The serodiagnosis of a lymphocystis disease virus infection using an antibody raised against a recombinant major capsid protein

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Lymphocystis is a viral disease of fish primarily in marine and brackishwaters. Here we report the cloning, expression, and the serological applications of the lymphocystis disease virus (LCDV) major capsid protein (MCP). The MCP gene was amplified by PCR from the genomic DNA of LCDV isolated from Schlegel's black rockfish, *Sebastes schlegeli*, and expressed in *E. coli*. Mouse antisera raised against the purified recombinant MCP (rMCP) reacted with the viral MCP in an immunofluorescence assay, indicating that this rMCP would be useful for serological studies of field samples.

Key words: Lymphocystis disease virus (LCDV), Major capsid protein (MCP), Immunofluorescence assay, Schlegel's black rockfish, *Sebastes schlegeli*.

Lymphocystis disease virus(LCDV) is the causative agent of lymphocystis disease. This disease is highly infectious and common in a culture or confined conditions (Plumb, 1993). The LCDV infection frequently appears in Pleuronectiadae (flatfish), such as flounder (*Platichthys flesus*), plaice (*Pleuronectes platessa*), and dab (*Limanda limanda*). The infected animals develop clusters of extremely hypertrophied fibroblasts of osteoblasts called lymphocystis cells. These cells are individually encapsulated by a hyaline extracelluar matrix (Tidona and Darai, 1997). The LCDV infection results in these papilloma-like lesions because of giant cell formation from putative dermal fibroblasts of the skin tissue but *in vitro*, the infection causes permissive cells, e.g. the flounder gill cell line, to exhibit an obvious cytopathic effect (CPE), including rounding of cells, cell detachment, and cell death. These changes resemble the process of apoptosis, in which a series of events are initiated such that the cell participates in its own death (Hu *et al*., 2004). Owing to massive skin lesions, the commercial value of the infected fish tends to decline resulting in an important economic problem. To confirm the present LCDV infection, the viral particle should be directly detected. So the goal of this study was to produce a recombinant major capsid protein (rMCP) for the production of LCDV MCP-specific antibody and to know whether this antibody can be used for serological studies of field samples.

Materials and Methods

E. coli strains and plasmids

E. coli strains, JM109 and BL21(DE3)pLysS, were purchased from Invitrogen (Carlsbad, USA). The pRSET vector (Invitrogen) was described pre-

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viously (Studier and Moffatt, 1986). *E. coli* manipulations were performed according to the manufacturer's instructions. Standard DNA and protein manipulations were carried out as described in elsewhere (Sambrook and Russell, 2001; Ausubel *et al*., 2002). Viral DNA was extracted from the liver, spleen, kidney, ovaries, and skin lesions of Schlegel's black rockfish, *Sebastes schlegeli*, which were infected with LCDV using a tissue lysis buffer as described previously (Wang *et al*., 2003).

Construction of bacterial expression vector

The primers used for PCR were designed from the nucleotide sequence in the GenBank database (AY823414). The coding region of LCDV MCP was amplified by PCR using genomic DNA as a template with a 5' -primer (5' -CGCG-<u>AGATCT</u>-TA-ATGACTTCTGTAGCGGGTTC-3') containing a *Bgl*II restriction site (underlined) and a 3' -primer (5' -GCGC-<u>AAGCTT</u>-CTAAAGTACAGGAAATCC-CA-3') containing a *Hind*III restriction site (underlined). The PCR products were purified from a gel using a PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, digested with two restriction enzymes (*Bgl*II and *Hind*III, B + H), and then cloned into the B + H-digested pRSET, generating the pRSET-LCDV-capsid. The recombinant DNA was transformed into chemically competent *E. coli* JM109 cells which were used for the propagation of a plasmid construct. The transformants were selected on Luria-Bertani (LB) plates containing 100 μg/mL ampicillin. A mini-scale isolation of plasmid DNA was used for the preparation of recombinant plasmid on the plasmid DNA QIAprepTM Spin Mini Kit (Qiagen). The presence of an open reading frame (ORF) was confirmed by restriction analysis (B + H) and by DNA sequencing.

Small-scale expression and time course studies

Once the sequence of pRSET-LCDV-capsid was verified by sequencing, the construct was transformed into BL21(DE3)pLysS host cells (Invitrogen). Transformants were grown on LB plates with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol at 37°C for 24 h. A single colony from the culture was grown in 25 ml of a LB medium with ampicillin and chloramphenicol to an optical density of 0.5. A protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration of 1 mM) with further growth at 37°C for 3 h. Samples were examined by a SDS-PAGE gel, which was performed on a 10 % polyacrylamide gel under denaturing conditions as described in Sambrook and Russell (2001).

Purification of recombinant MCP (rMCP)

rMCP was purified under a denaturing condition. To purify rMCP, 25 ml of a bacterial culture was pelleted by centrifugation at 4,000× g/10 min, and washed twice in PBS (150 mM NaCl, 10 mM NaH$_2$PO$_4$, pH 8.0). The pellet was lysed by the addition of a 6 M Guanidine lysis buffer (ProbondTM, Invitrogen) and the supernatant was used for affinity binding according to the manufacturer's instructions. Briefly, the supernatant was incubated with 1/4 volume of Ni^{2+}-charged resin for 1 h at room temperature, followed by the washing 3 times with washing buffer 1 (pH 6.0) and an additional washing 3 times with washing buffer 2 (pH 5.3). After elution, 1 ml fractions were analyzed with SDS-PAGE.

Preparation of a polyclonal antibody against rMCP and confirmation of immuno-reactivity of a polyclonal antibody

For the preparation of a polyclonal antibody, five 8-week-old ICR mice (Charles River, Yokohama, Japan) were immunized subcutaneously with puri-

fied rMCP (50 μg per mouse in 50 $\mu\ell$ PBS with 50 $\mu\ell$ of Freund's complete adjuvant). This was followed by two more booster injections of incomplete Freund's adjuvant at intervals of 2 wks. Blood samples were collected from the mice 7 days after the final injection. The antisera were separated from clotted blood, inactivated at 56°C for 30 min, and stored at -80°C until further use. Cryosections of skin lesions from fish with the LCDV infection were prepared on glass slides for an indirect immunofluorescence assay (IFA). Each slide, with a skin lesion section, was incubated with mouse antisera (diluted 1:100) for 1 h at room temperature in a moisture chamber. The control was incubated with normal mouse sera. After washing 3 times in cold PBS, slides were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG, IgM, and IgA (diluted 1:500). The nucleus was stained with propidium iodide. The cells were considered positive for the iridovirus capsid antigen when they showed specific cytoplasmic fluorescence distinct from any background reactions in controls.

Results

Detection of viral DNA

A 1,380 bp PCR product, which corresponded to the predicted size of the LCDV MCP gene, was amplified from the skin lesion sample (Fig. 1). No specific band was observed from the other samples.

Construction of bacterial expression vector

The pRSET-LCDV-capsid plasmid was constructed by cloning a B + H PCR fragment of the LCDV MCP gene (1,380 bp) into the B + H site of the pRSET. A clone containing the insert was selected and the plasmid was digested with B + H followed by DNA sequencing (data not shown).

Small-scale expression and time course studies

A small-scale (25 mL) expression study was used to confirm the expression of the rMCP (recombinant major capsid protein). Cell fractions were analyzed for protein production by SDS-PAGE. In the bands containing samples of cultured cells, clear evidence of production of a 52 kDa protein was seen on the SDS-PAGE gel (Fig. 2A). After purification, elution fractions were analyzed for LCDV rMCP production by SDS-PAGE. There was clear
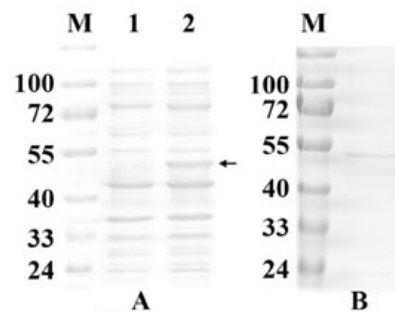
Fig. 2. **A**: The SDS-PAGE analysis of rMCP produced by *E. coli*. Lane 1, 10 $\mu\ell$ aliquot of culture lysate of BL21(DE3)pLysS(pREST-LCDV-cap) before induction; lane 2: after IPTG induction. M: molecular weight markers. After induction, the expression of the rMCP (arrowed, corresponds to 52 kDa) is markedly increased. **B**: The SDS-PAGE analysis of purified rMCP. Large amounts of purified rMCP without contamination by other proteins were recovered using a Ni^{2+}-charged resin affinity column following preparation of samples by a denaturing condition. M: molecular size marker.
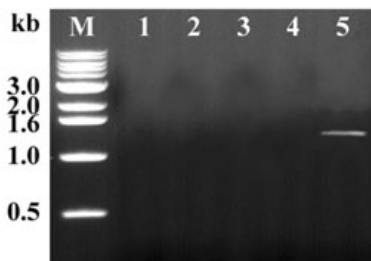

Fig. 1. The LCDV MCPgene was amplified by PCR from 5 different tissue samples: Lane 1, liver; lane 2, ovary; lane 3, spleen; lane 4, muscle; lane 5, skin. The PCR product (1.38 kb) was shown in the skin sample.
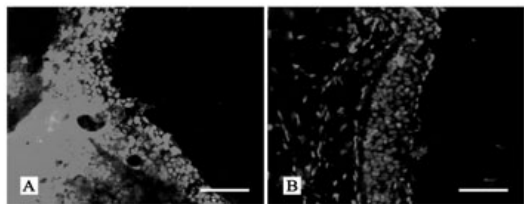
Fig. 3. The confirmation of the LCDV infection by IFA using a polyclonal antibody raised from mice. The confocal microscope of a mature lymphocystis cell with a mouse-polyclonal antiserum of iridovirus capsid proteins. **A**, The cells were stained with FITC-conjugated rabbit anti-mouse IgG, IgM, and IgA. Nucleus was stained with propidium iodide. Immunofluorescence was restricted to the cytoplasm; **B**, Negative control was stained with a normal mouse serum. Bar, A and B, 50 μm.

evidence of production of a 52 kDa protein on the SDS-PAGE gel (Fig. 2B) without contamination with other proteins.

Preparation of a polyclonal antibody against rMCP and confirmation of immuno-reactivity of a polyclonal antibody

Mouse antiserum, raised against the LCDV rMCP, reacted with viral particles in the indirect IFA (Fig. 3). Specific cytoplasmic fluorescence signals were detected at the peripheral zone of the hypertrophied cells cytoplasm indicating that the polyclonal antibody was reacting with viral particles at this site.

Discussion

The lymphocystis rarely causes death, but infected fish appear unsightly and cannot be sold, thus resulting in an important economic loss in aquaculture systems (Hu *et al*., 2004). Usually, LCDV infection results in the development of characteristic papilloma-like lesions on the skin of the infected fish due to the giant cell formation from putative dermal fibroblasts of the skin tissue. However, *in vitro*, the infec-

tion causes permissive cells (e.g., the flounder gill cell line) to exhibit an obvious cytopathic effect (CPE), including rounding of cells, cell detachment and cell death (Hu *et al*., 2004). Infections mainly occur on the skin and fin but can also be found in the eyes, kidney, spleen, liver, heart, ovaries, and mesenteries. Infections of the viscera are rare (Marcogliese *et al*., 2001). However, in this study, we failed to detect any positive signs in the detection of viral DNA by PCR in parenchymal tissues including liver, spleen, ovaries, and heart, but the skin lesion sample produced a strong band. We thought this to be because the propagation of LCDV occurred mainly in the skin region in rockfish.

LCDV infection seemed to induce a non-specific immune response (Marcogliese *et al*., 2001). Marcogliese *et al*. (12) found that the head kidney cells of American plaice, *Hippoglossoides platessoides*, showed an enhanced phagocytic activity, though no proliferation of those cells was found. Studies also suggested that there was some degree of immunity to the disease in the natural fish populations, as seroprevalence and antibody titers increased with age in flounder with no gross signs of the LCDV infection (Lorenzen and Dixon, 1991).

The immune response of fish to LCDV has received limited research attention. Though little information has existed on nonspecific cellular responses to infection with LCDV, flounder, *Platichthys flesus* L., produce antibodies to the virus with high titers noted in older fish not suffering from this pathology. This observation suggested that fish could recover and develop acquired immunity (Lorenzen and Dixon, 1991). Interestingly, antibodies have been detected in the uninfected fish and not all infected fish produced antibodies to the virus (Dixon *et al*., 1996). The presence of LCDV-specific antibodies could be explained as previous and/or present infections. So the viral particle itself

should be detected to diagnose and confirm the present infection in fish (for example, viral isolation, electron microscopic detection, and IFA). In this study, using mouse polyclonal antisera raised against the rMCP of the LCDV, viral particles were detected in the cytoplasma of cells infected with LCDV. The MCP was found to be a good target for the diagnosis of the LCDV infection (Cano *et al.*, 2007).

The LCDV particles were detected also using monoclonal antibodies raised against some viral antigens (Cheng *et al.*, 2006). They were located on the outer surface of virions and were thought not to be a MCP (52 kDa, while they are 116 kDa and 90 kDa). When these antigens shared some similarities with antigens from other viral species, false positive results might have occurred. It was found that the LCDV in Eastern Asia (China) was a separate species, which was different from the European LCDV (Zhang *et al.*, 2004). Also among host species, sequence similarities of some genes were low (Kim and Lee, 2007), indicating that many variants existed. So more specific and discriminative diagnostic tools were required, which was the main aim of this study.

Acknowledgements

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K.: Short protocols in molecular biology. Wiley, New York, 2002.

Cano, I., Ferro, P., Alonso, M.C., Bergmann, S.M., Römer-Oberdörfer, 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, 2007.

Cheng, S., Zhan, W., Xing, J. and Sheng, X.: Development and characterization of monoclonal antibody to the lymphocystis disease virus of Japanese flounder *Paralichthys olivaceus* isolated from China. J. Virol. Methods, 135: 173-180, 2006.

Dixon, P., Vethaak, D., Bucke, D. and Nicholson, M.: Preliminary study of the detection of antibodies to lymphocystis disease virus in flounder, *Platichthys flesus*L., exposed to contaminated harbour sludge. Fish Shellfish Immunol., 6: 123-133, 1996.

Hu, G.B., Cong, R.S., Fan. T.J. and Mei. X.G..: Induction of apoptosis in a flounder gill cell line by lymphocystis disease virus infection. J. Fish Dis., 27: 657-662, 2004.

Kim, T.J. and Lee, J.I.: Sequence variation in the genes encoding the major capsid protein, the ATPase, and the RNA polymerase 2 (domain 6) of lymphocystis disease virus isolated from Schlegel's black rockfish, *Sebastes schlegeli*. J. Fish Dis., 30: 501-504, 2007.

Lorenzen, K. and Dixon, P.F.: Prevalence of antibodies to lymphocystis virus in estuarine flounder *Platichthys flesus*. Dis. Aquat. Org., 11: 99-103, 1991.

Marcogliese, D.J., Fournier, M., Lacroix, A. and Cyr, D.G.: Non-specific immune response

associated with infections of lymphocystis disease virus in American plaice, *Hippoglossoides platessoides* (Fabricius). J. Fish Dis., 24: 121-124, 2001.

Plumb, JA.: Viral disease of marine fish. In: Athobiology of Marine Fish and Estuarine Organisms. Couch, J.A. and Fourine, J.W. eds. CRC Press, Boca Raton, pp. 25-52, 1993.

Sambrook, J. and Russell, D.W.: Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.

Studier, F.W and Moffatt, B.A.: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol.,189: 113-130, 1986.

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

Wang, C.S., Shih, H.H., Ku, C.C. and Chen, S.N.: Studies on epizootic iridovirus infection among red sea bream, *Pagrus major* (Temminck & Schlegel), cultured in Taiwan. J. Fish Dis.,26: 127-133, 2003.

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.