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Production of Carp Immunoglobulin-M Exposed with Whole Protein from Myxobolus koi Spore through Feed as an Immunostimulant

Moch Saad*
Department of Biotechnology
Fisheries and Marine
Postgraduate Airlangga
University
Surabaya, East Java 60115
Indonesia
amochsaad22@gmail.com

Gunanti Mahasri
Department of Fish Health
Management and Aquaculture
Faculty of Fisheries and
Marine
Airlangga University
Surabaya, Indonesia
mahasritot@gmail.com

Woro Hastuti Satyantini
Department of Fish Health
Management and Aquaculture
Faculty of Fisheries and
Marine
Airlangga University
Surabaya, Indonesia
woro_hastuti@yahoo.com

***Abstract*—Carp production in Indonesia in 2010-2015 has increased an average of 14.44%, a low average increase in production when compared with other main commodities such as shrimp, tilapia, catfish and others. One of the causes of the low average increase in production is the presence of disease attacks in some carp central production. One of the natural ingredients that can be used to control the body system against the disease is the whole protein from Myxobolus koi spore. The aim of this study was to analyze the effect of whole protein from Myxobolus koi spore on carp (Cyprinus carpio L.) through feed on increasing Immunoglobulin M (IgM) value in blood. This research was conducted by Randomized Complete Design with 5 replications. This study uses three kinds of treatment, control (100% commercial feed, 100% artificial feed and artificial feed + Immunostimulants (whole protein from Myxobolus koi spore as 1µg protein/gram fish) + adhesive Boster® Progol. The immunostimulan dose is of the whole protein from Myxobolus koi spores with a dose of 1µg protein/gram fish**
+ adhesive Boster® Progol with a volume of 5 ml/kg of feed. First step is identification of the pathogens that attack the carp before (first day) and after a given treatment (day 28). The results show that whole protein from Myxobolus koi spores given in feed as the immunostimulant have a significantly different effect ($p < 0.05$) on the number of pathogens attack and carp (Cyprinus carpio L.) IgM concentration. The immunostimulant can boost the immune system by increasing the IgM concentration of day 7 (1463,566µg/ml), day 14 (2189, 368µg/ml) and day 28 (1939, 087µg/ml) formed by proliferation of lymphocyte B and 99% survival rate.

***Keywords*— Whole Protein, *Myxobolus koi, Cyprinus carpio* L, IgM**

I. INTRODUCTION

Carp production in Indonesia in 2015 reached 445, 000 tonnes, and increased average 14.44% from 2010. This is the lowest average increase in production when compared with other major commodities, such as tilapia, patin, catfish and others which have an average hike of 19-30% [1]. One cause of the low increase in average carp production is disease in some of the central production [2].

According to [3], one of the pathogenic organisms often found in fish is from a class of parasites. One protozoan parasite that attacks carp is Myxosporea. Myxosporea is a parasitic protozoa capable of limiting the production of carp (Cyprinus carpio), mainly on seeds, and can cause death in more than 90% of the infected fish population. Myxosporea which attacks carp, including koi, are Myxobolus, M. toyamai and Thelohanellus caliporis [4].

Aside from the type of parasite, some bacteria such as Aeromonas sp [5], Pseudomonas anguilliseptica and Pseudomonas fluorescens [6] and Mycobacterium spp [7] is reported have pathogenic properties of the carp (Cyprinus carpio L.). diseases caused by pathogenic organisms in the environment mostly infectious and treatment may be needed to control disease outbreaks [8].

Efforts to control pathogens in cultivation over the last 20 years have been carried out with the use of antibiotics and chemicals, but long-term use can lead to resistance against multiple pathogens. Vaccination is a very effective treatment in
addressing the problem of pathogens in cultivation, but the cost is very expensive and can cause stress for the fish. One alternative technique to strengthen the immune system of the fish and counter attack by pathogens is by using immunostimulants [9].

Immunostimulants will stimulate macrophages to produce cytokines, such as interleukin, which will activate the lymphocytes which are then split into B lymphocytes and T lymphocytes. T lymphocytes in non-specific response would produce interferon that can activate performance macrophages through phagocytosis mechanism in the face of parasites, bacteria, viruses and foreign particles that are considered as an antigen [10]. Whereas, in the specific immune response, cytokines, including IL-4, IL-5 and IL-13 with the Th cells, will activate B lymphocytes and T lymphocytes [11].

B lymphocytes will establish an antibody mechanism called immunoglobulin (Ig). Mammalian immunoglobulin is divided into several types. including IgG, IgM, IgE and IGI, while immunoglobulins in teleosts are more limited than in mammals. As reviewed by [12], the most common immunoglobulin in serum IgM fish teleosts is tetramer, although some have teleost IgM monomer (H_2L_2) in the serum [13].

A material can be used as an immunostimulant if it has immunogenic properties. [14] managed to pull through the isolation, identification and characterization of surface *Myxobolus koi* glycoproteins as an immunogenic antigen for antibody production. [15] also successfully characterized each protein size of the whole protein from *Myxobolus koi* spore using SDS-PAGE (sodium dodecyl sulfate polyacrilamide gel electrophoresis). The results of SDS-PAGE of whole protein *Myxobolus koi* spores were obtained for a protein depicted in the form of ribbons on SDS-PAGE gel and obtained 6-band protein with molecular weight 68.1kDa, 38.5 kDa, 25.6 kDa, 23 kDa, 21.7 kDa and 18.9 kDa. These results showed that the protein was found have a high molecular weight. An immunogenic protein is a protein that has a molecular weight of 20000-100000 Dalton [16]. Good immunogen molecular weight has a size of <100 kilodalton. [17] suggested that a molecule has an immunogen characteristic when having a molecular weight of more than 10kDa.

Whole protein from *Myxobolus koi* spores can also boost the immune response and survival rate from 10% to 86% in koi fish and increase the defense against infections of *Myxobolus koi* [18]. Based on this background, it is necessary to develop ways of prevention against pathogen attack through the research by analyzing the immune response in carp (Cyprinus carpio L.) given whole protein from *Myxobolus koi* spores through the feed as an immuostimulant candidate. Thus, it can give protection against various types of pathogen that attack and is expected to increase the survival rate of carp.

II. PURPOSE

The research is aimed to know the concentration of Immunoglobulin M (IgM) in the blood and analyze survival rate after being exposed of whole protein from *Myxobolus koi* spores in carp (*Cyprinus carpio* L.) through feed.

III. MATERIALS AND METHODS

2.1. Time and Location

The research was conducted in August and September 2016 in Instalasi Budidaya Air Tawar (IBAT), Dlanggu, Mojokerto. Immune response tests were conducted in the ITD Leprosy laboratory Airlangga University, and pathogen identification test was conducted at the Microbiology Laboratory, Science and Technology Faculty, Airlangga University.

2.2. Research Methods

This research was conducted by Randomized Complete Design with five replications. This research uses three kinds of treatment, P_0/ control (100% commercial feed), P_1 (100% artificial feed) and P_2 (artificial feed + Immunostimulants whole protein from *Myxobolus koi* spore as 1µg protein / gram fish) + Boster® Progol adhesive. The immunostimulant dose of the whole protein from *Myxobolus koi* spores is 1µg protein / gram fish + Boster® Progol adhesive with a volume of 5ml / kg of feed.

2.3. Research Materials

Carp *(Cyprinus carpio* L.) were tested by using a pools sized1x7x8m with carp *(Cyprinus carpio* L.) measuring 3-5 cm by 3,300 fish (each pool contained 1,100 fish).

The main materials in the detection of bacteria, among others, were samples of carp *(Cyprinus carpio),* TSA (Trypticase Soy Agar) medium, 2.5% NaCl (sodium chloride), TSB (Tryptic Soy Broth) medium, TCBS (Thiosulfate Citrate Bile Salts sucrose) medium, GSP (Glutamate Starch Phenol) medium, distilled water, rubbing alcohol, and chemicals for analysis of morphological and biochemical tests. The materials used for the measurement of antibody titers used ELISA is washing liquids (PBS-tween 20 + preservative proclin 300 0.005%), a solution of conjugate *(phosphate buffered saline,* BSA and *stabilizers),* substrate *(tetramethyl-benzidine* with *citrate-phosphate buffer* containing H_2O2, *coating* buffer, 1% Casein PBS-T, *stopping solution* and distilled water.

The equipment used in the research included three pools sized 1x7x8m, three plastic tubs, nets, filter, digital scales , water quality gauges (pH pen, thermometer, DO meter, ammonia test kit), and

surgical instruments (scissors and tweezers). Hematological parameters were gauged using light microscopes, pipettes capillaries, syringes, Sahli haemometer, tube eppendorf, object glass, cover glass, sectio set, microplate, ose, petri dishes, pipettes, test tubes, micropipette,
beaker glass, tubes, microtube, centrifuges and haemositometer. Phagocytic index test equipment used a petri disk, a loopful, spectrophotometers, Bunsen burners, Erlenmeyer flask, a magnifying glass, a pipette, an analytical balance, gas stove, autoclaving and hotplate. Equipment for ELISA included a polystyrene 96 well microtiter plate (microplate), micropipette (100-200 mL), micropipette shaker, micro tube (1-1.5mL), micro plate washer, glass beaker and ELISA Reader.

TABLE 1. TOTAL RESULTS OF THE PATHOGENS THAT ATTACK THE CARP

Examination Time	Treatment	Pathogens type					
		Bacterial (CFU / g)	Parasites *Myxobolus koi*				
			Intensity	Healthy (0)	Low (1-4)	Medium (5-8)	Weight (> 8)
Before treatment (Day 1)	P_0	$2.02x10^6$	1.4		√		
	P_1	$1.15x10^6$	1.6		√		
	P_2	$1.3x10^6$	1.66		√		
After treatment (Day 28)	P_0	$11.95x10^5$	1		√		
	P_1	$3.2x10^5$	1		√		
	P_2	$4.7x10^4$	0	√			

2.4. Work Procedures

2.4.1 Feed Production + Immunostimulants

Manufacture feed was conducted in the IBAT Feed Laboratory. The feed was prepared for each treatment as much as 3% of the total weight of fish using each pool for one feeding. Commercial/ factory feed 100% was prepared for P_0 (control) treatment, and 100% artificial feed was prepared for P_1 treatment and artificial feed + immunostimulan of the whole protein *Myxobolus koi* spores with a dose of 1μg protein / gram fish + Boster® Progol adhesive with a volume of 5ml / kg of feed as P_2,treatment, according to research conducted by [15].

Spraying for mixing P_2 treatment was stirred to make it homogeneous, then dried with wind so that there was no moisture.

2.4.2 Pathogen Attack Identification

2.4.2.1 Bacteria

1) *Observations of external clinical symptoms (external examination)*

Observations were carried out against the external clinical symptoms. Signs or symptoms of disease seen from the outside are very varied, depending on the type or intensity of the disease. The clinical symptoms of infected fish included: blood vessels evident, especially on the fins, discoloration of red spots or hemorrhagic. weak looking, always at the surface or at the bottom, not eating, breathing fast, swimming irregularly, body position tilted, upright or inverted, or blind.

2) *Observations of internal symptoms (internal examination)*

Examination of the symptoms was done by dissecting carp and looking for indications present in internal organs such as gills, gastrointestinal tract, liver and kidneys.

3) *Isolation and purification of bacteria*

Isolation of bacteria from samples of carp was done through wound or aseptically from organ gills, kidney, liver and gastrointestinal tract using a sterile loop on TSA media with the addition of NaCl 2.5%, 2.5% NaCl GSP media and media TCBS.

Incubation was carried out at room temperature for 18-24 hours. Purification bacteria were performed by separating the bacteria that had a different colony morphology. Observations included form of colonies, colony morphology, elevation, shape, edge and color of the colony. Observations of colonies of bacteria included bacterial cell shape and Gram. Pure bacterial isolates were then stored in order to be tilted by sterile liquid paraffin.

4) *Bacterial examination*

Examination pathogens that cause disease in carp
include colony morphology, bacterial cell morphology and biochemical testing.

2.4.2.2 Myxobolus koi Parasites

Identification of parasites was done by parasite examination covering the outer parts. According to [19], examination of parasites in the outer parts is made by gills scrapings and skin mounting methods. Observations of parasites was carried out using a binoculars microscope and identification of parasites was made using *Parasites and Disease of Fish Cultured in the Tropics*, a guidebook [19]. Data from the research will be presented in tables, and analyzed

descriptively. After identification, then the degree of infestation and intensity of *Myxobolus koi* was measured. In determining the degree of infestation on *Myxobolus koi* , it is considered low when it is found 1-4 nodules, the degree of infestation is medium when 5-8 nodules are present, and heavy when there are more than eight nodules
[20]. Meanwhile, the intensity is calculated using the following formula:

$$\text{Intensity} = \frac{\text{Total } \textit{Myxobolus koi} \text{ infesting}}{\text{Total } \textit{Myxobolus koi} \text{ infested fish}}$$

2.4.3 The IgM concentration determination with indirect ELISA

IgM concentration derived from the blood serum of the three treatments carp was determined by indirect method with an ELISA Micro titer plate used as 96 micro titer plate wells. Each pitting condition had 100mL antigen solution with a concentration of 10ug / ml in coating buffer and was incubated at 40°C for one night. Microtiter plates were washed once with washing buffer in each pitting then added 100mL of fish blood serum for three treatments and diluted with PBS. Fish blood serum was diluted glow, and micro plates were incubated at 37°C for one hour followed by washing three times with wash buffer. Each of the wells was filled with 150 mL of a solution of conjugate anti-IgM fish. The conjugate was diluted with PBS in the ratio of 1: 4000. The micro titer plates were incubated at 37°C for one hour and then washed three times with wash buffer. Each of the wells was added the substrate solution pnpp 150mL. Micro titer plate was incubated in room temperature for five minutes. The antibody titer was read by spectrophotometer for ELISA at a wavelength of 405nm [21].

IV. Results and Discussion

3.1 Results

3.1.1 Pathogen Attacking

Identification of carp invading pathogens was carried out before (day 1) and after given treatment (day 28). Pathogens examined included parasites *(Myxobolus koi)* and bacteria *(Aeromonas hydrophilla).*Examination included colony morphology of bacteria, bacterial cell morphology and biochemical testing. Examination was made of previous parasite identification and then measured the intensity. Total results of the pathogens that attack carp can be seen in Table 1.

On the first day of observation or prior to treatment, the bacteria *(Aeromonas hidrophyla)* showed no significant difference in all treatments. The same thing happened on the intensity of parasite examination *(Myxobolus koi)* and the degree of infestation, which indicates to three treatments which experienced a mild infestation.

On the 28th day of observation, or after treatment, the bacteria *(Aeromonas hidrophyla)* and the intensity of the parasite *(Myxobolus koi)* at P_2 showed significant difference in P_0 and P_1 treatment. P_0 treatment was not significantly different from the P_1 treatment. The degree of infestation P_2 did not show a parasitic infestation. Meanwhile, P_0 and P_1 treatment had a mild infestation.

3.1.2 Concentration of IgM as an Immune Response with indirect ELISA

Measurement of the concentration of immunoglobulin M (IgM)was on days 1, 7, 14 and 28 in the research. In this research, sampling for IgM test was taken from fish blood serum. IgM concentration measurements were performed using *indirect* ELISA method. On the first day of observation, the concentration of Immunoglobulin M (IgM) P_0 treatment, P_1 and P_2 was not significantly different between treatments. The highest concentration was indicated in the treatment of P_2.

On the seventh day of observation, the concentration of immunoglobulin M (IgM) P_2 showed significant difference from the treatment of P_0 and P_1. The concentration of immunoglobulin M (IgM) P_2 treatment increased from the first day of observation.

On the 14th day of observation, the concentration of immunoglobulin M (IgM) P_2 treatment increased from the first and seventh days of observation. P_2 treatment showed significantly difference from the treatment of P_0 and P_1.

On the 28th day of observation, the concentration of immunoglobulin M (IgM) P_2 showed significant difference from the treatment of P_0 and P_1. The concentration of immunoglobulin M (IgM) treatment of P_2 decreased from the 14th day. Treatment P_0 was not significantly different from the treatment of P_1. Average chart IgM concentrations during the research can be seen in Fig 1.

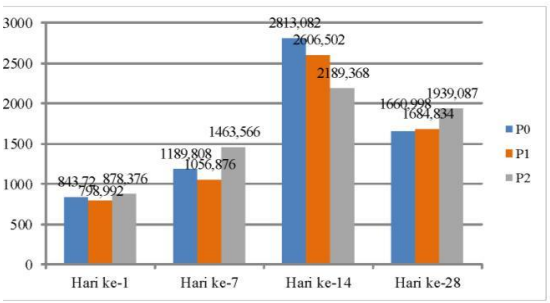

Fig 1. Average chart carp IgM concentrations during the research.

3.1.3 Water Quality

Water quality is an important factor for successful cultivation. The average yield checks of water quality parameters during the research are presented in Table 2.

During the research, water changed was changed once a day, every morning , by as much as 25% of the total water volume. The quality of water in this research ranged in the normal conditions for the maintenance of carp [22].

TABLE 2. THE AVERAGE YIELD MAINTENANCE INSPECTION OF WATER QUALITY PARAMETERS OF CARP DURING THE RESEARCH

Parameter	Average water quality during 30-day			Normal range value (Flajšhans & Hulata, 2007)
	P0 (control)	P1	P2	
Temperature (˚C)	29.5	30	29	27-30
pH	7.2	7.8	7.5	6.5 to 9
DO (mg/l)	5.6	6.6	5.9	>3-7
Ammonia (mg/l)	0,5	0,5	0, 5	0.5 to 0.52

3.2 Discussion

A material can be used as an immunostimulant if it has immunogenic properties. [14] managed to pull through the isolation, identification and characterization of surface *Myxobolus koi* glycoproteins as an immunogenic antigen for antibody production. [15] also successfully characterized each protein size of the whole protein from *Myxobolus koi* spore using SDS-PAGE (sodium dodecyl sulfate polyacrilamide gel electrophoresis). A.

The results of SDS-PAGE of whole protein *Myxobolus koi* spores were obtained for a protein depicted in the form of ribbons on SDS-PAGE gel and obtained 6-band protein with molecular weight 68.1 kDa;, 38.5 kDa, 25.6 kDa, 23 kDa, 21.7 kDa and 18.9 kDa. These results showed that the protein was found have a high molecular weight. An immunogenic protein is a protein that has a molecular weight of 20000-100000 Dalton [16]. Good immunogen molekuer has a size of <100 kilodalton. [17] suggested that a molecule has an immunogen characteristic when having a molecular weight of more than 10kDa.

Whole protein of *Myxobolus koi* spores can also boost the immune response and survival rate of 10% of koi fish to 86% and can increase defense against infection of *Myxobolus koi* [18].

As an immunostimulant substance, whole protein *Myxobolus koi* spores can enhance non-specific immune response, specifically by increasing the phagocytic cells. Phagocytic cells function by phagocytic activity against foreign objects that enter the host's body.

Immunostimulants will stimulate macrophages to produce cytokines, such as interleukin, which will activate the lymphocytes which are then split into B lymphocytes and T lymphocytes. T lymphocytes in non-specific response would produce interferon that can activate performance macrophages through phagocytosis mechanism in the face of parasites, bacteria, viruses and foreign particles that are considered as an antigen [10]. Whereas, in the specific immune response, cytokines, including IL-4, IL-5 and IL-13 with the Th cells, will activate B lymphocytes and T lymphocytes [11].

B lymphocytes will establish an antibody mechanism called immunoglobulin (Ig).

The results showed that carp were given an immunostimulant material of whole protein from *Myxobolus koi* spores capable of suppressing and lowering the pathogen attacks originating from bacteria (*Aeromonas hidrophylla*) and parasites (*Myxobolus koi)*.

This can be seen in the P_2 treatment day 28 following r artificial feed + immunostimulant whole protein from *Myxobolus koi* spores with a dose of 1µg protein /gram fish + booster® Progol adhesive volume of 5ml/kg of feed. *Aeromonas hidrophylla* attacks decreased from those before administration of treatment (day 1) of $1.3x10^6$ (CFU/g) be $4.7x10^4$ (CFU/g) after feeding artificial + immunostimulant whole protein from *Myxobolus koi* spore (day 28). Decline occurred in parasite intensity. Also, in *Myxobolus koi* checked before being given treatment (day 1) of 1.66, after feeding artificial + immunostimulant whole protein *Myxobolus koi* spore (day 28) the intensity of parasite did not exist. This is in accordance with the opinion of [10] that the introduction of immunostimulant into the body stimulates macrophages to produce cytokines, such as interleukin, which will activate the lymphocytes, which are then split into B lymphocytes and T lymphocytes. In response to non-specific, T lymphocytes will produce interferon, which is able to activate macrophage performance through the mechanism of phagocytosis in the face of bacteria, parasites, viruses and foreign particles, which are considered as antigens.

Blood is a tool used as a diagnosis of health status in an organism, including fish. Blood will undergo serious changes when exposed to infectious diseases. Blood tests can also be an indicator of the severity of a disease experienced by fish
[23]. Fish blood is composed of plasma fluid and blood cells consisting of red blood cells (erythrocytes), white blood cells (leukocytes) and blood clots (platelets). White blood cells
(leukocytes) in fish are part of the fish's body defense system. Factors that affect the number of leukocytes are the condition and health of the fish [24].

The fish body has a mutually supportive defense system to fight against incoming foreign bodies, both specific and non-specific immune systems. If the non-specific immune system is unable to eliminate the disease agent, immunoglobulin, as a specific defense mechanism, will help the body system as a second defense [25]. Fish have two primary lymphoid organs; the anterior portion of the kidney (analogous to the bone marrow in mammals) and the thymus. The kidneys function as secondary lymphoid organs along with the lymph [26]. The presence of incoming antigen stimulation makes cells in lymph produce an antibody / imuoglobulin mechanism. In this study, the results of IgM concentrations were tested using the ELISA (Enzyme-Linked Immunosorbent Assay) method. ELISA is a quantitative and qualitative test with the use of enzymes attached to specific antibodies [27]. [21] added that ELISA techniques are based on the use of antigen and antibody labeled as enzymes. The results of the ELISA test in Table 5.4 show that self-immunostimulant added feed of whole protein *Myxobolus koi* spores can induce the formation of antibodies in carp as seen by the results of IgM antibody concentration. The concentration of IgM on the seventh day of the study was highest in the treatment of P_2 (self-feed + immunostimulant) of (1463.566µg / ml) and significantly different from P_0 (feed / manufacturer) treatment of 1189.908µg / ml and P_1 (Feed independent) of 1056.876µg / ml.

Similarly, on day 28 the concentration of immunoglobulin M (IgM) P_2 (self feed + Immunostimulant) showed significant difference with treatment P_0 (feed manufacturer / control) and P_1 (self feed). This is because the whole protein spur increased lymphocyte proliferation. This is in accordance with the claims of [28] that the content of foreign substances in the body will be recognized and potentially increase the proliferation of lymphocytes, increase the number of T cells and B cells and increase the activity of IL-2. With the increase in immunoglobulin, IgM antibodies strive against the antigen contained in the fish body. Increased lymphocytes will either divide or differentiate into plasma cells, and plasma cells will produce and release thousands of antibodies that later enter the circulation.

4. Conclusion

The conclusion is that whole protein from *Myxobolus koi* spores can be used as an immunostimulant.

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