EXPERIMENTAL INFECTIONS OF MILKY HEMOLYMPH DISEASE IN SPINY LOBSTER
Panulirus homarus

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ABSTRACT

Milky hemolymph disease of spiny lobster (MHD-SL) is categorized as the most destructive disease in farming spiny lobster. Therefore, it is required to investigate the routes of milky disease infection in spiny lobster as a basic knowledge in order to prevent milky disease transmission. The aim of the present study was to perform an experimental infection of milky disease in spiny lobster *Panulirus homarus*. Experimental infection of milky disease was carried out by several modes of infection which were injection, immersion and per os exposure. Injection of each 0.2 mL undiluted and diluted hemolymph from the diseased lobster resulted in a cumulative mortality of 100% at 15 days post-infection (dpi), and 75% at 16 dpi, respectively. Experimental infection through water immersion caused in a cumulative mortality of 50% at 7 dpi. In contrast, no mortality was observed in per os exposure as well as in control groups. Results of this experimental study provided evidence for horizontal transmission of MHD-SL among *P. homarus*. Histopathological analysis exhibited that there were masses of Rickettsia-like bacteria (RLB) in the connective tissues of the gill, hepatopancreas, gonad, midgut, and muscle tissues of the affected lobsters. Mass of RLB was not only found in the moribund lobsters but also in the surviving lobsters with milky hemolymph appearance.

KEYWORDS: experimental infection; milky hemolymph disease of spiny lobster (MHD-SL); *Panulirus homarus*; spiny lobster

INTRODUCTION

A high priority for disease prevention and control in farming of spiny lobster has been set for several diseases including milky hemolymph disease of spiny lobsters (MHD-SL), red body, and black gill disease (Callinan & Corsin, 2009; Thuy *et al.*, 2010; Vu *et al.*, 2014). Among these diseases, milky disease is reported as the most devastating disease in farming of spiny lobster, which was reported first in Vietnam (Lightner *et al.*, 2008). The annual mortalities ranged from 50% to 100% in some regions, at the peak of the disease outbreaks in 2006 and 2007 (Callinan & Corsin, 2009).

In Indonesia, MHD-SL outbreaks were reported almost year-round in Lombok from 2012 to 2016, and in Pegametan Bay, North Bali in 2016. Morbidity caused by MHD-SL in Lombok reached up to 100%, and mortality was up to 60%-80% (pers. comm. with Joko Santosa). The worst outbreaks occurred in 2012 when most cultured lobsters in farming areas in Lombok were wiped out by MHD-SL. In Pegametan Bay, mortality of the farmed lobsters in net cages was also reported up to 80% from July 2016 to January 2017 (unpublished data). The economic losses due to the outbreaks were considerably high.

Considering the significant effects of milky disease in farming of spiny lobster, and limited available information of the disease in Indonesia, this study was conducted to perform an experimental infection of MHD-SL in *P. homarus* through several modes of infection. Findings in this study provide basic knowledge and understanding on milky disease for developing further practical prevention and sustainable control of the disease.

MATERIALS AND METHODS

Experimental Infection of Milky Disease

MHD-SL-infected lobsters used for experimental infection were collected from net cages in Pegametan

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Bay, North Bali during the outbreaks in November 2016, and healthy lobsters were obtained from Jembrana, South Bali. Confirmation for both milky disease infection and health status of the lobsters (free of MHD-SL) was assessed by PCR analysis. DNA was isolated from the muscle tissues using 10% chelex in TE buffer pH 8, in 1.5 mL microtubes. DNA was extracted by adding 200-250 μL chelex 10% into the microtubes and homogenized finely; then, 5-7.5 μL pK (20 mg/mL) was added and incubated at 55°C for 2.5 hour. The second incubation was done at 89°C for 8 minute. Finally, it was then centrifuged at 13.000 rpm for 5-7.5 min. and the supernatant was collected and stored at -20°C until used for PCR analysis. DNA extraction was completed following Möhlenhoff *et al.* (2001) with slight modification.

PCR analysis was performed using specific primers for RLB which were 254F and 254R for the expected PCR product (amplicon) size of 254 bp. The oligonucleotide sequences of the 254F primer were 5′-CGA-GGA-CCA-GAG-ATG-GAC-CTT-3′ and 254R primer were 5′-GCT-CAT-TGT-CAC-CGC-CAT-TGT-3′ (OIE Aquatic Animal Disease Cards, 2007; Thuy, 2011; DAFF, 2012; Lasmika, 2015). The reaction mix for PCR contained 2x KAPA 2G Fast Ready Mix, primer 254F (10 μM), primer 254R (10μM), NFW, and DNA template with the total reaction of 10 μL. DNA amplification was done in an automatic thermal cycler (Personal Thermalcycler, Biometra, 96 well fast). The thermal cycling program consisted of an initial denaturation at 96°C for 3 min.; followed by 30 cycles of denaturation at 96°C for 15 sec., annealing at 65°C for 30 sec., and extension at 72°C for 15 sec.; and the last step was a final extension at 72°C for 1 min. (modified from Koesharyani *et al.*, 2016). The PCR products were maintained at 4°C at the end of the cycling. The products were then separated by electrophoresis in a 1.5% agarose gel in 1X TBE buffer (trisborate-EDTA) containing 0.05 μL/mL of red safe DNA stain. The targeted product was measured using a 100bp DNA ladder under a UV-Transilluminator.

The experimental infection study was done for healthy lobster (mean weight = 141.15 ± 25.85 g) in 100 L tanks. The tested lobsters were assigned to 1 of 6 experimental treatments, four lobsters each: 1) injected with 0.2 mL undiluted infected hemolymph, 2) injected with 0.2 diluted (1:4) infected hemolymph, 3) immersed in 80 L sea water administered with 12.5 mL of undiluted hemolymph, 4) fed frozen (-20°C) heavily infected *P. homarus* tissue (per os exposure treatment), 5) injected with 0.2 mL PBS (as the negative control for injection exposure), and 6) fed trash fish (as the negative control for per os exposure and immersion treatment). The infected hemolymph was

diluted with sterile PBS. The injection was done in the first abdominal segment using a sterile 1 mL-syringe fitted with a 25-gauge 16 mm needle. The second addition of the infected hemolymph (5 mL) in the immersion method was done at day-6 after the first addition. In the per os exposure treatment, the lobsters were fed heavily infected *P. homarus* tissue from day-1 to day-6 with a total of 54.5 g infected tissue (Table 1). At day-7 afterward, the tested lobsters were fed trash fish due to the limitation of the infected tissue. The diseased lobster used as feed was confirmed for severe infection of MHD-SL by PCR analysis (Figure 1), and kept at -20°C until use. Trash fish was used to feed lobsters in other treatments. All lobsters were fed twice daily to satiation at 08:00 and 15:00. Signs of disease, mortality and moribund lobsters were observed and recorded daily until the experiment was terminated (33 days). Percentage of mortality was calculated as the number of lobsters died divided by the initial density. Cumulative mortality is the sum of mortality during the experiment (Hunt *et al.*, 1985). Dead lobsters were frozen for PCR analysis as described above, whereas moribund lobsters were preserved in Davidson's fixative for histopathological examination.

Histopathological Examination

Both milky disease-free lobsters (control groups) and moribund infected lobsters from the experimental infection study were collected for histopathological examination. Samples of hepatopancreas, muscle, gill, midgut, and gonad were preserved in bouin's fixative for histopathological examination. The samples were then transferred into 70% alcohol solution before tissue processing. The tissues were placed in a cassette embedding for tissue processing which was done through several steps. First, the tissues were dehydrated by immersing it in graded ethanol (70%, 80%, 90%, 95%, and 100%), and then xylene solution to allow complete penetration of wax into the tissues. Following that, the tissues were embedded in ParaPlast Plus paraffin, sectioned at 5 μm thickness, mounted onto microscope slides, and air dried overnight prior staining with haematoxylin eosin (HE) (Nunan *et al.*, 2010). Histopathological examination of the processed tissues was performed using standard light microscope.

Data Analysis

The data of mortality pattern resulted from the experimental infection was presented in a table and analysed descriptively. The results of the histopathological examination were also shown in a table, and histopathological changes in tissues of the infected lobster were described in figures.

Table1. The amount (g) of milky diseased *P. homarus* tissues used to feed the tested lobsters in the per os exposure treatment from day-1 to day-6

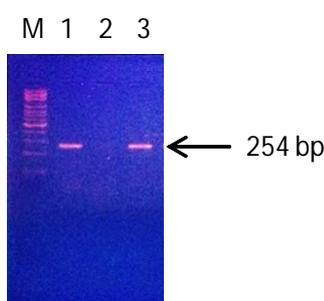| Day	Feed (g)
1	11
2	10
3	9
4	10
5	9
6	5.5
Total	54.5

Figure1. Electrophoresis banding patterns of milky diseased and healthy lobsters following PCR using a rickettsial primer set that amplifies a unique 254 bp region within the rickettsial DNA gene. M: 100 bp DNA ladder; lane 1: positive control; lane 2: healthy lobster; lane 3: heavily infected lobster that used as feed and its hemolymph was used for experimental infection.

RESULTS AND DISCUSSION

Experimental Infection Study of Milky Disease

All the experimentally infected lobsters showed milky hemolymph and exhibited a similar disease symptom and pattern of mortality in naturally infected lobsters. The mortality pattern of experimentally infected lobsters was found to vary with the method of disease introduction. Mortalities began at four days post-infection (dpi) when the pathogen was administered by intramuscular injection of 0.2 mL undiluted and diluted hemolymph. A cumulative mortality of 100% was observed within 15 days in lobsters injected with 0.2 mL undiluted infected hemolymph, and mortality of 75% occurred within 16 days in lobsters injected with diluted hemolymph (Table 2). It was clear that the healthy lobster could be infected by MHD-SL by intramuscular injection of 0.2 mL undiluted and diluted infected hemolymph. This infection was confirmed by PCR analysis (Figure 2). This finding indicated that dilution of hemolymph with sterile PBS at

a ratio of 1:4 caused in a relatively high mortality (75%) when compared to undiluted hemolymph which resulted in mortality of 100%. Mortality rate 100% was also obtained in a previous study when lobsters *P. ornatus* were injected with unfiltered hemolymph from the milky diseased lobster. The hemolymph was diluted with saline solution at a ratio of 1:3 and injected into healthy lobsters at a dose of 0.1 mL/lobster and 0.3 mL/lobster (Hoa *et al.*, 2009).

Furthermore, the present study found that milky disease could be transmitted through water immersion. Lobsters exposed to milky disease by water immersion experienced cumulative mortality of 50% within seven days post-infection (Table 2). The mortality did not occur after seven days because the addition of the infected hemolymph into the rearing tank was stopped. The additions of the infected hemolymph were only two times, 12.5 mL into the 80 L rearing water on day-1 and 5.0 mL on day-6. The two remaining lobsters were healthy and finally one of the tested lobsters moulted at the end of the ex-

Table 2. Details of mortality pattern recorded during the experimental infection of milky disease in spiny lobster *P. homarus* by several modes of infection. The mortality pattern indicated that the experimental infection by injection caused severe mortality than immersion method

Modes of infection	Mortality at day post infection (dpi)							Cumulative mortality (%)	Experimental extension (dpi)			Total time of experiment (days)
	0	4	5	7	10	15	16		17	28	33	
Injection of 0.2 mL undiluted hemolymph (n=4)	0	2	1*	0	0	1	0	100				16
Injection of 0.2 mL diluted hemolymph (n=4) (Tank A)	0	1	0	0	0	1	1	75	1**			17
Injection of 0.2 mL diluted hemolymph (n=4) (Tank B)	0	1*	0	0	1	0	0	50	2**			17
Water exposure (n=4)	0	0	0	2	0	0	0	50	0	0		28
Per os exposure (n=4) (Tank A)	0	0	0	0	0	0	0	0				28
Per os exposure (n=4) (Tank B)	0	0	0	0	0	0	0	0		1**		28
Injection of 0.2 mL sterile PBS (control) (n=4)	0	0	0	0	0	0	0	0			1***	33
Fed trash fish (control) (n=4)	0	0	0	0	0	0	0	0				33

Note: Dissection for histopathological examination:
* : Moribund lobster with symptoms of milky disease
** : The surviving lobster showing milky hemolymph appearance
*** : The control lobster with no signs of milky disease

periment. PCR analysis showed that the dead lobsters were infected by milky disease although the DNA band was not so clear compared to those infected by intramuscular injection of affected hemolymph (Figure 2).

Transmission of milky disease was also happened in per os exposure treatment. Lobster fed with infected tissue survived until 16 days. However, one lobster exhibited clinical symptom resembling milky hemolymph appearance (Figure 3). This result demonstrated that feeding with infected tissue could affect healthy lobster which was indicated by milky hemolymph appearance. This finding suggests that the natural route of milky disease introduction could occur through feeding infected lobsters. A further observation showed that milky hemolymph appearance decreased when the experiment was extended until day-28 (Figure 3). Although milky hemolymph appearance was lesser, the lobster was confirmed to be affected by milky disease based on PCR analysis (Figure 2). The decrease in milky appearance probably suggested that the lobster could recover from the infection when feeding with infected tissue was terminated. In addition, the concentration of RLB in the lobster's muscle was probably lower than those in hemolymph. Another reason may be that RLB in the muscle had been weakened due to storage in temperature of -20°C. Therefore, further study is required
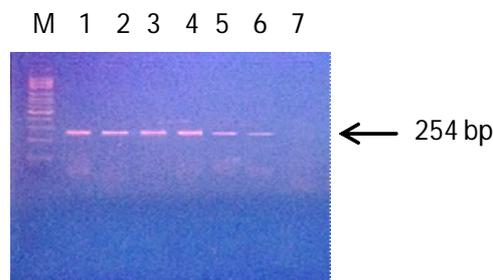

Figure 2. PCR results showed that experimental infection of milky disease by injection of 0.2 mL undiluted hemolymph (lane 2), 0.2 mL diluted hemolymph (lane 3 and 4); by water immersion (lane 5) and by per os exposure (lane 6) were successfully transmitted milky disease from the diseased lobsters to the healthy lobsters. In contrast, no DNA band was detected in the control group (lane 7). M: DNA ladder 100 bp marker; lane 1: positive control. DNA was extracted from the muscle tissue of the dead lobsters collected during the experiment.
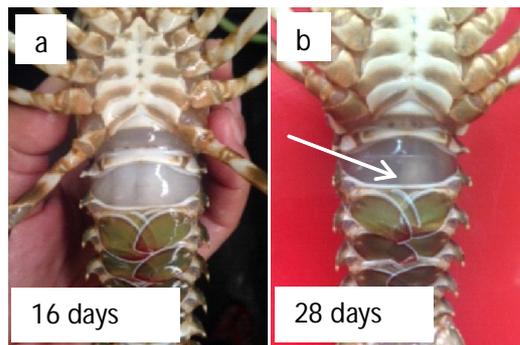
Figure 3.   Milky hemolymph appearance in lobster *P. homarus* fed infected tissues (per os exposure) after 16 days (a), and decreasing of milky hemolymph appearance (white arrow) after 28 days of rearing (b).

to investigate the effects of storage on the pathogenicity of RLB causing MHD-SL.

There was no mortality in the control groups (fed trash fish and injected with sterile PBS). The surviving animals in the control groups appeared healthy and did not reveal any signs of milky disease. Experimental lobsters showed normal feeding and activity until the end of the experiment (33 days) (Table 2) and confirmed free of milky disease by PCR (Figure 2, lane 7).

Milky disease transmission had also been experimented on other decapods crustacean. Intrahaemocoelic injection of 100-300 μL hemolymph from infected European crabs (*Carcinus maenas*) into healthy individuals caused in mortalities of 100% within only 24 hours post injection (Eddy *et al.*, 2007). In addition, experimental transmission of RLB from *P. monodon* to *P. vanamei* by injecting of RLB-infected frozen (-70°C) *P. monodon* tissue homogenate into the SPF *P. vannamei* resulted in a cumulative mortality of 97%. Contrastingly, per os exposure of frozen RLB-infected *P. monodon* to the SPF *P. vannamei* resulted in 93% survival rate at 21 dpi when the experiment was terminated (Nunan *et al.*, 2003b). Then, they concluded that per os exposure of infected *P. monodon* tissue to SPF *P. vannamei* did not transmit disease. In the study by Nunan *et al.* (2003b), the SPF *P. vannamei* was fed 2.5% body weight (4.5 g of the frozen *P. monodon* RLB-infected tissue collected during rickettsial disease outbreak in Madagascar). Feeding with infected tissue was done twice a day, for only two days. A similar study by Eddy *et al.* (2007) also showed that there was neither mortality nor white coloration of hemolymph when the disease-free European crabs fed hepatopancreas from milky diseased crabs

for up to eight weeks post-challenge. Their results were contradictory to the present study. Our results showed that milky disease could be transmitted through per os exposure which was confirmed by PCR analysis. Their conclusion should be revised, because the feeding rates examined in their experiment, might not adequate to result in the introduction of the disease.

Histopathological Examinations

Histopathological analysis showed the presence of masses of RLB in the gill, hepatopancreas, gonad, midgut, and muscle tissues (Table 3). Infection of MHD-SL was not only found in the moribund lobster but also in the surviving lobsters that were exhibited milky hemolymph appearance, white or pale coloration of gill and hepatopancreas. In contrast, there was no mass of RLB in the hepatopancreas, gill, gonad, midgut, and muscle of lobster in the control groups (Table 3).

Histopathological examinations confirmed a severe systemic RLB infection in a variety of tissues in the affected *P. homarus*. RLB fulfilled connective tissue cells and fixed phagocytes, causing enormous hypertrophy in the hepatopancreas, muscle, gill, gonad, and midgut. The infected connective tissue typically appeared to contain basophilic (with H&E) granular-like masses (rod shape) from small size to large masses (blue *) (Appendix 1, 2, 3).

The histopathological conditions of infected spiny lobsters, shrimp, and crabs were remarkable and mostly identical. A similar disease with similar gross and histopathological lesions particularly in connective tissues had been reported in captive-wild European shore crab (*Carcinus maenas*) (Eddy *et al.*, 2007)

Table 3. Histopathological examinations on hepatopancreas, gill, midgut, muscle, and gonad tissues of sub-adult spiny lobster *P. homarus* resulted from experimental infection of milky disease by several modes of infection. D: positive detection (showing mass of RLB); ND: not detected. NA: not available

Modes of infection	Histopathological examinations				
	Hepatopancreas	Gill	Mid gut	Muscle	Gonad
Injection of 0.2 mL undiluted hemolymph; moribund lobster	D	NA	D	D	D (♂)
Injection of 0.2 mL diluted hemolymph; surviving lobster with milky hemolymph;					
Sample-1	D	ND	NA	D	D (♂)
Sample-2	D	D	ND	ND	D (♂)
Sample-3	D	D	NA	ND	D (♂)
Injection of 0.2 mL diluted hemolymph; moribund lobster	D	ND	ND	ND	ND (♂)
Per os exposure; surviving lobster with milky hemolymph appearance	ND	NA	NA	ND	D (♀)
Control	ND	ND	ND	ND	NA

and in cultured black tiger shrimp (*Penaeus monodon*) (Nunan *et al.*, 2003a). The lesions caused by RLB infection of the respective RLB were essentially similar, as shown by sections through the HP and gills (Nunan *et al.*, 2010).

The causative agent of milky disease appeared to be replicated mainly in the fixed phagocytes observed in the hepatopancreas. The early stage of the replication was stated occurred within a large vacuole or in the cytosol of the fixed phagocytes and then released into the surrounding area. Subsequently, the remaining fixed phagocytes were enclosed by large amounts of bacteria comprised within the extracellular space adjoined by the uncommon perforated basement membrane. It was assumed that the bacteria circulate through these perforations to get into the general haemocoel, giving the characteristic milky colour symptomatic of this disease (Eddy *et al.*, 2007).

Results of the histopathological examinations in this study demonstrated that RLB did not only infect the hepatopancreas and gill, but also the muscle, gonad, and midgut. However, in the previous study of rickettsia-like infections in crab, *C. maenas* showed that there was no evidence of the presence of RLB in mid gut, gonad, and muscle tissues. It was unclear whether the RLB was capable of parasitizing within midgut epithelial cells of the crab (Eddy *et al.*, 2007).

CONCLUSION

The experimental infection indicated that milky disease could be transmitted horizontally from the diseased lobsters to the healthy lobsters by injec-

tion of undiluted and diluted hemolymph of the infected lobsters, by water immersion, as well as per os exposure. Histopathological analysis demonstrated that mass of Rickettsia-like Bacteria (RLB) was detected in the hepatopancreas, gill, midgut, gonad, and muscle tissues of the infected lobsters. The presence of RLB was not only observed in the moribund lobster, but also in the surviving lobsters with milky disease symptoms.

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Appendix 1. Histopathological changes in tissues of sub-adult spiny lobster *P. homarus* affected by milky hemolymph disease of spiny lobster (MHD-SL). (A) normal hepatopancreatic tubules in cross section showing fixed phagocytes (ff) surrounding an arteriole (black arrow) in the hemal sinus (hs). bm= basement membrane, rc= R-cell, h= free haemocytes, L= lumen of tubules. Scale bar, 30 μm, haematoxylin/eosin stained; (B) cross-sectioned hepatopancreatic tubules of a naturally infected lobster revealing the necrotic tubular structure (blue arrow), and darkly H&E stained granular-like masses of Rickettsia-like Bacteria (blue *) that are prominent in an arteriole in the haemal sinus. Scale bar= 30 μm; (C) hepatopancreatic tubules of an experimentally infected lobster in cross section showed the same necrotic tubular structure and mass of RLB (blue *) as the naturally infected lobsters; (D) high magnification of mass of RLB (blue *) in the hemal sinus of the hepatopancreatic tubule that appeared rod in shape, H&E stained
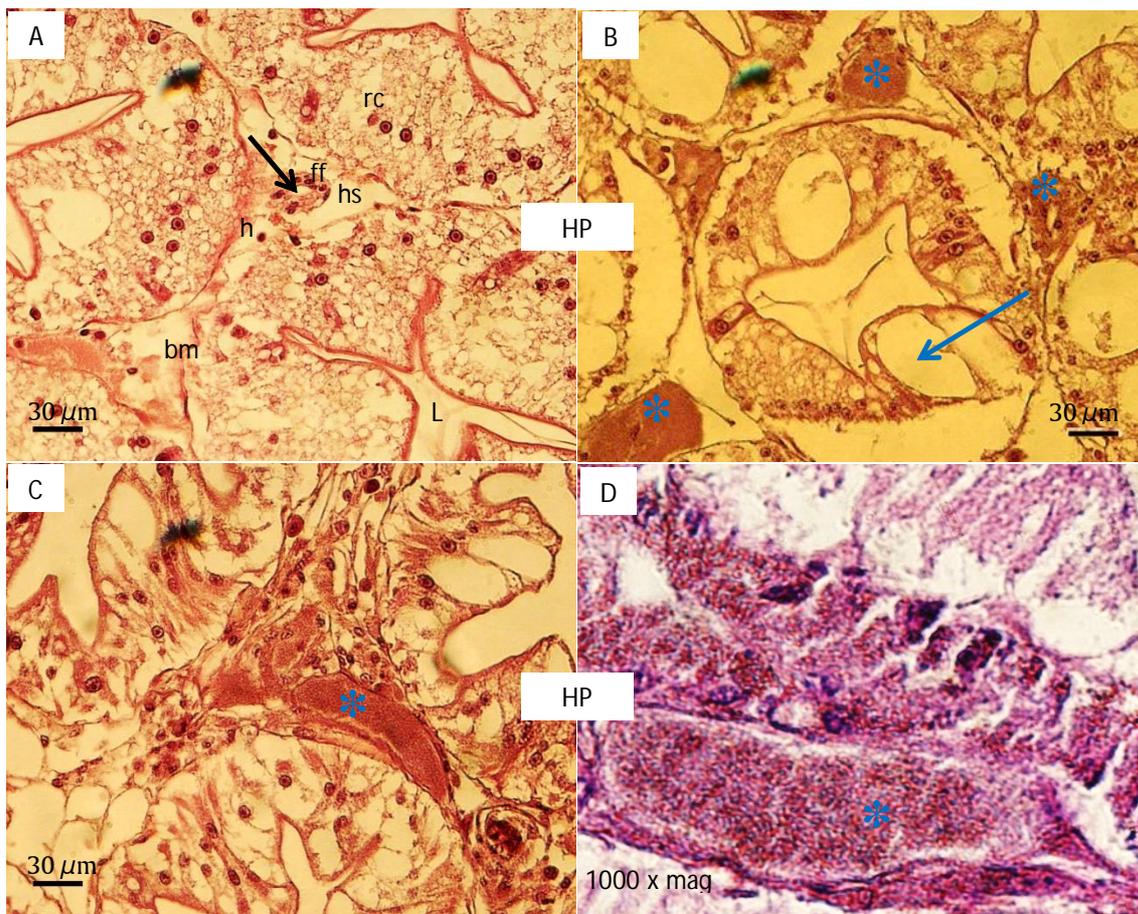
Appendix 2. Histopathological changes in tissues of sub-adult spiny lobster *P. homarus* affected by milky hemolymph disease of spiny lobster (MHD-SL). (A) longitudinal section of normal skeletal muscle (ms) with the hemal sinus (hs) passing longitudinally across the section, and fixed phagocytes (ff). Scale bar= 30 μm; (B) milky disease infected skeletal muscle displaying heavy infestation of RLB (blue *). Scale bar= 30 μm; (C) the normal structure of the gill (G) in cross section with spongy connective tissue (con), gill lamella (lam), and empty hemal sinus (hs). Scale bar= 100 μm; (D) longitudinal sections through the gills with MHD-SL. Large amounts of RLB appeared in granular-like masses (*) fulfilling in the hemal sinus. H&E stain, scale bar= 60 μm
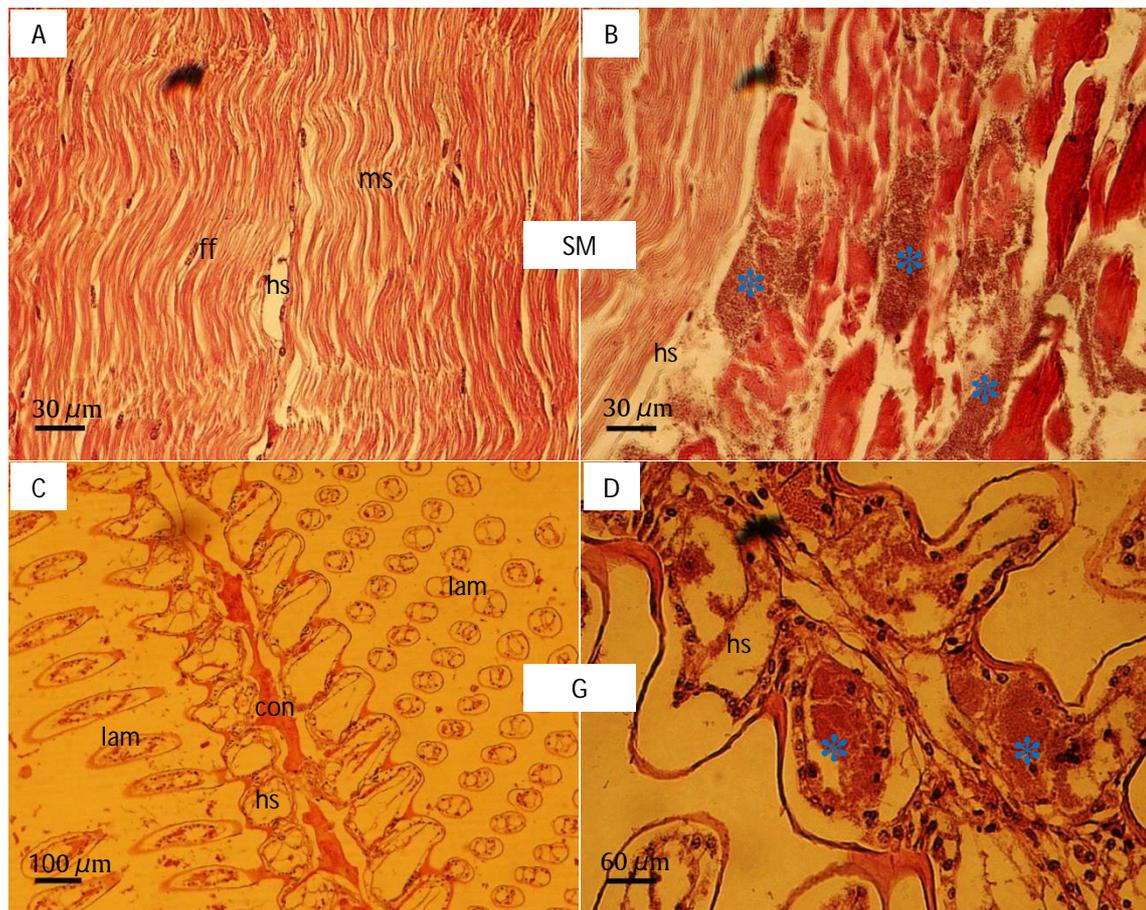
Appendix 3. Histopathological changes in tissues of sub-adult spiny lobster *P. homarus* affected by milky hemolymph disease of spiny lobster (MHD-SL). (A) the normal ejaculatory duct of male gonad exhibiting epithelial cells (ec), brush border (bb) skeletal muscle fibre (ms) and fibrous connective tissue (fcon). Scale bar= 100 μm; (B) milky diseased ejaculatory duct indicated by darkly H&E stained RLB (*) interspersed among fibrous connective tissue. Scale bar= 30 μm; (C) normal midgut (MG) exhibiting columnar epithelial cells (ec) with brush border (bb) supported on a basement membrane (m) overlying small muscle fibrils (m) and fibrous connective tissue (fcon). Brush border located at the apical end of the epithelial cells. L= lumen. Scale bar= 30 μm; (D) milky disease affected midgut indicated by darkly H&E stained granular-like masses (*) interspersed among muscle fibrils. Scale bar = 30 μm.