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First Report of Scale Drop Disease in Hatchery-produced Asian Seabass (*Lates calcarifer*) in Indonesia

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ABSTRACT

Mass mortality in early stage of cultured Asian seabass has become a major issue in aquaculture industry. This study aimed to investigate the causative agent of mass mortality associated of the scale drop syndrome in hatchery-produced Asian seabass juvenile. Multiple visceral tissues of natural sick fish were sampled for Polymerase chain reaction (PCR) analysis using specific primer for nervous necrosis virus (NNV), megalocytivirus and scale drop disease virus (SDDV). Findings of this study showed that natural sick fish were negative of ectoparasites, NNV and megalocytivirus infection, but were positive of SDDV (The GenBank accession number for DNA sequence: OR507239.1 and OR507238.1). The natural sick fish appeared to be co-infected by pathogenic bacteria shown by a high population of *Vibrio* sp. sampled in skin ulcer. These results were supported by histological observation showing hemorrhage and necrotized cells in hematopoietic tissue. The experimental challenge in healthy fish injected with SDDV inoculum showed 100% mortality at 10 days post challenge. The visceral tissue of experimental SDDV injected fish demonstrated similar histopathological changes to natural SDDV infected fish. The overall finding in this study suggests that mass mortality of Asian seabass in hatchery was primarily caused by SDDV, while bacteria were likely to be secondary causative agent.

Key words: Histopathology, *Lates calcarifer*, Pathogenic bacteria, Viral disease

INTRODUCTION

The Asian seabass is important global commodity from both aquaculture and capture fisheries in tropic and subtropic areas. In the wild, Asian seabass is widely distributed throughout the Southeast Asian region from west India to Taiwan and also to Papua New Guinea and Northern Australia (Yue et al. 2012; Vij et al. 2014). In the Indo-Pacific region, seabass is important species, both as food and recreational fishery (Farook and Ali 2021). The production of Asian sea bass, *Lates calcarifer*, from aquaculture has grown significantly since it was started in Southeast Asia in 1980s (Boonyaratpalin and Williams 2002). This has been reported due to Asian sea bass has a rapid growth rate and wide range of adaptation in cultured condition (Jesus-Ayson et al. 2014; Nurliyana et al. 2020). However, the growing aquaculture of Asian sea bass has also been accompanied by the record of some diseases causing mass mortalities of the cultured fish in some countries (Nurliyana et al. 2020; Zhu et al. 2021; Liu et al. 2022).

In Indonesia, cultured sea bass has been produced from ponds, estuaries, lagoons, coastal and offshore areas across the country, with major proportion was produced from sea cage (De Silva and Phillips 2007; Rimmer et al. 2013). The production of sea bass from aquaculture in Indonesia has been relying on the supply of fingerlings, spawned from wild-caught broodstock, from some hatcheries e.g., Bali, Lampung, East Java (Khotimah et al. 2022). However, the production of fingerlings from hatchery has been frequently reported to be impeded by disease outbreak causing mass mortality of the fingerlings. For example,

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100% mortality of Asian sea bass was reported in some hatcheries in Indonesia (i.e., Situbondo, Banyuwangi and Bali) (Zafran et al. 1998). A more recent case of mass mortality has been reported in North Bali during the period of 2018 – 2022, where about 70-80% of Asian sea bass juveniles (6-12cm) were reported to be dead, following the symptoms of decreased appetite, weak swim on the surface or on the bottom of the tank, dark body coloration, peeling scales and distended abdomen.

Prior studies have identified a number of diseases causing major impact on the Asian seabass hatchery production, including mass mortality of the fingerlings. For example, viral disease caused by nervous necrosis virus (NNV) infected larva to adult and caused up to 90% mortality of fish (Sembiring et al. 2018; Khotimah et al. 2022; Yang et al. 2022). Scale drop syndrome is also a disease reported to cause mass mortality in Asian sea bass in Southeast Asia (Domingos et al. 2021). Scale drop syndrome was reported with clinical signs of darkened bodies, scale loss and sometimes exophthalmia (Gibson-Kueh et al. 2012; de Groof et al. 2015; Senapin et al. 2019; Domingos et al. 2021). For bacterial diseases, streptococcus and bacterial septicemia, caused by *Streptococcus* sp. and *Vibrio* sp. have been reported to cause severe mortality in juvenile seabass (Creeper and Buller 2006; Kanchanopas-Barnette et al. 2009). Meanwhile, infection of parasites (e.g., monogenean and protozoan) has been reported to cause massive losses of up to 200,000 (~50 t) Asian sea bass from sea cage in Australia (Deveney et al. 2001).

In North Bali, Indonesia, mass mortality of sea bass are frequently observed in hatchery facilities in the last three years. The sick and dead fish showed symptoms of blackish body color and being inactive at the bottom of the tank. These conditions are followed by abdominal swelling as shown in fish infected by *Vibrio* sp. reported by Gibson-Kueh et al. (2021) and loss of scales Nurliyana et al. (2020). The flaking of scales is clearly visible on the area between the abdomen and near the tail of the infected fish, with brown color and excessive mucus (Gibson-Kueh et al. 2012; Senapin et al. 2019). Fish mortality occurs after three to fourteen days from the initial symptoms. Sea bass farmers suspect that the mass mortality of the fish might have been caused by a megalocytivirus which is more popularly known as iridovirus. This is due to the clinical symptoms are identical to the symptoms of Asian sea bass infected by scale drop disease in Batam Island, western part of Indonesia (Koesharyani et al. 2020). However, no investigation has been conducted to determine the causal agents of mass mortality, which has resulted in significant economic losses for the grow-out sea bass at net cage in this area. Therefore, this study aims to determine the causative agent of mass mortality and pathological changes in diseased Asian seabass juvenile from hatchery facilities.

MATERIALS AND METHODS

Ethic statement

Experiment conducted for this study followed the national guideline and was accepted by the animal ethics committee of Udayana University, Indonesia No. B/159/UN14.2.9/PT.01.04/2022.

Fish sample collection

Following a report of mass mortality from seabass hatchery owner, a total of 60 sick fish (7.2–12.2mm body length) showing lethargic, scale loses, abnormal swimming behavior and black body color were used for experiment. At the same time, a total of 90 healthy fish from the same hatchery were collected for later experimental challenge test. The fish were transported to the Laboratory of Pathology of the Institute for Mariculture Research and Fisheries Extension (IMRAFE) in a separated oxygenated plastic container for each sick and healthy fish for 20min. The sick fish were kept in a 100L concrete tank (80L volume) supplied with aeration and stagnant water system. The healthy fish were placed in two 500L concrete tank equipped with aeration and running water at 15mL/s. The water quality parameter of pH, DO, temperature and salinity were kept at 7.5–8.0, 5.0–6.0, 29.0–31.0°C, 32.0–33.0ppt respectively. The fish were fed *ad libitum* with commercial pellet (MS Megami GR 3) twice a day for two weeks. Siphoning and 50% of water change was carried out every morning.

Gross clinical and parasite observation

A total of 20 sick fish were anesthetized using a solution of 0.01% clove oil. Gill lamellae and skin mucus were isolated and were placed on glass objects with a drop of sterile seawater. The observation of parasite was conducted under a compound microscope (Olympus BH-2, Japan) at a magnification of 40–200x.

Bacterial observation

The skin ulcer and liver of ten sick fish (the same fish used for parasite observation) were isolated for bacterial observation. About 0.1mg of skin ulcer and liver was dissolved and homogenated in 0.9mL of sterile seawater. The homogenates were then diluted 100 and 10.000 time with sterile seawater. They were grown on media of tryptic soy agar (TSA) and thiosulfate citrate bile sucrose agar (TCBSA). These cultures were then subjected to 24h of 30°C incubation. Total bacteria and *Vibrio* sp. population in both organ skin and liver were calculated using a colony counter (Sibata CL560, Japan).

Blood smear and hematopoietic stamp

Five sick fish were used for blood and hematopoietic stamp observation. Blood smear samples were prepared by placing a drop of blood at the end of the slide glass. Meanwhile hematopoietic stamp samples were prepared by pressing a slice of the spleen and kidney on the glass object. The smears and stamps were then dried at room temperature and were placed in methanol for 1h of fixation. The smears and stamps were stained using Giemsa for 2min and rinsed in by tap water for 1min. Observation of hematopoietic cells was done under a microscope (Nikon Digital Camera DXM1200F, Japan).

Histological examination

Sick fish were dissected for skin-muscle, liver, spleen, anterior kidney and posterior kidney isolation. These organs were fixed in 10% buffered formalin for 24h prior to tissue processing in an automatic tissue processor (RH-12EP, Sakura, Japan). The paraffin embedded organs were sectioned at 5µm thickness and were stained

using hematoxylin and eosin. Histological observation was done under a microscope (Nikon Digital Camera DXM1200F, Japan).

Molecular identification of viral infection

Four organs (eyes, brain, spleen and kidney) of sick fish were used for molecular identification of viral infection following the method of Nurliyana et al. (2020). Each organ was pooled in an individual 1.5 micro tube. They were extracted using trizol following the protocol of Khumaidi et al. (2019). Reverse transcription of samples genomic RNA into DNA was carried out using GoScriptTM Reverse Transcription System (Promega, USA). GoTaq®PCR Core System (Promega, USA) was used for DNA amplification. Primers F2 and R3 at 426bp were used to amplify virus for NNV at amplification condition following the method of Nguyen et al. (1994). Genomic DNA from spleen and kidney were amplified using GoTaq®PCR Core System. Primers 1-F and 1-R at 570bp were used to amplify megalocytivirus at amplification conditions as described by Kurita et al. (1998). Meanwhile, the SDDV amplification conditions was applying the procedure and primers F and R at 643bp as reported by Koesharyani et al. (2020).

Five PCR products of positive SDDV were then purified by PCR purification kit (Innuprep PCR Pure kit 845-KS-5010050, Analytic Jena, Germany). Samples were then sequenced by 1st BASE provider (Singapore). DNA sequences were edited by BioEdit and analyzed by Mega-11 to know its distance. The BLAST analysis was used to know the homology with others known sequences. The DNA sequence was submitted to GenBank.

Viral inoculum preparation and challenge test

The specific inoculum for SDD was isolated from moribund or sick fish. Around 4mg of spleen and anterior kidney was ground using a sterile Grinder homogenizer (volume 20mL). The ground samples were then homogenated in 16mL of sterile phosphate buffer saline (PBS). The homogenate solution was centrifuged for 15min at 4°C and 6000g. The supernatant was separated using a 0.45µm microfilter, collected in 1.5mm Eppendorf tubes and kept in -80°C for later analysis.

A total of 90 healthy fish were divided equally into 3 groups of challenge treatments of intramuscular injection i.e., the first fish group (control) was injected with PBS, the second fish group was injected by 0-time dilution (100) of crude spleen suspension; the third fish group was injected by 10 times dilution (10^{-1}) of crude spleen suspension. Each group consists of 30 fish with 3 tank replicates (10 fish/tank). The observation of fish condition and mortality was conducted after 2 weeks of crude spleen suspension post-injection. Histological observations were conducted on the survived fish after 2 weeks of experimental SDDV challenge using similar method of histology for the natural sick fish.

Data analysis

All data were checked for normality and homogeneity of variance prior to parametric one-way ANOVA. The mean total bacteria and *Vibrio* sp. population was compared between skin ulcer and liver using a T-test analysis. The mean percentage of fish mortality following the experimental SDDV challenge was compared among different inoculum concentration using one-way ANOVA. When the analysis indicated significant difference at significance level of 0.05, further analysis of Tukey's post-hoc tests was used to identify which group is different to each other. A non-parametric analysis, Kruskal-Wallis test, was used when data violated the normality and homogeneity of variance.

RESULTS

Gross clinical and parasite observation

Clinical symptoms of sick fish included inability to maintain upright position, lethargic, decreased appetite and staying at the bottom tank, showing black body color, scale loss and exophthalmia. About 15 to 20% mortality was recorded three days from initial clinical signs. Parasitic infestation was not found on the mucus and gill lamellae of sick fish.

Bacterial observation

There was significant differences in the mean total bacteria ($\chi^2(1)=3.97$, $P<0.05$) and *Vibrio* sp. ($F(1,4)=412.19$, $P<0.05$) population isolated from liver and skin ulcer of the sick fish. The mean total bacteria and *vibrio* sp. population isolated in skin ulcer ($287.3\pm17.8x10^4$cfu.g^{-1} and $83.0\pm16.3x10^4$cfu.g^{-1}, respectively) were higher than liver ($2.6\pm0.1x10^4$cfu.g^{-1} and $0.5\pm0.1x10^4$cfu.g^{-1}, respectively) (Table 1).

Blood smear test

Sick fish in this study showed monocyte cells in blood (shown by arrow in Fig. 1a) and enlarged cells in in spleen (Fig. 1b).

Table 1: Mean bacterial population in the liver and skin ulcer of sick Asian seabass

Parameters	Population ($x10^4$)	
	Liver	Skin ulcer
Total bacteria (cfu g^{-1})	2.6 ± 0.1^a	287.3 ± 17.8^b
Vibrio sp. (cfu g^{-1})	0.5 ± 0.1^a	83.0±16.3b

Values (mean±SD) with superscript letters within the row are statistically different ($P<0.05$).

Histopathology observation of natural sick fish

The skin of sick fish in this study demonstrated lesions on scale loses areas (Fig. 2a) and cross section damage on muscles. The liver of the sick fish showed cell congestion (Fig. 2b) with cellular hemorrhage. The splenic tissue degeneration was shown with the presence of aggregation of multiple melanomacrophage centers (MMC) (Fig. 2c). Similar to liver, the anterior kidney of sick fish also showed cellular hemorrhage and degeneration (Fig. 2d). The posterior kidney was necrotic, showing fragmented interstitial cells and edema (Fig. 2e) and presented swollen nuclei with basophilic matter in cytoplasmic cell (Fig. 2f).

Viral observation

PCR test showed that the sick Asian seabass in this study were negative of both VNN and megalocytivirus shown by the missing bands at 426 and 570bp from gel electrophoresis. However, natural sick fish sampled in this study was positively infected with SDDV (shown by the presence of band at 643bp) (Fig. 3). The sequencing results

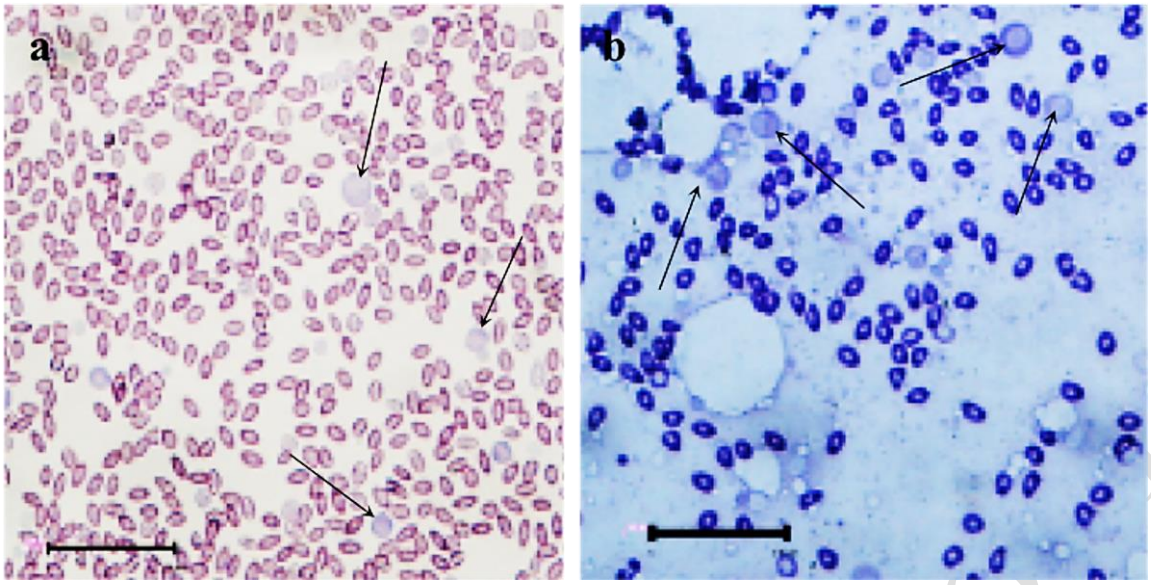

Fig. 1: Sick Asian seabass with monocyte cells in blood (arrow) (a) and enlarged splenic cells (arrow) (b) (scale bar: 50µm).

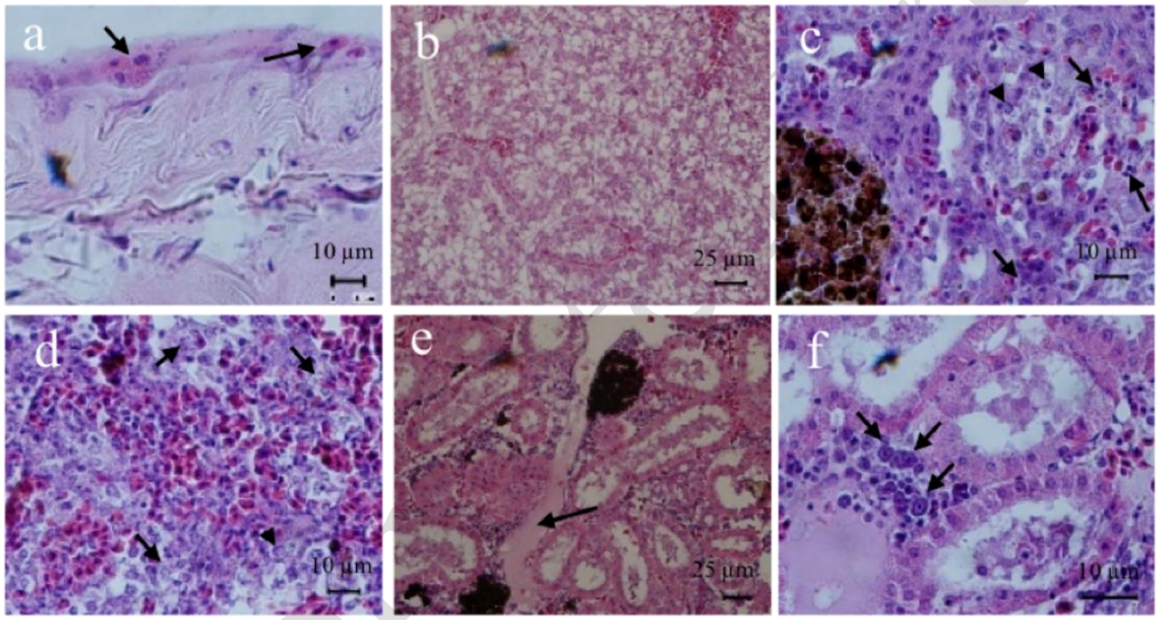

Fig. 2: Histopathology in organs of natural sick Asian seabass. Skin with perivasculitis (a), liver (b), spleen (c) and anterior kidney (d) with nuclear pyknosis (arrow) and marginal hyperchromatosis (arrow head), posterior kidney with oedema (e) and swollen nuclei (f). H and E Staining.

showed that three PCR products of 250, 298 and 598kb were 100% homologous. The GenBank accession number for DNA sequence data in this study were OR507239.1 (Strain Bali02) and OR507238.1 (Strain Bali03).

Experimental challenge test

The mean mortality of fish following injection by SDDV inoculum ranged from 0.0±0.0% to 100.0±0.0%. There was significance difference on the mean mortality of fish across treatment ($\chi 2=7.00$, $P<0.05$). The mortality of fish injected with 0 dilution of inoculum (96.7±0.0%) and 10 times inoculum dilution (100.0±0.3%) was not significantly different, but both were higher than the mortality of fish from control group (0.0±0.0%). The mortality of fish following viral injection started at day 5 post-injection (Fig. 4).

Experimental SDDV injected Asian seabass did not demonstrate any scale drop. A cross section of dorsal skin

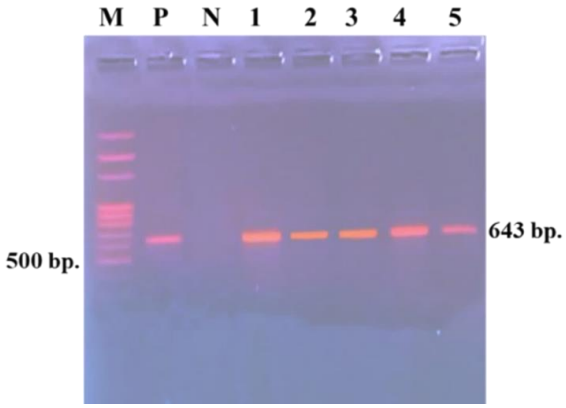

Fig. 3: Gel electrophoresis results using SDDV primer F 5' AACTTCAGTAGCGGGTG 3' and R 5' GACTCATCTCTACGGTGGCG 3' (M: 100bp marker, P: positive control, N: negative control, 1-5: sample of sick Asian seabass).

revealed normal dermis and muscle anatomy. The liver showed hemorrhage (Fig. 5a). The spleen revealed hemorrhage, cell degeneration, nuclear pyknosis, and marginal hyperchromatosis in hematopoietic cells (Fig. 5b). The anterior kidney revealed necrosis with nuclear pyknosis and many cells with marginal hyperchromatosis (Fig. 5c). The posterior kidney presented MMC, necrosis, and cells with marginal hyperchromatosis (Fig. 5d), but was absent of oedema.

DISCUSSION

The gross clinical examination on sick Asian seabass juveniles in this study showed that there were no parasites recorded in the mucus and gill lamellae. These findings are contradictory to the previous study of Rückert et al. (2008), where high parasitic infestation was found in Asian seabass collected from net cage in grow-out facilities in Lampung, Indonesia. The study reported that up to 19 species of parasites were found in the gill of living Asian seabass and the prevalence and intensity of infection was not seasonally dependent. The absence of parasite on the sick fish in the present study could be due to the fish were reared and grown in controlled rearing water in land based hatchery facilities. Therefore, parasite infestation is not likely to be the causative agent causing mass mortality event of Asian sea based sampled in this study.

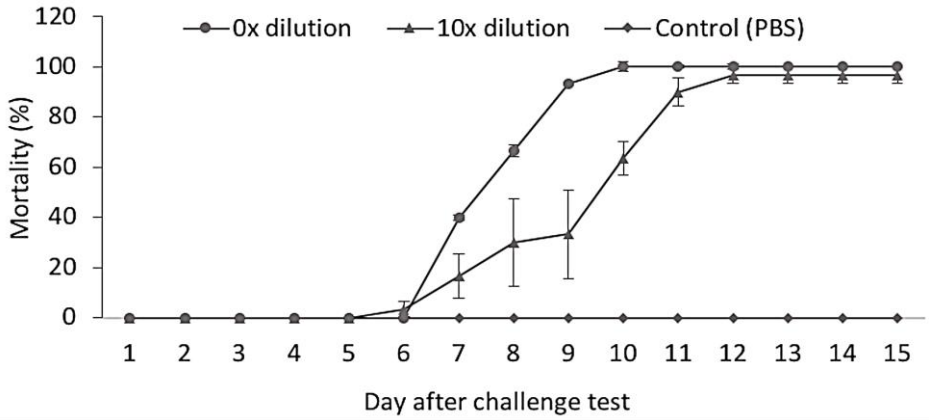

Fig. 4: Mean±SE percentage of daily mortality of Asian seabass following challenge test with SDD virus inoculum.

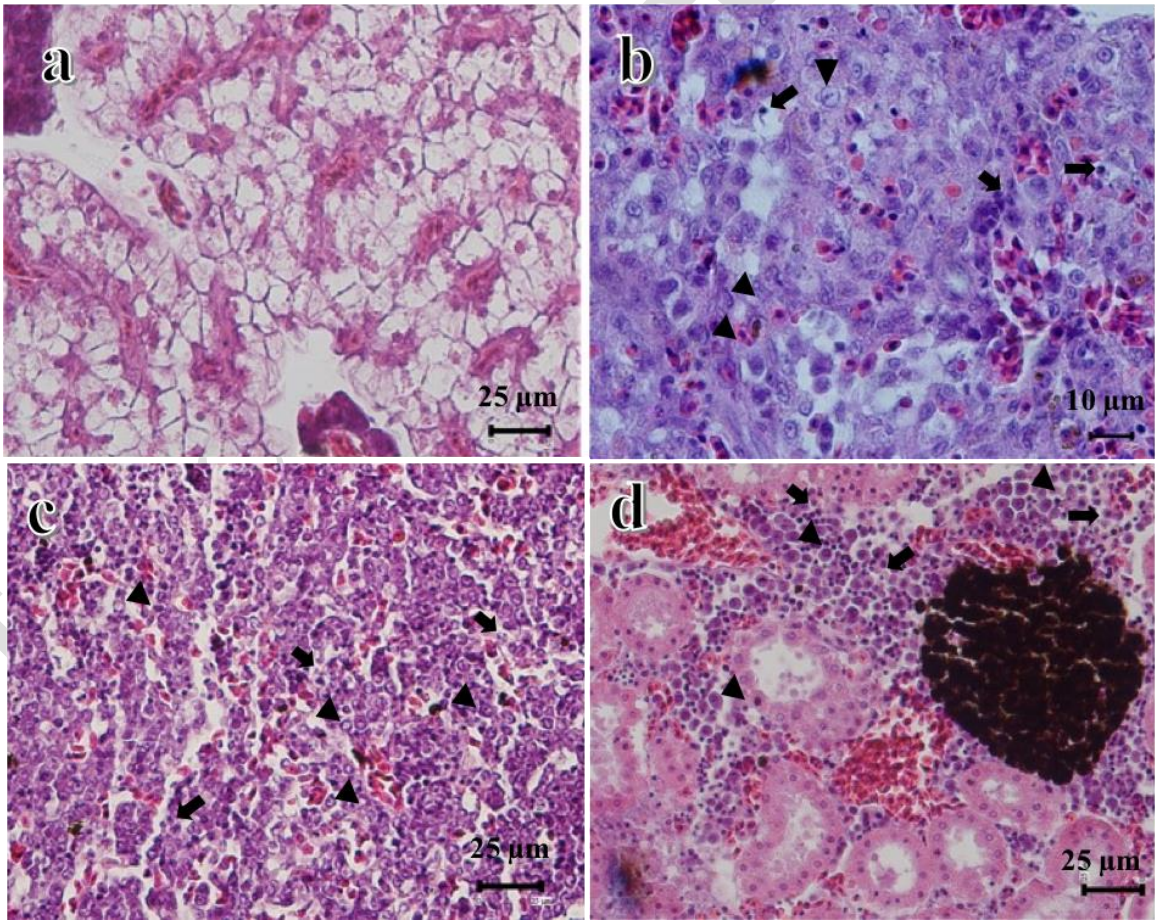

Fig. 5: Histopathology experimental SDDV injected Asian seabass. Liver with haemorrhage (a), spleen with cell degeneration, nuclear pyknosis (arrows) and marginal hyperchromatosis (arrow heads) (b), anterior kidney (c) and posterior kidney with cells necrotic and marginal hyperchromatosis (d). H and E Staining.

The natural sick fish in this study showed a severe detachment of scale which appeared to be the results of an intense dermal perivasculitis causing the inability of the skin tissue to support the scale bed. Vasculitis in major organs of fish e.g., skin, spleen, kidney has been reported to be the most distinctive histopathological condition of SDDV infection (Gibson-Kueh et al. 2012). Severe scale loss of the affected fish is likely to further compromise health of fish due to the increasing vulnerability to opportunistic pathogen infection. In this study, the total bacteria in skin ulcers were still within moderate limits, but the population of *Vibrio* sp. sampled from skin ulcer were high according to Mahardika et al. (2021).

Molecular detection for viral infection using specific primer in this study failed to detect the presence of NNV and megalocity virus. Therefore, NNV and megalocity virus is not likely to be the causative agents of the mass mortality event recorded in Asian seabass hatchery in Bali, Indonesia. The initial clinical examination on the sick fish showed several common symptoms of SDDV infection such as lethargy, scale loss, darkening body color as reported by Nurliyana et al. (2020). The infection of SDDV on the fish in this study was confirmed by molecular detection applying corresponding primer for the virus. The primer used in this research was retrieved from ORF 60 of SDD virus genome which was flanked at 643 unique region. In 2020, this primer was used to identify sick Asia seabass in Riau Island, Indonesia and the study found that the SDD isolate was identical to SDD found in Singapore (Koesharyani et al. 2020).

The SDDV infection on fish in this study appears to concur concurrently with the presence of *Vibrio* sp. in both skin and liver. The higher population of total bacteria and *Vibrio* sp. in skin than in liver indicates that pathogenic *Vibrio* sp. in this study is likely to be secondary agents following the detachment of scale and multifocal hemorrhage on the skin. Both SDDV and pathogenic bacterial infection has been reported to cause ulcerative lesions but they have a distinctive characteristic (Kerddee et al. 2020). The lesion damage caused by pathogenic bacteria is commonly limited to external organ (e.g., skin, gill, fin), while SDDV lesion affects both external and major internal organs (Pongnumpai and Chitmanat 2017; Kerddee et al. 2020). Furthermore, histopathological alteration associated with viral tropism for liver and hematopoietic organs (spleen and kidney) shown by tissue generation, hemorrhage and necrosis in natural and experimental SDDV injected fish presented in this study are distinctive feature of SDDV infection, consistent with the findings of previous studies (Gibson-Kueh et al. 2012; Senapin et al. 2019).

Conclusion

The current study showed that the main causative agent of mass mortality of Asian seabass from hatchery facilities is SDDV infection. The common symptom of SDDV infection was consistently shown from clinical assessment, molecular detection and histopathological observation. The presence of pathogenic bacteria, *Vibrio* sp., in sick fish appears to be mediated by the massive losses of scale following SDDV infection, which is likely to further compromise the health of fish leading to mortality. The overall findings in this study provide basis information for future studies in identifying appropriate treatments for the SDDV infected fish.

Conflict of Interest: All authors confirmed that there is no conflict of interest to disclose.

Author Contribution: Ketut Mahardika: Conceptualization, methodology, investigation, data analysis, visualization, writing-original draft. Indah Mastuti: Investigation-bacterial isolation and viral inoculum. I Gusti Ngurah Permana: Investigation-clinical examination and challenge test. Zafran: Investigation-challenge test. Isti Koesharyani: Investigation-molecular characterization of virus. Suko Ismi: Resource-providing experimental animals for challenge test. Ni Wayan Widya Astuti: Investigation-blood smear and hematopoietic examination. Ahmad Muzaki: Investigation-sample isolation for laboratory observation. Supono Supono: Writing-Original draft, writing-review and editing, data analysis, visualization. Rommy Suprapto: Writing-review. Warih Hardanu: review. I Made Merdana: Investigation-virus sequencing. I Gusti Ngurah Kade Mahardika: Supervision, writing-review and editing.

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