Bull. Eur. Ass. Fish Pathol., 19(2),53, 1999.

ISOLATION AND CHARACTERIZATION OF LYMPHOCYSTIS VIRUS (FLDV) FROM GILT-HEAD SEA BREAM (*SPARUS AURATA*, L.) USING A NEW HOMOLOGOUS CELL LINE

E. GARCIA-ROSADO[1], D. CASTRO[1], S. RODRIGUEZ[2], S.I. PEREZ-PRIETO[2] & J.J BORREGO[1]*
[1]Departamento de Microbiología, Facultad de Ciencias, Universidad de Malaga, 29071-Malaga, Spain [2]Centro de Investigaciones Biologicas, C.S.I.C., Velazquez, 144, 28006-Madrid, Spain
*email <jjborrego@uma.es>

Abstract

Five strains of lymphocystis virus (FLDV) isolated from gilt-head sea bream cultured in several fish farms of Andalusia (South of Spain) were studied. The culture characteristics of all viruses were compared using a new homologous cell line from *Sparus aurata*. In addition, the serological properties of the viral isolates were also determined comparatively by means of the indirect fluorescent antibody test (IFAT).

Introduction

Gilt-head sea bream (*Sparus aurata*, L.) is the most important cultured fish species in Mediterranean aquaculture. Several pathologies of infectious origin have been reported in different stages of development and culture of this species, the most relevant being the bacterial pathologies, which frequently provoke epizootic outbreaks (Balebona 1994, Sedano *et al.*, 1996). Unlike in other cultured fish species, only a few viral diseases have been described in S. aurata, including iridoviruses (Paperna *et al.*, 1982), reoviruses (Bandin *et al.*, 1995), nodaviruses (Comps & Raymond, 1996), and recently birnaviruses (Rodger *et al.*, 1997; Rodriguez *et al.*, 1997).

Lymphocystis virus (FLDV) presents a broad host-range, including freshwater and marine fish, although the most susceptible fish species are salmon and gilt-head sea bream, respectively. The disease is characterised 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 (Walter & Hill 1980). Although infected fish appear unsightly, the disease is rarely fatal, but provokes an important economic loss in aquaculture systems.

Although FLDV from several fish hosts have been cultured in vitro and in vivo, only a few studies on the isolation and characterisation of FLDV from sea bream have been conducted. In addition, methods to determine the in vitro production of infectious viruses and the viral yield from cell cultures have been poorly studied.

In this paper we have studied five different FLDV strains isolated from gilt-head sea bream cultured in several fish farms at Andalusia (South of Spain). The culture characteristics of the viruses using a new fish cell line derived from sea bream and the serological properties of all isolates were also

Table 1 Lymphocystis virus strains isolated from gilt-head sea bream and cultured on SAF-1 cell lin

Strains	Origin and date	Sensitivity to chloroform	Cytopathic effects (days post-inoculation)	$TCID_{50}$/ml	Abs against LDV2	
					1/1000	1/2500
LDV1	Granada, 1996	-	5	$>1 \times 10^8$	+	+
LDV2	Huelva, 1996	-	5-10	5×10^4	+	+
LDV3	Huelva, 1997	-	5	4×10^6	+	+
LDV4	Huelva, 1997	-	3	1×10^4	+	+
LDV5	Huelva, 1998	-	2-7	1×10^9	+	+

compared.

Materials and methods

Five isolates of FLDV were isolated from diseased gilt-head sea bream cultured in three fish farms located at the provinces of Granada and Huelva (Southern and South-western Spain) in a virological survey from 1996 to 1998. Samples of FLDV were collected from skin and fin nodules of the affected fish, and then they were homogenised in L-15 medium (Gifco) supplemented with 2% foetal bovine serum (FBS) to achieve a 1:10 dilution of crude extract. The suspension was sonicated at 40 W for 1.5 minutes, and centrifuged twice at 15,000 g for 20 minutes. The supernatants obtained were used to inoculate the fish cell line SAF-1 (Bejar *et al.*, 1997). To avoid the cytotoxic effects of raw supernatants, dilutions 1:5 to 1:50 in L-15 medium were carried out for each viral isolate. Each isolate of FLDV was also compared for its chloroform sensitivity following the method described by Oh *et al.*, (1995), and for its yield of virus cultures in vitro using SAF-1 incubated at 20ñ1øC. Cells were cultured and maintained using Dulbeccos Modified Eagles Medium/ nutrient mixture Hamïs F12 (DME/F12, Sigma), adjusted to pH 7.2 and supplemented with 1% antibiotic/antimycotic solution (10,000 IU penicillin G /ml, 10mg streptomycin/ml, 25 mg amphotericin B/ml) (Sigma), 2% L-glutamine (Sigma) and 15% FBS (Sigma). For virus titre assays, the cells were seeded into 24- or 96-well cell culture plates. The dilution of the virus infecting 50% of the cell cultures was considered the end point dilution ($TCID_{50}$/ml).

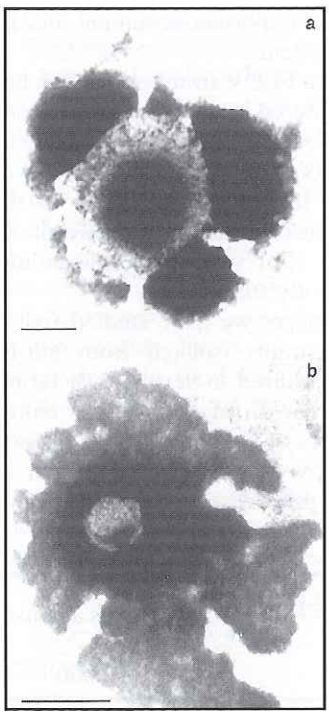

Figure 1. a) Negative staining of FLDV directly isolated from infected gilt-head sea bream
b) Negative staining of FLDV isolated from inoculated SAF-1 cell culture. Scale bar = 100nm

Following development of cytopathic effects (CPE) cell monolayers were scraped off and resuspended in the original medium. The cells were frozen and thawed three times and sonicated for 10 minutes in an ice bath. Then, they were centrifuged twice at 1,500g for 10 minutes. The final supernatant was negatively stained with 1% uranyl acetate and examined under transmission electron microscopy (TEM). Samples taken directly from skin nodules of fish were homogenised with L-15 medium and processed by TEM as above mentioned.

Flask supernatants harvested from cell cultures were purified following the technique described by Walker & Hill (1980), and the purified FLDV strain 2 was used to raise antibodies against lymphocystis viruses by subcutaneous inoculation to rabbits (New Zealand strain). The antigenic relationship among all the FLDV tested was performed by an indirect fluorescent antibody test (IFAT) following the protocol described by Hedrick *et al.* (1992).

Results and Discussion

Virus isolation from affected fish showing whitish nodules of tumour-like appearance on their skin or fins at three different places in the South of Spain is shown in Table 1. All the FLDV strains tested showed similar morphological characteristics under TEM (Fig. 1) and similar chloroform sensitivity

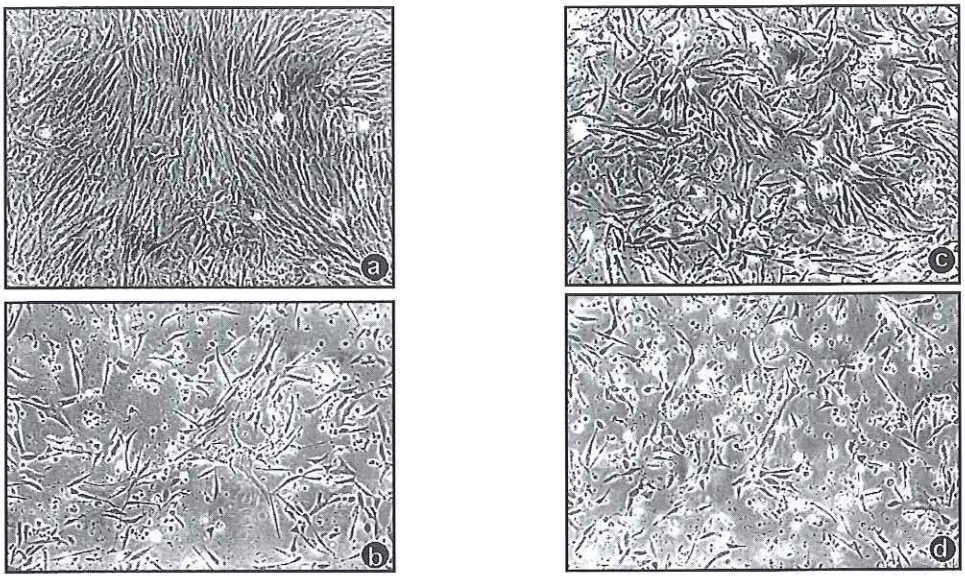

Figure 2. Cytopathic effects observed in SAF-1 cells inoculated with lymphocystis virus strain LDV5 at **a)** control, non-inoculated, **b)** post inoculation, and at **c)** 2 days and **d)** 7 days post inoculation

(Table 1). Negatively stained virions isolated directly from affected fish showed an icosahedral shape with a diameter between 200 and 250 nm. However, in the infected SAF-1 cells a smaller virus particle size (about 100 nm) with a rounded shape was observed. Similar results have been reported by Oh *et al.* (1995) studying other enveloped fish virus. Berthiaume *et al.* (1984) pointed out that the L-15 medium where the viral particles are suspended is responsible for the increase of viral size. However, more recently Peters & Schmidt (1995) established that a disintegration of virions occurred in lymphocystis infected cells, as well as anomalies in the virion assembly and budding process.

Marked cytopathic effects (CPE) were observed on the SAF-1 monolayers after 2-10 days of incubation at 20±1°C at a dilution 1/5 (Table 1), which is considerably lower than for BF-2 cell cultures (18 to 21 days) at the same temperature. The CPE consisting in rounding and enlargement of infected cells and formation of cytoplasmic inclusions (Fig. 2), and the isolated virus showed different infectivity degree ranging from 1×10^4 $TCID_{50}$/ml to $>1 \times 10^9$ $TCID_{50}$/ml (Table 1). Chloroform treatment of the viral isolates did not reduce their infectivity to SAF-1 cells.

SAF-1 cells obtained from gilt-head sea bream (Bejar *et al.* 1997) were successfully used to study the in vitro production of infectious lymphocystis virus. Previously, several authors had used different fish cell lines, including BF-2, MCT and BF-W, to quantify the viral yield from cell cultures, but lymphocystis infectious virus was only obtained from the latter (Walker & Hill 1980). The maximum yields of FLDV obtained in SAF-1 cell cultures were higher than those reported previously by other authors using non-homologous cell lines (Wolf *et al.* 1966; Midlige & Malsberger 1968). However, the strains LDV2, LDV3 and LDV4 showed similar titres to those obtained by Wharton *et al.* (1974) and Walker & Hill (1980). The different yield production obtained in this study for the three strains above mentioned may be explained by the fact that these strains remained cell-associated, and thus the viral release from infected cells and the cell to cell spread of virus cultures did not occurred. However, Walker & Hill (1980) sug-

gested that other mechanisms may be involved, such as inhibition of virus replication by interferon or the reduction of infectious viral yield by the presence of defective interfering particles.

Using the indirect fluorescent antibody test (IFAT), all the strains of FLDV tested showed similar serological characteristics, since all of them presented cross-reaction against the strain LDV2 antiserum at titres 1:1,000 and 1:2,500 (Table 1). Little is known about immunological aspects of the FLD in fish populations. Lorenzen & Dixon (1991) demonstrated the existence of seroprevalence and antibody levels against one strain of FLDV in seropositive fish of two populations of *Platichthys flesus* with no gross signs of disease in two separate geographical zones. Similarly, Hedrick *et al.* (1992) detected the presence of common antigens in three different iridovirus, which led the authors to suggest the existence of conserved group-specific antigens.

Acknowledgement

This work was supported by a Grant from C.I.C.Y.T (Ref. MAR95-1949-CO2-O1-O2) (Spanish Government) and from JACUMAR (Consejería de Agricultura y Pesca, Junta de Andalucia).

References

Balebona, M.C. (1994) *Caracterizacion y mecanismos de virulencia de cepas del genero Vibrio aisladas de patologias de doradas* (*Sparus aurata*, L.) *cultivadas*. PhD Thesis, University of Malaga.

Bandin, I., Rivas, C., Noya, M., Curtin, J.M., Barja, J.L. & Dopazo, C.P. (1995) Isolation of a new aquareovirus from gilthead sea bream cultured in Galicia (NW Spain). *Bull. Eur. Ass. Fish Pathol.*, **15**, 157-159.

Bejar, J., Borrego, J.J. & Alvarez, M.C. (1997) A continous cell line from the cultured marine fish gilthead seabream (*Sparus aurata*, L.). *Aquaculture*, **150**, 143-153.

Bethiaume, L., Alain, R. & Robin, J. (1984) Morphology and ultrastructure of lymphocystis disease virus, a fish iridovirus, grown in tissue culture. *Virology*, **135**, 10-19.

Comps, M. & Raymond, J.C. (1996) Virus-like particles in the retine of the sea bream, *Sparus auratus. Bull. Eur. Ass. Fish Pathol.*, **16**, 161-163.

Hedrick, R.P., McDowell, T.S., Ahne, W., Torhy, C. & de Kinkelin, P. (1992) Properties of three iridovirus-like agents associated with systemic infections of fish. *Dis. Aquat. Org.*, **13**, 203-209.

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

Midlige, F.H. & Malsberger, R.G. (1968) 'In vitro' morphology and maturation of lymphocystis virus. *J. Virol.*, **2**, 830-835.

Oh, M.J., Yoshimizu, M., Kimura, T. & Ezura, Y. (1995) A new virus isolated from salmonid fish. *Fish Pathol.*, **30**, 23-32.

Paperna, I., Sabnai, I. & Colorni, A. (1982) An outbreak of lymphocystis in *Sparus aurata* L. in the Gulf of Aqaba, Red Sea. *J. Fish Dis.*, **5**, 433-437.

Peters, N. & Schmidt, W. (1995) Formation adn disintegration of virions in lymphocystis cells of plaice Pleuronectes platessa. *Dis. Aquat. Org.*, **21**, 109-113.

Rodger, H.D., Muir, F. & Millar, S. (1997) Isolation of an aquatic birnavirus from sea bream *(Sparus auratus). Bull. Eur. Ass. Fish Pathol.*, **17**, 134-136.

Rodr¡guez, S., Castro, D., Perez-Prieto, S., Garcia-Rosado, E., Vilas, M.P., Gutierrez, M.C., Sarasquete, C. & Borrego, J.J. (1997) Patogenicidad de un aquabirnavirus en *Sparus aurata*. VI Congreso Nacional de Acuicultura, Cartagena (Spain), 1-7.

Sedano, J., Zorrilla, I., Mori¤igo, M.A., Balebona, M.C., Vidaurreta, A., Bordas, M.A. & Borrego, J.J. (1996) Microbial origin of the abdominal swelling affecting farmed larvae of gilt head seabream (*Sparus aurata*, L.). *Aquaculture Res.*, **27**, 323-333.

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

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