Pak. J. Biotechnol. Vol. 12(2) 131 - 137 (2015
www.pjbt.org
ISSN Print: 1812-1837
ISSN online: 2312-7791

MHC I MOLECULAR MARKER INHERITANCE AND FIRST GENERATION CATFISH (*CLARIAS* SP.) RESISTANCE AGAINST *AEROMONAS HYDROPHILA* INFECTION

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Article received August 21, 2015, Revised November 17, 2015, Accepted November 21, 2015

ABSTRACT

Three groups of *A. hydrophila*-challenged sangkuriang catfish strain were obtained, namely: resistant (R: no injuries and survive), recover from injuries and survive (L), and dead fish. In the present study, the inheritance of the major histocompatibility complex (MHC) 1 marker in the first generation (F1) of catfish and their resistance to *A. hydrophila* infection were evaluated. The F1 offsprings were produced by crossing female R and male R (abbreviated:RxR), LxL, RxL, LxR, and control fish (C) each was replicated twice (two brood pairs per crossing). Results of the PCR analysis using ClMHAh-01 primer showed that the percentage of F1 progenies from R and L broods carrying MHC I marker ranged between 62.5 and 83.4%. The challenge test with *A. hydrophila* 10^6 cfu/mL showed that the survival of F1 fish (76.8-87.0%) was more two times higher than that of C fish. Blood profiles and antibody titer were in line with high resistance of F1 offsprings from R and L broods against *A. hydrophila* infection. All the results indicated that *A. hydrophila* resistant catfish can be produced by MHC I marker based selection.

Keywords: Molecular marker, Inheritance, MHC I, *Aeromonas hydrophila*, catfish

INTRODUCTION

Sangkuring catfish (*Clarias* sp.) strain is produced by backcross of F2 and F6 of the introduced African catfish that conducted by Main Center for Freshwater Aquaculture Development, Sukabumi West Java, Indonesia (Sunarma *et al.,* 2005). This spesies is one of important freshwater cultured fish species in Indonesia. High mortality caused by *Aeromonas hydrophila* infection is still common in catfish farming, particularly in the rainy season. Farming using genetically disease resistant fish can be used in order to prevent the loss of production. Fish resistance to disease infection can be generated through the application of conventional selection method as it was done for the Krasnodar carp (*Cyprinus carpio*) resistant to dropsy (Kirpichnikov *et al*., 1993). However, it takes a relatively long time, for example, the dropsy resistance common carp is obtained at the 9th generation (Kirpichnikov 1999); it takes about 13 to 18 years. Another approach that can be used for saving time is marker assisted selection (MAS).

The MAS method has been successfully used to generate Indonesian common carp majalaya strain resistance to KHV infection using Cyca-DAB1*05 MHC II marker (Alimuddin *et al.*, 2011). MHC II plays a role in the activation of phagocytic cells to produce antibodies and activate the immune system; involve in the elimination of parasites and bacteria, and virus neutralization (Rakus 2008). Other MHC involves in the immune system is the MHC I. In a previous study, a candidate MHC I marker has been considered for catfish resistance to *A. hydrophila* infection (Azis *et al.,* 2015). In that study, *A. hydrophila* challenge test to catfish stocks show two categories of resistance, namely: resistant (no injuries and life) and wounds heal fish (wounds caused by infection, then recover and life). In order to produce catfish resistance to *A. hydrophila* infection through MAS, marker inheritance and the durability of the progeny against pathogen infection should be evaluated.

Blood is a very useful diagnostic tool in determining the health status of fish. Serious blood changes appear especially in case of disease infection. Blood parameters that can exhibit pathological changes are hematocrit, hemoglobin, erythrocytes and leukocytes counts (Bastiawan *et al.*, 2001). The purpose of this study was to evaluate the inheritance of MHC I marker in the first generation of *Clarias* sp. and the survival when challenged with *A. hydrophila*.

MATERIALS AND METHODS

First offspring (F1) production and maintenance: Parents "sangkuriang" catfish strain aging about 11 months were used in this research. Four females and males with "R" resistant category, and four females and males with "L" woundsand

healed category were selected. The average body weight of the female parent ranged between 968.3-973.6 g, while the male were 728.5-739.3 g. Parents fish without MHC I marker were obtained from Main Center for Freshwater Aquaculture Development (BBPBAT) Sukabumi West Java Indonesia and weighing between 1,672-1,980g (about 2 years old).

Ovulation and spermiation were induced by injecting ovaprim (Syndel Laboratories Ltd) at a dosage of 0.2 mL/kg body weight. Artificial fertilization was done by mixing sperm and eggs in a plastic bowl. Five cross combinations were made, namely: (1) R female *vs*. R male fish (abbreviated: RxR), (2) L female fish *vs*. R male (LxR), (3) R female *vs*. L male (RxL), (4) L female *vs*. L male (LxL), and (5) a cross between fish without the marker as a control (C). Two pairs of mating were made as replication for fish having the MHC I marker.

The eggs were incubated in 100-L glass aquaria. *Tubifex* sp. were provided *ad-libitum* from day 3 after the yolk absorbed until the day 13. In addition to *Tubifex* sp, at 11 to 15 days fish were also fed on commercial diet (protein content 40%). Furthermore, fish were fed on commercial diet (protein content 30%), three times daily and at satiation.

Analysis of MHC I marker inheritance: Genomic DNA was extracted from fin tail tissue of F1 fish using DNA isolation kit (Puregene, Minneapolis, USA) following the procedures in the manual. A total of 30 individuals were randomly taken from each crosses. DNA was diluted with 50mL sterile distilled water (SDW). Purity and DNA content was measured using spectrophotometer (Gene Quant) at a wave-length of 260 nm and 280 nm.

Final volume of PCR reaction used was 25mL, consisting of 2.50µL 10x buffer *Taq*DNA polymerase, 2µL of dNTP mix, 2µL of primers ClMHAh-01 reverse and forward, 0.25µL *Taq* DNA polymerase (KAPA Biosystems), 1 µL genomic DNA and 17.25µL SDW. Pre-denaturation of PCR process was performed at 94°C for 3min, 35 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 68°C for 20 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 5 min.

PCR products were separated by electrophoresis with 1.5% (w/v) agarose gel, at 70 volts for 90 min. DNA was visualized using red gel (Biotum Inc. California, USA) and UV transilluminator.

Challenge test of *Aeromonas hydrophila:* *A. hydrophila* AHL-110306-3 isolate was obtained from Research and Development Center for Freshwater Aquaculture, Bogor - Indonesia. A total of 25 fish (body weight 55±7 g) of each crosses were challenged by injecting 0.1 mL *A. hydrophila* (10^6 cfu/mL). As a negative control, fish was injected with 0.1 mL phosphate buffer saline. The fish were kept in glass aquaria100 L volume and each cross had three aquariums as replication.

The clinical symptoms: The diseased fish showed symptoms of increased respiration, slow swimming motion, hemorrhages on the base of fins and skin lesions such as white discoloration, shallow hemorrhagic ulcers or deep ulcers with exposed underlying muscle, other was decreased feed response.

Analysis of hematology: Blood samples were taken on day 0, 1, 3, 5 and 7 post-challenged test. Three fish samples from each treatments were taken to count the number of erythrocytes and leukocytes using Blaxhall and Daisley (1973), hematocrit (Anderson and Siwicki 1995), hemoglobin (Blaxhall, 1972) and anti-body titers using direct agglutination method (Anderson, 1974). Hematological data and fish survival were analyzed by one-way ANOVA using MINITAB ver. 16, with post-hoc Tukey HSD test at p= 0.05.

RESULTS

Inheritance of MHC I marker: Results of PCR analysis to evaluate the inheritance of MHC I marker is shown in Figure 1. Using ClMHAh-01 primer, fish carrying MHC I marker presented PCR products of approximately 300, 450, 1,000 bp (Figure 1). The percentage of F1 fish from RxR cross that having MHC I marker was 83.4%, as much as 66.7% in RxL, 75.0% in LxR and 62.5% in LxL fish. In control fish, DNA size of the PCR products were detected at about 450 and 1,000 bp, or no DNA band. The percentage of individuals having the marker in control fish was 25.0%.

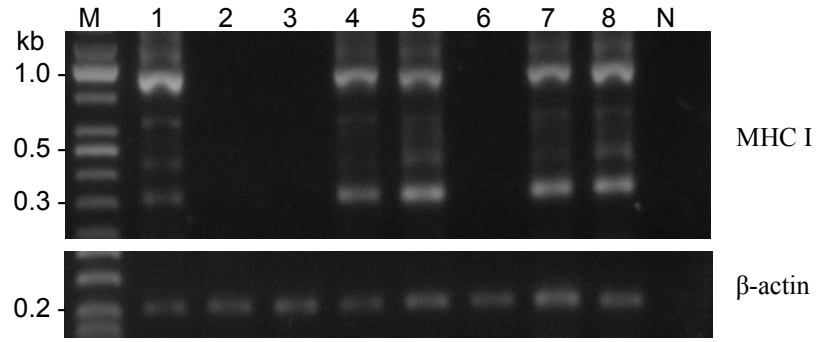

Figure 1.Electropherogram in identification of catfish first generation carrying MHC I marker using PCR method with ClMHAh-01 primer (picture above: MHC I). β-actin was used as an internal loading control DNA template (picture below: β-actin). M is DNA fragment size marker (KAPA Biosystems). Numbers 1 to 8 are the DNA of the test fish. N is PCR product without DNA template. Fish carry MHC I marker having PCR products of approximately 300, 450 and 1000 bp.

Survival and percentage of F1 resistant and wound-healed categories: Fish survival in challenge test with *A. hydrophila* was presented in Figure 2. The results showed that the survival of F1 fish derived from R and L broods crosses with the one carrying MHC I markers was more 2.0 times higher than the control fish. Survival of F1 from RxR cross was 87.20±4.01%, RxL was 76.80±7.21%, LxR was 82.40±8.94%, and LxL was 82.80±4.52%. Mean while, the survival of F1 control fish was 37.20±6.11%.

Among the life fish, fish grouping F1 by category "resistant" and "wounds heal" is presented in Figure 2. Resistant category most commonly found in offspring of RxR cross (77.2%), followed by LxR (70.8%), RxL (67.6%), and LxL (64.0%). In the control fish, resistant category was only 4.0%. The percentage of wounds heal fish category most commonly found in control (33.2%), followed by a crosses of LxL (18.8%), LxR (11.6%), RxR (10%) and RxL (9.2%).

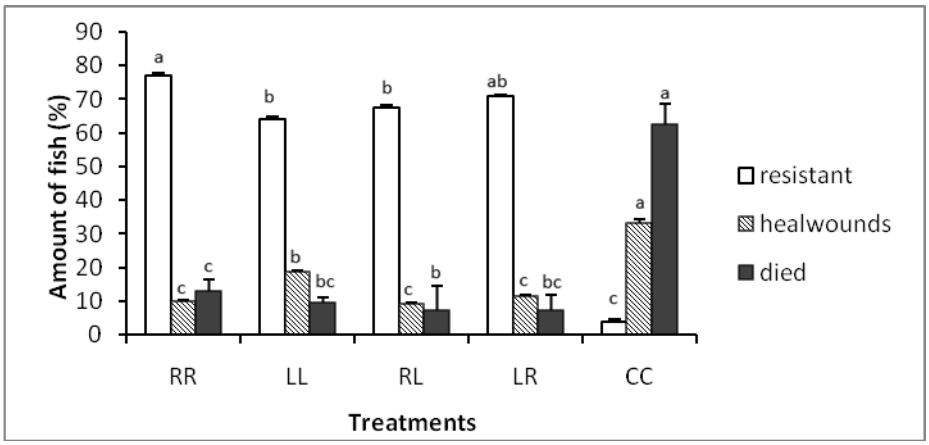

Figure-2: Percentage of resistant, heal wounds, and died catfish first generation post-challenged with 0.1 mL *Aeromonas hydrophila* (10^6 cfu/mL). (R = resistant fish ; L = healwounds fish and C = control fish)

Clinical symptoms: Overall, clinical symptoms of *A. hydrophila*-infected F1 fish from the parent carrying MHC I marker and the control without the marker were relatively the same, namely hemorrhage and injuries. However, the difference was the healing time. Thin skin as a sign of healing the wound area in F1 offspring from R and L broods was appeared on day 5, while in the control fish was on day 7.

External symptoms of *A.hydrophila* infected fish could be seen on the entire body, such as whitish skin, tentacle and fins, lower mucus, wound in the injection area, and some have holes because the muscle peeled off. Another symptom was a swollen in abdomen swelling, and yellowish-white and even red. The response of fish to disruption was slow, swim slower, and tends to dwell at the bottom or near water surface. In contrast, no hemorrhage and ulcers in resistant fish so their appetite and movement became normal on the second day of challenge test.

A. hydrophila infected fish shown internal symptoms as the yellow fluid in the abdominal cavity, intestinal yellow or pale, pale kidneys, black liver, stomach inflated with water, and the muscles become soft and easily damaged. However, some cases of *A. hydrophila* infection were not marked on external organ damage but causing death (Angka 2005).

Fish blood profiles post-challenged with *A. hydrophila:* Blood profile analyzed were total leukocyte count (Figure 3), erythrocytes (Figure 4), hemoglobin (Figure 5), hematocrit (Figure 6), and the antibody titer (Figure 7). On the negative control fish (C-), leukocyte count was relatively unchanged from day 0 up to day 7 (Figure 2). Meanwhile, first day post-challenge, leukocyte count increased in all treatments and the amount of leucocytes in control fish C+ ($4.12x10^5$ cells/mm^3) was higher than other treatments. Number of leukocytes on day 7 was decline, similar level with before challenge test, while leukocytes number in C+ fish was still the same as the first day to the 5th, and this was in line with signs of healing on day 7.

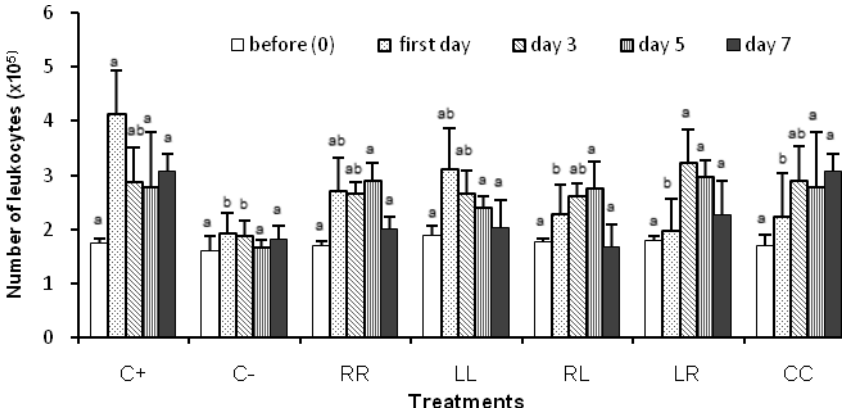

Figure-3: Leukocytes levels in catfish *Clarias* sp. post-challenged with *Aeromonas hydrophila*. Different letters on top of the baron the same day showed significantly different values by ANOVA ($p<0.05$). (R = resistant fish ; L = healwounds fish ; C = control fish ; C- = negative control fish and C+ = Positive control fish)

In general, number of erythrocytes decreased post-challenge (Figure 4). Lowest levels of erythrocytes found in C+ fish (1.15×10^6 cells/mm^3) at day 3 post-challenge, while the highest value was found in C- fish (3.56×10^6 cells/mm^3) at day 3 post-challenge. Subsequently, on day 7 the number of erythrocytes increased in all treatments, except RxL fish was relatively the same since day 3 to day 7.

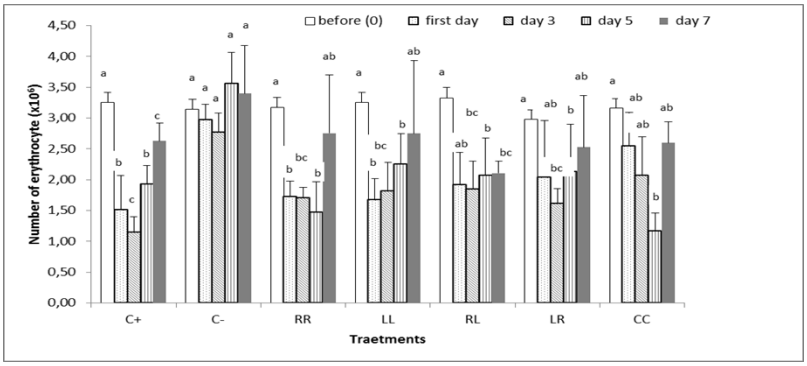

Figure-4: Erythrocyte levels in catfish post-challenged with *Aeromonas hydrophila*. Different letters on top of the bar on the same day showed significantly different values by ANOVA ($p<0.05$).(R = resistant fish ; L = healwounds fish ; C = control fish ; C- = negative control fish and C+ = Positive control fish)

Blood hemoglobin level is presented in Figure 5. Levels of hemoglobin before challenge test with *A. hydrophila* were similar in all treatments, ranging from 13.24 to 14.32 g%. Furthermore, hemoglobin levels decreased until day 3, and hemoglobin levels on day 7 were the same as before the challenge test, except C fish remains low.

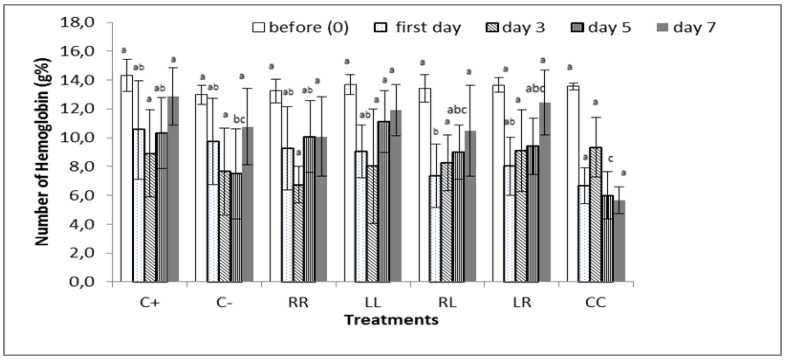

Figure-5: Hemoglobin levels in catfish post-challenged with *Aeromonas hydrophila*. Different letters on top of the bar on the same day showed significantly different values by ANOVA test ($p < 0.05$). (R = resistant fish ; L = heal wounds fish ; C = control fish ; C- = negative control fish and C+ = Positive control fish)

On day 5 challenge test, hemoglobin levels in offsprings of R and L broods were increased, while C+ and C was still low. Increased hemoglobin levels on day 5 to 7 post-challenge in offsprings of R and L broods indicated that fish had recovered from the infection. Decrease in hemoglobin levels on day 1 and day 3 challenge test showed haemolysis due to bacterial infection.

Hematocrit levels of fish were presented in Figure 6. Hematocrit levels before the bacterial challenge test were the same in all treatments, ranging from 40.00 to 42.92%. Hematocrit level in fish C+ was unchanged, while offsprings of R and L broods and C were decreased until 3rd day, slightly increased on 5th day and return to normal level on day 7 in all fish.

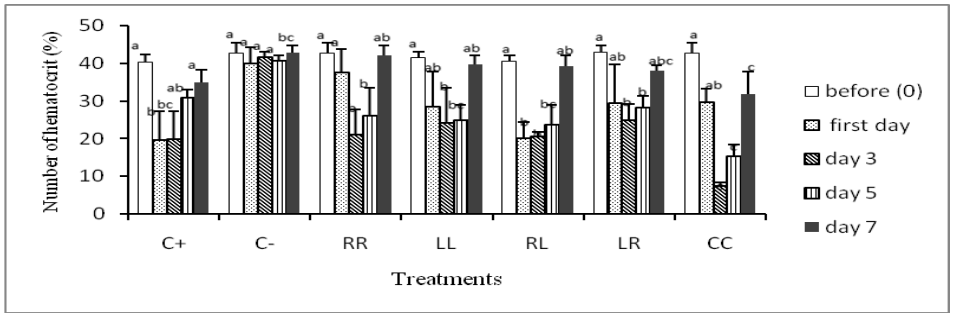

Figure-6: Hematocrit levels in catfish post-challenged with *Aeromonas hydrophila*. Different letters on top of the bar on the same day showed significantly different values by ANOVA ($p < 0.05$). (R = resistant fish ; L = healwounds fish ; C = control fish ; C- = negative control fish and C+ = Positive control fish)

Antibody titers of catfish on day 3 to day 14 post-challenged test were presented in Figure 7. Blood samples from three samples of fish were mixed in the same treatment. Antibody titers on the 3rd day of challenge test were relatively similar in all treatments, ranging from 0.3 to 0.9. On day 5, antibody titers in offsprings of RxR, RxL and LxR crosses were increased, while C fish and LxL were still the same as on day 3. Furthermore, antibody titers in offsprings of R and L broods on day 7 and 14 were higher than in the C fish.

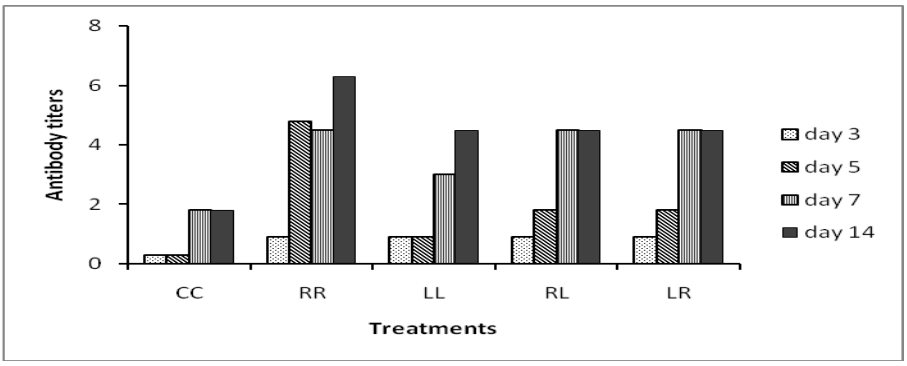

Figure-7:Antibody titers in catfish *Clarias* sp. first generation post-challenged with *Aeromonas hydrophila.* R = resistant fish; L = heal wounds fish; C = control fish; C- = negative control fish and C+ = Positive control fish. fish blood sampling at 3day, 5day, 7 day and 14 day after challenged *Aeromonas hydrophila.*

Water quality: Water quality during the research was within the tolerance limits for catfish, and within the standard water quality developed by Fleuren (2008). The water temperature ranged between 26-31°C, pH 6.8 to 7.5, dissolved oxygen 5.12-6.5 mg/L, and total ammonium nitrogen 0.01-2.9 mg/L.

DISCUSSION

High mortality caused by *A. hydrophila* bacterial infection is still common in intensive catfish farming. Application of molecular marker-based selection method is expected to accelerate the production of superior parent. In this study we used the MHC I marker to produce catfish F1 offspring resistant to *A. hydrophila* infection. These results indicate that the catfish F1 from parents R (resistance) and L (wounds healed) has viability of 2.21 times higher than the control fish when challenged with *A. hydrophila*. Furthermore, the survival of F1 fish from R and L broods were relatively the same. This shows a great potential to produce catfish resistant to *A. hydrophila* infection by crossing R and L broods. However, the consistency of endurance R and L fish to *A. hydrophila* infection needs to be tested further in the next few generations before being released to farmers. Higher number of resistant fish category in F1 offsprings was obtained by mating F0 parent R category ($p<0.05$), while the number of resistant F1 fish in RxL, LxR and LxL were similar (Figure 2). This is in line with the amount of F1 fish carrying MHC I markers; highest percenttage was obtained in RxR progeny (83.4%). However, in this study the DNA fragment from PCR products which are strongly associated with resistance to *A. hydrophila* is unknown. F1 fish from the parent R and L showed 3 DNA bands of PCR amplification products: 300, 450 and 1,000 bp. Furthermore, the size and number of those DNA bands is equal to the parent F0 catfish (Azis *et al.,* 2015). This suggests that the MHC I marker was passed on to offsprings. The percentage of F1 offspring carrying the marker vary among crosses (62.5-83.4%); highest value was obtained in RxR crossing, and the lowest was in LxL crossing. MHC I allele profile on R and L fish needed to be investigated further to explain the pattern of inheritance.

The number of F1 resistant fish resulting from RxR mating was about 70% higher than that obtained in F0 population (7%) (Azis *et al*., 2015). Selection of Indonesian common carp resistant to pathogen infection using Cyca-DAB1*05 MHC II marker showed that the percentage of fish carrying the marker increases from generation to generation: 50.0% in the F0 generation, 70.0% and 83.3% respectively in the F1 and F2 (Decree of Marine and Fishery Minister No.24/KEPMEN-KP/2015). The same pattern could be expected in catfish with the R category.

High resistance in F1 fish from the parent R and L to *A. hydrophila* infection was in line with the blood profiles (Figure 3, 4, 5, 6 and 7). On day 1 challenge test, the number of leukocytes increased. This showed the immune response due to pathogen infection (Li *et al*. 2013). Furthermore, the decrease in the number of leukocytes on day 7 in F1 fish from R and L broods were in line with signs of wound healing on day 5. Mean while, the number of leukocytes in control fish C and C+ were still high on the day 3,5 and 7 (Figure 3), and is also in line with clinical symptoms, signs of healing seen on day 7. Hemorrhagic ulceris the result of the hemolysin toxin from the bacteria on the surface of the fish body (Del Coral *et al*., 1990).

The number of erythrocytes (Figure 4), hematocrit (Figure 5), and hemoglobin (Figure 6) in *A. hydrophila* challenged catfish were lower than before the challenge test. Hemorrhage in infected fish resulted in rupture of blood vessels, and adecrease in the number of erythrocytes. *A. hydrophila* infection also caused hemolysis, which explains a decrease in the amount of hemoglobin on day 1 and 3. Increased number of erythrocytes and hemoglobin were in line with the signs of healing wounds due to pathogen infection. An increase in antibody titer in F1 fish from R and L parents was in line also with the fast wound recovery time and the higher survival than C control fish. Furthermore, the F1 fish from the parent groups R and L, and RxR fish antibody titer reached the same level on day 3 with antibody titers on day 7 or 14 on the fish RxL, LxR and LxL. It supports high survival in the RxR fish resistant category. Increase number of antibodies in the blood serum is as an antidote to attack disease agents that enter the body (Uthayakumar *et al*., 2012).

CONCLUSION

MHC I molecular marker can be inherited to the first generation (F1) catfish. The highest percentage of fish carrying the marker and the highest survival in challenge test was obtained in the RxR crossing.

Acknowledgements: This manuscript is the result of research work, which I have done at the Department of Aquaculture, Bogor Agricultural University (IPB) and Faculty of Fisheries and Marine Science, Borneo University-Tarakan, Main Center for Freshwater Aquaculture Development (BBPBAT) Sukabumi-West Java and graduate education scholarships (BPPS) from Directorate of Higher Education (Dikti) 2011. It was a great honor and pleasure for me to work with many people who have encouraged, supported and helped me a lot with my research.

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