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Research Article

Correlation of Nitrite and Ammonia Concentration with Prevalence of Enterocytozoon hepatopenaei (EHP) in Shrimp (*Litopenaeus vannamei*) on Several Super-intensive Ponds in East Java, Indonesia

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Abstract

Enterocytozoon hepatopenaei (EHP), a microsporidia parasite known to hinder shrimp growth by infecting its hepatopancreas, is recently an emerging infection for *Litopenaeus vannamei* farms in Indonesia. The present study was conducted to investigate the relationship between nitrite and ammonia concentration with EHP infection in the super-intensive ponds. Shrimps and water samples were collected from six ponds in aquaculture super-intensive ponds with a stocking density of 5,000 shrimps in each pond which is located in Pasuruan, Lamongan, and Tuban Regency, Indonesia. The water sample was taken to the laboratory for the measurement of ammonia and nitrite. The samples of ammonia and nitrite were detected by spectrophotometer, and the PCR was used to detect 18S rRNA of EHP. The PCR analysis that showed two positive samples of *L. vannamei*, from the ponds with a high concentration of ammonia and nitrite, were infected by EHP. The statistical analysis showed a significant correlation between ammonia and nitrite with the prevalence of EHP infections where the Pearson correlation (r) was 0.980 and 0.943, respectively. There was a high prevalence of EHP infection with the increase in nitrite concentration and ammonia in pond four and pond six. The concentration of over 1mg/l of ammonia and nitrite could influence EHP infection prevalence in the shrimp farms.

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1. Introduction

Shrimp farming in Indonesia has increased dramatically in the past decade to export between 220,000 and 260,000 tons in 2018. A total of 60%, 19%, and 5% of shrimp production were shipped to the United States of America, Japan, and the European Union, respectively. Furthermore, among the shrimp exported in 2018, around 80% was *L. vannamei* (Rubel et al., 2019). In 2001, Indonesia officially introduced *L. vannamei* as the primary culture species to replace the previous *Penaeus monodon* (Hanggono et al., 2019). The former native *P. monodon* declined because of the abrupt eruption of diseases such as white spot disease. *L. vannamei* has advantages over *P. monodon* due to its fast growth and high resistance to diseases (Mahasri et al., 2019), it can be cultured in high stocking density, it has a high selected viral-pathogen-free domesticated broodstock supply, lower demand for dietary protein, more effective use of plant proteins in formulated diets, greater adaptability to low salinity, better tolerance to ammonia and nitrite toxicity, and high larval survival and lower susceptibility to formulated diets (Biju et al., 2016; Liao and Chien, 2011). Despite the mentioned advantages of culturing *L. vannamei*, during the past few years, the emergency of microsporidians pathogen *E. hepatopenaei* (EHP) in Indonesia and other countries such as Thailand, China, Malaysia, Vietnam, and India has been reported (Caro et al., 2020; Sivakumar et al., 2018; Biju et al., 2016; Rajendran et al., 2016) which infects the hepatopancreases of the shrimp and causes low growth, chronic infection, and mortalities.

E. hepatopenaei is the fungal obligate intracellular microsporidia parasite (Tourtip et al., 2009). Previous studies reported that EHP can slough off the cells in the hepatopancreas of tiger shrimp and white leg shrimp (Hanggono et al., 2019; Aranguren et al., 2017). EHP is associated with severe growth retardation in shrimps (Rajendran et al., 2016) and yields a meager harvest. They proliferate in the shrimp's hepatopancreas and midgut, mature spore then spread out from the faces ready to infect other shrimps. EHP infects the hepatopancreases by sloughing the cells, which impacts reduction in feeding practice, less appetite, and causes slow growth (Tang et al., 2017; Jaroenlert et al., 2016). Previous studies showed that EHP is associated with white feces syndrome (WFS) which appeared floating on the surface of the shrimp's ponds (Caro et al., 2020; Anjaini et al., 2018; Tang et al., 2016). On the contrary, Alfiansah et al., (2020), Sumini and Kusdarwati (2020) reported that WFS is caused by bacteria such as *Alteromonas* and *Pseudoalteromonas* sp. due to the presence of a high number of mentioned bacterial

cells in the fecal matter of the shrimps. The associated EHP signs include the slow growth between the shrimp checked in the same period. Other gross symptoms include the appearance of a whitish hindgut and loose carapace (Tang et al., 2016). EHP can be transmitted mainly orally by ingestion of infected live feeds such as brine shrimp *Artemia salina*, polychaetes, and mollusks (Karthikeyan and Sudhakaran, 2020; Kesavan and Sudhakaran, 2019).

The previous studies stated that ammonia and nitrite are stress factors in aquaculture. High stocking density and poor management account for the stress factors that lead to a weak immune system (Pamenang et al., 2020). The exposure of ammonia and nitrite results in increases the risk of pathogens emergence (Han et al., 2017; Liu and Chen, 2004). According to Liu et al. (2020), there is a decrease in immune cell counts, the ratio of apoptotic cell increase, and so affect the immunity of the shrimp under ammonia exposure. Other study showed that water quality correlates with the growth of pathogen that affects the development of *L. vannamei*, such as *Acineta* sp., *Epistylis* sp., *Nematopsis* sp., and *Leucothrix mucor* (Gutiérrez-Salazar et al., 2011). It was reported that a high concentration of ammonia and nitrite exposure to shrimp can damage intestine barriers mucosal which act as shrimp defenses tissue against pathogens (Duan et al., 2018). In the ponds, shrimps themselves act as a source of ammonia as they tend to release nitrogenous wastes as the end products of protein catabolism (Liu and Chen, 2004). Also, excess feeds and decomposition of organic matter accumulate in the ponds add up to the ammonia concentration in the water. Zhao et al. (2020) stated that the intolerable concentration of ammonia and nitrite inhibits innate immune factors such as phenoloxidase and antimicrobial activities. It also impairs the expression of chitinase enzymes and hence interferes with molting which reduces the growth rate of the shrimps. Liu and Chen (2004) reported that 5.24 mg l⁻¹ ammonia-N was able to reduce the phenoloxidase activity after one week, which can account for the emergence of the EHP in the super-intensive ponds. The study also concluded that the high ammonia concentration led to depression in the immune which resulted in the emergence of pathogenic diseases. An intensive aquaculture system is essential to maintain and manage water quality parameters including ammonia and nitrite. Due to high stocking density of super-intensive ponds, which is above 250 shrimps per m³ (Nguyen et al., 2019), super-intensive pond requires intensive care of ammonia and nitrite to prevent the emergence of pathogenic diseases including EHP.

There was no study addressing nitrite and ammonia



Figure 1. Visual observation of *Litopenaeus vannamei* obtained during the sample collection from the shrimp's pond farms. A pale white, contracted, and shortened hepatopancreases of EHP infected shrimp in group A. In group B, the shrimp appeared to be large size compared to group A with normal hepatopancreases.

concentration with EHP infection in *L. vannamei* in super-intensive ponds. Thus, this study was conducted to investigate nitrite and ammonia's correlation in influencing EHP infection in super-intensive ponds.

2. Materials and Methods

2.1 Study area

The samples were collected randomly from six ponds located in Pasuruan, Lamongan, and Tuban Regency East Java, Indonesia, with two ponds in each regency. In this study, a total of 36 shrimps were enough to provide the relationship of each pond with the prevalence of EHP infection. Physicochemical water parameters and EHP diagnosis were made at the Center for Brackish Water Cultivation Fisheries laboratory, located at Jepara Indonesia.

2.2 Sample collection and sample size

Six samples of shrimp from six different shrimp ponds stocked in the same period were collected for diagnosis of EHP, making a total of thirty-six samples. The samples were collected according to *Standar Nasional Indonesia SNI (2000)* and the stocking density was estimated to be 5,000 shrimps on each pond. After a randomized collection of the sample they were categorized based on the degree of infection, from mild infected, mid-infected, and highly infected shrimps. The live sample was then supplied with an aerator and dry ice for transport to the laboratory.

2.3 DNA extraction, PCR amplification

The DNA was extracted from the hepatopancreas of the shrimp sample. Twenty-five milligram of obtained hepatopancreas samples was then grinded and then mixed with 500 μ L of lysis buffer (50 mM Tris, 1 M Methylene diamine tetra-acetic acid (EDTA), 500 mM NaCl, 1% SDS), and 0.1 mg proteinase, then, the mixture was centrifuged for 10 min at 4°C. Ethanol was added to the supernatant kept at -20°C for one hour and then centrifuged for 10 min at 4°C. The extracted DNA pellet was washed with alcohol and re-suspended in nuclease-free water (Thermo Fisher Scientific).

The amplification was done by PCR using forward and backward primers. The primer was designed according to Hanggono *et al.*, (2019) with a pair of primer (MF1-5'- CCGGAGAGGGAGCCTGAGA-3' and MR1-5'- GACGGGCGGTGTGTACAAA-3'). The reaction was done in a thermocycler (Eppendorf, USA). Denaturation was at 94°C for 20 sec followed by annealing at 24°C for 30 sec, and extension was done at 72°C for 5 min. The amplified products were then visualized in the 2% agarose gel.

2.4 Physicochemical parameters of the water

Physicochemical water parameters (pH, salinity, nitrate, nitrite, ammonia temperature, and dissolved oxygen) were measured. Nitrite and ammonia data were taken for analysis of the relationship with the EHP

infection in shrimp culture. Water sampling was done from each fish pond. The samples collected were used for the analysis of physicochemical water parameters. pH meter, mercury-filled thermometer with ranges from 0°C to 100°C, refractometer, and DO meter were used to measure pH, temperature, salinity, and dissolved oxygen respectively *in situ*. The water sample of 1,500 ml was taken using a water sampler and then preserved with pieces of ice in the cool box to limit chemical and biological activities. Furthermore, the water sample was transported to the laboratory for analysis. Ammonia was determined by using a spectrophotometer (Zitomer and Lambert, 1962), nitrate was determined by simple heterogeneous reduction followed by spectrophotometer (Morris and Riley, 1963), and nitrite was also done by spectrophotometric determination (Aydin et al., 2005).

2.5 Data analysis

Statistical analysis was performed with SPSS (ver. 26, IL, USA). Pearson correlation (r) and simple linear regression were used to analyze the relationship between the variables. The prevalence of diseases was calculated by the following formula:

$$\frac{\text{Number of EHP infected shrimp}}{\text{Total number of a sample shrimp}} \times 100 = \text{Prevalence (\%)}$$

3. Results and Discussion

3.1 PCR amplification analysis.

The sampled shrimps hepatopancreas PCR amplification results targeting the designed small subunit 18S rRNA (ssu rRNA) were analyzed. The PCR amplification results showed a product size of 510 bp from the two samples (Figure 2 A, B). *Litopenaeus vannamei* was collected from the six ponds in three different East Java regions (Pasuruan regency, Lamongan regency, and Tuban regency) two samples were found to be positive for EHP out of the 36 samples of Juvenile. The sequence analysis by BLAST search of 18S rRNA of EHP showed 99% similarity to the 18S rRNA gene of EHP from India (GenBank MH260592.1). Rajendran et al. (2016) showed 86% to 100% similarities of EHP from India with those obtained in Vietnam and Thailand.

When the samples were observed and compared, they were observed to be small compared to other shrimps stocked in the same period and with fewer clinical signs (Figure 1). The use of specific detection method such as PCR, LAMP, In-situ hybridization (ISH), histological examination of hepatopancreas is necessary to diagnose the EHP pathogens due to their less noticeable clinical signs (Hanggono et al., 2019; Rajendran et al., 2016; Jaroenlak et al., 2016; Tang et

al., 2015; Tourtip et al., 2009). It is tough to notice the EHP infected shrimps by using naked eyes except with high experience in observing EHP infection. Therefore, a highly sensitive detection method is suggested (Tang et al., 2015).

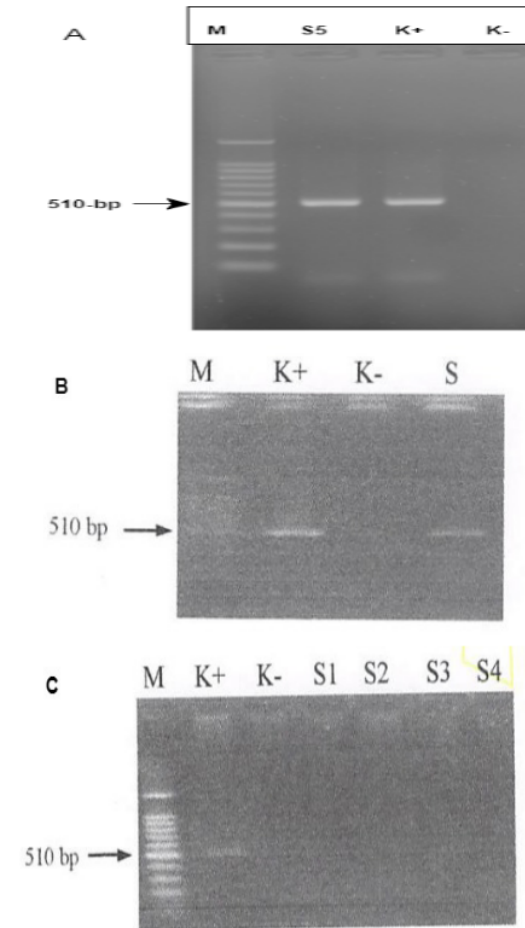


Figure 2. A & B. PCR detection analysis results showed 18S rRNA fragment in *Litopenaeus vannamei* hepatopancreas tissue sample. A PCR product size of 510-bp was obtained. S and S5 showed a positive EHP test from pond 4 and pond 6 respectively. C. PCR products showed negative EHP detection in the S1, S2, S3, and S4 hepatopancreas tissue samples. Note - M (100-bp) - marker, S, S1, S2, S3, S4, S5 are the shrimp hepatopancreas tissue samples from other ponds, and K- negative control; K+ positive control.

3.2 Prevalence of EHP disease

The results showed two positive samples of EHP from ponds four and six. From the formula, the prevalence of the disease was 16.7% for both shrimp ponds four and six. The samples from ponds one, two,

three, and five gave EHP negative results (Table 1). The physicochemical water parameters results showed that pond 4 and pond 6 had a range of 1 mg/l to 1.2 mg/l of ammonia and nitrite, which were higher than the rest of the ponds ranging from 0.2 mg/l to 0.5 mg/l. The higher deviation of ammonia and nitrite might explain EHP's positive results in pond 4 and pond 6. The results from the prevalence of EHP were in agreement with the results from a previous study that showed that ammonia and nitrite accumulation might impact shrimp production by reducing survival, growth performance, and damage of hepatopancreases of *L. vannamei*, which provides the chance for the opportunistic pathogen like EHP to infect the shrimp (Han *et al.*, 2017).

A growth variation of the shrimp stocked in the same period was observed in this study (Figure 1 A, B). There was an appearance of pale white color in the cephalothorax of the shrimp and hepatopancreas were shrunken (Figure 1 A). The observation made in this study agreed with the study of Rajendran *et al.* (2016) which stated that EHP causes severe growth retardation of the shrimps. EHP affects the shrimp's hepatopancreas causing severe necrosis seen by the sloughing of tubular hepatopancreases epithelial cells and they reported to accumulate spores in the tubular lumen of shrimps (Aranguren *et al.*, 2017). In another study, Tang *et al.* (2017) reported that EHP is often accompanied by other opportunistic pathogens, such as *Vibrio* spp. which colonizes the hepatopancreases after the slough damage of cells done by EHP. The study also stated that the susceptibility of acute hepatopancreatic necrosis disease (AHPND) and *Vibrio*-associated septic hepatopancreatic necrosis (SHPN) is higher when the shrimp is infected with EHP. According to Tangprasittipap *et al.* (2013), EHP could be associated with farmed based management practices, including contamination with infected shrimps or poor management of physicochemical water parameters. To better grasp EHP's leading source in these ponds, a screening survey that engages many ponds would give a better understanding.

3.3 Physicochemical water parameters

Ponds 4 and 6 showed a notable difference from other ponds (Table 2). The dissolved oxygen at pond 4 and pond 6 was the lowest of about 3.8 mg/ml and 3.7 mg/ml respectively. Ponds 1, 2, 3, and 5 had dissolved oxygen ranges between 4 mg/ml to 4.2 mg/ml. It was observed that ponds 4 and 6 had fewer aerators compared with other ponds which might explain the low dissolved oxygen to pond 4 and pond 6. Aeration in super-intensive ponds helps to supply and diffuse the oxygen from the air into the water, it also helps in circulating

the water, food and makes the water column clean. The low dissolved oxygen affected the immune system of shrimps. Flegel (1998) stated that shrimp's phagocytic activity reduced to 28.3% for oxygen-depleted shrimps from the control group, which were 34.7%. The study also reported that the higher maintenance of oxygen led to an increase in disease resistance.

The temperature was 29°C in ponds 1, 2, 4, and 6, while it was 28.5°C in ponds 3 and 5. The temperature difference might be due to the difference in the location of the pond with different weather conditions. According to Food and Agriculture Organization (FAO, 1986), water quality parameters influence the normal physiology of shrimps. Temperature influences the rate of biological reaction, as the temperature increase also the rate of biological reaction increases. Based on Fayer (2004) microsporidians depend on temperature. Microsporidians can remain inactive in low temperatures at 30% artificial seawater.

Pond 4 and 6 had the lowest pH of 6.8, while the rest ponds ranged from 7 to 7.5. pH has effects on physiology and the immunity of the shrimps. The exoskeleton of the shrimps is made of calcium carbonate which reacts with the acid in the water and makes the exoskeleton soft and easy for pathogenic attack (Furtado *et al.*, 2015). The factors for pH variation in shrimp aquaculture were studied by Luna (2017).

The higher water salinity was recorded in pond 4 and 6 which were 19 ppt and 18 ppt, respectively, while the other pond had water salinity 17 ppt changed in salinity levels affected the growth, physiology, and survival of the shrimps, which made it vulnerable to pathogenic infection (Bœuf and Payan, 2001).

The nitrate concentration was higher at ponds 4 and 6 (20 mg/ml), while the remaining ponds had nitrate concentrations ranged from 10 mg/ml to 15 mg/ml. Nitrate is one of nitrogenous waste which includes ammonia and nitrite. Their harmful nitrogenous waste which should be kept low at all time during shrimp farming (Furtado *et al.*, 2015). Nitrate is reported to cause gills abnormalities, circular dark lesions on the carapace of the shrimp, and generally reducing shrimp health which makes it vulnerable to EHP.

The nitrite concentration was lower at ponds 1, 2, 3, and 5 (0.3 mg/ml, 0.5 mg/ml, 0.2 mg/ml, and 0.5 mg/ml respectively) while it was higher in pond 4 and 6 (1 mg/ml and 1.2 mg/ml). The ammonia concentration was higher at pond 4 and 6 (1.2 mg/ml and 1 mg/ml, respectively), while it was 0.5 mg/ml at pond 1, 2, 3, and 5. Other studies have reported the effect of ammonia

Table 1. EHP prevalence estimated from the PCR test results

Pond No.	Total sample	Number of the positive sample	Results	Prevalence of EHP % = Number of positive sample / total sample
1.	6	0	Negative	0
2.	6	0	Negative	0
3.	6	0	Negative	0
4.	6	1	Positive EHP (<i>Enterocytozoon hepatopenaei</i>)	16.7
5.	6	0	Negative	0
6.	6	1	Positive EHP (<i>Enterocytozoon hepatopenaei</i>)	16.7

Table 2. Data of physicochemical water parameter

Pond No.	DO (mg/L)	Temperature(°C)	pH	Salinity (ppt)	Nitrate (mg/L)	Nitrite (mg/L)	Ammonia (mg/L)
1	4.2	29	7.5	17	15	0.3	0.5
2	4.1	29	7.2	17	10	0.5	0.5
3	4.2	28.5	7	17	15	0.2	0.5
4	3.8	29	6.8	19	20	1.0	1.2
5	4.0	28.5	7.5	17	15	0.5	0.5
6	3.7	29	6.8	18	20	1.2	1.0

Table 3. Correlation (r) summary between the ammonia and nitrite concentration with the prevalence of EHP diseases in *Litopenaeus vannamei*

	EHP		
	Pearson Correlation	Sig. (2-tailed)	N
Ammonia	0.980**	0.001	6
Nitrite	0.943**	0.005	6

Note; **. Correlation is significant at the 0.01 level (2-tailed); N- the total number of the sample; Dependent variable is the prevalence of EHP.

Table 4. Regression analysis model to summarize the relationship of EHP prevalence with the ammonia and nitrite concentration

Variable	B-coefficients	β	t - values	P (significance level)
Constant value for Ammonia	-13.14		-6.36	0.003
Ammonia	26.72	0.98	9.8	0.001
Constant value Nitrite	-7.02		-2.73	0.053
Nitrite	20.36	0.94	5.66	0.005

Note; R-squared for Ammonia = 0.960; R squared for Nitrite = 0.889; Dependent variable is prevalence of EHP

and nitrite due to their toxicity reduce hemocytes cells production (Liu *et al.*, 2020) weaken the immune system, cytoskeleton remodeling, and destruct anti-oxidative system and metabolic pathways in *L. vannamei* (Xiao *et al.*, 2019). Other studies stated that nitrite and ammonia cause physiological instability in crustaceans hemolymph (Waikhom, 2017; Gomes *et al.*, 2016). In the present study, ponds 1, 2, 3, and 5 tested negative for EHP, and the ammonia and nitrite levels were lower than 1mg/ml. In ponds 4 and 6, the ammonia and nitrite concentrations were higher than 1 mg/ml. The present study agrees with Gomes *et al.* (2016) which stated that the safe concentration for ammonia and nitrite is estimated to be 0.834 mg TAN/L and 0.328 mg NO₂-N/L for better growth and survival of the shrimps.

3.4 The Relationship between the ammonia and nitrite concentration with the prevalence of EHP infections in *Litopenaeus vannamei*

EHP showed a strong significant positive correlation with ammonia ($r = 0.980$) of $p < 0.001$. It also showed a strong positive significant relationship with nitrite ($r = 0.943$, $p < 0.001$) (Table 3). This study suggests that it is important to monitor and manage shrimp ponds in intensive aquaculture to control the spread of EHP infections. According to Thitamadee *et al.* (2016), poor management and low biosecurity measures led to the EHP spread resulting in slower growth and fewer returns.

Significant regression analysis for ammonia was carried out with the result of $f = 96$, $p < 0.001$, with an R^2 of 0.960. It was found that EHP was increased by 26.27% for each increase in 1mg/l of ammonia (Table 4). Based on Liang *et al.*, (2016), ammonia exposure 20 mg TAN/L induced stress factors and led to apoptosis of cells in the hepatopancreas. This condition makes the shrimp vulnerable to pathogenic infection including EHP. A simple linear regression was also calculated to predict EHP infection based on nitrate concentration in the ponds. The simple regression analysis showed $f = 32.038$ ($p < 0.001$) with an R^2 of 0.889 for nitrite. It was also found that EHP was increased by 20.34% for each increase in 1mg/l of nitrite (Table 4). The results were consistent with Ariadi *et al.* (2019) who stated that nitrite has a high impact on the growth rate and physiology of the shrimps.

4. Conclusion

Ammonia and nitrite concentration could influence the prevalence of EHP infection to the shrimps. This study concluded that there was a strong correlation between ammonia and nitrite concentration with the

prevalence of EHP infection. The concentration of over 1 mg/l of ammonia and nitrite could influence EHP infection prevalence in the super-intensive shrimp farms. Despite the detection of EHP infection, the infection source was unknown in these super-intensive ponds. It is recommended to investigate the source of this EHP infection.

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Authors Contributions

All authors have contributed to the final manuscript as follows, Anord; collected the data and wrote the original manuscript. Gunanti; designed, supervised, reviewed and edited the research and also managed project administration. Nunuk; reviewed, edited, and supervised the research. Mwendolwa; proofread, reviewed, and edited the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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