HISTOPATHOLOGY OVERVIEW OF THE LIVER OF SULAWESI MEDAKA FISH (*ORYZIAS CELEBENSIS*) INFECTED BY VIBRIO CHOLERA BACTERIA

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ABSTRACT

This study aims to determine the histopathological analysis of the liver of Sulawesi medaka fish (*Oryzias celebensis*) infected with Vibrio cholerae. The sample was divided into three groups, namely group 1 as a negative control group (G1) without treatment, group 2 namely the LD50 treatment group (G2) by giving V. cholerae bacteria 0.02 ml with a concentration of 5.73×10^5 CFU/ml into 600 ml water, group 3 as the experimental dosage treatment group (G3) with the administration of 0.2 ml V. cholerae bacteria with a concentration of 6×10^7 CFU/ml into 600 ml water. After soaking the bacteria for 5 hours, the fishes were kept in containers and maintained for seven days, and then on the 7th day samples were taken and histological examination were carried out. No symptoms were observed in any group of fishes with in 24 hours. From day 3rd fishes starts showing clinical signs like solitary swimming, weakness and swimming with the body upside down on the surface of the water and on day seven more than half of the fish population with bacterial immersion treatment is found dead. Histological examination results revealed no pathological changes in group G1 infected with V.cholerae, in group G2 there found necrosis, fat degeneration, hemorrhage and melanomacrophage. In contrast, in group G3 histological examination revealed more severe degenerations, hemorrhages and necrosis as compared to other two groups.

***Key words*:** Sulawesi medaka fish, Oryzias celebensis, *Vibrio cholerae*, liver, histopathology

Introduction

Indonesia is an archipelago country with the highest level of biodiversity after Brazil. In the field of fisheries, Indonesia has a biodiversity that lives in the sea and freshwater waters. Sulawesi Island is one of the large islands in Indonesia. There are 69 known fish species in Sulawesi, and 53 species are endemic. The complex of deep lakes, swift currents, and rivers that form Malili lakes in South Sulawesi, has at least 15 endemic species (Supriatna, 2008). Medaka fish is a group of freshwater fish from the Actinopterygii class, one of the endemic medaka fishes in South Sulawesi is the Sulawesi medaka fish (*Oryzias celebensis*) (Risnawati *et al*., 2015). Medaka fish or rice fish (*Oryzias* sp.) are widely known as model fish or experimental fish in biology (Sari *et al*., 2018).

Histopathological examination in fish can provide a picture of changes in tissue infected with pathogens and detect the presence of pathogenic components that are infective through micro observation of abnormal changes at the tissue level. The liver is the center of the body's metabolism, and the liver produces bile as an emulsifier of fat which plays an essential role in the process of digestion of food (Safratilofa, 2017). According to Helpern and Izaki (2017), *V.cholerae* is a pathogenic bacterium that causes cholera and lives in the marine and freshwater environments. So far, *V.cholerae* has been isolated from 30 species of fish (22 freshwaters; 9 marines). It can be detected in most cases in the intestines, skin, kidneys, liver, and brain tissue of fish. Based on this, it is necessary to observe pathological changes of the liver of medaka fish infected with *V. cholerae*.

Materials and Methods

This research was conducted from June to July 2019, which includes sampling from Pattunuang River, Bantimurung, Maros Regency. Maintenance of test fish and infection of Vibrio cholera bacteria were carried out in the Hatchery Laboratory of the Faculty of Marine and Fisheries Sciences. Histopathological examination were carried out in the Laboratory of Animal Clinical Diagnostics Laboratory of Hasanuddin University. The research ethics committee has approved this research for the use of animals by number 738/UN.4.6.4.S.31/PP.36/2019.

Sulawesi medaka fish are caught directly using a fish trap in the Pattunuang River, Bantimurung, Maros Regency. Minimum 100 samples were taken, each with a length of 1.672 cm - 3.721 cm and stored in plastic containers while still giving him oxygen to maintain life. After the acclimation process, samples were divided into three groups, namely group 1 (G1) as a negative control group without treatment, group (G2) as lethal dose treatment group by introducine V. cholerae 0.02 ml with a concentration of 5.73×10^5 CFU/ml into a container with 600 ml water and the experimental dosage group (G3) by giving *V. cholerae* bacteria 0.2 ml with a concentration of 6×10^7 CFU/ml into 600 ml water. After soaking for 5 hours, fish are kept in 3 liter containers (Dong *et al*., 2016). Bacteria infected fishes are kept for 2-7 days, and clinical symptoms were noted (Runft *et al*., 2014). During maintenance, dead fishes were kept in 10% formalin for preservation. On day 7 all live fishes were anesthetize by applying 2 drops of clove oil to reduce stress and pain and put in 10% formalin.

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For histopathological examination liver tissue of 3 fishes from each group were taken as suggested by Marwati *et al*. (2015). All tissue samples were fixed using 10% formaldehyde and after that processing and embedding were carried out by standard methods. Then the samples were cut using a microtome with a thickness of 4 mμ. After the deparaffinization process, samples were stained using haematoxylin eosin and observed under light microscope.

Results and Discussion

The liver is an organ that is very susceptible to the influence of chemicals and is the main target organ of the effects of toxic chemicals or toxins. The typical morphological response of the fish liver to toxicity is loss of liver glycogen and lipids (Wolf and Wolfe, 2005). The part of the dead hepatocyte cell has a shrinking core, irregular boundaries, and dark in color with a dye often used by anatomic pathologists. This process is called pycnosis. A destroyed nucleus characterizes Karyorrexis with cells losing the ability to be colored (pale) or appear vaguely hollow and disappear (Laily *et al*., 2018).

V. cholerae infects humans by releasing an enterotoxin that causes mild diarrhea to severe diarrhea, vomiting, rapid fluid loss, and can cause death in a relatively short time (Said and Marsidi, 2005). Sulawesi medaka fish (Fig. 1) were easily available fishes and out of 100 fish, only 21 fishes were able to survive after the acclimatization process. The size of all the fishes varying ranging from body lengths 1.672 cm - 3.721 cm, width 0, 23-0.883 cm and body weight 0.007 to 0.66 grams. At the time of sampling, the water conditions at the study site looked turbid, and several piles of garbage were found downstream of the river.

The bacteria used in the study were obtained from the Microbiology Laboratory of the Faculty of Medicine, Hasanuddin University, which had previously been tested for pathogenicity and then diluted using bacterial dilution calculations based on the McFarland standard to reach the required concentration of 5.73×10^5 CFU/ml and 6×10^7 CFU/ml. The bacteria were also inoculated to Thiosulfate-citrate-bile salts-sucrose agar (TBCS agar) medium as a selective media (Fig. 2). The samples were divided into three treatment groups namely: negative control group (G1), group with 0.02 ml concentration of 5.73×10^5 based on the LD 50 value into a container with 600 ml water and the group 3 with 0.2 ml *V. cholerae* with a concentration of 6×10^7 CFU/ml in 600 ml water.

Fig. 1: Medaka Sulawesi fish (Oryzias celebensis)

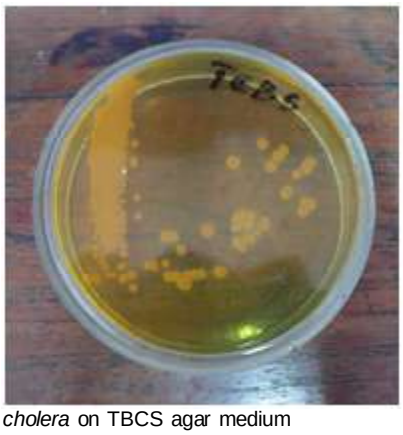

Fig. 2: *Vibrio cholera* on TBCS agar medium

Results after bacteria immersion

Clinical symptoms were observed in test fishes 24 hours after bacterial infection and no symptoms were observed in test group. From day 3rd post infection, fishes starts showing clinical signs of solitary swimming, weakening and swimming with the body upside down on the water surface as shown in Fig. 3, and on day seven more than half of the population of fishes with bacterial infection were found dead in slimy conditions. This is in agreement with Zamrud *et al*. (2017) that fishes that are attacked by *Vibrio* bacteria, often swims on surface of the water, shows lack of appetite, mucus decreases unevenly, shows solitary behavious and there are wounds on the surface of the skin. The disease can eventually cause death. Remaining fishes that are still alive are anesthetized using clove oil. Anesthetized fish immediately showed clinical symptoms of swimming as fast as possible until panting and then lost consciousness in an upside-down body condition in the water base.

Fig. 3: Fish condition after five days of bacterial infection (fish were seen swimming upside down).

Microscopic examination of the liver

Microscopic examination of the negative control group showed relatively normal appearance of the liver, clearly visible central veins and typical vacuoles. Histopathological examinatio of liver in the negative control group (G1) showed no degeneration and necrosis (score 0).These observations indicate that the negative control group's liver condition is still in a normal state. The research by Rauwaty and Morina (2012) states that in the healthy liver, the hepatocytes are visible, the nucleus is round, the location is central, the sinusoid is visible, and the central vein is evident as the center of the lobule appears round and empty.

Histopathological picture of Sulawesi medaka liver in treatment group 2 (G2) showed mild damage as there were more than 1- 20% degeneration and necrosis in part that was observed (score 1). Injuries that occur in this treatment group are

necrosis, fat degeneration, hemorrhage and melanomacrophages. This is in line with research conducted by Rauwaty and Morina (2012) and Asniatih *et al*. (2013).

Histopathological picture of Sulawesi medaka fish liver in treatment group 3 (G3) also showed mild damage as in the previous treatment group. However, in this treatment group, minor damage occurred more than 21-50% due to degeneration, hemorrhage, and necrosis than previous groups (score 2).

Table 1: Table of the average scores for hepatocyte cell damage

Group	Damage score	Note
G1	0	One field of view was not found degeneration and necrosis in the observed part
G2	1	One visual field was found to be 1-20% degeneration and necrosis in the observed part
G3	2	One field of vision found 21-50% degeneration and necrosis in the observed part

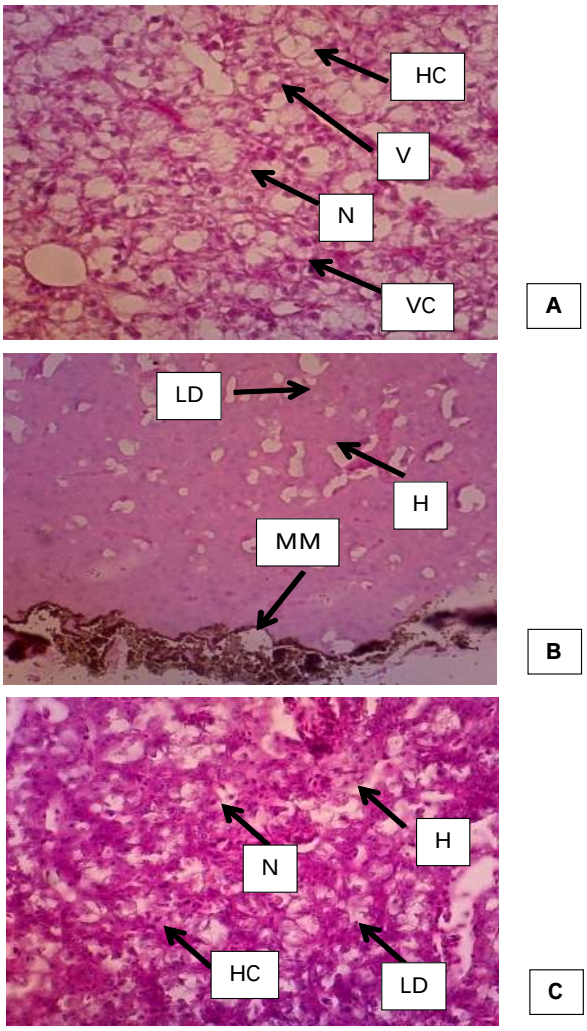

Fig. 4: Histopathological picture of Sulawesi medaka fish liver with negative control group 1 G1 (A), treatment group G2 with infectious lethal dose bacteria (B), group G3 with infecting experimental dose bacteria (C). Hepatocyte (HC) cells, Central Veins (VC), vacuoles (V), Necrosis (N), Fat Degeneration (LD), Hemorragi (H), Melanomacrofag (MM) (HE, 100x).

Qualitative data on liver damage in Sulawesi medaka fish are determined on the level of hepatocyte cell damage. The results showed that there were differences in the level of hepatocyte damage between treatments (Table 1). The negative control group (G1) showed no damage, group (G2) showed mild injury with an average score of 1, group (G3) showed mild damage with an average score of 2 due to liver degeneration and necrosis. Similar findings were also reported by Safratilofa (2017). Most of the toxic compounds enter the body through the gastrointestinal system. After absorption, the toxic material is carried by the portal vein to the liver. Blood flow that carries drugs or foreign organic compounds slowly passes through the liver cells (Siswandono and Bambang, 1995). Based on observations, changes in liver pathology of Sulawesi medaka fish that have been infected with *V. cholerae* bacteria are fat degeneration, melanomacrophages and necrosis, as seen in Fig. 4.

Degeneration is an inflammatory reaction that occurs when cell damage is not immediately deadly, and the changes are reversible (can be recovered after the source of damage is removed) that can be caused by injury due to bacteria. Degeneration is characterized by swelling of the tubular epithelium, which progresses to necrosis (Latifah *et al*., 2014). Fat degeneration is a pathological change that is also found in all treatment groups (G1, G2, and G3). Fat accumulation occurs due to decreased cellular enzyme activity resulting in the inability of non-adipose tissue to metabolize a number of lipids. This fatty degeneration occurs in marine animals due to excessive toxic amounts, especially the accumulation of nickel-metal, which decreases cellular enzyme activity, resulting in the formation of fat accumulation (Fahmi *et al*., 2019). Melanomacrophage was found in the treatment group by infecting lethal-dose bacteria (G2). Melanomacrophage can be interpreted as a solid round cell that has a varying amount of pigment found in healthy fish, but the number increases in cases of chronic stress, as shown in Figure 4.

In histopathological observations, necrosis was found in treatment groups 2 and 3. Necrosis is cells that have deficient activity and eventually experience tissue cell death resulting in loss of function in the area undergoing necrosis (Asniatih *et al*., 2013).

The *Vibrio* genus is one of the most significant pathogenic bacteria in marine fish. Species of this genus have also been isolated from freshwater environments and have been found to cause fish disease outbreaks (Rehulka *et al*., 2015).

V. cholerae shaped like a bent coma-like rod with a length of 2-4 μm. This bacterium can move very actively because it has a smooth polar flagellum (Amelia, 2005). All types of *Vibrio* members are motile (move) (Aulia, 2018) and do not form spores. In culture, there are convex colonies (convex), smooth and round, which are turbid (opaque) and granulated when irradiated (Amelia, 2005). *V. cholerae* has capsules of polysaccharides, lipopolysaccharides, pili, and produce toxins. The toxin produced by *V. cholerae* is similar to the toxin produced by *Escherichia coli.* According to Runft *et al*. (2014), fish and shellfish have long been known to play a role in cholera transmission. *V.cholerae* can contaminate the marine animals through water or through human feces contaminated water. *V. cholerae* bacteria are also found in 60% of fish species sampled in the Sea of Galilee and 50% of fish species sampled in rivers. In contrast, only one out of 44 fish species (2.3%) sampled

in the Mediterranean Sea were positive for *V. cholerae*. A report shows that *V. cholerae* can cause death in Ayu fish in Japan (Senderovich *et al*., 2010).

Pathogenic bacteria of the Vibrio group can cause diseases in the form of vibriosis and can cause death in fish, reaching more than 80% in fish farming in floating nets. Vibriosis disease attacks almost all types of fish that are cultivated and eventally can cause death (Aulia, 2018).

Conclusion

Based on the results of research conducted, it can be concluded that there is a difference between the histopathological picture of the Sulawesi medaka liver infected by the *V. cholerae* bacterium in the negative control group with the histopathology picture of the Sulawesi medaka fish treatment group with LD50 dose and group with a trial dose. The difference lies in the liver tissue of the negative control group having no tissue damage (score 0), whereas in the treatment group, the LD50 dose shows mild impairment because more than 1- 20% degeneration and necrosis were observed (score 1). In contrast, in the treatment group, minor damage occurred more than 21-50% due to degeneration, hemorrhage, and necrosis (score 2).

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