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Histopathology of *Spodoptera Litura* larva infected by Multiple Nucleopolyhedrosis Virus (*Splt*MNPV) in photo-protectant formulation

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Abstract

Spodoptera litura Multiple Nucleopolyhedrosis Virus (*Splt*MNPV) is a viral pathogen with the potential to control *Spodoptera litura* pests. The virus is effective in controlling *S. litura*, with mortality of 80–90%. Histopathologic preparations were made over the course of two ways using paraffin with hematoxylin-eosin staining and IHC methods. *Splt*MNPV was detected in *S. litura* and histopathologically characterized by the existence of polyhedral inclusion bodies of adistinctive color (dark brown), which had spread throughout the midgut lumen and epithelium. Tissues and organs were infected by *Splt*MNPV in vitro in a photo-protectant formula with incubation times of 0, 1, 2 and 3 days, using the paraffin method. Virus infected the lumen, midgut epithelium, epithelial skin, trachea, blood vessels, muscle cells and Malpighian tubules but not the cuticle or nerve cells. In addition, IHC revealed no *Splt*MNPV in muscle cells.

Keywords: Immunohistochemistry, *Spodoptera litura* Multiple Nucleopolyhedrosis Virus (*Splt*MNPV) (*Splt*MNPV), Photo-protectant

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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, 1997). One virus that is being developed as a bio-pesticide is *Spodoptera litura* multiple nucleopolyhedrovirus (*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 2009). 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 the virus' polyhedrin and primary antibody produced by the infected host and visualized by adding a secondary antibody labeled streptavidin-biotin with a brown chromogen (Mustafa et al. 2015).

MATERIALS AND METHODS

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.

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 and Nur 2009). As 10 larvae were incubated at room temperature (28-30°C) for 24 hours, 10 larvae for

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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).

Histological Preparation using Paraffin Method

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 *Splt*MNPV propagated in vitro using a modification of the paraffin method (Safdari *et al.* 2013). 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 microns 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 hematoxylin 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 with a cover glass and glued with entellan.

Histological Preparation using IHC

Samples were fixed with formalin buffer solution for 24 hours, then soaked in 70% ethanol for at least 24 hours. Dehydration is performed on an aspirator using alcohol solution series of 70%, 80%, 96% and absolute alcohol for 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-polyhedron from Sigma-Aldrich) was dripped on a specimen with a 1: 100 dilution (anti-polyhedron 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 (2 min), 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.

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 color. Cell or tissue with blue-purple indicated that it is not infected by *Splt*MNPV because not detected polyhedron protein.

RESULTS

Tissues and Organs of S.litura Larvae Infected by *Splt*MNPV in Photo-protectant Formula in the Body of S. litura Larvae Prepared through Paraffin and Immunohistochemistry Methods

Deployment *Splt*MNPV 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**.

Table 1. The tissues and organs of *S. litura* larvae infected by *Splt*MNPV through photo-protectant (kaolin and 20% of EPMS) which irradiated for 12 hours, and prepared through the paraffin method

No	Midgut location	Incubation (hours)	Repetition	Infected organs									
				Lm[a]	PM[b]	EM[c]	Tr[d]	BV[e]	TM[f]	N[g]	BF[h]	Ct[i]	M[j]
1	Negative Control	24	1	-	-	-	-	-	-	-	-	-	-
2	Front midgut	24	1	+	+	+	+	-	Null[k]	Inv[l]	-	-	-
			2	+	+	+	+	-	Null	Inv	-	-	-
	Middle midgut	24	1	+	+	+	+	-	+	Inv	+	-	-
			2	+	+	+	+	-	+	Inv	+	-	-
	Back midgut	24	1	+	+	+	+	+	+	Inv	-	-	+
			2	+	+	+	+	+	+	Inv	-	-	+
3.	Front midgut	48	1	+	+	+	+	+	Null	Inv	+	-	-
			2	+	+	+	+	+	Null	Inv	+	-	+
	Middle midgut	48	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Back midgut	48	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	Null	Inv	+	-	+
4.	Front midgut	72	1	+	+	+	+	+	Null	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Middle midgut	72	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+
	Back midgut	72	1	+	+	+	+	+	+	Inv	+	-	+
			2	+	+	+	+	+	+	Inv	+	-	+

[a]Lm = Lumen
[b]PM = Peritrophic membrane,
[c]EM = Epithelia Midgut,
[d]Tr = Trachea,
[e]BV = blood vessel
[f]TM = Tubules Malpighi
[g]N = Neuron
[h]BF = body fat,
[i]Ct = Cuticle
[j]M = muscle
[k] Null = no organ
[l] Inv = invisible organ
CE = cuticle epithelia

Table 2. 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 inclusion bodies)

No	Sample	Incubation (days)	Midgut section	Infected organs							
				Lm[a]	PM[b]	EM[c]	Tr[d]	BV[e]	TM[f]	M[g]	BF[h]
1	Negative Control			-[l]	-	-	-	-	-	-	-
2	In Vivo	2	CS[i]	+[m]	+	±[n]	-	-	+	-	+
			LS[j]	+	+	±	-	-	+	-	+
		4	CS	+	+	±	+	±	+	-	±
			LS	+	+	±	+	±	+	-	±
		6	CS	+	+	±	+	±	±	-	±
			LS	+	+	±	+	±	±	-	±
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%[k]	2	CS	+	+	+	+	-	-	-	+
			LS	+	+	+	+	-	-	-	+
		4	CS	+	+	+	+	-	+	-	+
			LS	+	+	+	+	-	+	-	+
		6	CS	+	+	+	+	+	+	+	±
			LS	+	+	+	+	+	+	+	±

[a]Lm = Lumen
[b]PM = Peritrophic membrane,
[c]EM = Epithelia Midgut,
[d]Tr = Trachea,
[e]BV = blood vessel
[f]TM = Tubules Malpighi
[g]M = Muscle
[h]BF = body fat,
[i]CS = cross section
[j]LS = longitudinal section,
[k]EPMS 15% = Ethyl-P Metoksinamat 15%
[l]+ = PIB found *Splt*MNPV
[m]- = PIB not found *Splt*MNPV
[n]± = found or not found PIB *Splt*MPNV

*Splt*MNPV 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.

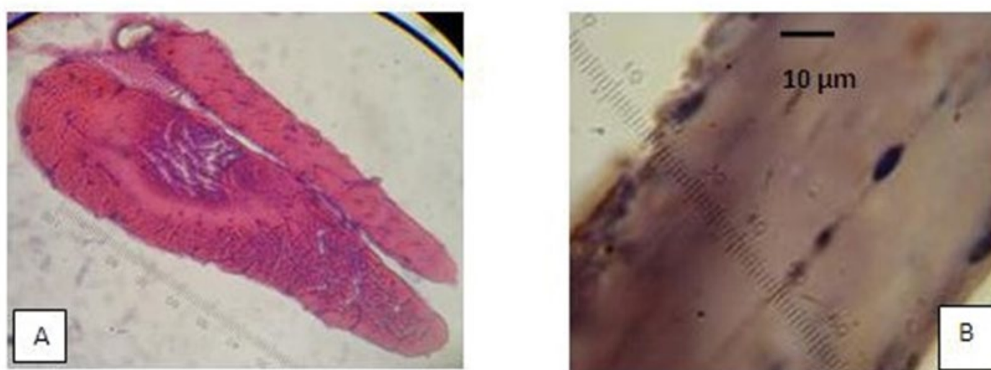

Fig. 1. Uninfected muscle cells uninfected (a) stained with he (400x) and (b) stained with ihc-dab (1000x) cells did not have pib but the edges showed a brown part containing polyhedron

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 (**Fig. 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.

DISCUSSION

Tissues and Organs of *S.litura* Larvae Infected by *Splt*MNPV in Photo-protectant Formula in the Body of *S. litura* Larvae Prepared through the Method of Paraffin and Immunohistochemistry

After 24 hours of incubation, PIB SpltMNPV was visible in the lumen of the midgut, peritrophic membrane midgut, epithelial midgut and trachea. Using electron microscopy, Lucarotti (2012) obtained mature PIB / OB from the Balsam fir sawfly (*Neodiprion abietis*) *Gammabaculovirus* (Baculoviridae: NeabNPV) at 72 hpi; these were seen in the 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 *Sl*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 grow pale; movement slowed. In this incubation, almost all organs in the midgut, anterior, middle, or posterior, were already infected, except the cuticles. 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 (Rohmann, 2008). Asri *et al*. (2013), said that PIB *Splt*MNPV saw in all organs except the epithelium of the skin and nerve cells at 72 h incubation.

Infected *S. Litura* Larvae Viewed Using Methods Paraffin and Immunohistochemistry

*The Splt*MNPV propagation mechanism begins with oral infection. The virus enters the midgut lumen, penetrates the peritrophic membrane and basal lamina, infects cells in the epithelial midgut and spreads out towards the hemolymph through tracheoblasts in the trachea. The virus then spreads to the Malpighian tubules, muscle, and fat cells as well some blood vessels (Jawad Hassan et al. 2014).

S. litura which was on the second-day treatment of 6 days incubation entered pre-pupa 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, fat and muscle).

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 polyhedron with primary antibody anti-polyhedron given at the time of immunohistochemistry, then colored by dye chromogen dark brown chromogen (DAB) so that the cell or polyhedron containing dark brown (Hayat, 2001). The linkage between the antigen polyhedron with anti-polyhedron (the primary antibody of polyhedrin) are

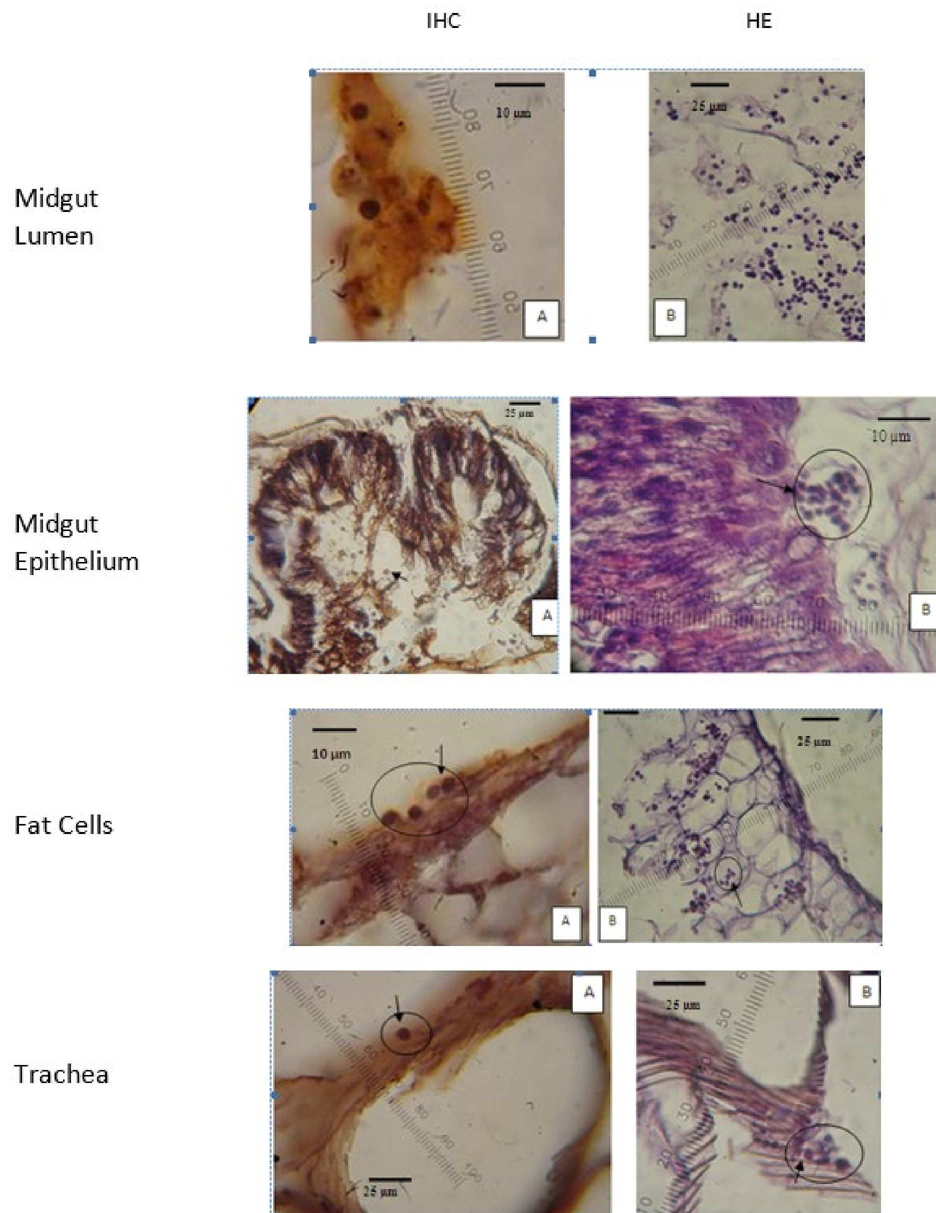

Fig. 2. Midgut lumen: (A) PIB *Splt*MNPV brown in midgut lumen (IHC) (1000X). B. *Splt*MNPV PIBs were purple-blue (400X). Midgut epithelium: (A) Damaged midgut epithelium cells had PIBs (black arrow) (400X). (B) PIB *Splt*MNPV blue-purple in the midgut epithelium (1000X). Fat cells: Trachea: tracheal epithelial cells infected mostly been separated so that no one in the picture is left only the cilia, their brown color indicates that the polyhedrin expressed by cilia.

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 *et al*. 2001).

Infected organs were mainly midgut lumen and epithelium, trachea, and fat cells except muscle (**Fig. 1**). There was midgut fluid in the lumen brown because of the polyhedron 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 (**Fig. 2**).

We conclude that *Splt*MNPV virus can be detected in the larvae of *S. litura* using IHC method. The PIB appear as round-oval, dark brown structures detected by HE staining as blue-purple. These structures appear as scattered ovals in the lumen, epithelial midgut, fat cells and trachea after immersion in photo-protectant formula for 0, 1, 2 or 3 days. The virus is a type of *Spodoptera litura* Multiple Nucleopolyhedrosis Virus (*Splt*MNPV) because it can specifically bind antigen polyhedrin

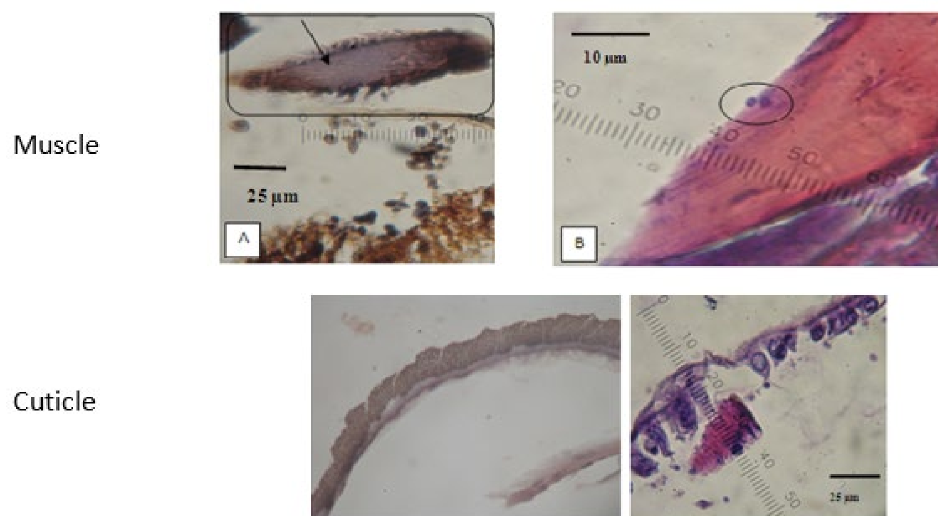

Fig. 2 (continued). Muscle: There is no *Splt*MNPV PIBs so purple (A) (400X), but on the edge of the muscle cells look brown. This suggests that the muscle cells already express polyhedron in the cell membrane. In with HE staining (B) (400X), *Splt*MNPV PIBs seen at the edge of the muscle cells (blue). 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

existing between the outer cases using anti-polyhedrin primary antibody.

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AUTHOR CONTRIBUTION

The MTA was done to conceived and designed the exriment, wrote the manuscripts, agree with the manuscripts result and conclusion. The MTA also made the critical revisions and procced final revision. MTA was reviewed and approved of the final manuscripts.

DISCLOSURES AND ETHICS

The author declare there are no conflicct of interest. This paper not recognized for publication in another journal and submit for Virology: Research and Treatment only.

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