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Phagocytosis Index (IF) and Total Vibrio Count (TVC) of Vaname Shrimp (*Litopenaeus vannamei*) in Intensive Ponds

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ABSTRACT

Whiteleg shrimp (*Litopenaeus vannamei*) is a commercially important fishery commodity of high economic value. Intensive shrimp culture is done with high stocking density and large amount of feed requirement. Intensification can trigger stress, increase the possibility of physical contact among shrimps, and accelerate the spread of disease. The most common disease of shrimp is vibriosis that caused by *Vibrio sp*. However, shrimp has hemolymph, an innate immunity system that has an important role in body defense. This study was conducted from August to September 2023 using a purposive random sampling with 50 whiteleg shrimp samples. Assay of phagocytosis index (PI) that determines a cellular response of the shrimp body defense system was done at the Center for Brackish Water Aquaculture (BBPBAP) Jepara by applying a bacterial suspension of *Staphylococcus sp.* which has been attenuated, and bacterial staining was also carried out. Calculation of Total Vibrio Count (TVC) was done by using TCBS (Thiosulfate Citrate Bile Salt Sucrose Agar) agar media from *Vibrio sp*. bacteria cultured from the hepatopancreas of shrimp samples, and this activity was conducted at the Laboratory of Bacteriology and Mycology, Faculty of Veterinary Medicine, Universitas Airlangga. The results showed that whiteleg shrimp cultured in the intensive ponds in Situbondo was infected with *Vibrio sp.* with a TVC value of $3.3x10^5$ cfu.ml^{-1} and Phagocytosis index of 0.87.

Keywords: *Litopenaeus vannamei*, phagocytosis index, *total vibrio count*

INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) is widely cultivated aquatic commodity that has several advantages its adaptability to a higher salinity range 5 - 39 g.l^{-1} (Bray *et al.*, 1994), stability of production, enhanced disease resistance, low feed conversion ratio, and capable of being cultivated at high stocking densities. Considering these advantages, many whiteleg shrimp producers culture this species in intensive ponds. Intensive ponds are characterized by high shrimp stocking densities 100 - 300 shrimps.m^{-2} (Purnamasari *et al.*, 2017).

High stocking densities in whiteleg shrimp cultivation will lead to decreased space for movement and increased physical contact between shrimp, increased cannibalism, and decreased water quality (Suwoyo dan Hendrajat, 2021), and the competition for food and oxygen consumption increases (Sun *et al.*, 2016). Decreased water quality in whiteleg shrimp rearing can cause the growth of *Vibrio sp.* which is an opportunistic bacterium to be more rapidly so that it can turn out to be pathogenic. In addition, shrimps are becoming stressed and can cause a decrease in the immunity of shrimp hence they tend to be susceptible to disease (Rum *et al.*, 2022).

The disease in whiteleg shrimp (*Litopenaeus vannamei*) can be caused by viruses, fungi, parasites and bacteria that are pathogenic. The most common diseases

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found in shrimp farming are Vibriosis. Vibriosis is an infectious disease that caused by *Vibrio sp.* bacteria which is pathogenic and can cause mortality in vaname shrimp up to more than 90% within 24 - 48 hours after infection. (Yang *et al.*, 2022). *Vibrio sp*. bacteria are pathogenic when the number exceeds $1x10^3$ cfu.ml^{-1} (Anjasmara *et al.*, 2018).

The number of *Vibrio sp*. can be calculated by Total Vibrio Count (TVC). The organ observed for TVC examination is the hepatopancreas as a target organ of *Vibrio sp.* bacteria where it grows in it (Widanarni *et al.*, 2016). The range of colonies of bacteria that can be counted as TVC is between 25-250 cfu.ml^{-1} (Anjasmara *et al.*, 2018). The immune defense response of whiteleg shrimp is characterized by phagocytosis activity. An increase in phagocytosis activity indicates an increased shrimp immune response. Phagocytosis activity can be seen from the phagocytosis index value where the PI value less than 1, it indicates immunosuppressive activity, which is a state of decreasing the immune system. Whereas PI value is more than 1 indicates immune system enhancing activity (Aldi *et al.*, 2014).

This study was conducted to determine the Phagocytosis Index (PI), and the Total Vibrio Count (TVC) in white leg shrimp cultivated in intensive ponds. Decreased water quality, and high stocking density could cause shrimp susceptible to infectious diseases, particularly the vibriosis. The value of TVC and PI of white leg shrimp representing the immune response that affected its health could give information to the farmers for them to be able to do the next action as an attempt to control this disease. Thus, failure in White leg shrimp production due to pathogenic disease could be avoided.

MATERIAL AND METHODS

Study Site and Duration

The Phagocytosis index (PI) analysis was conducted at the Center for Brackish Water Aquaculture in Jepara, and Total Vibrio Count (TVC) calculation at the Bacteriology and Mycology Laboratory, Faculty of Veterinary Medicine, Airlangga University for a duration of one month from August to September 2023.

Materials and Equipment

The whiteleg shrimp at a stage of Post Larvae 25 day with vibriosis infection was obtained from the intensive ponds in Situbondo, East Java. A total of 50 shrimps were taken as samples, and the determination of the sampling quantity refers to Cameron (2002) that the sample was taken 5% of the total population of whiteleg shrimp in intensive ponds with a stocking density of 1.000 shrimps/pond. Sodium citrate ($Na_3C_6H_5O_7$) 10% as an anti-coagulant for shrimp hemolymph, ethanol 96%, sodium chloride (NaCl) 2%, physiological sodium chloride (0.9%), alcohol 70%, cotton, aluminum foil, plastic wrap, safranin 10% as a bacterial stain, TCBS agar as a growth medium for *Vibrio sp*., sterile petri dishes, *Bacillus sp*., formalin 1% to dilute *Bacillus sp*. during PI analysis, immersion oil. An autoclave was used in the sterilization of all equipment used during the TVC analysis, 1 ml syringe for the shrimp hemolymph collection, mortar pestle, incubator for the incubation of *Vibrio sp*. that had been grown on TCBS agar, vortex, preparation glass, staining jar, micro well plate, binocular microscope, and fume hood.

Preparation of TCBS Agar Media

The first procedure that was carried out before the TCBS media preparation was the sterilization of equipment using an autoclave. The TCBS agar media was

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prepared by measuring 88 grams of TCBS powder and dissolved in 1 liter of sterile aquadest, and the TCBS agar media was sterilized by autoclaving. The process of dissolving TCBS media powder in sterile aquadest was done by heating (temperature less than 100°C) for 1 minute and shaking until the media was completely dissolved. The dissolved TCBS media solution was then poured into sterile petri dishes with a thickness of 15-20 ml in a fume hood so that the process of making TCBS media remains sterile. Let it set for a while until the TCBS agar media was formed, and then stored in the refrigerator at a temperature of 8 – 15 °C, and ready for use.

TVC Calculation

TVC calculation was done by counting the number of *Vibrio sp.* colonies that grew on TCBS agar media. Colonies count ranged from 35-300 colonies. If the calculation of the total colonies from all dilutions is less than 30, then the number of bacterial colonies from the lowest dilution will be reported. Otherwise, if the total of bacterial colonies from all dilutions is more than 300, the number of bacterial colonies from the highest dilution will be reported. The unit used for TVC calculation is cfu.ml^{-1} (Duan et al., 2017).

The organ taken for the calculation of Total Vibrio Count (TVC) is the hepatopancreas since *Vibrio sp.* grow and develop in these organs (Widanarni et al., 2016). In this calculation process, 1 gram of hepatopancreas was collected, and crushed using a sterile pestle and mortar. The crushed hepatopancreas was put in a tube with 2% NaCl solution, homogenized by vortexing, and then diluted 4 to 5 times.

After that, 1mL of the dilute was poured onto the TCBS agar medium and spread over the entire surface of the agar medium using an L bar respectively. The agar media was then incubated at (36°C) for 3-5 hours. After incubation, the *Vibrio sp.* colonies were calculated using the following formula:

TVC = *Total Bacteria colonies* X *dillutions*

Phagocytosis Index Calculation

The PI analysis was carried out by extracting 20 µl of shrimp hemolymph using a 1 ml syringe treated with sodium citrate as an anticoagulant (1:1). The hemolymph extract was collected and transferred to a micro-well plate. Inactivated *Bacillus sp.* treated with 1% formalin for 24 hours, were added to the extract, and mixed in the plate. The mixture was then incubated at 25°C for 20 minutes. After incubation, the mixture suspension was placed on the preparation glass for analysis. The plate was soaked in physiological NaCl solution (0.9%) for 20 minutes, then rinsed and dried.

Fixation was done by soaking it with methanol for 10 minutes and stained with 10% Safranin for 20 minutes. Afterwards, this was washed with flowing water and dried by aeration. After drying, the preparation was covered with cover glass, and observed under the microscope with a magnification of 1,000x using immersion oil to calculate the phagocytosis index with the following formula:

PI = number of phagocytizing cells / number of cells observed (100)

RESULT AND DISCUSSION

The analysis results of the 50 samples of whiteleg shrimp (*Litopenaeus vannamei*) obtained from the intensive ponds in Situbondo showed that whiteleg shrimp was infected with *Vibrio sp.* as indicated in the average TVC value at 3.3x10^5 cfu.ml^{-1} (Table 1).

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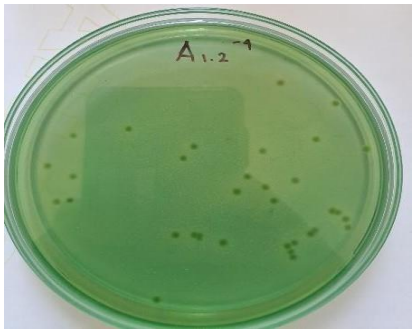

Fig. 1. TVC graph of 50 shrimp samples

The TVC value indicates that the value exceeds the normal threshold for the number of *Vibrio sp.*, which is 1x10^3 cfu.ml^{-1} (Anjasmara *et al.*, 2018). This was further supported by KEP.75/MEN/2016 in Madonsa *et al.* (2022), that the maximum TVC is less than 10^3 cfu.ml^{-1}. In addition to TVC calculations (Fig. 1), clinical phenomena that appeared from shrimp in this study were pale-colored hepatopancreas, soft exoskeleton at some points, and lack of appetite (Fig. 2). This condition is also supported by the opinion of Kumar *et al.* (2021), that the symptoms that arise when shrimp are infected with *Vibrio sp.* are pale-colored bodies, empty shrimp intestines, brownish-red tails, reddened legs, soft groupers, and the emergence of black spots on the shrimp body.

Table 1. TVC Calculation of all sample

No Samples	TVC (x10^5 CFU.ml^{-1})	No Samples	TVC (x10^5 CFU.ml^{-1})
U1	2,94	U26	3,43
U2	3,05	U27	3,44
U3	2,85	U28	3,65
U4	3,05	U29	3,25
U5	4,21	U30	3,53
U6	3,24	U31	2,98
U7	3,58	U32	3,83
U8	3,08	U33	4,04
U9	3,25	U34	2,76
U10	2,87	U35	3,25
U11	2,76	U36	3,31
U12	3,07	U37	4,46
U13	3,14	U38	3,64
U14	3,22	U39	3,25
U15	3,35	U40	4,24
U16	3,35	U41	4,09
U17	2,07	U42	3,15
U18	2,09	U43	3,25
U19	2,33	U44	3,15
U20	4,62	U45	4,52
U21	2,38	U46	4,25
U22	2,78	U47	3,08
U23	2,98	U48	3,22
U24	3,01	U49	3,14
U25	3,14	U50	3,51

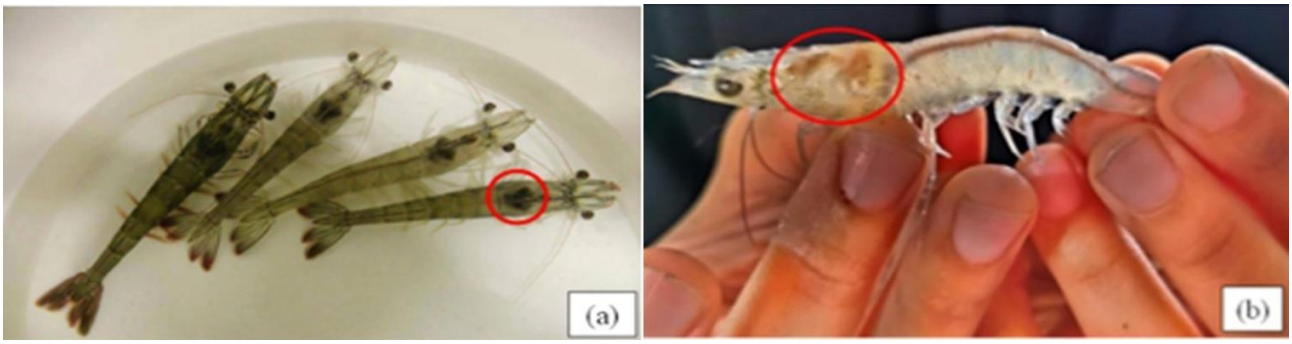

Fig. 2 Shrimp hepatopancreas color; healthy (a) and Vibrio sp. infected (b)

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The PI value obtained from whiteleg shrimp samples in intensive ponds amounted to 0.871±0,045 (Fig. 3). Based on Aldi *et al.* (2014), PI value < 1 indicates a decrease in the immune system (immunosuppressive). The data also indicate that the immune system of whiteleg shrimp has decreased, making the shrimp vulnerable to stress, and can easily be attacked by pathogens which can cause infection or disease, and even death.

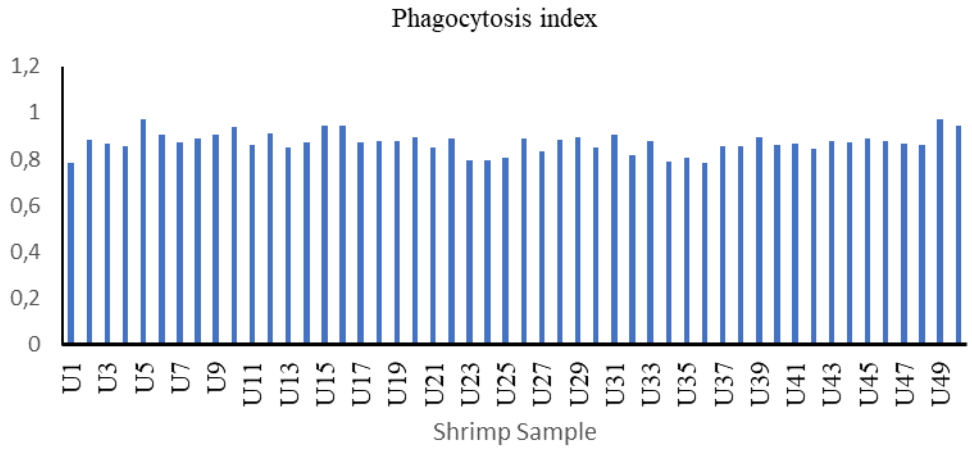

Figure 3. Phagocytosis index 50 shrimp samples

CONCLUSION

In conlucion, the whiteleg shrimp sampled from the intensive ponds in Situbondo has been attacked and infected by vibriosis disease as indicated in TVC of $3.3x10^5$ $cfu.ml^{-1}$ exceedingthe normal threshold of *Vibrio sp*. The PI value 0.87 indicates that the shrimp are immunosuppressed (<1).

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AUTHORS' CONTRIBUTIONS

The contribution of each author is as follows, PGEM; collected the data, drafted the manuscript, and designed the table as well as the graph. GM and LS; devised the main conceptual ideas and conducted a critical revision of the article. All authors discussed the results and contributed to the final manuscript.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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