Response of kutuklin hemagglutinin protein adhesion in koi fish (*Cyprinus carpio*) infected by *Myxobolus* sp.

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Abstract. Koi fish (*Cyprinus carpio*) cultivation is quite easy to maintain if it can look carefully at fish health well. The difficulty in maintenance due to parasitic attacks is *Myxobolus* sp., and this parasite is capable of causing death in fish. Treatment of parasites attached to koi fish can be done by giving chemicals such as Pyrethrum. This research aims to determine the reaction of kutuklin hemagglutination adhesin to koi fish infected by *Myxobolus* sp. The method used is an explorative and descriptive by conducting an In-Vivo test on koi fish. The treatment is, healthy fish without drug as a control, healthy fish with a drug dose of 1 μl/g feed, and fish were infected with a drug dose of 1 μl/g. Observation is done by taking fish blood plasma in each treatment. The results showed that kutuklin was able to contribute to treating *Myxobolus* sp. However, it cannot restore such a healthy state. This is indicated by high (up to 1/1024 dilution) in the hemagglutination (HA) test of koi fish blood plasma. So, that kutuklin can be used as an immune suppressor (immunosuppressor).

1. Introduction

One of the successes in the production of koi fish cultivation can be seen from the quality and health of fish itself. However, a problem that often appears in fish maintenance activities is the disease. The disease that usually attacks koi fish (*Cyprinus carpio*), especially in the larval phase, is from the parasite group, *Myxobolus* sp., particularly *Myxobolus koi*. Based on reports from koi fish farmers in Blitar, from 2009 to 2012, this disease could attack 3-5 cm koi with a mortality rate of 90% [1]. *M. koi* infect the gills, and in the lamella, this parasite grows by forming cysts in the form of a collection of reddish-white nodules [2]. Thus, increasing the number of buds that develop it will disrupt the process of respiration because the nodules cover the gills (operculum). A severe infection will cause the death of fish. The effort to control parasites can be made by using chemicals, including botanical insecticides such as pyrethrum. Pyrethroid insecticide that can be used is deltamethrin. Deltamethrin is the 4th generation group of pyrethroid that is easily degraded in soil and cannot be traced to microflora and microfauna. This insecticide is lipophilic and generally difficult to dissolve in water. In the aquatic environment, these compounds will be very quickly partitioned into sediments and biota [3].

Kutuklin is a type of practical medicine that is often used to treat parasites by traditional koi fish farmers in the Nglegok District, Blitar Regency, East Java. The maximum limit of deltamethrin residues according to the Committee for veterinary Medicinal Products [4], which is allowed in finfish based on Annex III of Council Regulation (EEC) No. 2377/90 is 10 μg/kg. In general, the provision of kutuklin by farmers is by mixing it with feed. The absorption rate of deltamethrin orally is until 75% and can be excreted quickly through urine and faeces (Standing Committee on Biocidal Products) [5]. The effect that can be shown by fish due to kutuklin treatment is the formation of adaptive immune responses in fish. This response is characterized by the creation of IGM and the establishment of
hemagglutinin proteins in koi fish. However, in the long-term, there will be environmental risks in the form of antibiotic residues. As an alternative, the development of fish body endurance needs to be done by immunostimulation (immunization-vaccination) [6]. Hemagglutination is the process of clumping red blood cells. A hemagglutination test is carried out to measure the number of antigen titers. A hematological examination is needed to determine the status of erythrocytes and leukocytes in fish blood that responds to the treatment. The purpose of this study was to assess the response of the hemagglutination test results against fish infected by *Myxobolus* sp. Based on the background stated, it is necessary to develop appropriate and accurate prevention methods through research by analyzing the immune response of koi fish through a hemagglutination test.

2. Materials and methods

The method used in this study is exploratory and descriptive based on observations and responses that occur through the In-Vivo test on koi fish. Treatment of kutuklin was given to the fish by mixing it with feed for 14 days in a controlled maintenance shelter. The treatments offered include (A) healthy fish, (B) infected fish, (C) infected fish with 1 μl/g kutuklin treatment, and (D) infected fish with 1.5 μl/g kutuklin treatment. Furthermore, blood was taken from live fish as samples of erythrocytes, leukocytes, and hemagglutination serological tests [7]. This blood sampling was carried out using a 0.5 mL syringe that has previously been added with Ethylene Diamine Tetra Acetic Acid (EDTA) at a dose of 1.50 ± 0.25 mg/mL of blood. The fish was placed with the head on the left side. Blood samples were taken using a syringe that pierced the muscles in the midline of the body behind the anal fin.

2.1 Erythrocyte calculation

The procedure of erythrocytes calculation was measured according to Blaxhall and Daisley [7]. First, blood was sucked with a pipette containing red stirrer grains to scale 1 (a pipette to measure red blood cells count), then hayem’s solution was added to scale 11. The stirring of the blood in a pipette was done by swinging a hand holding a pipette like forming a number, precisely number 8, for 3-5 minutes so that the blood was mixed evenly. The first two drops of the blood solution in a pipette were removed, then the drops were placed on a Neubauer hemocytometer and were covered with a glass cover. Then, erythrocytes cell count was calculated with the help of a microscope with 400x magnification. The following formula can calculate the number of erythrocytes [8]:

\[
\sum \text{erythrocytes found} \times 10^4 \text{cells/mm}^3
\]

2.2 Leukocyte calculation

The procedure of leukocytes calculation was measured according to Blaxhall and Daisley [8], blood samples were sucked with a pipette containing white stirrer grains to a scale of 0.5. Then, truk’s solution was added to scale 11. The stirring of the blood in a pipette was done by swinging a hand holding a pipette like forming a number, specifically number 8, for 3-5 minutes so that the blood was mixed evenly (the same as stirring for the calculation of red blood cells count). After that, the first two drops of blood solution from the pipette were removed, then the solution was dropped to the haemocytometer, after which it was closed with a glass cover. The number of erythrocytes can be calculated by the following formula [8]:

\[
\sum \text{leukocytes found} \times 50 \text{cells/mm}^3
\]

2.3 Hemaggglutination test

The hemaggglutination test performed the best results according to the effects of hematological observations. The blood sample used is the erythrocytes sample in treatment C (infected fish with "kutuklin" treatment dosage 1 μl/g). The hemaggglutination test is as follows [9]. Isolate the blood using 1 ml 26 GX 1/2 (Therumo) syringe that was previously moistened with 10% EDTA solution. Add PBS, then homogenize and centrifuged at 3,500 rpm for 10 minutes. Dilute plasma using PBS by using (1: 200) for use in HA testing. Use a microplate V-bottom 96 well for HA testing. It is inserting *Myxobolus* sp. 50 μl to be diluted using a lysis buffer solution. Review all diluted plasma into each V, homogenized, and discuss the response reaction after 20 minutes. Positive results are characterized by the absence of plasma sedimentation (in the form of dots) in the microplate V.
3. Results and discussion

3.1 Erythrocytes Calculation

Total erythrocyte observation in fish blood can be used to determine the physiological and health conditions of fish. This is indicated if the total erythrocytes are too high, then the fish can be stated to be under stress [10]. Based on observations and calculation of koi erythrocytes (C. carpio), data on the total number of erythrocytes that have been observed can be seen in Figure 1.

![Figure 1. Results of erythrocytes calculation. (A) normal fish, (B) infected fish, (C) doses of 1 μl/g feed and (D) doses of 1.5 μl/g feed](image)

In treatment (C), it can be seen that the results of erythrocytes obtained are close to the value (A) of regular fish, which has the lowest total erythrocytes. This shows that the treatment has erythrocyte levels that are still classified as good because the average number of erythrocytes in Teleostei fish is $1.05-3 \times 10^6$ cells/mm$^3$. Whereas in treatment (D), an increase in erythrocyte values was obtained. The more fish attacked by Myxobolus spores, the number of erythrocytes will also increase due to stressful fish and indicate the infection [11]. High erythrocytes indicate the fish are experiencing high stress [12].

3.2 Leukocytes Calculation

If the total leukocytes are below the standard value, it means that fish show symptoms of anemia [13]. Meanwhile, if the total leukocytes are above the average amount, the fish shows resistance to foreign antigens. The value of leukocytes in the blood can be used to determine the fish's body's defense system from external disturbances, including pathogens [11]. Based on observations and calculation of koi leukocytes (C. carpio), data on the total number of leukocytes that have been observed can be seen in Figure 2.
Figure 2. Results of leukocytes calculation. (A) normal fish, (B) infected fish, (C) doses of 1 μl/g feed and (D) doses of 1.5 μl/g feed

The lowest result of total leukocytes obtained in treatment (A) which is a healthy control fish without treatment obtained to 31717 (cells/mm$^3$) different from (B) which fish infected by *Myxobolus* sp. without treatment of leukocytes of 389200 (cells/mm$^3$), show an increase in the number of leukocytes is due to phagocytosis which causes the immune system of the fish to be automatically activated due to the entry of antigens into the body. Based on these results, it can be seen that the treatment (C) is the best because the number of fish leukocytes is close to the value of healthy fish leukocytes. Whereas in treatment (D), the number of leukocytes increases when it compared to (C), this is because the number of dosage of deltamethrin is too large so that it can be toxic to the body of the fish and will also affect the number of leukocytes in the fish. Changes in the total number and type of leukocytes can be used as an indicator of certain infectious diseases that occur in fish. Leukocytes in the blood component are one that functions as a specific body defense that will neutralize and destroy pathogens through phagocytosis [14]. Normal leukocyte counts in Teleostei fish range from 20,000-150,000 cells/mm$^3$ [15]. The number of leukocytes in the treatment C is still at the standard number of leukocytes of koi fish (*Cyprinus carpio*). As for other treatments showing leukocyte levels, this can indicate that fish are under stress due to infection with *Myxobolus* sp.

3.3 Hemaglutination Test

Kutuklin treatment on maintenance media is expected to increase the survival and growth of seeds so that the availability of koi fish seeds can increase. The use of kutuklin now is an alternative in overcoming problems related to fish diseases. The effect that arises in fish pertaining to the administration of kutuklin is the formation of an adaptive immune response in koi fish (*C. carpio*). One of the molecular response mechanisms formed by the fish's body in defending itself from attack by pathogenic microorganisms is through the formation of immune cells, both interleukin and MHC (Major Histocompatibility Complex). MHC (Major Histocompatibility Complex) is one of the molecules that play an essential role in the adaptive defense system [9]. Hemagglutination is the agglutination process of red blood cells by various components of microorganisms [16]. This adaptive immune response besides IGM, is also marked by the formation of hemagglutinin protein, as shown in Figure 3.

![Figure 3. The result of haemagglutination test](image)

Note:
A. PBS (50 μl) + Normal Erythrocytes
B. PBS (50 μl) + Kutuklin Erythrocytes
C. PBS (50 μl) + Myxo Protein Antigen + Kutuklin Erythrocytes

In sample A there was an erythrocyte deposition, or it could be said that the titer in the sample was 20. In sample B, there was no erythrocyte deposition, or it could be said that the titer in the example was 20. In sample C, there was a hemagglutination block in hole three or was able to agglutinate erythrocytes until dilution two times (1/4). The results of the hemagglutination test have a dot size. The results obtained indicate that the coughs in sample C are close to the figures in the normal sample (A) and are considered capable of contributing to treating *Myxobolus* sp. But cannot restore like a healthy fish.
Table 1. Results of hemagglutination test analysis

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | + | – | – | – | – | – | – | – | – | – | – | – |
| C | – | – | – | – | – | – | – | – | – | – | – | – |
| E | + | + | – | – | – | – | – | – | – | – | – | – |

Note:  
- (-) Dot is formed  
- (+) No dot is formed

4. Conclusion

Treatment of “kutuklin” at different doses can have very different effects on the amount of koi fish hematology parameters infected with *Myxobolus* sp. The dosage of 1 μl/g “kutuklin” (treatment C) obtained the number of Erythrocytes and leukocytes lowest than treatment (D), which is the most tolerable for infected fish and can be suspected that the dosage can be used to improve fish health. Haemmaglutination test from erythrocytes sample of infected fish with a dosage of 1 μl/g “kutuklin” shows that this treatment is approaching the result in the standard sample (healthy fish) and is considered capable of contributing to treating *Myxobolus* sp. Still, it cannot restore like a healthy fish. Based on the result, it can be said that the condition of koi fish pond cultivation is still in reasonably controlled circumstances based on the hemagglutinin test results showed fish infected with *Myxobolus* sp. react positively.

5. References

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