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Short Communication

Molecular Expressions: Scanning Electron Microscope (SEM) in Gills of Common Carp (*Cyprinus carpio*) Infected Myxobolus sp.

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Abstract

The biggest problem that is often considered to be an obstacle to Common carp culture is the emergence of disease attacks. One type of disease that often attacks the seeds of *Cyprinus carpio* is *Myxobolus* (a systemic parasite that can cause harm to fish farming). The aim of this study was to determine the molecular expression through the smear test on *C. carpio* gills, to determine the image of the gill organs of *C. carpio* using the SEM test, and to determine the description of the spores of *Myxobolus* sp. Data were analyzed using descriptive methods. Descriptive method used was comparative descriptive comparing molecular expression in the test of gill mucosal smear of fish using a light microscope and gill organ testing using Scanning Electron Microscope (SEM) on *C. carpio* infected by *Myxobolus* sp. In this study, the results showed that in *C. carpio* infected with true *Myxobolus* found the presence of *Myxobolus* in the mucosal smear test and SEM test on gill tissue, but not found in the blood smear test.

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1. Introduction

Common carp (*C. carpio*) is one type of freshwater fish that has a bright prospect to be cultivated and is one type of freshwater fish that has high economic value (Wang *et al.*, 2017). Besides common carp is one of the leading commodities of freshwater fisheries because most of Indonesian people are fond of these fish, the increasing demand for fishery products for domestic and export needs has now placed the fisheries sector in an important position. Carp production also plays an important role in improving the fisheries sector in Indonesia (Maftuch and Nurin, 2020).

In aquaculture, the disease is one of the obstacles for the farmers, especially diseases that attack *C. carpio* and the cause of *C. carpio* being attacked is due to an imbalance of interactions between the fish as the host, water as the environment, and the causative agent of the disease (pathogen) (Mulyani *et al.*, 2013). As a result of an imbalance between the host, the environment, and pathogens, the fish are easily stressed, so the defense mechanisms of the fish's body are weakened and susceptible to disease (Wang *et al.*, 2017).

Disease is a major obstacle in cultivation business because it can cause harm, such as causing mass mortality which causes a decrease in production. The disease can be caused by several types of pathogens such as viruses, parasites, fungi, and bacteria that commonly attack freshwater fish. Diseases caused by bacteria show symptoms such as loss of appetite, wounds on the surface of the body, bleeding in the gills, dilated stomach filled with fluid, loose scales, and loose tail fins (Maftuch and Nurin, 2020). If surgery is performed, swelling and damage to the liver, kidneys, and spleen will be seen. This can cause death above 80% in a relatively short time. Fish disease arises due to disharmony between fish as a host of pathogens (microorganisms that cause disease) and the environment. The body's defense system can be disrupted due to environmental changes and the development of pathogens in a culture container (Maftuch *et al.*, 2017).

Diseases that attack fish can generally cause stunted growth, longer maintenance periods, high feed conversion, and low stocking density. One type of parasite that often attacks the seeds of *C. carpio* is *Myxobolus*. *Myxobolus* is a systemic parasite that can cause harm to fish farming. This parasite is often found to be a cause of disease in common carp (Oumouna and Hoffmann, 2011). Among the *Myxosporeans*, species of the genus *Myxobolusare*, so far, the foremost unremarkably found in fish, with about 856 known species throughout the world (Eiras *et al.*, 2014).

The complex development of the parasite following penetration of salmonid fish or ingestion of the spores by the oligochaete host has been described. This has enabled investigations on parasite development among different fish host species. Throughout the growth of *Myxobolus* in the skin of fish, it seems as though some of the parasites are killed, probably by a humoral response in the fish's skin. Indeed, susceptibility/ resistance to *Myxobolus* differs significantly between salmonid species (Saleh *et al.*, 2020).

Myxobolusis is the largest of the myxosporid genera. The members of this genus parasitize a variety of organs in both marine and fresh water fishes. The morphological characteristics of the spores include two polar capsules, generally piriform, located together; bilateral symmetry; polar capsules each with a polarfilament; ellipsoid, oval or rounded spores; absence of caudal projections; binucleated protoplasm with or without an iodinophorous vacuole (Velasco *et al.*, 2012). When present *Myxobolus* in sufficient numbering the gill, myxosporeans can compromise respiratory capacity, because the gill is the major respiratory organ and plays an important role in nitrogenous waste excretion and in the ionic balance (Mathews *et al.*, 2016).

In order to detect the presence of the *Myxobolus* parasite by using a smear test on the mucosa, in addition parasites will be more visible if using SEM. The use of SEM has advantages such as the shape of the parasite will be clearly seen compared to the light microscope because SEM has a magnification of more than 1000x (Shimoda and Isogai, 2012).

Therefore, the aim of this study was to determine the molecular expression through the smear test on *C. carpio* gills, to determine the image of the gill organs of *C. carpio* using the SEM test, and to determine the description of the spores of *Myxobolus* sp.

2. Materials and Methods

The research method used in this research was comparative descriptive. The purpose of the comparative descriptive method is to solve the actual problem by collecting data, compiling, classifying, analyzing, and studying the relationship that compares the results of the research that has been done. The research was conducted at the Laboratory of Parasites and Diseases, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya Malang to take blood and organ samples to be tested. The gill mucosa testing of fish was carried out at the Parasite and Disease Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University. SEM testing was done at the Bioscience Laboratory, Brawijaya University.

2.1 Research Preparation

2.1.1 Fish Aquarium Preparation

Aquarium with a size of 80cm x 50cm x 40cm was prepared. In the aquarium, an aerator was installed as a source of oxygen to be used by fish. After the aerator was installed, the aquarium was filled with water as much as 70% of the aquarium volume so that the water was not excessive.

2.1.2 C. carpio preparation

The fish used were from farmers in Ngelegok, Blitar, East Java, Indonesia. The size of the fish used ranges from 4-6 cm, because the average fish suspected in Ngelegok, Blitar of being exposed to *Myxobolus* are fish with a size of 4-6 cm. Fish was acclimatized for approximately 3-5 days to prevent fish from being stressed.

2.1.3 Research Implementation

a. Mucosal Smear Test

In the mucosal smear test, the samples were obtained from the gills of fish by dissecting and removing their organs. The organs obtained are applied to an object glass using tweezers until they are even. Object glass with mucosal samples were dried, then washed using methanol for 10 minutes, then added 9-10 drops of Giemsa, and left for 5 minutes, then washed with distilled water and allowed to dry, after that observed under a microscope (Jasmanindar, 2011).

Blood smear testing used blood samples of *C. carpio* infected with *Myxobolus* (fish size ranged from 5 to 7 cm). Blood was drawn from the tail near the literal linea of the fish. Blood sampling used a 2 ml syringe. Blood samples that had been obtained were dripped on an object glass as much as 1-2 drops by removing the needle on the syringe (Syafar *et al*., 2017).

b. Scanning Electron Microscope (SEM) Test

Histologic analysis of fish organs, gill, muscle, intestine, liver, and kidney preparation was based on Stoskopf's method. The samples experienced fixation process for 24 h using 10% formalin solution. The next steps were dehydrating, clearing, paraffin infiltration, blocking, cutting/sectioning, staining, and mounting of the samples. The histology analysis followed a method by Aydin and Ciltas (2004). Light microscope was used to analyze the samples. The histopathology analysis was based on a journal by Aydin and Ciltas (2004).

2.1.4 Test Parameters

The test parameters observed in this study were samples of *C. carpio* gills infected by *Myxobulus*. Then observed using SEM.

2.15 Data Analysis

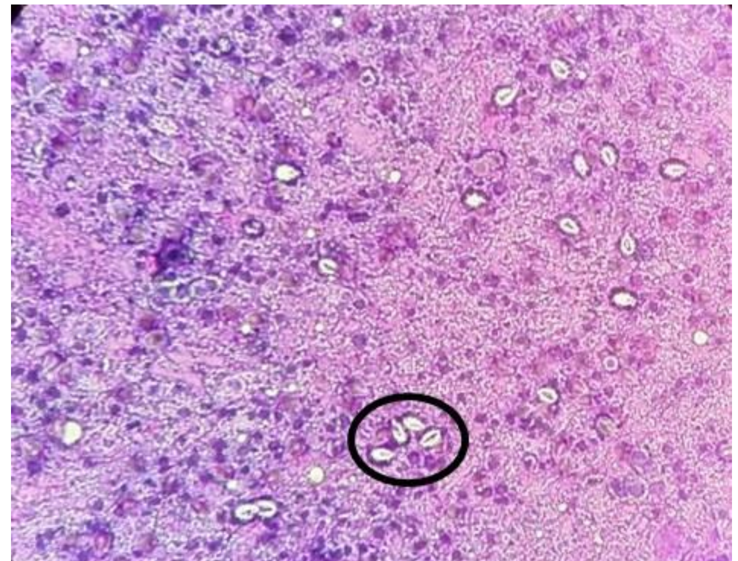

Figure 1. Testing of Fish Gills Mucosa Spread with Giemsa Staining with 400x magnification

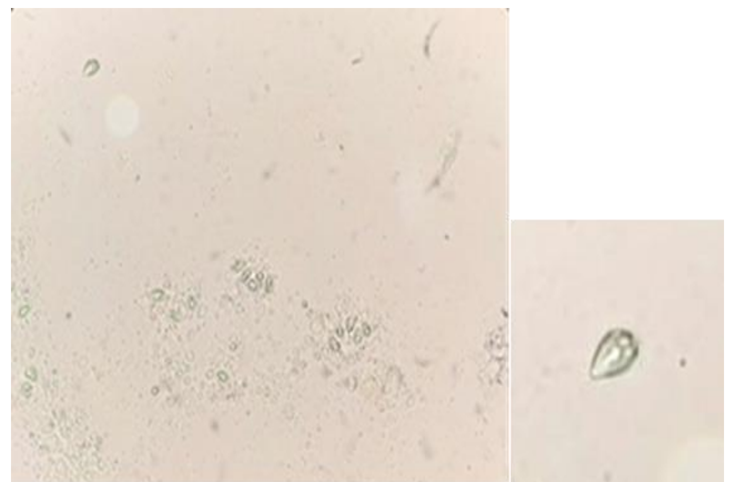

Figure 2. Testing of gill mucosa spread without Giemsa staining with 400x magnification

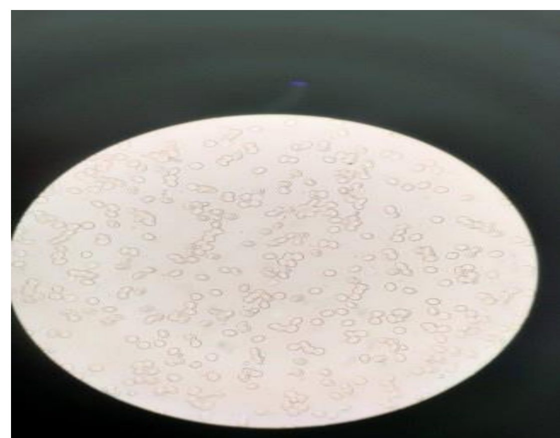

Figure 3. Blood Smear Testing without Giemsa Staining with 400x magnification

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Data were analyzed using descriptive methods. Descriptive method used was comparative descriptive comparing molecular expression in the test of gill mucosal smear of fish using a light microscope and gill organ testing using SEM on *C. carpio* infected by *Myxobolus* sp.

3. Results and Discussion

3.1 Gills Mucosal Test Analysis

The mucosal smear test is obtained from the mucous or mucosal gills of *C. carpio* infected with *Myxobolus* (Figure 1 and Figure 2). *Myxobolus* is imperfectly round with one side tapered or can be called an oval with a line in the middle. This shape resembles a watermelon seed that has a line in the middle. This is consistent with the statement of Özak *et al.* (2012), that *Myxobolus* has a shape like a pear or a watermelon seed wrapped in a nodule containing thousands of spores.

Although fish mucus is a pote nt defensive barrier, Kallert *et al.* (2012) proved with in vitro inactivation assays that sporoplasms are able to penetrate susceptible fish host tissue without being affected by host immune factors, that are present in fish mucus (e.g. lysozyme, antimicrobial peptides or complement). Studies show that mucus from both susceptible and non-susceptible fish is ineffective in breaking down actinospore sporoplasms of *Myxobolus* (Kallert *et al.*, 2012).

There is an uneven color difference in the results of mucosal smear test results caused by the use of coloring agents. In this study, the object was dyed with Giemsa so that it can be clearly observed under a microscope. Giemsa coloring is intended for testing blood samples containing parasites.

3.2 Blood Smear Test Analysis

. In the blood smear test of *C. carpio* infected with *Myxobolus*, there was no *Myxobolus* found in the blood (Figure 3), this is in accordance with the study of El-Matbouli *et al.* (1999). The intrapiscine development of *Myxobolus* starts with the attachment of actinospores to the gill epithelium or to the fin or skin epidermis of the host. Previous histologic and electron microscopic examinations of experimentally exposed fish revealed essential details regarding the intrapiscine development of *Myxobolus*. Results of these examinations showed that after the actinospore successfully attaches to the fish, the sporoplasms of the parasite penetrate and multiply mitotically in the epithelial cells of the host. Approximately two days post exposure (p.e.), the secondary cells of sporoplasms migrate to the subcutis, then move to the cells of nerve tissues within the peripheral nervous system, and a few weeks p.e., they reach the cartilaginous tissue of central nervous system, where sporogony takes place (Nurekawati *et al.*, 2016). El-Matbouli *et al.* (1999) did not find any evidence that in blood during the route of migration within the host.

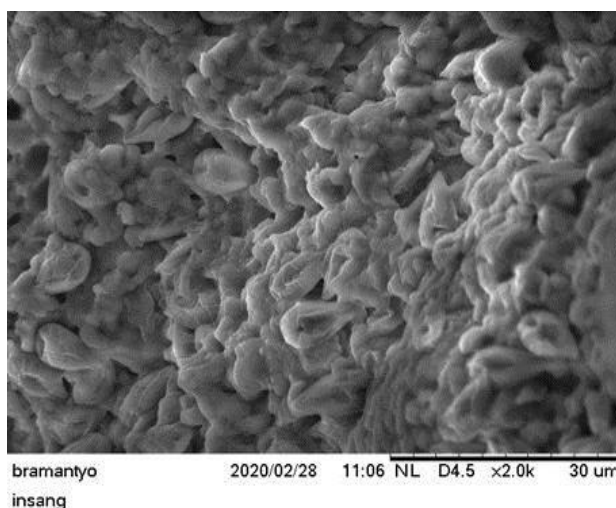

Figure 4. Testing of gill organs using SEM with 2000x magnification

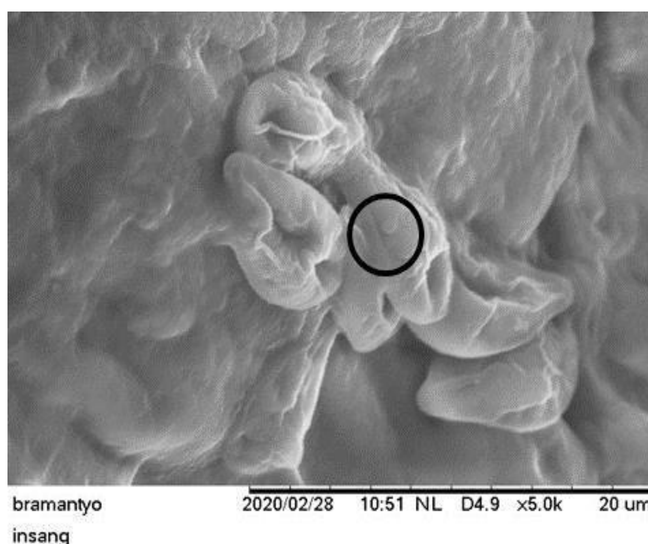

Figure 5. Testing of gill organs using SEM with 5000x magnification

However, in the research of Sipos *et al.* (2018), that *Myxobolus* enters the blood at the onset of infection and will disappear within a certain period of time, although the blood smears of all experimentally exposed fish were examined, no evidence of the parasite occurring in blood was detected and thereby, no potential contact of the parasite with immunocompetent cells, which could trigger an immune response. The findings of current study, based on qPCR analysis, supplied essential details on the host specificity of the examined histozoic *Myxobolus* sp. This study proved that Myxobolus does enter the bloodstream in the early stage

of infection. For the non-pathogenic *M. pseudodispar* lineage GER, instead of the original host common roach, the significantly highest infection intensity was detected in rudd a likely "dead end" for parasite development. Current study found impressive disparity in the infection dynamics of the two examined *Myxobolus* sp. The observed divergence in time trend may be due to their distinct pathogenicity or site preference. Current study's findings supply further evidence that host specificity is not determined during the "migration stage" of development, thus early parasite stages in blood are not host specific, similar to the fish- invading actinospores. So that in this research it can be said that when taking blood for a blood smear test, *Myxobolus* has migrated from the blood so it is not found when viewed on a microscope. The results of current study are corroborated by Chaidir *et al.* (2012). On the other hand, it is likely that the quantity of *Myxobolus* blood stages was below the detection limit of microscopy. This is not necessarily an exceptional case, certain pathogenic bacteria stains (e.g. *Mycobacterium* sp.) could not be detected microscopically using traditional techniques either. Although with PCR and isolation methods, their presence in host could be confirmed. Other myxozoans than sphaerosporids, it can be assumed that no myxozoans were seen or detected with traditional microscopy in blood. These developmental stages, if they are present in blood, do not, presumably, replicate and develop in the vascular system, they just simply use the blood stream for transport (Holzer *et al.*, 2013).

3.3 Scanning Electron Microscope (SEM) Test Analysis

In gill organ testing using SEM, *Myxobolus* is found in gills (*C. carpio*) (Figure 4 and Figure 5). The shape of *Myxobolus* resembles an imperfect ball with a cavity in the middle. This is consistent with statement of Anshary (2008) which explained that the *Myxobolus* sporoplasm has two nuclei (binucleate) with an iodinophilous vacuole located in the posterior part of the spore. *Myxobolus* has two pyriformis shaped capsules of the same size and are located in the anterior part of the spore.

Myxobolus sp. specifically attacks the gills on the arch and filaments gill. According to some studies, arch and gill filaments which are infected by *Myxobolus* sp. discrete blobs of pus (cyst) with irregular shapes (Özak *et al.*, 2012). In this study, *C. carpio* infection by *Myxobolus* was caused by external factors, the residual feed and residual sediment metabolism at the bottom of the waters, because it is left alone too long to turn into ammonia which is toxic to fish. According to the statement by Maftuch *et al.* (2017), the causes of the disease can be from the fish body itself or from outside. The internal causes of disease in fish are heredity (genetics), internal secretion, immunodeficiency, and neurological or metabolic disorders. While the external causes of disease and histology organs include pathogen attack due to environmental influences, mismanageme nt of fish feed, and antibiotic application.

4. Conclusion

In this study the results showed that in *C. carpio* infected with true *Myxobolus* found the presence of *Myxobolus* in the mucosal smear test and SEM test on gill tissue, but not found in the blood smear test.

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Authors' Contributions

All authors have contributed to the final manuscript. Maftuch; provide directions and suggestions for the manuscript. Bramantiyo; researched and collected data on this manuscript, Febi and Andhang; drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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