Jurnal Riset Veteriner Indonesia
Journal of the Indonesian Veterinary Research
P-ISSN: 2614-0187, E-ISSN:2615-2835
Volume 5 No. 2 (July 2021), pp. 66-79
journal.unhas.ac.id/index.php/jrvi/

The identification of gram-negative bacteria and their effects on liver histopathology of Amazon Sailfin Catfish (Pterygoplichthys pardalis) in Lapompakka and Sidenreng Lakes, Wajo.

Hairul Afzan1[1], Andi Magfira Satya Apada[2*], Wa Ode Santa Monica[2], Andi Rianti Rhasinta Alifha R[3]

[1]Student of Veterinary Medicine Hasanuddin University. Jl. Perintis Kemerdekaan kampus Tamalanrea Km. 10 Makassar, Indonesia
[2]Lecturer of Veterinary Medicine Hasanuddin University, Jl. Perintis Kemerdekaan kampus Tamalanrea Km. 10 Makassar, Indonesia
[3]Laboratory Staff of Veterinary Medicine Hasanuddin University, Jl. Perintis Kemerdekaan kampus Tamalanrea Km. 10 Makassar, Indonesia

*Corresponding authors: A. Magfira Satya Apada (andimagfirahsatyaapada@gmail.com)

Abstract

Amazon Sailfin Catfish (Pterygoplicththys pardalis) or commonly known as Plecos is originated from Amazon River in South America Continent. The aims of this study are to identify and isolate pathogenic gram-negative bacteria in the liver of the plecos from Lapompakka and Sidenreng Lakes and to observe the histopathological changes in liver identified with gram-negative bacteria infection. 10 samples were collected from both lakes with each 5 representing each lake. Identification and Isolation of gram-negative bacteria was performed through culture of pleco's liver swab results in MacConkey media using gram staining method and were continued by biochemical testing using VITEK 2 System. The acquired results indicated that there were 8 different species of gram-negative bacteria isolated and all of them were pathogenic to the plecos. The species of bacteria includes Klebsiella pneumoniae, E.coli, proteus sp., Acinetobacter baumannii, Aeromonas sobria, Enterobacterb cloacae complex, Citrobacter freundii, Enterobacter aerogenes. Specimen preparation for liver histopathology was carried out by 10% neutral buffered formalin (NBF) fixation, graded ethanol series dehydration, paraffin wax embedding, 4 μm sectioning, Hematoxylin and Eosin Staining. After that, the specimen was observed under a microscope. Data analysis employed qualitative descriptive method. From the observation, liver damages such as necrosis, inflammatory cell infiltration, hemorrhage, and cell hyperthrophy were identified. Liver damages were most probably caused by the infection of gram-negative bacteria.

Keywords *: Sidenreng Lake, Lapompakka Lake, Liver, Histopathology, Amazon Sailfin Catfish*

Introduction

Sidenreng and Lapompakka Lakes are located near from Tempe Lakes. These three lakes were surrounded by three regions of Wajo, Soppeng, and Sidrap. The three lakes are interconnected and located in the central part of South Sulawesi (Husnah et al., 2008). Lapompakka and Sidenreng Lakes are 4 meters deep and Lapompakka Lake covers approximately 300 Ha area while Sidenreng lakes cover approximately 3000 Ha area (Husna *et al.*, 2008). The three lakes become interconnected during the rainy season and cover a total area of 35.000 Ha. This enables different species of fish to migrate from one lake to the other lakes. The fauna composition from the three lakes does not indicate significant variability (AndiOmar, 2010). Amazon Sailfin Catfish or commonly known as Pleco is one species inhabiting the lakes. Their bottom-dwelling behaviour caused them susceptible to disease by viruses, bacteria and other pathogens. One of the fish organs that may serve as the indicator of the contaminated body of water is the liver (Manin *et al.,* 2012). The utilization of plecos as a food source must pass the standardized requirement and procedure to ensure whether the fish is free from harmful compounds or pathogenic microorganisms (Rahayu *et al.*, 2019). Based on the above discussion, the authors consider that it is important to investigate the potential pathogen in the plecos from Lapompakka and Sidenreng lakes, primarily the gram-negative bacteria which commonly infect aquatic animals.

Materials and Methods

This study employed a primary data descriptive analysis. Research site is located in Sidenreng Lake, Wette'e District, Panca Lautang Sub-District, Sidrap and Lapompakka Lake located in Tanasitolo District, Wajo. The research was conducted for 2 (months) from February to March 2021. The data source in this descriptive study was collected from laboratory observation performed by the Municipal Health Service Laboratory in Makassar and The Laboratory of Veterinary Clinic of Hasanuddin University to observe the histopathological damages on pleco's liver. Sample collection employed a random sampling technique at one stage from each lake. The data in this study is primary data. The method of data collection includes direct observation, review of literature, and documentation.

Results and Discussion

a. Identification of Gram-Negative Bacteria

The identification of gram-negative bacteria was performed by biochemical testing with *Vitek MS*. Before conducting biochemical testing, isolation on the samples was carried out on *MacConkey* agar culture media to obtain bacterial isolates with gram-negative characteristics.

From 10 samples of plecos *(Pterygoplichthys pardalis)*, 5 from Sidenreng Lake and the remaining 5 from Lapompakka Lake were identified to be contaminated by 16 strains of gram-negative bacterial isolates. From 16 bacterial isolates, 7 isolates were identified from Sidenreng Lake Plecos and 39 other isolates were identified from Lapompakka Lake. Such result was obtained after the testing of isolate selection found in plecos' liver (*Pterygoplichthys pardalis*) and overall, there were 8 pure isolates identified with gram-negative characteristics.

Bacteria Colony Morphology and Potential Gram-negative Bacteria Cell in Plecos' liver (Pterygoplichthys pardalis)

The characteristics of potential gram-negative isolates obtained from pleco's liver (*Pterygoplichthys pardalis*) isolation can be observed from the colony morphology which consists of colour, size, hemolysis, and shapes as well as the morphology of bacterial cells on MacConkey media. MacConkey culture media is a differential selective media containing carbohydrate and specific dye substances in distinguishing colonies based on lactose fermentation capability. Therefore, during the bacteria strain identification, gram-negative bacteria colonies will grow easily (Gunnarsson *et al.*, 1998). The morphological characteristics of 7 isolated colonies are presented in Tables 1 and 2.

Table 1. Morphological Characteristics of Liver Bacteria Isolate from Sidenreng Lake (SL) Plecos

No.	Sample Code	colony	color	size	shape	Elevation	edge	nature
1.	SL Isolate I	1	red	Big	round	Convex	flat	wet
		2	Pink	small-currently	round	Convex	flat	dry
2.	SL Isolate II	1	Pink	currently	round	Convex	flat	dry
		2	white	Big	widen	Irregular	flat	wet
		3	trnsprent	small	round	fine	flat	wet
3.	SL Isolate III	1	gray	Big	round	Convex	flat	wet
		2	Metallic red	small	round	Convex	flat	dry
		3	pink	small	round	fine	flat	wet
4.	SL Isolate IV	1	Transparent	currently	round	Convex	flat	wet
		2	Pink	currently	round	Convex	flat	wet
		3	gray	Big	round	fine	flat	wet
5.	SL Isolate V	1	Pale red	currently- big	round	Convex	flat	wet
		2	Metallic pink	currently-big	round	Convex	flat	wet
		3	Pale Pink	Big	widen	fine	flat	wet

Table 2. Morphological Characteristics of The Liver Bacteria Isolate from Lapompakka (LL) Plecos

No.	Sample Code	colony	color	size	shape	Elevation	Edge	nature
1.	LL Isolate I	1	Pink	currently-big	round	Convex	flat	wet
		2	Pink	currently	round	Convex	flat	wet
2.	LL Isolate II	1	Pink	Big	round	Convex	flat	wet
3.	LL Isolate III	1	white	currently	round	Convex	flat	wet
4.	LL Isolate IV	1	White	Currently	round	Convex	flat	wet
		2	Pink	small	round	fine	flat	wet
5.	LL Isolate V	1	Red	currently	round	Convex	flat	wet

2	Pink	currently	round	Convex	flat	wet
3	Pink	Big	round	Convex	flat	wet

Characterization and Identification of Gram-negative Bacteria in Pleco

The isolated bacteria colony in *MacConkey* media was further tested using VITEK MS and VITEK 2-compact. This device employed the principles of biochemical testing with a highly automatic system to perform bacterial identification and antimicrobial susceptibility testing according to the principles of advanced colourimetry and turbidimetry. Therefore, this will enable the anti-microbial identification and sensitivity testing results accomplished within 5-8 hours (Prihatini *et al.*, 2018). Meanwhile, VITEK MS using MALDOTOF MS method (Matrix-assisted laser desorption ionization–time of flight mass spectrometry) is well known for its quick and cost-effective identification process. The aim of this study is to identify the identification result using automatic biochemical testing using (VITEK® 2) dan MALDI- TOF MS (VITEK® MS). The result of biochemical testing testing on gram-negative bacteria is presented in the following Table 3. VITEK Bacteria Identification Results on Sidenreng and Lapompakka samples are presented as follows:

Table 3. Bacteria Identification Results on Sidenreng and Lapompakka samples

Sample type	colony	Garam stain	Type of bacteria	confidence
Sidenreng lake fish liver sample 1	1	Gram negative bacilli	*Klebsiella pneumoniae*	99.9%
	2	Gram negative bacilli	*Escherichia coli*	99.9%
	3	Gram positive bacilli	-	-
Sidenreng lake fish liver sample 2	1	Gram negative bacilli	*Klebsiella pneumoniae*	99%
	2	Gram negatuve bacilli	*Acinetobacter baumanni*	99%
Sidenreng lake fish liver sample 3	1	Gram negatuve bacilli	*Aeromonas sobria*	94%
	2	Gram positive bacilli	-	-
	3	Gram positive coccus	-	-
Sidenreng lake fish liver sample 4	1	Gram positive bacilli	-	-
	2	Gram positive coccus	-	-
	3	Gram positive coccus	-	-
Sidenreng lake fish liver sample 5	1	Gram negatuve bacilli	*Enterobacter cloacae complex*	99%
	2	Gram negatuve bacilli	*Klebsiella pneumoniae*	99%
	3	Gram positive bacilli	-	-
Lampopakka lake fish liver sample 1	1	Gram negatuve bacilli	*Klebsiella pneumoniae*	99.9%
	2	Gram negatuve bacilli	*Proteus sp*	99%
Lampopakka lake fish liver sample 2	1	Gram negatuve bacilli	*Klebsiella pneumoniae*	97%
Lampopakka lake fish liver sample 3	1	Gram negatuve bacilli	*Aeromonas sobria*	92%

Lampopakka lake fish liver sample 4	1	Gram negatuve bacilli	*Enterobacter cloacae complex*	98%
	2	Gram positive bacilli	-	-
Lampopakka lake fish liver sample 5	1	Gram negatuve bacilli	*Enterobacter cloacae complex*	98%
	2	Gram negatuve bacilli	*Citrobacter freundii*	91%
	3	Gram negatuve bacilli	*Enterobacter aerogenes*	94%

a. Klebsiella pneumoniae

Klebsiella pneumoniae is one of the bacteria species identified from the pleco's livers. The presence of such bacteria can be observed from the pleco's liver damages such as necrosis in which some parts of the liver showed blackish-red colour. This is in line with the study performed by Yu and Xia (2013) that observes the infection of Klebsiella pneumonia on dolphin's lungs and liver causing necrosis and similar blackish-red spots on some parts of the lungs and liver. This bacteria colonize the respiratory and digestive system and will cause tissue damage. The bacteria sometimes found in the fish liver if the fish has systemic infection where the pathogen disrupt the host immune system and eventually, cause liver, kidney, and lung damages. The above description is also in accordance with the study performed by Salahudin (2016) reporting that *klebsiella pneumonia* is sometimes found in the fish liver with structural damages.

b. Escherichia coli

From the observation of pleco's liver infected by Escherichia coli, the infected liver had a red spots. This is in line with the study by Maruka et al., (2017) confirming that the clinical symptoms of fish liver infected by E. coli are the presence of red spots and swellings. This was because of E.coli's ability to invade blood vessel walls and cells triggering inflammation and swelling. In a study performed by Haile and Temesgen (2018), E Coli colony was also obtained from the fish liver in Zeway Lake in Ethiopia. Similarly, Hamidah et al., (2019) found E. coli in fish liver with structural damages.

c. Proteus sp

Proteus sp. may also be found in both fish digestive systems and liver. *Proteus* sp.may emit distincitive smell by transforming acid and gasses from phenylalanine glucose into phenylalanine pyruvic acid. They are also capable of hydrolyzing urea rapidly because of the presence of the urease enzymes. According to Jaweetz (2005) *Proteus* sp. is a responsible pathogen for urinary tract infection, pus-forming disease such as abscess, wound infection and they can be found in fish organ, primarily liver, kidney, and intestines. In addition, Proteus. sp may also cause diarrhea in children and human infection. According to Manurung and Darna (2017), *Proteus* sp. is a pathogenic bacteria to fish and may be isolated from Tilapia organs such as kidneys, liver, and intestines.

d. Acinetobacter baumannii

Acinetobacter baumannii is also found in pleco's liver observed in this study and the bacteria are considered to be gram-negative and pathogenic in fish. A similar report was confirmed by Sutandhio *et al.,* (2016) who found *Acinetobacter baumannii* in fish and observed its ability to cause death in fish. In addition, Massinai *et al,m* (2017) also confirmed the toxic traits of *A.*

baumannii found in the fish liver where the bacteria can cause death in fish. The above mentioned studies are in accordance with the study performed by Sumarno *et al.,* (2020), stating that *A. baumannii* is a species of bacteria that colonzie fish liver and they are categorized as gram-negative bacteria.

e. Aeromonas sobria

Aeromonas sobaria was also identified in pleco's liver with SD-3,1 sample code. From the observed colony morphology of *Aeromonas sobaria* on MacConkey media, it was identified that the colony had a spherical and flat shape with a creamy colour. This is in line with the finding in the study by Salikin *et al.,* (2014) where the colony had a spherical and flat shape with creamy colour as well as growing well on MacConkey media. The bacteria *Aeromonas sobaria* is a pathogenic bacteria to fish and they may cause wounds to the skin, digestive systems, and internal organs. Nurjannah *et al.,* (2014) stated that Aeromonas sobaria is also found in Carp's liver causing liver lesions that eventually lead to fish death. Those studies provided similar details with the study performed by Azizah (2020) that concludes the gram-negative and pathogenic traits of Aeromonas sobria as well as their possibility of colonizing fish liver.

f. Enterobactercloacae complex

From the observation, *Enterobacter clocae* infection on pleco's liver was also identified. Yuningtyas (2011) also found similar bacteria in her experiment. *Enterobacter cloacae* is a gram-negative, anaerobic facultative, and motile bacteria with rod shape. Their motility uses a petritic flagella distributed over the entire cell surface. If the *Enterobacter cloacae* is grown on artificial media, they will perform glucose transformation into acid and gas. The bacteria reduce nitrate to nitrite. The bacteria cause nosocomial infection (an infection transmitted to fish living in less healthy or hygene environemnt) and they have endotoxin and exotoxin as a primary requirement of fish pathogen. The bacteria may be isolated from liver and fish kidney organ. The above mentioned studies has corrlated finding with the study by Mahendra (2016) where Enterobacter cloacae were identified to colonize fish liver and had gram-negative as well as pathogenic traits to fish.

g. Citrobacter freundii

In this study, *Citrobacter frefundii* was able to be identified with the morphological characteristics of metallic pink colour, medium size, shperical and flat shape and colony alkaline condition. Under the microscope observation, the bacterial colony has spherical shape and pink colour. *Citrobacter freundii* is a species of *enterobactericeae* that can be found in the animal digestive system, especially fish. They may be found in the fish liver because the bacteria colonize and cause lesions in liver. This is in accordance with the study performed by Angreni *et al* (201*). In addition, according to the study by Apriliana *et al.,* (2014), a diverse range of bacteria can be found in Pening marshes including citrobacter frefundii in fish that falls under the category of gram-negative bacteria.

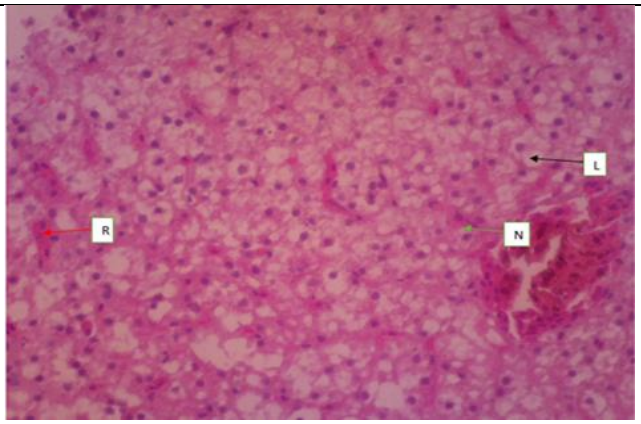
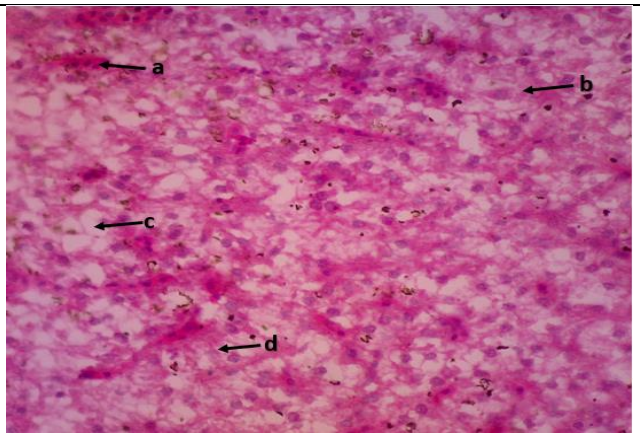
h. Enterobacter aerogenes

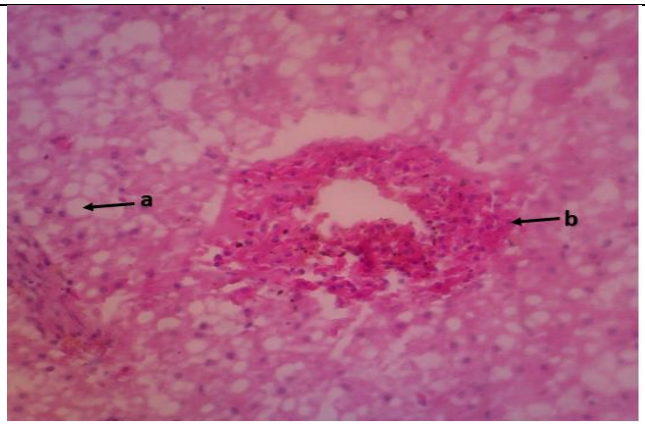
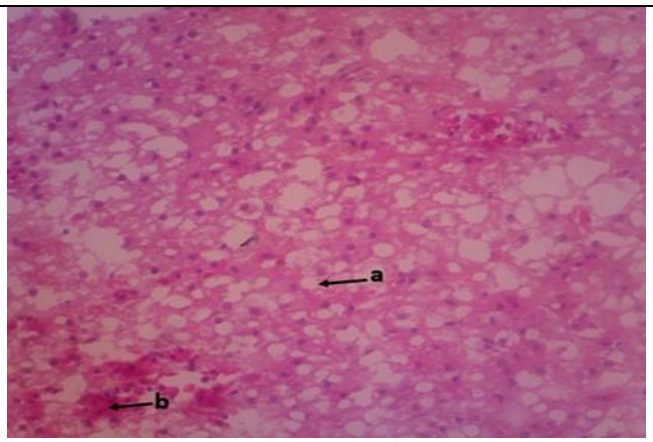
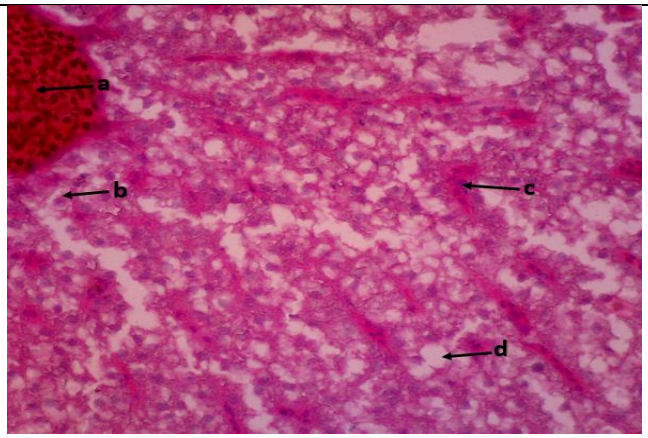
Enterobacter aerogenes is from the large family of *Enterobacteriaceae* and they were identified from the observation on DB-5.3 samples. Their colony morphological characteristics included pink colour, convex and shperical shape, and alkaline characteristic. Such morphological characteristics have been previously identified from a study by Mahendra (2016) where the morphological characteristics of *enterobacteria aerogenes* had a spherical and convex shape and slightly alkaline characteristics. *Enterobacter aerogenes* can be found in the liver of plecos from

Lapompakka lakes. The apparent symtomps included lesions in the pleco's fin. Similar results were identified by Purwani *et al.*, (2009) who managed to isolate *enterobacter aerogenes* from Tilapia liver that causes death in Tilapia. In line with the above mentioned studies, Milo *et al.*, (2015) confirmed that *enterobacter aerogenes* is a gram-negative bacteria and may be isolated from fish liver and they are pathogenic to fish.

Pleco's (*Pterygoplichthys pardalis*) Liver Histopathology

Based on the biochemical testing of VITEK system in this study, from 10 samples, 5 from Sindenreng and remaining 5 from Lapompakka Lakes, 9 samples were identified to be infected by gram-negative bacteria and from the 9 samples, there were 8 bacteria species identified. Pleco's liver Histopathology is presented as follows:

isolate	Bacteria name	Histopathological picture
SL-1,1	*Klebsiella pneumonia*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). R: hemorrhage; N: necrosis; L: fat degradation
SL-1,2	*E. coli*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: hemorrhage, b: necrosis, c: fat degeneration, d: inflammatory cell infiltration.
SL-2,2	*Acinetobacter baumanni*	

		 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: fat degeneration, b: hemorrhage.
SL-3,1	*Aeromonas sobria*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: fat degeneration, b: hemorrhage
SL-5,1	*Enterobacter cloacae complex*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: necrosis, b: inflammatory cell infiltration, c: hemorrhage dan d: fat degeneration

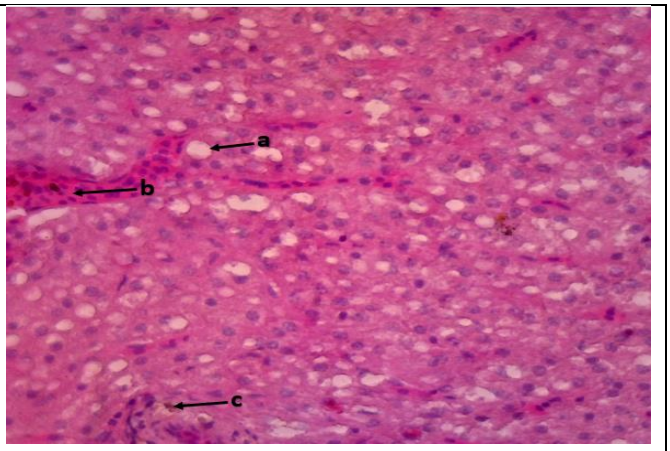
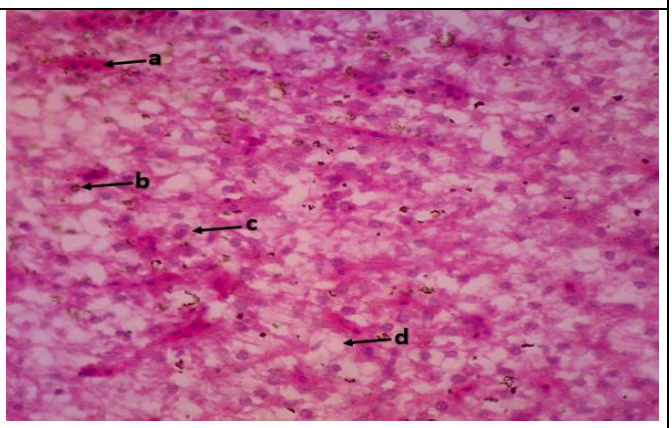
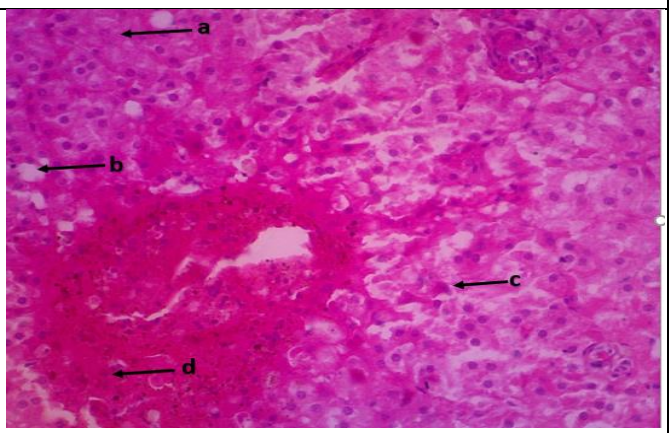
LL-1,1	*Klebsiella pneumoniae*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: fat degeneration, b: hypertrophy, c: necrosis.
LL-5,2	*Citrobacter freundi*	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: hemorrhage, b: necrosis, c: inflammatory cell infiltration d: fat degeneration.
LL-1,2	*Proteus* sp.	 Caption : liver histology of Suckermouth catfish at sidenreng lake location. HE staining (40x10 magnification; Bar= 10 μm). a: degeneration hidrofilik, b: fat degeneration, dan c: inflammatory cell infiltration, d: hemorrhage..

Figure 1. Pleco's (*Pterygoplichthys pardalis*) liver Histopathology identified with gram-negative bacteria

Fish liver is a very important organ for detoxification and therefore, it is very succeptible to bacterial toxin. Metabolism process will get disrupted if exposed to inflammatory agent. The

degree of liver damages may encompass congestion, fatty degeneration, necrosis and inflammatory cell infiltration and the scale of 0-6 depends on the degree and the area of damages namely (0): no changes, (2): minor damages, (4): medium damages, and (6): severe damages. Necrosis and cell death fall under the category of severe damages. Hemorrhage and congestion are categorized as medium damages. Fatty degeneration and cellular inflammation are categorized as minor damage (Bernet *et al.,* 1999).

Inflammatory cell infiltration was identified in the SL-5,1, SL-1,2, LL-1,2 dan LL-5,2 samples and all of them were infected by *Enterobacter cloacae complex*, *E. coli*, *Proteus* sp. and *Citrobacter freundi.* Such finding is in accordance with the explanation by Lukistyowati (2011), stating that inflammatory cell infiltration is a body response against harmful agent's stimuli such as pathogen and it serves as the non-specific immune system that localizes pathogen through phagocytosis. Similar findings by Abdelhamed *et al* (2017) also confirmed that infected catfish liver by *E. coli* and *Proteus* sp. suffers from inflammatory cell infiltration marked by the lymphocytes leakage from blood vessel. According to Meidiza *et al,* (2017), inflammatory cell infiltration is a body response against harmful agents stimuli such as pathogen and it serves as the non-specific immune system that localizes pathogen through phagocytosis.

In this study, fatty degeneration was observed from the pleco's liver samples of SL-1,2, SL-2,2, SL-3,1, SL-5,1, LL-1,1, LL-5,2 dan LL-1,2. Kalaiyarasi *et al.,* (2016) stated that fatty degeneration may be marked by swelling tissue and vacuolation. If within a cell, fat massively accumulates and lost its ability to perform fat metabolism properly, fatty degeneration will occur. Swelling empty spaces or vacuoles in the cytoplasm pushes the nucleus to the edge of the cell. Fatty degeneration is considered to be a minor damage exposure. fatty degeneration observed in this study is in line with the study performed by Laith & Najiah (2013), where (*Clarias gariepinus*) catfish liver also suffers from fatty degeneration marked by the presence of vacuoles in the liver. Liver and Intesine Histopathology of Catfish infected by the bacteria with vacuoles in catfish liver. All of the previous studies supported Andayani *et al.,* (2017) statement that liver damages such as white vacuoles from the liver histological description are due to a fatty degeneration stimulated by fat accumulation in the liver.

Tissue damage such as necrosis was also found in pleco's liver with sample codes of SL 1,1, SL-1,2, SL-5,1, LL-1,1, dan LL-5,2. This was in line with the study performed by Takashima & Hibiya (1995) reporting that necrosis may be marked by the loss of tissue structure and the presence of pyknosis and karyolysis. Pycnosis is characterized by the reduction in size and increase in staining of a cell or its nucleus, while Kariolysis is marked by the complete dissolution of the chromatin of a dying cell or necrosis due to the cytolysis or phagocytosis from lymphocytes or histiocytes causing nuclei reduction in size completely. Andayani *et al.,* (2017) stated that liver necrosis was caused by the dying cell of liver cells and Cell death occurs along with the rupture of the plasma membrane. Necrosis is started by the liver inflammatory reaction such as hepatocyte swelling and tissue death. Such symptoms can also be observed in Tilapia (*Oreochromis aureus*) liver marked by the cell nucleus reduction in size (Pyknosisc). According to Hastari *et al.,* (2014), fish infected by the pathogenic agents such as bacteria will show a histological changes such as necrosis where the necrosis only have low activity and in the end, tissue death occurs resulting in dysfunction in the impacted area.

Hemorrhage which also occurs in liver pathology may cause complex damages resulting in liver failure. According to Asniatih *et al.*, (2013), hemorrhage can be characterized by internal or external blood leakage from the blood vessel. Sign of hemorrhage includes the existing red spots in the mucous layer of a body organ. In African sharp tooth catfish liver injected with suspended pathogenic bacteria indicates histopathological changes of hemorrhage. In addition to that, Andayani *et al.*, (2020) further explained that hemorrhage can be categorized as

medium damage in the infected fish liver and it may be started from congestion. If it becomes worse, the blood vessel will leak out and the blood will not flow properly. Hemorrhage can also be characterized by blood leakage from the blood vessel, either outside of the body or inside the body tissue. Mangunwardoyo *et al.*, (2010) also stated that hemorrhage is a reaction to bacteria pathogenicity caused by hemolysin toxin secreted by the bacteria. Hemolysin toxin causes lysis of red blood cells by disrupting the cell membrane resulting in blood leakage from the blood vessel.

From the liver histopathology of plecos infected by negative-gram bacteria, the existing damages such as necrosis, fatty degeneration, hemorrhage, and inflammatory cell infiltration was similarly identified by Dong *et al.,* (2018) stating that the observable histopathological changes in liver infected by both gram-negative or positive bacteria cannot be distinguished significantly since both bacteria contributed to the similar damages such as necrosis, inflammatory cell infiltration, hydrolysis degeneration, hemorrhage, and fatty degeneration. From the study performed by Chang and Plumb (1996), they found that histology of liver infected by bacteria may have particular characteristics such as necrosis, a massive amount of inflammatory cell infiltration, fatty degeneration, and hemorrhage that can be observed in catfish.

Conclusion and Recommendation

There were 5 bacteria identified as gram-negative bacteria in plecos (*Pterygoplichthys pardalis*) from Sidenreng including klebsiella pneumonia, *E. coli, Acinetobacter baumanni, Aeromonas sobria,* dan *Enterobacter cloacae complex* and there were 3 species of gram-negative bacteria in plecos from Lapompakka lakes including *Klebsiella pneumoniae, Citrobacter freundi,* dan *proteus* sp. Gram-negative bacteria found from Sidenreng Lake and Lapompakka lake were pathogenic to fish. Micro-anatomy changes observed from the liver included necrosis, inflammatory cell infiltration, hypertrophy, and hemorrhage.

As a recommendation for further research, research on water quality from both lakes are also necessary to identify their toxicity level. Therefore, there will be more information available concerning the consumption feasibility of water biota from Sidenreng and Lapompakka Lakes in Wajo.

Acknowledgement

The authors would like to express their gratitude to the Municipal Health Service of Makassar, the Agricultural and Livestock Service of Sidram and Wajo as well as other parties involved in supporting this research. The authors also would like to express their gratitude to all lecturers from the Faculty of Veterinary Medicine that always encouraged and assisted the authors until the completion of this research. The authors would like to express their gratitude to the study club of “haji somad”, “philia”, and “sapu-sapu squad”. The authors have no conflicts of interest to declare in relation to the involved parties in this research.

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