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Immunogenization of Heat-Killed Vaccine Candidate from *Aeromonas hydrophila* in Catfish (*Pangasius hypophthalamus*) using Strain of Banjar, South Kalimantan, Indonesia

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ABSTRACT

Aeromonas hydrophila often attacks cultured catfish and causes motile aeromonad septicemia (MAS) disease outbreak in South Kalimantan, Indonesia. Deaths from *A.hydrophila* attacks could reach 100% so that prevention needs to done through vaccination. This study aimed to examine the potential immunogenicity of 6 heat-killed *A.hydrophila* vaccine candidates, a strain of Banjar, South Kalimantan, Indonesia. *A.hydrophila* strains obtained from infected catfish in aquaculture ponds around the Banjar District, South Kalimantan, Indonesia. From 10 fish infected with MAS, obtained 14 isolates of bacteria, ie, 8 isolates (AGC-1, AGC-2, AGC-3, AGC-4, AGC-6, AKC-2, AKC-3, and AKC-5) of Sungai Batang village, and 6 isolates (AGC-8, AGC-9, AKC-7, AKC-8, AKC-9, AKC-10) from Cindai Alus village. AGC signifies *Aeromonas* isolated from the gills, and AKC means *Aeromonas* isolated from the kidney. The antigen that used as a candidate for the heat-killed *A.hydrophila* vaccine made by inactivation through a heating process at 100 ^{o}C for 60 minutes. The results showed antigens from AGC-2 and AGC-8 strains had high immunogenicity because they could increase antibody titers compared to other strains and controls. The antibody titer in catfish, two weeks after booster vaccination, increased and showed the same value. The results of the cross-reaction assay showed that the antigens from the AGC-2 and AGC-8 strains were able to cross-react with strain AGC-1, AKC3, AKC-5, but unable to cross-react with AKC-7, so that AGC-2 and AGC-8 could be recommended as vaccine candidates for MAS disease in South Kalimantan, Indonesia.

INTRODUCTION

The catfish (*Pangasius hypophthalmus*) are freshwater fish and widely cultured in South Kalimantan. However, catfish culture often constrained by the disease that appears periodically every year. One of the diseases, namely MAS (motile aeromonad

septicemia). This disease caused by the bacterium *Aeromonas hydrophila* **(de Figueiredo and Plumb, 1977; Stratev and Odeyemi, 2017)**.

Aeromonas sp. was reported to have attacked catfish that cultured in South Kalimantan in 2007. At that time, it was not informed that the invading bacteria, namely *A. hydrophila*. Based on an annual report on the results of health and environmental monitoring of the Freshwater Aquaculture Fisheries Center, Mandiangin, South Kalimantan, Indonesia from 2009 to 2018, it was reported that *A.hydrophila* attack catfish in the South Kalimantan aquaculture environment **(Aisiah *et al.*, 2019)**.

Aeromonas hydrophila was always present in water and an opportunistic pathogen that would attack fish when the fish's body condition weak **(Stratev *et al.*, 2012)**. *A. hydrophila*, including pathogens with high virulence **(Chopra *et al.*, 2000; Rasmussen-Ivey *et al.*, 2016)**. *A. hydrophila* attacks host cells and spreads to many organs through blood vessels and could produce virulence factors, which contribute to the pathogenesis of *Aeromonas* infection **(Al-Fatlawy and Al-Ammar, 2013)**. The ability of some *A.hydrophila* strains to ward off complement-mediated lysis could cause bacteremia and other diseases associated with *Aeromonas* infections **(Merino *et al.*, 1996; Merino *et al.*, 1998)**.

Motile aeromonad septicemia (MAS) was an acute disease, infects all ages and types of freshwater fish, and could cause death up to 100% **(Osman *et al.*, 2009; Igbinosa *et al.*, 2012)**. Literature studies showed reports of *A. hydrophila* attack freshwater aquaculture, including tilapia (*Oreochromis niloticus*) **(El-Ashram, 2002; Sugiani *et al.*, 2012; Rodrigues *et al.*, 2019)**, snakehead (*Channa striata*) **(Olga, 2012; Minh *et al.*, 2013)**, climbing perch (*Anabas testudineus*) **(Hossain *et al.*, 2011; Hayati *et al.*, 2012)**, gouramy (*Osphromenus gouramy*) **(Mulia, 2010; Rozi *et al.*, 2018)**, catfish (*Pangasius hypophthalamus*) **(Olga and Aisiah, 2007; Aisiah *et al.*, 2020)**, african catfish (*Clarias gariepinus*) **(Olga *et al.*, 2004; Aly *et al.*, 2019; Wulandari *et al.*, 2019)**.

Aeromonas hydrophila infection cause considerable losses in catfish culture because this could cause fish death as much as 80-100% in about three days **(Hardi *et al.*, 2018; Nurruhwati *et al.*, 2019)**. *A. hydrophila* would decrease cause the quality and quantity of aquaculture products, as well as an impact on reduced production and economic losses.

Efforts to overcome the MAS disease have been carried out both through prevention and treatment. Prevention could do by administering immunostimulants and vaccines **(Assefa and Abunna, 2018)**. **Mulia *et al.* (2016); Ma *et al.* (2019)** reported that the success of vaccination for the prevention of MAS was highly dependent on many factors, including the quality, bacterial strains of various vaccine candidates, the method of manufacture, the type of antigen used, dosage, booster administration and the method of vaccination. Thus, efforts are needed to find quality and immunogenic vaccine materials in controlling MAS disease.

Vaccines made from bacterial cells that are inactivated by heating (heat-killed) that cause the lipids to be hydrolyzed during heating called the O antigen **(Dehghani *et al.*, 2012)**. O antigen was a constituent of lipopolysaccharide compounds and is capable of eliciting an immune response in animals **(Lerouge and Vanderleyden, 2001)**.

Previous studies have reported an increased immunogenicity of the vaccine on bacterial isolates from Indonesia inactivated by heat, including *A. hydrophila* vaccine of african catfish **(Mulia *et al.*, 2016)**, *A. salmonicida* vaccine of carp **(Wintoko *et al.*,**

2013), and *Streptococcus* sp. vaccine from tilapia (*Oreochromis niloticus*) **(Taukhid and Purwaningsih, 2011)**. Based on the description above, vaccines made from Kalimantan strain bacterial isolates are essential in overcoming the MAS disease that attacks freshwater fish farming in South Kalimantan. Thus, this research aimed to analyze the ability of heat-killed antigens from the Banjar, South Kalimantan *A.hydrophila* strain, in cross-reaction and immunogenic response of catfish (*Pangasius hypophthalamus*) as vaccine candidates for MAS disease control.

MATERIALS AND METHODS

The isolate of *A. hydrophila* used as a vaccine candidate material and was the screening of cultured catfish affected by MAS disease. Catfish with MAS obtained from the aquaculture ponds of Sungai Batang village and Cindai Alus village, Banjar Regency, South Kalimantan, Indonesia. Catfish (*P. hypophthalamus*) suspected of being infected with MAS can be seen in **Fig. 1**.

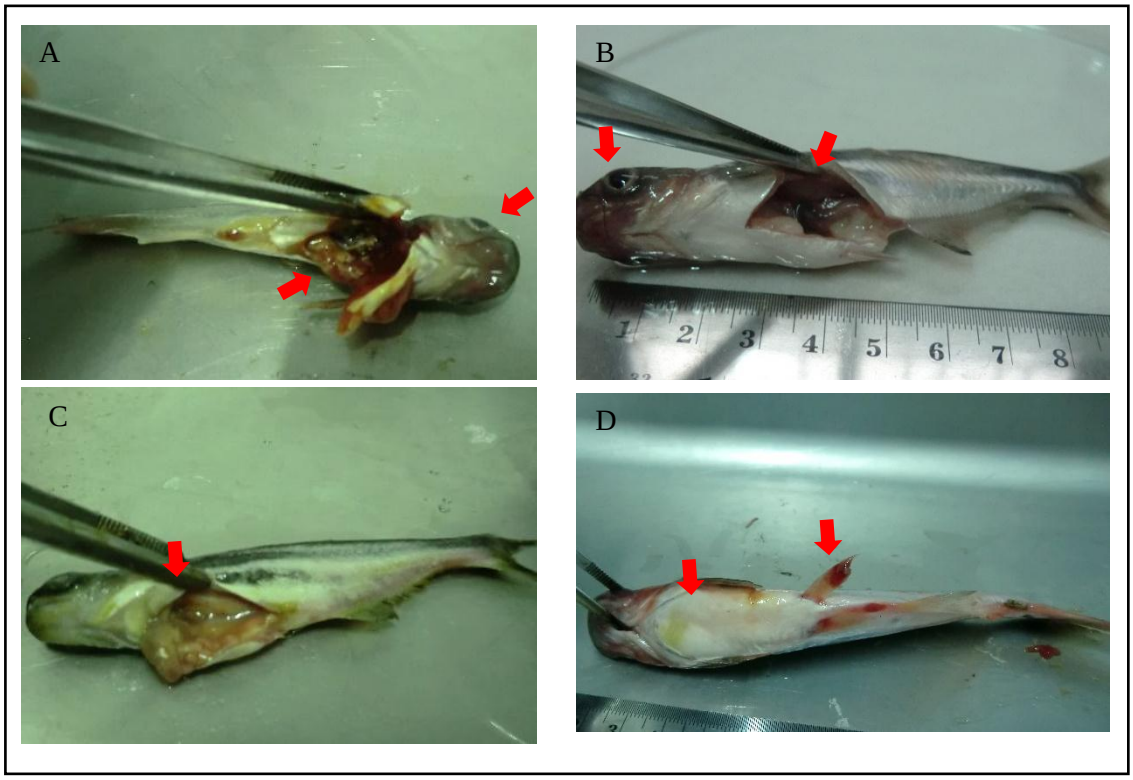

Fig. 1. Catfish (*P. hypophthalamus*) suspected of being infected with MAS disease
Note : reddish eye and mouth surface (A and B); reddish kidney (A and B); yellow fluid in the pectoral and abdominal fins (C and D); and hemorrhagic ulceration at the fin base (D).

Fig. 1 shows the clinical and pathological signs of catfish infected with *A. hydrophila*, ie, bilateral exophthalmia with reddish eye and mouth surface, hemorrhagic ulceration at the base of the fin, reddish kidney, and the presence of yellow fluid in the pectoral and abdominal fins **(Sarker and Faruk, 2016; Nahar *et al*., 2016)**.

The number of fish samples suspected of being infected with MAS was 15 fishes, consisting of 9 fish from catfish ponds in Sungai Batang village and 6 fish from Cindai

Alus village. After that, the screening results showed that only 6 fish from Sungai Batang village and 4 fish from Cindai Alus village positively identified as having MAS. From 10 fish infected with MAS, obtained 14 isolates of bacteria, ie 8 isolates (AGC-1, AGC-2, AGC-3 , AGC-4, AGC-6, AKC-2, AKC-3 and AKC-5) of Sungai Batang village, and 6 isolates (AGC-8, AGC-9, AKC-7, AKC-8, AKC-9, AKC-10) from Cindai Alus village. From these 14 isolates, 6 isolates identified as pathogenic *Aeromonas hydrophilla* as could be seen in **Table 1**.

Table 1. *A. hydrophila* isolates used in the research

Strain Code	Origin of the Strain	Origin of Isolation
AGC-1	SB-BR-SKI	Catfish, gill
AGC-2	SB-BR-SKI	Catfish, gill
AKC-3	SB-BR-SKI	Catfish, kidney
AKC-5	SB-BR-SKI	Catfish, kidney
AGC-8	CA-BR-SKI	Catfish, gill
AKC-7	CA-BR-SKI	Catfish, kidney

AGC = *Aeromonas* isolated from the gills of catfish; AKC = *Aeromonas* isolated from the kidney of catfish; Number 1-8 = Catfish Sample Numbers, SB-BR-SKI = Sungai Batang Village, Banjar Regency, South Kalimantan, Indonesia; CA-BR-SKI = Cindai Alus Village, Banjar Regency, South Kalimantan, Indonesia

The catfish used for assay were 12 - 15 cm long and obtained from fish farming ponds in Cindai Alus village. Catfish acclimatized for one month in the laboratory so that they are really in a healthy condition before being used as test animals.

Isolate *A. hydrophila*, before being made a vaccine candidate (antigen), was first reinfected and re-isolated to healthy fish to increase its virulence. Procedure for increasing bacterial virulence refers to **Mulia and Purbomartono (2007)**. The isolates cultured in TSB (tryptone soya broth, Merck-Germany) media, incubated at 37 ^{o}C for 18-24 hours. Bacterial culture on TSB media was injected intramuscularly at a dose of 0.1 ml (10^{11} cfu/ml) on five catfishes. Bacteria were isolated again on GSPA media (glutamate starch phenile agar, Merck-Germany) from assay fish showing symptoms of MAS. Then reinfection and re-isolation carried out three times. The procedures for making heat-killed vaccines refer to **Mulia *et al*. (2016); Saadh *et al*. (2017)**, i.e., bacterial cells in sufficient quantities harvested, and activated by heating (heat-killed) at 100 ^{o}C for 60 minutes, then washed with PBS pH 7.4 and centrifuged at a speed of 3000 rpm three times for 15 minutes. Furthermore, a viability assay performed to determine whether the bacterial cell was inactive.

This study used a completely randomized design (CRD) with 6 treatments (ie antigens from AGC-1, AGC-2, AKC-3, AKC-5, AGC-8, AKC-7) and 3 replications. For comparison, 1 control with 3 replications was used. The fish used for the assay were catfish that have been acclimated for one month. The experimental unit was in the form of 10 catfish in a maintenance tank filled with 15 liters of water. Catfish used were 12-15 cm long. Each antigen injected intramuscularly to fish at a dose of 0.1 ml (with a density of 10^{7} cells/ml). Adjustment of turbidity of bacterial suspension compared with McFarland Hi-Media standard. A booster carried out for the next week, while fish blood sampling to determine the increase in antibody titer during the immunogenicity test carried out at the beginning before vaccination and every week. The working procedure

of the antibody titer assay refers to **Hastuti (2013)**. The main parameters include antibody titers for immunogenic and cross-reaction testing. The antibody titer assay data for the immunogenic assay transformed into logarithmic form (Log2 Data). Data analyzed with analysis of variance (ANOVA). If there was a difference between treatments, then it continued with the Duncan Multiple Range Test (DMRT) at the 5% level. Observation of the cross-reaction assay and water quality analyzed descriptively.

RESULTS

Six strains of *Aeromonas hydrophila* were successfully selected and isolated from catfish, which showed symptoms of MAS and were pathogenic. Based on the results of Gram staining, these 6 strains belong to the Gram-negative (-) form of the rod. When referring to the genus *Aeromonas* phenotypically, the six strains have a circular colony, the elevation of the convex-shaped colony, and the color of the bacterial colony in the GSP (Glutamate Starch Phenol) agar medium was yellow, and inhibitory zones on the media around the colony. In addition, the isolates after being assayed by catalase and oxidase were positive, which meant that all bacteria were able to produce the enzyme catalase and oxidase. Their O/F assay results were fermentative, which proves that these bacteria are facultative anaerobes. More specifically, these 6 strains were motile and could produce H_2S on TSIA media (triple sugar iron agar) that characterize the species of *A.hydrophila* (this identification data has been published in the manuscript, which was published in the proceedings of the National Seminar on Fisheries and Marine Affairs VI, Faculty of Fisheries and Marine, University of Lambung Mangkurat, Indonesia. Proceedings were also temporary the process of publishing). Then, these six strains reproduced and made antigens to tested for their immunogenicity. The results of the measurement of antibody titers could be known based on the ability of antibodies in agglutinating antigens of *A. hydrophila*. At the beginning of the study, precisely at week 0, all antibodies in the assay fish were not formed, which is indicated by the value 1.00 - 1.33 in the antibody titer assay **(Table 2.)**. This was because the assay fish have not vaccinated, so the fish have not formed memory cells to produce specific antibodies.

Table 2. Catfish Antibody Titer After being vaccinated with the heat-killed vaccine candidate from *A.hydrophila*, strain of Banjar, South Kalimantan, Indonesia

Vaccination treatment	Sampling/Week			
	0	**1**	**2**	**3**
AGC-1	1.33^{a}	128.00^{c}	341.33^{c}	341.33^{b}
AGC-2	1.33^{a}	106.67^{bc}	1024.00^{d}	1706.67^{c}
AKC-3	1.00^{a}	85.33^{b}	144.00^{b}	170.67^{b}
AKC-5	1.00^{a}	85.33^{b}	192.00^{b}	256.00^{b}
AGC-8	1.33^{a}	149.33^{c}	853.33^{c}	1706.67^{c}
AKC-7	1.33^{a}	96.00^{b}	192.00^{b}	256.00^{b}
Control (PBS)	1.00^{a}	1.66^{a}	1.66^{a}	1.00^{a}

Note: Different letters on the table considered significantly different for each group at $p<0.05$.

Table 2 shows one week after booster vaccination; that is, on the second week, there was a trend towards a significant increase in antibody titer ($P<0.05$). Fish vaccinated with AGC-2, and AGC-8 antigen strains showed a significant increase in antibody titer compared to fish immunized with antigens from other strains.

Two weeks after booster vaccination, ie, third sampling, there was still an increase in antibody titers in the vaccine treatment, except for the AGC-1 strain. No increase in antibody titer in the AGC-1 strain could be caused by the ability of the fish's weak immune response to aggravating the presence of antigens. Furthermore, fish vaccinated with AGC-2, and AGC-8 antigen strains produced the same antibody titer, which was 1706.67. AGC-2 and AGC-8 antibody titers were higher than fish antibody titers vaccinated with antigens from strains AGC-1, AKC-3, AKC-5, and AKC-7. The lowest antibody titers on AKC-3 (170.67).

The results of a cross-reaction assay conducted to determine the immunogenicity of heat-killed *A. hydrophila* antigens given to catfish when there is an attack from other pathogenic strains, namely by looking for antigens that could react positively with themselves or with other antigens. Bacterial strains selected for cross-reaction assays were strains that provide high antibody titers in the immunogenicity assay, namely AGC-2, and AGC-8. Both of these strains produced antibody titers, which continue to increased after booster vaccination. In this cross-reaction assay, the serum of fish vaccinated with AGC-2 and AGC-8 antigens acts as antibodies reacted with antigens from all selected pathogenic strains, namely strains of AGC-1, AGC-2, AKC-3, AKC-5, AGC-8, and AKC-7. The results of the cross-reaction assay presented in **Table 3**.

Table 3. Cross-Reaction Assay Between *A. hydrophila* Strain from Banjar, South Kalimantan, Indonesia

Serum	**Antigens**					
	AGC-1	**AGC-2**	**AKC-3**	**AKC-5**	**AGC-8**	**AKC-7**
AGC-2	32,00	1024,00	32,00,	16,00	512,00	1,33
AGC-8	256	512,00	64,00	256,00	2.048,00	1,00
Control	1,00	1,00	1,00	1,00	1,00	1,00

Table 3 shows the strain that has the highest immunogenicity ability was the AGC-8 strain. In addition to being able to recognize strains of fish that vaccinated with AGC-8 antigens themselves, AGC-8 strain was also able to recognize antigens from strains of AGC-1, AGC-2, AKC-3, and AKC-5. But the AGC-8 strain was unable to recognize the antigen from the AKC-7 strain. The fish serum that vaccinated with antigens from AGC-2 strains was also able to recognize antigens from strains of AGC-1, AGC-2, AKC-3, and AKC-5. AGC-8 and AGC-2 strains were unable to recognize and couldn’t cross-react with antigens from AKC-7 strains. This study only looked at antibody titers produced in the cross-reaction between antibodies and strains without challenge assays (or infecting vaccinated fish with different bacterial strains). Challenge assay will be carried out at the next stage after getting potential strain candidates.

Water quality data measured in this study include temperature, pH, and dissolved oxygen. The measurement results showed the temperature of the water during the study ranged from 28-30 ^{o}C, pH 6.8 - 7.0, dissolved oxygen 6.6 - 6.8 mg/L.

DISCUSSION

The heat-killed/ O antigen vaccine is a constituent of immunogenic lipopolysaccharide compounds. The heat-killed / antigen O vaccine was capable of eliciting an immune response that is indicated by the formation of antibodies after the fish were vaccinated. Antibodies were serum proteins that formed in response to antigens that enter the fish's body and increase humoral defenses. **Thomas (2004)** reported that antibodies in the form of proteins that look like Y (roughly Y-shape) used as an immune system to identify, neutralize and kill foreign objects or pathogens such as bacteria and viruses. In this study, the ability of fish vaccinated with antigens from various *A.hydrophila* strains to produce antibody titers varies. The results of the antibody titer assay in **Table 2** confirm the findings and opinion that *A.hydrophila* isolates have genetic variation **(Aguilera-Arreola *et al*., 2007; Khor *et al*., 2015)**. Genetic variation of *A.hydrophila* was not only on the type of antigen but also on the value of the antibody titer formed **(Sha *et al*., 2002 and Tomas, 2012)**.

Vaccination in catfish triggers a humoral immune response in the form of antibodies. The requirements of a vaccine were immunogenic, which must be able to stimulate the formation of antibodies **(Clem, 2011)**. Microorganisms have many different antigens, and each antigen could recognize different lymphocyte clones. On stimulation, the clones proliferate with differentiation of daughter cells that have specialized functions depending on the presence of lymphocyte clone populations. There are two specific immune responses, namely humoral immunity (antibodies) and cell-mediated immunity (CMI). Exposure to antigens results in the stimulation of a small number of virgin lymphocytes that could recognize antigens through specific antigen receptors **(Zhao and Elson, 2018)**. Specific lymphocytes were clones and consist of two main populations of lymphocytes. T lymphocytes were originating from the thymus, and B lymphocytes were originating from the fabricius exchanges in the mammalian bone marrow. The source of this type in teleost fish is uncertain, but it was thought to be in the kidney **(Parra *et al*., 2013)**.

The formation of memory cells to produce specific antibodies, according to **Pennock *et al*. (2013)**, was a collaboration between T and B cells in responding when there is primary exposure to antigens. B lymphocytes differentiate into plasma cells that produce specific antibodies for stimulating antigens or into cells that were capable of becoming plasma cells on subsequent antigen exposure, known as memory cells. T cells have different functions. T cells that work together are called helper cells. Helper T cell clones stimulated by antigens, so helper T cells multiply into helper memory cells that are long-lived.

A week and two weeks after booster vaccination, fish antibody titer production in all vaccination treatments was increasing. The highest increase in antibody titers in fish vaccinated with antigens from AGC-2 and AGC-8 strains. **Table 2** explained that booster vaccination could stimulate the production of antibody titers to increase faster. The high value of antibody titers in fish treated with AGC-2 and AGC-8 showed that both of them have the opportunity to be selected as vaccine candidates compared to the four other

strains. Furthermore, both expected to protect catfish in a culture environment when exposed to MAS. According to **Smith (2012)** , if there is further antigen exposure, there would be an increase in the number of helper T cells to work together with an increase in the number of memory B cells. This would stimulate antibody production faster on secondary antigen exposure and reach higher concentrations than after primary exposure. **Sun *et al*. (2014)** said memory cells were able to produce immune responses quickly as measured by increased resistance after exposure to pathogens or vaccines. Furthermore, according to **Clem (2011)**, specific antibodies would provide immunity during ideal conditions, which is two or three weeks after stimulation.

Pasetti *et al*. (2011) said the high value of antibody titers does not fully reflect the level of absolute protection against target pathogens. But in general, it could be stated that the higher antibody titer values indicate the formation of an immune response that was positively correlated with the ability to ward off-target pathogen infections **(Swayne *et al*., 2015)**.

Assay of antigen cross-reactions with fish serum showed that AGC-2 and AGC-8 strains were potential vaccine candidates because they were immunogenic by being able to recognize more antigens than other strains. **Table 3** explained that the antigen-antibody molecular configuration between AGC-1, AKC-3, AKC-5 antigen strains, and antibodies from fish serum vaccinated using AGC-2 and AGC-8 antigen strains were such that only antibodies appear in response to just a specific antigen that matches the surface of the antigen while reacting with it. Additionally, clumping of antigen particles could be carried out because the structure of the antibody makes it possible to bind more than one antigen. Antibody molecules have at least two binding sites for antigens that can join with adjacent antigens. Bacterial clumps will make it easier for phagocytic cells (macrophages) to phagocyte bacteria quickly. Thus the antibody molecule could bind to the tested antigen in addition to the AKC-7 strain antigen.

Antigens from AGC-2 and AGC-8 strains could be further assayed if selected as a vaccine candidate. Fish vaccinated with antigens from AGC-2 and AGC-8 strains could be assayed with *A. hydrophila* strains that they recognize in cross-reaction assays so that the relative percent survival of vaccinated fish can be measured. Cross-reaction is an inherited trait of cells in the adaptive immune system **(Vergani *et al*., 2002)**. Derivatives from T and B lymphocytes to recognize some potential non-self antigens do not have to go through a primary exposure process to obtain information about their structure **(Vergani *et al*., 2020)**. An antibody has specific amino acid strands and sequences (Fab region) that determine its affinity for specific antigens. Cross-reactivity between antigens occurs when antibodies generated against one specific antigen have a high affinity that competes against different antigens. This often occurs when two antigens have similar structural regions that are recognized by antibodies.

Assefa and Abunna (2018) reported the level of protection by vaccination highly depends on the type and quality of the vaccine, the method of vaccination, and the condition of the fish and water quality. Thus the *A. hydrophila* antigen, which has high immunogenicity in the cross-reaction assay, has the better ability as a material for making vaccines **(Lei *et al*., 2019)**. **Hayati *et al*. (2012)** also reported that the heat-killed/O antigens of *A. hydrophila* could increase the immunogenicity of climbing perch fish.

Water quality data showed that the range of temperature, pH and oxygen was still normal for catfish growth **(Ayoub *et al*., 2012)**. According to **Ariyanto *et al*. (2008)**

temperature, pH and dissolved oxygen were important water quality parameters for catfish growth. The optimum water temperature for fish appetite between 22-29 ^{o}C. While the ideal pH for the optimal growth of catfish ranges from 6.5-9.0 **(Andriyanto *et al*., 2012)**. **Manunggal *et al*. (2018)**, reported at low pH, the dissolved oxygen content will decrease. The lack of dissolved oxygen content results in increased respiratory activity and decreased appetite for fish. Dissolved oxygen levels were good for fish ranging from 7.0 to 8.4 mg/L, but the dissolved oxygen content of 5 mg/L was still possible for fish life.

CONCLUSION

From the research, it could be concluded that the AGC-2 and AGC-8 antigen strains were immunogenic because they could increase the catfish antibody titer and could cross-reaction with other strains. AGC-2 and AGC-8 strains could be recommended as vaccine candidates for MAS disease in South Kalimantan, Indonesia. However, the AGC-8 strain was the best candidate to be made into a monovalent vaccine.

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