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NOTE

Lymphocystis disease virus (LCDV-Sa), polyomavirus 1 (SaPyV1) and papillomavirus 1 (SaPV1) in samples of Mediterranean gilthead seabream

Alejandro M. Labella[1], Rocío Leiva-Rebollo[1], Alí Alejo[2], Dolores Castro[1], Juan J. Borrego[1,*]

[1]Universidad de Málaga, Departamento de Microbiología, Campus Universitario Teatinos, 29071 Málaga, Spain
[2]Centro de Investigación en Sanidad Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Valdeolmos, 28130 Madrid, Spain

ABSTRACT: Lymphocystis disease, caused by the iridovirus lymphocystis disease virus (LCDV), is characterized by the appearance of tumour-like lesions on the skin of affected animals associated with several environmental factors and/or with stress due to the intensive culture conditions of fish farms. In a previous study, the genomes of a new LCDV species, LCDV-Sa, were detected, together with 2 previously unknown viruses, Sparus aurata papillomavirus 1 (SaPV1) and Sparus aurata polyomavirus 1 (SaPyV1). Gilthead seabream from 17 fish farms in Spain, Italy and Turkey were sampled between 2009 and 2015 to investigate the role of the newly described SaPV1 and SaPyV1 viruses in lymphocystis disease development. Our results show that in diseased fish, either or both of the new viruses are almost invariably detected together with LCDV (98%). In asymptomatic fish, these viruses were detected in a much lower percentage (28%) and mostly in concurrence with LCDV (24%). These data confirm the suspected association among the 3 different viruses during lymphocystis disease development in gilthead seabream and warrant future studies to establish their respective contributions.

KEY WORDS: Lymphocystis disease virus · LCDV-Sa · New fish viruses · Papillomavirus 1 · Polyomavirus 1 · Gilthead seabream · *Sparus aurata* · Virus survey

1. INTRODUCTION

Lymphocystis is a widely distributed disease affecting over 100 different marine and freshwater fish species. It is characterized by the appearance of tumour-like lesions on the skin and fins, developed over extended periods of time (weeks or even months). At the histopathological level, the disease is characterized by the formation of heavily enlarged dermal fibroblasts, named lymphocysts, surrounded by a thick hyaline capsule and containing cytoplasmatic basophilic inclusion bodies, granular cytoplasm and a prominent nucleolus. Single cells may coalesce to form larger nodules corresponding to the typical lesions observed on the fish skin, which may become vascularised or pigmented (Borrego et al. 2017). The aetiological agent is the lymphocystis disease virus (LCDV), a double-stranded DNA virus of cytoplasmic

*Corresponding author: jjborrego@uma.es

replication with complex icosahedral particles ranging from 130 to 300 nm in diameter, belonging to the genus *Lymphocystivirus* (family Iridoviridae) (Chinchar et al. 2011).

At present, complete genome sequences are only available for 3 distinct isolates, LCDV-1, isolated from European flounder *Platichthys flesus* (Tidona & Darai 1997), LCDV-China, collected from Japanese flounder *Paralichthys olivaceus* (Zhang et al. 2004), and LCDV-Sa, from gilthead seabream *Sparus aurata* (López-Bueno et al. 2016). According to the sequences of the conserved viral major capsid protein (MCP), 9 different genotypes have been proposed, with clustering related to host species rather than geographic location (Kitamura et al. 2006a, Hossain et al. 2008, Cano et al. 2010, Palmer et al. 2012).

Gilthead seabream is one of the most important cultured fish species in the Mediterranean and off the East Atlantic coast. Lymphocystis disease was first described in gilthead seabream in Israel in 1982 (Paperna et al. 1982), and since then it has been frequently reported in several countries from the same geographic area. The disease follows the typical course observed in other fish species, where lesions on skin, fins and tail usually resolve within 1 mo, while viral DNA can be detected beyond this period of time in those tissues as well as in internal organs including gills, liver, spleen and kidney (Kvitt et al. 2008, Cano et al. 2009), suggesting the systemic nature of infection and the establishment of an asymptomatic carrier state in recovered individuals. Genetic analyses have shown that all LCDV isolates obtained from gilthead seabream (LCDV-Sa) are very closely related to each other and constitute a single novel genotype distinct from previously reported isolates from other hosts and locations (Cano et al. 2010).

López-Bueno et al. (2016) examined the virome of lymphocystis disease-affected gilthead seabream to obtain the complete genome sequence of LCDV-Sa. This virome also included the complete sequence of 2 other abundant viruses (SaPyV1 and SaPV1) that might represent novel fish viruses of the families *Polyomaviridae* and *Papillomaviridae*, respectively. The concurrence of either or both of these viruses with LCDV-Sa in diseased and asymptomatic individuals suggests that they may play a previously unsuspected role in the development of lymphocystis disease in fish. Therefore, the aim of this study was to perform an epizootiologic survey of the 3 viruses (LCDV-Sa, SaPV1 and SaPyV1) in samples of gilthead seabream collected from different geographical locations throughout the Mediterranean area over a period of 7 yr.

2. MATERIALS AND METHODS

2.1. Fish samples

A total of 130 juvenile gilthead seabream specimens were collected from 17 fish farms in Spain, Italy and Turkey between 2009 and 2015 (see Table 2). Both diseased and asymptomatic fish were analysed. Fish were anaesthetized with MS-222 in seawater (Sigma) at a final concentration of 30 mg ml^{-1}. Samples of caudal fin (approximately 1 cm^2 in size) were aseptically cut off, frozen immediately at −20°C in dry ice and sent to the lab.

To determine whether the new LCDV-Sa, polyoma- or papillomaviruses, could be detected in samples from Senegalese sole *Solea senegalensis* — a co-cultured fish species — a short sampling (7 specimens) was conducted at a fish farm in southern Spain during 2015, following the same procedure explained above.

2.2. DNA extraction and virus detection

For DNA extraction, an EZNA Tissue DNA Kit (VWR, Omega bio-Tek) was used following the manufacturer's instructions. For the detection of different viruses, we used a combination of conventional PCR and nested PCR protocols, using a GoTaq® G2 Flexi DNA Polymerase kit (Promega), following the manufacturer's instructions in a 50 µl reaction volume (5 µl of 0.2 mM dNTPs [Roche Diagnostics]; 10 µl Colourless GoTaq Flexi Buffer 5×; 3 mM $MgCl_2$; 2 µl of each primer [15 pmol $µl^{-1}$] and 1.25 U GoTaq DNA Polymerase).

For LCDV detection, a 609 bp fragment of the viral MCP gene was amplified by PCR using the primers LCDVs-F and LCDVs-R described by Kitamura et al. (2006b). The amplification program consisted of an initial 5 min at 95°C denaturation, 35 cycles of 1 min at 95°C denaturation, 30 s at 50°C annealing and 1 min at 72°C extension steps, with a final 10 min at 72°C extension. For the nested PCR, specific primers were designed (RT-LCDV-F and RT-LCDV-R2), which generate a 238 bp amplicon within the 609 bp fragment of the MCP gene (nucleotide positions 173–410 of the LCDV-Sa 9 MCP gene, GenBank accession no. GU320728). The amplification program was 2 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 60°C and 45 s at 72°C, with a final extension of 5 min at 72°C.

For SaPyV1 and SaPV1 detection, PCR and nested PCR protocols using specific primers were designed to amplify a fragment of 602 bp of the large T antigen gene (Polyo602-F and Polyo602-R) and an internal

420 bp amplicon (Polyo420-F and Polyo420-R) within the 602 bp fragment for detection of polyomavirus, as well as a fragment of 650 bp of the L1 gene (L1-3′-F and L1-3′-R) and an internal 152 bp amplicon (NL1-3′-F and NL1-3′-R) within the 650 bp fragment for detection of papillomavirus. The amplification program for polyomavirus detection, both PCR and nested PCR, consisted of 2 min at 95°C, 30 cycles of 1 min at 95°C, 45 s at 58°C and 1 min at 72°C s, with a final 5 min extension at 72°C. The PCR program for papillomavirus was 5 min at 95°C, 30 cycles of 1 min at 95°C, 45 s at 52°C and 1 min at 72°C, and a 5 min extension at 72°C. For nested PCR, the protocol was 5 min at 95°C, 30 cycles of 1 min at 95°C, 45 s at 60°C and 1 min at 72°C, and 5 min at 72°C. Primer sequences are listed in Table 1.

2.3. Statistical analysis

A *t*-test (asymptomatic: p1; diseased: p2) was performed to establish whether the differences found in the detection of viruses between diseased and asymptomatic fish were statistically significant or not. The analysis was conducted using the differences in absolute values between each pair of results |p1 − p2|, corrected by the standard error [p (1 − p)/(1/n1 + 1/n2)1/2], where p is the arithmetic media = (p1 + p2)/2, and n1/n2 are the sample sizes. Moreover, the standard errors (SE) were multiplied by the statistical parameter Z with a level of significance of $\alpha = 0.05$. When $|p1 - p2| > Z\alpha = 0.05 \times SE$, the results were considered significantly different.

Table 1. Primer sets used in this study to detect lymphocystis disease virus (LCDV-Sa), polyomavirus 1 (SaPyV1), and papillomavirus 1 (SaPV1) in gilthead seabream and Senegalese sole

Primer	Primer sequence (5′–3′)	Amplicon size (bp)
LCDVs-F	YTG GTT CAG TAA ATT ACC RG	609[a]
LCDVs-R	GTA ATC CAT ACT TGH ACR TC	
RT-LCDV-F	ACG TTT CTC GAG GCG GAG AT	238[b]
RT-LCDV-R2	ACG CGT TTA GAA CCG CAC AT	
Polyo602-F	ATG TGG GTT TGC GAC AGA TG	602[b]
Polyo602-R	CTC TGC TTG ATG GAA TCT GGA A	
Polyo420-F	GTG GCT GCT GCT TAC CTT CTG	420[b]
Polyo420-R	CAC CCA GGG AGG GAA GTA CA	
L1-3′-F	CAG AGA CAG ATA ATC TGC G	650[b]
L1-3′-R	CTC CTT GAT GTG TCG CAG	
NL1-3′-F	AGG ATC AGC AGA CAT CGT CAC A	152[b]
NL1-3′-R	ATG GAC AAT GAC AAT ACT GGA GAC A	

[a]Kitamura et al. (2006a); [b]primers designed in this study using Primer Express Software v3.0 (Applied Biosystems)

3. RESULTS AND DISCUSSION

In an earlier study, López-Bueno et al. (2016) described the detection of LCDV-Sa DNA in all fish analysed showing lymphocystis symptoms. In addition, either SaPV1 or SaPyV1 (or both) were detected in all samples from diseased fish, with 60% positive for the 3 viruses concurrently. Neither SaPV1 nor SaPyV1 was detected in asymptomatic fish, while most of the examined individuals (83.3%) contained LCDV-Sa DNA as confirmed by nested PCR. However, the number of specimens (both diseased and asymptomatic) in the previous work was low and limited to only a few fish farms. We therefore decided to carry out a more detailed survey.

Of the 130 gilthead seabream analysed, 59 were diseased and 71 were asymptomatic. All samples from diseased fish were positive for at least 1 of the 3 viruses (LCDV-Sa, SaPV1, SaPyV1), whereas 15 samples of asymptomatic fish (21.1%) were free from the viruses (Table 2). However, most of the asymptomatic animals (75%) were positive for LCDV and, as expected, this increased to 100% of the diseased animals. Strikingly, in 98% of the diseased animals, either SaPV1 or SaPyV1, or both, were additionally detected, in comparison to 24% of the asymptomatic individuals. This result agrees with López-Bueno et al. (2016), showing concurrence of the viruses in lymphocystis-diseased gilthead seabream. More specifically, while only 1 out of 59 asymptomatic fish (1.4%) contained DNA of all 3 viruses, this percentage increased to 78% in the case of diseased animals, making a further case for the potential role of the newly described SaPV1 and SaPyV1 viruses in lymphocystis pathogenesis.

Several viral detection profiles were recorded. LCDV-Sa single detections were made in 1.7 and 50.7% of diseased and asymptomatic fish, respectively; in the case of SaPyV1, only 1.4% of the asymptomatic fish were exclusively infected with the virus, and 2.8% with SaPV1 (Table 3). The most frequently observed viral profiles were LCDV-Sa + SaPyV1 (15.2% from diseased and 2.8% from asymptomatic fish) and LCDV-Sa + SaPV1 (5.1% from diseased and 19.7% from asymptomatic fish). No significant differences ($\alpha < 0.05$) were obtained between diseased and asymptomatic fish for the cases of detection of SaPyV1 and SaPV1. In

Table 2. Detection of lymphocystis disease virus (LCDV-Sa), polyomavirus 1 (SaPyV1), and papillomavirus 1 (SaPV1) from diseased (D) and asymptomatic (A) gilthead seabream (GS) and Senegalese sole (SS). Each batch is a set of samples taken from the same fish farm, n refers to the number of samples taken on a particular date. +: positive detection; –: negative detection

Batch	Origin/year	Fish species	Status	n	LCDV–Sa	SaPyV1	SaPV1
1	Spain/2009	GS	D	10	+	+	+
2	Spain/2010	GS	A	13	+	–	–
				12	+	–	+
				1	+	+	+
				1	–	–	+
				4	–	–	–
3	Spain/2011	GS	D	1	+	–	+
4	Spain/2011	GS	D	1	+	+	+
5	Italy/2012	GS	D	1	+	+	–
6	Italy/2012	GS	D	6	+	+	+
7	Spain/2013	GS	D	1	+	–	–
				2	+	–	+
				3	+	+	+
8	Spain/2013	GS	A	1	+	+	–
				1	+	–	+
				1	+	–	–
9	Turkey/2014	GS	A	4	+	–	–
				1	–	–	+
				10	–	–	–
10	Spain/2014	GS	A	2	+	–	–
11	Spain/2014	GS	A	2	+	–	–
				1	–	+	–
12	Turkey/2014	GS	A	3	+	–	–
				1	+	–	+
				1	–	–	–
13	Spain/2014	GS	D	1	+	+	+
14	Spain/2015	GS	A	2	+	–	–
				1	+	+	–
15	Spain/2015	GS	D	2	+	+	–
				1	+	+	+
16	Spain/2015	GS	D	6	+	+	–
				24	+	+	+
17	Turkey/2015	GS	A	9	+	–	–
18	Spain/2015	SS	A	2	+	–	–
				4	+	+	–
				1	+	+	+

Table 3. Statistical analysis (*t*-test) performed to establish the significance level at $\alpha = 0.05$ for the differences in virus detection between diseased (n = 59) and asymptomatic (n = 71) fish. LCDV-Sa: lymphocystis disease virus; SaPyV1: polyomavirus 1; SaPV1: papillomavirus 1. p is the arithmetic media between p1 and p2

Virus detection profile	Asymptomatic			Diseased			p1–p2	p	1–p	1/n1+ 1/n2	SE	$Z\alpha \times$ SE	Significant at $\alpha = 0.05$
	n	Percentage	p1	n	Percentage	p2							
LCDV-Sa	36	50.7	0.507	1	1.7	0.017	0.490	0.262	0.738	0.03098	0.077	0.1517	Yes
LCDV-Sa+SaPyV1	2	2.8	0.028	9	15.2	0.152	0.124	0.090	0.901	0.03098	0.050	0.0987	Yes
LCDV-Sa+SaPV1	14	19.7	0.197	3	5.1	0.051	0.146	0.124	0.876	0.03098	0.058	0.1137	Yes
LCDV-Sa+SaPyV1 +SaPV1	1	1.4	0.014	46	78.0	0.780	0.766	0.397	0.603	0.03098	0.086	0.1688	Yes
SaPyV1	1	1.4	0.014	0	0	0	0.014	0.007	0.993	0.03098	0.015	0.0289	No
SaPV1	2	2.8	0.028	0	0	0	0.028	0.014	0.986	0.03098	0.021	0.0406	No
Negatives	15	21.1	0.211	0	0	0	0.211	0.106	0.894	0.03098	0.054	0.1060	Yes

the remaining profiles, significant differences between diseased and asymptomatic fish were observed.

Gilthead seabream is extensively farmed in the Mediterranean area, and severe economic loss due to lymphocystis disease is a serious problem. However, LCDV isolates from this species and location have been poorly characterized to date. Senegalese sole *Solea senegalensis* is frequently co-cultured with gilthead seabream, and Alonso et al. (2005) have previously shown LCDV-Sa to be able to infect this species. All specimens of Senegalese sole showed no clinical signs of lymphocystis disease, and 3 virus detection profiles were found: in 14.3% of samples, all 3 viruses were detected, 57.1% were positive for both LCDV-Sa and SaPyV1, and in 28.6% of the samples only LCDV-Sa was detected. This shows for the first time that the newly described polyoma- and papillomaviruses are not restricted to a single fish host. Interestingly, neither SaPyV1 nor SaPV1 could be detected in the absence of LCDV-Sa, possibly indicating an interdependence among these viruses. In this case, concurrence of all 3 viruses was not associated with obvious clinical signs, suggesting a species-specific clinical outcome of co-infections, although this interpretation requires further observations.

In this study, we have expanded the previous data (López-Bueno et al. 2016) showing the concurrent presence of LCDV-Sa with SaPV1 and SaPyV1 in lymphocystis-diseased juvenile gilthead seabream, in contrast to the almost absolute lack of detection of either SaPV1 or SaPyV1 in asymptomatic gilthead seabream in the absence of LCDV. This raises several intriguing questions that need to be addressed in the future. It might be possible for co-infection with either SaPV1 or SaPyV1 to trigger LCDV-Sa replication and disease. Alternatively, active replication of LCDV-Sa might favour secondary infections with SaPV1 or SaPyV1 as a consequence of locally dampened immune responses or might enhance the replication of those polyoma- and papillomaviruses (SaPyV1 or SaPV1) already present at undetectable levels. The isolation and culture of each of the 3 viruses appears to be a critical step towards understanding the relative contribution of each virus to lymphocystis disease. SaPyV1 belongs to a diverse group of fish-associated polyomaviruses that appear to be highly species-specific (Van Doorslaer et al. 2018). To date, only 2 polyomaviruses isolated from eels have been propagated *in vitro* using primary cultures or cell lines derived from their original host species (Mizutani et al. 2011, Pao et al. 2018). On the other hand, SaPV1 is the first papillomavirus species described from fish. Although there is a well-established cell culture system for the isolation of LCDV using SAF-1, a cell line derived from gilthead seabream (Garcia-Rosado et al. 1999), this is not the case for the other 2 viruses, as neither SaPyV1 nor SaPV1 replicate in this cell line. Therefore, the development of primary cell cultures from this fish species will be necessary in order to propagate these new viruses.

In summary, we have expanded on previous observations (López-Bueno et al. 2016) to show that the concurrent presence of SaPV1 and SaPyV1 together with LCDV-Sa is a hallmark of lymphocystis-diseased seabream, underscoring the potential relevance of this interaction among different viruses in disease development. These results warrant further studies to establish the respective replicative cycles, and the interaction between them, as well as the epidemiology of these viruses and their contribution to lymphocystis disease development in gilthead seabream and other fish species.

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