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The Occurance of *Aeromonas hydrophila* and Infectious Spleen and Kidney Necrosis Virus Pathogenicity Test in the Nile Tilapia *Oreochromis niloticus*

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ABSTRACT

This study aimed to isolate and identify *Aeromonas hydrophila* and to test the pathogenicity of this bacterium and the infectious spleen and kidney necrosis virus (ISKNV) in the Nile tilapia. The single infection experiments aimed to determine the LD50 of *A. hydrophila* and ISKNV infections. The infections were conducted in two stages: a single infection with *A. hydrophila* and another with ISKNV, each at different doses. Co-infection doses of *A. hydrophila* and ISKNV were derived from the LD50 of the single infections. Infections with either *A. hydrophila* or ISKNV resulted in mortality rates exceeding 50% in each treatment group. The treatments included the *A. hydrophila* control (K) and IB7, as well as the ISKNV control (K) and P0. The third treatment was co-infection with *A. hydrophila* and ISKNV (K and P0+IB7). All treatments were observed for 14 days. The results showed that single infections with *A. hydrophila*, ISKNV, and co-infections produced significantly different outcomes among treatments ($P<$ 0.05). In the *A. hydrophila* infection, the survival rate of the Nile tilapia in the IB7 treatment was 33%, while it was 100% in the control (K). In the ISKNV infection, the survival rate in the P0 treatment was 20% compared to 90% in the control (K). In the co-infection of *A. hydrophila* and ISKNV, the survival rate in the P0+IB7 treatment was 10% compared to 90% in the control (K). This study concluded that co-infection with *A. hydrophila* and ISKNV increases and accelerates mortality compared to single infections with either *A. hydrophila* or ISKNV. Additionally, hematological immune responses showed significant differences in co-infections compared to single infections.

INTRODUCTION

The Nile tilapia *(Oreochromis niloticus)* is a freshwater commodity with high prospects and is one of the most widely cultivated freshwater aquaculture commodities. Its production increased significantly from $186.682 in 2000 to $2.079.517 in 2018 **(FAO, 2020)**. Moreover, the global aquaculture production has increased to 223.2 million tons, with 185.4 million tons coming from aquatic animals and 37.8 million tons from

algae. About 89 percent of aquaculture production is used for human consumption, which is equivalent to approximately 20.7kg per capita in 2022. Aquaculture production is expected to increase 10 percent by the year 2032 **(FAO, 2024)**. According to **Miao and Wang (2020)**, the global Nile tilapia production accounted for 5.27% of the total global aquaculture production in 2018, with a value of $11.2 billion, contributing 4.5% of all aquaculture commodities. One of the most common practices is intensive farming systems, and one of the key challenges of intensive farming is disease. A common bacterial disease found in the Nile tilapia is Motile Aeromonad Septicemia (MAS), caused by *Aeromonas hydrophila* **(Wahjuningrum *et al.*, 2008; Younis *et al.*, 2023)**. The *Aeromonas hydrophila* is a Gram-negative, non-spore-forming bacterium with a single flagellum, and it is both aerobic and facultatively anaerobic. *Aeromonas* bacteria thrive in environments with temperatures ranging from 25–30°C. Motile Aeromonad Septicemia (MAS) is characterized by symptoms such as the loss of appetite, gill hemorrhages, damaged fins accompanied by red spots, swelling, and damage to several internal organs **(Alimuddin *et al.*, 2018; Austin, 2022)**. *Aeromonas hydrophila* is a virulent and contagious disease that caused mass mortality in freshwater fish in Indonesia in 1980 **(Nuryati *et al.*, 2006)**.

The infectious spleen and kidney necrosis virus (ISKNV) infects various fish species and causes significant mortality. In the mandarin fish (*Siniperca chuatsi*), ISKNV infection results in 100% mortality within 7–10 days post-infection. **Murwantoko *et al.* (2018)**, in their study, elucidates that ISKNV has been successfully detected in Indonesia. ISKNV infections have been identified in both marine and freshwater fish species, leading to mass mortality within a relatively short period of 1–2 weeks in aquaculture systems, causing economic losses for fish farmers. ISKNV infection is transmitted horizontally and spreads through water or infected tissues **(He *et al.*, 2002; Go *et al.*, 2006; Subramaniam *et al.*, 2012)**. The infection is marked by damage and swelling of the spleen and kidneys, ultimately leading to host mortality **(Sung *et al.*, 2010)**. In addition to being caused by *A. hydrophila* and ISKNV, diseases in the Nile tilapia can also be caused by the bacterium *Streptococcus*. Streptococcosis in fish caused by *Streptococcus agalactiae* can lead to septicemia **(Sukenda *et al.*, 2015)**.

The Minister of Marine Affairs and Fisheries of the Republic Indonesia, decree number 80 of 2015 postulates that the infectious spleen and kidney necrosis virus (ISKNV) is classified as a Category 1 Quarantine Fish Pest and Disease. Consequently, this disease is a priority, requiring strict monitoring and prevention measures to avoid its spreading to areas that are still free from infection **(KKP, 2015)**. Furthermore, a rapid and an accurate testing method for early detection of this disease is highly necessary. Therefore, the readiness of provincial, district, or city governments is essential to support efforts in preventing the transmission of this disease, thereby minimizing economic losses in both marine and freshwater aquaculture industries. According to **Kurita and Nakajima (2012)**, a total of 30 infectious spleen and kidney necrosis virus (ISKNV)

species infect marine fish. Based on information from the Minister of Marine Affairs and Fisheries of the Republic of Indonesia, as stated in decree number 81 of 2015, the regions affected by infectious spleen and kidney necrosis virus (ISKNV) in Indonesia includes North Sumatra, West Sumatra, Lampung, Bali, and the Riau Islands.

Recently, there has been a mass mortality of the Nile tilapia fingerlings in Bogor. The affected fish exhibited clinical symptoms such as necrosis on the body and fins, protruding eyes, reddened gills, and swollen abdomens. Preliminary examinations revealed the presence of both bacteria and viruses simultaneously. Therefore, this study aimed to isolate and identify *Aeromonas hydrophila* and the infectious spleen and kidney necrosis virus (ISKNV) (obtained from diseased fish) and to test their pathogenicity in the Nile tilapia.

MATERIALS AND METHODS

The test subjects used in this study were the Nile tilapia (*Oreochromis niloticus*) with an average weight of 3.56 ± 1.58 grams and an average size of 7.31 ± 2.15cm, sourced from fish farmers in Bogor, West Java. The bacterial isolate used was *Aeromonas hydrophila*, obtained from the bacterial isolation of wounds found on the Nile tilapia. Single infections were conducted to determine the LD50 of *Aeromonas hydrophila* or the infectious spleen and kidney necrosis virus (ISKNV) in the Nile tilapia.

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Isolation, identification and pathogenicity of *Aeromonas hydrophila*

The isolation of *Aeromonas hydrophila* was carried out aseptically. The medium used was tryptic soy agar (TSA) on petri dishes. The streaked media were then incubated in an incubator at a temperature of 29°C–37°C for 24 hours. Biochemical characterization of the bacteria included Gram staining, catalaze test, oxidaze test, and other biochemical tests. The results of the biochemical characterization were compared with Cowan and Steel's manual **(Barrow & Feltham, 2003)**.

Sample preparation of *Aeromonas hydrophila*

The identification of *Aeromonas hydrophila* using the PCR (Polymerase chain reaction) method included the preparation of liquid cultures of *A. hydrophila* with a concentration of 10^7 CFU/ml. Specific primers for *Aeromonas hydrophila* were used with the following sequences: forward primer F: 5'-GAAAGGTTGCCTAATACGTA-3' and reverse primer R: 5'-CGTGCTGGCACCAAAGGAGAG-3' **(Altinok *et al.*, 2008)**.

LD50 of *Aeromonas hydrophila*

The concentration of *Aeromonas hydrophila* was determined using the lethal dose 50 (LD_{50}) test. *A. hydrophila* aged 24 hours were inoculated into a test tube containing

10mL of tryptic soy broth (TSB) media and then incubated with a shaker for 24 hours. The stock culture watstested for total plate count (TPC). Fish were intramuscularly injected with *A. hydrophila* at concentrations of 10^6, 10^7, and 10^8 CFU mL^{-1}, while the control group was injected with 0.1mL of PBS solution per fish. The LD_{50} of *A. hydrophila* was determined using the method outlined by **Reed and Muench (1938)**.

LD50 infection *Aeromonas hydrophila*

Two treatments were pepared for experiment: IB7 and K. In the IB7 treatment, fish were infected with *Aeromonas hydrophila* at a concentration of 10^7 CFU mL^{-1}, with 0.1mL administered. In the K treatment, the Nile tilapia were intramuscularly injected with 0.1mL of PBS. The Nile tilapia in both treatments were observed for 14 days post-challenge and were fed a commercial feed and libitum three times a day (08:00, 12:00, and 16:00 WIB).

Identification and pathogenicity of infectious spleen and kidney necrosis virus sample preparation and ISKNV polymerase chain reaction (PCR)

The infectious spleen and kidney necrosis virus (ISKNV) amplification was carried out using specific ISKNV primers, with the forward primer sequence as follows: F: 5′-GGTGGCCGGCATCACCAACGGC-3′ and the reverse primer sequence for ISKNV: R: 5′-ACGGGGTGACTGAACCTG-3′ **(Kurita & Nakajima, 2012)**. Electrophoresis was performed using 1% agarose with 1x TAE buffer for 40 minutes. The results were documented using a gel documentation camera. Infected samples showed bands corresponding to ISKNV at 415bp. The DNA sequencing method was used following the method of **Sanger *et al.* (1977)**.

Product sequencing

The polimerase chain reaction (PCR) products showed positive results that were sent for DNA sequencing by 1st Base. The sequencing results were analyzed using BLAST search of the nucleotide sequence against available databases on NCBI. The program used for sequence alignment was Clustal W **(Tamura *et al.*, 2011)**. The nucleotide sequence was aligned with reference sequences from other species obtained from the NCBI website database (http://www.ncbi.nlm.nih.gov). Evolutionary analysis was performed using the MEGA X software **(Kumar *et al.*, 2018)**.

The infectious spleen and kidney necrosis virus (ISKNV) preparation

The infectious spleen and kidney necrosis virus (ISKNV) isolation was obtained from the positive PCR results. ISKNV isolation was performed using infected fish tissue (kidney, spleen, and liver) weighing one gram, which was homogenized with sterile

phosphate-buffered saline (PBS, Gibco) at pH 7.4 in a volume of nine milliliters. The tissue suspension was centrifuged at 1500rpm for 15 minutes at 4°C and was then filtered using a 0.45µm membrane filter (Millipore, USA). The resulting supernatant homogenate was stored at -86°C **(Gardenia *et al.*, 2020; Sukenda *et al.*, 2020)**.

The infectious spleen and kidney necrosis virus

The Nile tilapia, sourced from Bogor, had an average weight of 3.56 ± 1.58g and an average size of 7.31 ± 2.15cm. They were maintained in 500L fiberglass containers equipped with aeration, and the water temperature was kept between 25 & 30°C. The pathogenicity test, as described by **Tran *et al.* (2013)**, was conducted over 14 days. The LD_{50} was determined by injecting various dilutions of the virus stock concentration (10^{-1} – 10^{-3}) over a 7-day period.

Pathogenicity of *Aeromonas hydrophila* and infectious spleen and kidney necrosis virus (ISKNV)

The pathogenicity begins with the intramuscular injection of *Aeromonas hydrophila* and the infectious spleen and kidney necrosis virus (ISKNV) in the Nile tilapia measuring 7-8cm. The dose of *A. hydrophila* and ISKNV injected in the Nile tilapia was 0.1mL per fish. The injections of *A. hydrophila* and ISKNV were performed using a 1mL syringe, with the needle positioned at a 45°. The pathogenicity test was conducted for 14 days **(Tran *et al.*, 2013)**.

Test parameters

Survival rate

Survival rate is the ratio of the number of fish that survive from the beginning till the end of the maintenance period **(Norazmi *et al.*, 2020)**.

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Description:

SR = Survival rate (%)
Nt = Quantity of fish the end studi (heads)
No = Quantity of fish the beginning (heads)

Hematology
Hemoglobin

Blood was collected in a volume of 0.2mL using a Sahli pipette and placed into an Hb-meter tube. Then, 0.1 N HCl was added until the scale reached 10 (red scale). The blood was homogenized and allowed to stand for five minutes until the solution changed color. Distilled water was added to the tube and was stirred with a stirring rod until the color matched the Hb-meter indicator. The hemoglobin level was read by observing the scale on the Hb-meter tube, expressed in grams per 100cc of blood (g%) **(Walter, 1988; Fazio, 2018)**.

Hematocrit

Blood was placed into a hematocrit tube up to ¾ of the tube's length, and the end of the tube was sealed with crytoceal wax. The tube was then centrifuged for 5 minutes at 5,000rpm. After centrifugation, the blood pellet and total plasma were visible. The length of the blood pellet and total plasma were measured using a ruler **(Fazio, 2018)**.

$$\text{Hematocrit (\%)} = \frac{\text{Length of blood pellet}}{\text{Length of total volume}} \times 100$$

Total erythrocytes

The preparation method used in the current study followed the guidelines of **Blaxhall and Daisley (1973)**. Blood was drawn from an Eppendorf tube using a Sahli pipette to the red blood cell scale (0.5), then Hayem's diluent solution was drawn in the same way until the scale reached 101. The end of the Sahli pipette was sealed with a finger, and the blood was homogenized in the pipette by making a motion for 2-3 minutes (Fig. 8). 1-2 drops of blood were dicarded to eliminate any unmixed blood, and then the blood was returned to a new Eppendorf tube. The erythrocyte count is done using a hemocytometer, where a drop of blood was placed on the hemocytometer and covered with a cover slip. The number of erythrocytes was observed under a microscope and was counted in five visible sample squares.

$$\Sigma TE = \Sigma \text{ Erythrocytes} \times \frac{1}{\text{volume of large square}} \times \text{dilution factor}$$

Total leukocytes

The preparation method was achieved according to the details in the study of **Blaxhall and Daisley (1973)**. Blood was drawn into an Eppendorf tube using a Sahli pipette to the red blood cell scale (0.5). Hayem's diluent solution was then drawn in the same manner until the scale reached 101. The end of the Sahli pipette was sealed with a finger, and the blood was homogenized in the pipette by making a motion for 2-3 minutes (Fig. 8). One to two drops of blood were discarded to remove any unmixed blood, and the remaining

blood was returned to a new Eppendorf tube. Leukocyte counting is performed using a hemocytometer. A drop of blood was placed on the hemocytometer and covered with a cover slip. The number of leukocytes was then observed under a microscope and counted in five visible sample squares.

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$$\Sigma TL = \Sigma \text{ leukocytes x } \frac{1}{\text{volume of large square}} \text{ x dilution factor}$$

Histopathology

Observations were made using an Olympus CX23 microscope with 40x10. Moreover, observations were conducted in 5 different fields of view, and then counted using the following formula **(Wolf *et al.*, 2015)**.

$$P\ (\%) = \frac{\Sigma\ KS}{\Sigma\ TS} \text{ x } 100$$

Description:
P (%) = Percentage of cells experiencing necrosis
∑KS = Number of necrotic cells in 5 fields of view
∑TS = Total number of cells in 5 fields of view

The polymerase chain reaction (PCR) analysis

The polymerase chain reaction (PCR) analysis was then performed to detect the presence of *Aeromonas hydrophila* and the infectious spleen and kidney necrosis virus (ISKNV) in the Nile tilapia. The primers used were ISKNV (F) and ISKNV (R) with an amplicon size of 415bp, and *A. hydrophila* (F) and *A.hydrophila* (R) with an amplicon size of 685.

Data analysis

The research data obtained were tabulated using Microsoft Excel. The data were analyzed using SPSS 25 software. If the ANOVA results showed significant differences ($P< 0.05$), a Duncan test was performed with a 95% confidence interval. Data on survival rate, hematological parameters including hemoglobin, hematocrit, total erythrocytes, total leukocytes, and histopathology were analyzed quantitatively. While, data on PCR results were analyzed qualitatively.

RESULTS

Isolation, identification and pathogenicity of *Aeromonas hydrophila*

Isolation and identification of Aeromonas hydrophila

Biochemical test

Aeromonas hydrophila isolates were identified as Gram-negative bacteria. DNA was extracted using the Geneaid KIT for PCR testing with specific primers based on the OIE (Office International des Epizooties) guidelines. A DNA fragment of 685bp was successfully amplified.

Table 1. Biochemical test of *Aeromonas hydrophila* bacteria in the Nile tilapia

Isolate	Gram	Form	SIM	O/F	Catalase	Oxidase	Bacteria type
P7	-	Basil	+	F	+	+	*A. hydrophila*
P8	-	Basil	+	F	+	+	*A. hydrophila*

Description: P7: *A. hydrophila* density 10^7; P8: *A. hydrophila* density 10^8.

Single infection of *Aeromonas hydrophila*

Infection of *Aeromonas hydrophila* survival rate of the Nile tilapia at a concentration of 10^7 (IB7) and the control (K) showed significantly different results ($P< 0.05$). Single infection treatment *Aeromonas hydrophila* (IB7) treatment showed a survival rate of 33%, while the control treatment (K) had a survival rate of 100% (Fig. 1).

Fig. 1. The survival rate of the Nile tilapia after single infection *Aeromonas hydrophila*. Description: control (K), *Aeromonas hydrophila* 10^7 CFU/mL (IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$)

Hematology

The research results showed that on day 1 post-infection, there was no significant difference between the IB7 and control treatments ($P > 0.05$). Day 3 after infection, a decrease in hemoglobin and hematocrit levels was observed in all treatments infected with *A. hydrophila*, while total erythrocytes and leukocytes showed an increase from day 3 to day 14 (Figs. 2- 5).

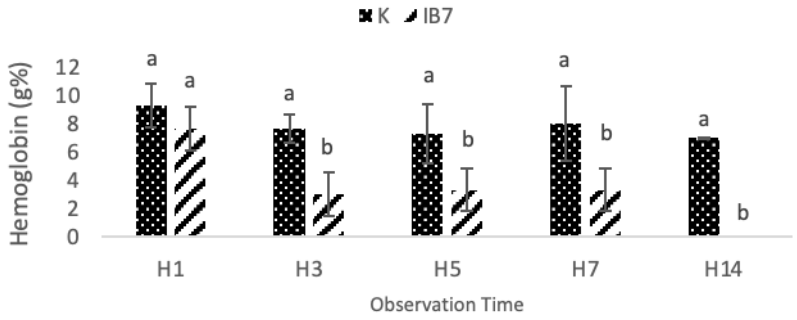

Fig. 2. Hemoglobin of the Nile tilapia after single infection *Aeromonas hydrophila*. Description: control (K), *A. hydrophila* 10^7 CFU/mL (IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$).

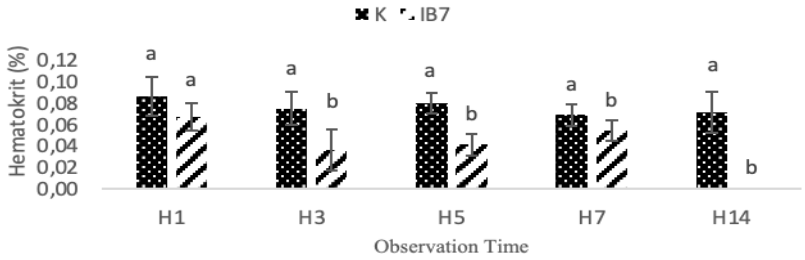

Fig. 3. Hematocrit levels of the Nile tilapia after single infection *Aeromonas hydrophila*. Description: control (K), *A. hydrophila* 10^7 CFU/mL (IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$).

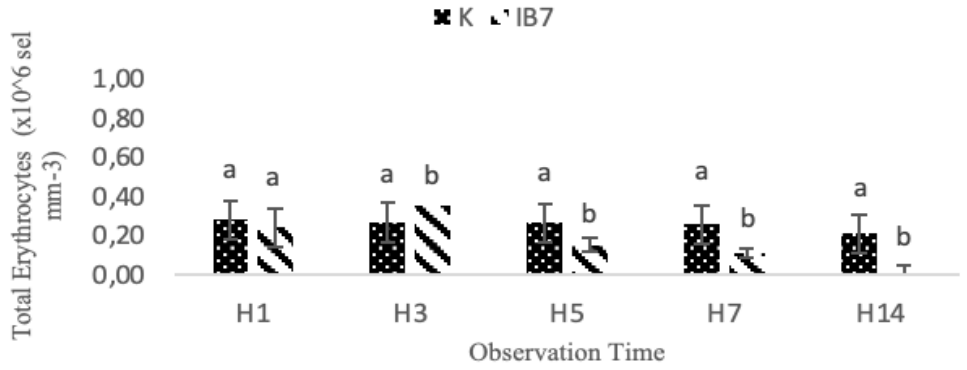

Fig. 4. Total erythrocytes of the Nile tilapia post-infection *Aeromonas hydrophila*. Description: control (K), *A. hydrophila* 10^7 CFU/mL (IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

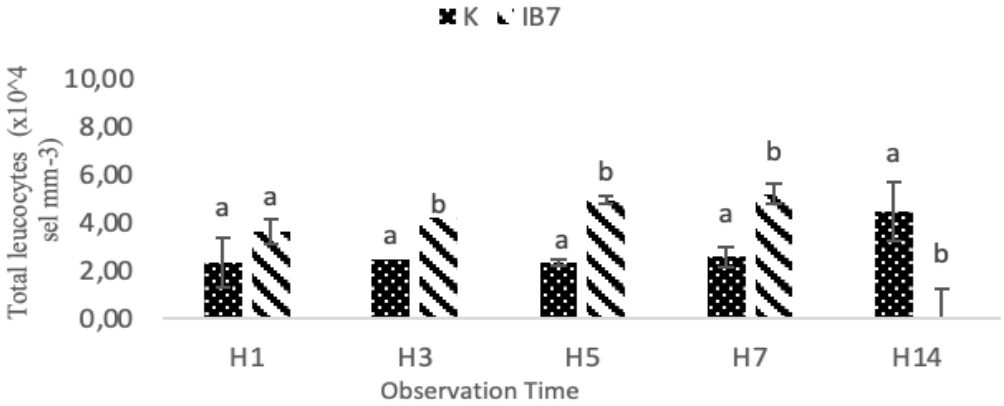

Fig. 5. Total leucocytes of the Nile tilapia post-infection *Aeromonas hydrophila*. Description: control (K), *A. hydrophila* 10^7 CFU/mL (IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

Histopathology

The level of damage in the kidney, spleen, and liver organs post-infection with *A. hydrophila* showed significant differences compared to the control treatment ($P< 0.05$). The highest percentage of kidney damage was observed in the IB7 treatment at 40.91 +

1.58 %. Moreover, the highest spleen damage was in the IB7 treatment at 34.55 + 1.14%, and the highest liver damage was in the IB7 treatment at 36.36 + 1.58 % (Table 2).

Table 2. Percentage of tissue demage in the kidney, spleen and liver of the Nile tilapia post infection *Aeromonas hydrophila*

Treatment	Kidney (%)	Spleen (%)	Liver (%)
K	10.91 ± 1.14 [a]	12.71 ± 2.17 [a]	10.00 ± 1.92 [a]
IB7	40.91 ± 1.58 [b]	34.55 ± 1.14 [b]	36.36 ± 1.58[b]

Description: Different *superscript* letters in each column (mean value ± standard deviation) indicate statistically significant difference ($P< 0.05$).

Isolation, identification and pathogenicity of infectious spleen and kidney necrosis virus (ISKNV)

Table 3. Detection of infectious spleen and kidney necrosis virus (ISKNV) using polymerase chain reaction (PCR) in the Nile tilapia

Type of fish	Sample	Kidney		Spleen		Liver	
		Positive	Negative	Positive	Negative	Positive	Negative
NK	10	10	0	10	0	10	0
NS	10	10	0	10	0	10	0
NB	10	10	0	10	0	10	0
Total	**30**	**30**	**0**	**30**	**0**	**30**	**0**

Description: NK = Small Nile tilapia (1-5 cm), NS = Medium Nile tilapia (5-10 cm) and NB = Large Nile tilapia (>10 cm).

Pathogenicity of infectious spleen and kidney necrosis virus (ISKNV)

Detection of infectious spleen and kidney necrosis virus (ISKNV)

The detection of Infectious Spleen and Kidney Necrosis Virus (ISKNV) was conducted on 30 tilapia samples of various sizes: small (1–5cm), medium (5–10cm), and large (10–20cm), targeting the MCP gene with an amplicon length of 415bp. Tables (3, 4) present the ISKNV detection results from tilapia farmed in Bogor. All 30 samples tested positive for the virus in the kidney, spleen, and liver organs across the different tilapia sizes (Fig. 6).

Confirmation of infectious spleen and kidney necrosis virus (ISKNV) using PCR

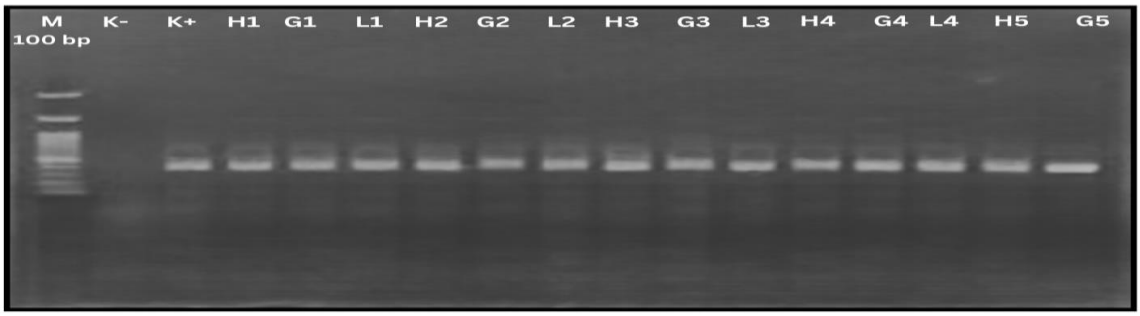

Fig. 6. Visualization results of infectious spleen and kidney necrosis virus (ISKNV) samples from PCR amplification. Description: Marker, K (+) (Positive control +), K (-) (Negative control -), H (Liver sample), G (Kidney sample), L (Spleen sample)

The phylogenetic tree analysis (Fig. 11) showed that the tested samples of the infectious spleen and kidney necrosis virus (ISKNV) from the Nile tilapia are closely related to the RSIV virus. The RSIV virus tested was positioned on the same branch as the reference RSIV virus sequence. These results suggest that the ISKNV and RSIV viruses found in Indonesia, despite originating from different regions, share a close genetic relationship. This finding is consistent with the study of **Murwantoko *et al*. (2018)** (Fig. 7).

The infectious spleen and kidney necrosis virus (ISKNV) phylogenetic tree

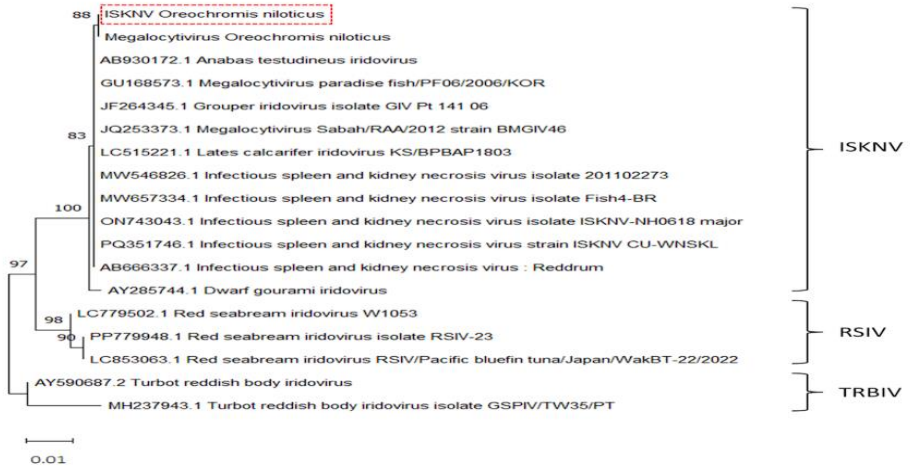

Fig. 7. Phylogenetic tree of ISKNV, RSIV, TBRIV viruses detected. All ISKNV isolates were closely related, as were RSIV and TBRIV isolates. A phylogenetic tree was constructed using the neighbor-joining method, with a 1000× boostrap targeting the MCP gene. For the phylogenetic tree analysis, the MEGAX software was utilized.

The survival rate of tilapia infected with ISKNV at the P0 concentration (without dilution) was significantly different from the control (K) ($P< 0.05$). The survival rate in the P0 treatment was 20%, while the control (K) showed a survival rate of 90% (Fig. 8).

Infectious spleen and kidney necrosis virus (ISKNV) infection

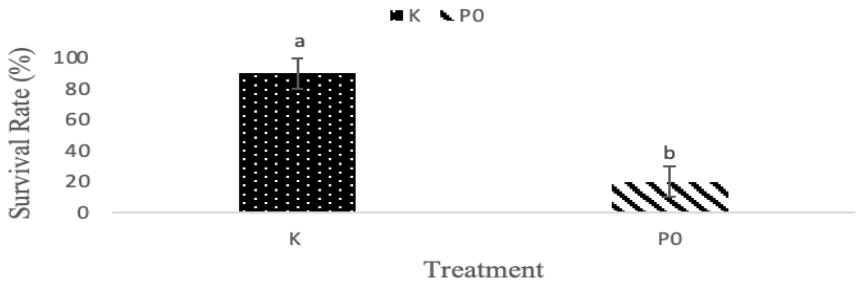

Fig. 8. Survival of the Nile tilapia post-infection with infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), without virus dilution (P0). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

Hematology

Hematological parameter measurements revealed significant differences between the P0 treatment and the control ($P< 0.05$). On the third day after infectious spleen and kidney necrosis virus (ISKNV) infection, hemoglobin and hematocrit levels decreased, while the total erythrocytes and leukocytes increased in the P0 treatment. In the control group (K), no decrease was observed, and the values remained stable until day 14 of maintenance (Figs. 9-12).

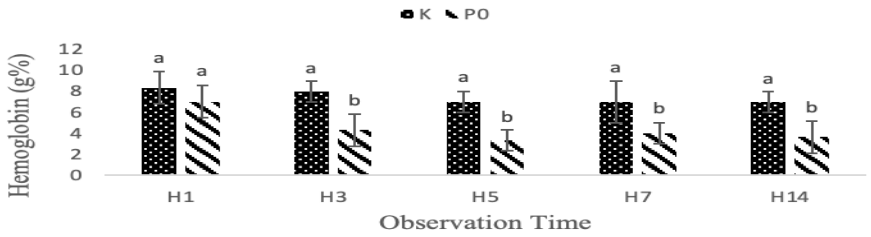

Fig. 9. Hemoglobin of the Nile tilapia post-infection with infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution (P0). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

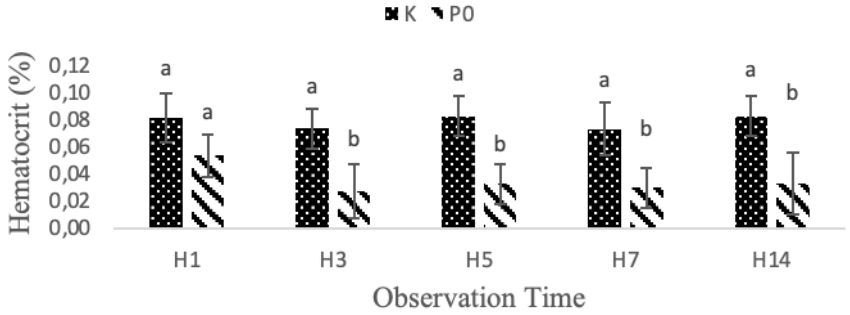

Fig. 10. Hematocrit levels of the Nile tilapia post-infection with infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution (P0). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

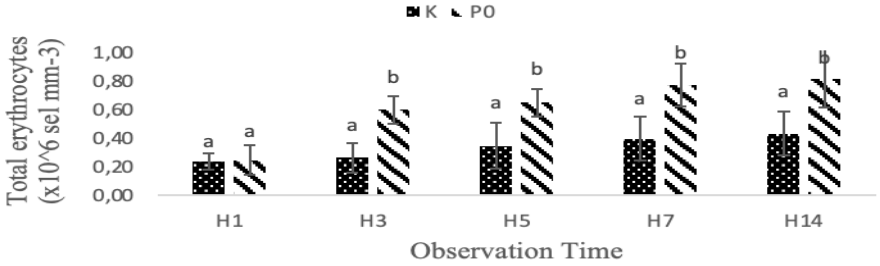

Fig. 11. Total erythrocytes of the Nile tilapia post-infection with infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution (P0). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

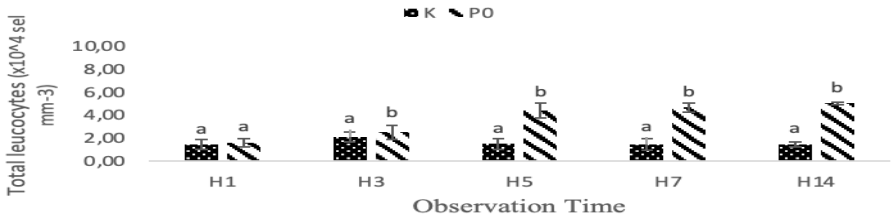

Fig. 12. Total leucocytes of the Nile tilapia post-infection with infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution(P0). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

Histopathology

The level of damage in the kidney, spleen, and liver organs post-infection with infectious spleen and kidney necrosis virus (ISKNV) showed significant differences compared to the control treatment ($P< 0.05$). The highest percentage of kidney damage was observed in the P0 treatment at 32.73 + 2.70 % (Table 4).

Table 4. Percentage of tissue damage in the kidney, spleen and liver post-infection with infectious spleen and kidney necrosis virus (ISKNV)

Treatment	Kidney (%)	Spleen (%)	Liver (%)
K	10.91 ± 2.30 [a]	12.73 ± 2.39 [a]	10.00 ± 1.92 [a]
P0	32.73 ± 2.70 [b]	26.36 ± 2.59 [b]	26.36 ± 2.59 [b]

Description: Different *superscript* letters in each column (mean value ± standard deviation) indicate Statistically significant difference ($P< 0.05$).

Co-infection of *Aeromonas hydrophila* and ISKNV

Survival rate

Co-infection treatment of *Aeromonas hydrophila* and infectious spleen and kidney necrosis virus (ISKNV) revealed a survival rate of 10% for the Nile tilapia in the P0+IB7 treatment, whereas the control (K) group showed a survival rate of 90%. There was a significant difference in survival rates between the K and P0+IB7 treatments ($P< 0.05$) (Fig. 13).

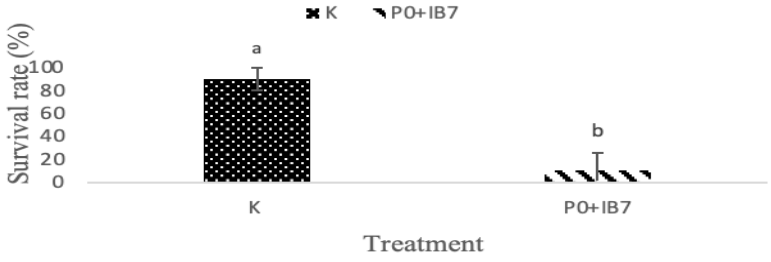

Fig. 13. Survival of the Nile tilapia after co-infection with *Aeromonas hydrophila* and infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution with 107 *A. hydrophila* (P0+IB7). Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P< 0.05$).

Hematology

Study results indicated that on day 1 post-infection with *A. hydrophila* and infectious spleen and kidney necrosis virus (ISKNV), there was no significant difference between the P0+IB7 and K treatments ($P > 0.05$). Day 3 post-infection, a decrease in hemoglobin and hematocrit levels was observed in the P0+IB7 treatment. Additionally, an increase in total erythrocytes and total leukocytes was noted from day 3 to day 14 post-infection (Figs. 13- 16).

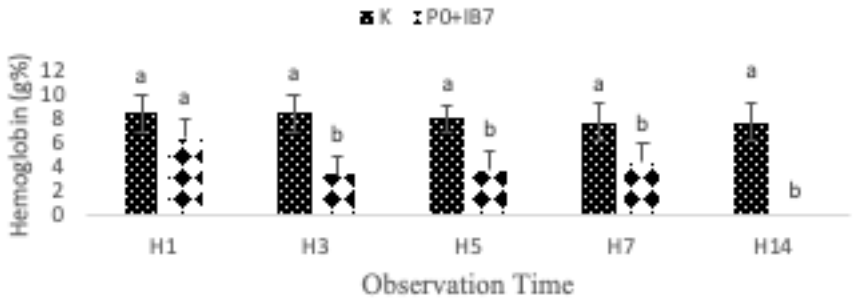

Fig. 14. Hemoglobin of the Nile tilapia after co-infection with *Aeromonas hydrophila* and infectious spleen and kidney necrosis virus (ISKNV). Description: control (K), no dilution with 10^7 *A. hydrophila* (P0+IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$).

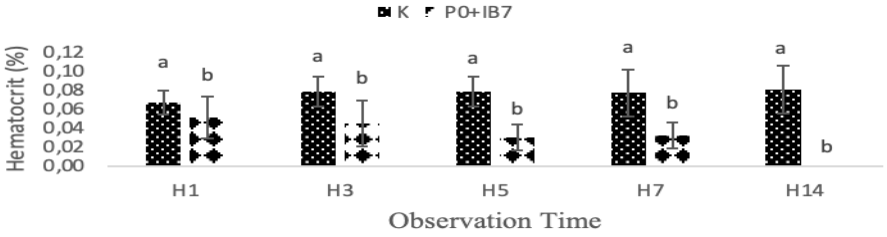

Fig. 15. Hematoctit level of the Nile tilapia after co-infection *Aeromonas hydrophila* and Infectious Spleen and Kidney Necrosis *Virus* (ISKNV). Description: control (K), no dilution with 10^7 *A. hydrophila* (P0+IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P<0.05$).

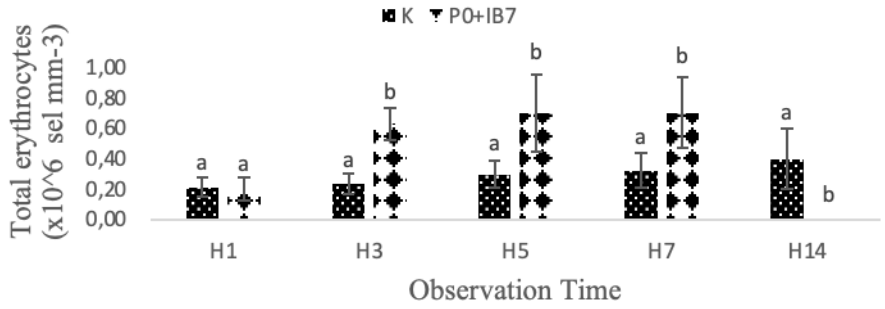

Fig. 16. Total erythrocytes of the Nile tilapia after co-infection with *Aeromonas hydrophila* and infectious spleen and kidney necrosis Virus (ISKNV). Description: control (K), no dilution with 10^7 *A. hydrophila* (P0+IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P<$ 0.05).

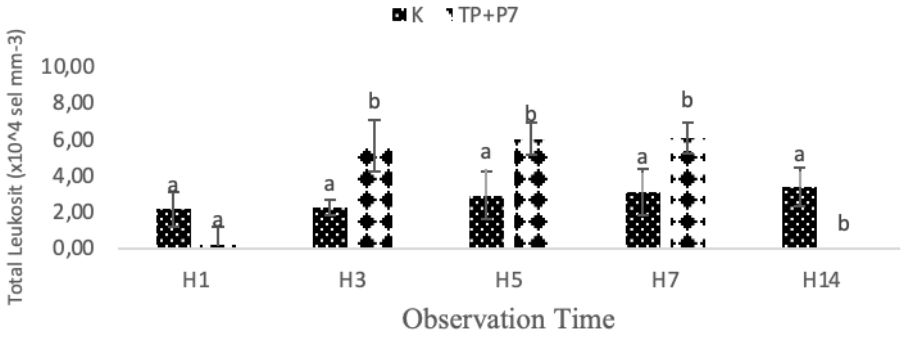

Fig. 17. Total leucocytes of the Nile tilapia after co-infection *Aeromonas hydrophila* and Infectious Spleen and Kidney Necrosis Virus (ISKNV). Description: control (K), no dilution with 10^7 *A. hydrophila* (P0+IB7). Different *superscript* letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P<0.05$).

Histopathology

The percentage results and histopathological scoring values for the kidney, spleen, and liver are presented in Table (5). The level of damage to the kidney, spleen, and liver organs post-infection with *A. hydrophila* and ISKNV showed significant differences between treatment P0 + IB7 and the control treatment ($P<0.05$). The highest percentage of kidney damage was observed in treatment P0 + IB7 at 33.33 ± 1.57%, the highest

spleen damage in treatment P0 + IB7 at 37.50 ± 1.58%, and the highest liver damage in treatment P0 + IB7 at 33.33 ± 1.58% (Table 5).

Table 5. Percentage of tissue damage in the kidney, spleen and liver post-infection with *Aeromonas hydrophila* and infectious spleen and kidney necrosis virus (ISKNV)

Treatment	Kidney (%)	Spleen (%)	Liver (%)
K	10.91 ± 2.30 [a]	12.73 ± 2.39 [a]	10.00 ± 1.92 [a]
P0+IB7	33.33 ± 1.58 [b]	37.50 ± 1.58 [b]	33.33 ± 1.58 [b]

Description: Different *superscript* letters in each column (mean value ± standard deviation) indicate Statistically significant difference ($P< 0.05$).

DISCUSSION

Infectious diseases, particularly those caused by *Aeromonas hydrophila* infection, pose a major challenge in the aquaculture sector, whether due to bacterial or viral infections **(Austin, 2022)**. The characteristics of bacterial infections include lesions or wounds on the skin and gills of fish. The IB7 bacterial isolate indicates that the identified bacteria belong to the *A. hydrophila* genus, as referenced in **Barrow and Felthan (2003)**. Molecular detection using the PCR method is one of the simplest ways to determine bacterial species. PCR method utilized specific primers, *Aeromonas hydrophila* 685F and 685R, on the obtained bacterial isolates. These findings are consistent with **Altinok *et al.* (2008)**, who stated that the DNA fragment length for *Aeromonas hydrophila* using specific primers is 685bp. Post-infection *Aeromonas hydrophila* in the Nile tilapia showed variations in mortality timing across treatments. The survival rate of the Nile tilapia post-infection with *A. hydrophila* in the IB7 treatment was 33%, whereas the control group (K) exhibited a 100%. The study revealed that on the third day of *A. hydrophila* infection, the fish experienced stress and a decrease in erythrocyte, hemoglobin, and hematocrit levels, attributed to the bacterial infection. Fish infected by the bacteria undergo a phagocytosis process that requires oxygen to digest bacterial particles by phagocytic cells, leading to a reduction in erythrocyte levels **(Mohammadi *et al.*, 2020)**. According to **Mohammadian *et al.* (2019)**, leukocytes protective role against pathogenic bacterial infections by activating the nonspecific defense system. An increase in leukocyte cells facilitates a faster recovery process from *A. hydrophila* infection **(Aluta *et al.*, 2021)**. Histopathological of kidney, spleen, and liver tissues in the Nile tilapia infected with *A. hydrophila* in the IB7 treatment group showed mild to moderate organ damage compared to the control (K) group. According to **El-Kady *et al.* (2022)**, organ development in fish greatly influences their growth, feed utilization, resistance to diseases, and stress factors.

Diseases caused infectious spleen and kidney necrosis virus (ISKNV) infections, which lead to high mortality rates in cultured fish, have been widely reported. According to **Dong *et al.* (2013)**, several factors influence the prevention and control of disease outbreaks caused by viral infections. ISKNV PCR detection was performed on all fish samples with the target gene MCP at a target length of 415bp. Samples from Bogor that were tested showed positive results in the PCR test. The phylogenetic tree analysis (Fig. 7) shows that the ISKNV tilapia samples tested are included in the ISKNV group. The results of this study are consistent with those of **Murwantoko *et al.* (2018)**, who stated that ISKNV isolates from Indonesia are closely related. The results of the ISKNV infection study showed that the survival rate in treatment P0 was 20%, while in treatment control (K) was 90%. Fish health status and immune responses in aquatic animals can be monitored using hematological parameters. According to **Shen *et al.* (2018)**, leukocytes are an essential component of the fish immune system, and they also express genes related to immunity. The entry of microorganisms into the host activates the host's defense mechanisms, both cellular and humoral, as part of the innate immune system **(Magnad'ottir, 2006)**. The results of the histopathological examination showed that the spleen, kidney, and liver exhibited clear damage, while the intestines and gills showed only minor damage. This is in line with previous research, where it was found that the most severe organ damage due to ISKNV infection occurs in the spleen, liver, and kidneys **(Shinmoto *et al.*, 2009)**. The highest percentage of kidney damage was observed in treatment P0 (no dilution) at 32.73 + 2.70%. The clinical symptoms in fish infected with the infectious spleen and kidney necrosis virus (ISKNV) include behavioral changes, such as a decrease in feed consumption, causing the body to appear emaciated. The fish swim weakly near the water surface or remain stationary at the bottom of the tank, lying on one side of their body. The gills and body become pale, and the fish lose their balance, often staying at the bottom, floating, and typically dying within a day after symptoms appear **(Weber, 2009)**. The fish's body appears darker, both on the surface and on the fins and tail **(Mahardika *et al.*, 2004)**.

Fish infected with *Aeromonas hydrophila* undergo phagocytosis, which requires oxygen to digest bacterial particles by phagocytic cells, leading to a decrease in erythrocyte levels. **Mohammadi *et al.* (2020)** added that the decrease in erythrocyte values is related to a decrease in hemoglobin and hematocrit, or vice versa. Leukocytes play a key role as the primary defense against *A. hydrophila* pathogenic infection by activating the non-specific defense system **(Mohammadian *et al.*, 2019)**. The histopathological observation results showed the percentage and scoring values for the histopathology of the kidneys, spleen, and liver. The level of damage to the kidneys, spleen, and liver after bacterial and viral infection was significantly different between the P0 + IB7 treatment and the control (K) treatment ($P<0.05$). The highest percentage of kidney damage was found in the P0+IB7 treatment at 33.33 + 1.57%, while the highest

spleen damage was in the P0+IB7 treatment at 37.50 + 1.58%, and the highest liver damage in the P0+IB7 treatment was 33.33 + 1.58.

CONCLUSION

The disease agents responsible for mortality in Nile tilapia include *Aeromonas hydrophila* and viruses that infect the Nile tilapia, with a new member of the infectious spleen and kidney necrosis virus (ISKNV) species identified. Single infection with ISKNV results in higher mortality compared to *A. hydrophila* infection, although no statistically significant difference was observed. Co-infection with both agents further increases and accelerates mortality compared to single infection treatments.

ETHICAL APPROVAL

All experiments in this study associated with fish complied with animal welfare was conducted according to protocol number 258-2024, approved by the Ethics Committee on Animal Use of the IPB University, February 2024.

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