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Blood cells as biomarkers of koi (*Cyprinus carpio*) infected by *Myxobolus* sp. with treatment of diflubenzuron in the water culture of quality

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ABSTRACT

Myxobolus sp. often attacks koi (*Cyprinus carpio*). Therefore, to cure that disease using natural or chemicals treatment, such as Diflubenzuron. Haematology is one part of the fish that can be used as an indicator to determine the health level of fish. The research to determine the status of haematology after being treated by Diflubenzuron. The method used was experimental with 5 treatments and Diflubenzuron dose that used is A (0.01 mg/5 L of water), B (0.02 mg/5 L of water), and C (0.03 mg/5 L of water). Haematological parameters were erythrocytes, leukocytes, haemoglobin, and hematocrit. The data analysis using ANOVA. The results of the research on the *Myxobolus sp.* with Diflubenzuron 0.02 g treatment is the best treatment. The erythrocytes at doses A lower (2.29 x 106- 2.40 x 106 cell/mm^3) than dose B and C because higher erythrocyte proves to indicate stress. Erythrocytes obtained is directly proportional to the hematocrit value. Dose A of leukocytes also lower (125.800-131.250 cells/mm^3) than dose B and C because the higher leukocytes prove that fish will try to protect the body from foreign objects. The results of water quality were obtained, the temperature 24.9 °C; pH 7.79; dissolved oxygen 5.8 mg/L; CO_2 4.9 mg/L. Based on the results obtained values of temperature, pH, DO, and CO_2 still in optimal condition because the water quality is still controlled with sufficient aeration. In conclusion, the Diflubenzuron that given to Koi fish infected *Myxobolus sp.* is a very significant effect on haematological.

Key words : *Cyprinuscarpio, Myxobolus Sp., Diflubenzuron*

Introduction

Koi carp is a type of freshwater ornamental fish that has a high economic result, in national and international markets (Azmi and Rini, 2013). Poor water quality is one of the factors that cause stress. That's can make koi carp decreased of immune system, so they will be easily infected by parasites, virus, bacteria and fungi. One of the diseases that can attack the koi is *Myxobolus*. Generally, it can be found in larval stage of fish. The presence of *Myxobolus* nodules on the gills can disturbed respiration process. The impact of *Myxobolus* sp. depends on the level of infestation and the location of the cyst. Large infestations that occur in the gills cause tissue death (necrosis) and respiratory dysfunction (Prihartini and Alifiyah, 2017). The koi carp farmers have done a variety of ways to avoid contracting Myxobolus infection, natural treatment and chemicals. Treatment of parasites that attach to the koi can be done through the granting of chemicals such as organophosphatess, potassium permanganate and

diflubenzuron (Dimilin) (Oge, 2002). Diflubenzuron is a pesticide, which acts by disrupting the process of formation of the outer skin (exoskeleton) during the period of growth and development (Steckler and Yanong, 2012). After giving this diflubenzuron resulted blood response also changes. Blood tests conducted to confirm a diagnosis of a disease, because if the occurrence of physiological disorders in fish, it will cause changes in the components of the blood of the fish. The next thing will be able to determine the condition or health status of the fish as a biomarker. Based on the explanation above, this study was conducted to see the haematological status of Koi carp (*Cyprinus carpio*) infected with *Myxobolus* sp. after giving dimilin.

Methods

Koi carp samples and *in vivo* Treatment

The method used in this research is the experimental method, but for haematology calculation performed by ANOVA analysis and measurement of water quality to support research data. Fish sampels from farmer group koi (*Cyprinus carpio*) in Kemloko village, Nglegok, Blitar City, East Java. In this research, fish samples of 7-12 cm were included in 15 treatment tanks. This treatment used 180 fish samples which are the treatment of 12 fish. The treatment using the immersion method by dimilin for 6 hours. Adding the dimilin to the soaking tub using treatment A doses of 0.01 mg/5 L of water, treatment B 0.02 mg/5 L of water, treatment C 0.03 mg/5 L of water, treatment D (normal fish) and E (fish infected with *Myxobolus*). Furthermore, the process of taking blood and haematological status consisting of erythrocytes, leukocytes, haemoglobin, hematocrit. As well as the analysis of water quality parameters as temperature, pH, DO, and CO_2.

Haematological Response Analysis

Observations of measured hematologic responses consisted of erythrocytes, leukocytes, haemoglobin and hematocrit. Blood samples were taken once at the end of the study. The method of blood sampling in fish was carried out according to Svobodova *et al.* 2006. 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.

Erythrocyte Calculation

The procedure for calculating erythrocytes count was measured according to Blaxhall and Daisley (1973), firstly, 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, specifically 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 haemocytometer and were covered with a glass cover. Then red blood cells count was calculated with the help of a microscope with 400x magnification. Red blood cells (erythrocytes) count can be calculated by the following formula. According to Blaxhall and Daisley (1973):

$$\Sigma \text{ erythrocytes found} \times 10^4 \text{ cells/mm}^3 \quad .. (1)$$

(Blaxhall and Daisley, 1973)

Leukocyte Calculation

The procedure for calculating leukocyte count was measured according to Blaxhall and Daisley (1973), 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 white blood cells (leukocytes) count can be calculated by the following formula. According to Blaxhall and Daisley (1973):

$$\Sigma \text{ leukocytes found} \times 50 \text{ cells/mm}^3 \quad .. (2)$$

(Blaxhall and Daisley, 1973)

Hemoglobin Calculation

Measurement of haemoglobin levels was done by sampling the blood with a Sahli pipette up to a scale of 20 mm^3 or on a scale of 0.2 mL. Then the tip of the pipette was cleaned with tissue paper. The blood in the pipette was transferred into a Hb-meter filled

with 0.1 N HCl to a scale of 10 (red). The blood was then stirred with a stirring rod for 3-5 minutes. The distilled water was added to the tube until the colour of the blood was like the colour of the standard solution present in the Hb-meter. The haemoglobin scale can be seen on the gr % (yellow) pathway scale, which meant the amount of haemoglobin in grams per 100 mL of blood.

Hematocrit Calculation

The examination of hematocrit values was performed using the microhematocrit method. Microhydematocrit with heparin was inserted into the collected blood sample until the blood-filled approximately three quarters (3/4) of the capillary tube. In addition, one end of the capillary tube was blocked by sticking it in the wax stopper. Then it was centrifuged for 5 minutes using a microhematocrit centrifuge with a speed of 1,500 rpm. In addition, the results were read using a hematocrit reader and were expressed in % (Vonti, 2008).

Results and Discussion

A sampling of erythrocytes was carried on day 14 after the koi carp (*Cyprinus carpio*) is treated on day 3, day 7, and day 10. Observations on the red blood cells (erythrocytes) normal koi carp and koi that infected *Myxobolus* sp. was observed using a microscope with a magnification of 400x.

Based on observations of the number of erythrocytes in koi fish with 5 treatments obtained the number of koi red blood cells in the treatment (E) fish infected with *Myxobolus* sp. of 2,913,333 cells / mm^3 and erythrocytes in (D) normal fish of 1,623,333 cells/mm^3. In the treatment (A) dosage of 0.01 mg, the number of erythrocytes was 2,496,667 cells/mm^3. In the treatment (B) dosage of 0.02 mg, the number of erythrocytes was 2,343,333 cells / mm^3. In treatment (C) the dosage of 0.03 mg showed erythrocyte counts of 2,730,000 cells/mm^3. This is consistent with the statement of Insivitawati *et al*. 2015, which states that the more fish attacked by *Myxobolus* sp. spores, then result of erythrocytes will increase as well due to the fish in a state of stress. Meanwhile, in normal fish (which is not affected by disease or other disorders) the result of red blood cells ranges from 1.05 - 3.00 x 10^6 cells/mm^3. Treatment of dimilin at different doses can have very different effects on the amount of erythrocyte koi fish infected with *Myxobolus* sp. In the negative control the number of erythrocytes was obtained under normal conditions and in the positive control the erythrocytes were very high. The more fish attacked by *Myxobolus* spores, the number of erythrocytes will also increase due to stressed fish.

A sampling of leukocytes carried on day 14 after the koi carp (*Cyprinus carpio*) is treated on day 3, day 7, and day 10. Observations on the white blood cell (leukocytes) were observed using a microscope with a magnification of 400x. Based on observations of the number of leukocytes in koi fish with 5 treatments obtained the white blood cell count of koi fish in the treatment (E) fish infected with *Myxobolus* sp. amounted to 197,183 cells/mm^3 and leukocytes in (D) normal fish was 69,950 cells/mm^3. In the treatment (A) dosage of 0.01 mg, the number of leukocytes was 149,167 cells/mm^3. In the treatment (B) dosage of 0.02 mg, the number of leukocytes was 128,283 cells/mm^3. In treatment (C) the dosage of 0.03 mg obtained the number of leukocytes of 163,883 cells/mm^3. Treatment of dimilin at different doses can have very different effects on the number

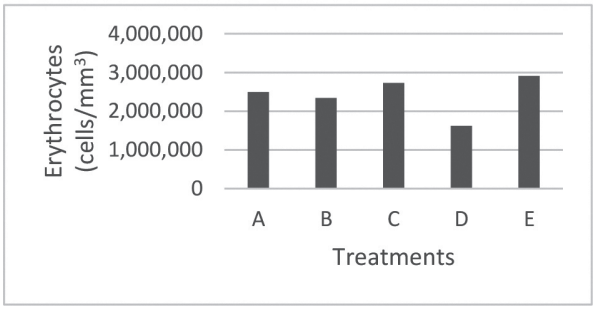

Fig. 1. Results of erythrocytes calculation. (A) doses of 0.01 mg, (B) doses of 0.02 mg, (C) doses of 0.03 mg, (D) normal fish and (E) fish infected with Myxobolus sp.

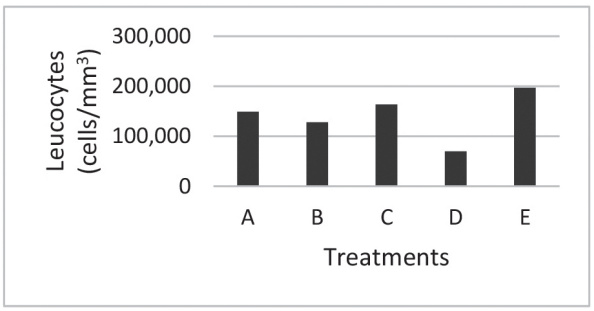

Fig. 2. Results of leukocytes calculation. (A) doses of 0.01 mg, (B) doses of 0.02 mg, (C) doses of 0.03 mg, (D) normal fish and (E) fish infected with *Myxobolus* sp.

of leukocytes of koi fish infected with *Myxobolus* sp. This is consistent with the statement of Syawal *et al.,* 2008, that an increase in the number of leukocytes can be used as a sign of infection, stress or leukaemia. The response will also arise due to several factors such as trauma, chemicals, toxins, parasites, bacteria, and viruses. In addition, Rastogi (2007), states that increasing the number of leukocytes is a signal of infection caused by certain chemicals that enter the body.

Based on observations of the amount of haemoglobin in koi fish with 5 treatments obtained the amount of koi fish haemoglobin in the treatment (E) fish infected with *Myxobolus* sp. of 3.7 gr/100 mL and haemoglobin in (D) normal fish of 7.9 gr/100 mL. In the treatment (A) dosage of 0.01 mg, the amount of haemoglobin was 5.1 gr/100 mL. In treatment (B) the dosage of 0.02 mg obtained amount of haemoglobin of 5.4 gr/100 mL. In the treatment (C) dosage of 0.03 mg, the haemoglobin concentration was 4.7 gr/100 mL. Based on the results obtained it can be concluded that the administration of dimilin with different doses can have very different effects on haemoglobin levels of koi fish infected with *Myxobolus* sp. The normal haemoglobin levels in fish ranged from 5.05 to 8.33 g/100 mL of blood (Safitri dan Suryaningsih, 2013). In the normal condition indicate that fish are able to bind oxygen well, in accordance with the main function of haemoglobin, in accordance with the function The main of haemoglobin that binds oxygen which is then used to process catabolism to produce energy and prevent blood acidity is too high (Indriastuti, 2006). and then, if the haemoglobin level is normal, so it can be seen that the protein content of the feed, vitamin

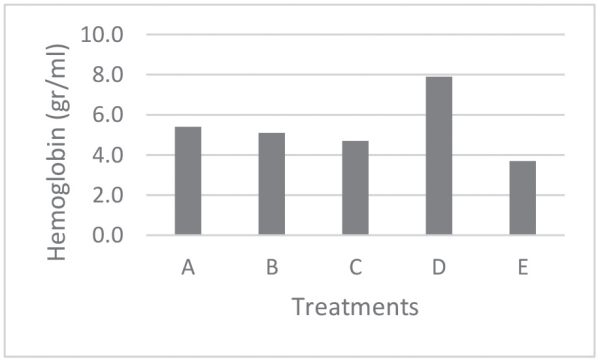

Fig. 3. Results of haemoglobin calculation. (A) doses of 0.01 mg, (B) doses of 0.02 mg, (C) doses of 0.03 mg, (D) normal fish and (E) fish infected with *Myxobolus* sp.

deficiency and poor water quality or fish have received an infection from the outside.

Based on observations of the count of hematocrit in koi fish with 5 treatments obtained the count of hematocrit koi fish in the treatment (E) fish infected with *Myxobolus* sp. by 36% and hematocrit in (D) normal fish by 17%. In the treatment (A) dosage of 0.01 mg, the amount of hematocrit was 30%. In the treatment (B) dosage of 0.02 mg obtained hematocrit amount of 28%. In treatment (C) the dosage of 0.03 mg obtained the amount of hematocrit of 34%. Based on the results obtained it can be concluded that the administration of dimilin with different doses can have very different effects on hematocrit levels of koi fish infected with *Myxobolus* sp. If the hematocrit result of the fish is less than 20%, then the fish are anaemic and if the fish hematocrit result of more than 60% indicates that the fish is experiencing stress on the environment. Decreased hematocrit result can be related to low protein content, vitamin or fish deficiency associated with infection (Mudjiutami *et al.,* 2007).

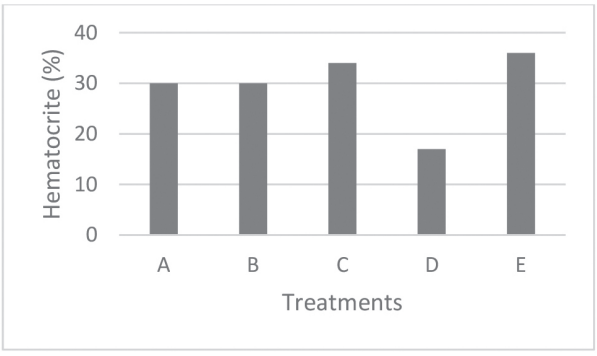

Fig. 4. Results of hematocrit calculation. (A) doses of 0.01 mg, (B) doses of 0.02 mg, (C) doses of 0.03 mg, (D) normal fish and (E) fish infected with *Myxobolus* sp.

Based on these results, the average use of the treatment dose of 0.01 mg / 5 L of diflubenzuron water is a dose that can still be tolerated based on the hematologic response. The use of a dose of 0.01 mg/5 L of diflubenzuron water is a pretty good dose among others. This can be due to the nature of diflubenzuron, including quite hard chemical compounds. thus, high dose use is not recommended.

Temperature is one of the parameters that determine the survival of fish. Temperature can affect the respiratory system, growth and reproduction in fish. In addition, a significant increase in temperature will reduce the activity of enzymes in the body

of koi fish (Yusriah and Kuswytasari, 2013). The results of temperature measurements during observations obtained an average of 24.9 ºC. Meanwhile, according to Fitriadi *et al.*, 2014, the effect of a very drastic increase in water temperature can cause a decrease in dissolved oxygen. Based on the graph shows that the distribution of daily water temperature fluctuations is almost the same, following the fluctuations in air temperature in the control room. Room temperature is in the range of 23-34.9ºC (Saputra, 2011).

Table 1. Water quality parameters during experiment

Temperature	24.9^{0}C
pH	7.79
DO (Dissolved Oxygen)	5.8 mg/L
CO_2 (Carbon dioxide)	4.9 mg/L

Measurement of pH in waters is needed because pH is an indicator to determine the concentration of hydrogen ions in the waters. Measurement of pH in the maintenance ponds obtained results of 7.79. The results of the pH measurement are still quite good. This is consistent with the statement of Sutiana *et al*. (2017), pH 7-7.9 tends to be neutral and good for koi fish growth. Changes in the pH value in the pond maintenance is strongly influenced by the process of respiration. Water that contains a lot of carbon dioxide has a pH value of less than 7 or is acidic. A decrease in pH can also occur because of the remaining metabolism of fish (Saputra, 2011).

Dissolved oxygen is one of the factors that influence in fish maintenance related to fish metabolism. Measurement of DO in maintenance ponds obtained results of 5.8 mg/L and is still relatively good. This is in accordance with Saptarini (2010), that the optimal temperature for fish development is 5 mg/L. If the oxygen level in the water is very low, it will cause death in carp. Therefore, the oxygen content in the culture tank needs to be maintained at optimal conditions with aerator added. In addition, the problem of low oxygen concentration can also be reduced through feeding arrangements. Excess feeding is usually followed by a decay process that utilizes oxygen from water and the result is inorganic material (Saputra, 2011).

Carbon dioxide comes from the process of overhaul of organic material by microorganisms and is the result of the process of respiration of aquatic organisms at night. Measurement of CO_2 in rearing ponds obtained results of 4.9 mg/L and can still be tolerated for fish growth. This is in accordance with the statement of Pramleonita *et al.* (2018), that a good CO_2 content for fish is at least 4 mg/L. Carbon dioxide (CO_2) in water in the form of gas and the measured value in water are usually free CO_2. The levels of free 10 mg/L kabon dioxide can still be tolerated by aquatic organisms to grow as long as it is balanced with sufficient oxygen levels. Most aquatic organisms can survive until free carbon dioxide reaches 60 mg/L (Kadarini *et al.,* 2015).

Conclusion

Based on the analysis of the haematological status of koi fish (*Cyprinus carpio*) infected with *Myxobolus* sp. by giving dimilin with different doses, it can be concluded that the haematological status in the treatment of koi affected by *Myxobolus* sp. by giving diflubenzuron, at a dose of 0.02 mg better than other doses. The cause of changes in the haematological status of koi fish (*Cyprinus carpio*) itself such as in red blood cells, white blood cells, etc. due to an infection in koi fish, namely *Myxobolus* sp.

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