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Sequential development of histopathological manifestations in response to experimental infection of *Vibrio alginolyticus* in Asian Seabass

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Original Article

Abstract

Asian seabass (*Lates calcarifer*), an important species for mariculture in the Asia-Pacific region, has been threatened by various infectious diseases hampering the profitability of its aquaculture. Vibriosis is the most common disease leading to considerable mortality and economic loss. Recent studies revealed that *Vibrio alginolyticus* is one of the most common species that causes vibriosis in farmed aquatic animals, including *L. calcarifer*. Despite our understanding of the aetiology, there is little information on the subsequent histological changes following experimental infection in fish. The present study fills this gap by investigating the sequential histopathological alterations in Asian seabass post intraperitoneal injection with *V. alginolyticus*. Significant organ changes were seen in the spleen, kidneys, liver, and to a lesser extent, the gills. The findings revealed an initial latency before major tissue responses occurred, with the kidney exhibiting the earliest and most severe changes. The spleen and liver also showed substantial alterations, while the gills showed minor changes. Experimental *V. alginolyticus* infection resulted in 90-95% mortality within 24-48 hours post-challenge. Our findings contribute to a more thorough understanding of histological changes in Asian seabass during *V. alginolyticus* infection, highlighting the importance of disease progression, and mitigation measures.

Keywords: *Vibriosis, Lates calcarifer, disease, aquaculture, histopathology*

Introduction

With the escalation of global food insecurity (Mnisi *et al*., 2021), which is further amplified by changing climates and pandemics such as COVID, enhancing food production to feed ever increasing population has become imperative. Aquaculture, known to be the fastest-growing food-producing sector, has drawn increased attention for its potential to meet a substantial part of human nutrient requirements. In recent years, the Asian Seabass (*Lates calcarifer*) has emerged as a prominent species for cage aquaculture, especially in the Asia Pacific due to its euryhaline nature and meat quality (Ail and Bhatta, 2016; Venkatachalam *et al*., 2018). While the growth of aquaculture is encouraging, it is challenged by the occurrence of diseases, predominantly those caused by bacteria that can threaten the sustainability of farming (Mohd Yazid *et al*., 2021)

Vibriosis, a prominent bacterial disease in marine aquaculture, affects many fish and shellfish species. Several species under the genus *Vibrio*, *eg. V. anguillarum, V. damsela, V. alginolyticus, V. vulnificus, V. harveyi V. salmonicida* and *V. ordalii,* cause mortality and subsequent economic loss in fish farms (Sanches-Fernandes *et al*., 2022). Of particular concern is *Vibrio alginolyticus*, a halophilic Gram-negative bacterium, which is generally considered to be a part of marine bacterial flora (Carli *et al*., 1993). It is pathogenic to humans (Bauer and Young, 2000) and several marine animals, including trout (Austin *et al*., 1993), grouper (Lee, 1995), seabream (Balebona *et al*., 1998), sea horse (Martins *et al*., 2010), Asian Seabass (Krupesha *et al*., 2013) and cobia (Rameshkumar *et al*., 2014, 2017). Infection of cultured fish due to *V. alginolyticus* is associated with stress factors inflicting the cultured animals. Thus, *V. alginolyticus* represents a serious pathogen that not only raises public concerns but also incurs considerable economic losses in aquaculture. Infection and pathology of cage-cultured fin fish caused by *V. alginolyticus* have been reported in Crimson snapper

(Cai *et al.*, 2013) and large yellow croaker (Chen *et al.*, 2008). In India, natural infection with *V. alginolyticus* has been reported in cage reared Asian seabass (Krupesha *et al.*, 2013) and cobia (Rameshkumar *et al.*, 2014, 2017).

Understanding histopathology and lesion development has significantly contributed to elucidating the pathogenesis of vibriosis in finfish. Several studies have explained the microscopic lesions in vital organs of fin fish infected naturally with *V. alginolyticus*, which were characterized by hyperaemia and haemorrhages. Experimental infections in the above cases were conducted to prove the Koch's postulates.

Since 2012, a highly virulent *V. alginolyticus* strain has caused severe acute mortality (5% of the total population) in cage-cultured Asian seabass in India (Krupesha *et al.*, 2013). Researchers report that typical clinical symptoms of affected fish include anorexia, weariness, skin darkening, tail and fin rot, eye opacity, generalized erythema, inflammation, liquefaction of internal organs, hemorrhagic ulcers, and necrosis across vital organs (Ransangan *et al.*, 2012; Ransangan and Mustafa, 2009; Talpur *et al.*, 2013; Talpur and Ikhwanuddin, 2012). Though there have been a few studies undertaken on the clinical signs and descriptions of internal lesions in naturally and experimentally infected fishes, information on the histopathological changes during the progression of this disease is scarce, such information is valuable for making predictions about the prognosis. Therefore, our study presents a sequential in-depth characterization of the histopathological changes in Asian seabass from zero hours to 48 hours after the intraperitoneal injection challenge.

Material and methods

Experimental samples

A total number of 100 healthy Asian Seabass (*Lates calcarifer*) (14 ± 3 g, 8 ± 2 cm length) were collected from the Rajiv Gandhi Centre for Aquaculture (RGCA) and transported to the laboratory. The fish were acclimated to the laboratory conditions for two weeks before the experiment (water quality parameters were observed as pH: 7.6±0.31, salinity: 35±0.5%, dissolved oxygen: 6.1±0.3 mg/L). Random specimens were taken for bacteriological examination to ensure that the fish was non-infected.

Preparation of inoculum

V. alginolyticus (CMFRI-JF260912), isolated from moribund sea cage cultured Asian Seabass (Krupesha *et al.*, 2013), was used for the experiment and prepared as I/P injections, according to Austin and Austin (1999). Briefly, the bacterial isolate was sub-cultured on trypticase soya agar plates with 2% NaCl and incubated at 30 °C for 24hrs. A typical isolated colony was picked up and inoculated into trypticase soya broth with 2% NaCl and incubated at 30 °C for 24 hrs. The broth culture was centrifuged and the supernatant was decanted out. The sediment was resuspended in PBS and adjusted to an optical density (OD) of 1 at 600 nm (each ml contained approximately 1×10^8 bacterial cells). The LD_{50} of a particular *V. alginolyticus* strain to Asian seabass was found to be $10^{4.05}$ CFU/g fish by Reed and Muench (1938).

Experimental design

A total number of 90 healthy juvenile Asian Seabass were selected after the period of acclimation and divided into three groups consisting of 30 fish in each group. Fish were kept in prepared glass aquaria (90 x 50 x 35 cm). Each group consisted of triplicate tanks of 10 fish per tank. Continuous aeration was maintained in each aquarium using electric air-pumping compressors. Fish were fed on a commercial fish diet containing 45% crude protein. The diet was provided daily at a fixed feeding ratio of 5 % body weight of the fish. The first two groups were inoculated intraperitoneally with *V. alginolyticus* suspension at a dose rate of 0.1 ml (10^4 CFU/ml) per fish. The third group of fish which were injected with 0.1 ml of PBS served as experimental control.

Histopathology

Three fish from each group were collected for histopathological investigation at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h post-challenge (HPC) after euthanization using an overdose of tricaine methanesulfonate-MS222 (Sigma-Aldrich, USA) (300 mg/l). Representative samples of liver, kidney, spleen, and gill from challenged and control fish were preserved in 10% neutral buffered formalin for histopathology for 24-48 hours and stored in 70% ethanol until processing, following standard histological procedures. Paraffin sections were cut (5-6 µm thickness) using a Leica Microtome (Leica, Wetzlar, Germany), deparaffinised, and stained with Harris Hematoxylin and Eosin (H&E). Sections were examined under a research microscope at various magnifications and conspicuous images were captured (Nikon Eclipse 80i, Tokyo, Japan).

Bacterial isolation and identification

Post the injection challenge, attempts were made for bacterial isolations from the liver, kidney, spleen, and blood on tryptone soya agar supplemented with 2% NaCl and thiosulphate citrate bile salt sucrose agar (TCBS; Himedia). The samples were incubated at room temperature for 24-48 hours to obtain

visible bacterial growth. Total genomic DNA was extracted from bacteria grown on LB broth for 24 h using phenol-chloroform extraction method (Sambrook and Russell, 2006). The 16S rRNA gene from the genomic DNA was amplified using universal primers. The amplified products were purified and sequenced. The bacterial identity was determined by searching the GenBank database using the BLASTN algorithm (Altschul *et al.*, 1997).

All experimental procedures involving live fish were carried out following the ARRIVE guidelines (Percie Du Sert *et al.*, 2020), ensuring the ethical treatment and welfare of the animals. The protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee of ICAR-Centrral marine Fisheries Reseafch Institute, Kochi, to ensure compliance.

Results

Gross signs and clinical pathology

Clinical signs observed in experimentally infected Asian seabass included lethargy, anorexia, abnormal swimming patterns, or spinning within 24 hrs. Haemorrhagic lesions on the skin, abdominal distension, exophthalmia, darkened skin, and mortality were seen within 48 hours.

Bacterial isolation and characterization

Repeated bacterial isolation attempts on TCBS plates from different diseased fish at various time intervals (2, 4, 6, 8, 10, 12, 24, and 48) gave morphologically similar bacterial isolates. Molecular identification confirmed the isolate as *V. alginolyticus.*

Histopathological findings

No significant changes were observed between 0 to 10 HPC. From 12 h and until 48 h spleen, liver, and kidney showed lesions in the majority of examined fishes. The spleen of the control fish revealed a normal structure of the red pulp with compact ellipsoids and a few melano-macrophage centres (MMC) (Fig. 1A). At 10-48 HPC, splenomegaly with a massive expansion of red pulp by many erythrocytes and ellipsoidal compression of white pulp were observed. At 12 HPC, approximately 95–100% of the spleen showed an increase in red pulp and accumulation of erythrocytes in the white pulp, increased MMC and hemosiderin deposits (Fig. 1B). At 24 HPC, loosening of tissue structure and haemorrhage in splenic parenchyma in addition to hemosiderosis were seen (Fig. 1C). While at 48 HPC, severe splenitis with diffuse

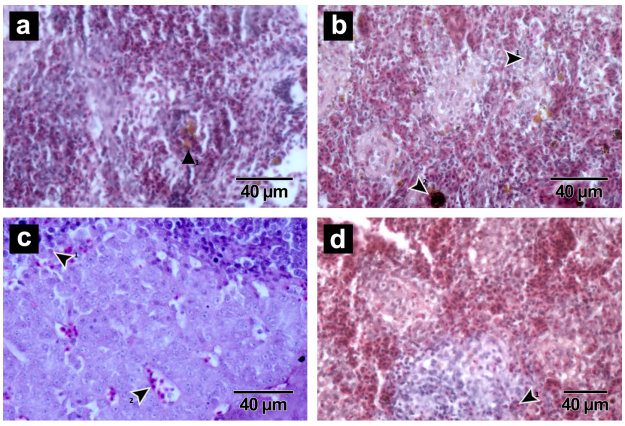

Fig. 1. Photomicrograph of control and infected Asian Seabass spleen. (A) Photomicrograph of the control spleen showed normal architecture and no significant changes were observed. Only few melano-macrophage centres (MMC) (arrow 1), 400, H&E. (B) Infected spleen showed increase in red pulp and accumulation of erythrocytes in white pulp (arrow 1), increased MMC and hemosiderin at 12 HPC (arrow 2), 400, H&E (C) Moderate splenitis with loosening of tissue structure (arrow 2) and haemorrhage in splenic parenchyma in addition to hemosiderosis (arrow 1) at 24 HPC, 400, H&E. (D) Severe splenitis with diffuse fibrinoid necrosis of ellipsoidal sheath and aggregation of inflammatory cells at 48 HPC (arrow 1), 400, H&E

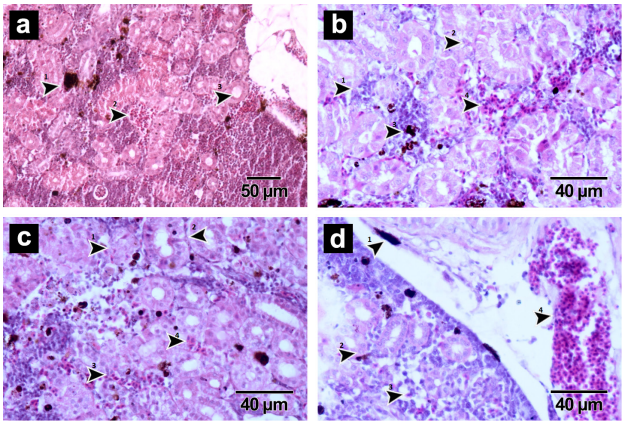

Fig. 2. Photomicrograph of control and infected Asian Seabass kidney. (A) Normal organization of control kidney with mild changes present, haemorrhage (arrow 2), MMC (arrow 1) and tubular degeneration (arrow 3), 500, H&E. (B) Infected kidney showing haemorrhage (arrow 3), infiltration with inflammatory cells in the interstitium (arrow 1), and tubular degeneration (arrow 2), hemosiderin deposits (arrow-4) at 12 HPC 400, H&E. (C) Diffuse necrosis of renal tubule (arrow 2), increase in number of hemosiderin deposits, loosening of tissue structure and severe haemorrhage (arrow 1, 3&4) at 24 HPC, 400, H&E. (D) Severe interstitial nephritis replaced the renal tubules (arrow 2), haemorrhage in white pulp (arrow 4), increased MMC and hemosiderin deposits (arrow 1), extensive congestion and haemorrhage (arrow 3) at 48 HPC 400, H&E

fibrinoid necrosis of ellipsoidal sheath and aggregation of inflammatory cells were observed (Fig. 1D). Hemosiderosis were seen in the majority of examined fish at different time points.

Kidneys of the control group exhibited normal organization of nephrons and tubules with a few MMC scattered in the parenchyma (Fig. 2A). At 12 HPC, approximately 75% of kidneys

showed haemorrhage, infiltration with inflammatory cells in the interstitium, tubular degeneration, and hemosiderin deposits (Fig. 2B). At 24 HPC, diffuse necrosis of renal tubule, increase in the number of hemosiderin deposits, loosening of tissue structure and severe haemorrhage were seen (Fig. 2C). At 48 HPC, severe interstitial nephritis, haemorrhage, increased MMC, hemosiderin deposits, extensive congestion and haemorrhage were seen (Fig. 2D).

Normal sinusoids and polygonal hepatocytes with mild fatty change were seen in the control group (Fig. 3A). Only mild lesions were observed in the liver at 2–10 HPC. At 12 HPC, the liver showed moderate congestion, and haemorrhage (Fig. 3B). At 24 HPC, there was pyknosis, severe haemorrhage and congestion (Fig. 3C). After 48 HPC, liver tissue showed hydrophic degeneration of hepatic cells, cellular swelling, and hepatic congestion with extremely dilated sinusoidal space and congestion in the pancreatic tissue (Fig. 4D).

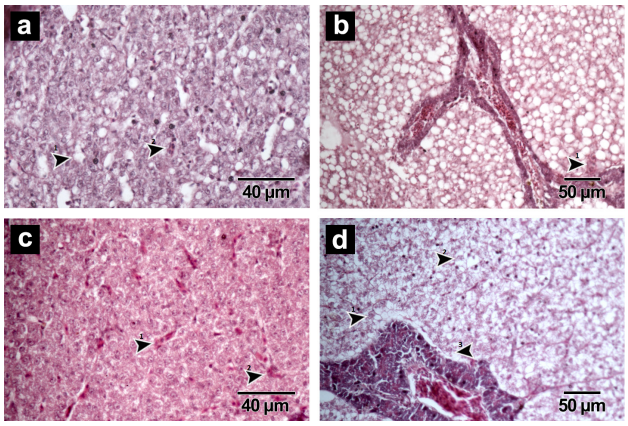

Fig. 3. Photomicrograph of control and infected Asian Sea bass liver. (A) The control fish liver shows the normal organization of polygonal hepatic cells (arrow 1) with mild congestion (arrow 2); some fatty changes appear due to feeding habits, 400, H&E. (B) Infected liver showed moderate congestion, moderate haemorrhage and large blood accumulation in hepatic cells (arrow 1) at 12 HPC, 500, H&E. (C) At 24 HPC shows pyknosis (arrow1), severe haemorrhage and moderate congestion (arrow 2), 400, H&E (D) After 48 HPC liver tissue shows hydrophobic degeneration of hepatic cells, cellular swelling (arrow 2), hepatic congestion with extremely dilated sinusoidal space (arrow 1) and congestion in the pancreatic tissue

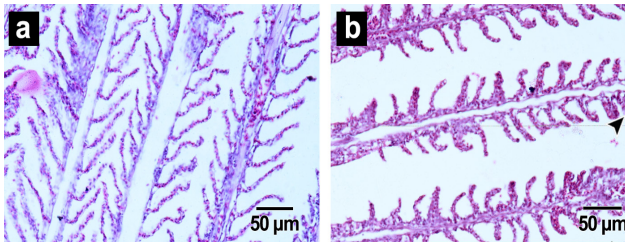

Fig. 4. Photomicrograph of control and infected Asian Seabass gills. (A) Control gills showed normal primary lamellae (PL) and secondary lamellae (SL), 500, H&E. (B) Infected gills showed thickening and clubbing of the primary and secondary lamellae (thick arrow) at 48 . HPC 500, H&E

Normal primary and secondary lamellae were seen in the gills of the control group (Fig. 4A). At 2–10 HPC, no detectable lesions were observed in any of the examined Seabass. From 12 to 24 HPC, sloughed epithelial cells of secondary lamellae were seen in the majority of examined gills. At 24 and 48 HPC, thickening of both primary and secondary lamellae in 75 – 80% of the gill lamellar surface, in addition to clubbing of the secondary and primary lamellae, were observed (Fig. 4B).

Discussion

Diseases caused by opportunistic bacteria are a major bottleneck in the economic sustainability of marine cage fish farming. Natural disease outbreaks in aquaculture are normally associated with concurrent infections with a homologous or a heterologous pathogen, which either enhances or suppresses the severity of the disease caused by the primary pathogen (Kotob *et al.*, 2016). Experimental challenge studies enable us to understand the time series analysis of sequential pathological manifestations in the tissues due to bacterial invasion. In this context, the present paper attempts to describe the sequential histopathological changes in the vital organs of a commercially important marine fish candidate species, *L. calcarifer* injection challenged with a virulent strain of *V. alginolyticus*. Experimental infection, while being fundamentally different from the natural infection pathway, provides a considerable advantage. It enables consistent replication of infections as well as the tracking of histopathological changes over time, offering crucial insights into the disease progression (Conforto *et al.*, 2021). The selected pathogen is a pervasive marine bacterium, known to induce vibriosis in numerous marine fish species, including Asian seabass (Lim *et al.*, 2021). While *Vibrios* infect fish in both hatchery and grow-out facilities, juveniles are reported to be more susceptible to *Vibrio* infections possibly due to low resistance to pathogens (Ina-Salwany *et al.*, 2019; Mohamad *et al.*, 2019; Shen *et al.*, 2017). Hence, juvenile fish were conveniently selected for inducing experimental disease in the present study.

In the present study, Asian seabass subjected to infection showed various clinical manifestations including reduced activity, loss of appetite, atypical swimming behaviour, and spinning within 24 hours. Additionally, within 48 hours, there were observable clinical signs such as hemorrhagic skin lesions, swollen abdomen, protruding eyes, and darkened skin. 90 -95% mortality occurred within 24-48 hours post-challenge. Martins *et al.* (2010) noted necrosis on the mouth epithelium and 100% mortality within 24 hours after the experimental infection of a seahorse (*Hippocampus reidi*) with *V. alginolyticus*, by immersion. In the case of cobia, the initial clinical manifestations encompassed decreased appetite, slow swimming, frequent surfacing, and bilateral

exophthalmos, when juveniles were experimentally infected with *V. alginolyticus* (Rameshkumar *et al*., 2017). Similar clinical signs were also noted when various *Vibrio* species were introduced via intraperitoneal injection into multiple fish species. *V. harveyi* was capable of causing mortality in white snook (*Centropomus viridis*) within 10 hours (Soto-Rodriguez *et al*., 2019). In the case of sea bass (*Dicentrarchus labrax*), mortality manifested within 48 hours (Firmino *et al*., 2019). Additionally, *V. ponticus* resulted in mortality within 25 hours for white snook and *V. cholerae* led to mortality in Indian major carp (*Labeo rohita*) within 84 hours (Devi *et al*., 2022).

The experimental challenge in the present study led to an acute to sub-acute nature of vibriosis. It is reported that haemorrhagic lesions in the internal organs with high mortality mark the sub-acute disease (Shen *et al*., 2017), while acute vibriosis is marked by lethargy, abnormal swimming behaviour and petechiae on the skin, indicating septicemia, with low mortality (Mohi *et al*., 2010). Asian seabass naturally infected with *V. alginolytics* displayed the presence of widespread hemorrhagic patches and ulcers on the body surface and haemorrhagic lesions in the liver, kidney, intestines, viscera, and enlargement of visceral organs (Krupesha *et al*., 2013). Conversely, in the case of experimental infections, no hemorrhagic lesions were observed on the external body surface.

In the present study, four vital organs, *viz* spleen, kidney, liver and gills were subjected to detailed sequential histopathology following intraperitoneal injection of pathogenic *V. alginolyticus*. In all the organ studies, the initial histo-morphological response to pathogen challenge consisted of circulatory changes like blood congestion, haemorrhage and hemosiderin deposits. Inflammatory reactions were seen only in the kidneys and spleen. However, the spleen revealed inflammatory reactions at 48 hours post the challenge, while these responses were seen at the 12th hour in the case of the kidney. In a previous report, the experimentally infected European seabass tissues with *V. alginolyticus* showed decreased number of melano-macrophages and severe congestion of blood vessels in the spleen (Mahmoud *et al*., 2018). Additionally, *L. calcarifer* experimentally infected with *V. anguillarum* revealed thickened ellipsoids and proliferation of MMCs in the spleen (Azad *et al*., 2004). An increased presence of MMCs, the contraction of renal tubules, and the shedding of tubular epithelial cells have also been documented in the kidney of *L. calcarifer* infected with *V. harveyi* (Dong *et al*., 2017).

No inflammatory reactions were observed in the liver and gills in the present study. In a previous study conducted by Dong *et al.* (2017) the liver of the infected seabass displayed blood congestion and haemorrhage in *V. harveyi* infection. Significant histopathological alterations were observed in the liver, including haemorrhage, varying degrees of hepatocellular degeneration, hepatocyte necrosis, and the occurrence of many melano-macrophage centres, Severe liver haemorrhage was documented in tilapia (*Oreochromis niloticus*) naturally infected with *V. vulnificus* (Sumithra *et al*., 2019). Inflammation in the visceral organs has been reported in white snook experimentally infected with *V. harveyi* (Soto-Rodriguez *et al*., 2019). However, no inflammatory lesions were seen in the liver in the present study. This may be because the host and the *Vibrio* species could significantly affect the outcome and severity of the infection. Moreover, bacterial colonization of the host's mucosal surface represents the initial stage in bacterial pathogenesis. The achievement of effective colonization is also impacted by factors originating from both the host and the pathogen (Manchanayake *et al*., 2023).

In our study, mild and transient changes and tissue alterations were observed in the gills. *Vibrio alginolyticus* immersion challenge in *H. reidi* causes the gills significant alterations including the loss of cellular integrity, particularly seen in hyperplastic epithelial cells and the secondary lamellae experienced effects like displacement and fusion, indicating substantial impacts on the gill structure following the infection (Martins *et al*., 2010). On the contrary, milder changes in the gills in this study may be attributed to the intraperitoneal administration of the bacterial pathogen, which likely caused a more direct and intense effect on the internal organs compared to the gills.

The *Vibrio* pathogen and its toxins are present in the circulation of the infected fish due to the septicemic nature of the infection. Hence, the toxins can exert their action on all the internal organs. The substantial histopathological changes caused in the spleen, kidneys, and liver emphasise the pathogenicity of the *Vibrio* strain and underline the need for further research into disease prevention strategies, prophylactic measures, as well as effective treatments for *V. alginolyticus* infections in Asian seabass. Good aquaculture farming practices, selective utilization of controlling agents, and proper vaccination programs are crucial in improving fish health, reducing disease outbreaks, and decreasing the economic impact on aquaculture farming (Ina-Salwany *et al*., 2019).

Conclusion

The discernible histopathological modifications observed in the head kidney, spleen, and liver underscored their pivotal roles in mounting immune responses during infection. While minor changes were detected in the gills, significant transformations predominantly unfolded between the 12-hour

and 48-hour intervals. These findings provide insight into the host-pathogen interactions in Asian seabass against vibriosis and lay the foundation for a more detailed study focusing on cellular and molecular responses, to pave the way for potential strategies aimed at disease management in aquaculture.

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References

Ail, S. K. S. and R. Bhatta. 2016. Small-scale Cage Culture of Asian Seabass in Kundapur Region, Karnataka, India. *WORLD AQUACULTURE*, 47 pp.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.*, 25 (17): 3389-3402.

Austin, B., M. Stobie, P. A. W. Robertson, H. G. Glass, J. R. Stark and M. Mudarris. 1993. *Vibrio alginolyticus*: The cause of gill disease leading to progressive low-level mortalities among juvenile turbot, *Scophthalmus maximus* L., in a Scottish aquarium. *J. Fish. Dis.*, 16 (3): 277-280.

Azad, I. S., A. R. Thirunavukkarasu, M. Kailasam and J. J. S. Rajan. 2004. Virulence and histopathology of *Vibrio anguillarum* like (VAL) bacterium isolated from hatchery produced juveniles of *Lates calcarifer* (Bloch). *Asian Fish. Sci.*, 17 (1-2) : 101-110.

Balebona, M. C., I. Zorrilla, M. A. Moriñigo and J. J. Borrego. 1998. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Aquaculture*, 166 (1-2): 19-35.

Bauer, J. C. and C. M. Young. 2000. Epidermal lesions and mortality caused by vibriosis in deep-sea Bahamian echinoids: A laboratory study. *Dis. Aquat. Org.,* 39 (3): 193-199.

Cai, S., Y. Lu, J. Jian, B. Wang, Y. Huang, J. Tang, Y. Ding and Z. Wu. 2013. Protection against *Vibrio alginolyticus* in crimson snapper *Lutjanus erythropterus* immunized with a DNA vaccine containing the ompW gene. *Dis. Aquat. Org.*, 106 (1): 39-47.

Carli, A., L. Pane, L. Casareto, S. Bertone and C. Pruzzo. 1993. Occurrence of *Vibrio alginolyticus* in Ligurian Coast Rock Pools (Tyrrhenian Sea, Italy) and Its Association with the Copepod *Tigriopus fulvus* (Fisher 1860). *Appl. Environ. Microbiol.*, 59 (6): 1960-1962.

Chen, Q., Q. Yan, K. Wang, Z. Zhuang and X. Wang. 2008. Portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker Pseudosciaena crocea, and characteristics of bacterial adhesion to mucus. *Dis. Aquat. Org.*, 80: 181-188.

Conforto, E., L. Vílchez-Gómez, D. Parrinello, M. G. Parisi, M. A. Esteban, M. Cammarata, and F. A. Guardiola. 2021. Role of mucosal immune response and histopathological study in European eel (*Anguilla anguilla* L.) intraperitoneal challenged by *Vibrio anguillarum* or *Tenacibaculum soleae. Fish. Shellfish. Immunol.*, 114: 330-339.

Devi, M. S., P. Paria, V. Kumar, P. K. Parida, P. Maurye, B. K. Behera and B. K. Das. 2022. Molecular identification and pathogenicity study of virulent *Vibrio cholerae* non 01/ 0139 serotype associated with mortality of farmed *Labeo rohita* (Hamilton, 1822), in India. *Aquaculture*, 547: 737529.

Dong, H. T., S. Taengphu, P. Sangsuriya, W. Charoensapsri, K. Phiwsaiya, T. Sornwatana, P. Khunrae, T. Rattanarojpong and S. Senapin. 2017. Recovery of *Vibrio harveyi* from scale drop and muscle necrosis disease in farmed barramundi, *Lates calcarifer* in Vietnam. *Aquaculture*, 473: 89-96.

Firmino, J., M. D. Furones, K. B. Andree, C. Sarasquete, J. B. Ortiz-Delgado, G. Asencio-Alcudia and E. Gisbert. 2019. Contrasting outcomes of *Vibrio harveyi* pathogenicity in gilthead seabream, *Sparus aurata* and European seabass, *Dicentrachus labrax. Aquaculture*, 511: 734210.

Ina-Salwany, M. Y., N. Al-saari, A. Mohamad, F. Mursidi, A. Mohd-Aris, M. N. A. Amal, H. Kasai, S. Mino, T. Sawabe and M. Zamri-Saad. 2019. Vibriosis in Fish: A Review on Disease Development and Prevention. *J. Aquat. Anim. Health*, 31 (1): 3-22.

Kotob, M. H., S. Menanteau-Ledouble, G. Kumar, M. Abdelzaher and M. El-Matbouli. 2016. The impact of co-infections on fish: A review. *Vet. Res.*, 47 (1): 98.

Krupesha Sharma, S. R., G. Rathore, D. K. Verma, N. Sadhu and K. K. Philipose. 2013. *V ibrio alginolyticus* infection in Asian seabass (*L ates calcarifer* , Bloch) reared in open sea floating cages in India. *Aquac. Res.*, 44 (1): 86-92.

Lee, K. K. 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch et Schneider. *Microb. Pathog.*, 19 (1): 39-48.

Lim, K. C., F. Md. Yusoff, M. Shariff and M. S. Kamarudin. 2021. Dietary astaxanthin augments disease resistance of Asian seabass, *Lates calcarifer* (Bloch, 1790), against *Vibrio alginolyticus* infection. *Fish. Shellfish. Immunol.*, 114: 90-101.

Mahmoud, S. A., Z. M. El-Bouhy, M. E. Hassanin and A. H. Fadel. 2018. *Vibrio alginolyticus* and *Photobacterium damselae* subsp. Damsel: Prevalence, Histopathology and Treatment in sea Bass *Dicentrarchus labrax. J. Pharm. Chem. Biol. Sci.*, 5(4): 354-364.

Manchanayake, T., A. Salleh, M. N. A. Amal, I. S. M. Yasin and M. Zamri-Saad. 2023. Pathology and pathogenesis of Vibrio infection in fish: A review. *Aquaculture Reports*, 28: 101459.

Martins, Ml., Jlp. Mouriño, Gf. Fezer, Cc. Buglione Neto, P. Garcia, Bc. Silva, A. Jatobá and Fn. Vieira. 2010. Isolation and experimental infection with *Vibrio alginolyticus* in the sea horse, *Hippocampus reidi* Ginsburg, 1933 (Osteichthyes: Syngnathidae) in Brazil. *Braz. J. Biol.*, 70 (1): 205-209.

Mnisi, C. M., M. Marareni, F. Manyeula and M. J. Madibana. 2021. A way forward for the South African quail sector as a potential contributor to food and nutrition security following the aftermath of COVID-19: A review. *Agric. Food. Secur.*, 10 (1): 48.

Mohamad, N., M. Mustafa, M. N. A. Amal, M. Z. Saad, I. S. Md Yasin and N. Al-saari. 2019. Environmental Factors Associated with the Presence of Vibrionaceae in Tropical Cage-Cultured Marine Fishes. *J. Aquat. Anim. Health*, 31 (2): 154-167.

Mohd Yazid, S. H., H. Mohd Daud, M. N. A. Azmai, N. Mohamad and N. Mohd Nor. 2021. Estimating the Economic Loss Due to Vibriosis in Net-Cage Cultured Asian Seabass (*Lates calcarifer*): Evidence From the East Coast of Peninsular Malaysia. *Front. Vet. Sci.,* 8: 644009.

Mohi, M. M., M. Kuratani, T. Miyazaki and T. Yoshida. 2010. Histopathological studies on *Vibrio harveyi* – infected tiger puffer, *Takifugu rubripes* (Temminck et Schlegel), cultured in Japan. *J. Fish. Dis.*, 33 (10): 833-840.

Percie Du Sert, N., V. Hurst, A. Ahluwalia, S. Alam, M. T. Avey, M. Baker, W. J. Browne, A. Clark, I. C. Cuthill, U. Dirnagl, M. Emerson, P. Garner, S. T. Holgate, D. W. Howells, N. A. Karp, S. E. Lazic, K. Lidster, C. J. MacCallum, M. Macleod and H. Würbel. 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Exp. Physiol.*, 105 (9): 1459-1466.

Rameshkumar, P., C. Kalidas, G. Tamilmani, M. Sakthivel, A. K. A. Nazar, V. Maharshi, K. S. Rao and G. Gopakumar. 2014. Microbiological and histopathological investigations of *Vibrio alginolyticus* infection in cobia Rachycentron canadum (Linnaeus, 1766) cultured in sea cage. *Indian J. Fish.,* 61 (1): 124-127.

Rameshkumar, P., A. K. A. Nazar, M. A. Pradeep, C. Kalidas, R. Jayakumar, G. Tamilmani, M. Sakthivel, A. K. Samal, S. Sirajudeen, V. Venkatesan and B. M. Nazeera. 2017. Isolation and characterization of pathogenic *Vibrio alginolyticus* from sea cage cultured cobia (*Rachycentron canadum* (Linnaeus 1766)) in India. *Lett. Appl. Microbiol.*, 65 (5): 423-430.

Ransangan, J., T. M. Lal and A. H. Al-Harbi. 2012. Characterization and Experimental Infection of *Vibrio harveyi* Isolated from Diseased Asian Seabass (*Lates calcarifer*). *Mal. J. Microbiol.,* 8 (2): 104-115.

Ransangan, J., and S. Mustafa. 2009. Identification of *Vibrio harveyi* Isolated from Diseased Asian Seabass *Lates calcarifer* by Use of 16S Ribosomal DNA Sequencing. *J. Aquat. Anim. Health*, 21 (3): 150-155.

Reed, L. J. and H. Muench. 1938. A Simple Method Of Estimating Fifty Per Cent Endpoints12. *Am. J. Epidemiol.*, 27 (3): 493-497.

Sambrook, J. and D. W. Russell. 2006. *The condensed protocols from molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press. 800 pp.

Sanches-Fernandes, G. M. M., I. Sá-Correia and R. Costa. 2022. Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System. *Front. Microbiol.*, 13: 904815.

Shen, G. M., C. Y. Shi, C. Fan, D. Jia, S. Q. Wang, G. S. Xie, G. Y. Li, Z. L. Mo and J. Huang. 2017. Isolation, identification and pathogenicity of *Vibrio harveyi* , the causal agent of skin ulcer disease in juvenile hybrid groupers *Epinephelus fuscoguttatus* × *Epinephelus lanceolatus. J. Fish. Dis.*, 40 (10): 1351-1362.

Soto-Rodriguez, S., R. Lozano-Olvera, S. Abad-Rosales, J. Martínez-Brown and L. Ibarra-Castro. 2019. Susceptibility of Pacific white snook *Centropomus viridis* to Vibrio species. *Dis. Aquat. Organ.*, 134 (3): 189-195.

Sumithra, T. G., K. J. Reshma, V. N. Anusree, P. Sayooj, S. R. K. Sharma, G. Suja, P. V. Amala, S. Joseph and N. K. Sanil. 2019. Pathological investigations of Vibrio vulnificus infection in Genetically Improved Farmed Tilapia (*Oreochromis niloticus* L.) cultured at a floating cage farm of India. *Aquaculture*, 511: 734217.

Talpur, A. D. and M. Ikhwanuddin. 2012. Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch). *Aquaculture*, 364–365: 6-12.

Talpur, A. D., M. Ikhwanuddin and A. M. Ambok Bolong. 2013. Nutritional effects of ginger (Zingiber officinale Roscoe) on immune response of Asian sea bass, *Lates calcarifer* (Bloch) and disease resistance against Vibrio harveyi. *Aquaculture*, 400–401: 46-52.

Venkatachalam, S., K. Kandasamy, I. Krishnamoorthy and R. Narayanasamy. 2018. Survival and growth of fish (*Lates calcarifer*) under integrated mangrove-aquaculture and open-aquaculture systems. *Aquaculture Reports*, 9: 18-24.