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SNP2-Lys-C markers inheritance, genes expression, and the resistance of second-generation Catfish *Clarias gariepinus* against *Aeromonas hydrophila* infection

Pewarisan marka SNP2- LYS-C, ekspresi gen, dan daya tahan ikan lele *Clarias gariepinus* generasi kedua terhadap infeksi *Aeromonas hydrophila*

Yudha Hanggara, Alimuddin Alimuddin*, Dinar Tri Soelistyowati, Sri Nuryati

Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java 16680, Indonesia
*Corresponding author: alimuddin@apps.ipb.ac.id

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ABSTRACT

High mortality rates in catfish seed caused by motile aeromonas septicemia disease caused by *Aeromonas hydrophila* infection. The primary focus is on the genetic selection of the second generation (G2) catfish for resistance to *A. hydrophila*, utilizing the type-C lysozyme gene at SNP-2 genotype TT. The objective is to assess the inheritance of the SNP2-Lys-C genetic marker from the first generation (G1) Sangkuriang broods and to evaluate the resistance of the G2 seed to infection. In the experiment, G1 broods with the TT genotype were spawned, and the resulting G2 seed were challenged with *A. hydrophila*. The findings indicate that all G2 seed possess the TT genotype and exhibit a higher survival rate compared to the dominant CT genotype seed. The expression of the Lys-C gene in G2 seed is elevated at the onset of infection, decreasing after the seventh day. The expression of the MHC1a and IL-1b genes is also associated with a higher survival rate. The survival of G2 seed correlates with the white blood cell and lysozyme activity, along with an increase in phagocytic activity peaking on the seventh day post-challenge. The conclusion of this study suggests that G2 catfish seed with the TT genotype have stronger resistance to *A. hydrophila* infection compared to seed with the dominant CT genotype.

Keywords: *Aeromonas hydrophila*, catfish, genotype, lysozyme, resistance

ABSTRAK

Mortalitas tinggi yang terjadi pada benih lele yang disebabkan oleh penyakit motile aeromonas septicemia akibat *Aeromonas hydrophila*. Fokus utama adalah pada seleksi genetik generasi kedua (G2) lele untuk resistensi terhadap *A. hydrophila*, menggunakan gen lisozim tipe-C pada SNP-2 genotipe TT. Studi ini bertujuan menilai pewarisan marka genetik SNP2-Lys-C dari induk sangkuriang generasi pertama (G1) dan mengevaluasi ketahanan benih G2 terhadap infeksi. Dalam eksperimen, induk G1 genotipe TT dipijahkan dan benih G2 yang dihasilkan diuji dengan bakteri *A. hydrophila*. Hasil menunjukkan bahwa semua benih G2 memiliki genotipe TT dan menunjukkan tingkat kelangsungan hidup yang lebih baik dibandingkan dengan benih dominan genotipe CT. Ekspresi gen Lys-C pada benih G2 tinggi pada awal infeksi, menurun setelah hari ketujuh. Ekspresi gen MHC1a dan IL-1b juga terkait dengan tingkat kelangsungan hidup yang lebih tinggi. Kelangsungan hidup benih G2 berkorelasi dengan jumlah sel darah putih dan aktivitas lisozim, serta peningkatan aktivitas fagositik yang mencapai puncak pada hari ketujuh. Kesimpulan studi ini menunjukkan bahwa benih lele G2 dengan genotipe TT memiliki resistensi yang lebih kuat terhadap infeksi *A. hydrophila* dibandingkan dengan benih dominan genotipe CT.

Kata kunci: *Aeromonas hydrophila,* genotipe, ikan lele, lisozim, resistan

INTRODUCTION

The production of catfish (*Clarias gariepinus*) in Indonesia has been increasing from 2017 to 2021, with an average growth rate of 10.9% (DJPB, 2022). The catfish strains cultivated in Indonesia include Sangkuriang catfish, Dumbo catfish, Phyton catfish, and Pearl catfish. Intensified catfish farming has led to a decline in water quality and a higher chance of infection by disease-causing organisms that can result in the death of catfish fry. One example is the bacterium Aeromonas, which causes motile aeromonas septicemia (MAS) (Zhang *et al*., 2016; Oladele *et al*., 2021; Sumitro *et al*., 2021; Russell *et al*., 2023). According to Bakiyev *et al*. (2022), the bacterium *Aeromonas hydrophila* can cause up to 100% mortality in catfish farms.

MAS infections also result in mass deaths ranging from 87-100% in carp (Khumaidi & Hidayat, 2018). Efforts have been made to reduce the impact of *A. hydrophila* infections using immunostimulants (Astria *et al*., 2017; Abdallah *et al*., 2019), vaccines (Mulia *et al*., 2015; Taukhid *et al*., 2018), and bacteriophages (Choudhury *et al*., 2017; Le *et al*., 2018). Additionally, to lessen the impact of *A. hydrophila* infections and environmental stress, it is necessary to use seeds that are resistant to disease infections (Nasrullah *et al*., 2020). Disease-resistant seeds can be obtained through either conventional or molecular selection (Das & Sahoo, 2014). Conventional selection methods are time-consuming and require many fish, whereas molecular selection is faster and more effective.

Molecular selection is a method used to select fish based on their genetic information. In fish, molecular selection can utilize single nucleotide polymorphisms (SNPs). SNPs are single nucleotide variations in DNA that can be used to identify genetic differences between individuals related to specific traits. SNP-based selection methods are often used to select fish with specific traits, such as disease resistance (Liu *et al*., 2015) or fast growth (Zhou *et al*., 2022; Chen *et al*., 2020). SNP marker selection has already been applied to *Litopenaeus vannamei* shrimp for ammonia tolerance. The study found that 12 out of 49 SNPs were strongly associated with ammonia tolerance, as evidenced by significant differences in 10 loci with different alleles and genotypes between ammonia-tolerant (AT) and ammonia-sensitive (AS) groups (Lu *et al*., 2018).

Selection of catfish using SNP markers for resistance to *A. hydrophila* infection has been conducted by Nasrullah *et al*. (2020), which showed that the lysozyme type-C gene can differentiate between catfish that are resistant and susceptible to *A. hydrophila* infection. The difference between resistant and susceptible catfish lies in the genotype of SNP variant 2. The TT genotype (thymine thymine) represents catfish that are resistant to *A. hydrophila* infection, while the CC genotype (cytosine cytosine) represents catfish that are susceptible, and the CT genotype (cytosine thymine) is intermediate between resistant and susceptible. Applying SNP-based selection for lysozyme type-C gene at SNP 2 with TT genotype needs to be evaluated across generations to assess the consistency of resistance levels to *A. hydrophila* infection and the inheritance pattern of resistance genes in their offspring.

MATERIALS AND METHODS

Preparation of test material

Catfish seeds were obtained from the spawning of selected catfish with the TT genotype from the first generation (G1), with four pairs, and from the spawning of Sangkuriang catfish without selection from the Sukabumi Freshwater Aquaculture Center and local catfish farmers in Bogor (Table 1). The resulting seeds were raised until they reached a size of 8-9 cm for testing their resistance to *A. hydrophila* infection.

Table 1. Spawning scheme of selected TT genotype catfish (G1) and non-selected Sangkuriang Catfish.

Perlakuan	Genotype
A	♀ TT × ♂ TT (Selected Sangkuriang G1)
B	♀ Sangkuriang × ♂ Sangkuriang (Non-selected)

SNP marker-based genotype selection

Potential broodstock with the TT genotype (G1) was selected using the polymerase chain reaction (PCR) method. Genotyping analysis began with DNA extraction. The DNA extraction procedure involved taking tail fin samples from the catfish, which were then extracted using the Genomic DNA Mini Kit (Geneaid, USA). PCR amplification was performed using primers based on Nasrullah *et al*. (2020). PCR products were visualized using electrophoresis on a 1% agarose gel. Four pairs of confirmed TT genotype parents

were then used for spawning to produce second-generation (G2) seeds.

Subsequently, the catfish seeds from the spawning of selected ♀ TT × ♂ TT parents and non-selected ♀ Sangkuriang × ♂ Sangkuriang parents underwent SNP2 marker-based genotyping analysis. A total of 10 fish from each spawning batch were analyzed.

Resistance testing to *A. hydrophila*

Resistance testing to *A. hydrophila* was conducted by intramuscularly injecting the bacteria at an LD50 dose of 0.1×10^7 CFU/mL per fish. Test fish, measuring 8-9 cm, were used in groups of 30 per treatment with four replicates. The fish were then maintained for 15 days, and mortality was observed. Red and white blood cell parameters were analyzed periodically on days 0, 3, 7, and 15.

Red blood cells

Red and white blood cell analysis was performed before and after bacterial injections on days 0, 3, 7, and 15. Blood collection was preceded by anesthesia with clove oil (0.15 mL/L). Blood was drawn using a 1 mL syringe pre-rinsed with an anticoagulant from the base of the tail. The method for blood cell testing followed Hesser (1960). Fish blood samples were aspirated with a Sahli pipette to the 0.5 scale, then diluted with Hayem solution to the maximum scale of 101. The solution was homogenized by gently shaking the pipette in a figure-eight motion for one to two minutes. Two drops of the blood solution were discarded, and the remaining blood was placed on a hemocytometer with a coverslip. Observations were made using a microscope at 400× magnification.

White blood cells

White blood cells in the *A. hydrophila* test treatments were measured on days 0, 3, 7, and 15. The white blood cell measurement method followed Hesser (1960). Fish blood samples were taken using a white cell pipette to the 0.5 scale. The pipette tip was cleaned with tissue, then the sample was mixed with Turk's solution to the 11 scale. The mixture was homogenized by gently shaking the pipette in a figure-eight motion for one to two minutes. Two drops of the blood solution were discarded, and the remaining blood was placed on a hemocytometer with a coverslip. Observations were made using a microscope at 400× magnification.

Phagocytic activity

Post-injection phagocytic activity of *A. hydrophila* was measured on days 0, 3, 7, and 15, according to Anderson and Siwicki (1993). A 50 µL blood sample was added to a microtube and mixed with 50 µL of *Staphylococcus aureus* (density 10^7 CFU/mL) diluted with PBS, then homogenized. The blood-bacteria solution was incubated at 28°C for 20 minutes. Then, 5 µL of the solution was placed on a glass slide to prepare a smear. The smear was fixed in methanol for 10 minutes and air-dried, then stained with 10% Giemsa for 10 minutes and air-dried. After rinsing with running water, the smear was observed under a microscope. Phagocytic activity was calculated based on the percentage of 100 cells showing phagocytic activity using the following formula:

$$\text{Phagocytic activity (\%)} = \frac{\sum \text{Phagocytizing cell}}{\sum \text{Phagocytic cell}} \times 100$$

Lysozyme activity

Serum was collected from blood samples without anticoagulant. The samples were left at room temperature for two hours, then stored in a refrigerator at 4°C for a full day. Serum was then centrifuged at 5,000 rpm for three minutes to obtain serum. The lysozyme activity was measured according to Hanif *et al*. (2005). A 100 µL sample was mixed with 0.2 mg/mL Micrococcus luteus suspension in PBS (pH 7.4). Absorbance was measured twice using a microplate reader at 450 nm after 30 seconds of mixing and repeated after 30 minutes. The lysozyme activity unit was determined based on the enzyme's capacity to reduce absorbance by 0.001 per minute. Lysozyme activity was calculated using the following formula:

$$\text{Lysozyme activity (unit/mL)} = \frac{\dfrac{\text{(initial OD}-\text{final OD)}}{\text{final time measurement}}}{\text{sample volume}}$$

Gene expression analysis

Total RNA was extracted using GENEzol reagent (Geneaid, Taiwan) according to the manufacturer's manual. RNA concentration and purity were measured using a spectrophotometer on a Pharmacia Genequant RNA/DNA Calculator (Pharmacia Biotech, UK). cDNA synthesis was performed from the total RNA extract with a concentration of 50 ng/µL using ReverTraAce qPCR RT Master Mix with gDNA Remover (Toyobo, Japan), following the manufacturer's protocol. The obtained cDNA solution was

diluted 10 times with NFW and stored at -20°C until analysis.

Genes analyzed included β-actin, Lysozyme type-C, Interleukin-1β, and MHC-1α. cDNA amplification was performed with a final PCR reaction volume of 20 µL consisting of 10 µL SensiFAST SYBR NO-ROX (Bioline, UK); 4 µL cDNA (5 ng/µL); 0.8 µL of each primer (10 µM); and 4.4 µL NFW. qPCR analysis was conducted on a Linegene K Plus machine (Bioer, China). The primer sequences used are shown in Table 3. Gene expression levels were analyzed after normalizing each gene expression to β-actin expression. Gene expression levels were calculated using the $2^{-\Delta\Delta ct}$ method (Schmittgen & Livak, 2008).

Data analysis

Data were analyzed using Microsoft Excel 365 and IBM SPSS 26 software for analysis of variance (ANOVA) with a 95% confidence interval, followed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Result

Gene inheritance

The G2 catfish seeds resulting from the TT×TT spawning showed 100% TT genotype. The spawning of Sangkuriang catfish from local farmers in Bogor (PT) produced seeds with 60% TT genotype and 40% CT genotype. Meanwhile, the spawning of Sangkuriang catfish from the Sukabumi Freshwater Aquaculture Center (SB) produced seeds with 40% TT genotype and 60% CT genotype. A total of 10 seeds from each treatment were examined.

Survival rate

The highest survival rate of catfish seeds at the end of the post-challenge period with *A. hydrophila* was observed in the TTA spawning, and it was not significantly different from PT (Figure 1). The lowest survival rate was observed in SB, which was 51.67%

Red blood cells

The total red blood cell counts in seeds from TT genotype parents tended to be more stable after the challenge test. The total red blood cells in seeds from Sangkuriang parents (PT and SB) increased on day 3 post-challenge. The total red blood cell counts in the TTA, TTB, and PT treatments were significantly higher before the challenge test ($p<0.05$, Figure 2) compared to SB.

White blood cells

The total white blood cell count increased in all treatments on day three (H3) and began to decrease on day 14 (H14). Treatments TTA and PT had the highest white blood cell counts on day 0 (H0) or before the challenge ($p<0.05$) compared to TTB and SB (Figure 3). The highest white blood cell count was observed on day seven.

Phagocytic activity

Phagocytic activity (PA) increased and was higher on day seven (H7) compared to before the challenge (H0) in all treatments: TTA, TTB, PT, and SB (Figure 4). Subsequently, the PA on day

Table 2. Primer sequences used for real-time PCR.

No	Gene	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')
1	β-aktin	ACCGGAGTCCATCACAATACCAGT	GAGCTGCGTGTTGCCCCTGAG
2	Lisozim type-C	AAGGTATGATCGGTGTGAGCTGG	CGGTTCTGGGCGTTGGTATTGA
3	Interleukin1β	GCAGTGCACCATTTGCGATA	GGGTTATACGCGCTCAGGTT
4	MHC-1α	AGACTCTGACCTGGATCGCT	CCAGTCGATACACTCCTTCTCC

Table 3. Results of marker inheritance in catfish seeds.

Treatment	Genotype (%)		
	TT	CT	CC
TT × TT (TTA)	100	-	-
TT × TT (TTB)	100	-	-
Non Selected Farmer (PT)	60	40	-
Non Selected (SB)	40	60	-

Note: TTA = Spawning result of TT × TT pair A; TTB = Spawning result of TT × TT pair B.

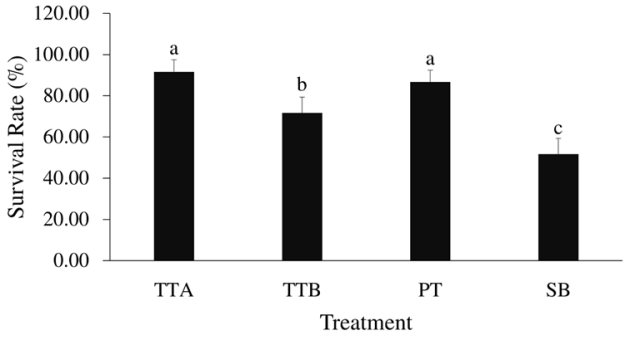

Figure 1. Survival rate of G2 catfish seeds challenged with *Aeromonas hydrophila* bacteria. Different letters above the bar chart indicate significant differences (Duncan's test: $p<0.05$).

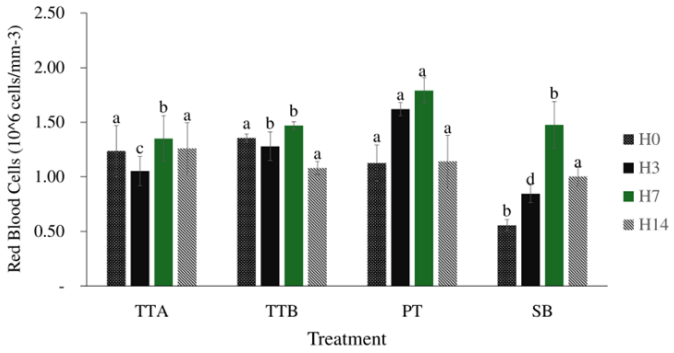

Figure 2. Total red blood cells of G2 catfish challenged with *A. hydrophila*. Different letters above the bar chart indicate significant differences (Duncan's test: $p<0.05$). H0, H3, H7, and H14 represent the total red blood cells after challenging them with *A. hydrophila*.

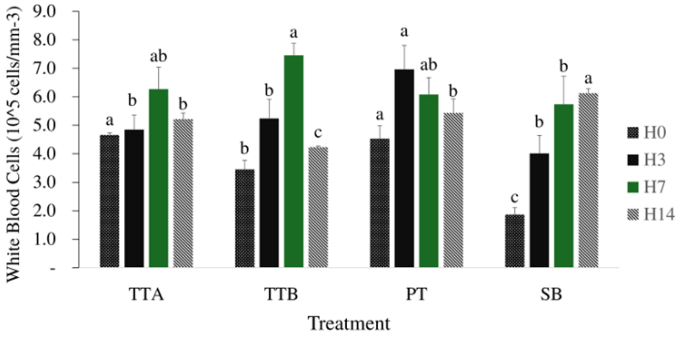

Figure 3. Total white blood cells of G2 catfish challenged with *A. hydrophila*. Different letters above the bar chart on the same day indicate significant differences (Duncan's test: $p<0.05$). H0, H3, H7, and H14 represent the total white blood cells after challenging with *A. hydrophila*.

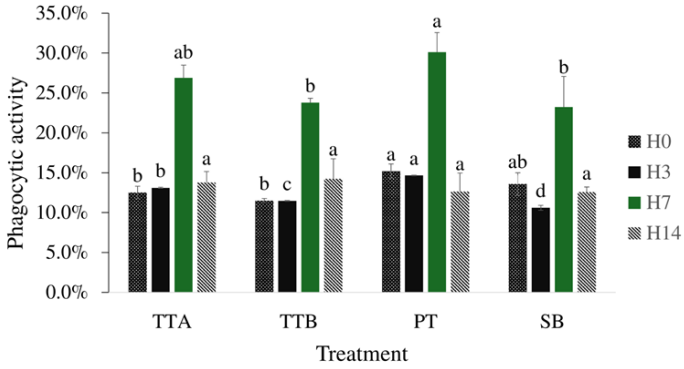

Figure 4. Phagocytic activity of G2 catfish challenged with *A. hydrophila*. Different letters above the bar chart indicate significant differences (Duncan's test: $p<0.05$). H0, H3, H7, and H14 represent phagocytic activity after challenging with *A. hydrophila*.

14 (H14) decreased and returned to levels like those on day 0 (H0). In G2 seeds, PA values for treatments TTA and TTB were higher than for SB ($p<0.05$).

Lysozyme activity

Lysozyme activity exhibited varying patterns across treatments. The highest lysozyme activity was observed in treatment TTB on day 0 (H0) (Figure 5). The lowest lysozyme activity was recorded across all treatments on day 7 (H7), with levels increasing again on day 14 (H14).

Gene expression

Expression of the Lys-C gene (Figure 6A) was high on days 1 and 3, and then decreased on days 7 and 14 post-infection. The expression patterns of the IL-1b gene (Figure 6B) and MHC1a gene (Figure 6C) varied among different crosses, but generally, the expression levels were higher on day 1 for catfish with higher survival rates.

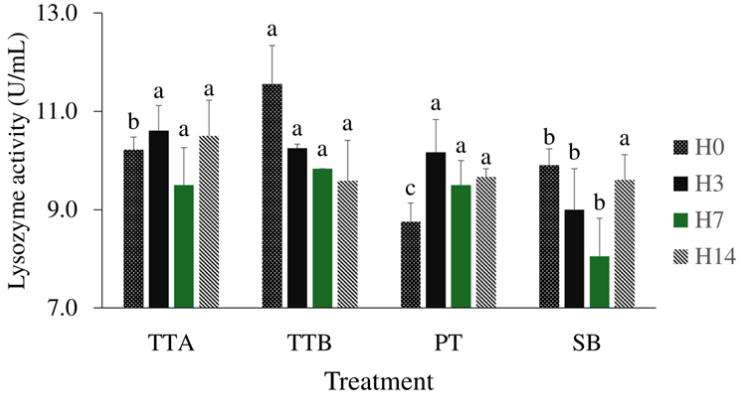

Figure 5. Lysozyme activity of G2 catfish challenged with *A. hydrophila*. Different letters above the bar chart indicate significant differences (Duncan's test: $p<0.05$). H0, H3, H7, and H14 represent lysozyme activity after challenging with *A. hydrophila*.

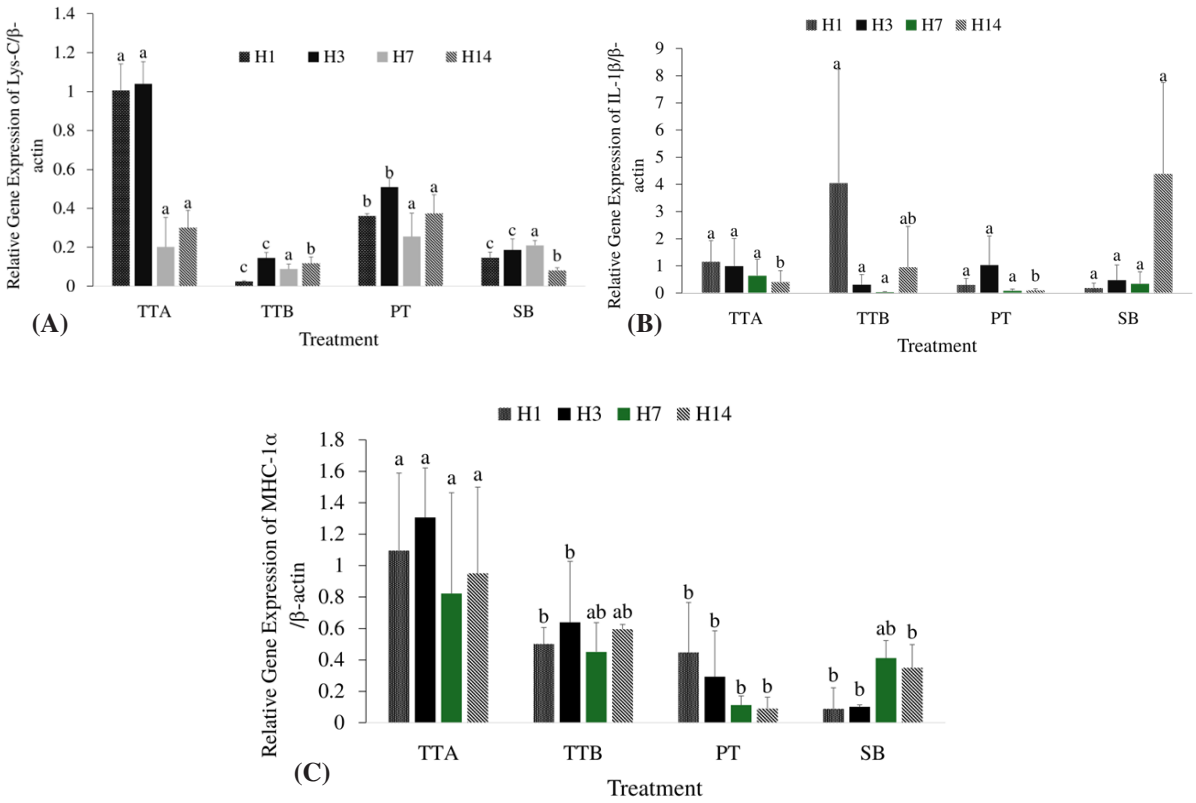

Figure 6. Gene expression of lysozyme-C (A), interleukin-1b (B), and MHC-1a (C) in G2 catfish challenged with *A. hydrophila*. Different letters above the bar charts indicate significant differences (Duncan's test: $p<0.05$). H0, H3, H7, and H14 represent relative gene expression levels after challenging with *A. hydrophila*.

Discussion

The inheritance of genotype markers in treatments TTA and TTB showed that 100% of the resulting seeds had the TT genotype. This result is consistent with Mendel's law, which states that crossing two homozygous individuals will produce 100% homozygous offspring. The highest survival rate was observed in treatment TTA with a survival rate of 91.67%, while the lowest was from the SB treatment with a survival rate of 51.67%. Seeds with the dominant TT genotype tend to have a higher survival rate.

The total red blood cell counts in treatments TTA and TTB was relatively stable compared to treatments PT and SB. An increase in red blood cells was noted due to an infection with *A. hydrophila*. According to Yu *et al*. (2014), an increase in red blood cells can assist in immune responses by recognizing antigens. The total white blood cell count on day 0 in the treatment with the highest survival rate was higher than in other treatments. Overall, white blood cell counts increased on days three and seven after challenge testing, indicating that white blood cells play a role in the immune system.

The increase in white blood cells occurs as an effort to defend the body against pathogen attacks (Shen *et al*., 2018). The highest phagocytic activity was observed on day 7, which corresponds with the peak in white blood cell counts. This increase in phagocytic activity indicates the process of destroying foreign particles or disease-causing bacteria by macrophages (Nainggolan *et al*., 2021). The expression patterns of the lysozyme-C, MHC-1a, and IL-1b genes in seeds with high survival rates showed high gene expression at the early stages of the challenge test.

Lysozyme-C is an enzyme that functions as part of the nonspecific immune system (Mai *et al*., 2014). Interleukins are a group of cytokines that play a role in immune responses (Elmowalid *et al*., 2023). MHC-1a functions as a gene presenting cells to cytotoxic T-cells (Yamaguchi & Dijkstra, 2019). Based on the results, seeds with high survival rates exhibited a strong immune response at the early stages of the challenge with *Aeromonas hydrophila*.

CONCLUSION

Crossbreeding of TT genotype catfish (G1) with SNP2-Lys-C marker-based selection resulted in a second generation (G2) with resistance to *A. hydrophila* infection (TT), achieving a survival rate of 72-92%.

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