupa stage, PIBs were not observed because the organ is undergoing the transition from larva to imago form the digestive system change food. Larvae are in phase eating solid objects, leafs, while the imago fed to liquid like honey. In the third treatment, *Splt*MNPV was formulated with Kaolin and EPMS 15% on a 6-day incubation, PIBs were in all observed organs i.e: lumen, peritrophic membrane, blood vessels, trachea, epithelial midgut and fat body and muscle. In observation using these immunohistochemical PIBs typical dark brown colour (Hayat, 2001) while in preparations paraffin purple PIBS (Elazar, 2001).?

Comment [IA13]: Ini hasil pengamatan sendiri atau bagaimana? Kenapa pakai sitas

Comment [A14]: Hasil pengamatan sendi tetapi diperkuat dengan teori yang mereka buat, bagaimana menyajikannya pak? Apa hayat dan elasarnya di delete saja karena sudah ada di penjelasan yang 3.2?

3.2 Infected S. litura Larvae Viewed Using Methods Paraffin and Immunohistochemistry

The tissue and organs, mainly in the midgut lumen had visible signs of *Splt*MNPV PIBs. Midgut lumen was light brown and purple midgut epithelial cells (Figure 1). Those colors can be seen that the primary antibody was dripped on midgut not find its substrate like polyhedrin which only present in the outer sheath of PIBs.

The specificity of the reaction at IHC marked by images of PIB *Splt*MNPV or part of a cell such as cell membranes is dark brown. The dark brown color of this occurs because of the specific reaction between antigen polyhedrin with primary antibody anti-polyhedrin given at the time of immunohistochemistry, then colored by dye chromogen dark brown chromogen (DAB) so that the cell or polyhedrin containing dark brown (Hayat, 2001). The linkage between the antigen polyhedrin with anti-polyhedrin (the primary antibody of polyhedrin) are then bound by biotinylated anti-chicken and blocked with peroxidase (H_2O_2)

to produce $H_2 + O_2$ so that by the time gave DAB dye made it darker than the uninfected cells. *Splt*MNPV was purple according to the opponent is Mayer's hematoxylin blue-purple with PIB *Splt*MNPV nucleic acids which absorb blue-purple dye derived from a hematoxylin (Elazar, 2001).

Infected organs were mainly midgut lumen and epithelium, trachea, and fat cells except muscle (Figure 1). There was midgut fluid in the lumen brown because of the polyhedrin was dissolved under lumen alkaline conditions. In paraffin slide, *Splt*MNPV PIBs were purple-blue since it absorbed the hematoxylin dye. The *Splt*MNPV PIB penetrated into larvae through the channel midgut lumen containing alkaline liquid. In the midgut epithelium, trachea, fat cells and cuticle had the same condition (Figure 2).

Comment [A15]: Tidak semua organ kondisinya lakalin yang alkaline hanya di lumen midgut. Bagaimana pak? Apa kalima ini dihilangkan saja?

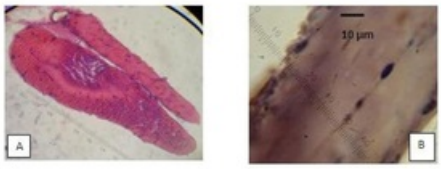
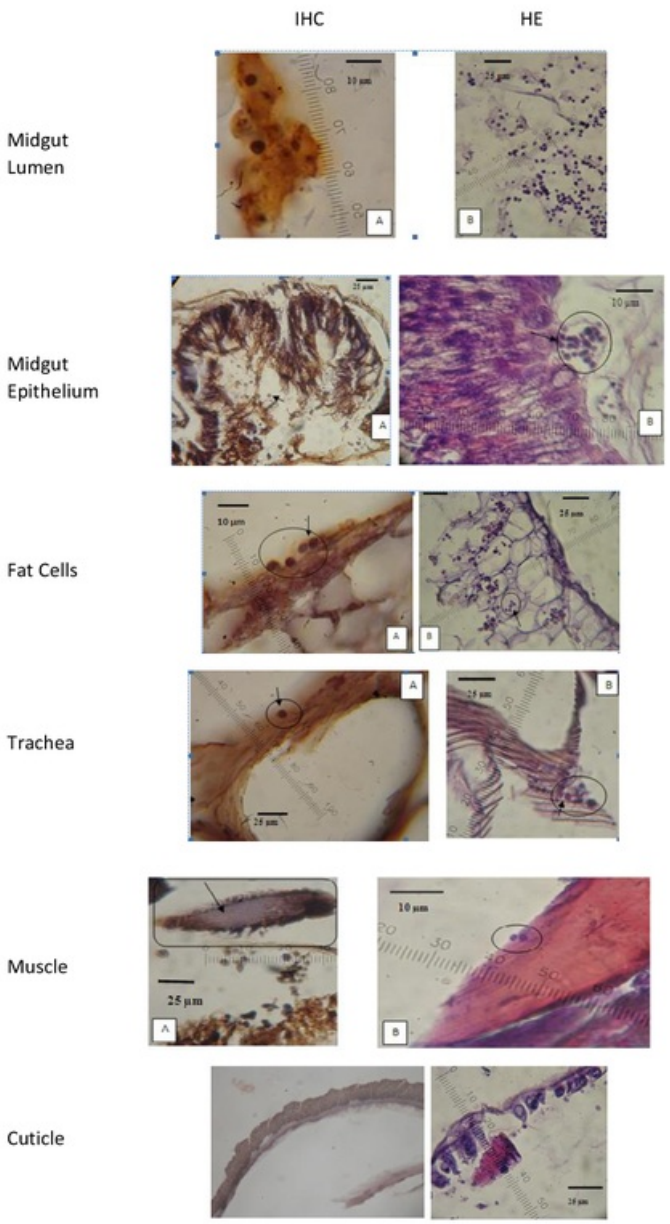


Figure 1. Uninfected muscle cells uninfected (A) stained with HE (400X) and (B) stained with IHC-DAB (1000X) cells did not have PIB but the edges showed a brown part containing polyhedrin.



Comment [IA16]: Perbesaran yang dipaka tidak homogeny sehingga kurang ideal untu komparasi. Lebih baik yang dipakai publikas pakai gambar yang semua perbesarannya sama.

Comment [A17]: Tidak semua gambar ya saya punya bagus dengan perbesaran yang sama makanya dibuat garis bar, tergantung besar preparat yang bisa diamati, dan kuali preparat yang kami punya, bagaimana pak? Kalau seadanya seperti ini, apa yang harus ditambahi?

Figure 2. Midgut lumen: (A) PIB *Splt*MNPV brown in midgut lumen (IHC) (1000X). B. *Splt*MNPV PIBs were purple-blue (400X). Midgut epithelium: (A) Damaged midgut epithelium cells had PIBs (back arrow) (400X). (B) PIB *Splt*MNPV blue-purple in the midgut epithelium (1000X). Fat cells: Trachea: tracheal epithelial cells infected mostly been separated so that no one in the picture is left only the cilia, their brown colour indicates that the polyhedrin expressed by cilia. Muscle: There is no *Splt*MNPV PIBs so purple (A) (400X), but on the edge of the muscle cells look brown. This suggests that the muscle cells already express polyhedrin in the cell membrane. In with HE staining (B) (400X), *Splt*MNPV PIBs seen at the edge of the muscle cells (blue). Cuticle: epithelial cuticle has been infected by *Splt*MNPV, but the chitin layer is not infected, (A) visible layers of chitin are still intact and (B) epithelial cuticle is broken.

4. Conclusion

*Splt*MNPV virus can be detected in the larvae of *S. litura* through IHC method, with the PIB are round-oval, dark brown and detected by HE staining blue-purple and scattered oval in lumen, epithelial midgut, fat cells and trachea in the formula photo-protectant the incubation time of 0, 1, 2 and 3 days. The virus is a type of *Spodoptera litura* nuclear polyhedrosis virus because it can specifically bind antigen polyhedrin existing between the outer cases with the anti-polyhedrin primary antibody.

Acknowledgement

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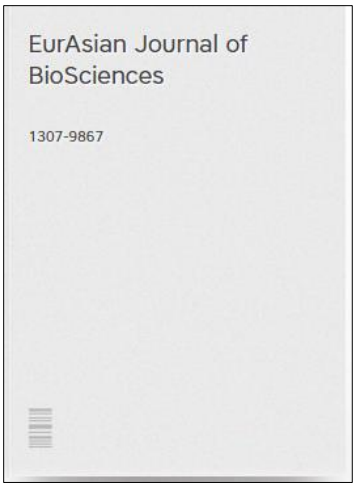

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We declare that the manuscript is original, has not been published before and are not currently being considered for publication elsewhere. We also declare that there is no competing financial interest.

We hope you find the manuscript suitable for publication in your journal and we look forward to hear from you.

Best Regards,
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Title of Paper: HISTOPATHOLOGY OF SPODOPTERA LITURA LARVA INFECTED BY MULTIPLE NUCLEAR POLYHEDRONS VIRUS (SPLTMNPV) IN PHOTO-PROTECTANT FORMULATION

Authors: Mahanani Tri Asri

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We would like to thank you for your submitted paper.

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