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Control Strategy for Viral Diseases of Salmonid Fish, Flounders and Shrimp at Hatchery and Seed Production Facility in Japan

Mamoru Yoshimizu*

Laboratory of Biotechnology and Microbiology, Faculty of Fisheries Sciences, Hokkaido University Hakodate, Hokkaido 041-8611, Japan

ABSTRACT—Salmonid fish are important species for hatchery reared and released fish. Flounders and shrimp are also important species for seed production and sea-farming in Japan. Viral disease is one of the limitations of successful propagation of these species. Methods currently used to control viral diseases are 1) hygiene and sanitation in facilities, 2) disinfection of rearing and waste water using U. V. irradiation, ozonization and electrolyzation, 3) selection of pathogen-free brood stock by cell culture isolation and detection of specific antibody against important pathogens with ELISA or viral gene with PCR, 4) health monitoring of hatched fry by cell culture isolation and detection of pathogens by immunological and molecular biological methods, 5) control of normal intestinal flora by feeding bacteria producing antiviral substances, and 6) temperature manipulation. Under these circumstances, hatched fish and shrimp are healthy and specific pathogen free, but there is still a possibility of infection by some pathogens in environmental waters after they are moved to ponds or net pens outside of facilities. For prospective studies, development of effective vaccines, vaccine injection machines and immunological tools for evaluation of vaccination effect are necessary.

Key words: salmonid fish, flounder, shrimp, disinfection, UV, ozone, electrolyzation, vaccination

Salmonid fish including chum salmon *Oncorhynchus keta*, pink salmon *O. gorbuscha* and masu salmon *O. masou* are important species for hatchery reared and released fish. Flounders such as Japanese flounder *Paralichthys olivaceus* and barfin flounder *Verasper moseri* and kuruma shrimp *Penaeus japonicus* are also important species for seed production and sea-farming in Japan, because of their rapid growth and relatively high market prices. Viral diseases; infectious hematopoietic necrosis (IHN), *Oncorhynchus masou* virus disease (OMVD), erythrocytic inclusion body syndrome (EIBS), lymphocystis disease (LCD), hirame rhabdovirus disease (HIRRVD), viral epidermal hyperplasia (VEH), viral nervous necrosis (VNN), viral hemorrhagic septicemia (VHS) and penaeid acute viremia (PAV) (=white spot disease: WSD) are serious problems and one of the limitations of successful propagation and aquaculture of these species.

We have developed different control methods against these diseases, which will be described below. Among them, a special emphasis is put on the development of different types of vaccines, although they have not yet been used in the field: recombinant vaccines against IHN, formalin-inactivated or attenuated vaccine against OMVD and/or LCD, and recombinant oral vaccine against PRDV (=WSSV) for kuruma shrimp.

* Corresponding author
E-mail: yosimizu@fish.hokudai.ac.jp
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1) Hygiene and sanitation

General sanitation measures are standard practice in hatchery and seed production facilities (Kimura and Yoshimizu, 1991). Special care must be taken to avoid the movement of equipment from one tank to another and all should be disinfected after use. Methods to sanitize a rearing unit should be carefully developed with respect to chemical toxicity for fish, effects of water temperature and their repeated use. It should be remembered that workers themselves might serve as efficient vectors for pathogens and proper disinfection of hands and boots are required to prevent dissemination of viruses. Although it may be difficult to sanitize a rearing unit during use, tanks and raceways should be disinfected with chlorine before and after use (Ahne *et al.*, 1989; Kasai *et al.*, 2005). Equipment, nets and brushes are disinfected with ozonated or electrolyzed sea water

containing 0.5 mg/L of total residual oxidants (TROs) or chlorine for 30 min and are separately used for individual tanks (Watanabe and Yoshimizu, 1998, 2001).

2) Disinfection of water supplies and waste water

Water systems provide an efficient means for introduction and spread of infectious diseases. Pathogen-free water sources are often essential for success in aquaculture. Waters commonly used in aquaculture, which come from domestic bay, contain fish pathogens. Such open water supplies should not be used without treatment to kill fish pathogens. Usually, treatment systems make use of highly efficient sand filters to remove particulates before treatment with ultraviolet (UV) light or ozonation (Yoshimizu and Hyuga, 1992). Fish viruses are divided into two groups based on the sensitivity to UV and TROs (Yoshimizu *et al*., 1986a, 1995). Sensitive types are IHNV, OMV, LCDV and HIRRV (Fig. 1). These viruses are inactivated by treatment with 10^4 $\mu W \cdot sec/m^2$ (UV dose) or 0.1 mg/mL (TROs) for 1 min. Resistant types are infectious pancreatic necrosis virus (IPNV), marine birnavirus, reovirus and fish nodavirus, and these are inactivated at the doses of 10^6 $\mu W \cdot sec/m^2$ (UV dose) or 0.5 mg/mL (TROs) for 1 min (Yoshimizu *et al*., 1990; Yoshimizu, 1992; Ito *et al*., 1996a). In the case of ozonated sea water that contains TROs, toxicity to the fish may be observed. The TROs should be removed by charcoal before use (Ito *et al*., 1996b). Disinfection by electrolysis is very effective to treat large volumes of seawater (Kasi *et al*., 2000, 2001a, b).

3) Pathogen-free brood stock

Monitoring the health condition and management is very important for seed production in aquaculture (Yoshimizu, 1998). Since some viruses are transmitted vertically from adult to progeny via contaminated eggs or sperm, disinfection of the surface of fertilized eggs has been proven to be effective in breaking the infection cycle for several viruses, such as rhabdovirus, herpesvirus, and nodavirus (Yoshimizu *et al*., 1989; Yoshimizu, 1998; Watanabe *et al*., 2000). Health inspections of brood stock are conducted to insure that fish are free from certain important diseases. Routine inspections and specialized diagnostic techniques are required to make specific pathogen free brood stock (Yoshimizu and Nomura, 1989).

For salmonid fish, ovarian fluid is collected by the methods of Yoshimizu *et al*. (1985) and routinely inspected with the cell culture isolation method. Fertilized eggs were disinfected with iodofore 25 ppm for 20 min or 50 ppm for 15 min. Inside the egg membrane of eyed eggs is pathogen free (Yoshimizu *et al*., 1989). Because viruses and bacteria such as IHNV, OMV, *A. salmonicida* and *R. salmoninarum* can grow well in the embryo. It is very important to disinfect the surface of eyed eggs with iodofore (Yoshimizu and Nomura, 1989) (Fig. 2).

At flounder hatcheries, tagging is applied for identification of individual fish in the brood stock. For

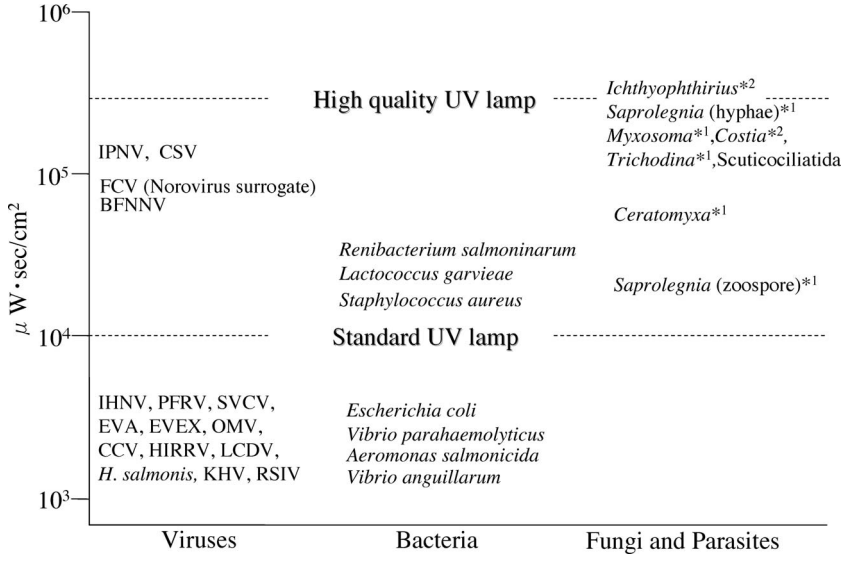

Fig. 1. U. V. susceptibility of fish pathogenic microorganisms and food-born microorganisms. IPNV: infectious pancreatic necrosis virus, FCV: feline calicivirus, BF-NNV: barfin flounder nervas necrosis virus, IHNV: infectious hematopoietic necrosis virus, PFRV: pike fry rhabdovirus, SVCV: spring viremia of carp virus, EVA: eel virus from America, EVEX: eel virus from Europe X, OMV: *Oncorhynchus masou* virus, HIRRV: hirame rhabdovirus, LCDV: lymphocystis diseases virus, *H. salmonis*: herpesvirus salmonis, KHV: koi herpesvirus, RSIV: red sea bream irridovirus, *1: Hoffman (1974), *2: Vlasenko (1969).

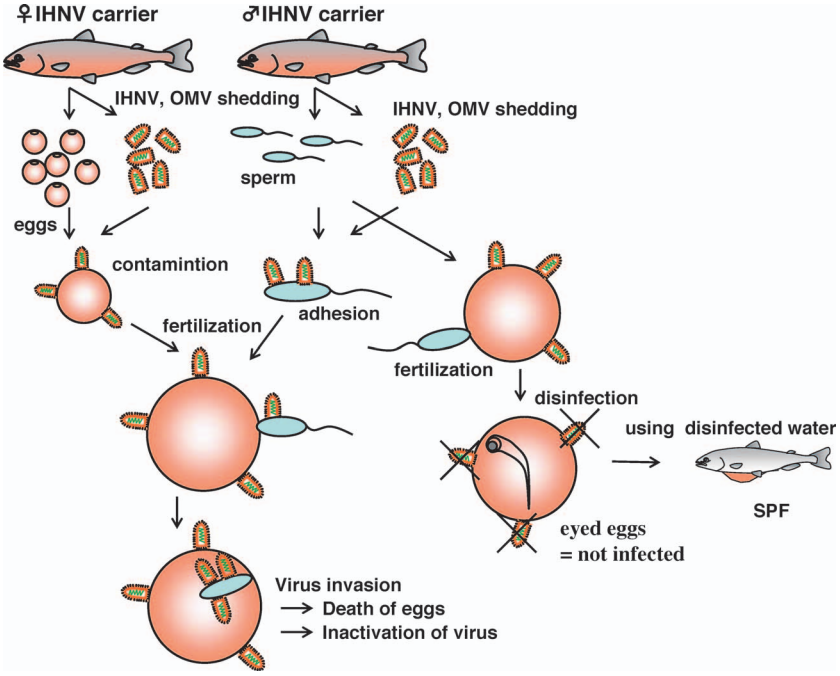

Fig. 2. Prevention method of vertical transmission from matured fish to progeny by disinfection of egg surface.

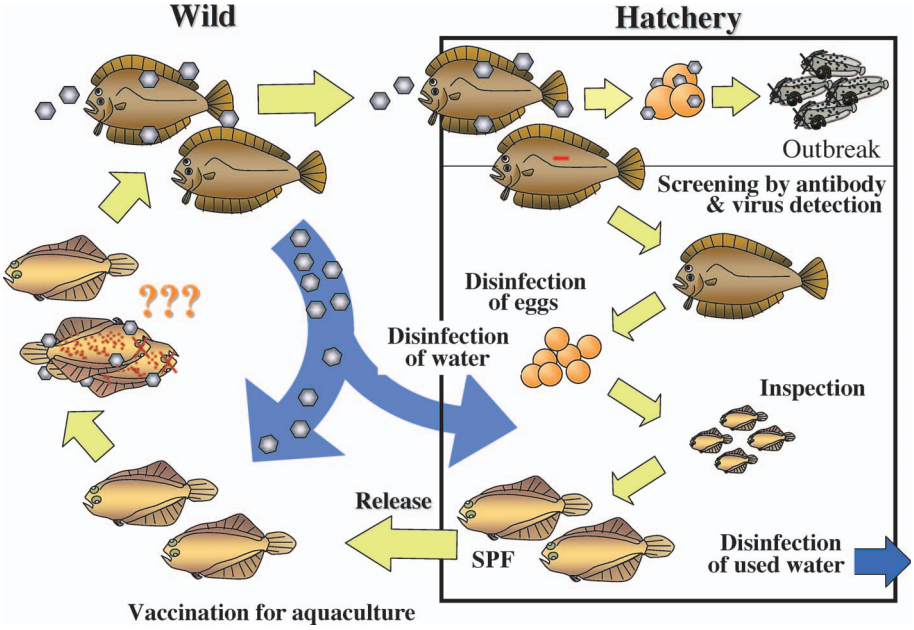

Fig. 3. Disease control procedures in flounder hatchery.

example, to control VNN caused by barfin flounder nervous necrosis virus (BFNNV) and Japanese flounder nervous necrosis (JFNNV), a standard sandwich ELISA using an expressed protein of partial BFNNV coat protein as an antigen to capture the specific antibodies (Yoshimizu *et al*., 1997, 2001; Watanabe *et al*., 2000) and RT-PCR to detect striped jack nervous necrosis virus (SJNNV) specific gene sequences are used for brood stock selection. ELISA is done 3 months before spawning and negative fish in the ELISA test are reared as brood stocks. Eggs and sperm are tested by RT-PCR, and specimens inoculated to SSN-1 cells at the same time. The eggs or sperm that were positive in the RT-PCR test are removed (Watanabe *et al*., 1998). Eggs are disinfected with ozonated seawater (0.5 mg/L of TROs for 10 min) at the morula stage (Watanabe and Yoshimizu, 2000). In 2008, 1,000,000 barfin flounder fry were released successfully in Hokkaido (Fig. 3).

4) Health monitoring of hatched fry

In flounder hatcheries, different from salmonid hatcheries, it is possible to culture fry from each spawner in separate tanks. When fry show abnormal swimming or disease signs, they should be picked up and brought to the laboratory for diagnosis as soon as possible. Moreover, health monitoring should be done regularly. There are many methods for viral detection such as cell culture, fluorescent antibody techniques (FAT), immunoperoxidase stain (IPT), antigen detecting ELISA and PCR test. RT-PCR is suitable to detect the fish nodavirus and flounder ascites virus. FAT is commonly used to diagnose viral epidermal hyperplasia, lymphocystis disease, HIRRVD and reovirus infection (Yoshimizu, 1995).

5) Control of normal bacterial flora

Generally, normal bacterial floras play an important role to inhibit the growth of pathogenic bacteria in the intestine or on the skin, and also to stimulate the immune response of the host animals. Bacterial flora of larvae cultured in the disinfected water, is not normal. It is important to establish normal bacterial flora of the fish before they are released to the river or ocean. Many bacterial strains that produce anti-viral substances against fish viruses have been reported (Yoshimizu *et al*., 1986b; Yoshimizu and Ezura, 1999). Rainbow trout and masu salmon fed with the bacteria having an anti-IHNV activity, which were isolated from normal intestinal flora of fish, showed more resistance to artificial infection with IHNV (Yoshimizu *et al*., 1992). In the case of barfin flounder and Japanese flounder, *Vibrio* spp. which showed the anti-viral activity against IHNV, OMV and/or BFNNV were isolated from normal intestinal flora and added to *Artemia* or rotifer which were disinfected at the egg stage and hatched out in disinfected water. Anti-IHNV, OMV and/or BFNNV activities were observed in homogenates of intestine of fish fed with the *Artemia* and rotifer (Yoshimizu and Ezura, 1999; Yoshimizu *et al*., 2006). These barfin flounder fed with *Artemia* containing *Vibrio* sp. showed more resistance to natural infection by BFNNV.

6) Temperature manipulation

It is well known that many diseases of aquatic animals are temperature dependent. In the case of HIRRV, natural outbreaks of infections disappear when the water temperature increases to 15°C. Cumulative mortalities of Japanese flounder challenged by an intraperitoneal injection ($10^{5.3}$ $TCID_{50}$/fish) of HIRRV and reared at 5°C, 10°C, 15 and 20°C were 40%, 60%, 10% and 0%, respectively. The highest virus infectivity was obtained from the fish that were cultured at 5°C, followed by 10°C (Oseko *et al.,* 1988). We strongly recommended rearing of Japanese flounder at water temperatures above 18°C, and outbreaks of HIRRV infection have not been reported since 1988. Now, temperature control treatment is done against IHN.

7) Vaccination

Immunization represents the most effective method to control the diseases for which avoidance is not possible. Commercial vaccines have now become available to protect fish against important pathogens. We tested formalin-inactivated OMV or recombinant IHNV-G protein expressed by yeast. When we injected the OMV vaccine to mature rainbow trout, incidence of OMV in ovarian fluid decreased. A bathing vaccine using IHNV-G protein expressed by yeast prevented IHN infection (Nishizawa and Yoshimizu, 2004). Apparently healthy Japanese flounder that showed low titers for ELISA antibodies against JF-LCDV were injected with three types of inactivated JF-LCDV vaccines including a formalin-killed JF-LC cell suspension, a heat-killed JF-LC cell suspension and a formalin-killed purified JF-LCDV. Three months after injection, ELISA antibody titers increased to 1:40, while in control fish without vaccine injection, titers remained low (1:5 or 1:10). Outbreaks of LCD were observed at that time. LCD cells did not grow and gently disappeared among the immunized fish that showed high ELISA titers. In contrast, LCD was observed in 53% of control fish at 6 months post-vaccination (Yoshimizu and Iwamoto, 2001). Recently, a quaji-immuno system was reported in kuruma shrimp and oral vaccination with recombinant WSSV proteins (rVP 26 and rVP 28) was effective against WSD in kuruma shrimp (Sato *et al.,* 2008).

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