한국어병학회지 제24권 제2호 (2011)
J.Fish Pathol., 24(2) : 47~51

Propagation of lymphocystis disease virus (LCDV) in the FFN cell line originated from olive flounder *Paralichthys olivaceus* fin

Mosharrof Hossain*,**, Wi-Sik Kim***, Sung-Ju Jung* and Myung-Joo Oh*†

*Department of Aqualife Medicine, Chonnam National University, Yeosu 550-749, Korea

**Department of Zoology, Rajshahi University, Rajshahi-6205, Bangladesh

***The Fisheries Science Institute, Chonnam National University, Yeosu 556-901, Korea

The present study demonstrated lymphocystis disease virus (LCDV) propagation through cytopathic effects (CPE) formation and LCDV detection in olive flounder fin (FFN) cells by polymerase chain reaction (PCR) and fluorescent antibody technique (FAT) methods. Tissue filtrates from the cluster cells produced CPE in FFN cells, which initially cells became enlarged and gradually underwent fusion en masse. Infectivity of culture grown LCDV using the FFN cells reached $10^{2.3}$ $TCID_{50}$/ml at 4 days post infection and the highest titer was measured $10^{6.5}$ $TCID_{50}$/ml at 12 days. The viral DNA was detected in the cell culture supernatants showing CPE and the CPE cells by PCR. Antigen specific strong fluorescence reacting with monoclonal antibody against the virus revealed the presence of viral antigen in the cytoplasm of infected FFN cells. These results suggest that the FFN cell line originated from the olive flounder has a susceptibility of the LCDV.

Key words : Lymphocystis disease virus, *Paralichthys olivaceus*, FFN cells, Propagation

Lymphocystis disease (LCD) is a chronic and benign viral disease occurring in marine, brackish and fresh water fish worldwide. Affected fish with LCD show a typical external symptomology with clusters consisting of enormously hypertrophied cells on the skin and fins (Nigrelli and Smith, 1939; Wolf, 1988). The hypertrophied cells have a thick hyaline capsule, an enlarged nucleus, and prominent basophilic cytoplasmic DNA inclusions (Samalecos, 1986). The causative agent of LCD, LCDV is a large icosahedral DNA virus in the genus *Lymphocystivirus* of the family *Iridoviridae* (Chinchar *et al.*, 2005). In Korea, LCD is a common fish disease, especially in olive flounder (*Paralichthys olivaceus*) (Kitamura *et al.*, 2006a). Although the disease is not fatal to the flounder, the unsightly appearance may cause important economic losses.

Since an electron microscopic study on LCDV by Walker (1962), a lot of studies have been conducted, including morphological and molecular characteristics of LCDV (Zwillenberg and Wolf, 1968; Schnitzler and Darai, 1993; Tidona and Darai, 1997; Zhang *et al.*, 2004; Cano *et al.*, 2006a; 2006b; Kitamura *et al.*, 2006a; 2006b). However, a few studies on the *in vitro* production of LCDV have been conducted, although *in vitro* virus culture is an important to virus study. The major obstacle

†Corresponding author: Myung-Joo Oh
Tel : +82-61-659-7173 Fax : +82-61-659-7173
E-mail : ohmj@chonnam.ac.kr

to progress is the lack of an efficient cell culture system for propagation of this virus. In the present study, we report that flounder fin (FFN) cell line originated from the olive flounder has a susceptibility to LCDV.

Materials and Methods

Preparation of LCDV inoculums

Lymphocystis tissues were sampled from the tumor or cluster cells from our previous experimentally LCDV infected olive flounder (Hossain *et al.*, 2009). The cluster cells were washed with phosphate buffered saline (PBS) and homogenized with 10-fold volume of minimum essential medium (MEM). The homogenized solution was centrifuged at 2000 rpm at 4℃ for 10 min to remove the cell debris, and the supernatant was collected and filtered with 0.45 μm Super® syringe filter (Acrodisc, USA). All these filtered solutions were used for cell lines infection and stored at -80°C for further use.

Cell line susceptibility

Three fish cell lines were used for LCDV susceptibility test; FHM (fathead minnow; Gravel and Malsberger, 1965), CHSE-214 (Chinook salmon embryo; Lannan *et al.*, 1984) and FFN (olive flounder fin; Kang *et al.*, 2003) which were maintained at 20°C with MEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. The cell lines were seeded at 1×10^5 cells/ml either in 25 cm^2 tissue culture flasks or in 24-well plates and incubated at 20°C for overnight. The culture medium was discarded the following day and 500 μl (100 μl in 24-well plates) of virus inoculums were applied in all cell lines. After overnight adsorption, cells were washed with MEM 5 times and MEM_{10} was then added. The cytopathic effect (CPE) was checked every day post inoculation. Similar batches of controls were also maintained to compare the CPE by light microscopy. After CPE production, cell culture supernatants and CPE cells were collected for detection of LCDV by polymerase chain reaction (PCR) and fluorescent antibody technique (FAT) methods.

Virus productivity of the cell lines ($TCID_{50}$/ml)

Monolayer cultures of FFN cell line were prepared in 25 cm^2 tissue culture flasks and inoculated with the virus inoculums by the above mentioned condition. After overnight adsorption, cells were washed with MEM 5 times and MEM supplemented with 10% FBS was then added. The culture was incubated at 20°C. At 4, 8, 12 and 16 days post-inoculation, the virus infectivity was determined using 96 well microplates and the end point was calculated by the method of Reed and Muench (1938).

Detection of LCDV by PCR

PCR was conducted using the method described by Kitamura *et al.* (2006a). A 20 μl aliquot of proteinase K (1 mg/ml, Takara, Japan) was added to 200 μl of the virus-cultured medium and CPE cells. The mixture was then incubated at 56°C for 90 min. DNA was isolated using phenol and chloroform. The nucleic acids were precipitated with isopropanol, resuspended with distilled water and stored at −20°C until use. The specific primer pairs LCC-F (5'-CAA GTG TTA CTA GCG CTT T-3') and LCC-R (5'-ATC CCA TTG AAC CGT TCT-3'), targeted a 1,347 base region of LCDV major capsid

protein (MCP) gene, were used for PCR amplification. The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 40 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. The PCR product was analyzed in 1% agarose gels containing ethidium bromide and visualized under ultraviolet (UV) light.

LCD viral antigen detection by FAT

CPE cells were fixed with cold acetone (-20°C) for 15 min, followed by rinsing with PBS. Undiluted monoclonal antibody (MAb) against LCDV prepared in our laboratory was applied to these cells and incubated at 37°C for 1 h. After having been washed with PBS 3 times, the cells were stained with 100 μl goat anti-mouse IgG conjugated to FITC (fluorescein isothiocyanate, Sigma, USA), diluted 1:200 in PBS containing 1% BSA (bovine serum albumin) (w/v) and incubated for 1 h at 37°C. The FITC stained cells were washed with PBS, mounted with 1:1 volume glycerol to PBS, and finally examined under fluorescence microscope.

Results and Discussion

The present study demonstrated LCDV propagation through CPE formation and LCDV detection in FNN cells by PCR and FAT. Tissue filtrates from the tumor or cluster cells produced CPE in FFN cells, while CPE was not observed in FHM and CHSE-214 cells and mock infected cells. The detectable signs of CPE were observed 4 days post infection and continue onward upto 16 days. Initially cells became enlarged and gradually underwent fusion en masse (Fig. 1), but cell lysis was not observed until 20 days post infection. Infectivity of culture grown LCDV using the FFN cells reached $10^{2.3}$ $TCID_{50}$/ml at 4 days post infection and the highest titer was measured $10^{6.5}$ $TCID_{50}$/ml at 12 days (Fig. 2). The specific PCR products corresponding to the 1347 bp fragment of the MCP gene were detected in the cell culture supernatants showing CPE and the CPE cells (Fig. 3). Antigen specific fluorescence reacting with MAb against the LCDV revealed in the cytoplasm of infected FFN cells (Fig. 1C). In contrast, negative results were observed in the FHM and CHSE-214 cells by PCR and FAT (data not shown). These results suggest that FFN cells are susceptible to the LCDV and the virus propagates in the cells. The FFN cells may be useful for studies on *in vitro* production of infectious LCDV.

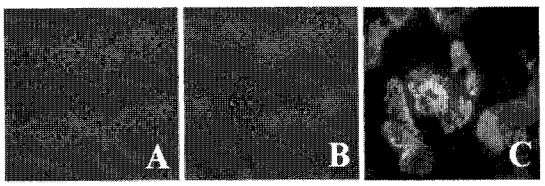

Fig. 1. Cytopathic effect (CPE) observed in olive flounder fin (FFN) cells inoculated with lymphocystis disease virus. (A) normal cells, (B) infected cells at 12 days post inoculation, (C) immunofluorescence in infected FFN cells.

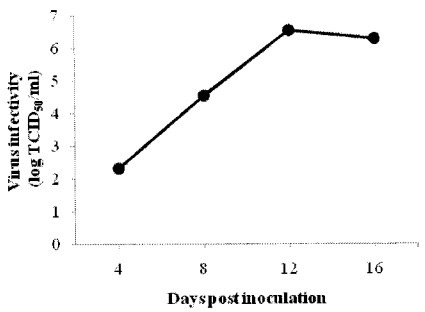

Fig. 2. Infectivity titers of lymphocystis disease virus in the FFN cells.

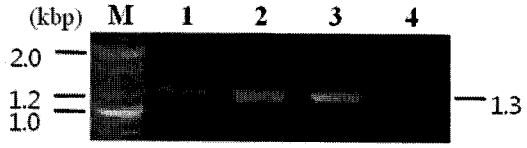

Fig. 3. Detection of lymphocystis disease virus in culture supernatants and pellet of FFN cells showing CPE by polymerase chain reaction (PCR). (1) cell culture supernatants, (2) cell pellet, (3) positive control: filtrates of nodule homogenates, (4) negative control: normal FFN cells.

Many efforts have been taken to *in vitro* propagation of LCDV. A few cell lines such as BF-2 (blue gill fibroblast), BF-W (bluegill fibroblast, Weymouth), SAF-1 (gilt-head sea bream fin) and HINAE (hirame natural embryo) were found to be susceptible to LCDV (Wolf *et al.*, 1966; Walker and Hill, 1980; Garcia-Rosado *et al.*, 1999; Kasai and Yoshimizu, 2001). These cell lines produced CPE of rounding and enlargement of infected cells. These CPE was similar to that of infected FFN cells, but cell fusion en masse after enlargement of cells was only observed in the present study. Although LCDV is characterized by the development of macroscopic clusters of hypertrophied cells, lymphocystis cells formation is not yet clearly understood. The FFN cells might help to study development of lymphocystis cells because cell fusion by LCDV was observed in FFN cells.

Acknowledgments

This work was supported by Korean Research Foundation grants (KRF-2006-F00022). A part of the first author's doctoral research program in Korea was supported by a grant from KRF-2006-11-F00015.

References

Cano, I., Alonso, M.C., Garcia-Rosado, E., Rodriguez Saint-Jean, S., Castro, D. and Borrego, J.J.: Detection of lymphocystis disease virus (LCDV) in asymptomatic cultured gilt-head sea bream (*Sparus aurata* L.) using an immunoblot technique. Vet. Microbiol., 113: 137-141, 2006a.

Cano, I., Ferro, P., Alonso, M.C., Bergmann, S.M., Romer-Oberdorfer, A., Garcia-Rosado, E., Castro, D. and Borrego, J.J.: Development of molecular techniques for detection of lymphocystis disease virus in different marine fish species. J. Appl. Microbiol., 102: 32-40, 2006b.

Chinchar, V.G., Essbauer, S., He, J.G., Hyatt, A., Miyazaki, T., Seligy, V. and Williams, T.: Family Iridoviridae. In Favquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A., editors. Virus taxonomy: Eighth report of the international committee on taxonomy of viruses. Elsevier/Academic Press, London United Kingdom. pp.145-162, 2005.

Garcia-Rosado, E., Castro, D., Rodriguez, S., Perez-prieto, S.I. and Borrego, J.J.: Isolation and characterization of lymphocystis virus (FLDV) from gilt-head sea bream (*Sparus aurata*, L.) using a new homologous cell line. Bull. Eur. Ass. Fish Pathol., 19:5 3-56, 1999.

Gravel, M. and Malsberger, R.G.: A permanent cell line from the fathead minnow (*Pimephales promelas*). Ann. N. Y. Acad. Sci., 126: 555-565, 1965.

Hossain, M., Kim, S.R., Kitamura, S.I., Kim, D.W., Jung, S.J., Nishizawa, T., Yoshimizu, M. and Oh, M.J.: Lymphocystis disease virus persists in the epidermal tissues of olive flounder, *Paralichthys olivaceus*

(Temminch & Schlegel), at low temperatures. J. Fish Dis., 32: 699-703, 2009.

Kasai, H. and Yoshimizu, M.: Establishment of 2 Japanese flounder embryo cell lines. Bull. Fish Sci. Hokkaido University, 52: 67-70, 2001.

Kang, M.S., Oh, M.J., Kim, Y.J., Kasai, K. and Jung, S.J.: Establishment and characterization of two new cell lines derived from flounder, *Paralichthys olivaceus* (Temminck & Schlegel). J. Fish Dis., 26: 657-665, 2003.

Kitamura, S.I., Jung, S.J., Kim, W.S., Nishizawa, T., Yoshimizu, M. and Oh, M.J.: A new genotype of lymphocystivirus, LCDV-RF, from lymphocystis diseased rockfish. Arch. Virol., 151: 607-615, 2006a.

Kitamura, S.I., Jung, S.J. and Oh. M.J.: Differentiation of lymphocystis disease virus genotype by multiplex PCR. J. Microbiology, 44: 248-253, 2006b.

Lannan, C.N., Winton, J.R. and Fryer, J.L.: Fish cell lines: establishment and characterization of nine cell lines from salmonids. In Vitro, 20: 671-676, 1984.

Nigrelli, R.F. and Smith, G.M.: Studies on lymphocystis disease in the orange filefish, *Ceratacanthus schoepfi* (Walbaum) from Sandy Hook Bay, New Jersey. Zoologica, 24: 255-264, 1939.

Reed, L.J. and Muench, H.: A simple method of estimating fifty percent endpoint. Am. J. Hyg., 27: 493-497, 1938.

Samalecos, C.P.: Analysis of the structure of fish lymphocystis disease virions from skin tumors of *Pleuronectes*. Arch. Virol., 91: 1-10, 1986.

Schnitzler, P. and Darai, G.: Identification of the gene encoding the major capsid protein of fish lymphocystis disease virus. J. Gen. Virol., 74: 2143-2150, 1993.

Tidona, C.A. and Darai, G.: The complete DNA sequence of lymphocystis disease virus. Virology, 230: 207-216, 1997.

Walker, R.: Fine structure of lymphocystis disease virus of fish. Virology, 18: 503-505, 1962.

Walker, D.P., and Hill, B.J.: Studies on the culture assay of infectivity and some *in vitro* properties of lymphocystis virus. J. Gen. Virol., 51: 385-395, 1980.

Wolf, K.: Lymphocystis disease. In Wolf, K., editor. Fish viruses and fish viral diseases. Cornell University Press, Ithaca, New York, USA. pp. 268-291, 1988.

Wolf, K., Gravell, M. and Malsberger, R.G.: Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science, 151: 1004-1005, 1966.

Zhang, Q.Y., Xiao, F., Xie, J., Li, Z.Q. and Gui, J.F.: Complete genome sequence of lymphocystis disease virus isolated from China. J. Virol., 78: 6982-6994, 2004.

Zwillenberg, L.D. and Wolf, K.: Ultrastructure of lymphocystis virus. J. Virol., 2: 393-399, 1968.

Manuscript Received : April 22, 2011
Revised : July 6, 2011
Accepted : August 16, 2011