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A Filterable Agent Caused the Hemorrhagic Syndrome on Giant Gourami (*Osphronemus goramy* Lac.) at Yogyakarta, Indonesia

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Abstract

Giant gourami (*Osphronemus goramy* Lac.) is one of the important freshwater fish commodities in Indonesia. Disease infection is one of the constraints in the production of this fish. The mortality of giant gourami has been reported in Yogyakarta, Indonesia. This study describes the disease based on observations of external and internal signs, along with the histopathology of several tissues, and determine the causative agent disease. The diseased fish were sampled from Gamping and Moyudan Districts, Sleman Regency; and Wates District, Kulon Progo Regency. The internal organs were collected and storage in fresh dan fixed forms. Postulate river is used to prove the causative disease of the filterable agents. Polymerase Chain Reaction (PCR) and Reverse Transcriptase PCR (RT-PCR) are applied to confirm the presence of the virus. Sick fishes show the hemorrhage over the entire body surface, rotted fins, exophthalmia, petechiae, pale liver, visceral adhesions, and enlarged kidneys. Histopathological analysis shows lipidosis in the liver; bleeding in the liver, kidneys, spleen, and brain; and multiple necrosis in the kidneys, spleen, and brain. Based on these signs, we designated the disease to be Hemorrhagic Syndrome. The virus was strongly suspected the causative agent, as infecting healthy fish with a bacteria-free filtrate homogenate from diseased fish organs resulted in the same clinical signs observed in a natural outbreak. PCR tests for Megalocytivirus and EHNV, along with RT-PCR tests for VHSV, SVCV, TiLV, IHNV, and IPNV, did not show any DNA bands, indicating that these viruses were not present. A filterable agent, potentially representing a new virus species or strain, causes hemorrhagic syndrome in giant gourami.

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1. Introduction

The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. The likelihood of major disease problems occurring increases as intensifying aquaculture activities.

Giant gourami (*Osphronemus goramy* Lac.) is one of the important freshwater fish commodities and has been widely cultivated for consumption in several locations in Southeast Asia, including Indonesia (Slembrouck *et al.,* 2020). Giant gurami can be cultured in concrete ponds, earthen freshwater ponds, and floating net cages. The best performance of Sago strain of giant gourami was found in the floating net cages culture system (Azrita *et al.,* 2020)

The rapid growth of aquaculture and a prolific global trade of the live aquatic animals and their products have led to the emergence of many new diseases in fish (Patil *et al.,* 2025). Aquaculture industry has been overwhelmed with its share of diseases and problems caused by parasite, bacteria, fungi, and viruses (Senthamarai *et al.,* 2023). *Heneguya* sp. parasitic infection was reported in snakeskin gourami *Trichopodus pectoralis* in Thailand (Dinh-Hung *et al.,* 2022). Several species of bacteria have been reported infect on the giant gourami such as *Streptococcus suis* (Nguyen *et al.,* 2023), *Aeromonas hydrophila* (Febrianti *et al.,* 2021; Fitria *et al.,* 2021), *Nocardia* sp. (Chen *et al.,* 2019), and *Mycobacterium* sp. (Agriandini *et al.,* 2021). The Infectious Spleen and Kidney Necrosis Virus (ISKNV) which recognized as Giant Gourami Iridovirus (GGIV) have been reported to cause mass mortality giant gourami in Indonesia (Sukenda *et al.,* 2020). The ISKNV have been reported attacks in India and caused the mass mortality of gourami (Swaminathan *et al.,* 2021). Jaemwimol *et al.* (2018) demonstrated that using artificial infection Tilapia Lake Virus (TiLV) is a novel orthomyxo-like virus causes clinical signs of infection including skin erosion, pale skin, exophthalmia and skin haemorrhage, histopathological changes of hepatocellular necrosis and syncytial formation in the liver and lymphoid depletion in the anterior kidney, and a cumulative mortality up to 100%.

The mortality of broodstock giant gourami with typical hemorrhagic clinical symptoms was reported in several places in Yogyakarta at different times. Cases of gourami broodstock mortality with typical clinical symptoms have been reported repeatedly in Yogyakarta, but there has never been a study to confirm the causative agent. Therefore, this study focuses on revealing the main causative agent through a molecular approach, River's postulates, and histopathological images due to infection. This study focuses on confirmation the causative agent of mortality of giant gourami broodstock in Yogyakarta using virological, molecular, and histopathological approaches. The study determines a definite relationship between the observed clinical symptoms and the causative agent.

2. Materials and Methods

2.1 Materials

2.1.1 The equipment

The equipment used are tissue dehydration (TSP-6A/TSP-6B Bioevopeak Automated Tissue Processor, Infitek, China), microtome (BK-2258 rotary microtome, Bioevopeak, China), 0.22-µm filter (Millipore Filter, Merck, Germany), centrifuge (Sorval Legend 21R, ThermoScientific, USA), spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA), light microscope (CX23, Olympus Corporation, Japan), and thermal cycler (TM 100, Bio-Rad, USA).

2.1.2 The materials

The materials used are anesthetic agent (MS-222, Sigma-Aldrich, USA), preservative agent (Ethanol, Merck, Germany), preservative agent (RNAlater, Thermo Fisher Scientific, USA), fixative agent (Formaldehide, Merck, Germany), histological stains (Hematoxylin and Eosin, HiMedia, USA), Tryptic Soy Agar (TSA, Merck, Germany), paraffin (Paraffin flakes, Indopath, Indonesia), nucleic acid extraction kit (Viral Nucleic Acid Extraction Kit II VR300, Geneaid, Taiwan), RNA extraction kit (High Pure RNA Isolation Kit, Roche, Germany), RT-PCR kit (MyTaq™ One-Step RT-PCR Kit, Bioline, USA), PCR master mix (MyTaq™ HS Red Mix, Bioline, USA), agarose gel electrophoresis reagent (Agarose, Merck, Germany), DNA stain (FluoroSafe DNA Stain, 1st BASE, Singapore), and DNA ladder (100bp DNA Ladder, Geneaid, Taiwan)

2.1.3 Ethical approval

The dissection and utilization of giant gourami (*Osphronemus goramy* Lac.) as experimental animals and samples in this study were performed under the supervision of ethical clearance no.00007/04/LPPT/III/2021.

2.2 Methods

2.2.1 Case report

An outbreak of giant gourami occurred in earthen ponds in several districts in the Special Region

of Yogyakarta, Indonesia, includes farms in Gamping District, Sleman Regency, in October 2021; Moyudan District, Sleman Regency, in October 2021; and Wates District, Kulon Progo Regency, in April 2023 and July 2024. From 2021 until the last sampling in 2024, this outbreak has recurred every year. The pattern of this outbreak occurred during the transition from the dry season to the rainy season (inter seasonal period). This outbreak occurred in giant gourami broodstock. The first report of mortality appeared three days after the onset of clinical symptoms and continued to occur gradually until a total of 15 broodstock died in 10 days.

2.2.2 Sample collection

Six broodstock giant gourami (*Osphronemus goramy* Lac.) weighing approximately 2-2.5 kg showed clinical symptoms of bleeding and weak swimming on the surface from each location. They were collected from earthen ponds in Gamping District, Sleman Regency; Moyudan District, Sleman Regency; and Wates District, Kulon Progo Regency. Fish samples were euthanized by immersing in an MS-222 overdose (250 mg/mL) according to the American Veterinary Medical Association (AVMA, 2020) protocol. Necropsies were performed on antemortem and postmortem examinations. Liver, kidney, and spleen samples were fixed in 75% ethanol and RNA-Later for molecular analysis and 10% normal buffered formalin for histopathological analysis. Kidney and spleen tissues were also stored at -80°C for postulate River.

2.2.3 Histopathological observation

Liver, kidney, and spleen tissues were fixed in 10% NBF for 24 hours. Fixed tissues were cut into 0.5 cm cubes. The Tissue were proceed using Bioevopeak Automated Tissue Processor by dehydration in graded alcohol and infiltration of paraffin. The sample was embedded in paraffin and sliced into 5 µm sections using a manual rotary microtome. Tissue sections were stained with hematoxylin eosin (Alturkistani *et al.,* 2016). Examination of pathological change was performed under a light microscope.

2.2.4 Postulate River

Preparation of bacteria-free kidney filtrate followed Murwantoko *et al.* (2016). The kidneys were homogenized in PBS with a ratio of 1:9 and centrifuged at 3,000 ×*g* for 10 minutes at 4°C. The supernatant was filtered with a 0.22-µm Millipore filter. The virus filtrate was inoculated into Tryptic Soy Agar (TSA) to ensure it was free from bacteria.

The 30 healthy *O. goramy* weighting 150-160 grams (length 15 - 18 cm) with active movement and shiny scales were collected from commercial farms that had no history of disease infection, totaling 30 fish for River postulation. Fish were reared at a density of one fish per one and a half liters of water with a water change every two days with contionuous aeration at the Laboratory of Fish Health and Environment, Universitas Gadjah Mada. Fish were acclimatizced for one week and fed commercial feed adlibitum in the morning and evening.

Fish were anesthetized by immersion in 4°C water until movement slowed down (AVMA, 2020). Intraperitoneal injection of 0.1 mL of bacterial-free filtrate was performed in the infection group and injection of PBS in the control group. Post-infection, fish were maintained for 14 days with continuous aeration and water changes every two days. The fish behaviour and mortality were recorded daily. Liver, kidney, and spleen were collected and preserved in ethanol for further analysis.

2.2.5. DNA and RNA extraction

Tissue samples were rinsed with PBS (pH 7.4). Approximately 10 mg of tissue was homogenized with a micropestle in 300 µL of PBS and centrifuged at 10,000 ×*g* for 5 min. DNA was isolated with the Geneaid Viral Nucleic Acid Extraction Kit II following the manufacturer's protocol. RNA was isolated using the High Pure RNA separation kit following the manufacturer's protocol. The final step of DNA and RNA was eluted in 75 µL of elution buffer. The concentration and purity of DNA and RNA were measured using a NanoDrop 2000 spectrophotometer (García-Alegría *et al.,* 2020). Good quality DNA and RNA with absorbance values of 260/280 in the range of 1.8–2.0 were stored at −80°C until use.

2.2.6 PCR and RT-PCR amplification

DNA amplification was performed for several possible viruses, including Megalocytivirus, Viral Haemorrhagic Septicemia Virus (VHSV), Spring Viraemia Carp Virus (SVCV), Tilapia Lake Virus (TiLV), Infectious Haematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), and Epizootic Haematopoietic Necrosis Virus (EHNV). The Primers targeting beta-actin, EF1α genes were used as housekeeping gene (HKG) for RNA template, and CO1 gene for control marker of DNA template. Target DNA was amplified using Thermal Cycler TM 100 with primer pairs listed in Table 1. This addresses the possibility of identifying potential etiologies, especially pathogens that have been reported to infect teleost species. Several pathogens, such as Megalocytivi

Table 1. List of primers targeting suspect viruses and internal controls used in this study

Name	Targeted	Sequence	Tm ^{0}C	Reference
MgCyst G1 F	Megalocytivirus	GCCTGTATATGCATCGT	55	This Study
MgCyst G1 R		GCACATCGCTGATTGTGT		
VN-F	VHSV	ATGGAAGGAGGAATTCGTGAAGCG	55	Snow *et al.*, 2004
VN-R		GCGGTGAAGTGCTGCAGTTCCC		
SVCV F1	SVCV	TCTTGGAGCCAAATAGCTCARRTC	50	Ip *et al.*, 2016
SVCV R2		AGATGGTATGGACCCCAATACATHACN-CAY		
Pair 4R	TiLV	GCCCAGAGCCTCTTGTCAAT	55	This Study
Pair 4F		GCCCAGAGCCTCTTGTCAAT		
IHNV-NF	IHNV	TGAAGTACCCCACCCCGAGCAGCATCC	60	Popova *et al.*, 2008
IHNV-NR		GTTCAACTTCAACGCCAACAGG		
VP2-R	IPNV	CCGCAACTTACTTGAGATCCATTATGC	60	Ballesteros *et al.*, 2012
VP2- F		CGTCTGGTTCAGATTCCACCTGTAGTG		
N gene R	EHNV	AAAGACCCGTTTTGCAGCAAAC	55	WOAH, 2022
N gene F		CGCAGTCAAGGCCTTGATGT		
EF1A F	EF1A	GCACGCTCTGCTGGCCTTT	59	Wang *et al.*, 2015
EF1A R		GCGCTCAATCTTCCATCCC		
B. Actin F	Beta actin	CAGCAAGCAGGAGATA	60	This Study
B. Actin R		TGTGTGGTGTGTGGTTGTTTTG		
COI F	CO1	TCAACCAACCACAAAGACATTGGCAC	46.5	Soliman *et al.*, 2017
COI R		TAGACTTCTGGGTGGCCAAAGAATCA		

rus (Kurita and Nakajima, 2012), VHSV (Dale *et al.*, 2023), SVCV (Souto *et al.*, 2024), IHNV (Yong *et al.*, 2019), IPNV (Robles *et al.*, 2022), and EHNV (Becker *et al.*, 2016), have been reported to cause significant morbidity and mortality in farmed and wild fish.

3. Results and Discussion

3.1 Results

3.1.1 Anatomical pathology

Typical clinical symptoms observed in giant gourami samples from Wates District, Kulon Progo Regency, are bleeding those spreads over the entire body surface (Figure 1A). Pathological anatomical changes seen include rotted fins (Figure 1A), exophthalmia (Figure 1B), petechiae and pale liver (Figure 1C), visceral adhesion (Figure 1D), and enlarged kidneys (Figure 1E).

3.1.2 Postulate River

Behavioral changes, clinical symptoms, and mortality occurred in giant gourami after infection. Fish swam limply on the surface and anorexia was observed three days after injection (dpi). Clinical symptoms of hemorrhage on the body surface (Figure 2A), rotted fins (Figure 2B), and visceral adhesions (Figure 2C) were clearly visible in six dpi. The control group showed no behavioral changes during the observation period.

Mortality patterns were clearly observed in giant gourami from Wates District, Kulon Progo Regency fish after infection. Mortality of the fish was first observed at five dpi at 20% of the population and increased gradually until seven dpi at 25%. A high increase in mortality was observed at 8 dpi at 60%. The

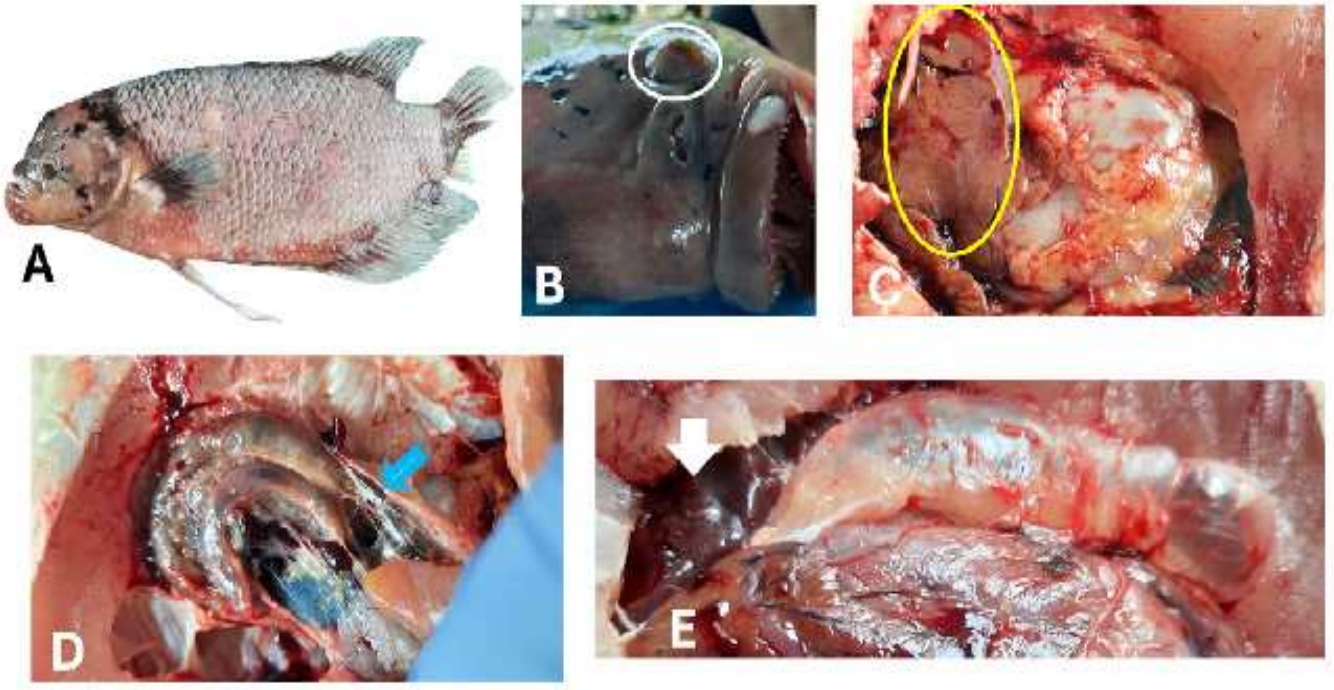

Figure 1. Anatomical pathology of sick giant gourami (*Osphronemus goramy* Lac.) from Wates District. (A) Hemorrhagic on the entire body surface and rotted fins, (B) exophthalmia (white circle), (C) petechiae characterized by black spots on the entire liver surface and the liver color is pale (yellow circle), (D) visceral adhesion with white fascia fibers (blue arrow), (E) enlarged kidneys (white arrow).

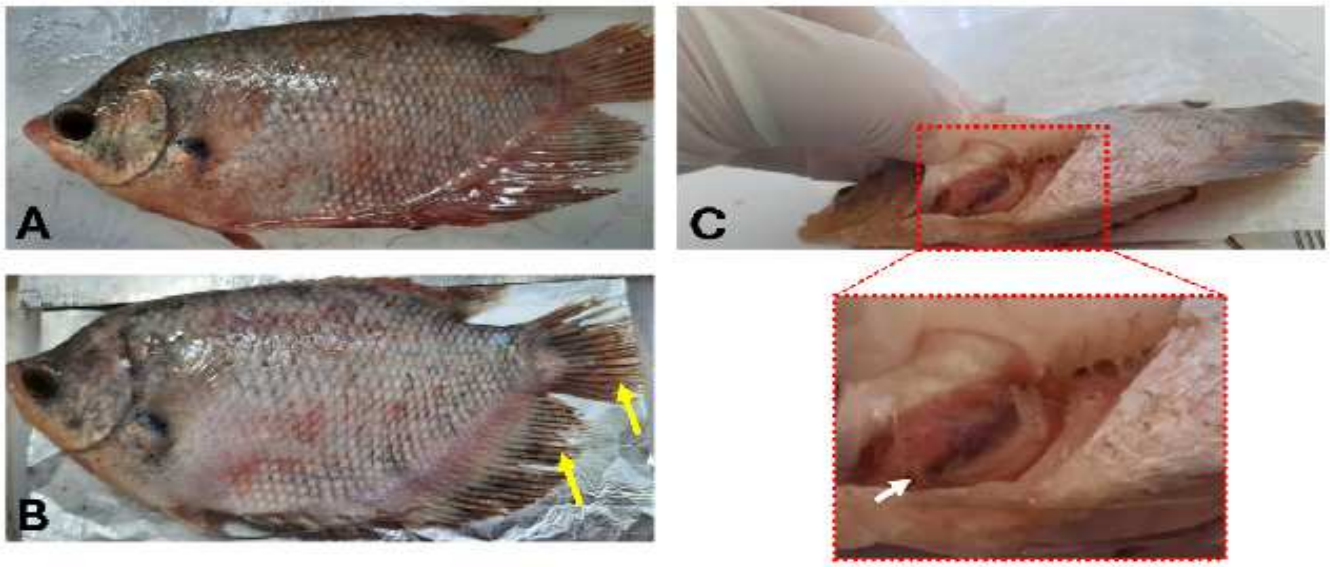

Figure 2. Clinical symptoms of giant gourami (*Osphronemus goramy* Lac.) postulate River. (A) Hemorrhagic on the body surface, (B) rotted fins, and (C) visceral adhesion.

peak of mortality was observed at nine dpi, indicating cumulative mortality of up to 90% of the total population. The control group did not experience mortality during the observation period (Figure 3). Postulate River result using giant gourami samples from Gamping and Moyudan Districts showed the similar clinical signs and mortality patterns with the samples from Wates District.

3.1.3 Histopathological observation

Histopathological changes were observed in the liver, kidney, spleen, and brain of sick giant gourami from Wates District. Most of the hepatocyte cells showed lipidosis characterized by spaces in the form of fat droplets in the cytoplasm and pushed the nucleus to the edge of cell. Liver tissue showed local hemor-

rhage and sinusoidal enlargement (Figure 4A). Kidney tissue showed the normal glomeruli, tubules, however the hematopoietic area displayed multiple necrosis, and the intertubular area showed hemorrhage (Figure 4B). Spleen tissue showed diffuse red and white pulp, hemorrhage, and multiple necrosis (Figure 4C). Brain tissue showed multiple necrosis, and hemorrhage in gray matter (Figure 4D). The histopathology of Gamping and Moyudan District samples revealed the similar changes as in Wates District samples.

The liver, kidney, spleen, and brain all showed histopathological changes in River postulate giant gourami. Liver tissue was observed with hepatocyte cells that were mostly lipidosis with fat droplets in the cytoplasm. Hemorrhage with sinusoid enlargement and multiple necrosis were also observed in liver tissue (Figure 5A). Kidney tissue showed hemorrhage and melanomacrophage center (mmc) in the intertubular (Figure 5B). Spleen tissue showed hemorrhage and multiple necrosis (Figure 5C). Brain tissue showed hemorrhage in the white matter (Figure 5D).

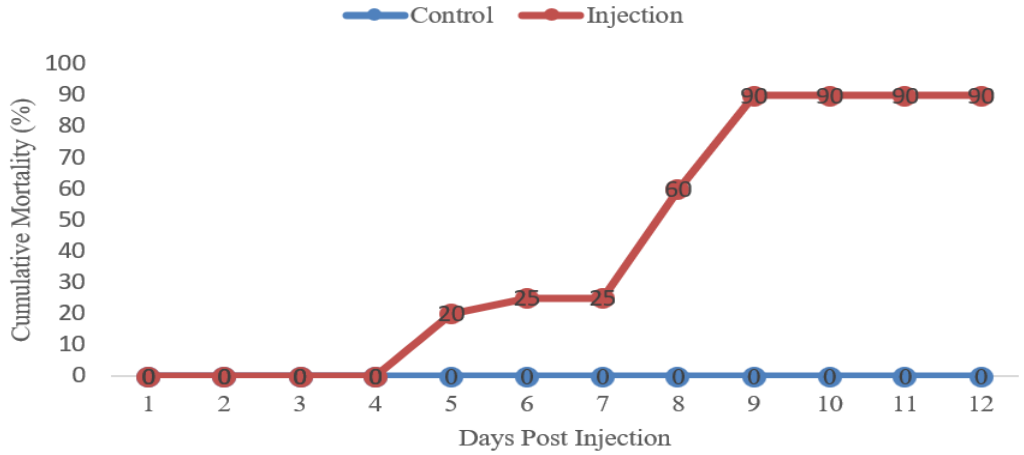

Figure 3. Cumulative mortality (%) of giant gourami (*Osphronemus goramy* Lac.). Fish were injected intraperitoneally with kidney homogenate, and control fish were injected with sterile PBS.

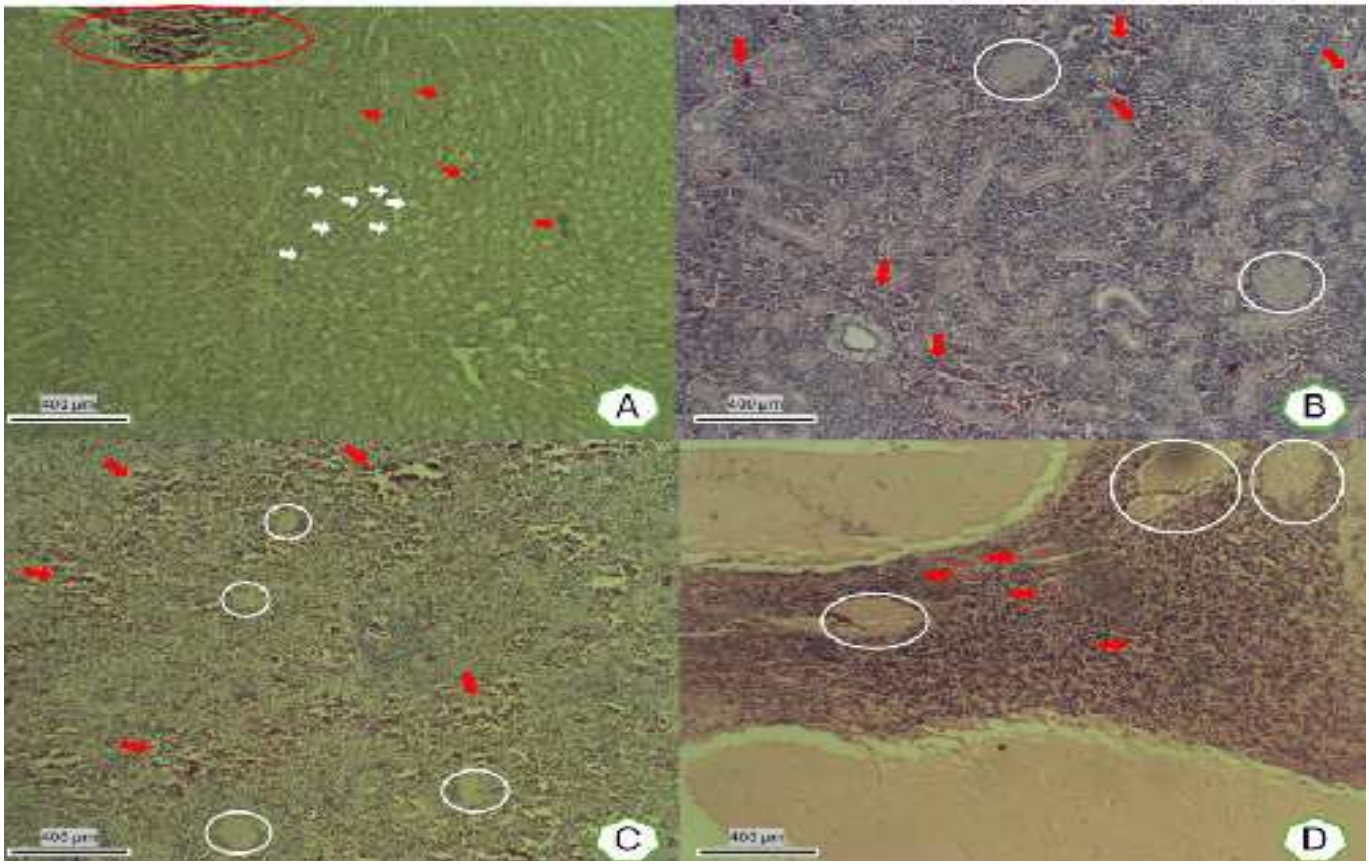

Figure 4. Histopathology of diseased giant gourami (*Osphronemus goramy* Lac.) from Wates District. (A) liver shows lipidosis (white arrow), congestion (red arrow), and local hemorrhage (red circle); (B) kidney shows hemorrhage (red arrow) and multiple necrosis (white circle); (C) spleen shows hemorrhage (red arrow) and multiple necrosis (white circle); (D) brain shows hemorrhage (red arrow) and multiple necrosis (white circle).

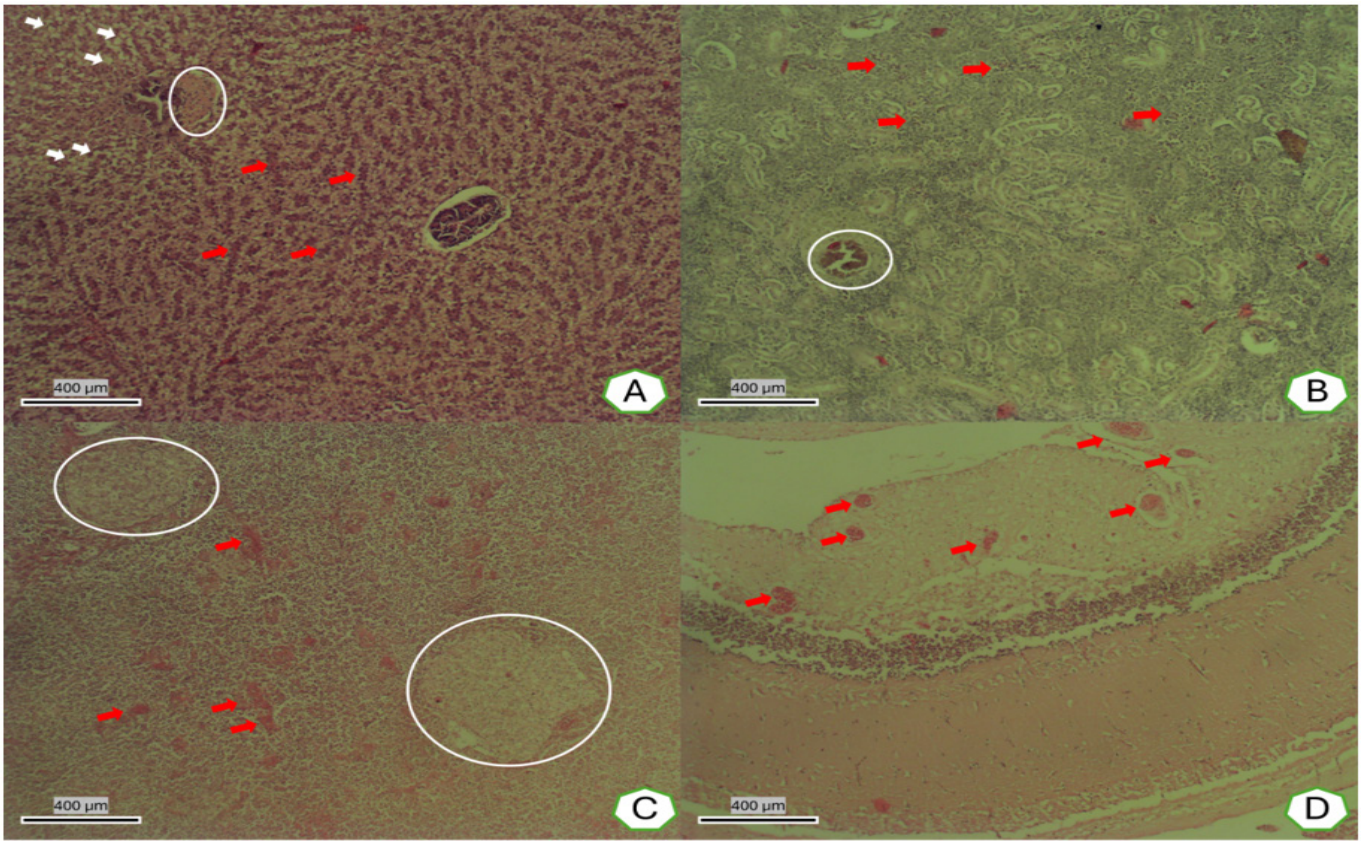

Figure 5. Histopathology of postulate River giant gourami (*Osphronemus goramy* Lac.) tissue. (A) liver shows lipidosis (white arrow) and congestion (red arrow); (B) kidney shows hemorrhage (red arrow) and mmc (white circle); (C) spleen shows hemorrhage (red arrow) and multiple necrosis (white circle); (D) brain shows hemorrhage (red arrow).

3.1.4 PCR and RT-PCR

Molecular detection using PCR and RT-PCR was carried out to identify viruses as etiological agents of disease outbreaks in giant gourami. Molecular detection results were negative for Megalocytivirus, VHSV, SVCV, TiLV, IHNV, IPNV, and EHNV infections. The house keeping genes as beta-actin and EF1α, also control marker of CO1 were amplified in all RNA and DNA samples, respectively.

3.2 Discussion

3.2.1 Anatomical pathology

Every year, seasonal disease outbreaks affect giant gourami (*Osphronemus goramy* Lac.), an important freshwater fish commodity in Indonesia. Yogyakarta reports the outbreak in Gamping District, Sleman Regency; Moyudan District, Sleman Regency; and Wates District, Kulon Progo Regency. The most reported typical clinical symptoms include hemorrhagic (Figure 1A), rotted fins (Figure 1A), and visceral adhesions (Figure 1D).

Hemorrhagic clinical symptoms refer to bleeding conditions that can occur in various tissues and organs. Various types of pathogens, including viruses, can cause this pathogenic condition (Ahmadivand *et al.*, 2016). Visceral adhesions are pathological conditions in which internal organs stick together in the body cavity due to the formation of fibrous tissue (Deynez *et al.*, 2023). This condition has been reported to occur in response to parasitic infection (Nagasawa *et al.*, 2022), injury (Liakakos *et al.*, 2001), ischemia (Liakakos *et al.*, 2001), and chronic inflammation (Deynez *et al.*, 2023).

The anatomical pathology of sick fish also shows exophthalmia (Figure 1B), petechiae and pale liver color (Figure 1C), and kidney enlargement (Figure 1E). Exophthalmia is abnormally protruding eyes caused by various infectious and non-infectious factors (Noor El Deen *et al.*, 2012). Viral infection is one of the causes of exophthalmia, as reported due to VHSV infection (Ahmadivand *et al.*, 2016) and SVCV (Ashraf *et al.*, 2016). Petechiae are purplish-red spots caused by bleeding; pathogen infection is one of the causes (Ahmadivand *et al.*, 2016). Researchers have reported that VHSV infection (Ahmadivand *et al.*, 2016) and TiLV (Pierezan *et al.*, 2020) cause pale liver and petechiae. Pale liver and enlargement of kidney were reported on the ISKNV infection (Murwantoko

et al., 2018). Kidney enlargement in fish has been previously reported to be caused by pathogen infection (Bunnajirakul *et al.,* 2015; Gorgoglione *et al.,* 2020) and environmental stress (Mishra and Mohanty, 2009).

These clinical symptoms have been reported in megalocytivirus infections, including hemorrhage on the skin and fins, rotted fins, pale body color, decreased swimming activity, enlarged abdomen, and visceral adhesions due to chronic inflammation (Kurita and Nakajima, 2012; Subramaniam *et al.,* 2016). The histopathological analysis of the kidney, liver, and spleen organs of the fish carried out by the postulate River.

3.2.2 Mortality

Rivers (1937) proposed his postulate to establish a causal relationship between virus and disease, i.e., that specific virus should be associated with disease, it should present occurrence in sick individuals as a cause of the disease under investigation instead of a coincidence or accidental finding, and a virus acquired from a sick individual can cause the same disease and symptoms when administered to a healthy individual. Bacterial-free tissue homogenate infection caused the mortality of giant gourami. The first mortality of fish in this study occurred at five dpi, with peak mortality observed at eight dpi (Figure 3). The mortality pattern in the postulate River shows a clear trend, increasing slowly and reaching a total of 90% by nine dpi, indicating that the disease incubation is between five and nine days after infection. Mortality due to virus infection shows a predictable pattern. The mortality pattern of fish after infection is related to the incubation of the virus, which depends on the type of virus, immune system, and environmental conditions (Ludwig *et al.,* 2011).

3.2.3 Histopathological alterations

Bacterial-free tissue homogenate infection caused clinical symptoms of external hemorrhage on the superficial body, rotted fins, and visceral adhesions (Figure 2). Histopathology observation of infected fishes demonstrated the congestion and hemorrhagic in the kidney, liver, and spleen and fatty degeneration in the hepatic organs. Those result shows the similarity with the histopathological change on outbreak sick giant gourami. Based on those above results of Postulat River's experiment on the mortality, clinical symptoms and anatomical pathology gave similar signs from outbreak diseased fish supported the conclusion on the fulfills the requirements of River's proposition. The disease outbreak attacking giant gourami at Yogyakarta was caused by filterable agent or may a virus.

Hemorrhage in surface and internal organs were important sign in this disease case (Figure 1, 2). The hemorrhage of fish has been reported in the infection by several viruses, including ISKNV (Nguyen *et al.,* 2024), VHSV (Dale *et al.,* 2023), Megalocytivirus (Kurita and Nakajima, 2012), Iridovirus (Qin *et al.,* 2023), SVCV (Souto *et al.,* 2024), TiLV (Tattiyapong *et al.,* 2017), IHNV (Yong *et al.,* 2019), IPNV (Robles *et al.,* 2022), and EHNV (Becker *et al.,* 2016).

3.2.4. Molecular detection

Molecular detection using PCR and RT-PCR was carried out to identify the virus might causing disease in the giant gourami disease outbreak. The electrophoresis results showed that no target genes were amplified, so they were negative for several possible diseases caused by Megalocytivirus, VHSV, SVCV, TiLV, IHNV, IPNV, and EHNV. The expression of housekeeping genes and control marker needs to be known to ensure the quality of the template (Wang *et al.,* 2015). EF1α and beta-actin are one of the optimal housekeeping genes in fish for precise and accurate gene expression analysis during pathogen infection (Wang *et al.,* 2015). CO1 is important as a control marker of DNA templates (Asgharian *et al.,* 2011). In this study, PCR and RT-PCR tests successfully amplified control marker and all housekeeping genes, including COI, beta-actin, and EF1α, indicating that the DNA and RNA samples were in good condition.

4. Conclusion

The outbreak disease on giant gourami (*Osphronemus goramy* Lac.) at Yogyakarta gave pathognomonic symptoms of hemorrhagic in the superficial body and in several internal organs designed as Hemorrhagic Syndrome. The same pathological changes of artificial infected fish with outbreak sick fish supported the conclusion on the fulfills the of Postulate Rivers' proposition, and the Hemorrhagic Syndrome was caused by filterable agent or may a virus. It is plausible that a novel, unidentified viral strain to cause this disease. Multiple indicators of diagnosis for etiological agents, such as behavioral anomalies, clinical signs, histopathological changes, incubation period, and mortality patterns, indicate the existence of a pathogen as the etiological agent. Nevertheless, molecular testing has not yet detected the pathogen. Consequently, a thorough, interdisciplinary investigation strategy is required to ascertain the explanation of this phenomenon.

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Authors' Contributions

NLFN: provided the main conceptual ideas, developed the methods, conducted laboratory and field experiments, handled the experimental animal, analyzed the data, designed the figures, and improved the manuscript. Mw: developed methods, analyzed the data, earned funds for the laboratory and field activities, and finalized the manuscript. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare no competing interest.

Declaration of Artificial Intelligence (AI)

This entire research does not use artificial intelligence (AI) tools, services, or technologies in the creation, editing, or refinement of this manuscript. All content presented is the result of independent intellectual efforts that guarantee originality and integrity.

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