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Detection of Antibody against Lymphocystis Disease Virus in Japanese Flounder by Enzyme Linked Immunosorbent Assay

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Enzyme linked immunosorbent assay (ELISA) was applied to detect the antibody of Japanese flounder (*Paralichthys olivaceus*) against lymphocystis disease virus (LCDV). Purified Japanese flounder LCDV was used as a capture antigen of ELISA. Monitoring the immune response of Japanese flounder to LCDV was done using the sera obtained from apparently healthy, LCD-diseased and LCD-recovered fish. ELISA absorbances of these three groups were clearly different. Using the serum with high ELISA absorbance, the optimum ELISA condition was set up. Apparently healthy fish were injected with inactivated LCDV. One to three months after the injection, the antibody against LCDV was detected by ELISA established for LCDV and their ELISA antibody titer increased during that period.

Key words: lymphocystis disease, LCDV, Japanese flounder, antibody, ELISA

Japanese flounder (*Paralichthys olivaceus*) is economically important fish and recently it's culture is popular in different parts of Japan. In Hokkaido, there are some hatcheries and fish farms that culture the Japanese flounder. Almost every year, lymphocystis disease (LCD) occurred in the fish cultured in Hokkaido and the economic loss from this disease is severe to the farms. The disease is a worldwide in water environments (Anders and Darai, 1985) and it's infection is widely spreading. It was reported that LCDV infected not only Japanese flounder *Paralichthys olivaceus* (Tanaka *et al.*, 1984), more over it spontaneously infected many other fish species, such as sea bass *Lateolabrax japonicus* (Miyazaki and Egusa, 1972), yellowtail *Seriola quinqueradiata* (Matusato, 1975) and also some other commercial fishes. It was also reported that the cultured black rockfish *Sebastes schlegeli* was infected by LCDV in Hokkaido (Tanaka *et al.*, 1984). The causative agent is the fish lymphocystis disease virus which induces some characteristic nodules on the body and fins of some fish species (Nigrelli and Ruggieri, 1965). Infected fish showed poor growth and anemia, in the worst case die of hunger. But most of the infected fish were able to recover from the disease and would not be infected again in the next year. It has been reported that seroprevalence and antibody activity of the fish increased with age (Lorenzen and Dixon, 1991). Therefore, protective immunization to LCDV in flounder may be effective. For the immunization, it is necessary to monitor the immune response against the antigen in the fish.

The purpose of this study is to examine the detection of antibody against LCDV in cultured Japanese flounder by ELISA and to compare the ELISA antibody titers between apparently healthy, LCD-diseased, LCD-recovered and immunized fish.

Materials and Methods

Fish

Japanese flounder used in this study was cultured at Kumaishi Fisheries Station in Hokkaido. Firstly, the antibody-monitoring test was done in the spring using the 3 types of each 10 fish cultured in different tanks; apparently healthy, LCD-diseased and recovered fish. Average body weight of these fish was about 40 g. Following this experiment, 5 healthy fish (45 g) were injected with LCDV by the method described below. Two 1^+ year-old Japanese flounder, weighting about 800 g, were also injected and detected their ELISA antibody titers against LCDV.

Purification of LCDV antigen

Lymphocystis cells were collected from the infected

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Japanese flounder. The specimens of lesion (10 g) were washed with PBS (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, and 1.47 mM KH_2PO_4), homogenized and suspended to TE (50 mM Tris-HCl, 2 mM EDTA). The suspension was repeated freezing and thawing for 3 times, then centrifuged for 20 min at 1,800 $\times g$. The supernatant was centrifuged for 2 h at 81,000 $\times g$. The pellet was resuspended in TE, overlaid on 15, 20, 30, 40 and 50% sucrose steps and centrifuged for 2 h at 81,000 $\times g$. Virus bands were harvested and diluted in TE. After 2 h centrifugation at 81,000 $\times g$, the pellets were resuspended in TE.

ELISA to detect the antibody against LCDV in Japanese flounder

Sandwich ELISA based on the method reported by Yoshimizu *et al.* (1992, 1997) was used. Briefly, microtiter plate (Greiner) was coated with 50 μl of purified LCDV antigen diluted to 10 μg/ml and left overnight at 4°C. Following 3 times washes with PBS containing 0.05% Tween 20 (T-PBS), the plate was blocked with 2% skim milk for 1 h at 37°C. After 3 times washes with T-PBS, 50 μl of fish serum diluted to 1:40 in T-PBS were applied and incubated for 2 h at 37°C. Followed by the T-PBS wash, 50 μl of the anti-flounder IgM rabbit serum (the 2 nd serum; Suzuki *et al.*, 1998) diluted to 1:400 in T-PBS was added to each well and incubated for 1 h at 37°C. Washed by the T-PBS and 50 μl of peroxidase conjugated anti-rabbit IgG swine serum (the 3 rd serum; Dako) diluted to 1:500 in T-PBS was added to each well and incubated for 30 min at 37°C. Followed by the wash with T-PBS for 5 times, 50 μl of substrate (*o*-phenylenediamine) in citric acid buffer (0.2M Na_2HPO_4 : 0.1M citric acid = 103:97) was added and incubated for 15 min at room temperature. The reaction was stopped by adding of 50 μl /well of 2N H_2SO_4. The absorbance was read at 492 nm, using a micro plate reader (MTP-120, Corona).

Selection of the antigen concentration and antisera dilution in ELISA

The capture antigen was prepared ranging from 1 ng/ml to 1,000 ng/ml, anti-Japanese flounder IgM rabbit serum was diluted from 1:100 to 1:16,00, and peroxidase conjugated anti-rabbit IgG swine serum was diluted from 1:100 to 1:1,000 and examined for the ELISA assay.

Absorbed test with LCDV

Fish serum which had high ELISA absorbance was reacted with homogenized lymphocystis cells *in vitro* overnight at 4°C. Then the suspension was centrifuged and the supernatant was collected to use for the ELISA assay.

Injection of inactivated LCDV to Japanese flounder

Five healthy fish (0^+ year-old) that had negligible ELISA titers were injected with formalin-inactivated purified virus (250 μg/ml) at pterygiophore region, just below the dorsal margin and 2 healthy fish (1^+ year-old) were injected at the blood vessel. Fish were tagged for monitoring individually and 1 and 3 months later, their ELISA antibody titers were measured. Control fish were injected with Hanks' BSS by the same method.

Results

Comparison in ELISA absorbances among healthy, LCD- diseased and recovered fish

ELISA absorbance of the 3 groups of healthy, LCD-diseased and recovered fish was different from each other. Average absorbance of healthy, LCD-diseased and recovered fish was 0.004, 0.032 and 0.082, respectively (Fig. 1).

Determination of ELISA conditions to detect the antibody against LCDV

Reactivity of the purified LCDV diluted to 1 ng/ml was enough to detect the antibody against LCDV and could distinguish the positive serum from the negative one (Fig. 2). Reactivity of the anti-sera were decreased with increase of the dilution rate of the 2nd and 3rd sera for the ELISA assay. Reactivity of the 2nd and 3rd sera diluted to 1:400 or 1:500 were enough to detect the antibody against LCDV (Fig. 3) and could distinguish the positive serum from the negative one (data not shown). Thus, 1:400 dilution was selected for 2nd serum and 1:500 dilution was selected for the 3rd serum.

Determination of the ELISA base line to detect the antibody against LCDV in Japanese flounder

ELISA absorbance of the serum from ELISA positive fish, absorbed with homogenized lymphocystis cells was decreased to approximately a rage from 0.005 to 0.010, almost the same as to the absorbence of healthy fish. Besides, ELISA absorbance of the ELISA positive serum was decreased with increase of the dilution rate and became flat at the same point of the absorbed serum

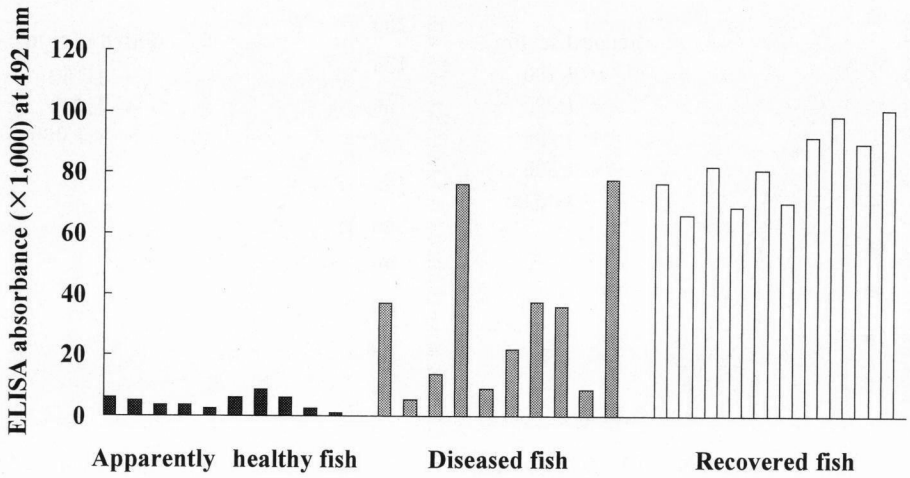

Fig. 1. Comparison of ELISA absorbance among 3 groups of fish; healthy, LCD(lymphocystis disease)-diseased and recovered fish. Ten fish of each group were tested.

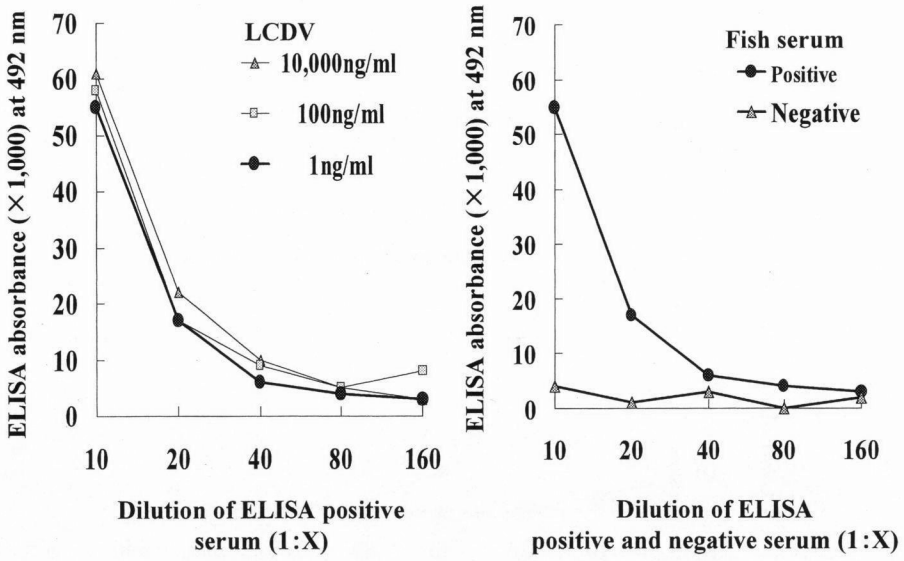

Fig. 2. Effect of the antigen concentration of LCDV on ELISA absorbance.

about 0.005 (Fig. 4). Therefore, ELISA base line to determine the antibody borderline between positive or negative ones was chosen to 0.010.

Immune response of Japanese flounders

One month after the injection with inactivated LCDV, two 1+ year-old Japanese flounder which were injected into blood vessel showed 1:640 and 1:1280 ELISA antibody titers. Three months after the injection, ELISA antibody titer of 5 juvenile Japanese flounder injected at pterygiophore region increased to a rage from 1:20 to 1:40. ELISA antibody titers of both control fish remained 1:5 (Table 1).

Discussion

Recently, ELISA methods to detect the antibody against fish pathogens such as *Aeromonas salmonicida*

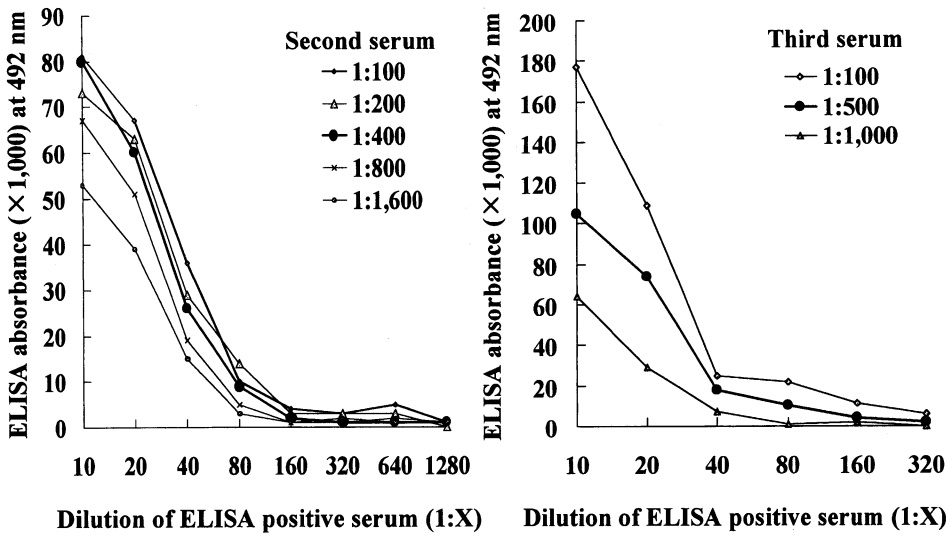

Fig. 3. Effects of the concentration of anti-Japanese flounder IgM rabbit serum (the 2nd serum) and peroxidase conjugated anti-rabbit Ig swine serum (the 3rd serum) on ELISA absorbance.

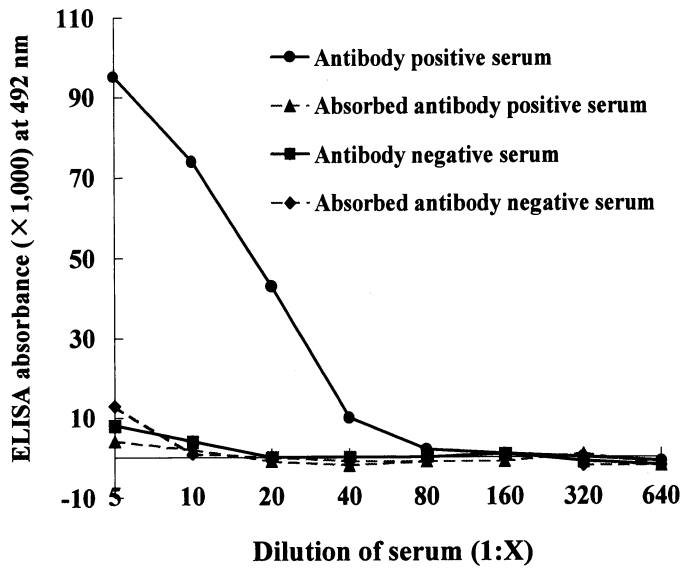

Fig. 4. Comparison of ELISA absorbance of the sera from LCD-recovered (●, ▲) and healthy (■, ◆) fish between non-absorbed and absorbed with homogenized lymphocystis cell.

(Yoshimizu *et al.*, 1992), striped jack nervous necrosis virus (SJNNV) (Mushiake *et al.*, 1992) and *Edwardsiella tarda* (Bang *et al.*, 1992) were reported. Determination of the activity responding with cell-mediated immunity is thought to be difficult and then monitoring of the humoral immune response was selected and examined by ELISA. The ELISA established in this study is useful to detect the antibody against LCDV in Japanese flounder without killing the fish. But in general, the ELISA methods to detect the antibody are not common to all the pathogens. Detection of the antibody against *Renibacterium salmoninarum* that causes bacterial

Table 1. ELISA antibody titer of Japanese flounder injected with the purified formalin inactivated LCDV 1 month and 3 months after injection

Age	Group	Month after injection	Number of fish	ELISA antibody titer
1 year-old	Control	1	2	1:5
1 year-old	Injected (Blood vessel)	1	2	1:640 ~ 1:1,280
6 month-old	Control	3	5	1:5 ~ 1:10
6 month-old	Injected (Pterygiophore region)	3	5	1:20 ~ 1:40

kidney disease has not been regarded as a standard method (Thoesen, 1994). But detection of antibody by ELISA against *Aeromonas salmonicida* that causes furunculosis and fish nodavirus that causes viral nervous necrosis (VNN) were reported to be useful for the detection of the antibody and applied to select the nodavirus carrying broodstocks of berfin flounder (*Verasper moseri*) (Yoshimizu *et al.*, 1992, 1997).

Lymphocystis disease appears in Japanese flounder in almost every year in Hokkaido and establishment of the protection method is requested. ELISA absorbance of healthy fish was low and that of the fish recovering from the disease was high. Most of LCD-diseased fish showed a low level ELISA absorbance but some of the fish showed high absorbance as the recovered fish. It was thought that the diseased fish with high ELISA absorbance was going to recover from the disease at that time. And then, the fish immunized with inactivated LCDV developed the ELISA antibody titer up to 1:20 to 1:1280 after 1 to 3 months while the control fish remained 1:5. These results suggest that the humoral immune response against LCDV reflect the disease progression of LCD in Japanese flounder. Moreover, vaccination for protecting from LCDV infection will be effective.

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