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Disease control measures in hirame hatchery: A case of hirame aquareovirus infection

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Extended Abstract: Japanese flounder *Paralichthys olivaceus*, commonly known as "hirame" in Japan, is a widely produced fish species mainly for stock enhancement in Japan. Approximately 20 million juveniles are produced annually for stock enhancement and aquaculture. Outbreaks of various diseases, such as viral nervous necrosis, birnaviral infection, and bacterial enteritis, have been hampering the production of hirame in hatcheries. In addition, mass mortality events associated with reovirus-like pathogens have been reported in some hirame hatcheries since the 2000s, which drastically impacts the production. Gross signs of the diseased fish are dark body color, abdomen swelling, and/or opaque viscera (clouding of the intestinal tract). The disease occurs in 20 to 50-day-old juveniles with cumulative mortality rates exceeding 80%. In contrast, mortality in adult fish has not been reported. The water temperature at which the outbreak has been reported is broad, ranging between 14.5 and 21°C. Hatchery staff members attempted to develop measures to mitigate mortalities associated with the disease by rearing fish in semi-saline water, lowering fish density, or treating them with sodium nifurstyrenate, however, none of them was effective.

Our research team has identified the causative agent as hirame aquareovirus (HAqRV) and developed several diagnostic tools. HAqRV is classified into the genus *Aquareovirus* of the family *Reoviridae*. This virus has double-stranded RNA as the viral genome in a non-enveloped virion. The virus actively replicates in the intestinal tract and liver, forming syncytia in which multiple cells are fused. Furthermore, we obtained findings suggesting that the vertical transmission from broodstock to juveniles is a primary transmission route, resulting in mass mortality events in juveniles. So, this study aimed to develop measures to prevent the vertical transmission of HAqRV.

To estimate the risk of vertical transmission, we checked the production process in hirame hatcheries. In hatcheries, wild fish are used as broodstock to maintain genetic diversity. Our previous study revealed that HAqRV was detected in approximately 30% of the wild fish caught for the broodstock. In general, hirame broodstock in a hatchery are maintained in a tank for years, so that we speculated that most of the broodstock are horizontally infected with HAqRV and become carriers of the viral agent. In addition, fertilized eggs, naturally spawned in a broodstock tank, are collected from the rearing water, and hence, the fertilized eggs could be contaminated with HAqRV released from the carrier broodstock. Based on these presumptive risks, we developed a series of control measures against HAqRV infection.

Our first approach was to develop a sublethal testing method for broodstock selection to detect and eliminate the virus-infected broodstock because our previous testing methods needed to kill broodstock. Our previous findings indicated that the intestinal tract was a major target organ of the virus. So, a medical swab was inserted

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from the anus for about 5 cm into the intestine of anesthetized broodstock and rubbed the intestinal wall several times to scrape the epithelium and syncytia of the intestinal tract. In order not to collect feces, fish were fasted prior to sampling. RNA was extracted from the swab specimens and a viral gene was detected by reverse transcription real-time PCR method. The intestinal swab test successfully detected the virus-infected fish, but we realized that the diagnostic sensitivity of this method was lower than that of the lethal sampling method which sacrifices broodstock and uses a portion of the intestine. Thus, the effectiveness of the broodstock selection using the intestinal swab test was assessed in an experimental setting by detecting and quantifying the viral loads in raring water and fertilized eggs from broodstock that had received the test. Wild hirame (approximately 1.0-4.0 kg), which were candidates for the broodstock, were separated into HAqRV-negative (n = 33) and HAqRV-positive (n = 43) groups by the intestinal swab test. These fish were housed in a 50-t tank, respectively. Then, spontaneous spawnings of both groups were induced by elevating water temperature. The HAqRV was detected in the raring water and fertilized eggs from the HAqRV-positive group, but not from the HAqRV-negative group, suggesting that broodstock selection using the intestinal swab test was effective in reducing viral loads in fertilized eggs and rearing water in broodstock tank.

The second approach was to disinfect fertilized eggs using electrolyzed seawater. Although the intestinal swab test was able to eliminate virus-infected broodstock shedding a large number of viral particles, the disinfection procedure for fertilized eggs was still required to minimize the risk of viral transmission from virus-infected broodstock that cannot have been detected by the broodstock selection. Electrolyzed seawater mainly contains oxidants of hypochlorous acid produced by electrolysis equipment and has strong bactericidal and virucidal effects. Electrolyzed seawater has been used in some hirame hatcheries in Japan as it has been reported to be effective in inactivating some fish pathogens. Hence, we decided to focus on optimizing a condition of electrolyzed seawater for treating fertilized eggs, which inactivates HAqRV but does not compromise the hatching of the fertilized eggs. To determine the concentration for inactivating the virus, the infectivity of HAqRV was confirmed using a cell line after treatment with electrolyzed seawater at a range of oxidant concentrations. HAqRV was inactivated by the electrolyzed seawater at an oxidant concentration of 0.25 mg/L for 1 minute. Next, we determined an appropriate oxidant concentration and treatment time of the electrolyzed seawater that does not affect the hatching of the fertilized eggs. We set initial oxidant concentration supplied by the electrolysis equipment at 0.75 mg/L based on previous reports. The oxidant concentration drastically decreased to approximately 0.25 mg/mL immediately when fertilized eggs were immersed in the electrolyzed seawater due to organic substances derived from the eggs and the seawater. The oxidant concentration was not recovered to the initial concentration although the electrolyzed seawater was continuously supplied for at least 5 min at 1.0 L/sec which was the maximum condition of the water supply in this study. In any case, up to 5 min of treatment time did not affect the hatching of the fertilized eggs. Given the significant decrease of oxidant concentration, the fertilized eggs should be treated with electrolyzed seawater for maximum treatment time at optimum oxidant concentration to reduce the risk of vertical transmission of HAqRV. Thus, the optimum condition for disinfection of hirame fertilized eggs with electrolyzed seawater was determined to be as 5 min immersion in electrolyzed seawater at the nominal oxidant concentration of 0.75 mg/L with continuous supply at 1.0 L/min.

Finaly, we applied a comprehensive biosecurity plan against HAqRV in hirame hatcheries. In the plan, our newly developed measures were incorporated into conventional disease control protocols, which includes the use of UV-treated seawater and appropriate zoning in hatcheries. To confirm the effectiveness of the plan, we chose a hirame hatchery where an outbreak of HAqRV infection had occurred in the spring of 2021. We provided technical advice to the staff members in the hirame hatchery regarding the preventive measures against HAqRV, and the tools and equipment for the intestinal swab testing and disinfection were prepared. In November of the same year, all incoming and resident broodstock were tested by the intestinal swab test, and positive individuals were eliminated. Broodstock started to spawn in the winter, and the fertilized eggs were disinfected by the method developed in this study. In this batch, hirame were successfully raised to juvenile stages and released into the ocean for stock enhancement without an outbreak of HAqRV infection. The measures were further disseminated to seven additional

fish hatcheries, and we successfully prevented the outbreak of the virus. These results indicate that our disease control measures are highly effective in preventing outbreaks of HAqRV infection in hirame hatcheries.

Key words: hirame, aquareovirus, broodstock selection, electrolyzed seawater

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Annotated Bibliography of Key Works

(1) Nishioka T, Furusawa T, Mizuta Y (1997) Diseases Occurring in Marine Fish and Shellfish Hatcheries in Japan (1989-1994). *Aquacult. Sci.*, **45(2)**, 285-290 (in Japanese with English abstract).

This valuable paper summarized fish and shellfish diseases that had occurred in hatcheries in Japan. The highest number of cases were reported in hirame, and the types of diseases that occurred in hirame hatchery were diverse.

(2) Nishioka T, Fujimoto H, Oka M, Arimoto M (2009) Diseases of marine fish and shellfish in hatcheries in Japan. *J. Fish. Technol.*, **2(1)**, 57-65 (in Japanese with English abstract).

As the above article, this paper also summarized fish and shellfish diseases that had occurred more recently (FY, 2000-2006) in hatcheries in Japan. The highest number of reports were recorded again in hirame, indicating that the diseases in hirame continued to be serious.

(3) Kawato Y, Mekata T, Nishioka T, Kiryu I, Sakai T, Maeda T, Miwa S, Koike K, Sadakane M, Mori K (2021) Isolation and characterization of hirame aquareovirus (HAqRV): A new Aquareovirus isolated from diseased hirame *Paralichthys olivaceus*. *Virology*, **559**, 120-130.

They isolated a novel *Aquareovirus* (hirame aquareovirus: HAqRV) from hirame and determined the complete genome of this virus. A comparison of the entire genome between the new virus and other aquareoviruses suggested that HAqRV is likely a new aquareovirus species. Virulence of HAqRV was demonstrated by experimental infection using hirame juveniles and HAqRV was reisolated from diseased fish. In immunohistochemistry, syncytial cells in the intestinal tract and liver of the experimentally infected fish were stained with the antiserum against HAqRV.

(4) Kawato Y, Maeda T, Nishioka T, Kiryu I, Mekata T, Matsuyama T, Tensha K, Yamashita I, Kawamura Y, Raku A, Senbokuya K, Yanagi S, Hayashi K, Kumagai A, Mori K (2022) Asymptomatically infected broodstock are a potential infection source for aquareovirus outbreaks in hatchery-reared Japanese flounder *Paralichthys olivaceus*. *Fish Pathol.*, **57(1)**, 11-19.

This paper estimated the route of transmission in hirame hatcheries where outbreaks occurred due to HAqRV. Quantitative PCR analysis of organs from broodstock revealed that more viral genes were detected in the intestinal tract than in the gonads. In addition, the viral sequences from broodstock and juveniles died by HAqRV were consistent among facilities. These results suggest that vertical transmission is the primary route of transmission, and the virus released from the intestinal tract of the broodstock might be the source of infection in the juveniles.