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# Histopathological Observation of Lymphocystis Disease and Lymphocystis Disease Virus (LCDV) Detection in Cultured Diseased *Sebastes schlegeli*

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**Abstract** Lymphocystis nodules occurring in the cultured sting fish *Sebastes schlegeli* were observed under light and electron microscope. Lymphocystis disease virus (LCDV) in the tissues of diseased fish was detected with indirect immunofluorescence test (IFAT). Results showed that lymphocystis cells had overly irregular nuclei, basophilic intracytoplasmic inclusion bodies with virions budding from the surface, and hyaline capsules outside the cell membrane. Numerous virus particles about 200 nm in diameter scattered in the cytoplasm, electron-dense particles 70-80 nm in diameter filled in perinuclear cisterna, and membrane-enveloped particles with electron-dense core of 70-80 nm appeared around cellular nucleus. IFAT using monoclonal antibody against LCDV from *Paralichthys olivaceus* revealed that specific green fluorescence was present in the cytoplasm of lymphocystis cells, epithelium of stomach, gill lamellae, and muscular fibers under epidermis of *S. schlegeli*, just as that in the cytoplasm of lymphocystis cells of *P. olivaceus*, suggesting the presence of LCDV in these tissues.

**Key words** sting fish *Sebastes schlegeli*; lymphocystis disease; histopathology; ultrastructure; indirect immunofluorescence test

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## 1 Introduction

Lymphocystis disease virus (LCDV), a member of family Iridoviridae, is the causative agent of lymphocystis disease (LCD) (Anders, 1989; Wolf, 1988; Sano *et al.*, 1994). It can infect over 125 fish species belonging to 42 families, causing the development of whitish, reddish or grayish nodules of hypertrophic fibroblastic cells in the dermis and occasionally in visceral organs (Colorni and Diamant, 1995). Outbreaks of LCD have been reported worldwide (Tidona and Darai, 1997; Garcia-Rosado *et al.*, 2002); however, little is known about the process of virus spread. In recent years, nearly 10 fish species have been infected by LCDV in China, of which the cultured *Paralichthys olivaceus* is reported more frequently (Sun *et al.*, 2000; Zhang *et al.*, 2004). Several strains of monoclonal antibodies (Mabs) against LCDV from *P. olivaceus* have been produced (Cheng *et al.*, 2006), and LCDV in *P. olivaceus* has been detected using indirect immunofluorescence test in our laboratory (Xu *et al.*, 2004).

In July 2003, LCD occurred in sting fish *Sebastes schlegeli* in a farm of Shandong Province, China. Clinically, the presence of grayish, whitish or reddish nodular lesion was noticed, mainly on the body skin and fins. To our knowledge, this is the first report of LCD in *S. schlegeli* in China. Tanaka (1984) described the histopathological structures of lymphocystis disease of *S. schlegeli* in Japan. This work was undertaken in order to provide better understanding about histopathological features of lymphocystis and to detect LCDV antigen in the tissues of *S. schlegeli*.

## 2 Materials and Methods

### 2.1 Materials

Diseased fish were obtained from a farm of Shandong Province, China. The body length of fish averaged about

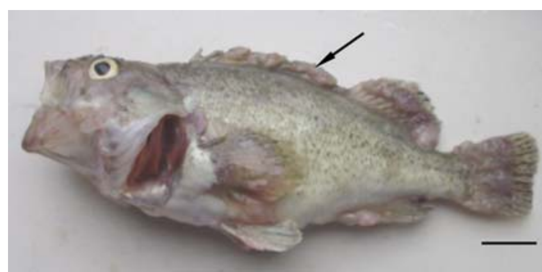

Fig.1 Lymphocystis disease virus infected sting fish *Sebastes schlegeli*, the nodules on fins and body surface being shown (arrow).

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15cm. Lymphocystis nodules in different sizes occurred mainly on the fins and the body skin of the fish (Fig.1).

### 2.2 Paraffin Sectioning

Lymphocystis nodules with the connected skin and the tissues (gill, stomach, pylorus, intestine, spleen, liver, kidney and head kidney, and heart) were fixed with Bouin's fixative. Paraffin sections (5μm in thickness) were prepared with a routine procedure and stained with hematoxylin and eosin (H-E) for histopathological study.

### 2.3 Ultrathin Sectioning

Lymphocystis nodules were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) ($0.1 \mathrm{mol L^{-1}}$, pH7.4) at 4℃ for 2h, post-fixed in 1% osmium tetroxide in PBS at 4℃ for 1h, dehydrated in an ascending ethanol series and finally embedded in Epon epoxy resin following standard procedures. Ultrathin sections were double-stained with uranyl acetate and lead citrate, observed and photographed with an H-7000 transmission electron microscope (TEM).

### 2.4 Immunodetection of LCDV

Mabs against LCDV from *P. olivaceus* produced in our laboratory were used for the detection of LCDV antigen in the tissues of *S. schlegeli*. Tissue samples (skin nodules, liver, spleen, head kidney, kidney, stomach, pylorus, intestine, heart, and gill) were taken from the diseased *S. schlegeli*, washed with PBS and embedded at −80℃. Cryosections (in 5μm thickness) were prepared on a Leica microtom, fixed with acetone for 10min, and then stained with IFAT. The lymphocystis nodular sections from *P. olivaceus* were used as the positive control, while the tissue sections from the healthy *S. schlegeli* were used as the negative control.

IFAT was performed as follows: the sections were incubated either with PBS (negative control) or with Mabs against LCDV from *P. olivaceus* in a humid chamber set at room temperature for 45min. After three washes in PBS (5min each), sections were labeled with goat anti-mouse IgG-FITC (fluorescence-isothiocyanate) (Sigma) in a humid chamber set at 37℃ for 45min, then washed three times in PBS and covered with the glycerin, observed and photographed with an Olympus fluorescence microscope.

## 3 Results

### 3.1 Microstructures and Ultrastructures of Lymphocystis

Lymphocystis cells scattered in the connective tissues beneath the epidermis and between the scales of fish (Fig.2a). They contained irregular nuclei. The inner areas near the nuclear membrane of these cells were heavily stained with hematoxylin because chromatin was marginated. Abundant basophilic inclusion bodies in massive or cordlike shape located at the cell periphery. The cytoplasm was stained unevenly in color by hematoxylin and eosin, yielding a granular texture. A thick hyaline capsule appeared outside the cell membrane (Fig.2b). The senile lymphocystis cells became irregular. Their nuclei disappeared and their cytoplasms were released partly or entirely (Fig.2c). No lymphocystis cells were observed in other tissues of *S. schlegeli*.

Under TEM, the nuclear membranes of lymphocystis cells were overly folded and invaginated, and showed a multi-lobular appearance (Fig.2d). High electron-dense particles of 70–80nm in diameter were observed in the perinuclear cisterna, which may be released into the cytoplasm. Membrane-enveloped particles with high electron-dense concentric core of 70–80nm and hexagonal or irregular profiles scattered in the cytoplasm around the nucleus (Fig.2e). The typical virions had hexagonal profiles about 200nm in diameter with a concentric core of 135–140nm, which budded from the surface of the inclusion bodies (Fig.2f). Virus particles were usually in crystalloid arrays and scattered throughout the cytoplasm, but not in the nucleus (Fig.2g). The cytoplasmic organelles were crowded somewhere within the cytoplasm, and mitochondria became swollen or empty because the cisterna disappeared partly or entirely (Fig.2h). The hyaline capsule enclosing lymphocystis cell was homogeneous with low electron density.

### 3.2 IFAT

IFAT showed that the specific green fluorescence was present in the cytoplasm but not in the nuclei of lymphocystis cells of *S. schlegeli* and *P. olivaceus*, suggesting that the Mabs against LCDV of *P. olivaceus* could react with epitopes of LCDV from *S. schlegeli* (Fig.3a, 3b). No green fluorescence was observed in the negative controls, which were treated with PBS instead of primary antibody.

For the detection of LCDV antigen of *S. schlegeli*, various tissues were stained with the Mabs. It was found that the specific fluorescence was present in the epithelium of stomach (Fig.3c), gill lamellae (Fig.3e), and muscular fibers under epidermis (Fig.3g), revealing the presence of LCDV antigen in these tissues. No fluorescence was observed in other tissues and in the negative control (Fig.3d, 3f and 3h, respectively).

## 4 Discussion and Conclusions

Lymphocystis disease of *S. schlegeli* reported in Japan (Tanaka *et al.*, 1984) showed that the lymphocystic cells have the common characteristics of fish lymphocystis, which include cellular hypertrophy, cell enclosure by a distinctive hyaline capsule, enlarged nucleus and granular-appearing cytoplasm with prominent inclusions. The results of the present study are consistent with these descriptions. In addition, high electron-dense particles 70-80nm in diameter were observed within the perinuclear cisterna, and membrane-enveloped particles with high electron-dense concentric core of 70-80nm were found to scatter in the cytoplasm around the nucleus.

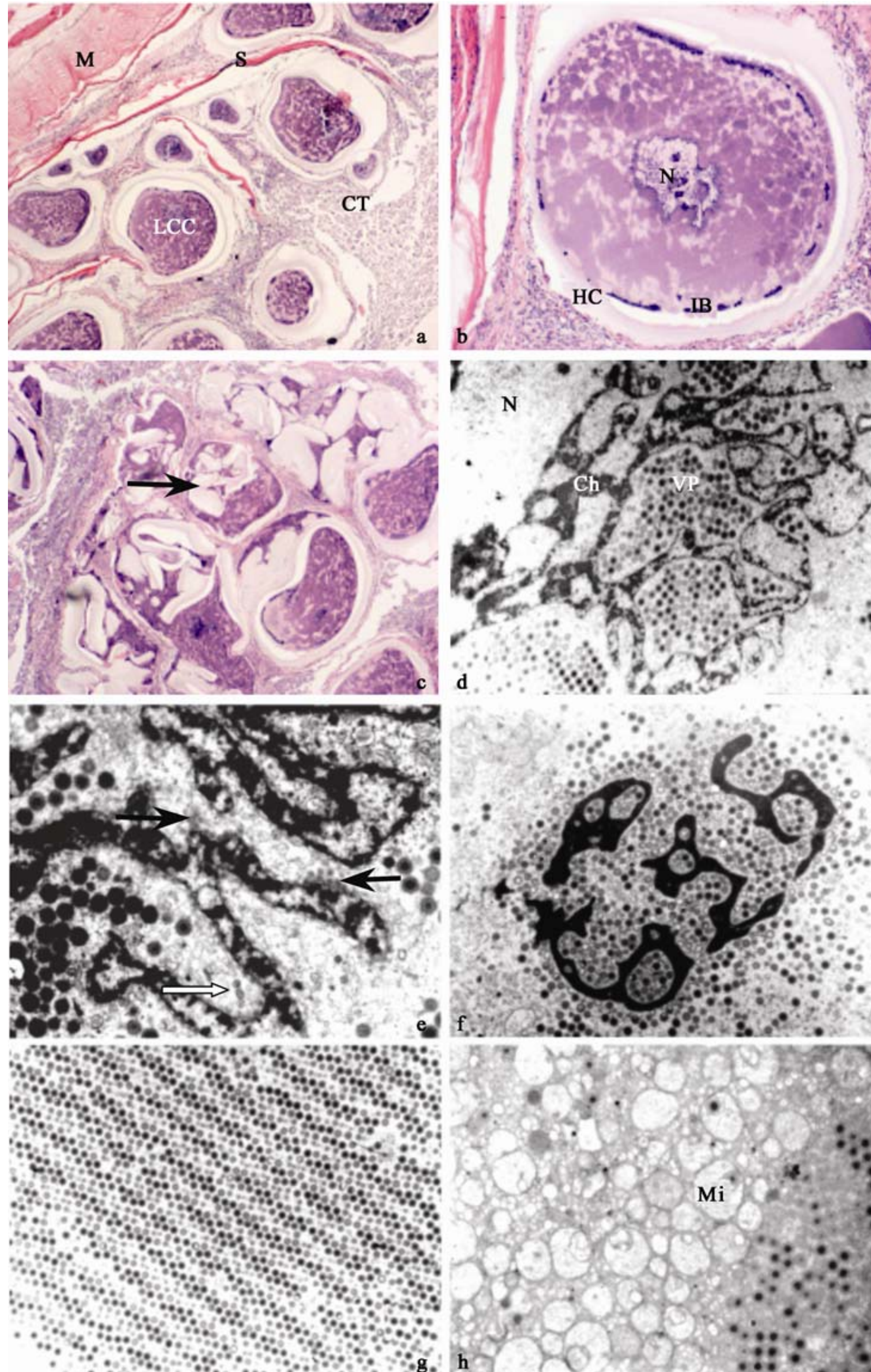


Fig.2 Histopathological features of lymphocystis of sting fish *Sebastes schlegeli*.

(a) Micrograph of lymphocystis nodules, showing lymphocystis cells (LCC) in connective tissue (CT) and between the scales (S). M: muscle.

(b) Magnification of LCC, showing irregular nucleus, marginated chromatin, unevenly stained cytoplasm, inclusion body (IB) at the cell periphery, and hyaline capsule (HC) outside the cell membrane.

(c) Collapse of the senile lymphocystis cells and the released cytoplasm (arrow).

(d) TEM photograph of LCC, showing extremely folded nuclear membrane (NM) (arrow), hexagonal virus particles (VP), and the marginated chromatin (Ch) connected with nuclear membrane.

(e) Magnification of nuclear membrane, showing electron-dense particles (arrow) in perinuclear cisterna and membrane-enveloped particles (empty arrow) in the cytoplasm around the nucleus.

(f) Micrograph of TEM, showing the electron-dense inclusion body and virus particles around it.

(g) Virus particles in crystalloid array and scattering throughout the cytoplasm.

(h) Mitochondria. It becomes swollen or empty because the cisternae partly or entirely disappear.

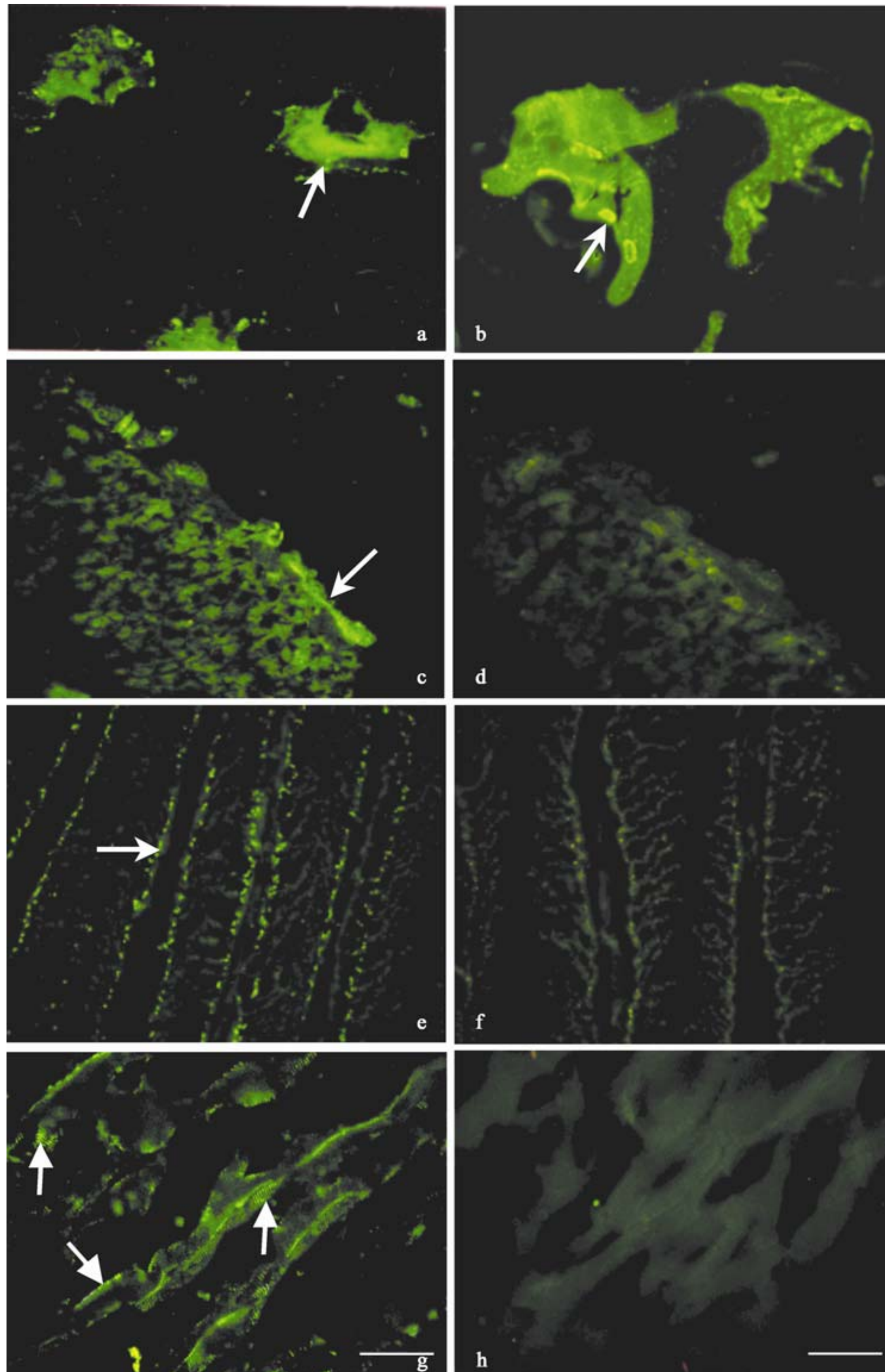


Fig.3 Detection of LCDV antigen in the tissues of sting fish *Sebastes schlegeli*. Viral antigens were detected in the cytoplasm of lymphocystis cells (a), epithelium of stomach (c), gill lamellae (e), and muscular fibers under epidermis (g). (b): Positive control (lymphocystis cells of *Paralichthys olivaceus*). (d), (f), (h): Negative controls of (c), (e), (g), respectively.

Goorha *et al*. (1989) reported that DNA replication in Frog Virus 3 (FV3) occurred in the nucleus as well as in the cytoplasm, about 30% of the viral DNA was synthesized in the nucleus and the majority of these viral DNA (about 90%) was transported into the cytoplasm. In *S. schlegeli*, the high electron-dense particles in the perinuclear cisterna may be the viral DNA synthesized in the nucleus, which then were released into the cytoplasm through the nuclear membrane, forming the membrane-enveloped particles. The same characteristics have been observed in *P. olivaceus* (Sheng and Zhan, 2004). Therefore, DNA of LCDV is replicated in both nucleus and cytoplasm. However, the mature LCDV particles are about 200 nm in diameter, and the viral maturation takes

place in the sites of inclusion bodies. More work is needed in order to clarify the relationships between the membrane-enveloped particles and the mature LCDV.

Lymphocystis nodules mainly develop on the fin and body surface of fish, but occasionally in visceral organs (Colorni and Diamant, 1995). In *P. olivaceus*, the typical hypertrophic lymphocystis cells are observed in the gill, submucosa of intestine, spleen, peripheral portion and the surface of head kidney, as well as the adjacent mesenteries of liver and intestine (Sheng and Zhan, 2006). However, in this study, the typical lymphocystis cells are found only in the connective tissues beneath the epidermis in *S. schlegeli*, not in other tissues. The possible reason is that the fish in the present study are young and the course of the disease is short.

Immunoflurescence test has been extensively used for the detection of viral antigen in cell cultures and fish tissues; the method is rapid and sensitive, and the observation of cytopathic effects is not required (LaPatra *et al*., 1989). Garcia-Rosado *et al*. (2002) detected the LCDV antigen in the SAF-1 cells and fish leukocytes by IFAT using polyclonal antiserum. LCDV antigen in the tissues of *P. olivaceus* by IFAT of Mabs against LCDV from *P. olivaceus* was detected in our laboratory (Xu *et al*., 2004), and we found that positive green fluorescence was present in the cytoplasm of lymphocystis cells, epithelium of stomach and intestine, gill, and muscular fibers under epidermis. In *S. schlegeli*, LCDV antigen was detected in the cytoplasm of lymphocystis cells, epithelium of stomach, gill lamellae, and muscular fibers under dermis. These results suggest that LCDV can penetrate into the fish through the gill, digestive tract (*e.g.* stomach) and epidermis of body skin. The connective tissue cells beneath the epidermis is the main target of LCDV infection; however, how the virus particles spread among these tissues is not clear yet. Further efforts are needed to make the mechanism underlining such spreading clear.

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