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PREVENTION AND TREATMENT OF DISEASES CAUSED BY FISH PATHOGENS

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peptide, Disease resistance.

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Summary

This chapter describes methods to prevent and/or protect fish from infectious diseases. Chemotherapy using antimicrobial agents and criteria is effective but users should pay attention to avoid the increases of multiple drug resistant strains of fish pathogenic bacteria. Vaccination by injection, immersion and oral methods is important to prevent diseases. Besides formalin-killed and heat-treated vaccines, there are several other types of vaccines, such as attenuated, subunit, and DNA vaccines. Fish rely more on their innate immunity to prevent diseases and immunostimulants generally stimulate innate immune components. Many immunostimulants such as glucans, levamisole, chitin, lipopolysaccharides and nucleotides have been reported to increase protection against bacterial, viral and parasitic diseases in fish.

Diagnostic methods are indispensable to fish farm management and will help in identifying proper therapeutic measures and preventing the spread of diseases. Diagnostic methods currently used are antibody-based diagnosis, detection of specific genes in the target pathogen by polymerase chain reaction (PCR) and the loop mediated isothermal amplification (LAMP) method. In aquaculture, one way to prevent fish diseases is to develop disease-resistant strains of fish through the use of marker-assisted selection (MAS). MAS requires an understanding of the linkage between quantitative trait loci of a target trait and DNA markers. Transgenic technology is applicable to obtain disease-resistant strains of fish. Recent advances in the fish transgenesis for disease-resistance are discussed.

1. PREVENTION AND PROTECTION AGAINST INFECTIOUS DISEASES

1.1. Prevention

Mamoru Yoshimizu and Hisae Kasai

1.1.1. Synopsis

Methods currently used to prevent infectious diseases in hatcheries and seed production facilities are: 1) good hygiene and sanitation, 2) disinfection of culture and waste water, 3) selection of pathogen free brood stock, 4) washing and disinfection of eggs, 5) monitoring the health of hatched fry, 6) temperature control, 7) vaccination, and 8) control of intestinal flora.

1.1.2. Introduction

Fish aquaculture is economically important worldwide. Infectious diseases, which include viral, bacterial, fungal, and parasitic diseases, are one of the limiting factors in the successful propagation of cultured fish. Methods currently used to prevent infectious diseases in hatcheries are: 1) good hygiene and sanitation, 2) disinfection of culture and waste water, 3) selection of pathogen free broods tock, 4) washing and disinfection of eggs, 5) monitoring health of hatched fry, 6) temperature control, 7) vaccination, and 8) control of normal intestinal flora. Disinfection of water and eggs is especially important. This chapter will focus on the first five methods mentioned above. (Yoshimizu, 2003, 2009)

1.1.3. Hygiene and Sanitation

General sanitation measures are standard practice in hatchery and seed producing facilities. Special care must be taken to avoid the movement of equipment from one tank to another and all articles should be disinfected after use. Methods used to sanitize a rearing unit should take into account chemical toxicity to fish, effects of temperature and consequences of prolonged use. It should be remembered that workers themselves often act as vectors for pathogens and therefore proper disinfection of hands and boots is required to prevent dissemination of pathogens. Although it may be difficult to sanitize a rearing unit during use, tanks and raceways should be disinfected with chlorine before and after use. Equipment, nets, brushes may be disinfected with ozonated or electrolyzed sea water containing 0.5 mg/l of total residual oxidants (TROs) or chlorine for 30 minutes in separate tanks.(Ahne et al, 1989; Kasai et al, 2005)

1.1.4. Disinfection of Water Supplies and Waste Water

Water supplies for seed production and aquaculture may also be pathways for the introduction and spread of infectious diseases. A pathogen free water source is essential for success in aquaculture. Water commonly used in aquaculture comes from coastal waters or rivers and may contain fish pathogens. Such open water supplies should not be used without prior treatment. Disinfection of wastewater before discharging is necessary to avoid contamination of the environment with pathogens. Below are examples of studies on the use of ultraviolet (UV), oxidants produced by ozonization of seawater, and hypochlorite produced by electrolyzation of seawater for disinfection of water. In addition to evaluating the disinfection efficacy of these three methods for a hatchery water supply and wastewater, their effects on survival of cultured fish was assessed. (Kasai et al, 2002)

1) Susceptibility of fish pathogens to U.V and its efficacy for disinfection of hatchery water

The disinfectant effects of UV irradiation on fish pathogenic bacteria, viruses, and fungi were determined using cell suspensions of bacteria, punched agar medium disk covered with aquatic fungi, and cell free suspensions of viruses. Of the viable bacterial cells of Gram negative bacteria and Gram positive bacteria, 99.9% or more were killed by UV

irradiation at doses of 4.0×10^3 and 2.0×10^4 μW・sec/cm^2, respectively. The phyphae of aquatic fungi showed relatively lower susceptibility to UV irradiation, levels that inhibited the growth of phyphae were 1.5×10^5 to 2.5×10^5 μW・sec/cm^2. Fish rhabdoviruses, herpesviruses and iridovirus were found to be sensitive to UV irradiation. The dose that resulted in a 99 % or more infectivity decrease (ID_{99}) was observed at the dose of 1.0 to 3.0×10^3 μW・sec/cm^2. Susceptibility of birnaviruses, reovirus and nodavirus was found to be lower with an observed ID_{99} of 1.5 to 2.5×10^5 μW・sec/cm^2 (Figure 1.1.1). (Kasai et al, 2002)

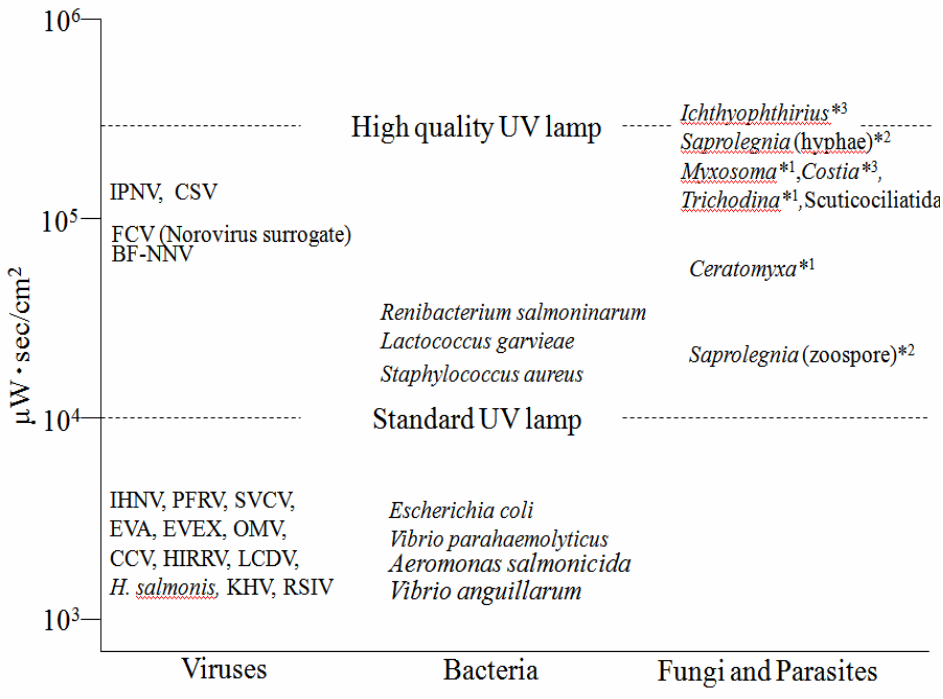


Figure 1.1.1. UV susceptibility of fish pathogens. (see Ahne et al, 1989)

In the studies on infectious hematopoietic necrosis virus (IHNV), infectivity in virus contaminated river water and pond water, was 0.56 and 5.6 $TCID_{50}$/l, respectively, when measured using the molecular filtration method. UV treatment of river water with 10^4 μW・sec/cm^2 a UV dose prevented an IHN outbreak. Furthermore, UV treatment of the hatchery water supply also decreased the viable bacterial counts and fungal infection rates in salmonid eggs. (Kasai et al, 2002)

2) Disinfectant effect of oxidant produced by ozonization of sea water on fish pathogens

Treatment of natural seawater with ozone produced oxidants that showed a disinfectant effect. Total residual oxidants (TROs) produced in seawater were stable for 1 h or more. Disinfectant effect of TROs against fish pathogenic organisms was observed at a dose of 0.5 mg/*l* for 15 to 30 s or 0.1 mg/*l* for 60 s, and killed more than 99.9 % of bacterial cells of *Vibrio anguillarum, Lactococcus garvieae, Aeromonas salmonicida, A. hydrophila and E. coli,* and inactivated 99 % or more of IHNV, hirame rhabdovirus (HIRRV) and *Oncorhynchus masou* virus (OMV). To inactivate or kill more than 99 % of yellowtail ascites virus (YAV), infectious pancreatic necrosis virus (IPNV), chum salmon virus

(CSV), and a Scuticociliatida (ciliata), higher doses of 0.5 to 1.0 mg/*l* for 1 min were required (Table 1).(Yoshimizu et al, 1995)

However TROs were toxic to fish. Barfin flounder (*Verasper moseri*) and herring (*Clupea pallasii*) died after 16 and 2 h exposure to TROs of 0.1 and 0.5 mg/*l*, respectively. Nevertheless, Japanese flounder could be cultured in ozonized seawater after the TROs were removed using charcoal, resulting in survival rates similar to fish cultured in UV treated or non-treated seawater. (Yoshimizu et al, 1995)

Fish Pathogens	TROs concentration (mg/l)	Treatment time (sec)	Reduction Rate (%)	Initial number (log)
Yellow ascites virus (YAV)	0.5	60	>99	4.3[1]
Hirame rhabdovirus (HIRRV)	0.5	15	>99	5.6[1]
Infectious pancreatic necrosis virus (IPNV)	0.5	60	>99	4.1[1]
Infectious haematopoietic virus (IHNV)	0.5	15	>99	4.1[1]
Onchorhynchus masou virus (OMV)	0.5	15	>99	3.1[1]
Chum salmon virus (CSV)	0.5	60	>99	4.1[1]
Vibrio anguillarum NCMB6	0.5	15	>99.9	5.6[2]
Lactococcus garvieae 538	0.5	15	>99.9	5.8[2]
Aeromonas salmonicida ATTC14174	0.5	15	>99.9	5.1[2]
Aeromonas hydrophila IAM1018	0.5	15	>99.9	4.6[2]
Scuticociliatida BR9001	0.8	30	>99.9	5.5[3]

[1]Initial viral infectivity ($TCID_{50}$/ml). [2] Initial viable bacterial number (CFU/ml). [3]Initial viable number.

Table 1. Effect of total residual oxidants (TROs) concentrations produced by ozonization of seawater on infectivities of fish pathogens

3) Disinfectant effect of electrolyzed salt water on fish pathogenic bacteria and viruses

The bactericidal and virucidal effects of hypochlorite produced by electrolysis of salt water were examined against pathogenic bacteria and viruses of fish. Sodium chloride solutions, ranging from 0.5 to 3 % were electrolyzed and the concentration of chlorine produced was measured. Similar concentrations of chlorine were produced when 1.0 % or higher NaCl solution and seawater were electrolyzed. A 3 % solution of sodium chloride containing pathogenic bacteria or virus was electrolyzed and the organisms were exposed to chlorine. Greater than 99.9 % of *V. anguillarum* and *A. salmonicida* cells were killed when the bacteria were exposed to 0.1 mg/l chlorine for 1 min. On the other hand, 99.9 % or higher yellow tail ascites virus (YTAV) and HIRRV were inactivated after treatment with 0.45 mg/l chlorine for 1 min (Table 2). (Kasai and Yoshimizu, 2002)

The bactericidal and virucidal effects of hypochlorite produced by electrolysis were greater than that of the chemical reagent. The purity of the sodium chloride used for electrolysis influenced the efficacy of hypochlorite produced. Sodium chloride obtained as a super grade chemical reagent was more effective than food-grade sodium chloride. Nevertheless, a sufficient disinfectant effect was observed even in electrolyzed seawater, a method which may have wide applications in aquaculture. To use electrolyzed seawater for culture, the chlorine has to be removed with charcoal because of its toxicity. (Kasai et al, 2002)

Fish Pathogens	Chlorine concentration (mg/l)	Treatment time (min)	Initial number (log)	Reduction Rate (%)
Vibrio anguillarum NCMB6	0.07	1	6.7[1]	>99.99
Aeromonas salmonicida ATTC14174	0.06	1	6.6[1]	99.96
Escherichia coli O-26	0.14	1	6.6[1]	99.98
Yellow ascites virus (YAV)	0.45	1	4.5[2]	99.92
Hirame rhabdovirus (HIRRV)	0.34	1	4.5[2]	99.97

[1] Initial viable bacterial number (CFU/ml). [2]Initial viral infectivity ($TCID_{50}$/ml)

Table 2. The chlorine concentration produced by electrolysis of salt water and treatment time required to reduce the viability of bacteria and the infectivity of viruses by 99.9 %

4) Disinfection of wastewater

In studies on the disinfection of hatchery wastewater, the bactericidal effect of hypochlorite produced using a continuous flow electrolyzer was investigated. The number of viable bacteria in the wastewater was reduced by more than 99 % when the water was treated with chlorine at a concentration of 0.5 mg/*l* for 1 min, and over 99.9 % of the bacteria cells were killed when treated with 1.28 mg/l for 1 min. Viability of bacteria was reduced greater than 99 % after treatment with 0.5 mg/l of chlorite for 1 min. The bactericidal effect of electrolysis was almost the same as that of ultraviolet irradiation (1.0×10^5 μW・sec/cm^2) or ozonization (TROs 0.5 mg/l, 1 min) of seawater. Electrolization can be used to treat larger volumes of wastewater compared to with the ultraviolet irradiation or ozonization.

All three disinfection methods above eliminated 96.6 to 99.8 % of bacteria in hatchery water supplies. Survival rate of Japanese flounder *Paralichthys olivaceus* and barfin flounder cultured in UV irradiated, ozonized and electrolyzed seawater have been compared. No statistically significant differences in survival rates were found between the three groups of fish cultured with treated water. Ozonized and electrolyzed seawater have been demonstrated to be effective for disinfecting equipment used in aquaculture and ozonized seawater is effective for disinfecting fertilized barfin flounder eggs contaminated with nervous necrosis virus. Therefore, ozonization and electrolization of seawater seem to be effective methods for disinfection of the water for fish culture. (Kasai et al, 2002)

1.1.5. Pathogen-Free Brood Stock

Monitoring the health of brood stock is very important for seed production in aquaculture. Health inspections of brood stock are conducted to insure that fish are free from certain important diseases. Specialized diagnostic techniques are required to make specific pathogen free brood stock for routine inspections. The tests have been made easier and more rapid by the development of enzyme-linked immunosorbent assay (ELISA). (Yoshimizu et al, 1997)

For salmonid fish, Yoshimizu et al, (1985) recommended a method for collection of ovarian fluid for routine inspection. Fertilized eggs were disinfected with 50 ppm iodofore for 20 min. It was also suggested that eyed eggs were an indication that inside the egg membrane is pathogen free (Yoshimizu et al, 1989). However, disinfection of the surface of eyed eggs with iodofore was considered important as viruses and bacteria like IHNV, OMV, *A. salmonicida* and *R. salmoninarum* can infect and grow well in the embryo.

At a flounder hatchery, tagging was used for identification of individual fish. For example, to control the barfin flounder and Japanese flounder nervous necrosis (BF-, and JF-NNV), a standard sandwich ELISA to use an expressed protein of partial BF-NNV coat protein for an antigen to capture the specific antibodies and RT-PCR to detect striped jack nervous necrosis virus specific gene sequences are using for healthy brood stock selection. ELISA was done 3 months before spawning and the negative fish by ELISA are reared for the brood stock (Watanabe et al, 2000). Eggs and sperms are tested by RT-PCR, and specimens inoculate to SSN-1 cells at the same time. The eggs or sperms that showed positive by RT-PCR were removed.

1.1.6. Washing and Disinfecting Eggs Before or Just After Fertilization and Eyed Stages

Since some viruses and bacteria are transmitted vertically from adult to progeny via infected eggs or sperms, washing and disinfection of eggs before or after fertilization has proven to be effective in breaking the infection cycle for several viruses, such as rhabdovirus, herpesvirus, and nodavirus. This method is also effective for controlling bacteria such as causative agents of bacterial kidney disease and cold water disease (Kohara et al, 2012). For salmonid eggs, disinfection with iodine (50 ppm for 20 min) just after fertilized and eyed stages is effective (Yoshimizu, 2009). For eggs of marine fish, disinfection with ozonized seawater (0.5 mg/l of TROs for 10 min) or iodine (10 to 50 ppm for 10 to 20 min) at the stage of eggs stable against chemical treatments is effective. Except for infections with pathogens causing BKD and cold water disease, eggs that reach the eyed stage are usually pathogen free on the inside and successfully yield healthy fry if the water is disinfected.

1.1.7. Monitoring Health of Hatched Fry

For monitoring purposes, it is advisable that fry from each spawner are cultured in separate tanks. Although this is difficult in a salmonid hatchery, it can be achieved for flounder. If fry show abnormal swimming or disease signs, they should be isolated for

diagnosis as soon as possible. Moreover, health monitoring should be done using a variety of methods for viral detection such as; cell culture, fluorescent antibody techniques (FAT), immuno-peroxidase stain (IPT), antigen detecting ELISA and PCR test. RT-PCR is suitable for detection of fish nodavirus and flounder ascites virus. FAT is commonly used to diagnose the viral epithelial hyperplasia and lymphocystis disease, and HIRRV, and reovirus (see Sections 1.1.4 & 1.1.8).

1.1.8. Temperature Control

It is well known that many diseases of aquatic animals are temperature dependent. In the case of HIRRV infection, natural outbreaks of infections disappear when the water temperature increases to 15 C. It is reported that cumulative mortality of artificially infected Japanese flounder (IP $10^{5.3}$ $TCID_{50}$/fish) which were reared at 5, 10, 15 and 20 ˚C, were 40%, 60%, 10% and 0%, respectively. The highest virus infectivity was obtained from the fish cultured at 5 ˚C, followed by the 10 ˚C. We strongly recommended that Japanese flounder be cultured at water temperatures above 18 ˚C. It is notable that outbreaks of HIRRV infection have not been reported since 1988 (Oseko et al, 1992). Currently, temperature control treatment is being used to control HIRRV infection.

1.1.9. Vaccination

Vaccination is the most effective method to control the diseases for which avoidance is not possible (see Sections 1.3 & 1.4). Several commercial vaccines are available to protect the fish against important pathogens. In Norway, mixed vaccines containing five pathogens are available. In Canada, DNA vaccine against IHNV is available. In Japan, vaccines against vibriosis, streptococcosis, pastureosis, red sea bream iridovirus disease are available. Tests have also been done with formalin-inactivated OMV, LCDV or recombinant IHNV-G protein expressed by yeast.

1.1.10. Control of Normal Bacterial Flora

Generally, normal bacterial flora plays an important role in inhibiting the growth of pathogenic bacteria in the intestine or on the skin, and also to stimulate the immune response of the host animals. Sometimes, bacterial flora of larvae cultured in the disinfected water is not normal. It is important to establish the normal bacterial flora of the fish before they are released to the river or ocean. Many bacterial strains that produce the anti-viral substances against fish viruses have been reported. In one study, rainbow trout and masu salmon fed with bacteria isolated from normal intestinal flora and showed anti-IHNV activity, and higher resistance to artificial infection with IHNV (Yoshimizu and Kimura, 1976; Yoshimizu et al, 1992). In another study, barfin flounder, disinfected at the egg stage and hatched in disinfected water fed with *Artemia* added with *Vibrio* spp. isolated from the normal intestinal flora, showed anti-viral resistance against IHNV, OMV and BF-NNV. Anti-IHNV, OMV and BFNNV activities were observed in homogenates of intestines of fish fed with the *Artemia.* These barfin flounder fed with *Artemia* containing *Vibrio* sp. also showed more resistance to natural infection by BFNNV (Yoshimizu and Ezura, 2002).

1.2. Chemotherapy: Antimicrobial Agents for Aquaculture in Japan

Takashi Aoki

1.2.1. Synopsis

Various antimicrobial agents have been used for treatment of bacterial infectious diseases of fish in freshwater as well as marine farms in the world. In this session, antimicrobial agents used and criteria for use in aquaculture in Japan are introduced. Negative effects of the use of antimicrobials, especially the increase of multiple drug resistant strains of fish pathogenic bacteria are also discussed.

1.2.2. Antimicrobial Agents and Mechanism of Antibacterial Activity

The antimicrobial mechanism of action is different depending on the kind antibacterial agent. The mechanisms of action can be classified into two types: bacteriostatic and bactericidal. Bacteriostatic action is to inhibit the growth of bacteria and then to prevent bacteria from proliferating, while bactericidal action is to kill bacteria in a relatively short period of time.

Antimicrobial agents on the other hand can be classified into 3 groups based on their mechanism of action: 1) inhibit cell wall synthesis, 2) inhibit biosynthesis of nucleotide and nucleic acid and 3) inhibit protein synthesis. Group 1 (inhibit cell wall synthesis) includes cell-wall synthesis inhibitors like bicozamycin benzoate, fosfomycin; inhibitors of bacterial peptidoglycan synthesis such as β-lactam antibiotics (amoxicillin, ampicillin, tobicillin, penicillin, cephalosporin); and those that interfere with bacterial cell membrane integrity like polymyxin B and colistin. Group 2（inhibit biosynthesis of nucleotide and nucleic acid） includes quinolones (oxolinic acid, piromicic acid, miloxacin, flumequine and nalidixic acid), rifampicin, nitrofuran derivatives (sodium nifurstyrenate and furazolidone) and novobiocin, and those that promote the inhibition of metabolic pathways: inhibition of folate-dependent of sulfonamids (sulfamonomrethoxine, sulfadimethoxine and sulfisozole) and sulfamonomethoxine combined with ormethoprim and trimethoprim. Group 3 (inhibit protein synthesis) includes tetracyclines (oxyteyracycline, doxytetracycline, chlortetracycline, tetracycline and minocycline), aminoglycosides (kanamycin, streptomycin), macrolides (erythromycin, josamycin, kitasamycin, oleandomycin, and spiramycin), lincomycin, amphenicol (chloramphenicol, florfenicol, thiamphenicol).

1.2.3. Drug Sensitivity Test

Drug sensitivity is important to chemotherapy; and since effectiveness differs for each microorganism and changes when time passes, it is necessary to determine the kind and the amount of drugs to be used in the treatment of infection by the microbial sensitivity test. The drug sensitivity test provides information about which antimicrobial agents are effective or not.

Minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a bacterium. There are two methods of MIC test: agar plate dilution method and broth dilution method (Revised Standard Method of

the Japanese Society of Antimicrobials for Animals in 2003; Miller *et al*., 2005) (Figures 1.2.1 and 1.2.2). In the agar dilution method a lot of bacterial strains can be tested at the same time. However, the antimicrobial activity of tested drug may be reduced because the test using the agar medium is kept at 50 °C.

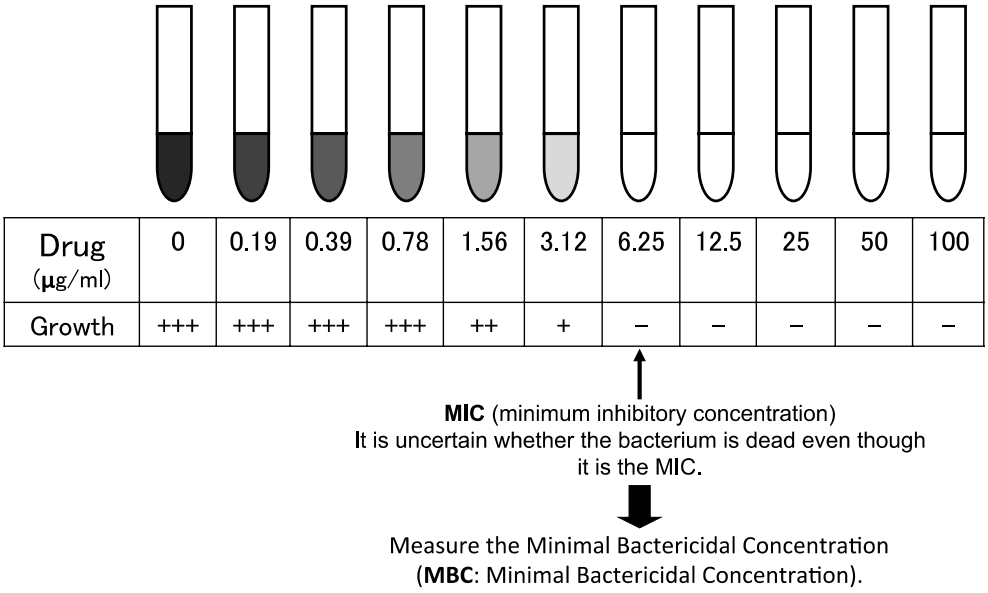
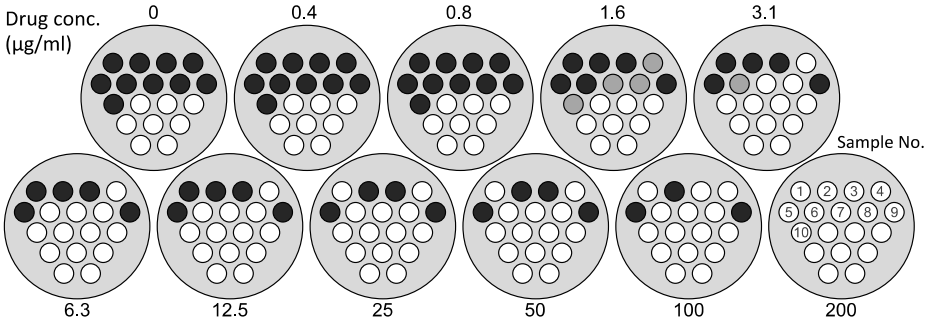


Figure 1.2.1. Determination of MIC by liquid (broth) dilution method



Sample No.	Drug concentration										
	0	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100	200
1	+++	+++	+++	+++	+++	+++	+++	-	-	-	-
2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
3	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-
4	+++	+++	+++	⑤	-	-	-	-	-	-	-
5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
6	+++	+++	+++	+++	①	-	-	-	-	-	-
7	+++	+++	+++	+	-	-	-	-	-	-	-
8	+++	+++	+++	④	-	-	-	-	-	-	-
9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
10	+++	+++	+++	9	-	-	-	-	-	-	-

The MIC value is enclosed with a circle. Fusion growth, single growth and values are indicated with +++, + and number, respectively. If the colony was 6 pieces or more, it was assumed +, and assumed that it was 5 pieces or less.

Figure 1.2.2. Determination of MIC by agar plate dilution method

In the disc method, the most effective drug against a clinical bacterial strain is obtained rapidly within 24 hrs, showing a very visible zone of inhibition on the agar medium (Figure 1.2.3). The size of the *zone* of *inhibition* indicates the degree of sensitivity of bacteria to a drug.

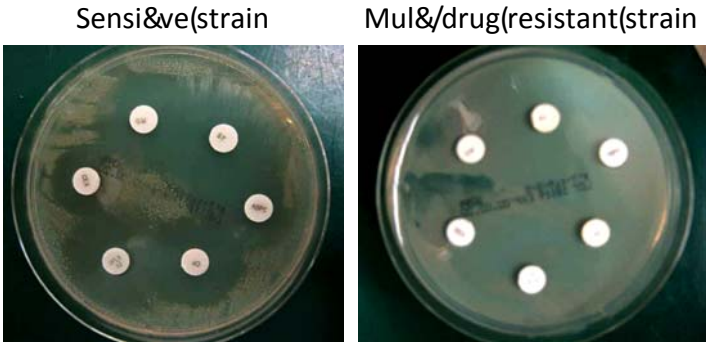


Figure 1.2.3. Microbial sensitivity test using antibiotic/drug sensitivity disk

The minimal bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent required to kill the bacteria. The MBC can be determined from broth dilution MIC tests by sub-culturing to broth without antimicrobial agent (Figues 1.2.4).

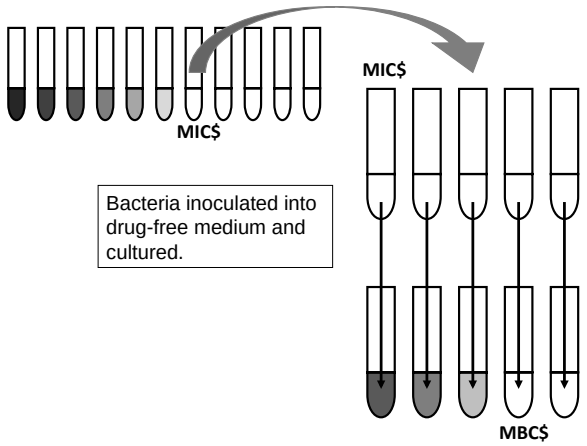


Figure 1.2.4. Minimum bactericidal concentration (MBC)

1.2.4. Methods of Administration and Dynamics of Antimicrobial Agent

Almost all antimicrobial agents are administered orally by incorporating them in feed pellets. The recommended period for oral administration of each drug to fish is about five to seven days. In addition, the continued use of some of the drugs for more than seven days is prohibited. Some antimicrobial agents have been administered by immersing the fish in a drug solution.

The antibacterial agent administered orally had most amounts of absorption in the liver and subsequently in order of absorption the kidney, blood, muscles, and skin mucus (Figure 1.2.5). Orally administered antimicrobial agent is absorbed in the intestines of fish and excreted in the urine, bile (intestine to feces) and gills. The pharmacokinetics of

absorption, distribution, metabolism, and excretion in the fish depends on the kinds of antimicrobial agents to be administered.

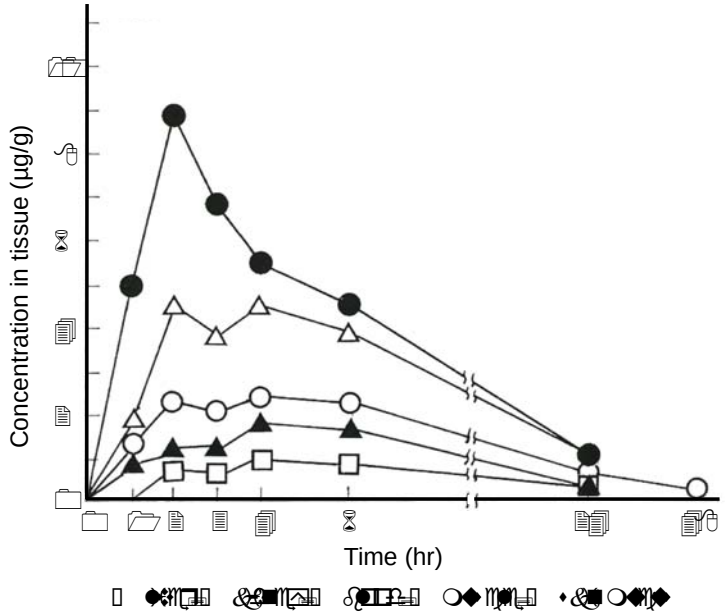


Figure 1.2.5. Concentration of transition curve in each tissue after medicine is administered in eel

1.2.5. Antimicrobial Use is allowed Against Fish Bacterial Infection in Japan

The rule of standard chemotherapy for bacterial infections of cultured fish was approved by Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries in Japan (The Use of Aquatic Medicine 25th Report, 2012) (In Japanese) www.maff.go.jp/j/syouan/suisan/suisan_yobo/pdf/suiyak25.pdf.

The antimicrobial compounds, routes of administration, dosages, disease treated, and withdrawal times of the antimicrobial agents were established in the treatment of fish (Aoki, 1992).

The withdrawal time has been decided based on the period from the ingestion of the medicine to its complete disappearance. It is possible that when the fish is shipped within the washout period, that the medicine remains in the fish, and it is necessary to avoid this completely. In the past, "Zero residues" was the internationally accepted standard. It was based on the maximum residues limit (MRL) of an object animal and each edible part. As for all veterinary products, acceptable daily intake (ADI) and MRL are being set in Japan. The positive list system was implemented to prohibit the distribution of foods that contain agricultural chemical for which ADI had not yet been decided. A uniform limit of 0.01ppm (concentration equivalent to 0.01mg of agricultural chemical in 1kg of food) is set as the tolerable quantity for agricultural chemicals that have not been evaluated. The distribution of foods which contain agricultural chemicals in excess of the determined residue limits is banned in principle. Recently, ADIs (value) of seven aquatic medicines were decided by the Ministry: Florfenicol (0.01 mg/kg BW/day), thiamphenicol (0.005

mg/kg BW/day), tetracycline (0.03 mg/kg BW/day), doxytetracycline (0.0053 mg/kg BW/day), lincomycin (0.0032 mg/kg BW/day), fosfomycin (0.019 mg/kg BW/day) and oxolinic acid (0.021 mg/kg BW/day). The ADI of the remaining aquatic medicines will be decided in the near future.

Acceptable daily intake (ADI) is measured as dosage per weight (mg/kg/day) of the medicine remaining on food that can be ingested (orally) on a daily basis over a lifetime without any appreciable health risk.

Antimicrobial agents approved for treatment of marine fish and shellfish; Perciformes (Chub mackerel, Greater amberjack, Japanese amberjack, Red seabream, yellowtail etc), Pleuronectiformes Tetraodontiformesin and kuruma shrimp in Japan is shown in Table 1.2.1. Antimicrobial agents approved for treatment of freshwater fish (Clupeiformes, Ayu [*Plecoglossus altivelis*], Cypriniformes and Anguilliformes in Japan is shown in Table 1.2.2.

Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Perciformes *(Chub mackerel, Greater amberjack, Japanese amberjack, Red sea bream, Yellowtail etc.)*				
Alkyltrimethyl ammonium calcium oxytetracycline	Oral	50 mg/kg	Streptococcicosis	20 days
			Vibriosis	
Amoxicillin	Oral	40 mg/kg	Pseudotuberculosis	5 days
Ampicillin	Oral	20 mg/kg	Pseudotuberculosis	5 days
Bicozamycin	Oral	10 mg/kg	Pseudotuberculosis	27 days
Doxycycline	Oral	50 mg/kg	Streptococcicosis	20 days
Erythromycin	Oral	50 mg/kg	Streptococcicosis	30 days
Florfenicol	Oral	10 mg/kg	Pseudotuberculosis	5 days
			Streptococcicosis	
Fosfomycin	Oral	40 mg/kg	Pseudotuberculosis	15 days
Josamycin	Oral	50 mg/kg	Streptococcicosis	20 days
Phosphomycin	Oral	40 mg/kg	Pseudotuberculosis	15 days
Lincomycin	Oral	40 mg/kg	Streptococcicosis	10 days
Oxytetracycline	Oral	50 mg/kg	Vibriosis	30 days
Oxolinic acid	Oral	30 mg/kg	Pseudotuberculosis	16 days
Spiramycin	Oral	40 mg/kg	Streptococcicosis	30 days
Sulfamonomethoxin	Oral	200 mg/kg	Vibriosis	15 days
		50 mg/kg	Nocardiosis	15 days
Thiamphenicol	Oral	50 mg/kg	Pseudotuberculosis	15 days
			Vibriosis	
Tobicillin	Oral	100,000 units	Streptococcicosis	4 days
Clupeiformes *(Coho salmon, Cherry salmon, Mountain trout, Rainbow trout, Red spotted masu trout, Willow minow etc.)*				

Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Oxolinic acid	Oral	20 mg/kg	Vibriosis	21 days
		10 mg/kg	Furunclosis	21 days
Sulfamono-methoxine	Oral	100 mg/kg	Vibriosis	30 days
Oxytetracycline	Oral	50 mg/kg	Vibriosis	30 days
Bronopol	Immersion (for 30 min)	0.1-0.2 ml/L	Fish egg disinfect	
Pleuronectiformes *(Japanese flounder, Mud dab, Spotted halibut etc.)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Alkyltrimethyl ammonium calcium oxytetracycline	Oral	50 mg/kg	Streptococcicosis	40 days
Oxytetracycline	Oral	50 mg/kg	Streptococcicosis	40 days
Sodium Nifurstyrenate	Immersion	10 g/1k	Flexibacteriosis	2 days
Tetraodontiformes *(Black scraper, Torafugu, Threadsail filefish etc.)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Oxytetracyclin	Oral	50 mg/kg	Vibriosis	40 days
Kuruma shrimp				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Oxolinic acid	Oral	50 mg/kg	Vibriosis	30 days
Oxytetracyclin	Oral	50 mg/kg	Vibriosis	days

Table 1.2.1. Chemotherapeutic agents approved for the treatment of marine fish in Japan (Bacterial infectious disease)

Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Clupeiformes *(Coho salmon, Cherry salmon, Mountain trout, Rainbow trout, Red spotted masu trout, Willow minow, except for Ayu)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Florfenicol	Oral	10 mg/kg	Furunculosis	14 days
			Vibriosis	
Oxytetracycline	Oral	50 mg/kg	Furunclosis	30 days
			Vibriosis	
			Streptococcicosis	
Oxolinic acid	Oral	10 mg/kg	Furunclosis	21 days
		20 mg/kg	Vibriosis	

Sulfamonomethoxine	Oral	150 mg/kg	Furunclosis	30 days
			Vibriosis	
	Immersion (for 10 min)	10 kg/t 1% saline solution	Furunclosis	15 days
			Vibriosis	
Sulfisozole	Oral	200 mg/kg	Vibriosis	15 days
			Cold-water disease	
2-Povidine-iodine	Immersion (for 15 min)	50 ml/10L	Fish egg disinfect	
Bronopol	Immersion (for 30 min)	0.1-0.2 ml/1L	Fish egg disinfect	
Clupeiformes *(Ayu)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Florfenicol	Oral	10 mg/kg	Vibriosis	14 days
Oxolinic acid	Oral	20 mg/kg	Vibriosis	14 days
	Immersion (for 5 hrs)	10 g/t water	Vibriosis	
Sulfamonomethoxine	Oral	100 mg/kg	Vibriosis	15 days
Sulfamonomethoxine : Ormethoprim (3:1) complex	Oral	50 mg/kg	Vibriosis	15 days
Sulfisozole	Oral	200 mg/kg	Vibriosis	15 days
			Cold-water disease	
Bronopol	Immersion (for 30 min)	0.1-0.2 ml/1L	Fish egg disinfect	
Cypriniformes *(Carp, Catfish, Crucian carp, Loach etc.)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Metrifonate (Trichlorfon)	Dispersal	0.3 g/1t	Lernaeosis	5 days
			Argulus Infestation	
Oxolinic acid	Oral	10 mg/kg	Aeromonasis	28 days
Sulfisozole	Oral	200 mg/kg	Chondrococcus Infection	10 days
Anguilliformes *(Eel etc.)*				
Chemotherapeutic agents	**Route of administration**	**Dosage**	**Disease treated**	**Withdrawal time**
Florfenicol	Oral	10 mg/kg	Edwardsiellosis	7 days
Metrifonate (Trichlorfon)	Dispersal	0.2 g/1t	Lernaeosis	5 days
Miloxacin	Oral	30 mg/kg	Edwardsiellosis	20 days
Oxolinic acid	Oral	20 mg/kg	Edwardsiellosis	25 days
			Red fin disease	
		5 mg/kg	Red spot disease	
Oxolinic acid	Immersion (for 6 hrs)	5 g/t	Edwardsiellosis	25 days

Oxytetracycline	Oral	50 mg/kg	Edwardsiellosis	30 days
Sulfamonomethoxine	Oral	200 mg/kg	Red fin disease	30 days
Sulfamonomethoxine : Ormethoprim (3:1) complex	Oral	50 mg/kg	Edwardsiellosis	37 days

Table 1.2.2. Chemotherapeutic agents approved for the treatment of fresh water fish in Japan (Bacterial infectious disease)

1.2.6. Evils of Aquatic Medicine Use

The administration of excessive aquatic medicine can cause fish to suffer neurotoxic and physiological disorders such as kidney, liver, hematogenetic tissues and gastrointestinal malfunctions, photosensitivity and immune suppression. Therapy using medicinal agents of broad antibacterial spectrum sometimes can induce microbial substitution, for example, bacterial infection change to fungal infection. For antimicrobials used frequently in fish farms, the most damage is the appearance of multiple drug resistant strains of fish pathogen and the emergence of pathogens that may affect humans and livestock and influence the environment around the farms.

1.2.7. Appearance of Multiple Drug Resistant Strains in Fish Farms

Multiple drug resistant strains of fish pathogenic bacteria have been reported in fish farms in South East Asia, North America and European countries (Aoki, 1988, 1992; Sørum, 2006). These drug resistant bacteria included *Aeromonas hydrophila, A. salmonicida, Edwardsiella ictaluri, E. tarda, Flavobacterium psychrophilum, Lactococcus garvieae, Photobacterium damselae* subsp. *piscicida, Streptococcus parauberis, Vibrio anguillarum, V. salmonicida* and *Yersinia ruckeri* (Castillo *et al*., 2013; Kim *et al*., 2008; Maki *et al*., 2009; Welch *et al*., 2009. These drug resistant strains encoded resistance to ampicillin, chloramphenicol, florfenicol, kanamycin, macrolide antibiotics, lincomycin, streptomycin, tetracycline, sulfonamides, and/or trimethoprim. Transferable R plasmids were detected in these drug resistant strains. Furthermore, quinolone resistant strains of Gram-negative fish pathogenic bacteria have increased (Rodkhum *et al*, 2008; Sørum, 2006). Almost all quinolone resistant strains have chromosomally mediated changes caused by point mutation in the DNA gyrase gene A or topoismeraze IV parC. Recently, transferable R plasmids mediated mechanisms of quinolone resistant were detected from *A. hydrophila* and *A. salomonicida* (Han, *et al.,* 2012a,b).

The genetic structures of various R plasmids and drug resistant genes from fish pathogenic bacteria have been elucidated. Drug resistance genes and R-plasmids have been detected not only in pathogenic bacteria but also in environmental bacteria. Based on the analysis of the structures of the R-plasmids and drug resistance genes, it was clarified that the drug resistance genes were transferred and spread between the pathogenic bacteria of humans, domesticated animals, and fish. In order to form a comprehensive approach to resolve the problem of the spread of drug resistance in medicine and animal culture, it is necessary to completely understand how drug resistance determinants are disseminated and transferred between bacteria from different sources.

Glossary

MIC: Minimal inhibitory concentration,

MBC: Minimal bactericidal concentration

1.3. Vaccination – Injection, Oral and Immersion

Mitsuru Ototake

1.3.1. Synopsis

There are three methods of vaccination for fish, namely: injection, immersion and oral methods. The characteristics of each method are shown in this sub-section.

1.3.2. Introduction

Vaccination has become a means of protecting fish, as well as human beings and livestock, from diseases. There are three methods of vaccine administration used today, namely: injection, immersion and oral methods. The characteristics of each method are shown in Table 1.3.1. Among these three methods, the injection method is the most frequently used at present because effectiveness is regarded as the most important point in fish vaccination. However, if a more effective vaccine, which has enough effectiveness even when it is administered by immersion or by oral, is developed in the future, oral administration will probably become the main stream method of vaccination.

	Injection	**Immersion**	**Oral**
Target diseases	many	a few	very few
Efficacy	very high	high	low
Adjuvants	many	a few	none
Labor	much	little	little
Accidents (Operators' side)	likely	unlikely	unlikely
Stress to the fish	much	little	none
Administration to juveniles	not possible	possible	possible
Necessary quantity of vaccine	small	large	large
Accuracy of administration quantity	accurate	not very accurate	inaccurate

Table 1.3.1. Characteristics of each method of vaccination

1.3.3. Oral Administration

1) Characteristics

Vaccine can be mixed into the feed and given to fish. The vaccine administered in this way is considered to be taken into the body through the intestine during the process of digestion.

(Advantage) This method can be applied to almost all sizes of fish. It gives no stress to

fish and requires little human labor because there is no need to catch fish for administration. Moreover, no additional or new tools are necessary. Oral administration is the ideal way of vaccination in aquaculture.

(Disadvantage) The oral administration of vaccine, however, often shows lower efficacy than that of injection, which is the biggest disadvantage of this method. The inferred reason for the lower efficacy is that the active substances, which should be taken into the body through intestine, are degraded or broken down by acid or digestive enzymes (pepsin) in the stomach. Some new steps are taken to improve this method. For example, vaccine is coated with acid-resistant membrane or microencapsulated, in order to prevent the vaccine from being digested. However, these measures are still in the developmental stage. Another disadvantage is that the amount of vaccine intake varies considerably among individuals because the amount of intake depends on the amount of feed actually eaten by individual fish. As a result, the efficacy of the vaccine is not stable.

(Precaution for use) The amount of the feed should be about 80% of the full feeding, so that there won't be any leftover. In order to prevent the vaccine from deteriorating, namely, being digested, decomposed or degraded by enzymes or bacteria, the feed should be given to fish immediately after the vaccine is added. The feed that does not adsorb the vaccine is not suitable.

1.3.4. Immersion/Bath Method

1.3.4.1 Characteristics

(Advantage) Vaccine is administered to fish by immersing the fish in vaccine solution, so it is possible to vaccinate a lot of fish at a time. All the labor required for this method is to capture the fish in the rearing pond or in the preserve and transfer them to the tank containing the vaccine solution. Therefore, this method is suitable for vaccinating a group of fish being cultured in aquatic farms. The efficacy of two vaccines, namely, vibriosis vaccine and enteric red mouth disease vaccine, administered to fish by this method has already been proven, and they are of practical use. There have also been reports on the efficacy of immersion vaccines, such as yellow tail Lactococcosis vaccine (Iida et al, 1982) and viral nervous necrosis (VNN) vaccine (Kai and Chi, 2008). There are several variations of this method such as prolonged immersion method (Nakanishi and Ototake, 1997), spray method (Gould et al, 1978), shower method, immersion-supersonic wave method (Zhou et al, 2002), and stamp method (Nakanishi et al, 2002). In prolonged immersion method, vaccine is directly added to the rearing water to immerse the fish for a prolonged period, so there is no need to capture the fish or transfer them into the tank containing the vaccine solution. In spray method, fish are taken out of the water and sprayed with vaccine solution. In immersion-supersonic wave method, fish are exposed to supersonic waves while being immersed in vaccine solution. In stamp method, fish are stamped with a multiple puncture instrument that has several short needles, while they are immersed in vaccine solution.

1.3.4.2. Factors That Have Influences on Antigen Uptake

There are seven factors that have influences on the uptake of antigen administered by

immersion, namely: (1) antigen concentration of vaccine solution, (2) salt concentration of vaccine solution, (3) Immersion time, (4) water temperature, (5) body weight of fish, (6) anesthetics, (7) salt concentration of rearing water (Fender and Amend, 1978; Thune and Plumb, 1984; Ototake and Nakanishi, 1992a). Among these seven factors, (1), (3), (4) and (5) are reported to have a positive correlation with the concentration of antigen in the blood or the body of fish after the immersion. When fish are treated with (6) before the immersion, the antigen uptake will be reduced. As for (7), antigen concentration in the blood of tilapia and salmon reared in sea water is lower and decreases more quickly after the immersion than that of Tilapia and Salmon reared in fresh water (Ototake and Nakanishi, 1992b).

1.3.4.3. The Sites of Antigen Uptake

When a rainbow trout is immersed in BSA solution for 2 minutes and then returned to the rearing tank, the concentration of BSA in the blood increases rapidly until 2 hours after immersion, and stabilizes at a certain level between 2 to 24 hours after immersion. The authors examined qualitatively and quantitatively the distribution of antigen taken up in the body. As a result, it is considered that soluble antigen is taken primarily into the skin and secondarily into the gills during immersion, and then within several hours, transferred from these organs by blood flow to the body kidney, head kidney, spleen, and secondary respiratory system. When fish is immersed in latex beads suspension, particulate antigen primarily sticks to micro wounds on the skin, and in the process of wound healing, is taken up into the body through ambulatory epithelial cells (Kiryu et al, 2000). It is considered that particulate antigens such as bacteria are also taken up into the body primarily through the skin and the gills.

1.3.4.4. Activation of the Immune System after Immersion Vaccination

When inactivated vaccine for pseudotuberculosis, which is sold in Europe, is administered to Mediterranean Sea bass (*Dicentrarchus labrax* L.) by immersion, specific antibody producing cells of the gills increase dramatically (dos Santos et al, 2001). It is also reported later that similar antibody producing procedures in the skin and gills are observed after immersion vaccination in rainbow trout (Swan et al, 2008), African catfish (Vervarcke et al, 2005), and European eel (Esteve-Gassent et al, 2003). These indicate that local humoral immunity plays an important role in immersion vaccination.

1.3.5. Injection

1.3.5.1. Characteristics

The vaccine is injected into the fish body, mainly into the peritoneal cavity, with an injector (Figure 1.3.1). Because the fish is taken out of water, it is not only exposed to the danger of suffocation, but also is likely to have its scales and mucosa ripped off during the treatment. In addition, the fish is injured by the injection needle. All together, this method gives the fish a lot of stress, and it is not suitable for vaccinating small fish. For aquatic farmers, a lot of labor is required, because the fish must be injected one by one. Moreover, some special tools such as continuous syringe are necessary to practice this method, and

there is a risk of needle-stick accident. As mentioned above, this method has a lot of disadvantage, but nevertheless, it is the best method at present from the viewpoint of vaccine efficacy. Though the amount of the vaccine administered to fish is small, we can expect a stable and definite effect. Furthermore, the efficacy of the vaccine can be reinforced by adding an immunopotentiating agent called adjuvant to the vaccine.

Figure 1.3.1. Photos showing administration of vaccine to fish via injection

1.3.5.2. Precaution

(For the fish) In this method, a lot of fish are injected with an identical needle, so if one of the fish is infected with some disease, all the other fish in the group may get infected. Therefore, this method should be used when fish are healthy and not infected with any disease. The size of the needle used for the injection must fit the size of the fish, so the farmers should know exactly the size of the fish prior to the treatment. Besides, there is a need for feed withdrawal at least 24 hours before the treatment. This is because if the stomach of the fish is filled up with feed, there won't be enough space left in the abdominal cavity, and the internal organs may be more vulnerable to needle-stick accident. When the stomach is empty, the fish needs less oxygen than when it is full, so the withdrawal is also favorable from the viewpoint of oxygen consumption during the treatment.

(For the operators and assistants) The operators and assistants must always keep in mind that there is a risk of needle-stick accidents or accidental injection of vaccines to themselves. They must always wear protective gear (goggle, mask, thick gloves, etc.) when they practice the treatment. The needle-stick accidents are likely to happen to the non-dominant hand, with which the operator usually holds the fish when injecting, so it is important to wear a thick glove on the non-dominant hand. If accidental injection to the operator is repeated, he might become allergic to the vaccine, and in the worst case, his life could be at risk. In order to carry out the vaccination procedure efficiently, there is a need for assistants who take over the transfer and anesthetic of the fish.

(Anesthetics) Anesthetics can be used if necessary. Anesthetics must be used very carefully, because it might kill the fish when used inappropriately. The effect of the anesthetic depends on the kind and the weight of the fish, as well as environmental factors such as water temperature and water quality, so the amount of the anesthetic should be

adjusted carefully. When the atmospheric temperature is high, we should pay attention to the temperature of the anesthetic solution and make sure it does not get too high.

(Injection) If there is air in the syringe, it should be pushed out before the injection, because such air may cause unstable pressure of the syringe, and thus, inaccuracy of the amount of vaccine solution injected to the fish. Air in the syringe is an obstacle to the efficient administration. In some fish species, scales stuck by the needle will pile up around it when injection is repeated. When this happens, the length of the needle that sticks into the fish body becomes practically shorter, and accurate injection is no longer possible. These scales should be removed if necessary, but that must be done very carefully not to stick your fingers or not to bend the needle. Moreover, the needle should be replaced by a new one every so often, because the needle tip becomes blunt as the injection is repeated. It requires a larger pressure to inject vaccine to a fish with a needle whose tip is blunt, which might result in the bending or breaking of the needle, and at the same time, might give a greater damage to the fish. If the broken needle remains stuck in the fish body (this is called residual needle), and fish is shipped to the market, it is not only dangerous as food but also seriously degrades the reliability of the product.

1.4. Vaccination – Recombinant and DNA Vaccines

Takashi Aoki

1.4.1. Synopsis

Besides formalin-killed and heat-treated vaccines, there are several other types of vaccines, such as attenuated, subunit, and DNA vaccines. In this subsection, current knowledge of the three vaccines is introduced, and the mechanism of action or effect of DNA vaccine is also explained.

1.4.2. Attenuated Vaccine

1.4.2.1. What is an attenuated vaccine?

Attenuated vaccine is used with a mutant that has lost or weakened its pathogenecity as an antigen. The mutant is attenuated conventionally by repeating a subculture for several generations in nutrient media, by chemical processing or radiation. Recently, the attenuated mutant is constructed by modification or mutation of the domain of pathogenic gene using genetic techniques. Such a mutant constructed by these techniques is called as the attenuated vaccine (pathogenic gene mutant vaccine). Generally, it is more effective to remove the pathogenic gene compare to expression of infectious protective antigen for the construction of vaccine since the genome sizes virus and bacteria which are big and have several infectious protective antigens.

Since attenuated vaccines use live virus or bacteria that only weakened its pathogenicity, it is still infectious and can possibly survive in the hosts. Furthermore, because the attenuated vaccine immunity lasts for a long time after inoculation, fewer booster shots are needed. Thus, the attenuated vaccine is effective against intracellular parasitism, bacteria, and viral infection because the immunogenicity of the live pathogenic microbe is maintained. In effect, the host continually produces antibodies and cell-mediated

immunity for the pathogen effectively attained, particularly in cell injury activity compared to inactivated vaccine (formalin inactivated vaccine etc.). Attenuated vaccines induce two immune mechanisms and those responses depend on the infected cells or attenuated vaccine phagocytosed cells (Figure 1.4.1). The cells infected by attenuated microorganism (attenuated vaccine) activate cytotoxic T cell by antigen presentation (Dijkstra et al., 2001; Woolard and Kumaraguru, 2010). Accordingly, infected cells are eliminated by cytotoxic activity. Furthermore, attenuated vaccine phagocytosed cells promote the differentiation of antibody-producing cells (matured B cells) by the activation of helper T cells (Leong, 1993). Due to this, the attenuated pathogenic microorganisms injected to the host are neutralized by the specific responses (Figure 1.4.1).

1.4.2.2. Attenuated Vaccine (Made By a Specific Gene Mutation) For Fish Pathogens

In fish pathogenic bacteria, the *aroA* gene, which is essential in the biosynthesis of aromatic amino acids is the most used gene as pathogenicity knock out attenuated vaccine. The kanamycin resistance gene is inserted in the *aroA* gene present in the chromosomal DNA of pathogenic strains, to produce *aroA* gene-deficient mutant strain (non-pathogenic strain) by homologus recombination. By inoculating the host with an aroA deficient pathogen, it acquires immunity through antibody production or cytotoxic activity to (Figure 1.4.1). In fact, *aroA* gene mutant strains are reported in fish pathogenic bacteria including *Aeromonas salmonicida* (Vaughan et al., 1993; Marsden et al., 1996; Grove et al., 2003; Martin et al., 2006), *A. hydrophila* (Moral et al., 1998; Vivas et al., 2004, 2005), *Yersinia ruckeri* (Temprano et al., 2005), *Photobacterium damsela* subsp. *piscicida* (Thune et al., 2003) (Table 1.4.1). It was reported that the production of B and T cells were strongly induced when *aroA* gene-deficient mutants of *A. salmonicida* described above inoculated was into Atlantic salmon (Marsden et al., 1996). In addition, the comprehensive analysis using microarray shows that gene expression increased in the gills of Atlantic salmon inoculated with *aroA* gene-deficient mutants and also an increase in the expression ofmolecules involved in iron metabolism in the head kidney and liver (anti-microbial protein, C-type lectin and chemokines) (Martin et al., 2006).

Other bacterial pathogens with mutants made for use as attenuated vaccines include: *purA* gene mutant strain (Lawrence et al., 1997),*crp* gene variant of *Edwardsiella ictaluri* (Santander et al., 2011); *esrB* gene mutant strain (Lan et al., 2007) and nutrition related mutant strain (*alr* and *asd* gene mutant) (Choi and Kim, 2011) in *E. tarda*; *exbD* gene mutant strain of *Flavobacterium psychrophilum* (Álvarez et al., 2008); *fur* gene mutant strain of *Pseudomonas fluorescens* variant (Wang et al., 2009); *pgm* gene mutant strain (Buchanan et al., and 2005) and *simA* gene mutant of *Streptococcus iniae* (Locke et al., is 2008) (Table 1.4.1). Attenuated vaccines for fish pathogenic viruses include NV mutated gene in Rhabdoviruses, VHSV and IHNV. NV gene-deficient IHNV strain infected rainbow trout did not show any symptoms of infection and the cumulative mortality was 0% (Thoulouze et al., 2004). It has been reported that infection of VHSV in zebrafish and rainbow trout was controlled the when recombinant virus glycoprotein protein gene, known as antigen protein (G protein), of VHSV and IHNV was substituted with GFP (green fluorescence protein) (Biacchesi et al., 2000, 2002; Romero et al., 2008, 2011; Novoa et al., 2006; Romero et al., 2005). It was also reported that the pathogenicity Koi

herpesvirus (KHV) was slightly weakened and its virulence was reducedwhen thymidine kinase gene was mutated (Costes et al., 2008) (Table 1).

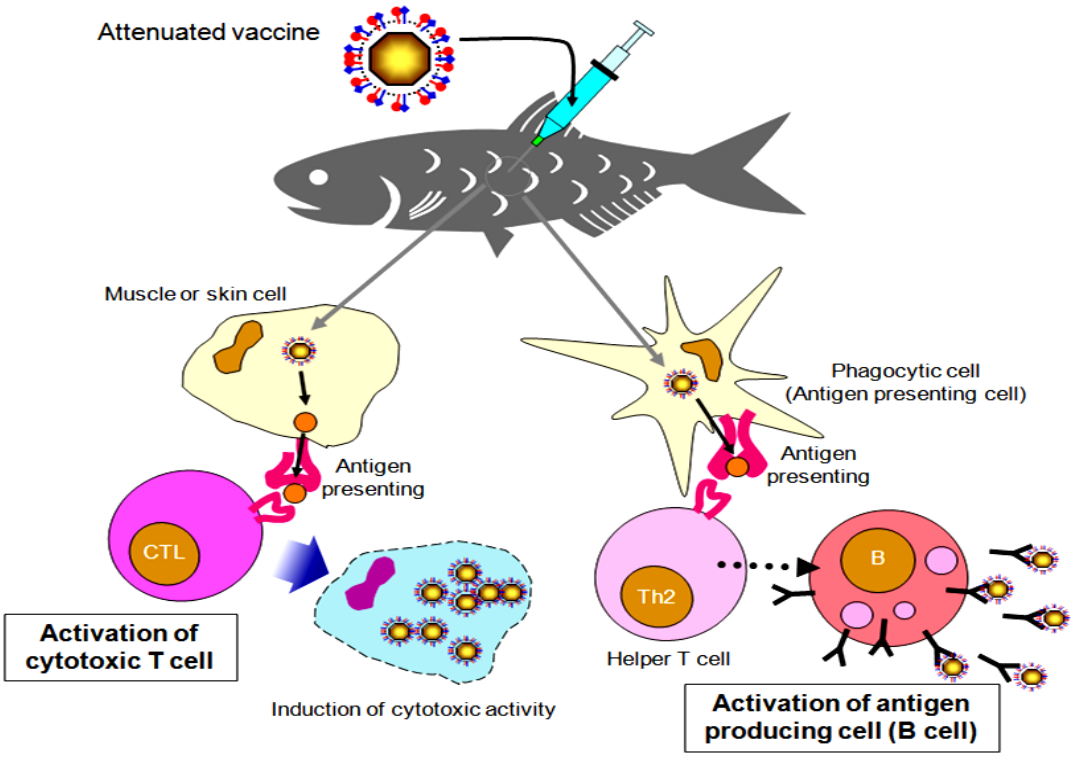


Figure 1.4.1. Immune response induced by attenuated vaccine

Pathogens	Target genes	Fish	Reference
Bacteria			
Aeromonas salmonicida	*aroA*	Atlantic salmon (*Salmo salar*), Brown trout (*S. trutta*)	Vaugahan et al., 1993
		Atlantic salmon (*S. salar*)	Martin et al., 2006
			Grove et al., 2003
		Rainbow trout (*Oncorhynchus mykiss*)	Marsden et al., 1996
	aroA mutants (Birvax I, Birvax II)	Rainbow trout (*Oncorhynchus mykiss*)	Marsden et al., 1998
A. hydrophila	*aroA*	Rainbow trout (*O. mykiss*)	Moral et al., 1998
			Vivas et al., 2005
		Rainbow trout (*O. mykiss*) infected with *A. salmonicida*	Vivas et al., 2004

Edwardsiella ictaluri	*purA*	Channel catfish (*Ictalurus punctatus*)	Lawrence et al., 1997
	crp	Channel catfish (*I. punctatus*)	Santander et al., 2011
E. tarda	*esrB*	Turbot	Lan et al., 2007
	alr and *asd*	Japanese flounder (*Paralichthys olivaceus*)	Choi and Kim, 2011
Flavobacterium psychrophilum	*exbD*	Rainbow trout (*O. mykiss*)	Álvarez et al., 2008
Photobacterium damselae ssp. *piscicida*	*aroA*	Hybrid striped bass*	Thune et al., 2003
Pseudomonas fluorescens	*fur*	Japanese flounder (*P. olivaceus*)	Wang et al., 2009
Streptococcus iniae	*pgm*	Hybrid striped bass*	Buchanan et al., 2005
	simA	Hybrid striped bass*	Locke et al., 2008
		Zebrafish (*Danio rerio*)	
Yersinia ruckeri	*aroA*	Rainbow trout (*O. mykiss*)	Temprano et al., 2005
Virus			
KHV	Thymidine kinase gene	Common carp (*Cyprinus carpio*)	Costes et al., 2008
IHNV	NV	Rainbow trout (*O. mykiss*)	Thoulouze et al., 2004
	rIHNV-Gvhsv	Rainbow trout (*O. mykiss*)	Romero et al., 2005, 2008, 2011
VHSV	rVHSV-ΔNV-EGFP	Japanese flounder (*P. olivaceus*)	Kim et al., 2011
*Hybrid striped bass（HSB）：Hybrid fish with *Morone saxatilis* and *M. chrysops*			

Table 1.4.1．Attenuated vaccines (mutated target gene) used for fish pathogens

1.4.3. Subunit Vaccine (Or Component Vaccine)

1.4.3.1. What Is A Subunit Vaccine?

Subunit vaccines (or component vaccines) makes use of antigenic proteins of pathogenic microorganisms which are extracted and purified from the pathogen, or are produced by genetic engineering using *Escherichia coli*, *Bacillus subtilis*, yeast and cultured animal cells. In theory, the subunit vaccine is more effective compared with inactivated vaccine and its main component is only the antigenic protein so that certain contamination of unwanted proteins is less; it is also very safe, inexpensive and can be mass produced. Immune response mechanism of this vaccine is different from the attenuated vaccines described above and it activates only the antigen presentation pathway (Figure 4). First, the recombinant antigen proteins derived from pathogenic microorganisms that were

produced by *E. coli* etc. is inoculated into the host as a subunit vaccine. Then macrophages and phagocytes such as dendritic cells (antigen presenting cells) capture it as a foreign protein and the helper T cells are activated by antigen presentation and co-stimulation (Leong, 1993; Christie, 1997). Differentiation of antibody-producing cells is promoted by this and the pathogenic microorganism infected to the host is neutralized by specific antibodies produced (Figure 1.4.2).

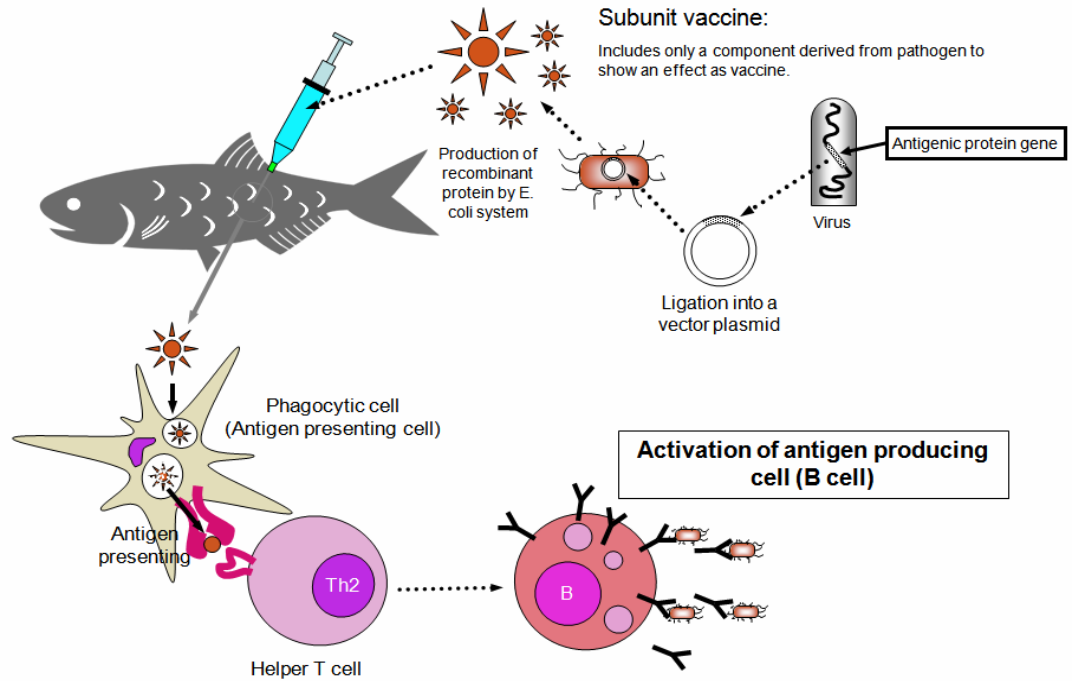


Figure 1.4.2. Immune response induced by subunit vaccine

2) Subunit vaccine for fish pathogens

Subunit vaccines that showed effectiveness against viral infections in fish is given in Table 1.4.2. As described in the section of attenuated vaccines, G protein used as a subunit vaccine is effective against infections with Rhabdoviruses. It has been shown that the Rhabdoviral G protein is highly effective as an antigenic protein (Winton, 1997). The recombinant G protein of infectious hematopoietic necrosis virus (IHNV) shows was also shown to be highly effective as an antigenic protein (Leong et al., 1987; Engelking and Leong, 1989a, 1989b; Gilmor et al., 1988; Oberg et al., 1991; Noonan et al., 1995; Cain et al., 1999a, 1999b; Simon et al., 2001). In addition, rainbow trout inoculated with recombinant G protein induces the expression of type I interferon (IFN) and IFN-γ gene and inflammatory cytokines (Verjan et al., 2008). Aside from recombinant G protein (Lorenzen et al., 1993; Lecocq-Xhonneux et al., 1994; Lorenzen and Olsen, 1997), CTL-like peptide (Estepa and Coll, 1993), and VHSV-G protein as a G4 peptide protein (Estepa et al., 1994; Lorenzo et al., 1995) were also effective against viral hemorrhagic septicemia virus (VHSV).

Capsid protein is used as a subunit vaccine for infection of birnaviruses or beta nodaviruses (Table 1.4.2). There are VP1 and VP2, VP3 in the capsid protein of infectious pancreatic necrosis virus (IPNV) (Dorson, 1988; Yao and Vakharia, 1998) and it is the recombinant protein of VP2 was effective against IPNV infection (Allnutt et al.,

2007; Min et al., 2012). Furthermore, it has been shown that the recombinant capsid protein rVP2 is effective against IPNV when inoculated into Atlantic salmon mixed into the oil adjuvant with glucan (Christie, 1997). It has been reported that Norvax Compact 6 which is combination vaccine with IPNV-rVP2 proteins (available from MERCK Co.) indicated a high protection (Ramstad et al., 2007).

As the vaccine against infection of beta Noda virus, it has been shown that a recombinant capsid protein recAHNV-C of Atlantic halibut (*Hippoglossus hippoglossus*) nervous necrosis virus (AHNV) (Sommerset et al., 2005) or a recombinant capsid protein of rT2 of striped jack nervous necrosis virus (SJNNV) (Húsgağ et al., 2001) were effective. In addition, it has been reported that inoculation of a virus-like particle (VLRs) of giant grouper (*Epinephelus lanceolatus*) viral nervous necrosis of (DGNNV) gives a high antibody titer against DGNNV and maintained for more than five months (Liu et al., 2006).

In the iridovirus and herpes virus family, which has a double-stranded DNA genomes, recombinant capsid protein (18R, 351R, MCP) has been reported as a vaccine against iridovirus disease of the red sea bream, although the protective effect is not so high (Shimmoto et al., 2010). On the other hand, recombinant major capsid protein (rMCP) of parrot fish iridovirus showed high protective effect even for a month after inoculation (Kim et al., 2008).

Various recombinant proteins have been used as subunit vaccines against fish pathogenic bacteria (Table 2). The outer membrane proteins of *A. hydrophila*, *E. tarda*, *V. alginolyticus*, *V. haveyi* etc. (Guan et al., 2011b; Khushiramani et al., 2012; Maiti et al., 2011; Qian et al., 2007; Ningqiu et al., 2008), flagellar protein *FlgK* of *A. hydrophila* (Yeh and Klesius, 2011), recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *E. tarda* (Liu et al., 2005) displayed protective efficiency. Specific antibody titers rises, with some individual difference, when A-layer protein (or At-R recombinant protein) which is an extracellular molecules involved in spontaneous aggregation of atypical *A. salmonicida* inoculated to Goldfish (Maurice et al., 2003; 2004) or the spotted wolffish (*Anarhichas minor*) (Grøntvedt and Espelid, 2004). A-layer protein-specific antibody reactions have been identified when strains have an A-layer protein gene inoculated into fish (Lund et al., 2003). In addition, it has been shown that the recombinant p57 protein of *Renibacterium salmoninarum* is effective as an epitope because of the antibody titer increased when it was inoculated to sock eye salmon (*Oncorhynchus nerka*) (Alcorn and Pascho, 2000); in rainbow trout inoculated with DNA vaccine containing p57 gene (*msa*), the expressions of IL-1β, Cox-2, and inflammatory cytokine genes such as TNFα were induced (Grayson et al., 2002).

It has been shown that outer membrane lipoprotein OspA (Kuzyk et al., 2001a, 2001b) as a sub-unit vaccines against rickettsial septicemia by salmonid fish (*Piscirickettsia salmonis*) infection, and mixed subunit vaccine of heat shock protein and flagellar protein (Hsp70, Hsp60 and FlgG) (Wilhelm et al., 2006) are highly effective in Atlantic salmon and coho salmon. Furthermore, the ChaPs (Epitope protein) of 57.3kDa in the heat shock protein family is also effective as an antigen (Marshall et al., 2007). Goldfish inoculated with the recombinant protein for 48kDa immobilization antigen gene (Clark et al., 2001) of *Ichthyophthirius multifiliis* which is a fish parasite showed protective response against

I. multifiliis (He et al., 1997). Recombinant proteins which derived from my32 (akirin-2 like gene) of a sea lice, *Caligus rogercresseyi* act effectively when inoculated into Atlantic salmon and the number of parasites in the body surface was reduced significantly (Carpio et al., 2011).

Pathogens	Recombinat protein	Fish	Reference
VIRUS			
Infectious hematopoietic necrosis virus (IHNV)	Glycoprotein (G protein)	Rainbow trout (*Oncorhynchus mykiss*)	Leong et al., 1987; Engelking and Leong, 1989a, 1989b; Oberg et al., 1991; Noonan et al., 1995; Cain et al., 1999a, 1999b
	G protein + *trp*E (fusion protein, *trp*E-G)		Gilmore et al., 1988; Xu et al., 1991
	G protein (184 amino acid residues)		Simon et al., 2001
Viral hemorrhagic septicemia virus (VHSV)	G protein	Rainbow trout (*Oncorhynchus mykiss*)	Lorenzen et al., 1993; Lecocq-Xhonneux et al., 1994; Lorenzen and Olsen, 1997; Noonan et al., 1995
	G protein (G4 peptide protein)		Estepa et al., 1994; Lorenzo et al., 1995
Infectious pancreatic necrosis virus (IPNV)	IPNV-VLPs (Vius-like particle)	Atlantic salmon (*Salmo salar*)	Shivappa et al., 2005
	Capsid protein (rVP2) + oil/glcan adjuvant		Christie, 1997
	IPNV-rVP2 (NC-4, NC-6)		Ramstad et al., 2007
	VP2	Rainbow trout (*O. mykiss*)	Leong et al., 1987
	rVP2-SVP		Allnutt et al., 2007
	VP2, VP3		Min et al., 2012
Yellowtail ascites virus (YAV)	VP2, NS-VP3	Yellowtail (*Seriola quinqueradiata*)	Sato et al., 2000
Atlantic halibut nodavirus (AHNV)	Capcid protein (recAHNV-C)	Atlantic halibut (*Hippoglossus hippoglossus*)	Sommerset et al., 2005
Striped jack nervous necrosis virus (SJNNV)	Capcid protein (rT2)	Turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*)	Húsgağ et al., 2001

Dragon grouper nervous necrosis virus (DGNNV)	Vius-like particles (VLPs)	Giant grouper (*Epinephelus lanceolatus*)	Liu et al., 2006
Rock bream iridovirus (RBIV)	Major capsid protein (MCP)	Rock seabream (*Oplegnathus fasciatus*)	Kim et al., 2008
Red sea bream iridovirus (RSIV)	Capcid proteins (18R, 351R, MCP)	Red seabream (*Pagrus major*)	Shimmoto et al., 2010
BACTERIA			
Aeromonas hydrophila	GAPDH (pETGA-pUTaBE)	Turbot (*Sc. maximus*)	Guan et al., 2011a
	Omp-G	European eel (*Anguilla anguilla*)	Guan et al., 2011b
	FlgK (Flagellar protein)	Channel catfish (*Ictalurus punctatus*)	Yeh and Klesius, 2011
	Omp48	Rohu (*Labeo rohita*)	Khushiramani et al., 2012
A. salmonicida (A-typical)	At-R (A-layer protein)	Goldfish (*Carassius auratus*)	Maurice et al., 2003
	At-R and At-MTS (Kaposi fibroblast growth factor)		Maurice et al., 2004
	A-layer protein	Spotted wolffish (*Anarhichas minor* Olafsen)	Grøntvedt and Espelid, 2004
A. sobria	Omp-G	European eel (*An. anguilla*)	Guan et al., 2011b
Edwardsiella tarda	rGAPDH	Japanese flounder (*Paralichthys olivaceus*)	Liu et al., 2005
	Esa1		Sun et al., 2010
	DnaJ (Hsp70)		Dang et al., 2011
	Sia10-DnaK		Hu et al., 2012
	Inv1 (invasin)		Li et al., 2012
	DegP		Jiao et al., 2010
	GAPDH	Turbot (*Sc. maximus*)	Mu et al., 2011a
	OmpA	Common carp (*Cyprinus carpio*)	Maiti et al., 2011
	A. hydrophila Omp48	Rohu (*L. rohita*)	Khushiramani et al., 2012
Photobacterium damselae sbsp. *piscicida*	HSP60, ENOLASE, GAPDH	Cobia (*Rachycentron canadum* L)	Ho et al., 2011
Streptococcus iniae	Sip11	Japanese flounder (*Pa. olivaceus*)	Cheng et al., 2010
	Sia10-DnaK		Hu et al., 2012

Vibrio alginolyticus	OmpW (outer membrane protein)	Large yellow crocker (*Pseudosciaena crocea*)	Qian et al., 2007
V. haveyi	OmpK (outer membrane protein)	Orange-spotted grouper (*Epinephelus coioides*)	Ningqiu et al., 2008
	DegQ (Vh)	Japanese flounder (*Pa. olivaceus*)	Zhang et al., 2008
V. vulnificus	epinecidin-1	Orange-spotted grouper (*Ep. coioides*)	Pan et al., 2012
RICKETTSIA			
Piscirickettsia salmonis	OspA (outer surface lipoprotein)	Coho salmon (*O. kisutch*)	Kuzyk et al., 2001a, 2001b
	Hsp70, Hsp60, FlgG	Atlantic salmon (*Sa. salar*)	Wilhelm et al., 2006
	ChaPs (57.3kDa epitopic protein)	Coho salmon (*O. kisutch*)	Marchall et al., 2007
Parasite			
Caligus rogercresseyi (Sea lice)	my32 (akirin-2)	Atlantic salmon (*Sa. salar*)	Carpio et al., 2011
Ichthyophthirius multifiliis	GST-iAgI fusion protein	Goldfish (*Carassius auratus*)	He et al., 1997

Table 1.4.2. Recombinant vaccines used for fish pathogens

1.4.4. DNA Vaccine

1.4.4.1. What Is DNA Vaccine?

In DNA vaccines, induction of immunity against pathogenic microorganisms is effected by injection of genetically engineered DNA (recombinant DNA) of pathogenic microorganisms into the body surface and muscle of fish by using a gene gun or syringe (Figure 1.4.3). DNA vaccine can effectively express epitope gene in tissues *in vivo*, induce cellular immune function and acquired immune function when inoculated into the fish. The DNA vaccine is a very excellent method compared to other vaccines with its high efficiency, lower dose, long term effect, and there are no side effects. It can easily be mass produced with lower cost and the efficacy is kept even when stored at room temperature. DNA vaccines for IHNV are already commercially available and used in farms in Canada (Salonius et al., 2007). However, since DNA vaccines (recombinant plasmid DNA) inoculated directly to fish and DNA is replicated in the fish body, it is not

permitted in countries other than Canada from the viewpoint of food safety (Myhr and Dalmo, 2005; Schild, 2005; Gillund et al., 2008a, 2008b; Gomez-Casado et al., 2011).

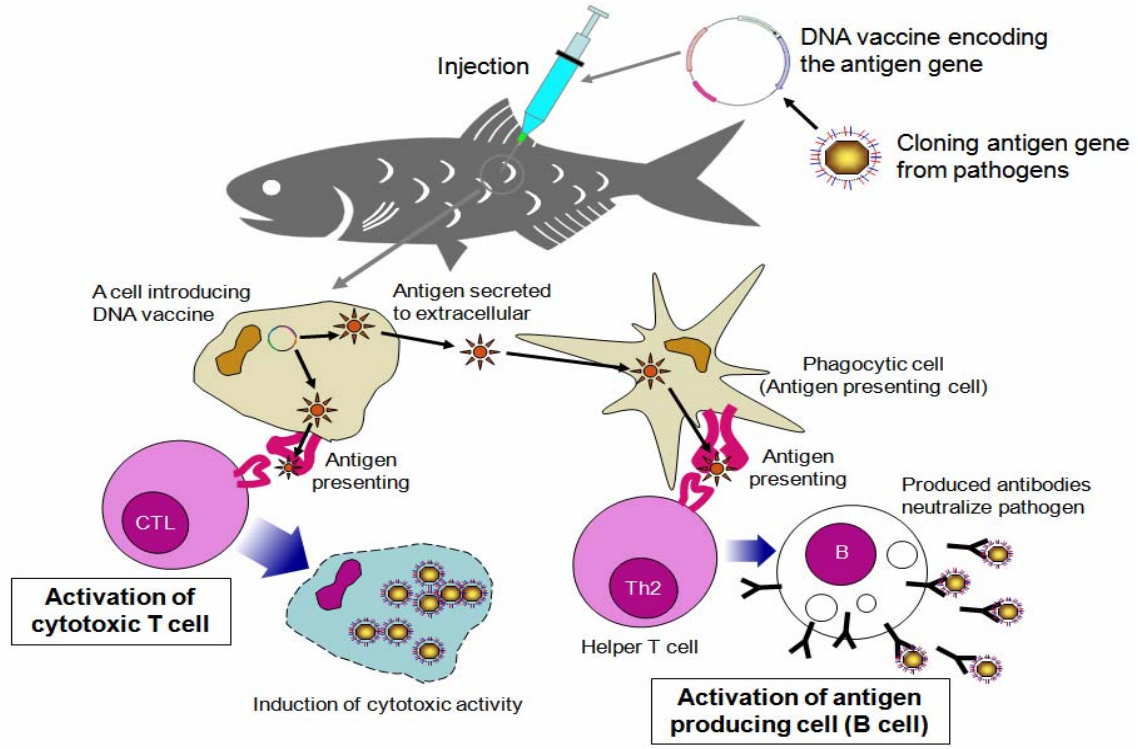


Figure 1.4.3. Immune response induced by DNA vaccine

1.4.4.2. DNA Vaccine for Fish Pathogens

So far, efficacy of DNA vaccines against many pathogenic microorganisms and parasites of fish have been reported (Kurath, 2008; Tonheim et al., 2008; Gomez-Casado et al., 2011) (Table 1.4.3). In fish pathogenic virus, effectiveness of DNA vaccine of G protein gene has been observed in flounder and salmonid fish against IHNV, VHSV and Hirame Rhabdovirus (HIRRV) (Oberg et al., 1991; Anderson et al., 1996; Corbeil et al., 1999; Traxler et al., 1999; Graver et al., 2005; Acosta et al., 2005; Byon et al., 2005; Takano et al., 2004; Seo et al., 2006; Yasuike et al., 2007). The vaccine effect has been also confirmed in carp for G protein of Spring Viraemia of Carp Virus (SVCV) (Kanellos et al., 2006; Emmenegger and Kurath, 2008). VP2 gene shows effectiveness against IPNV (Mikalsen et al., 2004; De las Hears et al., 2010). It have been identified that Major capcid protein (MCP) gene is used as a DNA vaccine for two Iridoviruses, Red Seabream Iridovirus (RSIV) and Lymphocystis disease Virus (LCDV), and it is effective when orally administered in a micro-capsule (Caipang et al., 2006a; Tian et al., 2008a, 2008b, 2008c; Tian and Yu, 2011). The other DNA vaccines, Hemagglutinin-Esterase (HE) of Infectious Salmon Anemia Virus (ISAV) (Mikalsen et al., 2005), Envelope glycoprotein (EG) of Channel Catfish Herpesvirus (CCV) (Nusbaum et al., 2002), capsid protein of Viral Nervous Necrosis Virus (VNNV) (Sommerset et al., 2003), and VP28 of White Spot Syndrome Virus (WSSV) (Kumar et al., 2008b) have also been reported.

Pthogens	Taget gene	Fish	Delivery method	Effects	Reference
VIRUS					
IHNV (Infectious hematopoietic necrosis virus)	Glycorotein (G protein)	Rainbow trout	Intramus-cular (i.m.)	Yes	Oberg et al., 1991; Anderson et al., 1996; Corbeil et al., 1999
	G protein	Atlantic salmon	i.m.	Yes	Traxler et al., 1999
	G protein	Chinook salmon	i.m.	Yes	Graver et al., 2005
	G protein	Sockeye salmon	i.m.	Yes	Graver et al., 2005
	G protein	Rainbow trout	Gene gun	Yes	Corbeil et al., 2000
	G protein	Rainbow trout	Intraperito-neal (i.p.)	Weak	Corbeil et al., 2000
	SVCV-G protein	Rainbow trout	i.m.	Yes	Kim et al., 2000
	SHRV-G protein	Rainbow trout	i.m.	Yes	Kim et al., 2000
	VHSV-G protein	Rainbow trout	i.m.	Yes	LaPatera et al., 2001
	G (M-type) protein	Rainbow trout	i.m.	Yes	Perelberg et al., 2011
	G protein (suicidal)	Rainbow trout	i.m.	Yes	Alonso et al., 2011
VHSV (Viral hemorrhagic septicemia virus)	G protein	Rainbow trout	i.m.	Yes	Lorenzen et al., 1998; Heppell et al., 1998; Acosta et al., 2005
	G protein	Japanese flounder	i.m.	Yes	Byon et al., 2005
	G protein	Atlantic salmon	i.m.	Yes	Acosta et al., 2005
	IHNV-G protein (gIHN)	Rainbow trout	i.m.	Yes	Boudunot et al., 2004
	Carp β-actin promoter + G protein	Rainbow trout	i.m.	Yes	Chico et al., 2009
	G protein	Rainbow trout (fly)	Immersion	Weak	Fernandez-Alonso et al., 2001
	VHSV **+** IHNV-G protein (bivalent vaccine)	Rainbow trout	i.m.	Yes	Boudinot et al., 1998; Eonnwe-jensen et al., 2009
HIRRV (Hirame rhabdovirus)	G protein	Japanese flounder	i.m.	Yes	Takano et al., 2004; Seo et al., 2006; Yasuike et al., 2007
IPNV (Infectious pancreatic necrosis virus)	VP2 (Large ORF polyprotein)	Atlantic salmon	i.m.	Yes	Mikalsen et al., 2004
	VP2	Rainbow trout	Oral (Aliginate micro-capsule)	Yes	De las Hears et al., 2010
RSIV (Red	MCP (Major	Red seabream	i.m.	Yes	Caipang et al.,

seabream iridovirus)	capsid protein)				2006a
	ORF569 (Transmembrane domin protein)	Red seabream	i.m.	Yes	Caipang et al., 2006a
	MCP + TD-569 (bivalent vaccine)	Red seabream	i.m.	Yes	Caipang et al., 2006a
LCDV (Lymphocystis disease virus)	MCP	Japanese flounder	Oral (PLGA* micro-capsule)	Yes	Tian et al., 2008b; Tian and Yu, 2011
	MCP	Japanese flounder	Oral (Arginine microspheres)	Yes	Tian et al., 2008a
	MCP	Japanese flounder	Oral (Chitosan microspheres)	?	Tian et al., 2008c
ISAV (Infectious salmon Anemia virus)	HE (Hemagglutinin-Esterase)	Atlantic salmon	i.m.	Yes	Mikalsen et al., 2005
SVCV (Spring viraemia of carp virus)	G protein	Common carp	i.m.	Medium	Kanellos et al., 2006
	G protein	Koi carp	i.m.	Yes	Emmenegger and Kurath, 2008
CCV (Channel catfish virus)	EG (Envelope glycoprotein : ORF59)	Channel catfish	i.m.	Yes	Nusbaum et al., 2002
	EG+MP (Membrane protein)	Channel catfish	i.m.	Yes	Nusbaum et al., 2002
AHNV (Atlantic halibut nodavirus, One of VNNV: Viral nervous necrosis virus)	Capsid protein	Atlantic halibut	i.m.	Weak	Sommerset et al., 2003
	VHSV-G protein (derived from Rainbow trout)	Atlantic halibut	i.m.	Yes	Sommerset et al., 2003
WSSV (White spot syndrome virus)	VP28	Black tiger shrimp	i.m.	Yes	Kumar et al., 2008
	VP28	Kuruma shrimp	i.m.	Yes	Kumar et al., 2008
RICKETTSIA					
Piscirickettsia salmonis	Unknown antigenic protein	Coho salmon	i.m.	Weak	Miquel et al., 2003
BACTERIA					
Aeromonas veroni	Omp38 (Major outer membrane protein)	Spotted sand bass	i.m.	Yes	Vazquez-Juarez et al., 2005
	Omp48	Spotted sand bass	i.m.	Yes	Vazquez-Juarez et al., 2005
	Omp38 + Omp48 (Bivalent vaccine)	Spotted sand bass	i.m.	Yes	Vazquez-Juarez et al., 2005

Edwardsiella tarda	Eta6 + FliC fusion gene（pCE6）	Japanese flounder	i.m.	Yes	Jiao et al., 2009
	Eta2	Japanese flounder	i.m.	Yes	Sun et al., 2011a
	Esa1 (D15-like surface antigen gene)	Japanese flounder	i.m.	Yes	Sun et al., 2011b
Mycobacterium marinum	Ag85A (Antigenic protein)	Hybrid striped bass	i.m.	Yes	Pasnik et al 2005, 2006
Streptococcus iniae	Sia10 (Putative secretory antigen)	Turbot	i.m.	Yes	Sun et al., 2010
Vibrio alginolyticus	flaA (flagellin)	Red snapper	i.m.	Yes	Liang et al., 2010
V. anguillarum	OMP38 (Outer membrane protein)	Barramundi (Asian seabass)	i.m.	Medium	Kumar et al., 2006
	OMP39	Barramundi (Asian seabass)	Oral (Chitosan nanoparticle)	Medium	Kumar et al., 2008
	EmpA (Extracellular zinc metalloprotease)	Japanese flounder	i.m.	Yes	Yang et al., 2009
	Streptococcus iniae のSia10 + EmpA (bivalent vaccine)	Japanese flounder	i.m.	Yes	Sun et al., 2012
V. parahaemo-lyticus	Serine protenase	Turbot	i.m.	Yes	Liu et al., 2011
V. harveyi	OmpU (Outer membrane protein)	Turbot	i.m.	Yes	Wang et al., 2011
	DegQ (Antigenic protein)	Japanese flounder	i.m.	Yes	Hu and Sun, 2011
	Vhp1 (Antigenic protein)	Japanese flounder	i.m.	Yes	Hu and Sun, 2011
	DegQ + Vhp1 (pDV: bivalent vaccine)	Japanese flounder	i.m.	Yes	Hu and Sun, 2011
PARASITE					
Crytobia salmositica	MP (Metalloprotease)	Rainbow trout	i.m.	Yes	Tan et al., 2008
	MP	Atlantic salmon	i.m.	Yes	
Cryptocaryyon irritans	iAg (Immobilization antigen)	Grouper	i.m.	Medium	Priya et al., 2012
*** PLGA: Poly(D,L-Lactic-Co-Glycolic Acid)**					

Table 1.4.3. DNA vaccines used for fish and shellfish pathogens

Further, unidentified genes encoding the antigenic proteins as DNA vaccine against Rikkechia (Riscirickettsiosis) have been used, but the protective effect is not high (Miquel et al., 2003).

In the fish pathogenic bacteria, DNA vaccines against infection of *A. veronii*, *E. tarda*, *Streptococcus iniae, Vibrio alginolyicus, V. anguillarum, V. parahaemolyticus, and V.*

harveyi, and Mycobacterium marinum has been reported and an antigen protein such as Outer membrane protein (OMP) are used. However, the effect is varied so that further confirmation is necessary.

Finally, the development of DNA vaccine against the parasite infection of *Crytobia salmositica* also has been studied and it has been confirmed that the Metalloprotenase (MP) gene vaccine shows protective capacity (Tan et al., 2008).

1.4.4.3. Machinery of DNA Vaccine Process in Fish

T cells are activated when recombinant plasmid DNA inserted with an antigenic gene (DNA vaccine) is inoculated into vertebrate muscle, further antibody production was observed, depending on the type of antigen. In fish, most of these defense reaction pathways are still unidentified. It is suggested that recombinant DNA in DNA vaccines inoculated in Atlantic cod is carried by the blood to endocardial endothelial cells and incorporated into the EEC by endocytosis through scavenger receptors (Seternes et al., 2007). So far, it has been experimentally confirmed that the expression of MHC class I, MHC class II, TCRα, and TCRβ and T cell activation-associated genes is induced in flounder inoculated with DNA vaccines encoding the G protein gene of HIRRV (Takano et al., 2004; Yasuike et al., 2011), from a microarray experiment it was shown that expressions of IgM, IgD, MHC class II, CD8α, CD20 receptor, CD40, B lymphocyte cell adhesion molecule and NK/ Kupffer cell receptor genes were induced in flounder t inoculated with the VHSV G protein DNA vaccine (Byon et al., 2005, 2006). Further, in rainbow trout vaccinated with VHSV G protein gene DNA vaccine, prominent expression of IL-1β and MHCIIα in spleen and MHCIα, IFN and Mx gene in spleen and blood were observed (Cuesta and Tafalla, 2009). In addition, the antibody titer after DNA vaccination of MCP of RSIV was increased and the expression of MHC class I gene is induced (Caipang et al., 2006a, 2006b). From these, it can be inferred that maturation and differentiation of B-cell antigen presentation to T cells, and differentiation, to the functional T cells occurred as an effect of the DNA vaccine in fish (Figure 1.4.3).

1.4.5. Conclusion

The focal point of these types of vaccine research is how to explore the antigenic determinants (epitope) to maximize the immune defense function of the host and how to activate efficiently the immune responses. Recombinant vaccine for rhabdoviruses etc. whose chromosome genomes are single-stranded RNA is relatively highly effective, but those of the iridovirus etc. whose chromosome genomes are double-stranded DNA is less effective. Further, it is considered to induce immunity with a combination of adjuvants because Subunit vaccine itself is pure antigenic protein so that its immunity induction is poor. In the future, further research of vaccines with immunological background and the development of more effective DNA vaccines are desired. Research is also required on efficient delivery or transport methods for vaccines to achieve higher effectiveness.

1.5. Fish Immunostilumants

Masahiro Sakai

1.5.1. Synopsis

Fish rely more on their innate immunity to prevent diseases and immunostimulants generally stimulate innate immune components. These immunostimulating substances mostly activate the phagocytes and their function along with production of acute phase proteins to provide protection against diseases. Many immunostimulants such as glucans, levamisole, chitin, lipopolysaccharides, lactoferrin, vitamins C and E, hormones, CpG-ODN and nucleotides have been reported to increase protection against bacterial, viral and parasitic diseases in fish.

1.5.2. Introduction

Immunostimulants increase resistance to infectious disease, not by enhancing specific immune responses, but by improving innate immune defense mechanisms. There is no memory component and the response is likely to be of short duration. These immunostimulants have been used in medical and veterinary sciences. Research on fish immunostimulants is developing and many agents are currently in use for the aquaculture industry. Use of immunostimulants, in addition to chemotheraputic agents and vaccines, has been widely accepted by fish farmers. However, several questions about the efficacy of immunostimulants from users still remain unanswered. In this review, the use of immunostimulants, particularly their dose, time of application and route of administration, will be described.

1.5.3. Immunostimulants Used in Fish and Shrimp

Immunostimulants which have been used or studied in fish and shrimp, include chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors, cytokines, CpG-ODN and nucleic acids etc. (Table 1.5.1). Glucan is one of the most extensively studied and applied in aquaculture.

Synthetic Chemicals	
Levamisole	
FK-565	
MDP (Muramyl dipeptide)	
Biological substances	
1) Bacterial derivatives	
β-glucan	
Peptidoglycan	(*Brevibacterium lactofermentum*)
	(*Vibrio* sp.)
FCA (Freund completed adjuvant)	
EF 203	
LPS (lipopolysaccharide)	

Clostridium butyricum cells
Achromobacter stenohalis cells
Vibrio anguillarum cells (*Vibrio* vaccine)
2) Polysaccharides
Chitin
Chitosan
Lentinan
Schizophyllan
Oligosaccharide
3) Animal and Plant Extracts
Ete (Tunicate)
Hde (Abalone)
Firefly sequid
Quillaja saponin（Soap tree）
Glycyrrhizin (licorice)
4) Nutritional Factors
Vitamin C
Vitamin E
5) Hormones and Cytokines
Lactoferrin
Interferon
Growth hormone
Prolactin
6) Antimicrobial components
Lactoferrin
Lysozyme
7) Nucleic acids
CpG DNN
PolyI:C
Nucleotides

Table 1.5.1. Main immunostimulants used in fish and shrimp (modified from Sakai, 1999)

1.5.4. Fish Defense System Enhancement by Immunostimulants

Generally, immunostimulants activate the innate and acquired immune systems. Fish treated with immunostimulants usually show enhanced phagocytic cell activities such as phagocytosis, killing and chemotaxis. Lymphocytes (T and B cells) and NK cells are also activated by immunostimulants. Furthermore, the humoral factors such as complement

activity and lysozyme can also be activated by immunostimulants (Sakai, 1999). However, the activated immune system by immunostimulants may not relate with the increased resistance to pathogen. Actually, immunostimulants do not increase resistance against *Renibacterium salmoninarum, Photobacterium damsela* or *Edwardsella ictaluri* infection. These bacteria are resistant to phagocytosis and can survive within macrophages. As already indicated, the main immunological function increased by immunostimulants is the activity of phagocytic cells. However, macrophage-resistant bacteria may escape from activated macrophages and thus in these situations, immunostimulants do not appear effective against such infections.

1.5.5. Field Application for Fish Immunostimulants

1.5.5.1. Effect of Time and Long Term Administration

The time of administration of any immunostimulant is an important issue to be considered. Unlike antibiotics that are applied usually after disease occurs, this substance should be applied before the outbreak of disease to reduce disease-related losses. Some immunostimulants can promote recovery from immunosuppression states caused by stress. Kitao and Yoshida (1986) reported that rainbow trout injected with cyclophosphamide or hydrocortisone showed suppressed phagocytic activity of peritoneal and kidney leucocytes, and this suppression was reversed by injection of FK-565.

As most of the immunostimulating substances have short-lived effect, continuous administration might be necessary to sustain effective results. However, the effects of long-term administration of immunostimulants still need to discuss. Matsuo and Miyazano (1993) reported that rainbow trout treated with peptidoglycan orally for 56 days did not show resistance after challenge with *Vibrio anguillarum*, although fish treated for 28 days showed increased resistance.

1.5.5.2. Route

Injection of immunostimulants can enhance the function of leucocytes and protection against pathogens. However, this method is labor intensive, relatively time-consuming and becomes impractical when fish weigh less than 15 g. Thus, another method such as oral administration or immersion should be used. Oral administration of immunostimulants is generally acceptable in fish farm as it is not stressful and ideal for mass application. The controversies on oral administration are wastage in environment, differential stimulation and above all no or poor stimulation in diseased fish that are under stress to accept feed. On the other hand, efficacies of immersion treatment have been reported by several authors. However, the dilution and the levels of efficacy require a more complete investigation.

1.5.5.3. Dose

The effective dose of immunostimulants should be determined carefully. Kajita et al. (1990) showed that the chemiluminescent effects of phagocytic cells in rainbow trout were increased by injection of levamisole at 0.1 and 0.5 mg/kg. However, they also

reported that the injection of 5 mg/kg of levamisole did not produce any immunostimulant effect. Similar results were reported in experiments using glucan (Robertsen et al, 1994). The effects of immunostimulants are not directly dose-dependent. High doses may suppress the immune function. Furthermore, an effective dosage will be further complicated by different feeding strategies adopted by farmers in culture operations.

1.5.5.4. Additional Effects of Immunostimulants

There are few studies on combination of antibiotics and immunostimulants. Some antibiotics such as tetracycline exhibit immunosuppressive effects. Tompson et al. (1995) investigated about the combination of oxytetracycline and glucan to examine the resistance to vibrio disease. Their results showed that the survival rate is higher than the single administration of each substance. The lysozyme activity in fish administered with oxytetracycline alone decreased compared with the control. However, this activity was recovered by concomitant use. It is necessary to investigate in detail the effectiveness of the combination of antibiotics and immunostimulants for future use.

Immunostimulants have been originally developed as an adjuvant. Thus, it has the function to enhance the ability of antibody production. Rorstad et al., (Rørstad et al, 1993) reported that the effect of the *Aeromonas salmonicida* vaccine is enhanced when yeast glucan is administered as an adjuvant. As a similar example, yeast glucan is also effective as an adjuvant of Vibrio vaccine (Baulny et al, 1996). Askre et al. (1994) reported that and the increase in antibody titer of vaccinated fish was observed, although the efficacy of the vaccine was not enhanced when *A. salmonicida* cell wall bacterin containing β-1, 3-M-glucan was administered as an adjuvant.

1.5.6. Conclusion

In this review, the use of immunostimulants was discussed as a means of controlling and preventing fish disease. To control fish diseases, vaccination, chemotheraputics and immunostimulants have been used in aquaculture. Immunostimulants may be able to compensate for the limitations of chemotherapeutics and vaccines. The advantages of immunostimulants are thought to be safer than chemotheraputics and their range of efficacy is wider than vaccination. The administration of immunostimulant as adjuvants may also increase the potency of vaccines. Thus, with a detailed understanding on the efficacy and limitations of immunostimulants, they may become powerful tools to control and prevent fish diseases.

Glossary

CpG-ODN: Cytosine-phosphate-guanine oligodeoxynucleotides,

NK cells: Natural killer cells

2. DIAGNOSIS OF DISEASES

2.1. Diagnosis -Antiserum Detection

Tae-Sung Jung

2.1.1. Synopis

In the advent of readily available diagnostic kits, coupled with the rapid advancement in the field of genetic manipulation, the application of antibody-based diagnosis seems to lose its significance (Cunningham, 2002). Not to mention the difficulty in producing specific antibodies and the time needed for it to be usable. However, it remains indispensable and essential in understanding immune response mechanisms and development of effective vaccines and has high efficiency in the aspect of rapid diagnosis without the need for any complicated machines and kits (Adams and Thompson, 2006). Here, some useful applications of antibody-based diagnosis will be introduced, from basic agglutination to immunochromatography assay.

2.1.2. Introduction

Animals immunized by antigens (or immunogens) produce antibodies in response to proteins or other molecules recognized as foreign by their immune system (Tizard, 2010). Of course, there are several factors to be considered in terms of immune intensity. Firstly, immunogens are composed of foreign proteins, carbohydrates, lipid, enzymes, virus and bacteria. Good immunogens are high molecular weight and highly purified proteins recognized as foreign body which has high digestibility but low solubility. Secondly, immunogenicity differs depending on the injection site, antigen processing and animals immunized. Particulate antigens are normally injected intravenously, but protein antigens and bacterial carbohydrates are immunized through I.M., I.D. or S.C. after mixing with proper adjuvant to enhance immune response without generating unwanted antibodies (Ellis, 1988). There is no rule of thumb on how many times the animal needs to be injected and how much antigen to be injected. In order to achieve high antibody response, it is usual to conduct repeated exposure to the immunogen, so a series of injections at regular intervals is useful to produce both high levels of antibody and antibodies of high affinity.

Recently, animal welfare issues are getting much attention, as an alternative, chicken IgY was made available (Nho et al, 2009). It has the advantage of producing large amounts of specific antibodies without sacrificing experimental animals and the IgY produced is more or less phylogenetically distant from others thus reducing non-specific reaction.

Concerning monoclonal antibodies (mAbs), even though there are several advanced methods developed in producing mAbs, it basically needs animals such as mice or rats to immunize antigens, fuse, and continue mass production depending on the purpose. It is well known that mAbs have high specificity compared with polyclonal antibodies, which makes it possible to differentiate between false positive and positive or negative results. Recently, mAbs have been applied in developing lateral imunochromatography assay for convenient and rapid diagnosis of some viral diseases (Lipman et al, 2005).

2.1.3. Diagnosis

2.1.3.1. Agglutination Reaction

Agglutination is an antigen-antibody reaction especially between particulate antigens and its specific or cross reactive antibodies, easily observed by the clumping of these particles. The reaction occurs quickly and is easy to produce making it a very useful tool in diagnosis. This reaction is also applied to detect unknown antigen with known antibody (direct method) or vice versa, unknown antibody with known antigen, usually to check for bacterial infection. An improved version (indirect method), wherein soluble antigens or antibodies are used to coat latex, bentonite, colloidon and bacteria to detect its antibody from sera or antigens from tissues etc., was also developed. There are several methods for agglutination depending on which tool used, such as plate agglutination, tube agglutination, and 96 (u or v type) agglutination tests. In the case of viral hemagglutination and hemagglutination inhibition tests, these are very limited in the aquaculture field but are applied for myxovirus, paramyxovirus, arbovirus and poxvirus (Roberson, 1993).

2.1.3.2. Fluorescent Antibody Test or Immunofluorescence Antibody Test

Fluorescent antibody test basically applies the same principle as the ones above. It is carried out using a fluorescence microscope having a different light source, usually a mercury lamp and using distinct wavelengths, which hits antibodies attached to luorescent dyes and visualized through specific color associated with antigen and antibody (Marja and Richard, 2006). It is a fairly easy technique and only needs a fluorescence microscope, which is why it is widely used in the field of diagnosis especially for viral infection in cells and tissues. A good example is immunohistochemistry that allows for the detection of the location of antibodies. In the introduction of new fluorophores and microscopes, especially the use of epifluorescence microscope and the confocal microscope, this technique has advanced considerably from the conventional IFA test. This test has several advantages: the capacity to use mAbs and polyclonals (Anderson, 1993); high sensitivity and specificity; can be applied for bacteria determination; can be used to label single cells; and it allows the use of different types of fluorescent-labeled antibodies to observe multiple cell types in one sample. However, it can give cross reactivity when polyclonal antibodies are used, so careful analysis of the results should be made to avoid false positive or negative conclusions. The application of this test has two different methods, direct and indirect.

2.1.4. Direct Method

Direct immunofluorescence uses a single antibody combined chemically with a fluorophore. The antibody recognizes the target molecule and binds to it, and the fluorophore gives specific color to examine the sample. Because it only involves one step, it can reduce the number of steps in the staining procedure and can reduce background signal by avoiding some issues with antibody cross-reactivity or non-specificity. However, it is very difficult to bind the fluorescent molecules with antibody and is known to be less sensitive than indirect immunofluorescence.

2.1.5. Indirect Method

Indirect immunofluorescence uses two antibodies, the primary antibody specifically binds to a target molecule, and the secondary antibody, carrying the fluorophore, recognizes the primary antibody and binds to it. The secondary antibody recognizes the constant region on the first antibody, which allows the indirect method to use a variety of secondary antibodies which are commercially available.

2.1.6. Flow Cytometry Analysis

Recently, cytometry analysis to differentiate cells based on phenotypes is gaining more importance. This technique is highly similar to IFA test in terms of antigen and antibody reaction (Thuvander et al, 1992). It uses laser light to hit the fluorescent molecules attached to antibodies giving rise to signals that can be detected by cytometry. There are direct and indirect methods depending on the antibodies attached on fluorescent molecules.

2.1.7. Virus Neutralization Test

Neutralization of a virus is defined as the loss of infectivity through reaction of the virus with specific antibody. Virus and serum are mixed under appropriate conditions then inoculated into cell cultures, eggs or animals. The presence of un-neutralized virus can be detected by reactions such as cytopathic effect (CPE), haemadsorption/haemagglutination, and plaque formation. The loss of infectivity is brought about by interference of the bound Ab with any one of the steps leading to the release of the viral genome into the host cells. Even though this method is labor intensive and dependent on cell cultures, it is highly sensitive and specific (Kim et al, 2011). Moreover, this test is very useful to recognize the immune response after vaccination for a virus in mammals.

2.1.8. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, is a technique that is extensively used for its rapidity, high sensitivity and high specificity even for small amounts of test samples (Alexandra, 2006). It is performed in 96-well plates known as ELISA plate which permits high throughput results. In order to perform ELISA test, first, either the antigens or antibodies need to be coated to allow them to stick to a polyvinyl plate, and then washed to prevent nonspecific reaction of unbound antigens or antibodies. The corresponding secondary antigen or antibody is then added which reacts with the antigens or antibodies fixed on the plate. An enzyme is tagged on the second antigen or antibody and this enzyme reacts with a suitable substrate when it is added, producing a color which is measurable as the quality or quantity of antigens or antibodies present in the given sample and thereby identified. When the enzyme reaction is complete, the entire plate is placed into a plate reader which measures the optical density (i.e. the amount of colored product) for each well. The intensity of the color produced is proportional to the amount present in the sample. Qualitative ELISA simply evaluates whether the results are positive or negative. Quantitative ELISA meanwhile, measures the optical densities or fluorescent units of the sample that are compared with a standard curve to determine the quantity. ELISA can be used to measure

serum antibody concentration, determine antigens and measure some toxin or allergens. ELISA can be used in different ways depending on the purpose. Direct ELISA uses only one set of antigens and one set of antibodies to react: Ag + Ab-E →Reaction color. Indirect ELISA uses additional antibodies added in the reaction: Ag or Ab + Ab or Ag + Ab-E → Reaction color. Sandwich ELISA is a kind of indirect ELISA, the only difference is that antigen is present between two antibodies: Ab + Ag + Ab-E→ Reaction color. Competitive ELISA is a slight modification of direct, indirect and sandwich ELISA. One more substance is added to compete with Ab or Ag to bind to the already added Ag or Ab during the reaction. The addition of this competitor substance prevents unnecessary binding of Ab or Ag, thereby promoting greater affinity between Ag or Ab. The process remains the same with other ELISA.

2.1.9. Immunochromatography Assay

Lateral flow tests also known as Lateral Flow Immunochromatographic Assays are simple devices aimed to identify the presence (or absence) of target antigens in the sample and doing so without specialized and costly equipments (Oh et al, 2006). The devices are initially developed for medical diagnostics either for home testing (as in the case of home pregnancy tests), point-of-care testing, or laboratory use.

2.1.10. Development of an Imunochromatography Assay for Fish ISAV

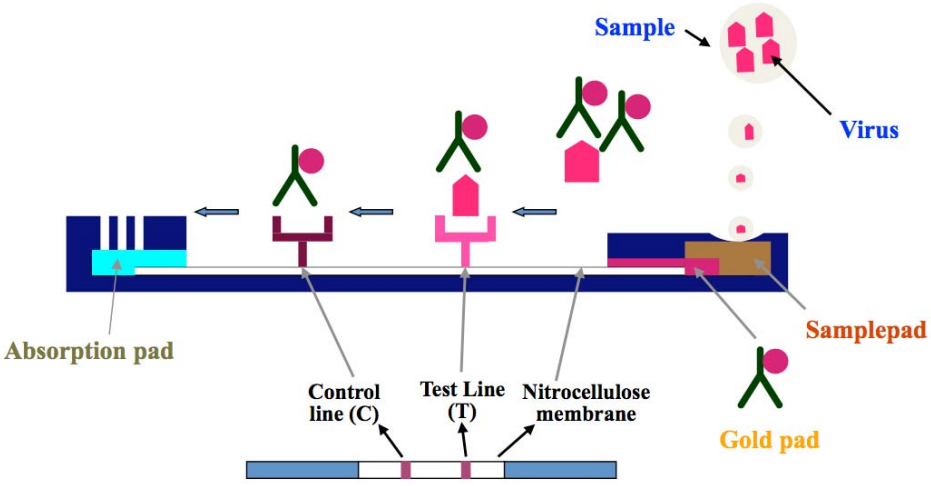


Figure 2.1.1. The principle behind the immunochromatography kit.

The technology is based on a series of capillary beds (sample pad, gold pad, and absorption pad) made of pieces of porous paper that can transport fluid spontaneously (Adams and Thompson, 2011). Usually, two monoclonal antibodies (mAbs) are applied, one is lined up on the nitrocellulose paper and the other is in the gold pad attached by gold particles. When a sample is poured into the hole on the sample pads, the pad acts as a sponge and once soaked, the fluid migrates to the gold pad in which mAbs are stored and reacts with a corresponding antigen. The antigen and antibody mixture is flows through the porous structure and reach to the mAbs on the nitrocellulose paper. The mAbs capture the mixture of antigen-mAbs attached to gold particles. As more fluid pass, the first stripe

where monoclonal antibodies are lined up, golden particles accumulate and the strip area changes color. If there is no reaction with the captured mAbs, the fluid will reach the second strip lined with capture polyclonal antibody which will react with the monoclonal antibody attached to gold particles but did not react with the target. The second stripe will indicate whether or the device is working fine or not. After passing these reaction zones, the fluid enters the final porous material, the absorption pad, which simply acts as a waste container. The principle behind this technology is shown in Figure 2.1.1 while Figure 2.1.2 shows an example of this test.

Figure 2.1.2. An immunochromatography kit showing negative, positive and invalid results.

2.2. Diagnosis – PCR Detection

Takashi Aoki and Jun-ichi Hikima

2.2.1. Synopsis

A quick, reliable, and efficient diagnostic method is indispensable to fish farm management and will help in identifying proper therapeutic measures and preventing the spread of diseases. The recent advances in genetic engineering and molecular biology made it possible to detect a specific gene in the target pathogen. The ability of polymerase chain reaction (PCR) to accurately detect viral or bacterial genes in a relatively shorter period compared to other diagnostic methods, made it one of the most widely used diagnostic tool for detecting viral and bacterial pathogens in fish. In this section, the basic principles of PCR assay, the bacteria and viruses in fish to which a PCR platform for detection and diagnosis was developed, and the target genes in these pathogens that were used for detection, will be introduced.

2.2.2. Introduction

As the number of fish and shellfish species for aquaculture increases to augment the need for cheaper food sources, the development of aquaculture technologies and diversification of demand for them also increases. With this increase however, comes the emergence of new diseases and disease-causing microbes associated with these new cultured species. Diseases caused by new bacterial and viral pathogens are causing huge damages and to address this concern, proper prophylactic and therapeutic methods are necessary. More importantly, rapid and accurate diagnostic tools to detect specific pathogens need to be developed.

Historically, morphological observation, biochemical and immunological (using antiserum) methods have been used for general identification and diagnosis of major pathogenic bacteria and virus including fish pathogens. It has also been done by comparison of nucleic acids, components and substances produced from the pathogens. However, since all of these methods are complicated and requires considerable time for detection, they are not suitable for use in aquaculture farms.

Recently, through the advances of technology in genetic engineering and molecular biology, it is possible to detect a specific gene in the target pathogen. The morphological and biochemical features of pathogens are basically determined by the genome (genes) derived from pathogens. By detecting a unique gene for a target pathogen, Polymerase Chain Reaction (PCR) diagnosis method, which is able to perform the rapid identification and precise classification, has been developed.

In this sub-section, the PCR method, currently one of the most popular diagnostic methods to identify fish pathogenic bacteria and viruses will be introduced.

2.2.3. The Basic Principles of PCR

PCR technology was developed by Dr. Karrie B. Mullis in 1987 using a thermophilic bacterial DNA polymerase (Taq DNA polymerase) that works efficiently even at high temperatures. Since the development of the PCR technique, research in the field of molecular biology improved in leaps and bounds. Now, it is one of the most widely used techniques not just in molecular biology, but also in most other scientific fields for its many advantages.

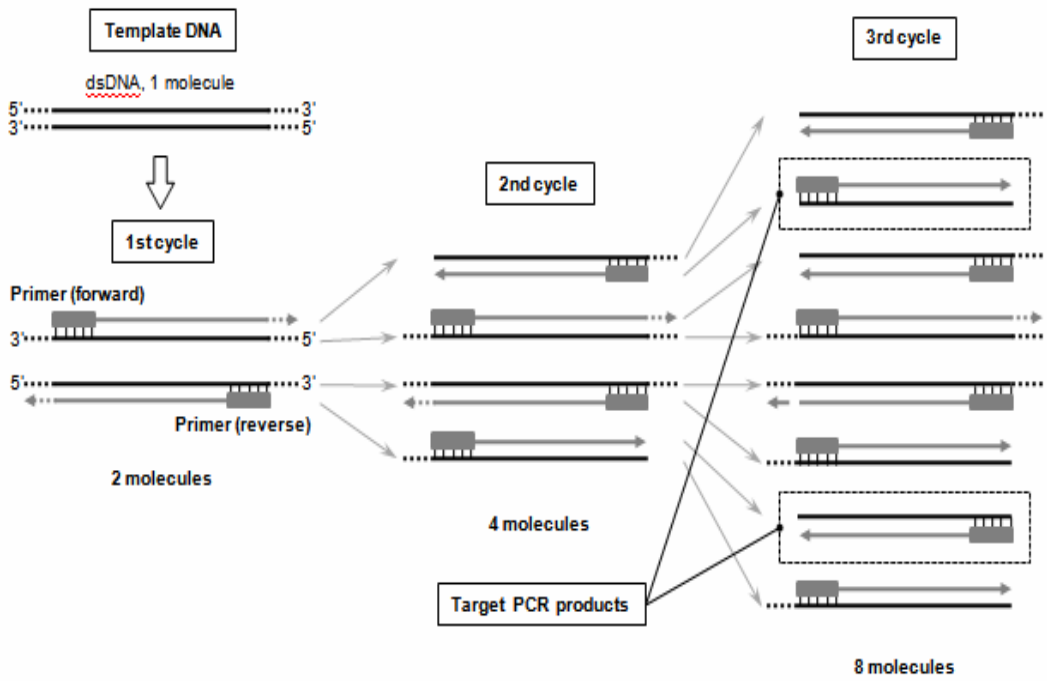


Figure 2.2.1. Schematic drawing of the basic principle of PCR technology. Grey box indicates primers, and grey arrow is the DNA fragment synthesized with Taq DNA polymerase, which synthesizes DNA from 5' to 3' direction. Target PCR products (specific DNA fragments) can be obtained in the 3rd cycle.

PCR functions to repeat only certain regions of DNA replication and amplification reaction and amplify DNA fragments with the same nucleotide exponentially, and generate large amounts of specific DNA fragments within a short time. PCR reaction is composed of the following steps: 1) dissociation (denaturing) of double-stranded DNA (dsDNA) (complementary DNA as template) by high temperature; 2) annealing of primers to the denatured template DNA (hybridizing the primer and the single-stranded DNA); and 3) synthesis of the complementary strand DNA with Taq DNA polymerase. By repeating these steps about 25 to 35 cycles, the certain area (*e.g.*, targeted gene) specified by two primers (*i.e.*, forward and reverse primers) is capable of amplifying 2^{25} to 2^{35} fold of the target DNA fragments (Fig. 2.2.1). Primers are 18- to 30-mer oligonucleotide DNA fragments, which specifically bind to the complementary strand of the target dsDNA by hydrogen bonds in each PCR cycle.

2.2.4. Diagnosis of Fish Bacterial Pathogens by PCR

PCR is a simple, rapid and more accurate, compared to other diagnostic techniques, method to identify specific genes of pathogenic bacteria and virus and is therefore widely used as a detection method for various fish and shellfish pathogenic bacteria and viruses. To date, the following bacterial pathogens in fish have been diagnosed by PCR using specific primer sets: *Edwardsiella ictaluri* and *Ed. tarda* (edwardsiellosis), *Tenacibaculum maritimum* (flexibacteriosis), *Flavobacterium columnare* (columnaris disease), *Renibacterium salmoninarum* (Bacterial kidney disease: BKD), pathogen of Bacterial hemolytic jaundice (unidentified species), *Pseudomonas anguilliseptica* (red spot disease), *Aeromonas salmonicida* (furunculosis), *Nocardia seriolae* (nocardiosis), *V. anguillarum, V. trachuri, V. vulunificu* (vibriosis), *Mycobacterium marinum* (mycobacteriosis), *Photobacterium damselae* subsp. *piscicida* (pseudotuberculosis), *F. psychrophilum* (cold water disease), *Y. ruckeri* (enteric redmouth disease), *Lactococcus garvieae*、*Streptococcus iniae*、*S. dysgalactiae*、*S. agalactiae*、*S. parauberis*、*S. difficilis* (streptococcosis) (Table 2.2.1).

Meanwhile, fish viruses detected by PCR method include, koi herpes virus (KHV), *Oncorhynchus masou* virus (OMV), channel catfish virus (CCV), red seabream iridovirus (RSIV), lymphocystis disease virus (LCDV), epizootic haematopoietic necrosis virus (EHNV), infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicemia virus (VHSV), spring viraemia of carp virus (SVCV), viral nervous necrosis virus (VNNV), salmonid alphavirus (SAV), grass carp reovirus (GCRV), European catfish virus (EGV), and hirame rhabdovirus (HRV) (Table 2.2.1).

2.2.5. Target Genes for PCR-Based Diagnosis

16s and 23S rRNA (or rDNA) genes and related genes (including ISR region or ITS genes located between 16S and 23S rRNA genes) has been frequently used as target genes to detect fish pathogens by PCR method. The following genes are also used for PCR diagnosis as the target gene: major outer membrane protein gene *p57* derived from *R. salmoninarum* (Brown *et al*., 1994; McIntosh *et al*., 1996; Miriam *et al*., 1997), surface array protein gene *vapA* in *A. salmonicida* (Gustafson *et al*., 1993), glutamine synthetase gene *glnA* in *Y. ruckeri* (Keeling *et al*., 2012), dihydropteroate synthase gene in *L.*

garvieae (Aoki *et al*., 2000), Lactate oxidase gene *lctO* in *S. iniae* (Hussein and Hatai, 2006; Mata *et al*., 2004a) (Table 1). In other pathogenic bacteria, such as the causative agent for vibriosis, there have been many evidences using genes related to virulence as the target gene for PCR detection namely: Hemolysin gene (Hirono *et al*., 1996), *rpoS* gene (Kim *et al*., 2008), *empA* gene (Xiao *et al*., 2009), *toxR* gene (Crisafi *et al*., 2011), cytotoxin-hemolysin gene (Coleman *et al*., 1996; Hill *et al*., 1991) (Table 2.2.1). Furthermore, a certain region in species-specific plasmid pZP1 (Aoki *et al*., 1997) and the species-specific sequences in the chromosomal DNA obtained by RAPD (Random Amplification of Polymorphic DNA)-PCR method or random cloning method (Aoki and Hirono, 1995; Aoki *et al*., 1995; Argenton *et al*., 1996; Iwamoto *et al*., 1995; Miyata *et al*., 1996) are also useful for PCR diagnosis.

Target genes for PCR detection of fish viruses on the other hand are: thymidine kinase and terminase for KHV (Bercovier *et al*., 2005; Yuasa *et al*., 2012); major capsid protein for OMV and LCDV (Aso *et al*., 2001; Kitamura *et al*., 2006; Cano *et al*., 2007; Palmer *et al*., 2012); ORF 8 for CCV (Gray *et al*., 1999); reductase, ATPase and DNA polymerase for RSIV (Ohima *et al*., 1996; 1998; Kurita *et al*., 1998); DNA polymerase for EHNV and ECV (Holopainen *et al*., 2011); VP1, VP3 and VP4 for IPNV (Willisms *et al*., 1999; Rodriguez *et al*., 2001; Orpetveit *et al*., 2010; Bowers *et al*., 2008); segment 8 for ISAV (Devold *et al*., 2000); the nucleoprotein or glycoprotein genes for IHNV, VSHV, SVCV and HRV (Williams *et al*., 1999; Arakawa *et al.*, 1990; Bruchhof *et al*., 1995; Miller *et al*., 1998; Liu *et al*., 2008; Lopez-Vazquez *et al*., 2006; Chico *et al*., 2000; Koutna *et al*., 2003; Sun *et al*., 2010); coat protein for VNNV (Dalla *et al*., 2000); NSP1 for SAV (Zhang *et al*., 2010); and segment 10 for GCRV (Hodneland and Endresen, 2006) (Table 2.2.1).

Because of its effectiveness, PCR has revolutionized modern pathogen-diagnostics and it has been developed to detect a wide variety of bacterial and viral pathogen. It is easy to use, rapid and accurate making it a very excellent diagnostic method for fish pathogens.

2.2.6. Conclusion

Farm-level diagnostic tools that are cost-effective, easy to use, and allows for rapid detection of well known pathogens will greatly improve aquaculture outputs. The PCR-based diagnostic method embodies these characteristics very well and has proven to be reliable if not more reliable than traditional methods for disease detection. Although this method has evolved and developed through the years, the need to optimize detection, sensitivity, and accuracy is required to expand its utility and versatility. The development according to the intended use and purpose of further diagnosis will be anticipated.

Diseases	**Causative agent**	**Target genes**	**Primer sequences（F: forward / R: reverse）**	**Products（bp）**	**References**
Edwardsiellosis	*Edwardsiella ictaluri*	Region between IVS-IRS genes	F:5'TTAAAGTCGAGTTGGCTTAGGG3', R:5'TACGCTTTCCTCAGTGAGTGTC3'	2,000	William and Lawrence, 2010
	Ed. tarda	*Eta1*（Species-specific DNA fragments）	F:5'AGTTCAGCGCCCAGTCATA3', R:5'CGCCAGATCCGCTGCCCGT3'	580	Aoki and Hirono, 1995
Flexibacteriosis	*Tenacibaculum maritimum* (Former name: *Flexibacter maritimus*)	16S rRNA gene	F:5'AATGGCATCGTTTTAAA3', R:5'CGCTCCTACTTGCGTAG3'	1073	Toyamaet al., 1996
		16S rRNA gene	F:5'TGTAGCTTGCTACAGATGA3', R:5'AAATACCTACTCGTAGGTACG3'	400	Bader and Shotts, 1998; Cepeda et al., 2003
		16S rRNA gene	F:5'AATGGCATCGTTTTAAA3', R:5'CGCTCCTACTTGCGTAG3', F(nested):5'AGAGTTTGATCCTGGCTCAG3', R(nested):5'AAGGAGGTGATCCAGCCGCA3'	1088	Avendano-Herrera et al., 2004
Columnaris disease	*Flavobacterium columnare*	16S rRNA gene	F:5'GCCCAGAGAAATTTGGAT3', R:5'TGCGATTACTAGCGAATCC3'	1,193	Bader et al., 2003
		16S rRNA gene	F:5'CAGTGGTGAAATCTGGT3', R:5'GCTCCTACTTGCGTAGT3'	679	Darwish et al., 2004
		ISR region between 16S-23S rRNA genes	F:5'TGCGGCTGGATCACCTCCTTTCTAGAGACA3', R:5'TAATYRCTAAAGATGTTCTTTCTACTTGTTTG3'	450~550	Welker et al., 2005
Bacterial kidney disease (BKD)	*Renibacterium salmoninarum*	16S rRNA gene	F:5'TGGATACGACCTATCACCGCA3', R:5'GCAAGTACCCTCAACAACCACA3'	312	Magnússon et al., 1994
		*p57*major outer membrane protein gene	F:5'CAAGGTGAAGGGAATTCTTCCACT3', R:5'GACGGCAATGTCCGTTCCCGGTTT3'	501	Brown et al., 1994
		*p57*major outer membrane protein gene	F:5'GCGCGGATCCAAAATAAAAAAAATTTTAGCGCTG3', R:5'GCGCGGATCCTTGGCAGGACCATCTTTGT3'	376	McIntosh et al., 1996
		*p57*major outer membrane protein gene	F:5'CGCAGGAGGACCAGTTGCAG3', R:5'GGAGACTTGCGATGCGCCGA3'	349	Miriam et al., 1997
		*p57*major outer membrane protein gene	F:5'CGCAGGAGGACCAGTTGCAG3', R:5'TCCGTTCCCGGTTTGTCTCC3'	372	Miriam et al., 1997

		16S-S23 rDNA ITS gene	F:5'CCGTCCAAGTCACGAAAGTTGGTA3', R:5'ATCGCAGATTCCCACGTCCTTCTT3'	751	Grayson et al., 1999
		16S-S23 rDNA ITS gene	F:5'CCGTCCAAGTCACGAAAGTTGGTA3', R:5'GTGGGTACTGAGATGTTTCAGTTC3'	895	Grayson et al., 1999
Bacterial hemolytic jaundice	Unidentified	16S rDNA gene	F:5'AGCACTTATGTATAGGTGTA3', R:5'GTATAAAACGCCAAACATAT3'	387	Mitsui et al., 2004 (In Japanese)
Red spot disease	*Pseudomonas anguilliseptica*	16S rRNA gene	F:5'GACCTCGCCATTA3', R:5'CTCAGCAGTTTTGAAAG3'	439	Blanco et al., 2002
Furunculosis	*Aeromonas salmonicida*	*vapA* gene	F:5'GGCTGATCTCTTCATCCTCACCC3', R:5'CAGAGTGAAATCTACCAGCGGTGC3'	421	Gustafson et al., 1992, 1993
		16S rRNA gene	F:5'CGTTGGATATGGCTCTTCCT3', R:5'CTCAAAACGGCTGCGTACCA3'	423	O'Brien et al., 1994
	A. salmonicida subsp. *salmonicida*	Species-specific region in chromosome (RAPD products)	F:5'AGCCTCCACGCGCTCACAGC3', R:5'AAGAGGCCCCATAGTGTGGG3'	512	Miyata et al., 1996
Nocardiosis	*Nocardia seriolae*	16S rRNA gene	F:5'ACTCACAGCTCAACTGTGG3', R:5'ACCGACCACAAGGGGG3'	432	Miyoshi and Suzuki, 2003
Vibriosis	*Vibrio anguillarum*	Hemolysin gene	F:5'ACCGATGCCATCGCTCAAGA3', R:5'GGATATTGACCGAAGAGTCA3'	490	Hirono et al., 1996
		rpoS gene	F:5'AGACCAAGAGATCATGGATT3', R:5'AGTTGTTCGTATCTGGGATG3'	689	Kim et al., 2008
		empA gene	F:5'CAGGCTCGCAGTATTGTGC3', R:5'CGTCACCAGAATTCGCATC3'	439	Xiao et al., 2009
		toxR gene	F:5'ACACCACCAACGAGCCTGA3', R:5'TTGTCTCTTCGGGTTGCGA3'	93	Crisafi et al., 2011
Vibriosis	*V. anguillarum*	16S rRNA gene	F:5'CCACGCCGTAACGATGTCTA3', R:5'CCAGGCGGTCTACTTAACGCGT3'	81	Crisafi et al., 2011
	V. trachuri	Species-specific region in chromosome	F:5'TGCGCTGACGTGTCTGAATT3', R:5'TGACGAACAGTAGCGACGAA3'	417	Iwamoto et al., 1995

	V. vulunificu	Cytotoxin-hemolysin gene	F:5'CCGGCGGTACAGGTTGGCGC3', R:5'CGCCACCCACTTTCGGGCC3'	519	Hill et al., 1991
		23S rRNA gene	F:5'CCACTGGCATAAGCCAG3', R:5'CTACCCAATGTTCATAGAA3'	978	Arias et al., 1995
		Cytolysin-hemolysin gene	F:5'CGCCGCTCACTGGGGCAGTGGCTG3', R:5'GCGGGTGGTTCGGTTAACGGCTGG3'	1416	Coleman et al., 1996
Mycobacteriosis	*Mycobacterium spp., Mycobacterium marinum*	16S rRNA gene	F:5'GRGRTACTCGAGTGGCGAAC3', F:5'GGCCGGCTACCCGTCGT3'	208	Kox et al., 1995, 1997; Puttinaowarat et al., 2002
Pasteurellosis (Pseudotuberculosis)	*Photobacterium damselae* subsp. *piscicida* (Former name: *Pasteurella piscicida*)	Species-specific region in chromosome	F:5'GTAGCTCTTGTGGAGTAATGCT3', R:5'CATTCGTAGTGCTTACTGCCCA3'	629	Aoki et al., 1995
		DNA fragment from pZP1	F;5'GCCCCCATTCCAGTCACACA3', R:5'TCCCTAAGCACACCGACAGG3'	484	Aoki et al., 1997
		16S rRNA gene	F:5'CGAGCGGCAGCGACTTAACT3', R:5'GATTACCAGGGTATCTAATC3'	~750	Matsuoka et al., 1997 (In Japanese)
Cold water disease	*F. psychrophilum* (Former name: *Cytophaga psychrophila*)	16S rRNA gene	F:5'CGATCCTACTTGCGTAG3', R:5'GTTGGCATCAACACACT3'	1073	Toyama et al., 1994
		16S rRNA gene	F:5'GTTAGTTGGCATCAACAC3', R:5'TCGATCCTACTTGCGTAG3'		Urdaci et al., 1998
Enteric redmouth Disease (ERM)	*Yersinia ruckeri*	Unidentified gene（RAPD-PCR products）	F:5'TCACGAATCAGGCTGTTACC3', R:5'TTCTGCCTGTGCCAATGTTGG3'	512	Argenton et al., 1996
		16S rRNA gene	F:5'GCGAGGAGGAAGGGTTAAGTG3', R:5'GAAGGCACCAAGGCATCTCTG3'	575	Gibello et al., 1999
		glnA gene	F:5'TCCAGCACCAAATACGAAGG3', R:5'ACATGGCAGAACGCAGATC3', Probe:5'CGCGATCAAGGCGGTTACTTCCCGGTTCCCGATCGCG3'(Real-time PCR)	ND	Keeling et al., 2012

Streptococcosis	*Lactococcus garvieae*	16S rDNA gene	F:5'CATAACAATGAGAATCGC3', R:5'GCACCCTCGCGGGTTG3'	1,100	Zlotkin et al., 1998a; Hussein and Hatai, 2006
		Dihydropteroate synthase gene	F:5'CATTTTACGATGGCGCAG3', R:5'CGTCGTGTTGCTGCAACA3'	709	Aoki et al., 2000
	Streptococcus iniae	16S rDNA gene	F:5'CTAGAGTACACATGTACTNAAG3', R:5'GGATTTTCCACTCCCATTAC3'	300	Zlotkin et al., 1998b
		ITS region between 16S-23S rRNA genes	F:5'GGAAAGAGACGCAGTGTCAAAACAC3', R:5'CTTACCTTAGCCCCAGTCTAAGGAC3'	373	Berridge et al., 1998
		Lactate oxidase (*lctO*) gene	F:5'AAGGGGAAATCGCAAGTGCC3', R:5'ATATCTGATTGGGCCGTCTAA3'	870	Mata et al., 2004a; Hussein and Hatai, 2006
		ITS region between 16S-23S rRNA genes	F:5'GAAAATAGGAAAGAGACGCAGTGTC3', R:5'CCTTATTTCCAGTCTTTCGACCTTC3'	377	Zhou et al., 2011
		16S rDNA gene	F:5'CTAGAGTACACATGTACTIAAG3', R:5'GGATTTTCCACTCCCATTAC3'	300	Roach et al., 2006
	S. dysgalactiae	ITS region between 16S-23S rRNA genes	F:5'TGGAACACGTTAGGGTCG3', R:5'CTTTTACTAGTATATCTTAACTA3'	270	Forsman et al., 1997
	S. dysgalactiae subsp. *dysgalactiae*	ITS region between 16S-23S rRNA genes	F:5'TGGAACACGTTAGGGTCG3', R:5'CTTAACTAGAAAAACTCTTGATTATTC3'	259	Hassan et al., 2003; Hussein and Hatai, 2006
	S. agalactiae	ITS region between 16S-23S rRNA genes	F:5'GGAAACCTGCCATTGCG3', R:5'TAACTTAACCTTATTAACCTAG3'	280	Forsman et al., 1997
	S. parauberis	23S rRNA gene	F:5'TTTCGTCTGAGGCAATGTTG3', R:5'GCTTCATATATCGCTATACT3'	718	Mata et al., 2004b
	S. difficilis	ITS region between 16S-23S rRNA genes	F:5'AGGAAACCTGCCATTTGCG3', R:5'CAATCTATTTCTAGATCGTGG3'	192	Mata et al., 2004b

Table 2.2.1 . Fish pathogenic bacteria detected by PCR methods

Glossary

PCR: polymerase chain reaction,
dsDNA: Double-stranded deoxyribonucleotide,
RAPD: Random Amplification of Polymorphic DNA,
KHV: Koi herpes virus,
OMV: *Oncorhynchus masou* virus,
CCV: Channel catfish virus,
RSIV: Red seabream iridovirus,
LCDV: Lymphocystis disease virus,
EHNV: Epizootic haematopoietic necrosis virus,
IPNV: Infectious pancreatic necrosis virus,
ISAV: Infectious salmon anemia virus,
IHNV: Infectious haematopoietic necrosis virus,
VHSV: Viral haemorrhagic septicemia virus,
SVCV: Spring viraemia of carp virus,
VNNV: Viral nervous necrosis virus,
SAV: Salmonid alphavirus,
GCRV: Grass carp reovirus,
EGV: European catfish virus,
HRV: Hirame rhabdovirus

2.3. Loop Mediated Isothermal Amplification (LAMP) Method

Masahiro Sakai

2.3.1. Synopsis

The LAMP (loop mediated isothermal amplification) can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions. The LAMP reaction employs a DNA polymerase and a set of four specific primers that recognize a total of six distinct sequences of the target DNA. In aquaculture, this technique has already been applied for detection of several fish and shrimp pathogens such as KHV, SVCV, IHNV, WSSV, *Edwardsiella tarda*, *Vibrio nigripulchritudo*, YHV, *Nucleospora salmonis* etc. According to these reports, the sensitivity of LAMP is almost the same as PCR and suggesting that this technique can be used for diagnosis of these diseases. Furthermore, the real-time LAMP method has been recently developed for quantitative detection of pathogens.

2.3.2. Principle of LAMP

Loop-mediated isothermal amplification is a sensitive strand displacement technique developed by Notomi *et al.* (2000). This method amplifies target DNA from a few copies to 10^9 copies in less than an hour under isothermal conditions. Briefly, four specific primers are designed from the target DNA, one set of primers anneal to the target region one after the other on the same strand and the primer which anneals at the later stage displaces the strand formed by the first primer using *Bst* DNA polymerase which has a strand displacement activity. This takes place on both strands and the primers are designed such that loops are formed. The reaction is carried out under isothermal conditions as denaturation of the strand takes place by strand displacement. The reactions produce a series of stem-loop DNAs with various lengths. The four primers hybridize against six distinct sequences in the target DNA making it highly specific.

2.3.3. Design of Primers

Designing primers for LAMP is a complex procedure compared with the PCR. The LAMP reaction requires four primers. The primers required are one pair each of inner-primers and shorter outer-primers (Figure 2.3.1). Although the design of each primer is very complex, it can be developed using Primer Explorer version 3 software (http://primerexplorer.jp/lamp3.0.0/).

2.3.4. Requirements for LAMP Reaction

The LAMP reaction is performed by the *Bst* polymerase along with dNTP's and reaction buffer. The reaction is carried out at 60-65^{o}C for 40 min to 1 hour and terminated at 80^{o}C for 2 min. The main advantage of the technique is that it does not need a thermocycler. As the amplification is done in an isothermal condition, a water bath or heating block is sufficient to maintain the required temperature.

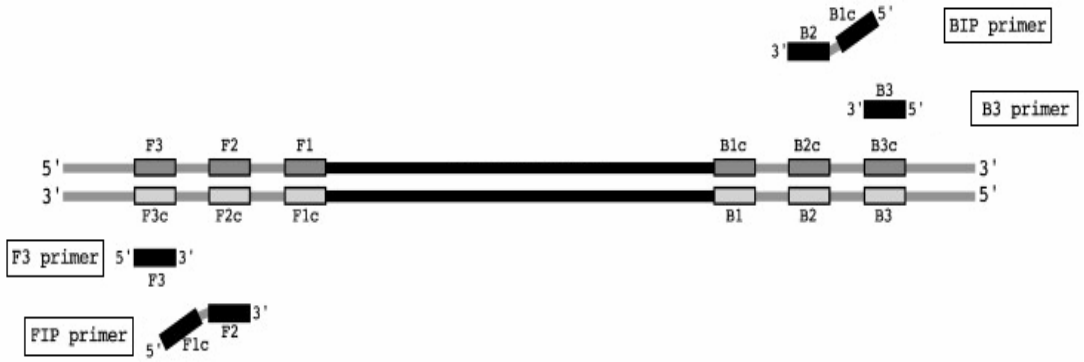


Figure 2.3.1. (A) Schematic diagram of two-inner (FIP, BIP) and -outer (F3, B3) primers for LAMP. This diagram was adapted from Eiken Chemical Co. Ltd (This Figure was modified and cited from Aquaculture, Vol. 288, p27-31 (2009).

2.3.5. Visualization of Amplified Products

The amplified products by LAMP are commonly visualized by agarose gel

electrophoresis stained with ethidium bromide. As the LAMP reaction produces products of various lengths of stem loop structures, the gel will show a smear and bands at the base of the gel (Figure 2.3.2). Furthermore, the large amount of product amplified by LAMP can be visualized on a UV-transilluminator by incorporating intercalating agents, such as SYBR Green I, directly into the LAMP-amplified tubes (Notomi et al, 2000). For alternative method, these products are assessed by the amount of white precipitate formed from magnesium pyrophosphate (Mori et al, 2001).

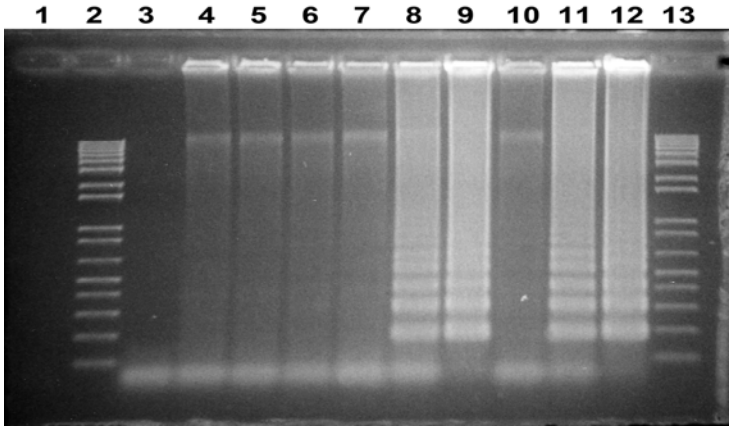


Figure 2.3.2. Determination of LAMP conditions. Effect of temperature and time on amount of LAMP product. Temperature: Lanes 2 and 13: molecular size marker (φ/X174/Hinc II digest), lane 3: blank, lanes 4–6: 60°C, lanes 7–9: 63°C, lanes 10–12: 65°C. Time: Lanes 4, 7 and 10: 30 min, lanes 5, 8 and 11: 45 min, lanes 6, 9 and 12: 60 min. Products were electrophoresed on a 2% agarose gels and stained with ethidium bromide (This Figure was cited from Aquaculture, Vol. 288, p27-31 (2009)).

2.3.6. Application of LAMP for Diagnosis of Fish Pathogens

Since the 2000s, LAMP method has been widely used for detecting human pathogens, because of its simplicity, rapidity, high efficiency, and outstanding specificity. In fisheries sciences, the first use of LAMP for detection of an aquaculture pathogen was reported by our group (Savan et al, 2004). Until now, more than 50 articles covering detection of virus, bacteria and parasitic pathogen have been reported.

2.3.7. Bacterial Pathogens

Many reports on LAMP mediated diagnostic methods have been developed for bacterial pathogens associated with fish and shrimp. The first use of LAMP for detection of aquaculture pathogen was reported for Edwardsiellosis (Savan et al, 2004). LAMP primers were designed by targeting the hemolysin gene of *Edwardsiella tarda*. The specificity of LAMP was tested for 5 different *E. tarda* strains and non-specific amplification was not seen in other bacteria. The optimum amplification was determined to be at 65^{o}C for 45 min. *E. tarda* could be detected from 10 CFU and 10^3 CFU by LAMP and PCR, respectively. LAMP method has also been applied for the detection of Nocardiosis (Itano et al, 2006). In Nocardiosis, the detection limits of LAMP and PCR were 10^3 CFU and 10^4 CFU, respectively. Compared to PCR, a ten fold higher sensitivity

is observed using LAMP. Furthermore, LAMP detection was superior to PCR, when spleen DNA extracted from infected fish was used as template. In shrimp pathogen, the detection of *Vibrio nigripulchritudo* was established by Fall et al. (Fall et al, 2008). Reaction time and temperature were optimized for 60 min at 63^{o}C, respectively and the detection limit of this bacterium by LAMP was 10^2 CFU. The application of LAMP method to diagnose other bacterial fish and shrimp pathogens is shown in Table 2.3.1.

2.3.8. Detection of Viruses

2.3.8.1. DNA Viruses

In the detection of fish viral pathogens, LAMP was first applied in koi herpesvirus (KHV). A set of four primers were designed based on the sequence of the thymidine kinases gene of KHV (Gunimaladevi et al, 2004). The time and temperature conditions for detection of KHV were determined for 60 min at 65ºC. The detection limit using LAMP was found to be similar to that of PCR. Detection of WSSV infecting kuruma shrimp, *Marsupenaeus japonicus,* was reported by Kono *et al*. (Kono et al, 2004). The detection limit of the viral DNA template was 10 fg level, while nested PCR mediated detection limit was 100 fg level. The study concluded that detection by LAMP was superior to PCR since it was faster and more sensitive. Yoshino *et al.* (Yoshino et al, 2006) reported the diagnosis method of KHV using additional sets of loop primers. Additionally, LAMP has also been used to detect red sea-bream iridovirus (Caipang et al, 2004) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al, 2006).

Pathogen	Method
Bacteria	
Edwardsiella tarda (Savan et al. 2004)	LAMP
Edwardsiella ictaluri (Yeh et al. 2005)	LAMP
Flavobacterium columnare (Yeh et al. 2006)	LAMP
Nocardia seriolae (Itano et al., 2006)	LAMP
Yersinia ruckeri (Saleh et al., 2008)	LAMP
Flavobacterium psychrophilum (Fugiwara-Nagata, Eguchi, 2009)	qLAMP
Renibacterium salmoninarum (Gahlawat et al. 2009)	LAMP
Mycobacterium sp. (Ponpompisit et al., 2009)	LAMP
Vibrio anguillarum（Hongwei et al., 2010）	LAMP
Francisella piscicida (Caipang et al., 2010)	LAMP
Vibrio nigripulchritudo (Fall et al., 2011)	qLAMP
Streptococcus iniae (Han et al., 2011)	LAMP
Viruses	
KHV (Gunimaladevi et al., 2004)	LAMP
WSSV (Kono et al., 2004)	LAMP
IHNV (Gunimaladevi et al., 2005)	RT-LAMP
YHV (Mekata et al., 2006)	RT-LAMP
SVCV (Shivappa et al., 2008)	RT-LAMP
VHSV (Soliman and El-Matbouli 2006)	RT-LAMP
IHHNV (Sudhakaran et al., 2008)	qLAMP

WSSV (Mekata et al., 2009)	qLAMP
YHV (Mekata et al., 2009)	qRT-LAMP
IPNV (Soliman at al., 2009)	RT-LAMP
Iridovirus (Caipang et al., 2004)	LAMP
NNV (Sung and Lu 2009)	RT-LAMP
Parasites	
Tetracapsuloides bryosalmonae (El-Matbouli and Soliman 2005)	LAMP
Myxobolus cerebralis (El-Matbouli and Soliman 2005)	LAMP
Nucleospora salmonis (Sakai et al., 2009)	LAMP
Clonorchis sinensis (Cai et al., 2010)	LAMP

Table 2.3.1. Fish and shrimp pathogens detected using LAMP

2.3.8.2. RNA Viruses

For detecting RNA viruses, the cDNA from the virus RNA must be synthesized by reverse transcription. After the development of the LAMP method, an extended application of RT-LAMP has been developed (Notomi et al, 2000). In fish, RT-LAMP was first reported for IHNV (Gunimaladevi et al, 2005). An RT-LAMP protocol for detection of IHNV was developed targeting the G-protein of the virus. A comparative analysis of RT-LAMP, LAMP and nested PCR was conducted. LAMP and nested PCR require an additional 30-40 min as cDNA should be synthesized first. However, RT-LAMP can directly use RNA as template, where the cDNA synthesis and target gene amplification is carried out in a single tube. In this study, LAMP was 10-fold more sensitive than nested PCR. Although real-time PCR is a superior method, RT-LAMP might be a good alternative as the former can be expensive as a routine diagnostic tool. The use of RT-LAMP has also been reported in the detection of viral hemorrhagic septicemia virus (VHS) of salmonid fish (Salivan and El-Matbouli 2006), yellow head virus in shrimp (Mekata et al, 2006), spring viremia of carp (SVC) (Shivappa et al, 2008) and infectious salmon anemia virus (ISAV) (Sakai, personal communication).

2.3.9. Parasitic Infections

LAMP method has also been applied for detecting fish parasitic diseases. A myxozoan spore, *Tetracapsuloides bryosalmonae*, is the causative agent of proliferative kidney disease (PKD). Et-Matbouli and Soliman (El-Matbouli and Soliman, 2005a) used LAMP for rapid diagnosis of (PKD) affected rainbow trout. Furthermore, a comparison of PKD-LAMP to PCR has been evaluated in this study. Four sets of primers along with loop primers were designed targeting SSU-rDNA of *T. bryosalmonae*. The loop primers were used for the acceleration of LAMP reaction. The PKD-LAMP was found to be 100-fold more sensitive and a low amount of DNA sample as template could also be amplified in 1 h. In addition, Et-Matbouli and Soliman (El-Matbouli and Soliman 2005b) have also reported a detection method based on LAMP for *Myxobolus cerebralis*, which is a causative agent of whirling disease.

2.3.10. Quantitative LAMP Method

A quantitative real-time LAMP method has been reported (Mori, et al., 2001). This method produces large amounts of the target DNA as well as an insoluble by-product, magnesium pyrophosphate, during the reaction making it possible to perform a real-time measurement of turbidity using an inexpensive photometer. Sudhakaran et al. (2008) reported the real-time LAMP assay to detect IHHNV in shrimp. The real-time LAMP method for IHHNV is simple and rapid with specific amplification within 60 min at 63ºC. The sensitivity analysis revealed this method is capable of detecting as few as 10^2–10^3 copies/μL. This method was also reported in the detection of WSSV (Mekata et al, 2009a) and YHV (Mekata et al, 2009b).

2.3.11. Conclusion

This review describes the application of LAMP method for detection of fish and shellfish pathogens. Various studies cited in this review have convincingly demonstrated that LAMP is a superior diagnostic tool compared to other methods. This method can be widely applied in clinical diagnostics, environmental monitoring and food safety in aquatic sciences.

Glossary

LAMP: Loop Mediated Isothermal Amplification,

dNTP: Mixture of dATP (deoxyadenosine triphospate) + dCTP (deoxycytidine triphospate) + dGTP (deoxyganosine triphospate) + dTTP (deoxytymidine triphospate)

3. SELECTION AND ESTABLISHMENT OF DISEASE-RESISTANT FISH

3.1. Development of Disease-Resistant Fish Using Marker-Assisted Selection

Takashi Sakamoto, Akiyuki Ozaki and Nobuaki Okamoto

3.1.1. Synopsis

In aquaculture, one way to prevent fish diseases is to develop disease-resistant strains of fish through the use of marker-assisted selection (MAS). MAS requires an understanding of the linkage between quantitative trait loci (QTL) of a target trait and DNA markers. Presently, detection of disease-resistant phenotypes requires artificial challenge tests, which are labor intensive and expensive. However, such tests are no longer needed once the linkage between disease resistance traits and DNA markers is known. So far, MAS has been used to develop Japanese flounder resistant to lymphocystis disease (LD) and Atlantic salmon resistant to infectious pancreatic necrosis (IPN).

3.1.2. Introduction

The majority of species and strains reared globally for aquaculture are relatively unimproved for commercially important traits. Presently, cultured and wild fish species

have high genetic diversity and thus have more opportunities and higher potential for genetic improvement than domestic livestock and crops which have already undergone selection over many centuries. DNA markers can be used for genetic improvement through selection of economically important traits, such as disease resistance. DNA markers detect DNA polymorphisms that can be used to trace the Mendelian inheritance of homologous chromosome segments. Economically important traits are generally thought to be controlled by many genes of small additive effects, which are known as quantitative trait loci (QTL). Construction of a genetic linkage map based on DNA markers at a large number of sites in the fish genome is necessary to identify QTLs controlling disease resistance. Once the markers associated with a QTL have been identified, it may be possible to improve other strains through introgression of the QTL through cross breeding.

One of the goals of modern selective breeding programs for aquaculture is to include the use of genetic markers from pedigreed brood stocks. This approach, called marker-assisted selection (MAS), is expected to improve the efficiency and accuracy of selection.

3.1.3. Marker-Assisted Selection

3.1.3.1. LD-Resistant Japanese Flounder (*Paralichthys Olivaceus*) In Japan

Japanese flounder is an economically important food fish that is widely cultured in Japan, Korea and China. Lymphocystis disease (LD), caused by LD virus (family Iridoviridae), has seriously damaged fish farms in these countries. There is no effective treatment for LD or a commercially available vaccine. To solve this problem, we have initiated a search for DNA markers associated with LD resistance. As a first step, our research group constructed a primary genetic linkage map in Japanese flounder using microsatellite (MS) markers. A first-generation linkage map was constructed using approx. 150 MS markers (Coimbra et al., 2003) and a more recent map has over 1000 markers (Sanchez et al., 2010).

Linkage of the DNA markers with LD resistance was analyzed in a backcross progeny (n =136) produced by crossing a susceptible male with a (susceptible x resistant) hybrid female. Fuji et al., (2006) detected a major locus (*Poli.9-8TUF*) for LD resistance on linkage group 15 on the map of Coimbra et al., (2003) (Figure 3.1.1). To introduce the trait and marker information linked to LD resistance into a commercial strain, we performed a cross between a resistant strain and a commercial strain, and generated F_1 hybrid families. The LD-resistant Japanese flounder stock produced by MAS was tested in two commercial fish farms. An allele (147bp) of *Poli9-8TUF* shows a dominant effect in Mendel's law. A new disease-resistant strain of Japanese flounder was produced by MAS using this allele. A female with LD-R that was homozygous for the favorable allele and a male from a commercial stock bred for higher growth rate and good body shape were selected as parents. A female was selected as the LD-R-bearing parent because the recombination rate of females is lower in the region where the LD-R locus is located. As expected, the favorable allele was transmitted as a heterozygote to the progeny (LD-R+ strain). The LD-R+ strain and a control strain (LD-R−) were tested at two commercial fish farms that had had LD outbreaks. The incidence of LD in the LD-R+ strain was zero

at both farms, while the incidences of LD in the control strain were 4.5% and 6.3% at the two farms (Fuji et al. 2007). LD-resistant flounder developed by MAS now account for about 35% of the retail sales of farm-raised Japanese flounder in Japan (Figure 3.1.2). Field tests of F_1 hybrid families demonstrated that LD resistance was successfully transmitted to the commercial strain.

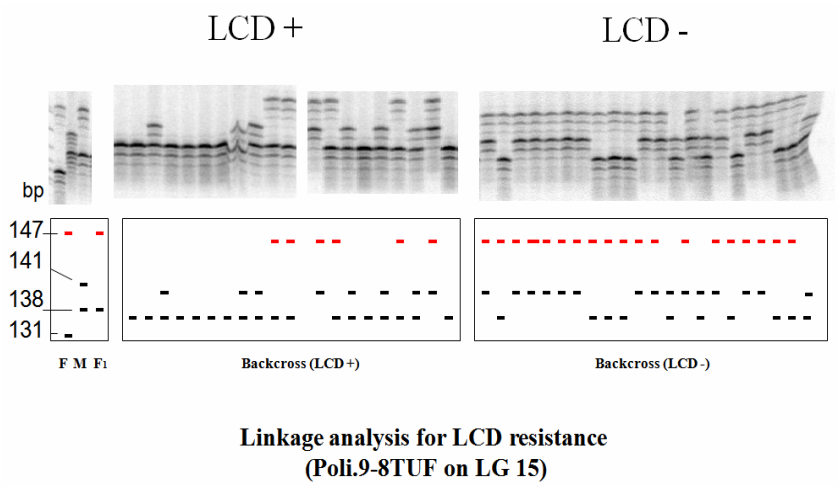


Figure 3.1.1. Autoradiograph of one marker (Poli.9-8TUF) associated with LD resistance on LG15. The upper red band (147 bp) from a resistant strain was confirmed to be responsible for LD resistance.

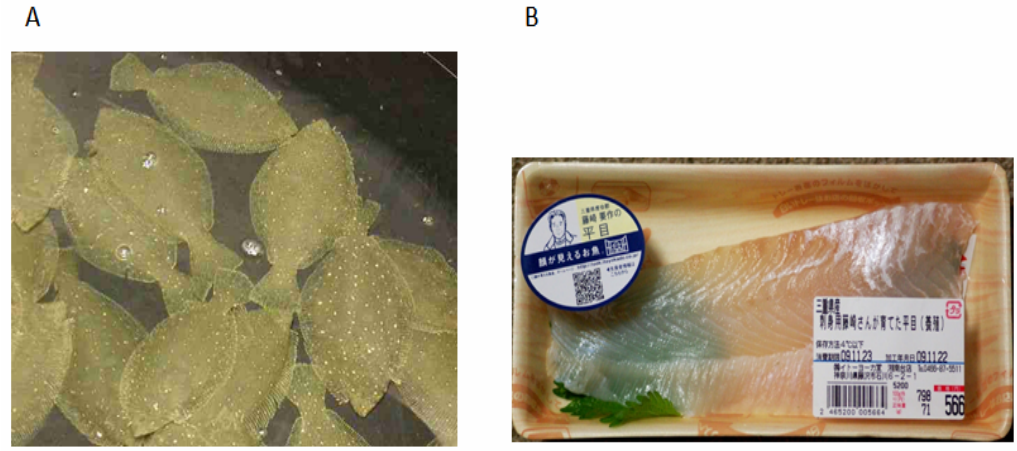


Figure 3.1.2. (A) LD-resistant Japanese flounder (*Paralichthys olivaceus*) by MAS in Japan. (B) LD-resistant Japanese flounder filet is sold at the supermarket.

3.1.3.2. IPN Resistant Atlantic salmon (*Salmo Salar*) In Norway

IPN is a viral disease that is a major problem in the production of Atlantic salmon, and other salmonid species, worldwide. In the freshwater phase of the salmon life cycle, IPN outbreaks in fry have been observed for several decades, with up to 70% mortality. In the marine environment, problematic IPN outbreaks (resulting in up to 40% mortality) have emerged more recently, coinciding with the dramatic expansion of salmon aquaculture (Houston et al., 2008). Several genetic linkage maps have been constructed for Atlantic salmon (Moen et al., 2004; Gilbey et al., 2004; Moen et al., 2008; Lien et al., 2011). One

major QTL for IPN-resistance was detected on linkage group 21 in Scottish and Norwegian Atlantic salmon populations (Houston et al., 2008; Moen et al., 2009; Houston et al., 2012). Challenge-tests showed that the QTL had the same beneficial effect on fry as on post-smolts, with the confidence intervals for the QTL positions in the two age groups overlapping. QTL genotypes based on MS markers and single nucleotide polymorphism (SNP) markers were deduced within most parents of breeding companies, providing a solid framework for linkage-based MAS within the whole population in subsequent generations (Moen et al., 2009; Houston et al., 2012).

3.1.4. Future Perspectives

The culture of some Japanese species such as Pacific bluefin tuna, yellowtail and Japanese eel is still based on the capture of wild fish. Recently, methods for propagating these species in captivity have been developed. Now there is a need to develop useful strains from the wild fish populations. We have attempted to combine classical selection and marker-assisted selection (MAS) in yellowtail (*Seriola quinqueradiata*) to develop strains resistant to the ectoparasite *Benedenia seriolae*, which causes secondary infections and reduced growth.

First, we constructed a genetic linkage map for this species. Second, we obtained the first generation by classical selection, examining 200 adult wild yellowtail individuals from coastal waters for *B. seriolae* and selecting a few fish with low numbers of parasites. These fish were one-on-one crossed to produce F_1 families. In the F_1 families, we searched for D NA markers associated with resistance to the parasitic infection and identified two QTLs (Squ2 and Squ20) (Ozaki et al., in press). Third, MAS was performed using QTL markers associated with parasitic resistance to produce F_2 families. F_1 siblings were placed in two groups according to whether or not they inherited the QTL alleles for resistance. Then, two types (putative resistant families and susceptive families) of F_2 families were established by one-on-one crossing. The two types of families were reared together and challenged by exposure to *B. seriolae*. In the F_2 families, the resistant family fish had significantly fewer parasites than the susceptive family fish in all tested cases. These results show that it is possible to establish a new strain with resistance to some diseases by combining classic breeding and molecular genetic breeding (MAS).

Glossary

IPN: nfectious pancreatic necrosis,

LD: Lymphocystis disease,

MAS: Marker-assisted selection, MS: microsatellite,

QTL: Quantitative trait loci, SNP: single nucleotide polymorphism

3.2. Establishment of Disease-Resistant Fish

Ryosuke Yazawa

3.2.1. Synopsis

The establishment of disease-resistant strains for aquaculture is important since

infectious diseases are the greatest problem in the aquaculture industry all over the world. There are several ways to prevent and control diseases. Drugs and vaccines are the most popular and direct methods to control infectious diseases; however targeted species and diseases have been limited, besides they are both expensive and labor-intensive. Although, selective breeding is the traditional way to establish new strains, the rate and consistency of genetic improvement might be unstable. Transgenic technology could be an alternative approach to prevent and control infectious diseases, since it is theoretically possible to integrate the foreign gene coding the protein or peptide that could provide a desirable trait to the host species. Most research to have successfully established disease-resistant transgenic strains is based on the idea of overproducing the antimicrobial peptide that possesses anti-bacterial or anti-viral activities derived from the transgene. In this section, recent advances in the fish transgenesis for disease-resistance are discussed.

3.2.2. Introduction

Infectious diseases are the one of the greatest problems for aquaculture and causes severe economic losses worldwide. Therefore, it is necessary to establish the disease-resistant strains for aquaculture species. To address this issue, transgenic technology could be an approach for prevention and control of infectious diseases as an alternative to the conventional methods, such as a selective breeding. Transgenesis is the process of introducing an exogenous gene, called a transgene, into a host species so that the host species acquire a new trait derived from the exogenous gene and transmit the trait to its offspring. Fish transgenesis could be a way to establish new strains more rapidly and consistently rather than the traditional selective breeding. Besides, selective breeding may also have the potential risk to retain undesirable traits. Transgenic fish with enhanced disease resistance would increase the production efficiency and benefit the aquaculture industry. To achieve this task, several researchers have been tried to establish disease-resistant fish strains (Dunham, 2009).

3.2.3. Transgenesis for Disease-Resistance

To date, most research to have successfully established disease-resistant transgenic strains is based on the idea of over-expressing an antimicrobial (anti-bacterial or anti-viral) peptide gene (summarized in Table 3.2.1). Although the inhibition of viral replication by antisense RNA is also a potential technique to prevent viral diseases, thus far there is only one report of the transient expression of an antisense of viral genes improving viral resistance in rainbow trout (Anderson et al, 1996).

Anti-bacterial or –viral peptides play important roles in the innate immunity of a wide range of organisms. Although fish possess their own antimicrobial peptides against infections from pathogenic organisms, antimicrobial peptides from different taxa tend to possess higher activities in a xenogeneic environment. Therefore, the genes coding these antimicrobial peptides were chosen as a transgene to produce disease-resistant transgenic fish. It seems reasonable that the host species do not have effective antimicrobial peptides against the pathogens possessing high virulence to the host species from the viewpoint of the evolutionary aspects to the host-pathogen relationship. Besides, antimicrobial peptides that possess activity against a wide spectrum of bacteria, such as cecropin,

lysozyme, hepcidin or lactoferrin, have been chosen as the transgenes in the previous studies (Table 3.2.1).

Species	Foreign gene	Promoter	Challenge test	Ref.
Channel catfish	Silk moth cecropin	CMV	*Flavobacterium columnare*	Dunham et al, 2002
Medaka	Silk moth cecropin Pig cecropin	CMV	*Pseudomonas fluorescens, Vibrio anguillarum*	Sarmasiket al, 2002
Grass carp	Human lactferrin	Carp beta-actin gene	*Carp haemorrhage virus*	Mao et al, 2004
Zebrafish	Chicken lysozyme	Japanese flounder keratin gene	*Flavobacterium columnare, Edwardsiella tarda*	Yazawa et al, 2006
Rare minnow	Rare minnow MX	CMV	*Grass carp reovirus*	Su et al, 2009
Zebrafish Convict cichlid	Tilapia hepcidin	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Hsieh et al, 2010
Zebrafish	Epinecidin-1	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Peng et al, 2010
Atlantic salmon	Rainbow trout lysozyme	Ocean pout antifreeze protein gene	-	Fletcher et al, 2011
Zebrafish	Tilapia hepcidin Giant tiger prawn chelonianin	Zebrafish myosin light chain gene	*Vibrio vulnificus, Streptococcus agalactiae*	Pan et al, 2011

* CMV: cytomegalovirus promoter

Table 3.2.1. List of the disease resistance transgenic fish reported to date.

For the effective action of the transgenes in the host species, it is essential to control their expression at a high level or tissue-specific manner using the regulatory region of the genes, called the promoter. In the beginning of the transgenic fish studies, the promoter derived from viruses, such as the CMV promoter, were frequently used due to their high activity in the broad range of species. It is preferable to use promoters derived from target fish (or closer species) as recent studies suggest, since the promoters derived from fish are thought to work more dependably in general.

These transgenic strains when challenged with bacteria showed resistance against the pathogens. In the case of our previous work, we established a transgenic zebrafish that expressed the chicken lysozyme gene under the control of the Japanese flounder keratin gene promoter (Su et al, 2009). In challenge experiments, 65% of the F2 transgenic fish survived an infection of *Flavobacterium columnare* and 60% survived an infection of *Edwardsiella tarda*, whereas 100% of the control fish were killed by both pathogens. Thus, new strains with enhanced disease resistance by genetic modification have been successfully established.

However, there is no study to establish the disease-resistant transgenic fish in marine

aquaculture species. The generation of transgenic fish targeted on marine aquaculture species is still not popular due to the difficulties associated with handling small and fragile pelagic eggs. Recently, our group has developed a feasible and reproducible microinjection method for the pelagic eggs of marine fish and to establish stable transgenic strains in Nibe croaker, *Nibea mitsukurii* that could be a model species for the marine aquaculture fish species spawning pelagic eggs (Yamamoto et al, 2011). Accumulation of these techniques will realize the production of disease-resistant transgenic aquaculture species in near future.

3.2.4. Risks and Benefits of Transgenic Fish

Although, fish transgenesis has great advantages for the breeding of aquaculture species, there are several potential risks, particularly environmental and human health concerns. If transgenic fish escape into the natural environment, it would cause problems ecologically and genetically. Sterilization of transgenic strains with the polyploidy treatment and/or physical containment by the land-based marine aquaculture with the closed re-circulating system could be realistic way to solve this problem (Dunham, 2009). Another issue is human health concerns. To settle this issue, it is important to select the targeted gene, to conduct food safety trials securely and to keep consumers informed. Since it might be possible that transgenic fish with enhanced disease resistance may decrease or suppress the drug usage in aquaculture, this would improve the aquaculture production more safely from the standpoints of the drug residues and the emergence of antibiotic-resistant pathogens. Although it is essential to guarantee the safety of transgenic fish as genetically modified food, the disease-resistant transgenic fish could be of great help to improve the aquaculture.

Glossary

CMV: Cytomegalovirus

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