Study on milky haemolymph diseases infection in wild and cultured of spiny lobster, *Panulirus homarus* in Indonesia

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Abstract. Milky Haemolymph Disease in Spiny Lobster (MHD-SL) is the most pathogenic diseases in spiny lobster (*Panulirus homarus*). Research on MHD-SL infection has not been undertaken in Indonesia. Therefore, present study aims to determine the infection of MHD-SL lobster. In 2016 a total of 240 lobsters for 30 each both from wild and cultured sample were collected from four locations (Candi Kusuma Bay of Bali Island, Gerupuk, Awang, and Telong-Elong Bays of Lombok Island) and in 2019, 50 lobster samples were collected for artificial infection study. While in January 2020, another 40 lobsters were collected from 2 different sites of culture (coastal and offshore cages) within Telong Elong Bay to determine infection of MHD-SL and for transmission study. The MHD-SL diseased was first check by clinical sign and confirmed by PCR-DNA molecular with specific primer of 254 bp. An experimental infection of MHD-SL was carried out by injection and cohabitation. The result showed that infected MHD-SL lobster shows inactive, loose appetite to eat, reddish and white colour of abdomen then moribund and all positive by PCR test. MHD-SL was found only in cultured lobster on the cages located at coastal water and no in the cages located at offshore within Telong-Elong Bay. In the experiment of artificial infection, either by injection or cohabitation, shows clinical sign of MHD-SL appeared at 8 days and all died after 14 days for both treatments. The present study approved that MHD-SL is pathogenic agent belonged to Rickettsia-like bacterium and infection occurred by horizontal transmission.

1. Introduction

Aquaculture is promising aquatic business in Indonesian industry. The Government of Indonesia through Ministry of Marine Affairs and Fisheries mentioned that lobsters that belonged to species of *Panulirus homarus* and *Panulirus ornatus* are priority species for aquaculture development. The development of economically, environmentally and sustainable aquaculture production will be important for many sectors in Indonesia and for global food security. In recent years, spiny lobster (*Panulirus sp*) is growing commercial value in Indonesia and all over the world because of market demand [1]. Development of spiny lobster culture is increasing interest all over the world, as demand increases and capture fisheries supply decreases. Tropical spiny lobster *Panulirus homarus* and *Panulirus ornatus* are the most interest candidates for commercial aquaculture in Indonesia [2] due to both species demand consistent and strong interest among international seafood markets. Lobster culture expanded considerably in Indonesia [2], [3].

Diseases are a crucial factor which inhibits the expansion of aquaculture. Various studies have mentioned on biological data such as source of wild seeds, larval, puelurus rearing technology, growth
in marine cages and tanks and feeds that contribute to further development [3], [5]. There are surprisingly no diseases reported from these lobsters culture in Indonesia. A wide variety of bacteria and virus agents have been recorded as significant problems in lobster aquaculture [6]. However, recent attempts to culture lobsters have failed because of mortalities associated with bacterial infections [7]. It was suspected that fresh foods (fishery by catch, mollusks and decapod crustaceans) fed to net-cage reared lobsters in Viet Nam are the source of the bacterial agent of the disease [8, 10]. In Indonesia, major gaps still exist in the knowledge of spiny lobster diseases. So, there is a great requirement of research for additional knowledge of diseases that may be of future significance for the lobster industry in Indonesia

The aim of present study is to determine infection of MHD-SL disease from wild and cultured lobster (Panulirus homarus). Furthermore, artificial infection by injection and cohabitation were also studied.

2. Materials and methods

2.1 Sample lobster
From January to April 2016 a total number of 240 spiny lobsters (Panulirus homarus) samples weighing between 200.5 to 215.8 g (120 wild and 120 cultured) were collected from four different lobster capture and cultured sites within Bali and Lombok Islands. In Bali from Candi Kusuma Bay while in Lombok Island, the sites are Gerupuk Bay, Awang Bay and Telong-elong Bay, (Figure 1). In January 2019 another 50 lobster’s weight of 202.1-219.5 g were collected for artificial injection study. The lobster samples were first check for clinical sign as described by [11] and 20 mg of tissue samples were then dipped in ethanol 90% for Polymerase Chain Reaction (PCR) analyses followed [12]. In January 2020, another 40 lobster samples weight of 205.7-209.8 g were collected from the net cages located in the coastal water near Island (coastal cage) and the net cages located far from the Island (offshore cage) within Telong-elong Bay (Figure 1). The samples also were check their clinical sign for MHD infection and confirm by PCR [11], [12].

![Figure1](image-url). Sampling sites of wild and cultured lobster in Bali Island (Candi Kusuma Bay) and Lombok Island (Gerupuk, Awang and Telong-elong Bays), also lobster samples from culture sites at coastal and offshore cage within Telong-Elong Bay
2.2 Polymerase chain reaction (PCR) analysis

Total DNA extraction was done by Kit DNAzol. Preparation of sample and PCR analyses followed [12]. Individual of sample which suspected infected by haemolymph milky diseases (MHD-SL) were placed in 1.5 ml clean micro tube then homogenized in 1 ml DNAzol. Homogenate used was centrifuged at 12,000 x g for 10 min. at room temperature. Supernatant was then transferred into new tube. This step removed in soluble tissue fragment, RNA and excess polysaccharides from the homogenate. DNA was precipitated from the homogenate with 0.5 mL of 100% ethanol per 1 mL DNAzol reagent used for the isolation. Mix sample by inversion and store them at room temperature (27°C to 30°C) for 1 to 3 min. DNA should quickly become visible as a cloudy precipitate. DNA precipitate was centrifuged at 8,000 x g for 3 min. At room temperature; carefully decant the supernatant leaving the DNA pellet. Wash the DNA precipitate twice with 0.5 mL of 95% ethanol. Washing was done by inverting the tubes 2-6 times. The tube was stored vertically for 0.5 to 1 min. to allow the DNA to settle to the bottom of the tubes and removed the ethanol by pipetting or decanting. Air dries the DNA by storing it in an open tube for 5 to15 second after removing the ethanol (If the DNA is exposed to air for more than a few seconds, it will be much more difficult to dissolve). Dissolve the DNA in 100-200 μL Triss EDTA (TE) or Water, Dnase, RNase free (Deionized water treated with 0.001 DEPC filtered through 0.2 micron filter and autoclaved), and as template DNA for PCR amplification and other investigation.

PCR amplification was done in SpeedSTAR™ HS DNA Polymerase TAKARA RRO 70A contained: Buffer I, dNTPs, SpeedSTARS HS DNA polymerase, each 1 μL reverse and forward primers 10 pMol and nuclease-free water. Primers were designed from geographic origin of lobster from Vietnam. Primer set designation: 254F/R.: 254F: 5’-CGA-GGA-CCA-GAG-ATG-GAC-CTT-3’ and254R: 5’-GCT-CAT-TGT-CAC-CGC-CAT-TGT-3’ the expected PCR product size was 254 bp (OIE, 2008). The optimization cycle condition program was pre-denaturation for 3 sec. at 96°C, 40 cycles for denaturation for 5 sec at 96°C; annealing/elongation for 30 sec. at 65°C then followed by an extension period for 3 sec at 72°C after the last cycle. Reaction PCR mixes prepared in 20 μL were amplified in an automatic thermalcycler (AB. system, AB. applied biosystem, verity. 96 well fast). Analysis of PCR products or amplification was running on a 1.5% gel electrophoresis in 1xTAE buffer and stained with ethidium bromide, the target product was measured using a fast ruler low range DNA ladder (50; 200; 400; 850; 1,500 bp) under UV-Trans illuminator [12].

2.3 Artificial infection

Artificial infection test were carried out by intramuscularly injection. The challenge tests for healthy lobster against MDH-SL were performed using 4 groups of lobster. Each group consisted of 10 lobster were reared in an aquarium with a water temperature of 28.8°C. Due to MHD could not be cultured in agar medium [13, 14] and impossible to create certain density, then haemolymph of diseased lobster was taken at amount of 0.5 ml and diluted directly by physiological salt water at 10⁻², 10⁻⁴, 10⁻⁶, and 10⁻⁸ and at amount of 0.1 mL diluted haemolymph were injected to 10 individuals of healthy lobster for each treatment. For control the lobster were similarly injected with 0.1 mL physiological saline water. The MHD-SL infected lobster was determined by PCR. The dead lobster were observed every day for all treatments and experiment was end at day 14.

2.4 Artificial transmission

A healthy lobster obtained from offshore cage in Telong-Elong Bay were kept in 500 L tank with aeration for 3 days prior to the experiment, while MHD-SL infected lobsters were collected from coastal cage culture of farmer farms also located in Telong-Elong Bay. A challenge tests for diseases transmission of healthy lobster were performed using 2 groups of lobster. One group consisted of 20 lobsters (15 healthy and 5 MHD-SL diseased) and other group consisted of 20 healthy lobster as control, the body weight of lobster ranged from 205.7-209.8 g and reared in tank with a water temperature of 28.8°C. During the course of the experiment, lobster were fed by mixed chopped trash fish (sardine, anchovy and small shrimp). The lobsters were observed by checking abdominal on the
ventral side every day. An affected MHD-SL lobster may appear white turbid or milky if drawn into a syringe. The experiment was ended after 10 days of cohabitation.

3. Results and discussion

In the present study a healthy, early infected and infected MHD-SL lobsters are shown in Figure 2. Infected spiny lobsters by MHD-SL were characterized not only by coloration of abdomen but also behavior. Infected lobster become increasingly inactive, loose appetite to eat, then moribund [11]. Early sign of infected MHD-SL, shows redish coloured of abdomen as in the present study. The gross sign of infected MHD-SL is swollen in abdominal (visible on ventral side) become apparent withn 3-5 days after early sign, while healthy lobster shows transparent coloration of abdomen (Figure 2).

When haemolymph drawn with a syringe will range from slightly cloudy or turbid to milky white and will not clot [14]. In the present study dissection of MHD-SL infected lobsters shows the presence of milky colored haemolymph. Clinical sign of MHD-SL were found only in cultured spiny lobster from Telong-elong Bay (Table 1).

Extracted DNA from suspected infected spiny lobsters (hepatopancreas, haemolymph and muscle) from Telong Elong Bay was amplified using specific primer pairs for MHD-SL followed [12] method (Figure 3). Meaning that for detection of MHD-SL in lobster using PCR analyses can be used hepatopancreas, haemolymph and muscle. All red color of abdomen and moribound samples were positive MHD-SL, also known as milky haemolymph syndrome (MHS) [7], [14], [15].

Based on the total number of spiny lobster examined for clinical sign and confirmed by PCR (Figure 4), No infected MHD-SL lobsters were detected from wild samples, and infected sample was found in cultured lobster at Telong-elong Bay only with prevalence of MHD-SL was 26,7 % (8/30) (Table 1). While the lobster sample collected in 2020 revealed that the only cultured lobster collected from coastal cage infected by MHD-SL diseases at amount of 11/38 (28.95%) and no infected MHD-SL found in the lobster cultured at offshore cages. Based on the present result the prevalence of MHD-SL diseases occurred in the lobster that was culture in the cage that placed in the coastal area, which closed to the farmer village. The lobster culture cages located in coastal water and near to the farmer villages may effects degradation of the water quality especially impact of excess or uneaten feed and farmers household sewage released to the coastal water, hence stimulate growth of diseases like bacteria as in this case MHD-SL [16], [17].
Figure 3. Amplification PCR product 254 bp of natural infection, Test MHD-SL from different tissues in 1.5% agarose gel, 1= hepatopancreas; 2= haemolymph; 3 & 4 muscle, M= marker Fast Ruler Low Range DNA Ladder (50; 200; 400; 850; 1,500 bp).

Figure 4. Samples PCR product No 1 and No 2 : not infected lobster (Negative) and No 3 and No 4 : infected lobster (positive) at 254 bp by MHD-SL infection; K (-): Control Negative; M:Marker 100 b.

Table 1. The prevalence of MHD-SL diseases among examined spiny lobster in 2016

| Site              | Telong Elong Bay | Awang Bay | Gerupuk Bay | Candy Kusuma Bay |
|-------------------|------------------|-----------|-------------|------------------|
| Type of Sample    | No of sample (ind) | Prevalency (%) | No of sample (ind) | Prevalency (%) | No of sample (ind) | Prevalency (%) | No of sample (ind) | Prevalency (%) |
| Wild              | 30               | 0         | 30          | 0                | 30               | 0         | 30               | 0 |
| Cultured          | 30               | 26.7 (8 Ind) | 30          | 0                | 30               | 0         | 30               | 0 |

The results of study confirm that causative agent of milky disease of spiny lobster in Telong-elong Bay- Lombok Indonesia is a strange bacteria, and based on the molecular analyses result with molecular weight of 254 bp this bacteria known as a Rickettsia-like bacterium [13]. Four distinct Rickettsia-like bacteria have been found, one of which is known to be associated with the disease is Rickettsia-like bacterium as in the present study [11]. This bacteria can not be isolated by commercial medium [13].

In the present artificial infection experiment, the treatment at $10^2$, shows first moribund of lobsters were appeared from day 8-10 days post injection and started died for 2 lobster at day 11th post injection, and in the treatments of $10^4$ and $10^6$ the lobster started died at day 12 and 14 post injection, respectively. No mortality were observed in dilution of MHD $10^8$ and control group. In MHD-SL injected lobsters exhibited abnormal swimming behavior, in active, lost apetite then moribund,
suggesting that lobster was affected by MHD-SL. No clinical signs were observed in the treatment of diluted MHD 10-8 and control group. The cumulative mortality (%) for all treatments at day 14th are presented in Table 2. The cumulative mortality the groups 10⁻² at day 14 was 100 % while group 10⁻⁴ and 10⁻⁶ were 50 and 20 respectively (Table 2).

In 2020, 40 samples from Telong-Elong Bay were collected for cohabitation experiment and PCR analyses, remaining spiny lobster in the cage at coastal site was continued to observe for one moth of their behaviour. One week observation in cage did not show any clinical sign of infected MHD-SL, but after 8 days some spiny lobsters shows MHD-SL diseased under swollen abdominal and all died within 14 days. This most probably MHD-SL disease is already existed in that cage, and horizontal transmission by contaminated water from adjacent net cage is suspected as in this study [7], [8], [18]. This founding has been approved in the present cohabitation experiment, healthy and diseased MHD-SL lobsters were kept in the same tanks and after 8 days of experiment, all of fifteen healthy lobster shows reddish coloured of abdomen and loose appetite to eat, while 5 diseased MHD-SL lobsters that were used in the experiment died within 3 days after cohabitation. The MHD-SL disease has been transmitted from diseased MHD-SL lobster into healthy lobsters by cohabitation [19]. It was suspected that horizontal transmission has been occurred by direct contact between diseased MHD-SL and healthy lobsters. Based on the present experiment has been confirmed that MHD-SL diseases has been transmitted horizontally among lobster by cohabitation. While [11] mentioned vertical transmission also frequently occurred due to MHD-SL infected the lobster from juvenile up to adult size [10]. This study also approved that MHD-SL is pathogen diseases for lobster culture and may cause mortality up to 70-100 % [10].

| Day Post Injection | Number of mortality lobster post injection of different dilution of MHD-SL | Dilution |
|--------------------|------------------------------------------------|----------|
|                    | 10⁻²      | 10⁻⁴      | 10⁻⁶    | 10⁻⁸    | Control |
| 6                  | 0          | 0          | 0        | 0        | 0        |
| 7                  | 0          | 0          | 0        | 0        | 0        |
| 8                  | 0          | 0          | 0        | 0        | 0        |
| 9                  | 0          | 0          | 0        | 0        | 0        |
| 10                 | 0          | 0          | 0        | 0        | 0        |
| 11                 | 2          | 0          | 0        | 0        | 0        |
| 12                 | 2          | 1          | 0        | 0        | 0        |
| 13                 | 4          | 3          | 0        | 0        | 0        |
| 14                 | 2          | 1          | 2        | 0        | 0        |

**Cumulative Mortality at Day 14th (%)**

| 100 | 50 | 20 | 0  | 0  |

Table 2. The mortality of spiny lobster *P. homarus* in the artificial experimental injection with various dilutions of MHD-SL

There are many different types of fish diseases caused by various pathogens in Indonesia including viral, bacterial, parasitic and fungus. The diseases caused by the combined pathogens are dominant cases. In order to prevent the occurrence of that diseases, various diseases control strategy have been applied in Indonesia. Basic strategies are the pathogen control with chemotherapeutics or medicinal herbs, and host control with vaccines and immunostimulants. The Indonesian government have been decided do not use any kind of antibiotic for fish culture activities.

4. Conclusion

From this study it was concluded that MHD-SL infected lobster were detected only from cultured lobster, where culture cages placed in coastal area and transmission of MHD-SL diseases horizontally.
The MHD-SL diseases is pathogenic agent and may cause 100% mortality within 14 days post infection. The present finding suggested that in other to avoid MHD-SL disease infection the culture site of lobster cages have to be set up in offshore and clean sea water.

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