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MOLECULAR DETECTION AND CLONING FOR RICKETTSIA-LIKE BACTERIA OF MILKY HAEMOLYMPH DISEASE OF SPINY LOBSTER *Panulirus* spp.

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ABSTRACT

Spiny lobster (*Panulirus homarus* and *Panulirus ornatus*) are important commodities for Indonesia. The aquaculture of lobster is susceptible for several diseases like parasite, fungi, bacteria, and virus. Among those diseases, milky haemolymph disease (MHD) is often seen as a symptom to mass mortality occurred at lobster farms in Gerupuk Bay of Lombok. The purpose of this study was to determine the lobster diseases on cage culture in Gerupuk Bay of Lombok, West Nusa Tenggara. The study was undertaken from January to March 2015. Diseases status was determined by application of molecular plat-form, polymerase chain reaction (PCR) with designation of specific primer for MHD (254F/R), 254F: 5'-CGA-GGA-CCA-GAG-ATG-GAC-CTT-3' and 254R: 5'-GCT-CAT-TGT-CAC-CGC-CAT-TGT-3' with PCR size product of 254 for cloned the pathogen was used TA-cloning Invitrogen for the DNA plasmid as positive control for other analysis. Several tissue samples i.e hepatopancreas, haemolymph, part of muscle hepatopancreas *P. homarus* and *P. ornatus* were taken from cage culture farms at Gerupuk Bay then preserved on 90% ethanol for further analysis by PCR and then the amplificated DNA were cloned into pCR®2.1 plasmid and transformed into competent *E. coli*. The result showed almost all lobster samples from Gerupuk Bay were positive infected by MHD, as the results of PCR amplification whereas the band appeared at 254bp. Also MHD plasmid has been successfully cloned and will be used for further examination. Histopathologically in hepatopancreas infection have seen necrosis that contain numerous of rickettsia-like bacteria.

KEYWORDS: lobster; diseases; MHD; rickettsia

INTRODUCTION

Indonesia mainly in Lombok, West Nusa Tenggara (NTB) is a major seed lobster producer and grow-out of wild lobster which have been exported to around the world. The price of lobster is high in both the domestic and export markets. Within a year, NTB produced 78.5 tons of lobster worth IDR 55.25 billion. While the economic value of lobster seed sales reached IDR 16 billion per year (Petersen *et al*., 2013). There are many species of lobster in Indonesia, such as *Panulirus homarus*, *Panulirus ornatus*, *Panulirus versicolor*, *Panulirus longipes*, *Panulirus polyphagus*, and *Panulirus penicillatus*. Among those species *Panulirus homarus* were 90% as a grow-out cultured lobster in cage (Jones, 2015; Mustafa, 2013). Intensive capture can lead to a decline in natural population while the lobster can not be cultured. So it needs the proper techniques for handling the seed, density, feed, and disease prevention during grow-out in cage. However, along the grow-out of lobster even with the right technique there always have problem of emerging disease. The disease that commonly attack lobster farming include, rickettsia-like bacteria, true bacteria (*Vibrio alginolyticus*), protozoa, black gill disease kind of fungi (*Fusarium* sp.), virus (*Panulirusargus* V1/Pa.V1), parasite, and big head syndrome. Those pathogens have had a major impact on the lobster culture (Diggles *et al*., 2000; Shields & Behringer, 2004; Behringer, 2012; OIE, 2007; Kiryu *et al*., 2009; Shield, 2011, Lightner & Redman, 2010; Stentiford *et al*., 2002; Stentiford *et al*., 2012; Jones, 2015). Among the pathogens, milky haemolymph disease or MHD often infects to several species of cultured spiny lobster such as *Panulirus homarus*, *Panulirus ornatus*, and *Panulirus polyphagus* at all life stages and could cause mortality that reached 70%-100% (Jones, 2015). Milky

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haemolymph disease (MHD) is a descriptive name for disease affecting spiny lobsters. The syndrome is given name according to the symptoms of the hemolymph of the affected a lobster appeared to be turbid or feculent/cloudy and turned to a milky liquid. The turbidity is caused by huge number of rickettsia-like bacteria (RLB) circulating in the hemolymph. MHD was reported also attack to black tiger shrimp *Penaeus monodon* farmed in East Africa and Madagascar (Nunan *et al*., 2003a), wild European shore crabs *Carcinus maenas* (Eddy *et al*., 2007) and in cultured spiny lobsters *Panulirus* spp. in Vietnam (Lightner & Redman, 2010). According information of pathogens that affect to lobster culture farming and associated with the mass mortality that occurred in Lombok, so the disease status was determined through standard identification by polymerase chain reaction (PCR) plat-form and DNA MHD of lobster will cloned by using vector plasmid for other research purpose. The purpose of this study was to determine the lobster diseases on cage culture in Gerupuk Bay of Lombok through molecular detection and rickettsia-like bacteria cloning.

MATERIALS AND METHODS

Samples

Lobster samples with disease symptom were collected from Gerupuk Bay of Lombok, Indonesia in periods of Januari-March 2015. A hepatopancreas, haemolymph, and muscle of *P. homarus* and *P. ornatus* were transported by keeping them in 90% ethanol for molecular investigation and in formalin buffer for histopathological purposes.

Polymerase Chain Reaction (PCR) and Cloning Analysis

Total DNA extraction was done by Kit DNAzol. A total of 25-30 mg tissue sample and or 100 μL of haemolymph from naturally infected lobster in 1.5 mL clean micro tube was homogenized in 1 mL DNAzol. Homogenate was centrifuged at 12.000 *x g* for 10 min. at room temperature. Supernatant was then transferred into new tube. This step removed insoluble 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 Speed STAR™ HS 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' and 254R: 5'-GCT-CAT-TGT-CAC-CGC-CAT-TGT-3' the expected PCR product size was 254 bp (OIE, 2007). The optimation cycle condition program was pre-denaturation for 3 sec. at 96°C, 40 cycles for denaturation for 5 secat 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 thermal cycler (AB. system, AB. applied biosystem, verity. 96 well fast). Analysis of PCR products or amplification was running on a 1.5% gel electrophoresis in 1x TAE 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.

Cloning, the PCR amplicons were ligated with TA cloning, DNA PCR product were ligase using the vector plasmid pCR®2.1-TOPO system (INVITROGEN). Ligation transformed into competent *E. coli* were plated onto Luria-Bertani (LB) media containing 100 μg antibiotic ampicillin and containing Xgal (5-bromo-4-chloro-3-indolyl-β–D-galactoside) and IPTG (isopropyl-1-thio-β-D-galactoside); Plates were incubated overnight at 37°C. White and single colonies were isolated then scratched at LB plate for mass culture in LB both, cultured overnight at 37°C put on a shaker. DNA was extracted from the cultures byusing Plasmid Mini Kit (Gene Aid). Plasmid DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios by Nanodrop. Ligation of correct products was confirmed by restriction enzyme digestion *Eco*RI (1 μL 10x Buffer RE; 0.5 μL *Eco*RI enzyme; 5 μL H2O; 6 μL DNA plasmid) and analysis of products on agarose gels. Right Plasmid DNA were store in mix 0.85 mL of culture with 0,15 mL sterile Glycerol and transfer to cryovial and store at -80°C.

Histopathology analysis, Infected tissue (Hepatopancreas organ) were fixed in Davidson's solution for 24 hours then transferred for refixation process in 70% ethanol solution for further histopathologycal analysis. Samples of lobster were analyzed at the Division of Veterinary Pathology Section, Faculty of Veterinary Medicine, Bogor Agricultural University.

RESULTS AND DISCUSSION

Mortality of spiny lobsters *P. homarus* and *P. ornatus* with similar MHD symptom were found in grow-out floating sea cages culture at Gerupuk Bay Lombok. The symptoms or gross sign of naturally infected spiny lobster were milky appearance in abdomen, in tail muscle, necrosis on hepatopancreas, lethargy, poor feed response, and significant mortality (Figure 1).

Severe infected lobster was characterized by special sign not only marked with white color like milk on the abdomen but also on part of hepatopancreas releasing a milky liquid, severely affected lobster become lethargic, lose appetite then moribund (Nunan *et al*., 2010). Extracted DNA from infected spiny lobsters (hepatopancreas, haemolymph, and both muscle of *P. homarus* and *P. ornatus*) from Gerupuk Bay were amplified using specific primer pairs for MHD (OIE, 2007). The thermal cycling PCR program have been optimized as shown in part material and method above. All samples were positive by rickettsia-like bacteria (Table 1). Infection, as evidenced from PCR examination, suggested that the intracellular rickettsia-like bacterium (RLB) was the cause of disease and resultant mortalities in the samples of lobster from Lombok Island. The causative agent of the disease was a genus Rikettsia Gram-negative bacterium obligate intracellular parasite that could not be cultured in artificial nutria environments. Attempts to isolate and grow the potential causative agent on a wide range of agar-based culture media failed despite the use of long incubation periods and a range of dilutions of the infected haemolymph (Eddy *et al*., 2007). Rikettsia was grown either in tissue or embryo culture *i.e*. chicken embryo. Rickettsia is obligate intracellular pathogenic bacteria that grow only within the cytoplasm, and occasionally the nucleus, of eukaryotic cells (Frutos *et al*., 1994 *in* Nunan *et al*., 2003a).

Amplification fragment from all tissue hepatopancreas, haemolymph, muscle of *P. homarus* and *P. ornatus* was ilustrated on a single 254 bp of PCR. Amplification product was run on 1.5% agarose gel and staning with ethidium bromide is shown in Figure 2.

Additional data of artificial infection of MHD by injection pathway, showed that the clinical symptom appear on the day fourth after injection with antigen of MHD-spiny lobster and infected lobster were began loss of appetite in day ten then finally mass mortality occurred at day fourteen (Lasmika, 2015). This is in conformity with the opinion of Nunan *et al*. (2010) which MHD-SL highly virulent and could cause mass mortality of up to 100%.

Figure 1. Lobster infected by MHD, (Black arrow) in part of abdomen looks white color like milk (milky) were found in grow-out floating sea cages culture in Gerupuk Bay, Lombok

Table. 1. Diagnostic amplification PCR result of MHD in natural infection of spiny lobster samples from Gerupuk Bay, Lombok

Organ	PCR tested
Hepatopancreas of *Panulirus homarus*	Positive
Haemolymph of *Panulirus homarus*	Positive
Muscle tissue of *Panulirus homarus*	Positive
Muscle tissue of *Panulirus ornatus*	Positive

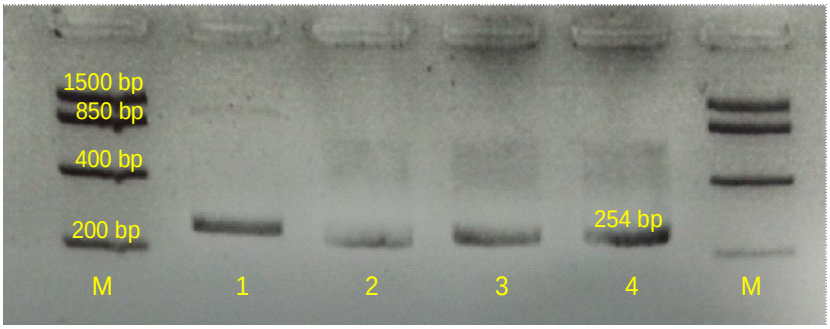

Figure 2. Amplification PCR product of natural infection MHD of spiny lobster from different tissue in 1.5% agarose gel, 1 = hepatopancreas; 2 = haemolymph; 3 = muscle *Panulirus homarus*; 4 = muscle *Panulirus ornatus*, M = marker FastRuler Low Range DNA Ladder (50; 200; 400; 850; 1,500 bp)

Several species of mollusc, fish, and crustacean were detected as infected with Rickettsia like Organism/RLO (Galvan *et al*., 2013). Black tiger shrimp *Penaeus monodon*, European shore crab *Carcinus maenas* and spiny lobster *Panulirus* spp. affected by milky haemolymph syndrome (Nunan *et al*., 2010 and Nunan *et al*., 2003a). The result of experimental infection of *P. vannamei* by a rickettsia-like bacterium (RLB) originating from *P. monodon*, showed that *P. vannamei* developed RLB infection when injected with either RLB infected *P. monodon* or *P. vannamei* tissue homogenates (Nunan *et al*., 2003b). Consequently, it is should be paid attention because the disease can infect to shrimps by artificial injection of homogenated RLO which shrimps were huge culture in Indonesia. This condition will be more dangerous because *P. monodon* is also very sensitive to WSSV infections that harmful virus. Therefore, prevention needs to be done for transmission route the MHD to other aquaculture by shorter cropping period, prophylactic antibiotics in feed, use of vitamins, immunostimulants, and use probiotics in feed.

Cloning DNA of MHD, *E. coli* bacteria bring the recombinant plasmid DNA of MHD were mass cultured in Luria Berani Broth (1% trypton, 0.5% yeast extract, 1% NaCl in pH 7 containing 100 μg/mL of ampicillin) and further purified. Confirmation of the success of the cloning was done by restriction enzyme *Eco*RI and PCR test against recombinant plasmid DNA purified. Plasmids of MHD were stored in -80°C as positive control and for further experiment, *i.e*. sequencing (Figure 3).

The histopathology analysis of MHD in spiny lobster, RLB appeared visible on the hepatopancreas tissue. Photomicrograph-histopathology of hepatopancreas organ (40x magnification) in ephitel tubulus hepatopancreas have seen necrosis and part of the lumen of hepatopancreas contain huge number of Rickettsia-like bacteria. Deparaffinized slide sections were stained with Haematoxyln-Eosin/H & E (Figure 4).

Furthermore studies on this pathogen are needed to classify this organism that was found from Indonesia. Comparison from the same gene in other rickettsia like-bacteria will be analyzed and placed in a phylogenic tree. Molecular diagnostic procedures developed in this study will be a standard after validate the specificity and potential utility of these techniques.

CONCLUSION

Milky haemolymph desease (MHD) was found in spiny lobster from Gerupuk Bay, Lombok, West Nusa

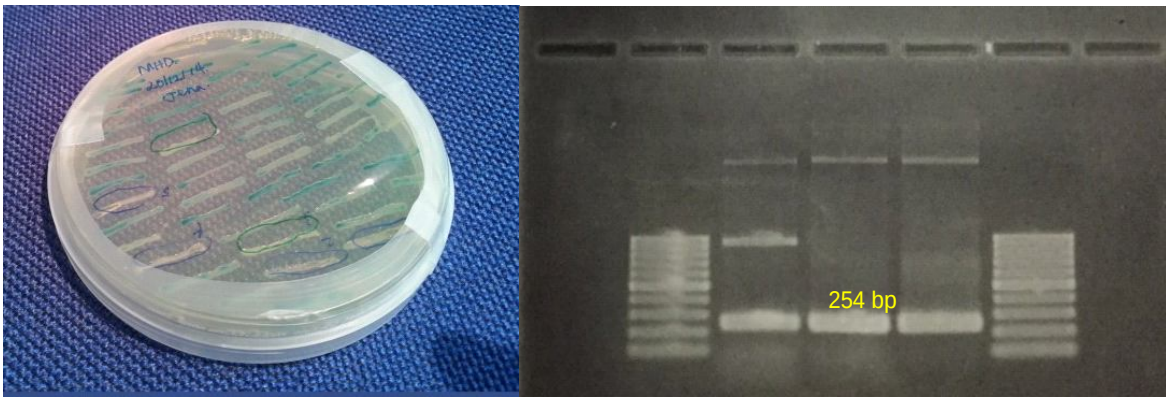

Figure 3. White colonies then scratched at LB plate for mass culture in LB broth and PCR product cloned DNA plasmid of MHD

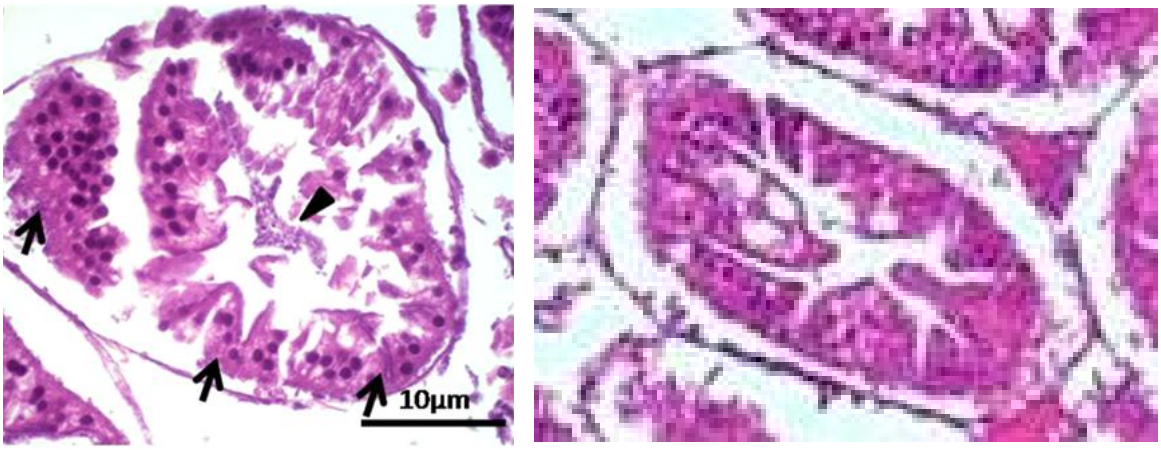

Figure 4. Photomicrograph-histopathology of hepatopancreas organ (40x magnification), (right); ephitel tubulus hepatopancreas with necrosis (black arrow) and the lumen of hepatopancreas contain huge number of bacteria (black triangle), (left); normal tissue; slide sections were stain with haematoxyln-eosin (H & E)

Tenggara, Indonesia. Histopathological analysis showed necrosis in hepatopancreas and huge number of Rickettsia-like bacteria in lumen of tubulus in hepatopancreas of infected lobster. Cloned of MHD from spiny lobster can be used for further experiment.

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