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The lymphocystis diseases in the Olive flounder, *Paralichthys olivaceus*

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Abstract: Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease, affecting more than 100 teleost species worldwide. Characteristically, LCD is chronic, self limiting and species specific. The greatly hypertrophied cells, called lymphocystis tumor cells, typically occur on the skin, fins and oral region. Lymphocystis cells were ovoid to circular and varied in sizes ranging from 200-250 mm. The lymphocystis disease infected flounder have unsightly appearances that discourage the commercial values. A PCR detection technique was developed to amplify a fragment of LCDV major capsid protein gene (1347bp) which is shortcoming and useful. The PCR result proved that the LCD-virus replicated in the epidermis (fins and skin) not in the spleen, kidney, intestine or brain of Paralichthys olivaceus.

Keyword: Lymphocystis disease, LCDV, PCR, Paralichthys olivaceus

Introduction

Lymphocystis disease (LCD) is a chronic, self-limiting, viral disease affecting many species of teleosts worldwide. Freshwater, estuarine, and marine fish in warm-water, and cold-water environments are susceptible to this disease. In general, lymphocystis is a disease of more evolutionarily advanced species of teleosts, like perches, seabreams and flounders. Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease, is double stranded DNA which is circularly permuted and terminally redundant (Darai *et al.,* 1983). Because of viral replication and subsequent inhibition of mitosis in the host's connective tissue cells, affected individuals develop macroscopic, nodular lesions that somewhat resemble warts or clusters of tumor (Wolf, 1962). The greatly hypertrophied cells, called lymphocystis tumor cells, typically occur on the skin, fins and oral region. Rarely, these lesions may develop in internal organs (Sindermann, 1979; Plumb, 1993). Lymphocystis is the most common viral infection of Japanese flounder, rock breams and sea breams in Korea (Kitamura *et al.,* 2006). Although lymphocystis disease has a low mortality rate, it may leave an individual disfigured. This disfigurement can have several consequences. If the gills are affected, the fish can have difficulty in breathing. When the lesion is located around the mouth, the fish may have difficulty in feeding.

Many fish populations have declined drastically over the last several decades. Scientists are attempting to discern the cause of these declines to prevent more fish from dying. Environmental pollution appears to be playing a major role in the development or exacerbation of disease. By understanding fish with respect to health and disease, fish populations can be used as sentinels to monitor water quality, to evaluate human impact on the environment, and to serve as harbingers of spreading of disease. Lymphocystis diseases have been isolated from more than 100 teleost species (Anders, 1989), however the infections and virus replication is unknown. LCDV has been studied for the different isolation and characterization techniques (Iwamoto *et al.,* 2002; Alonso *et al.,* 2005; Cano *et al.,* 2006) that helping shortcoming detection of the disease and to take initiatives to a disease free aquaculture farming. The recent emphasis on intensive fish culture has also brought a more acute concern for problems associated with pathogenic agents that cause diseases. The diseases, by slow continuous attrition or by sudden catastrophic epizootics can cause fish mortalities often resulting in great losses of the fish farming. Generally, infectious diseases of fish and other aquatic animals are caused by parasites, bacteria, fungi and viruses. The lymphocystis disease infected flounder have unsightly appearances that discourage the commercial values. In this study, we tried to detect the extent of lymphocystis disease virus in Olive flounder, *Paralichthys olivaceus*.

Materials and Methods

Sample collection: A sample of Olive flounder (12.6-14.3 g and 80.2-96.3 g) was collected from commercial fish farms situated in Yeosu on the southern part of Korea. The fish were examined carefully in the field and nodules consistent with lymphocystis disease were noted. After the sampling, flounder were immediately preserved in ice box and carried to the laboratory, where the lymphocystis nodules were cut off from the fish organ and washed with phosphate buffer solution (PBS) and preserved at -80℃ until used.

Histopathological examinations: For histological studies, the fin and skin nodules of lymphocysitis disease of infected flounder were immediately preserved in 10 % neutral-buffered formalin. After fixation, standard histological procedure was followed

for tissue dehydration and paraffin embedding. Tissue section was stained with standard procedure.

Virus detection by PCR: Polymerase chain reaction was performed using the method of Kitamura *et al*. (2005). Briefly, the fin, skin, spleen, kidney, brain and intestine samples of lymphocystis disease infected flounder were homogenized in a nine-fold volume of TE buffer(10mM Tris-HCl, 1 mM EDTA, pH 7.4) and centrifuged at 2000 g for 15 min at 4℃. A 20-$\mu\ell$ aliquot of proteinase K(1mg mL-1; TAKARA, Japan) was added to 200$\mu\ell$ of the supernatant in each tubes. The mixture was incubated at 56℃ for 90 min in an incubator. DNA was isolated by using phenol and chloroform. The nucleic acids were precipitated with isopropanol, re-suspended with double distilled water and stored at -20℃ for over-night. The PCR primer LCC-F (5´-CAAGTGTTACTAGCGCTTT-3´) and LCC-R (5´-ATCCCATTGAACCGTTCT-3´) was designed on the basis of nucleotide sequence of MCP (major capsid protein) gene open reading frame of LCDV-C (AY380826) for a 1347 base region for PCR amplification. PCR amplification was performed using a GeneAmp 2400 thermal cycler (Perkin Elmer, Foster City, USA) with 30 cycles (95℃ for 1min, 54℃ for 1 min, and 72℃ for 1 min). The PCR product was analyzed in 1.5% agarose gels containing ethidium bromide and visualized under UV light.

Results and Discussion

Lymphocystis disease is characterized by the development of abnormally enlarged cells usually evident macroscopically as single whitish nodules or clusters of nodules of tumour-like appearance on the skin and fins of infected fish (Nigrelli & Smith, 1939). In the sampled fish persisting tumor-like dermal lesions and clusters of tumor cells on the caudal, dorsal, ventral fins and the oral region (Plates 1 and 2) which were similar to the stated by Nigrelli & Smith (1939). Although the infected fish appears to be unsightly, the disease is rarely fatal (Templeman, 1965). Mature lymphocystis clusters appeared as swellings on the skin and fins to reveal of white masses or nodules (Plate 1).The gross lesions or nodules, and /or clusters of tumors were similar to that described by Wolf and Carlson (1965).

In Korea, lymphocystis disease is a common fish disease occurs mainly in Japanese flounder, rockfish and sea bass and it increases in summer months (Kitamura *et al.,* 2006, 2007). From the microscopic observations (Plate 2) the lymphocystis tumor was a cluster of hundreds of individual cells hypertrophied bi-layer at the initial stages. Lymphocystis cells were ovoid to circular and varied in sizes ranging from 200-250 mm (Walker, 1962; Murchelano & Bridges, 1976). Although, several immunological techniques have been previously used for detection of LCDV in different fish species (Garcia-Rosado *et al.,* 2002), however, PCR is a rapid, sensitive and highly specific detection method for fish viruses which can be powerful stool to detect iridovirus infections (Mao *et al.,* 1997). In this study, PCR detection we developed the technique to amplify a fragment of LCDV major capsid protein gene which is shortcoming and useful. The PCR result proved that the LCD-virus replicated in the fin but skin not in the spleen, kidney, intestine and brain (Fig. 1). Although some authors reported that LCDV can multiply in the internal organs (Sindermann, 1979; Plum, 1993).

Olive flounder is economically important food fish and recently culture of flounder has become popular in Asian countries like Korea, Japan and China. However, in these countries, the flounder farming aquaculture industry has many problems such as diseases (Murogo & Egusa, 1996) and color abnormalities (Venizelos & Benetti, 1999). There are presently no medicines or commercially available vaccines for LCD. Thus, it is important to detect the disease at early infection stage to have some protection and isolation of those fish from massive outbreaks. In conclusion, LCDV detected in Olive flounder collected from different fish farms suggesting that the flounder may be playing a key role as vector, and or reservoir for the virus in Korea. The role of these flounder in disease spreading is an important question to be investigation in future research on lymphocystis disease virus pathogenesis.

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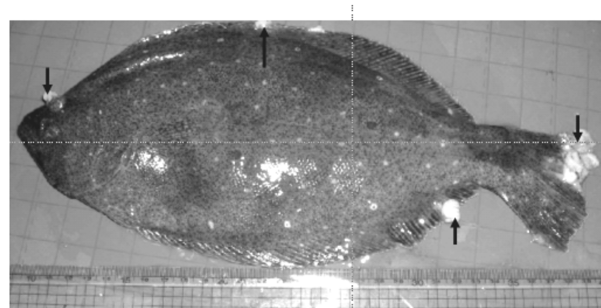

Plate 1. Lymphocystis disease affected Olive flounder, *Paralichthys olivaceus. A*rrows showing the virus infected organs that persisting clusters of tumor cells with lymphocystis disease virus (LCDV).

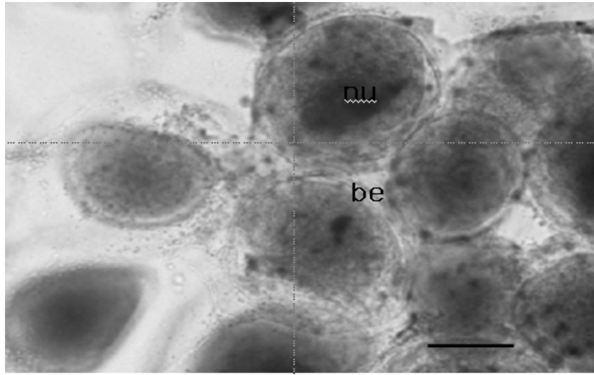

Plate 2. The lymphocystis disease in *Paralichthys olivaceus*. Lymphocystis clusters of tumor cells or hypertrophied cells with surrounded bi-layer epithelium (be) and enlarged nucleus (nu). (Scale bar =200㎛)

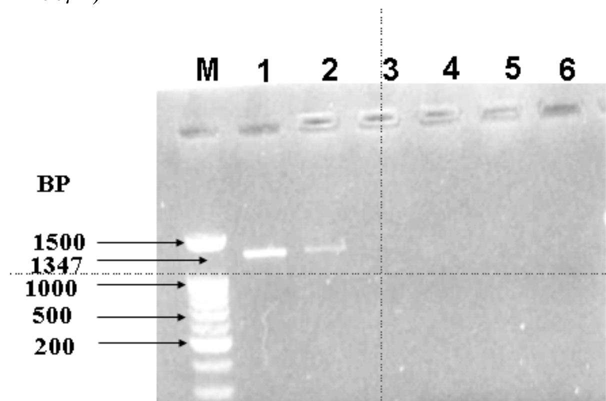

Fig 1. Agarose gel electrophoresis of polymerase chain reaction amplification product from the major capsid protein gene (1347 bp) of Olive flounder, *Paralichthys olivaceus* . The gel was stained with ethidium bromide. Lanes are as follows: M, the DNA molecular weight marker (Promega); Lane 1, skin isolates; Lane 2, fin isolates; Lane 3, spleen isolates; Lane 4, kidney isolates; Lane 5, brain isolates; Lane 6, intestine isolates; and BP, base pairs.

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