Comparison of Lymphocystis Disease Virus (LCDV) isolates obtained from different marine fish species and geographical areas

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Abstract

Seven lymphocystis disease viruses isolated from dab, flounder and plaice in Northern Europe, and from gilt-head sea bream, Senegalese sole and blackspot sea bream in Southern Europe have been comparatively studied by inoculation on different fish cell lines. Viral isolates induced cytopathic effects (CPE) only on fibroblastic cells, and differences in the incubation time necessary to develop CPE, as well as in viral titre were observed in relation to the geographical area of isolation rather than to the host fish species. In addition, the pattern of the major viral proteins from different fish species in the same area was similar; however, North European isolates showed three major proteins which were absent in the South European viruses. Immunohistochemistry techniques were used to detect LCDV in infected cells, the antibody used (developed against a gilt-head sea bream LCDV) did not react with any of the North European isolates, whereas those cells infected with the South European isolates showed an intensive labelling. The results suggest that these viral isolates constitute two homogeneous groups referring to the geographical area of isolation, rather than to the fish species affected.

Introduction

Lymphocystis disease virus (LCDV) is an iridovirus with a worldwide distribution, which has been isolated from many different fish species in marine, brackish and fresh waters (Anders, 1989). This virus is the causative agent of the lymphocystis disease, a chronic disease whose main symptom is the appearance of macroscopic nodules on fins and skin, which are formed by abnormally enlarged fibroblastic cells (Wolf, 1988).

The *Iridoviridae* family comprises four genera which may affect vertebrate and invertebrate animals causing diseases ranging from lethal to asymptomatic infections (Williams, 1996). Although a great variability among viruses in this family is expected, only some studies focused on genetic analyses have been reported (Hedrick et al., 1992; Hyatt et al., 2000; Goldberg et al., 2003). Several authors have described differences in genome structure, gene organization and DNA sequences of LCDV isolates depending on the host fish

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or the geographical location (Darai et al., 1983; Müller et al., 1995). Based on these results, LCDV has been classified in two species: LCDV-1, which occurs in flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*), and LCDV-2, usually found in dab lesions (*Limanda limanda*) (Darai, 1986). However, Kitamura et al. (2006) have proposed the existence of three genotypes in the genus *Lymphocystivirus* according to the nucleotide sequence of the gene coding the major capsid protein (MCP), and other LCDV isolate, LCDV-C (isolated from Japanese flounder in China), has been proposed as a new species (Zhang et al., 2004).

Gilt-head sea bream (*Sparus aurata*) is the main host for this virus in Southern Europe, since it is one of the main cultured fish species in this area, and stress factors derived from this activity may enhance viral transmission and provoke the appearance of the clinical signs (Paperna et al., 1982; Mellergaard & Nielsen, 1995). Recently, Alonso et al. (2005) have reported the isolation of LCDV from Senegalese sole (*Solea senegalensis*) and blackspot sea bream (*Pagellus bogaraveo*). On the contrary, in Northern Europe this virus is widely spread in wild fish, and it is mainly associated with flounder (*Platichthys flesus*), plaice (*Pleuronectes platessa*), and dab (*Limanda limanda*) (Russell, 1974; Reiersen & Fugelli, 1984; Lorenzen et al., 1991).

In this study a comparison among seven LCDV isolates obtained from different geographical locations and fish species has been performed by the analysis of the viral replication on several fish cell lines, the structural protein patterns, and the reaction with a specific polyclonal antibody.

Materials and methods

Viruses and cell cultures

Four LCDV isolates obtained in Southern Europe from cultured gilt-head sea bream (LCDV no. 3 and no. 9), Senegalese sole (LCDV no. 11), and wild blackspot sea bream (LCDV no. 7), as well as three isolates from Northern Europe collected from wild dab (LCDV no. 444), flounder (LCDV no. 445), and plaice (LCDV no. 446) were compared.

The TV-1, BF-2, SAF-1, CHSE-214, and EPC cell lines were tested for susceptibility to the LCDV isolates. Cells were cultured at 25°C (20°C for the TV-1 cell line) using Leibovitz (L-15) medium (Gibco) supplemented with 2% L-glutamine (Sigma), 1% penicillin-streptomycin (Gibco) and 10% foetal bovine serum until semi-confluence prior to inoculation. Inoculated cells were incubated at 20°C and daily examined for cytopathic effect (CPE). Titres, expressed as $TCID_{50}$ ml^{-1}, were considered as the viral dilution infecting 50% of the cell cultures (Cunningham, 1973).

Virus purification and analysis of structural viral proteins

Virions were purified on a sucrose gradient (20-60%) in TNE (0.1 M NaCl; 1 mM EDTA; 0.01 M Tris) as described by Cano et al. (2007) prior to the electrophoresis analysis. The protein concentration was determined using bicinchoninic acid (Sigma) and bovine serum albumin (BSA) as standard. Viral proteins (10 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4-20% linear

LCDV isolates	Cytopathic effects (days p.i.)					Viral titre ($TCID_{50}$ ml^{-1})				
	SAF-1	BF-2	TV-1	EPC	CHSE-214	SAF-1	BF-2	TV-1	EPC	CHSE-214
South European										
no. 3	6	9	6	-[a]	-	5×10^7	5×10^5	5×10^8	-	-
no. 7	6	9	6	-	-	5×10^7	5×10^5	5×10^8	-	-
no. 9	6	9	6	-	-	5×10^7	5×10^5	5×10^8	-	-
no. 11	6	9	6	-	-	5×10^7	5×10^5	5×10^8	-	-
North European										
no. 444	8	6	6	-	-	5×10^7	5×10^8	5×10^8	-	-
no. 445	8	6	6	-	-	5×10^7	5×10^8	5×10^8	-	-
no. 446	8	6	6	-	-	5×10^7	5×10^8	5×10^8	-	-

[a] No CPE observed at 12 days p.i.

Table 1. Replication of LCDV isolates on different fish cell lines.

gradient gels, and the approximate molecular weights estimated by comparison with the molecular standard (Biorad).

Immunohistochemistry

The immunohistochemistry technique was performed on inoculated SAF-1 cells using a polyclonal antibody raised against a gilthead sea bream LCDV isolate (Garcia-Rosado et al., 2002). Cells were cultured on silane-treated slides (Sigma) until semi-confluence and inoculated with virus suspensions containing 10^3 $TCID_{50}$ ml^{-1}. At day 5 post infection (p.i.) cells were fixed by heating at 100°C for 1 min, and immediately placed in 10% buffered neutral formalin (Merck) added with 0.5% Triton X-100. The slides were maintained in this solution at room temperature overnight. The endogenous alkaline phosphatase activity was subsequently removed by boiling for 5 min in 10 mM citrate buffer (pH 6). Blocking was carried out with 5% (w/v) BSA for 30 min, and cells were incubated with the primary antibody. The secondary antibody was anti-rabbit IgG conjugated to alkaline phosphatase developed in goat (Sigma). Antibodies were diluted in TBS (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl) with 0.1% BSA and incubated for 2 h. The alkaline phosphatase activity was developed with NBT/BCIP (Roche) for 1 h, and cells were counter-stained with a 0.5% Bismarck Brown Y solution (Sigma). Non-inoculated SAF-1 cells were used as negative controls.

Results and discussion

LCDV isolates obtained from several fish species in two different areas were comparatively studied in terms of their replication on several fish cell lines, resulting in the appearance of CPE only on fibroblastic cells (SAF-1, BF-2 and TV-1) at times ranging from 6 to 9 days p.i. (Table 1). The pattern of the CPE developed was the same regardless of the viral isolate, and it was characterized by the early appearance of rounded and enlarged cells which finally detached. Virus inoculation yielded titres between 10^5 and 10^8 $TCID_{50}$ ml^{-1} (Table 1).

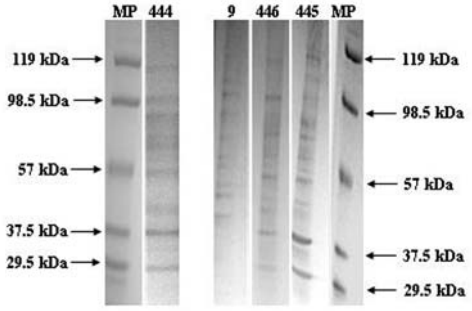

Figure 1. Structural protein profiles of LCDV isolates. LCDV nos. 444, 445, and 446 are North European isolates collected from dab, flounder, and plaice, respectively. LCDV no. 9 has been isolated from gilt-head sea bream in Southern Europe. MP: marker proteins.

The maximum titre was always obtained on the TV-1 cell line, which appears to be a highly permissive cell line for fish virus replication. The propagation of LCDV only on fibroblastic cell lines has previously been reported for South European isolates (Alonso et al., 2005), and seems to be a common characteristic for all LCDV isolates. This result is consistent with the fact that the main targets for virus replication in naturally affected fish are these kind of cells located in the skin, fins, mouth, and gills (Gonzalez de Canales et al., 1996).

Differences regarding both, the minimum time of incubation necessary for the CPE development and the viral titre obtained, were observed depending on the geographical origin, but not on the host fish within each geographical area. CPE was first observed in BF-2 and TV-1 cells for North European isolates, whereas the earliest development of CPE for isolates from Southern Europe was recorded on SAF-1 and TV-1 cells.

Lymphocystis replication on BF-2 cells has previously been reported showing CPE similar to those described in the present study (Walker & Hill, 1980; Faisal, 1989). In addition, Perez-Prieto et al. (1999) reported viral titres significantly lower on BF-2 than on SAF-1 cells for a LCDV isolated from gilt-head sea bream, which is in concordance with the results shown in this study. The replication of the South European LCDV isolates on SAF-1 cells yielded similar titres to those obtained by Garcia-Rosado et al. (1999) and Alonso et al. (2005).

The structural viral proteins were analyzed by SDS-PAGE electrophoresis. In this study, only one South European isolate (no. 9) has been included, since Alonso et al. (2005) reported identical protein profiles for gilt-head sea bream, sole and blackspot sea bream isolates collected in South Europe, and only differences in minor proteins were described by Garcia-Rosado et al. (2004). The comparison of the protein profiles revealed that the virions isolated from dab, flounder and plaice (LCDV nos. 444, 445, 446) showed a similar profile of major proteins, which was different from that obtained from the South European isolate (no. 9). Three proteins of approximately 57, 37 and 29 kDa were only present in the North European isolates, which constitutes the most important difference between the viral protein patterns from the North and South European isolates (Figure 1).

Differences in the number and molecular weights of the major proteins in virions isolated from different fish species in the same area were not observed, which is in concordance with the results reported by

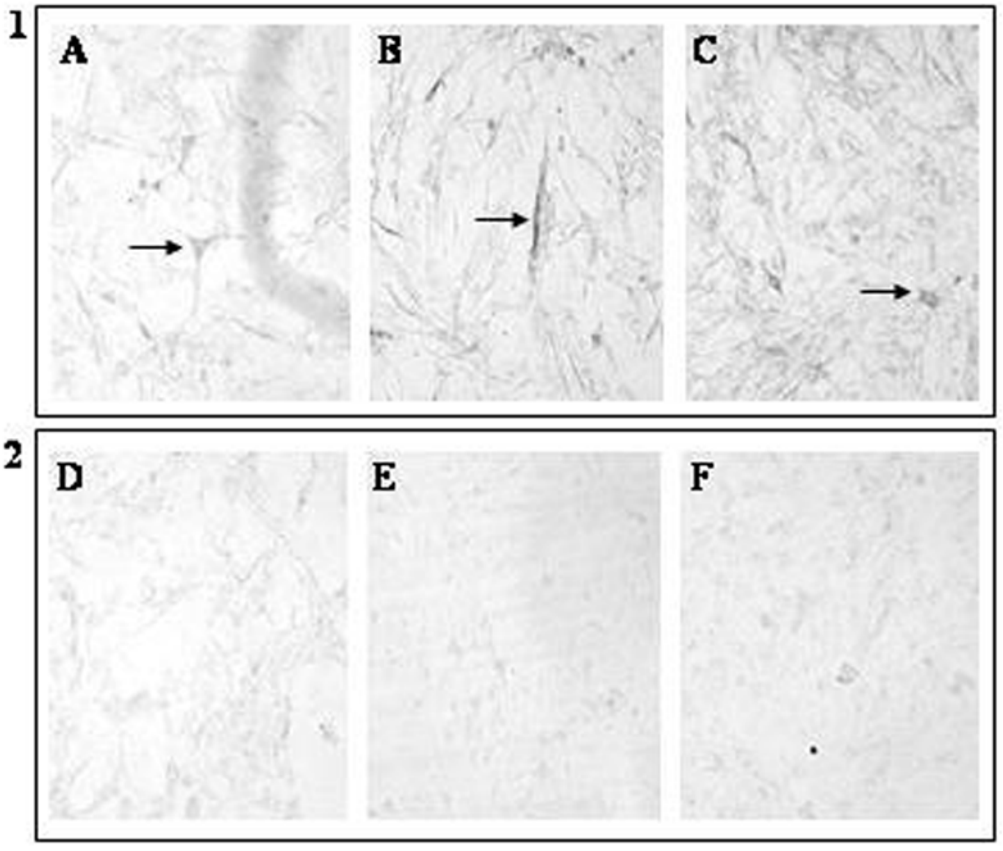

Figure 2. Detection of LCDV by immunohistochemistry on SAF-1 cells. 1: analysis of South European isolates obtained from gilt-head sea bream (A), blackspot sea bream (B) and Senegalese sole (C). 2: analysis of North European isolates obtained from plaice (D), flounder (E) and dab (F). Inoculated cells were incubated with a specific antiserum developed against a gilt-head sea bream viral isolate. Arrows show cells with intensive labelling in the cytoplasm.

Flügel et al. (1982). These authors described variations, particularly between viruses isolated from flounder and dab, regarding only the minor protein profiles, which are not clearly detected in the present study.

Several immunological procedures have previously been applied for LCDV detection in cell culture, such as indirect immunofluorescence assay, flow cytometry and dot blot hybridisation. However, Garcia-Rosado et al. (2002) reported the low sensitivity and specify of these methodologies, and recorded a non-specific signal when antibodies against whole viral particles were used to detect purified LCD virions by the immunoblot technique. Cano et al. (2006) improved the specificity of this technique by developing antibodies against a specific viral protein; however, these antibodies were only evaluated to detect South European LCDV isolates, and may not be appropriate to show general differences in antigenic proteins among the

LCDV under study. These previous results have led us to perform the immunohistochemistry technique to study the specific virus-antibody reaction. The viral antigen detection was conducted using antibodies against whole virions of a gilt-head sea bream LCDV isolate, and a non-specific labelling was not observed in any of the negative controls.

SAF-1 cells inoculated with the viral isolates from Southern Europe showed a strong labelling within the cytoplasm, whereas no signal was observed in cells inoculated with the North European viruses under the same conditions (Figure 2). Therefore, although the presence of conserved group-specific antigens has been described in the *Iridoviridae* family (Hedrick et al., 1992), the antiserum used in this study only detects the viral isolates from Southern Europe by the immunohistochemistry technique.

The pattern of antigenic proteins was previously analyzed by Alonso et al. (2005) by Western blotting using the same polyclonal antibody. These authors reported a complete similarity among viruses isolated from sole, blackspot sea bream and gilt-head sea bream in the same area, suggesting that the variability among LCDV isolates might be associated with the geographical location rather than with the host fish species.

In a previous study, Cano et al. (2007) suggested genetic differences between North and South European LCDV isolates on the basis of the sequencing of a fragment within the MCP gene. These authors proposed that South European isolates might constitute a different species of LCDV, since they showed a low nucleotide identity with the published sequences or with sequences obtained from North European isolates. In addition, some differences between the nucleotide sequences of this fragment in LCDV no. 445 and 446 (both belonging to the LCDV-1 species) and LCDV no. 445, which belongs to the LCDV-2 species, were reported by these authors (98.5% identity). It is important to note that, although these two groups of North European isolates show genetic differences, they are homogeneous in relation to the phenotypic characteristics analyzed in the present study.

In summary, this study shows important phenotypic differences (*in vitro* replication, major protein pattern and antigenic relationship) between LCDV isolates from different geographical areas. Isolates from the same location seem to form a relatively homogeneous group, even though they were isolated from different fish species. These results indicate that the virus has not been spread from a single or limited origin.

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References

Alonso MC, Cano I, Garcia-Rosado E, Castro D, Lamas J, Barja JL, Bergmann, SM & Borrego JJ (2005). Isolation of lymphocystis disease virus from sole, *Solea senegalensis* Kaup, and blackspot sea bream, *Pagellus bogaraveo* (Brünnich). *Journal of Fish Diseases* **28,** 221-228.

Anders K (1989). Lymphocystis disease of fishes. *In* "**Viruses of lower vertebrates**" (W. Anhe & D. Kurstak, Eds.), pp. 141-160. Springer-Verlag, Berlin.

Cano I, Alonso MC, Garcia-Rosado E, Rodriguez Saint-Jean S, Castro D & Borrego JJ (2006). Detection of lymphocystis disease virus (LCDV) in asymptomatic cultured gilt-head seabream (*Sparus aurata*, L.) using an immunoblot technique. *Veterinary Microbiology* **113,** 137-141.

Cano I, Ferro P, Alonso MC, Bergmann SM, Römer-Oberdörfer A, Garcia-Rosado E, Castro D & Borrego JJ (2007). Development of molecular techniques for detection of lymphocystis disease virus in different marine fish species. *Journal of Applied Microbiology* **102,** 32-40.

Cunningham CH (1973). Quantal and enumerative titration of virus in cell cultures. *In* "**Tissue culture: methods and applications**" (P.F. Kruse Jr & M.K. Patterson Jr, Eds.), pp. 527-532. Academic Press, Inc. New York.

Darai G (1986). Molecular biology of fish lymphocystis disease virus. *In* "**Pathology in marine aquaculture**" (C.P. Vivares & E. Jaspers, Eds.), pp. 261-280. Special publication no. 9. European Aquaculture Society. Bredene, Belgium.

Darai G, Anders K, Koch HG, Delius H, Gelderblom H, Samalecos C & Flügel RM (1983). Analysis of the genome of fish lymphocystis disease virus isolated directly from epidermal tumours of pleuronectes. *Virology* **126,** 466-479.

Faisal M (1989). Lymphocystis in the Mediterranean golden grouper *Epinephelus alexandrinus* Valenciannes 1828 (Pisces Serranidae). *Bulletin of the European Association of Fish Pathologists* **9,** 17-18.

Flügel RM, Darai G & Gelderblom H (1982). Viral proteins and adenosine triphosphate phosphohydrolase activity of fish lymphocystis disease virus. *Virology* **122,** 48-55.

Garcia-Rosado E, Castro D, Rodriguez S, Perez-Prieto SI & Borrego JJ (1999). Isolation and characterization of lymphocystis virus (FLDV) from gilt-head sea bream (*Sparus aurata,* L.) using a new homologous cell line. *Bulletin of the European Association of Fish Pathologists* **19,** 53-56.

Garcia-Rosado E, Castro D, Cano I, Perez-Prieto SI & Borrego JJ (2002). Serological techniques for detection of lymphocystis virus in fish. *Aquatic Living Resources* **15,** 179-185.

Garcia-Rosado E, Castro D, Cano I, Perez-Prieto SI, Alonso MC & Borrego JJ (2004). Protein and glycoprotein content of lymphocystis disease virus (LCDV). *International Microbiology* **7,** 121-126.

Goldberg TL, Coleman DA, Grant EC, Inendino KR & Philipp DP (2003). Strain variation in an emerging iridovirus of warm-water fishes. *Journal of Virology* **77,** 8812-8818.

Gonzalez de Canales ML, Muñoz-Cueto JA Arellano J, Garcia-Garcia A & Sarasquete C (1996). Histological and histochemical characteristics of the lymphocystis disease in gilt-head sea bream, *Sparus aurata* L., from the South-Atlantic coast of Spain. *European Journal of Histochemistry* **40,** 143-152.

Hedrick RP, McDowell TS, Ahne W, Torhy C & de Kinkelin P (1992). Properties of three iridovirus-like agents associated with systemic infections of fish. *Diseases of Aquatic Organisms* **13,** 203-209.

Hyatt AD, Gould AR, Zupanovic Z, Cunnigham AA, Hengstberger S, Whittington RJ, Kattenbelt J & Coupar BE (2000). Comparative studies of piscine and amphibian iridoviruses. *Archives of Virology* **145,** 301-331.

Kitamura SI, Jung SJ, Kim WS, Nishizawa T, Yoshimizu M & Oh MJ (2006). A new genotype of lymphocystivirus, LCDV-RF, from lymphocystis diseased rockfish. *Archives of Virology* **151,** 607-615.

Lorenzen K, Desclers SA & Anders K (1991). Population-dynamics of lymphocystis disease in estuarine flounder, *Platichthys flesus* (L.). *Journal of Fish Biology* **39,** 577-587.

Mellergaard S & Nielsen E (1995). Impact of oxygen deficiency on the disease status of common dab *Limanda limanda. Diseases of Aquatic Organisms* **22,** 101-114.

Müller M, Chnitzler P, Koonin EV & Darai G (1995). Identification and properties of the largest subunit of the DNA-dependent RNA polymerase of fish lymphocystis disease virus: dramatic difference in the domain organization in the family *Iridoviridae. Journal of General Virology* **79,** 1099-1107.

Paperna I, Sanai I & Colorni A (1982). An outbreak of lymphocystis in *Sparus aurata* L. in the Gulf of Aqaba, Read Sea. *Journal of Fish Diseases* **5,** 433-437.

Perez-Prieto SI, Rodriguez-Saint-Jean S, Garcia-Rosado E, Castro D, Alvarez MC & Borrego JJ (1999). Virus susceptibility of the fish cell line SAF-1 derived from gilt-head seabream. *Diseases of Aquatic Organisms* **35,** 149-153.

Reiersen LO & Fugelli K (1984). Annual variation in lymphocystis infection frequency in flounder, *Platichthys flesus* (L.). *Journal of Fish Biology* **24,** 187-191.

Russell PH (1974). Lymphocystis in wild plaice, *Pleuronectes platessa* (L.), and flounder, *Platichthys flesus* (L.), in British coastal waters. Histopathological and serological study. *Journal of Fish Biology* **6,** 771-1974.

Walker DP & Hill BJ (1980). Studies on the culture assay of infectivity and some *in vitro* properties of lymphocystis virus. *Journal of General Virology* **51,** 385-395.

Williams T (1996). The iridoviruses. *Advances in Virus Research* **46,** 345-412.

Wolf K (1988). "**Fish viruses and fish viral diseases**". Cornell University Press, Ithaca, New York.

Zhang QY, Xiao F, Xie J, Li ZQ & Gui JF (2004). Complete genome sequence of lymphocystis disease virus isolated from China. *Journal of Virology* **78,** 6982-6994.