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Pathogenicity Testing of Bacterial Disease in the Barramundi (*Lates calcarifer*) in North Aceh

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ABSTRACT

The barramundi (*Lates calcarifer*) is a fish of high economic value, a feature of which has increased its aquaculture. The expansion of aquaculture not only leads to higher profits but raises the risks involved in aquaculture as well. One of them is a bacterial disease. Pathogenicity testing is essential to understand how these bacteria infect their host, which can inform strategies for managing bacterial diseases. Preliminary tests identified two bacteria, which were subjected to molecular DNA identification and sequencing, revealing the presence of *Vibrio navarrensis* and *Acinetobacter lwoffii*. The two bacterial strains were then tested on barramundi fingerlings measuring 6- 7cm to determine the LD50 for subsequent pathogenicity assessment. The pathogenicity tests involved seven treatments with three replicates each, including treatment A, which involved the injection of *V. navarrensis* 10^7 CFU $mL^{-1;}$ treatment B with the injection of *V. navarrensis* at 10^6 CFU mL^{-1}; treatment C with the injection of *V. navarrensis* 10^5 CFU mL^{-1}; treatment D with the injection of A. *lwoffii* at 10^7 CFU mL^{-1}; treatment E with the injection of A. *lwoffii* at 10^6 CFU mL^{-1}; treatment F with the injection of *A. lwoffii* at 10^5 CFU mL^{-1}; and a control group injected with phosphate buffered saline (PBS). The LD50 results indicated that *V. navarrensis* and *A. lwoffii* bacterial densities were 1,7 x 10^5 CFU mL^{-1} dan 2,1 x 10^5 CFU mL^{-1}. Significant differences were noted in mortality rates, average time to death, and blood parameter profiles across all treatments compared to the control group ($P< 0.05$). Histopathological examination of the liver and spleen revealed hepatocyte damage, cell inflammation, melanomacrophage centers, and cell necrosis. Test of the intestine showed hemorrhage and hydropic degeneration in the muscular layer. A series of pathogenicity tests confirmed that these two bacteria are responsible for causing bacterial disease in Barramundi culture in North Aceh.

INTRODUCTION

North Aceh is a regency consisting of 27 districts. Among them, Dewantara is part of the coastal region of West Lancang, which includes a village (Gampong) known for its potential in aquaculture, developed by the local community. The primary commodity cultivated by aquaculture farmers in this area is barramundi (*Lates calcarifer*). Barramundi (*L. calcarifer*) is a fish of significant economic value, serving as an important export commodity with extensive markets in America, Europe, Malaysia, and Thailand.

Its production has been increasing annually. In 2018, barramundi production reached 30,000 tons, a substantial increase from the previous year's production of 25,051 tons **(Rayes *et al.*, 2013; Ministry of Marine Affairs and Fisheries, 2018)**. The tall trade request presents an opportunity for the advancement of barramundi aquaculture, as the angle is simple to develop and incorporates a tall resilience for changes in saltiness (**Hardianti *et al.*, 2016)**.

The rising demand for barramundi has led to a significant increase in aquaculture activities. However, the lack of knowledge about proper farming practices has resulted in several issues, notably disease outbreaks. Farmers in Dewantara District frequently report high mortality rates among their farmed barramundi (*Lates calcarifer*). The observable symptoms in the deceased fish include surface lesions, darkened body coloration, torn tail fins, and sloughing gills. Additionally, a decrease in appetite is a concern for farmers in Dewantara District. These symptoms are suspected to be caused by bacterial infections. **Kurniawan (2012)** elucidated that bacterial diseases in fish typically manifest as reddish lesions on the body surface. Bacterial infection typically results in abnormal changes (lesions) on the skin or fins, muscle tissues, and internal organs.

One effective and accurate method utilized to measure the pathogenicity of bacterial diseases is through pathogenicity testing and determining the *Lethal Dose* 50 (LD50) - the bacterial dose that causes mortality in 50 percent of the infected fish population. Additional methods include observing clinical symptoms, calculating mortality rates and average time to death, conducting histopathological examinations of tissue samples, analyzing the blood profiles of fish affected by bacterial diseases, and testing antimicrobial sensitivity to assess the effectiveness of antibiotic substances in inhibiting the growth of bacteria causing these diseases **(Izwar *et al.*, 2020)**. This study aimed to investigate the bacterial species causing bacterial diseases in barramundi and to test their pathogenicity.

MATERIALS AND METHODS

Description of study sites

This research was conducted at the Brackish Water Aquaculture Fisheries Center Ujung Batee, Aceh, Indonesia. Molecular analysis of the bacteria was conducted at PT Genetica Science Indonesia, Tanggerang City, Banten, Indonesia. Experimental fish was sent from PT Kembang Tani Farm, North Aceh, Aceh, Indonesia.

Study design

This research comprises preliminary and main studies. The preliminary study involves molecular analysis of bacteria from **Saragih *et al.* (2024)** to definitively identify the bacterial species. This study involved two bacterial samples, designated as isolate A and isolate B, which were sent to PT Genetica Science Indonesia for analysis. The PCR analysis conducted by PT Genetica Science Indonesia included the following steps: (1)

Genomic DNA extraction with Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005); (2) PCR amplification with (2x) Dream Taq PCR Master Mix (K9021; Thermo); and (3) Bi-directional sequencing. The sequencing results indicated that isolate A was *Vibrio navarrensis* and isolate B was *Acinetobacter lwoffii*. Subsequently, Koch's postulates were tested to confirm the virulence of the test bacteria. Koch's postulates were conducted by injecting each bacterium at a density of 10^8 CFU mL^{-1}. Observations regarding clinical symptoms such as changes in behavior, morphology, external symptoms, internal symptoms, and fish mortality were conducted over a period of 96 hours.

The main study commenced following the completion of the preliminary study. The pathogenicity test was conducted using a completely randomized design with 6 treatments and 1 control, each with 3 replicates. The experimental design for the treatments is outlined in Table (1).

Table 1. Experimental design for the pathogenicity test of bacterial diseases in barramundi

No	Treatment	Explanation
1	K	Control: fish injected with *Phosphate Buffered Saline* (PBS)
2	A	Fish injected with *V. navarrensis* 2.3×10^7 CFU mL^{-1}
3	B	Fish injected with *V. navarrensis* 2.2×10^6 CFU mL^{-1}
4	C	Fish injected with *V. navarrensis* 1.7×10^5 CFU mL^{-1}
5	D	Fish injected with *A. lwoffii* 2.1×10^7 CFU mL^{-1}
6	E	Fish injected with *A. lwoffii* 2.5×10^6 CFU mL^{-1}
7	F	Fish injected with *A. lwoffii* 1.7×10^5 CFU mL^{-1}

The research steps included preparing the containers, preparing the test fish, preparing the bacteria and conducting Koch's postulates, conducting the pathogenicity test, and making observations. Each bacterial strain was cultured for 24 hours before being injected into the barramundi fingerlings. The maintenance containers, which had a volume of 25 liters, were disinfected to prevent contamination. A total of 105 liters of seawater was used across all containers, with 105 barramundi fingerlings used for the study.

Preparation of test fish

The fish used in this study were barramundi measuring 7 cm in length. They were carefully selected to ensure they were in good health and free from any wounds or physical deformities in order to obtain accurate results.

Preparation of test bacteria and Koch's postulates test

The bacteria were obtained from the study conducted by **Saragih *et al.*, (2024)**. Both bacterial isolates were purified in TSA media + NaCl 0.9%. Three colonies of each bacterium were transferred into TSB and homogenized. Each bacterium, with a density of

10^8 CFU, was injected intraperitoneally into 10 barramundis. The injected fish were observed for changes in behavior and clinical symptoms over a period of 96 hours **(Sarjito, 2010)**.

LD50 test

The bacterial virulence level was assessed by calculating its LD50 value. LD50 testing was conducted using the Reed-Muench method (1938), with three different dosage treatments achieved through dilution methods and bacterial density calculations. The bacterial densities used in the LD50 testing were 10^5, 10^6, dan 10^7 cells per 0.1 mL per fish. The LD50 testing was conducted by injecting healthy barramundi fingerlings with bacteria at 0.1mL using the intraperitoneal injection method, while the control group was injected with phosphate buffered saline (PBS). Prior to infection, the test fish were first coated with wet cloth to minimize stress from handling. After injection, the fish were returned to the maintenance containers and observed at least every 12 hours. The LD50 testing results will assess bacterial virulence and mortality among the barramundi fingerlings **(Sunarto *et al.*, 2003)**.

$$\text{Proportion Odds Ratio (POR)} = \frac{50 - \textit{Deaths below } 50\%}{\text{Deaths above } 50\% - \text{Deaths below } 50\%}$$

Log negative LD50 = Log concentration in bacteria below 50% + (Proportion Odds Ratio × Log dilution)

LD50 = Anti Log × Log negative LD50

Pathogenicity test

The fish infected during the LD50 test were monitored to evaluate the pathogenicity of the test bacteria. Parameters observed included clinical symptoms, mortality rate, average time to death, and blood profile analysis. Changes observed during the LD50 test were recorded over a period of 7 days. Throughout the observation period, fish were fed commercial feed twice daily ad libitum. Fish that died during the study were disposed of by burial to prevent disease transmission.

Mortality rate

The observation of fish mortality was done every day after pathogen injection. Fish mortality was calculated by using the formula below **(Effendie, 1979)**:

$$\text{Mortality rate (\%)} = \frac{\text{The total dead fish}}{\text{The total fish}} \times 100$$

Meantime of death (MTD)

Fish that was already injected by pathogen was observed detecting the behavior every day, such as the clinical symptoms, fish mortality, and meant time to death. Meantime to death was calculated by using the formula below **(Hubbert, 1980)**.

$$MTD = \frac{\sum_{i-1}^{n} a1\ bi}{\sum_{i-1}^{n} bi}$$

Note:
MTD = Meantime of death (hour)
a = mortality time (hour)
b = number of dead fish

Blood profile parameters

Blood profile parameters analyzed include total erythrocytes, total leukocytes, haemoglobin, and haematocrit. Test fish injected with bacteria were subjected to blood profile analysis on days 1, 3, 5, and 7 according to the treatment. Blood sampling from the fish was conducted using the *Caudal Vessel Puncture technique*. The syringe needle was first moistened with an anticoagulant, ethylene diamine tetraacetic acid (EDTA), to prevent blood clotting during the analysis.

Total Erythrocytes

The erythrocyte count in barramundi was conducted using a hemocytometer. This device involves drawing a 0.5mL blood sample and mixing it with Hayem's solution up to the 101 mark. The mixture is then pipetted and gently mixed for 5 minutes. Subsequently, the blood is dropped into a Neubauer hemocytometer chamber. The erythrocyte count is observed and calculated using a microscope connected to a computer screen to facilitate observation **(Saparuddin, 2017)**.

Total leukocytes

The tool used to count white blood cells was a hemocytometer. This device was employed by drawing a blood sample up to the 0.5mL mark for each treatment, followed by the addition of Turk's solution up to the 1:11 ratio. The pipette was gently swung to ensure thorough mixing for 5 minutes. Subsequently, the blood was dropped into a Neubauer hemocytometer chamber. The number of white blood cells was then observed and calculated using a microscope connected to a computer screen for easier observation **(Saparuddin, 2017)**.

Hemoglobin

The procedure for hemoglobin measurement was conducted using the Sahli method. First, a blood sample was drawn into a pipette up to the 0.2 mL mark. The pipette tip was then wiped clean with tissue paper. Next, the blood from the pipette was transferred to an Hb-meter tube filled with 0.1 N HCl up to the 10 mL mark. Next, the blood was stirred for 3 to 5 minutes, followed by the addition of distilled water (aquades) into the tube until the blood color matches that of the standard solution in the Hb-meter. The hemoglobin level was expressed in g $100mL^{-1}$ (g dl^{-1}) **(Hartika *et al.*, 2014)**.

Haematocrit

Hematocrit levels are determined by filling a capillary tube approximately two-thirds full with blood from a porcelain dish. The tube's end is tightly sealed with wax and wrapped in tissue to prevent breakage during centrifugation. The sample is then centrifuged at 1500rpm for 5 minutes. The red portion of the contents in the tube is measured as the hematocrit level **(Sarkiah *et al.*, 2016)**.

RESULTS

Molecular analysis of bacteria

Both types of bacteria from the previous study were subjected to molecular testing and underwent sequencing to accurately identify their types. The DNA concentration of each bacterial isolate met purity standards with an Absorbance $_{260/280}$ 1.90 for bacterial isolate A, and 1.96 for bacterial isolate B (Table 2). A DNA sample is considered pure when the A260/280 ratio ranges from 1.8 to 2.0. If the ratio falls below 1.8, it indicates contamination by phenol. Conversely, if the ratio exceeds 2.0, contamination by proteins or other compounds is inferred **(Sambrook & Russell, 2001; Maulida & Lisdiana, 2024)**.

Table 2. Results of quantitative DNA testing

No	Sample Name	Conc (ng/µL)	$A_{260/280}$
1	Isolate 1	38.0	1.90
2	Isolate 2	21.5	1.96

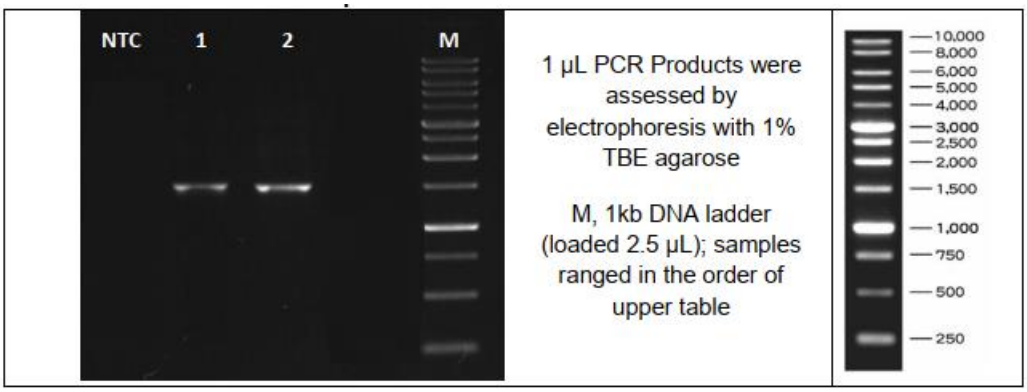

Fig. 1. Electrophoresis bands of rDNA gene fragments from bacterial isolate 1 and isolate 2

Observation of clinical symptoms

Prior to conducting the LD50 test, all treatment groups exhibited similar conditions: responsiveness to feed, normal behavior, active movement, silvery-white body color, smooth skin surface, intact fins, clear eyes, non-distended abdominal cavity, and well-preserved anatomical organs. However, following the LD50 test across all treatments,

barramundi showed signs of feed non-responsiveness, abnormal behavior, reduced activity, darkened body color, rough skin, frayed fins, yellowish-white eyes, distended abdominal cavity, and hemorrhaging in anatomical organs such as the intestines, lymphatic system, and kidneys. Detailed clinical symptoms of barramundi are presented in Table (3).

Table 3. Clinical symptoms of barramundi after LD50 test

Observation of morphology and anatomy	Treatment						
	K	A	B	C	D	E	F
Feeding response	Responsive	Non responsive	Non responsive	Non responsive	Non responsive	Non responsive	Responsive
Behavior	Normal	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal
Movement	Active	Passive	Passive	Passive	Passive	Passive	Passive
Body Color	White	Blackish	Blackish	Blackish	Blackish	Yellowing	Yellowing
Skin Surface	Smooth	Rough	Rough	Rough	Rough	Rough	Rough
Fins condition	Intact	Chipped	Chipped	Chipped	Chipped	Chipped	Chipped
Eyes Condition	Shiny White	Yellowing	Yellowing	Yellowing	Yellowing	Yellowing	Yellowing
Abdominal cavity Condition	Normal	Swollen	Swollen	Swollen	Swollen	Swollen	Swollen
Anatomical organ	Normal	Hemorrhage	Hemorrhage	Hemorrhage	Hemorrhage	Hemorrhage	Hemorrhage

Notes: K-control; A-Fish injected by *V. navarrensis* 2.3×10^7 CFU mL^{-1}; B- Fish injected by *V. navarrensis* 2.2×10^6 CFU mL^{-1}; Fish injected by *V. navarrensis* 1.7×10^5 CFU mL^{-1}; Fish injected by *A. lwoffii* 2.1×10^7 CFU mL^{-1}; Fish injected by *A. lwoffii* 2.5×10^6 CFU mL^{-1}, Fish injected by *A. lwoffii* 1.7×10^5 CFU mL^{-1}.

Fish injected with bacteria also exhibited body color changes to a darker shade and developed lesions on their bodies. This is caused by excessive mucus production, leading to a rough body texture and reduced pigmentation. Additionally, the bacteria damaged the fish's body surface and tissue or outer skin layer, resulting in skin decay and ultimately causing lesions.

LD50

Based on the research results, the LD50 testing on barramundi showed varying outcomes. The LD50 result for the bacterium *V. navarrensis* was 1,7 x 10^5 CFU mL^{-1}. The LD50 value for the bacterium *A. lwoffii* was 2,1 x 10^5 CFU mL^{-1}. The determination of the LD50 was performed by recording the number of test animal deaths that occurred within 24 hours after injection. The results of the LD50 test are presented in Table (4).

Table 4. Results of the LD50 test in bacterial pathogenicity testing

Bacterium	**LD50 (CFU mL^{-1})**
V. navarrensis	1,7 x 10^5
A. Lwoffii	2,1 x 10^5

Fig. 2. Comparison of clinical symptoms between control fish and diseased fish: a. Control fish with normal eyes; b. Treated fish with exophthalmia; c. Healthy control fish with intact scales; d. Treated fish with body lesions and frayed scales

Mortality Rate

Based on the results of the Kruskal-Wallis test analysis, significant differences in death rates were found across the treatments. The research results show an asymptotic significance value (asymp. sig.) of 0,003 meaning that the value is < 0,05. This indicates that the null hypothesis (Ho) is rejected and the alternative hypothesis (H1) is accepted, meaning there is an effect of the bacterial pathogen on the mortality of barramundi. This study demonstrates that bacterial pathogens significantly influence the mortality of barramundi (Fig. 3).

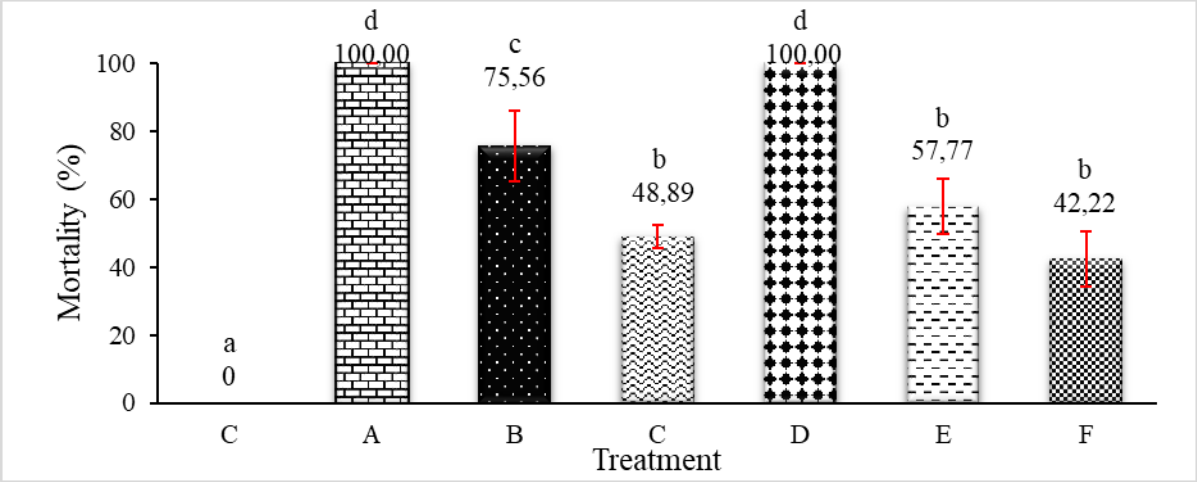

Fig. 3. Mortality of Barramundi (*Lates calcarifer*) in bacterial pathogenicity testing: K-control: Fish injected with PBS A: Fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B- Fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; Fish injected with bacteria *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}, Fish injected with bacteria *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

Mean time to death

Based on the results of the Kruskal-Wallis test analysis, significant differences were found in the mean time to death of barramundi *(L. calcarifer)* across different treatments. The study results showed an asymptotic significance value (asymp. Sig) of 0.003, indicating that this value is less than 0.05. As a result, the null hypothesis (H0) is rejected, and the alternative hypothesis (H1) is accepted, meaning there is an effect of the pathogenic bacteria on the average to death of barramundi. The mean times to death for each treatment are presented in Fig. (4).

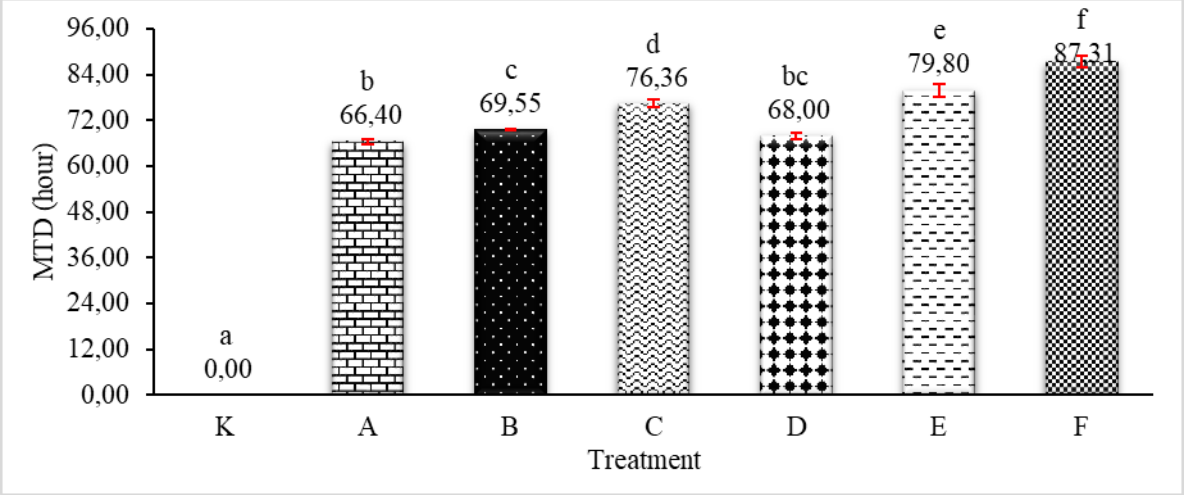

Fig. 4. The mean time to death of fish in the pathogenicity test for bacterial disease K-control.; A - Fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B - Fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; Fish injected with bacteria. *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; Fish injected with bacteria. *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; Fish injected with bacteria. *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}, Fish injected with bacteria. *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

Blood Profile

Hemoglobin levels tended to decrease in each treatment, and hematocrit values were directly proportional to the decreasing erythrocyte and hemoglobin levels over time, showing a significant difference from the control. The blood analysis can be seen in Figs. (5, 6, 7, and 8).

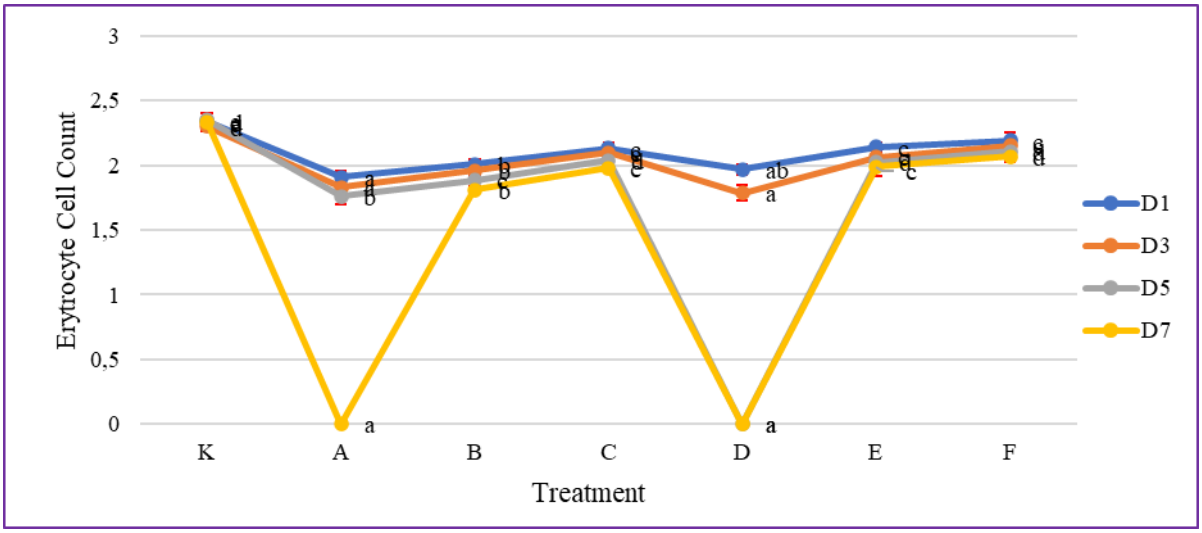

Fig. 5. Erythrocyte Cell Graph of Barramundi K-control; A- The fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B- The fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; The fish injected with bacteria *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; The fish injected with bacteria *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; The fish injected with bacteria *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}; The fish injected with bacteria *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

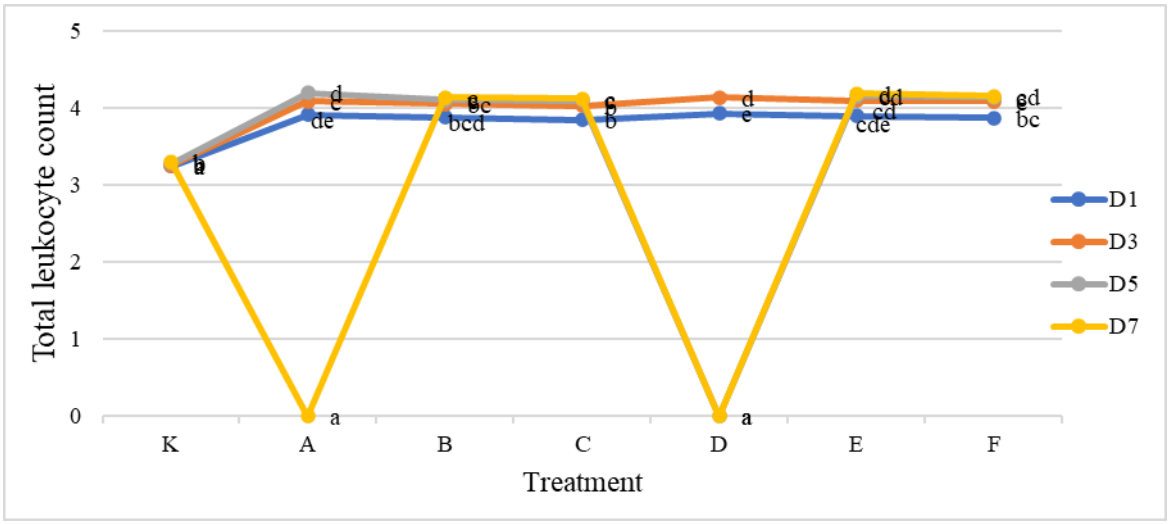

Fig. 6. Total leukocyte count in barramundi fish infected with K-control bacteria; A-Fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B- Fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; Fish injected with bacteria *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

The hemoglobin (Hb) levels observed in barramundi remain within the normal range, typically ranging between 5.47 &7.67 g% (Fig. 7). However, a significant distinction between diseased and healthy fish is evident, as the Hb levels in the treatment groups progressively decrease over time. This decline is attributable to reduced red blood cell counts caused by bacterial lysis, leading to diminished blood oxygen levels and thereby contributing to decreased Hb levels in barramundi.

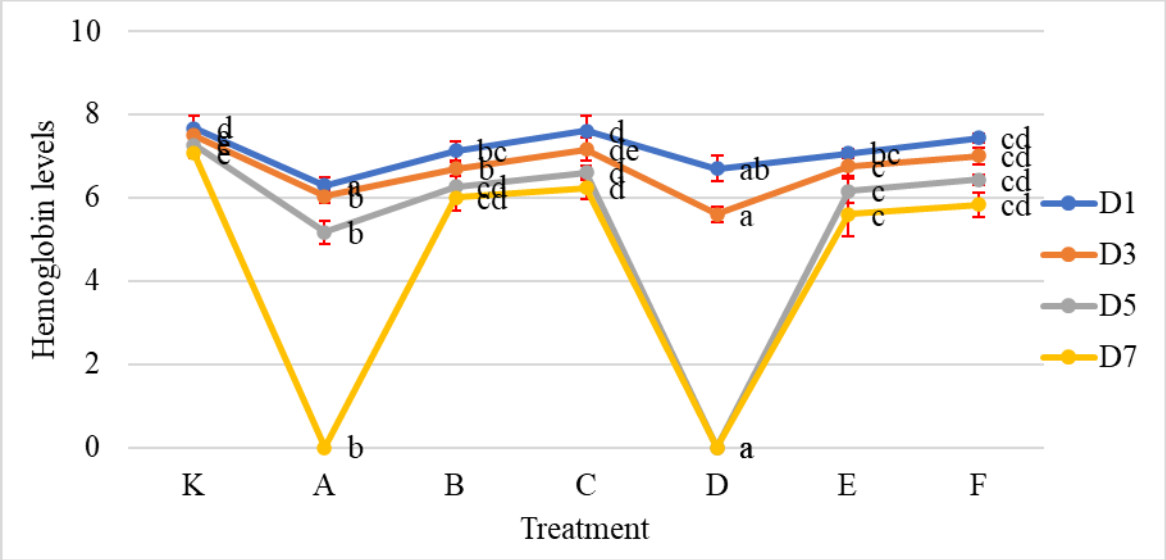

Fig. 7. Hemoglobin cell morphology in barramundi under K-control; A-Fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B- The fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; The fish injected with bacteria *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; The fish injected with bacteria *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; The fish injected with bacteria *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}, The fish injected with bacteria *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

Based on the observations, the hematocrit levels in barramundi infected with bacteria and those that are not, also exhibit a notable decline (Fig. 8). This is due to bacterial activity within the bloodstream, which reduces hematocrit levels.

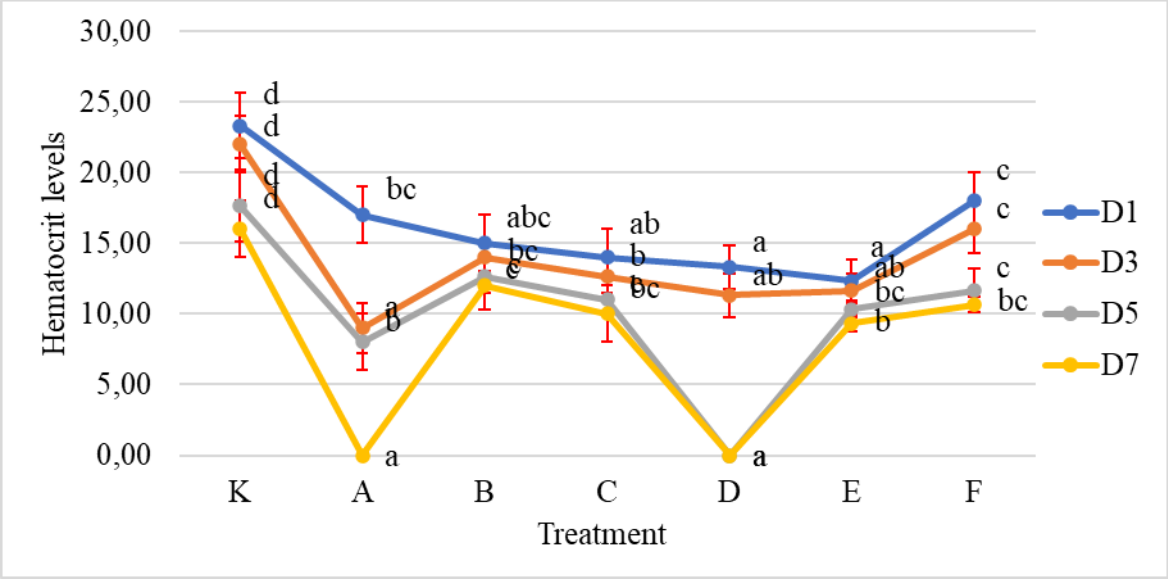

Fig. 8. Hematocrit cell morphology in barramundi under K-control; A-Fish injected with bacteria *V. navarrensis* 2.3 × 10^7 CFU mL^{-1}; B- Fish injected with bacteria *V. navarrensis* 2.2 × 10^6 CFU mL^{-1}; Fish injected with bacteria *V. navarrensis* 1.7 × 10^5 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.1 × 10^7 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 2.5 × 10^6 CFU mL^{-1}; Fish injected with bacteria *A. lwoffii* 1.7 × 10^5 CFU mL^{-1}.

DISCUSSION

Molecular analysis of bacteria

The results of electrophoresis presented in Fig. (1) show bands at the 1500bp marker. Each sample exhibits only one band, indicating the presence of a single type of DNA fragment molecule from bacterial isolates one and two, without any contaminants. This allows progression to the next stage, sequencing. Based on sequencing results matched with the NCBI GenBank database, isolate one was identified as *Vibrio navarrensis* bacteria with 100% similarity, and isolate two as *Acinetobacter lwoffii* bacteria, also with 100% similarity.

V. navarrensis is a gram-negative bacterium first discovered in 1982 from sewage water in Spain. **Gladney & Tarr (2014)** characterized V. *navarrensis* isolates from various sources, including blood, revealing its potential to become a human pathogen. Genetically, *V. navarrensis* shares phenotypic and genotypic similarities with Vibrio *vulnificus* and is considered an opportunistic bacterium with a 50% mortality rate associated with symptoms of septicemia **(Thompson *et al.*, 2005; Gladney & Tar, 2014; Ayuzar *et al.*, 2023)** mentioned that *vibriosis* infections can lead to mortality rates of 80-90% in barramundi. **Gladney *et al.*, (2014)** stated that *V. navarrensis* has the capability to thrive across a wide range of salinities. This is evidenced by its presence in freshwater, brackish water, and marine environments. Furthermore, isolations from humans and

household settings indicate that *V. navarrensis* inhabits a broad ecological niche, demonstrating extensive genetic diversity within the species.

Similar to *V. navarrensis*, *Acinetobacter* bacteria are also Gram-negative bacteria commonly found in various aquatic environments from freshwater to seawater, soil, and even in the air, capable of infecting diverse hosts **(Wang *et al.*, 2020)**. *Acinetobacter* spp. are not pathogenic; however, they are known to cause nosocomial infections in immunocompromised ones **(Baumann 1968; Berlau *et al.*, 1999; González *et al.*, 2000)**. In recent years, there have been several reports of fish infected by *Acinetobacter*. For instance, *Ictalurus punctatus* and *Channa striatus* have been infected by *A. baumannii* **(Lu *et al.*, 2008; Rauta *et al.*, 2011)**, and the rainbow trout (*Oncorhynchus mykiss*) has been infected by *Acinetobacter johnsonii* **(Kozińska *et al.*, 2014; Bi *et al.*, 2023)**. Recently, **Ledesma *et al.* (2017)** postulated that *A. lwoffii* can cause respiratory infections in the mammal *Lama glama* and *postencephalitic hydrocephalus* with blindness in dogs **(Kim *et al.*, 2017)**. It has also been linked to esophagitis in the common carp (*Cyprinus carpio*) **(Kozińska *et al.*, 2014)**, exophthalmos in the rainbow trout (*Oncorhynchus mykiss*) **(Dadar *et al.*, 2016)**, gill disease in the African catfish (*Clarias gariepinus*) **(Elsayyad *et al.*, 2010)**, and diseases in *Schizothorax* **(Cao *et al.*, 2018)**. To our knowledge, this is the first reported case of *V. navarrensis* and *A. lwoffii* infection in barramundi.

Observation of clinical symptoms

The clinical symptoms observed after the LD50 test across all treatments were nearly identical. These included unresponsiveness to feed, abnormal movement behaviors, passivity, darkening of body color, rough body surface, frayed fins, yellowish-white eyes, bloated abdomen, and bleeding in the organs of the barramundi. These symptoms are consistent with those of fish affected by bacterial infections or bacterial diseases. This finding aligns with the research conducted by **Napitupulu (2016)**, indicating that fish infected by bacteria exhibit clinical symptoms such as lesions on the body surface, detached scales, abnormal changes (lesions) on the skin or fins, muscle tissue damage, and damage to internal organs.

Fish injected with bacteria exhibited body color changes to a darker shade and developed lesions on their bodies. This is caused by excessive mucus production, leading to a rough body texture and reduced pigmentation. Additionally, the bacteria damage the fish's body surface and tissue or outer skin layer, resulting in skin decay and ultimately causing lesions. This observation is consistent with **Subachri *et al.* (2012)** research indicating that the darkening of fish body color is caused by bacteria, viruses, or parasites disrupting the fish's immune system and affecting mucus production. As a physiological response, fish infected by bacteria, viruses, or parasites will initially produce excessive mucus, followed by a drastic reduction in mucus production. This results in a rough skin texture and the darkening of the body color.

The subsequent symptoms exhibited by fish injected with bacteria include a rough skin surface, frayed fins, and eyes that turn yellowish-white. The rough skin is caused by excessive mucus production in response to bacterial infection. Frayed fins (Fig. 2a) result from bacteria attacking and adhering to the tissue or cells in the fin area. The yellowish-white eyes or exophthalmia (Fig. 2b) are due to cell damage caused by bacterial activity. **Hastari *et al*. (2014)** noted that the clinical symptoms in fish infected with bacteria are caused by inhibited cell formation, leading fish to respond by producing excess mucus. Lesions result from bacteria eroding cells and tissues and changes in eye color are due to cell damage, ultimately causing exophthalmia. This is supported by the study of **Cao *et al.* (2018)**, who reported that fish infected with A. *lwoffi* bacteria exhibit clinical symptoms such as lesions on the body surface, especially the dorsal region, and exophthalmia in the eyes.

In contrast to the six treatments, the clinical symptoms in the control group remained unchanged before and after the LD50 test. This is because the control fish were only injected with PBS (*Phosphate Buffered Saline*), a solution with ion conditions similar to those within the body, thus not causing any effects or clinical symptoms indicative of bacterial infection. **Luo (2020)** stated that PBS has good buffering capacity and can maintain osmotic pressure since it contains salts and amino acids. PBS functions to regulate pH and osmolarity balance by providing essential water and organic ions.

LD50

Wahjuningrum (2014) stated that the virulence of bacteria is influenced by the production of enzymes and toxins from the bacterial cells themselves. Differences in LD50 values are also attributed to variations in bacterial serotypes and biotypes. Furthermore, the differences in enzyme and toxin production due to the use of different isolates, may also play a role. A low LD50 value further demonstrates that these bacteria have the capability to cause infections in host cells, leading to subsequent death. This is supported by **Mangunwardoyo *et al.* (2016)**, who stated that death at low LD50 values is due to high virulence and pathogenicity, as the bacteria possess the ability to transmit, adhere to host cells, invade host tissues, and ultimately cause infections that lead to death. **Priyadi (2010)** further added that, generally, the smaller the LD50 value, the more toxic the compound or substance. Conversely, the larger the LD50 value, the lower the toxicity.

Mortality rate

The treatments with the highest mortality rates were Treatment A and D, with an average of 100%, followed by Treatment B with an average of 75.56%, Treatment E with an average of 57.77%, Treatment C with an average of 48.89%, and Treatment F with an average of 42.22%. The control treatment (K) showed a mortality rate of 0%. The mortality rates are illustrated in Fig. (2). Additionally, treatment C and F showed lower mortality rates compared to the other 4 treatments. The decrease in bacterial density is one of the factors contributing to the lower mortality rates in treatments C and F

compared to the others. This indicates that the reduction in the injected bacterial concentration affects the percentage of deaths. This is consistent with the work of **Susanti (2016)**, who stated that increasing bacterial concentration by 10 times increases the percentage of fish deaths, and conversely, decreasing bacterial concentration by 10 times decreases the percentage of fish deaths. The mortality rate in the control treatment was 0%, indicating that no fish died among those injected with PBS. The absence of mortality is due to the fact that the test fish were only injected with PBS, without introduction of any bacteria or foreign pathogens into their bodies. This ensured that there was no disruption to the fish's immune system or health, maintaining the control fish in a healthy state throughout the experiment.

Cao *et al.*, (2018) stated that *A. lwoffi* infection with a density of 10^7 CFU mL^{-1} resulted in 100% death in the *Schizothorax* fish genus. While in this study, *A. lwoffi* infection can kill 100% of barramundi with a density of only 10^6 CFU mL^{-1}. This is thought to be related to differences in fish species and differences in the environment, where the bacteria are located.

Mean Time of Death (MTD)

The treatment with the shortest MTD was treatment A, with an average time of 66.40 hours. This was followed by treatment D at 69 hours; treatment B at 69.55 hours; treatment C at 76.36 hours; treatment E at 79.80 hours; and treatment F, which had the longest average time to death at 87.31 hours. The control treatment did not exhibit any death throughout the maintenance period. Statistical analysis showed that *V. navarrensis* bacterial infection of 10^6 CFU mL^{-1}, 10^5 CFU mL^{-1}, and *A. lwoffii* bacterial infection with a density of 10^7 CFU mL^{-1} produced the same MTD. These three treatments had the fastest MTD value among the other treatments, namely 66.40 - 69.55 hours. This is in accordance with the finding of **Sivasankar *et al.* (2015)** postulating that vibriosis infection can kill fish populations within 1-3 days.

Blood Profile

Blood profile parameters have been used as health indicators in fish **(Sayed & Moneeb, 2015; Nugrahawati *et al.*, 2019)**. One of the blood parameters used to indicate the health condition of fish is the total erythrocyte count. The total erythrocyte count in the six treatments was significantly different ($P< 0.05$) compared to the control treatment. At all observation times, the total erythrocyte count in the control treatment was higher than in the six treatments (Fig. 4). The decrease in erythrocyte count is believed to be caused by the hemolytic properties of the bacteria and *Acinetobacter lwoffii* **(Kozińska *et al.*, 2014; Cao *et al.*, 2018)** and *Vibrio navarrensis* **(Schwartz *et al.*, 2017)** which can break down the red blood cells.

CONCLUSION

The bacteria that attack the white sea bass in North Aceh are *V. navarrensis* and *A. lwoffi*. The bacterial infections by *V. navarrensis* and *A. lwoffi* can effectively infect and induce bacterial diseases in barramundi, as evidenced by clinical symptoms, high mortality rates, relatively short average time to death, and distinct blood profiles compared to the control group. A series of pathogenicity tests confirmed that these two bacteria are responsible for causing bacterial disease in barramundi culture in North Aceh.

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