Molecular Detection, Histopathology, and Scanning Electron Microscopy of *Myxobolus koi* Infecting *Cyprinus carpio* Koi

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Abstract

Myxobolus koi is a type of parasite that infects many freshwater fish through myxospores, resulting in a disease called myxobolosis. This research aimed is to investigate the clinical symptoms that occur in the gills of infected Koi carp (*C. carpio*). The utilized methods in this study are molecular detection, histopathology, and Scanning Electron Microscopy (SEM). Koi carp samples that are suspected to be infected with *Myxobolus koi* show several clinical signs, such as swollen and pale-colored gills and gill covers (opercula) that do not close completely, as well as white spots that appear on the edge of the lamella. The results of this study showed that through histological observations, there were changes characterized by hyperplasia of the primary lamella cartilage that envelops parasitic cysts and the encapsulation process that surrounds the cysts by gill cartilage in infected fish; SEM showed oval and elongated spores of a size of ± 12 µm (800x). The DNA template from gill tissues in clinically infected and uninfected fish was examined by PCR testing with primers ERB 1 (Forward) and ERB 10 (Reverse); the results of electrophoresis in infected fish were detected at 2000 bp.

Keywords: *Cyprinus carpio*, Histopathology, Molecular Detection, *Myxobolus koi*, Parasite, SEM.

INTRODUCTION

Globally, the aquaculture industry in the ornamental fish sector has grown rapidly in recent decades [1,2]. The value of global trade in ornamental fish estimated at more than 15 million dollars, with an annual growth of 8%. One of these ornamental fish is koi fish, which is an ornamental strain of carp (*Cyprinus carpio*) [3,4].

With the increasing demand for trade in ornamental fish, there is an increased risk of cross-sectional spread of several pathogens. Trade in the ornamental fish industry has indirectly moved millions of fish every year around the world, in some cases resulting in myxozoan parasites moving to new areas that cause pathological changes and death among fish [5]. Myxozoan parasites are a type of cnidarian endoparasites that are widely distributed throughout the world and cause economic losses in fisheries and aquaculture. Deaths from *Myxobolus koi* have also been reported in koi that transferred from Asia to the United Kingdom and the United States [6]. *Myxobolus koi is* a type of myxozoan parasite that infects many freshwater fish, resulting in a disease called myxobolosis. Myxobolosis that infect the gills of carp in the juvenile stage results in large losses [7,8]. At present, there are 29 *Myxobolus* species known as parasites of *C. carpio*, of which 17 infect the gills [9]. Myxozoans are among the most abundant parasites in nature [10,11]. Their life cycles involve two hosts: an invertebrate, usually an annelid, and a vertebrate, usually a fish [12,13]. They affect fish species in their natural habitats but also constitute a menace for fish aquaculture [14,17].

Gill myxobolosis due to *Myxobolus koi* (Myxozoa; Myxosporea) (Kudo, 1920) of common carp (*Cyprinus carpio*) juveniles have been known to occur occasionally in Japanese carp culture and cause serious damage to fish farmers in 1978 [7]. Attacks of *Myxobolus* found in Indonesia in 1974 and 1978, which caused the death of up to 100% of koi fish, especially in the juvenile phase [18].

In 2013, *Myxobolus koi* was included in the list of Class I Quarantine of Fish Pests and Diseases in the parasite type by Ministry of Fishery and Maritime Affairs Decision Number 26/Kepmen-Kp/2013 on the Establishment of Quarantine of Fish Pest and Disease Types, Groups, Carrier Media, and Their Distribution, which means that if this kind of parasite is found in carrier media, destruction must be carried out. [19]. Fish diseases are usually difficult to control and cure; when the disease infection has taken place, it is often late for its management and the prevention of greater losses. It becomes very important to find out the health level of fish [20].

Clinical parameters and zootechnical indices have been regarded as insufficient to monitor fish health during early infection. Therefore, in addition to traditional markers (biochemistry,

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histology, morphology, and physiology), it is important to look for alternative parameters such as molecular biomarkers [21,22,23]. The genome strategy is revolutionizing scientific research in understanding fish physiology and gene evolution because it is relatively easy to isolate novel genes and homologs using public databases [24,25]. The aim of this research is to investigate the clinical symptoms that occur in the gills of Koi carp (*C. carpio*) infected by *Myxobolus* sp. The utilized methods are traditional methods (histopathology, SEM) as well as the molecular detection method, which are seen as the most representative and effective methods for checking the health level of fish.

MATERIALS AND METHODS

Sampling

Juvenile koi (*Cyprinus carpio*) of approximately 5-7 cm in length and 8-10 grams in weight were obtained from ponds in Kemloko and Kedungwaru Villages of Nglegok Sub-District in Blitar, Province of East Java. Eight samples were collected from December 2018 to January 2019. The live fish were transported to the Bioscience Laboratory of Brawijaya University and the Molecular Biology Laboratory of the Class I Fish Quarantine and Inspection Agency of Surabaya.

For Scanning Electron Microscopy and molecular identification, taking gills of the test fish and preserved in 95% ethanol solution steps was conducted according to the protocol [26,27]. Fish gills containing plasmids were fixed with Davidson solution, gradient-dehydrated, embedded in paraffin wax and sectioned at 3-5 µm, and given color with hematoxylin and eosin [28]. Ethanol-preserved plasmids were used for genomic DNA extraction according to the procedure recommended by the manufacturer [29].

Histopathology Study

Making of Histological Block Tissue

1. Specimens were fixed using Davidson solution for a maximum of 24 hours and then transferred to a 10% formalin buffer solution.
2. Samples were cut into small pieces measuring ± 1 cm. The samples were inserted into a cassette or specimen container and labeled according to the sample number.
3. These were inserted into a tissue processor container and the device was programmed accordingly.
4. The dehydration process used multilevel alcohol solutions ranging from 70%, 80% (2x replications), to 85%, each for 2 hours. Subsequent processing used pure alcohol, conducted 3 times each for two hours.
5. The clearing process used xylene, with 3 replications each for 30 minutes.
6. The embedding process utilized liquid paraffin at a temperature of 58°C, with 2 replications each for 2 hours.
7. Blocking was performed by removing the specimens from the cassette to be printed using a paraffin mold, then the block was put in the freezer for 5 minutes. The block was removed from the mold and trimmed by forming a 1.5 cm square.
8. By using a microtome, the tissue was cut to a thickness of 3-5 µ and immediately floated in a water bath filled with distilled water heated to 50°C. Paraffin tape was removed using a glass, and the object was air-dried and labeled.

Coloring of Histological Tissue

1. The tissue pieces that have been attached to the glass are arranged in a staining jar and then inserted into an incubator at 37°C for 1 hour, and the glass is dried.
2. The deproteination process was carried out using a xylene solution with 2 repetitions each for 5 minutes.
3. Dehydration was performed using alcohol starting from pure alcohol to 95% alcohol, with 2 repetitions 10 times for each dye, or ±1.
4. Rinsing was performed 10 times with distilled water.
5. Next, the coloring process used Hematoxylin dye for 2 minutes, before rinsing with running water for 5 minutes and followed by eosin staining for 10 minutes.
6. The dehydration process used alcohol starting from pure alcohol to 95% alcohol.
7. The clearing process used xylene, with 2 repetitions for 10 times or ± 1 minute.
8. For mounting, slides containing pieces of tissue were removed from the staining jar one by one and then covered with a glass cover that had been given an Entellan solution.
9. Tissue preparations were then observed under a microscope with 40x magnification for analysis.
10. Existing tissue abnormalities were observed [26,28].

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Scanning Electron Microscopy

For SEM analysis of the myxozoan parasites, 2.5% glutaraldehyde fixation solution was administered for two hours at 4 °C, followed by dehydration with ethanol and air-drying. The sample was then coated with metallic platinum in an IB-2 ion counter and examined with a ZEISS Scanning Electron Microscope at an accelerating voltage of 15.00 KV with 10.00 KX magnification. The host fish was examined and fixed in 2.5% glutaraldehyde solution for two hours at 4 °C, followed by dehydration with ethanol and rinsing with pure acetone and mixtures with amyl acetate in 3:1, 2:2, and 1:3 ratios, and finally with 100% amyl acetate. The tissues were then dried at critical point using CO_2 in a HCP:2 Critical Point Dryer (Hitachi), coated with metallic gold at accelerating voltages of 15 and 20 KV [26,27].

DNA Extraction

DNA was extracted using the Silica Extraction Kit (Gene) from tissues preserved in pure ethanol solution. Each gill and intestinal tissue sample identified as Myxobolus-infected or healthy was inserted into 1.5 mL microtubes, to which 900 µl GT Buffer is added before being mashed with a grinding pestle and centrifuged at a speed of 12000 rpm for 3 minutes. The 600 µl layers were put into new 1.5 mL microtubes, given 40 µL of silica, vortexed until homogeneous, and centrifuged at a speed of 12000 rpm for 15 seconds (no more than 20 seconds). After centrifuging, the solution was discarded and the silica pellets were washed with 500 µL GT Buffer, vortexed until a suspension was formed, and centrifuged at a speed of 12000 rpm for 15 seconds (no more than 20 seconds). The solution was discarded again and 1 ml 70% ethanol was added to rinse the silica pellets, vortexed until a suspension was formed and centrifuged at 12000 rpm for 15 seconds (no more than 20 seconds). The ethanol was removed and any remaining ethanol was taken by a micropipette. The silica pellet was re-suspended in 1 ml of ddH2O, vortexed until the silica pellet forms a suspension, incubated at 55°C for 10 minutes, homogenized with a vortex machine, and centrifuged at a speed of 12000 rpm for 2 minutes. 500 µl of the top solution was put into new microtubes and the total amplification process was performed with a PCR reaction mixture volume of 25 µL, which consists of Master Mix (KAPABiosytems, KK510) and 2 µL Forward Primer (ERB1), 2 µL Reverse Primer (ERB10), 2 µL Template DNA, and 19 µL Nuclease-free water [9,29]. The primer composition can be seen in Table 1.

Table 1. List of Primers for PCR

No	Primer	Sequence (5`-3`)	Size (bp)
1	ERB1 (F)	ACCTGGTTGATCCTGCCAG	2–20
2	ERB10 (R)	CCTCCGCAGGTTCACCTACGG	2079–2059

Source: Scientific journals [7]

Amplification

Pre-denaturation was performed at the temperature setting of 94°C for 2 minutes, followed by denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute 30 seconds for 35 cycles, and final elongation at 72°C for 5 minutes [29].

Electrophoresis

The results of DNA amplification were examined using agarose gel 1.5% soaked with 1X Tris Acetate-EDTA (TAE). The holes in the gel were filled sequentially with 100 bp DNA Ladder markers (Geneaid, Nexmark), 8 µL of amplification, and control blanks [29]. The PCR products were analyzed on 1.5% agarose gel containing 0.5 $mg.mL^{-1}$ ethidium bromide in 1X Tris Acetate-EDTA (TAE) buffer and the size was estimated by comparison with 100 bp of invitrogen DNA Ladder [30,31]. The electrophoresis process was carried out for 45 minutes with a voltage of 100 volts. The gel was placed in gel documentation, observed under UV light, and documented [29]. The tool for taking electrophoresis images is the BioDoc System Imaging tool.

RESULTS AND DISCUSSION

Fish Infection

Koi carp samples that were suspected to be infected with *Myxobolus koi* can be seen in Figure 1. The gills had swollen and become pale in color, and the gill cover (operculum) did not close completely (Fig. 1a). White spots also appeared on the edge of the lamella. Figure 1b shows the pale red gills along with spots on the gills. The observed primary and secondary lamellae are shown to have merged in the figure. One of the symptoms of *Myxobolus koi* infections in Koi carp is the appearance of parasite cysts in the gills [32]. The fish were in fair physical condition, though the gills were characterized grossly by widespread, multifocal coalescence, with 2–3 mm nodular white foci [9,33,34].

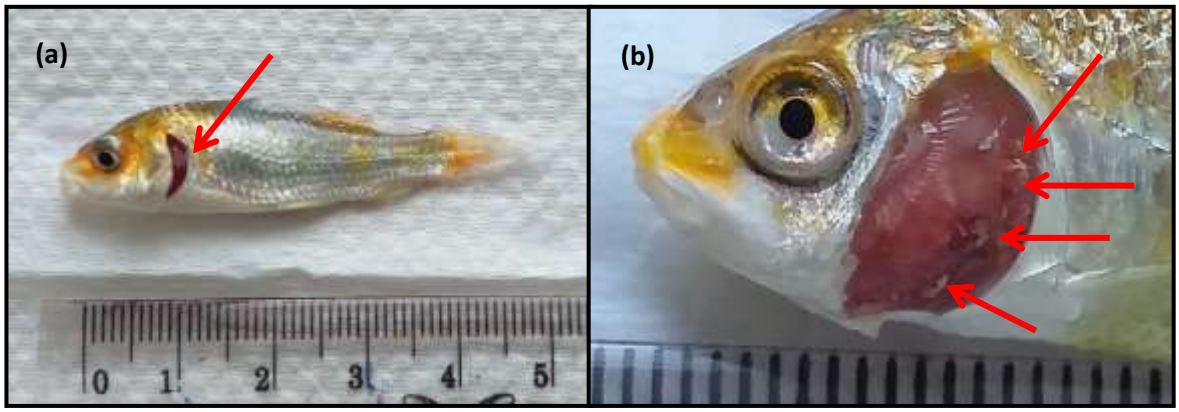

Figure 1. Koi gills infected by the *Myxobolus koi* parasite; (a) operculum did not close completely, (b) nodules in the gills

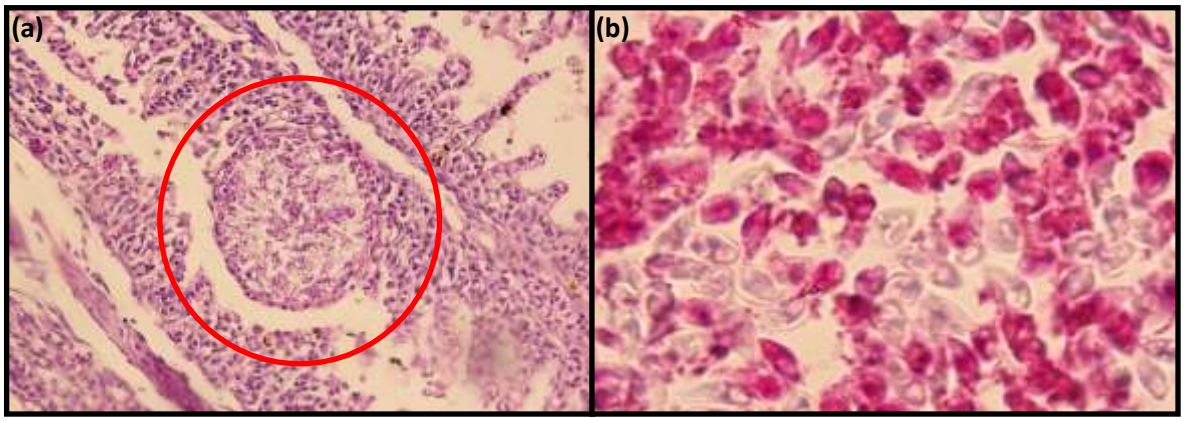

Figure 2. Histological sections of *Cyprinus carpio* gills showing location of *Myxobolus koi* tissue infection **(a)** Gills showing plasmodia in gill lamellae (100x) **(b)** *Myxobolus koi* spores in gill lamellae (1000x)

Gill Histopathology

The gills function as a breathing apparatus, but also function to regulate the exchange of salt and water between the body and the environment and play a role in removing nitrogen-containing wastes [34,35,36]. Structural damage in the gills of fish very much influences the regulation of osmosis, disrupting breathing and osmoregulation processes of fish [37,38].

Plasmodia are commonly found in gill filaments and are generally located in the middle of the gill lamella [39]. Gill histopathological studies show the number of plasmids and parasites in gills with little cell hypertrophy and epithelium [39]. Histological analysis showed that plasmodia developed in the lumen of flat capillaries and in the encapsulation process with gill filaments (Fig. 2a). *Myxobolus koi* spores in the gill lamella can be seen in Figure 2b. However, the rest of the gill lamellae and cartilage structure remained intact. There was no inflammation and swelling or infiltration of inflamed cells in one of the examined histological slides [33]. In the infected fish, there were only changes characterized by hyperplasia of the primary lamella cartilage that envelops parasitic cysts and the encapsulation process that surrounds the cysts by gill cartilage [46].

Scanning Electron Microscopy (SEM)

Some features of the myxozoan spores are more specifically disclosed through SEM. Plasmid spores that cover the gills will inhibit the process of absorption of oxygen by the gill lamella. *Myxobolus koi* specifically attacks the gills in the gill arches and filaments [32]. In Figure 3, the spores appear to break out of the gills and cover the lamella section. The spores are oval and elongated, with a size of ± 12 µm (800x).

Molecular Detection

Initially, identification with spore morphology was used for the basis of identification of myxozoan species. In the early 1990s, phenotypic features such as hosts, organs, and tissue specificity were used as important characteristics for specific identification [40,41,42]. Then,

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several studies confirmed the importance of molecular features, and in some cases, they have been found to correlate with the results of molecular data based on the 18S rDNA sequence comparison [34,43].

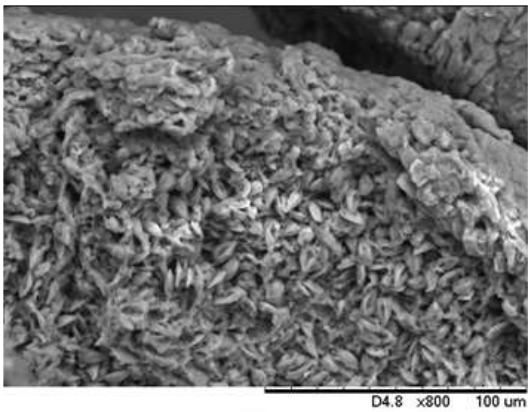

Figure 3. Scanning electron microscopy of *Myxobolus koi* spores, gill lamellae of *Cyprinus carpio* showing coverage with spores (Scale bar = 100 µm; 800x)

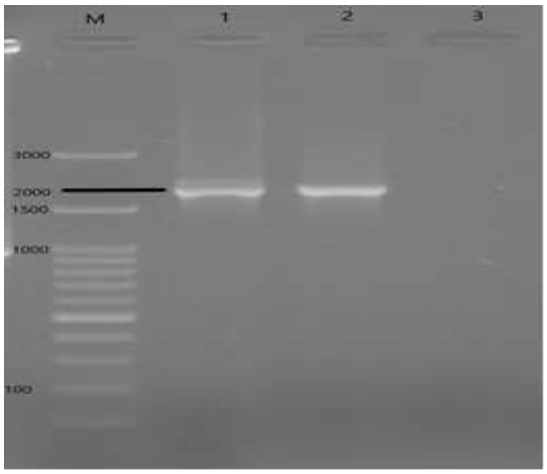

Figure 4. Agarose gel (1.5%) showing 18S r DNA gene amplification of *Myxobolus koi* DNA (2000bp) from *Cyprinus carpio*.
Description:
M: Marker/DNA ladder;
1: *Myxobolus koi* DNA (K+),
2: Positive *Myxobolus koi*,
3: Negative *Myxobolus koi*

PCR testing with specific primers in the DNA template amplification process will only display the appropriate band. DNA from gill tissue in clinically infected and uninfected fish was examined by PCR with specific primers (ERB1 Forward and ERB10 Reverse) to find the presence of the *Myxobolus koi* parasite. [44,45]. The results of electrophoresis can be detected at 2000 bp. DNA-based examination with molecular techniques has several advantages over microscopic observation because it is very specific and sensitive, and thus it can detect infections at an early stage and very mild infections [25]. In Figure 4, the appearance of the band in lane 2 shows the copy of *Myxobolus koi* genome in the target organ sample, whereas in lane 3, the band does not appear, presumably due to the absence of a copy of the *Myxobolus koi* genome or the very small volume of the genome that is undetectable with the nested PCR method.

CONCLUSION

For the present study, the myxosporean parasite *Myxobolus koi* was found to have infected the gill lamellae of *Cyprinus carpio* cultivated in Blitar, East Java. The present species was described based on morphology, SEM, histopathology, and molecular detection with specific primers. The *Myxobolus koi* parasite can be detected by positive samples marked by checking using Nested PCR, with positive control found at 2000 bp. Molecular detection with specific primers is the most effective method because it can detect early stages of infection, mild infection without clinical symptoms, and specific species that infect the host.

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