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Lymphocystis disease virus: its importance in aquaculture

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Abstract

Lymphocystis disease virus (LCDV) is the causative agent of a well-known fish viral disease that is characterized by hypertrophy of fibroblastic cells in the connective tissue. This viral disease affects more than 125 wild and cultured species of teleost fish from marine and freshwater environments and has a cosmopolitan geographical distribution. In aquaculture systems, the prevalence of LCDV infection is very high, likely reflecting the horizontal transmission of this virus. The incidence rate of the disease may reach 70%, causing significant economic losses for the aquaculture industry. This review provides information on the taxonomy, viral properties, epizootiology and pathogenesis, diagnostic methods and control measures of LCDV infection in fish.

Key words: aquaculture, control measures, diagnostic methods, Lymphocystis disease virus, pathogenesis.

Introduction

Large-scale aquaculture has been associated with environmental and microbial concerns worldwide as a consequence of its intensive culture and high-stocking density. Fish culture production mainly follows two procedures: (i) integrated management of coastal zones, performed mainly in marine and brackish waters using extensive and semi-intensive procedures, and (ii) the intensive farming of highly valuable fish and shellfish species, performed mostly in tanks and cages. In recent years, modern aquaculture has been characterized by a strong increase in production output because of the application of innovative technologies in fish farms and the optimization of management strategies. The potential of fish farming has been compromised by the emergence of infectious pathologies, being the most frequently reported of infectious in origin. Viruses are a limiting factor for the expansion of aquaculture because of the direct losses of fish production, costs derived from reduced productivity and disease management, and loss of export markets related to trade restrictions (Whittington & Chong 2007; Renault 2009; Rigos & Katharios 2010).

Members of the *Iridoviridae* family have been described as causal agents of high mortalities in a wide range of invertebrate and lower vertebrate animals (Chinchar 2002). According to the 9th Report of the International Committee on Taxonomy of Viruses, the family *Iridoviridae* is subdivided into five genera: *Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus* and *Megalocytivirus* (Jancovich *et al.* 2012). Three genera, *Lymphocystivirus, Megalocytivirus* and *Ranavirus*, have received attention due to the economic losses caused to the aquaculture industry (Chao *et al.* 2002; Dong *et al.* 2010; Whittington *et al.* 2010; Kuttichantran *et al.* 2012). Features that distinguish these genera are GC content, nucleotide sequence, cytopathological signs, the use of both host- and virus-encoded RNA polymerases and differences in the inferred amino acid sequence of key genes such as ATPase, methyltransferase or major capsid protein (MCP) (Sudthongkong *et al.* 2002; Wang *et al.* 2003; Do *et al.* 2005; Williams *et al.* 2005; Kuttichantran *et al.* 2012; Chinchar & Waltzek 2014).

Lymphocystis disease (LCD) is a well-known fish viral infection that is characterized by hypertrophy of fibroblastic cells in the dermis connective tissue of affected fish, occasionally proliferating as true epithelial tumours (Samalecos 1986). This viral disease affects a wide variety of freshwater, brackish and marine fish species. LCD was one of the first fish viral diseases reported in the 19th century (Wolf 1988), and its viral aetiology was demonstrated by electron microscopy by Walker (1962) and the subsequent virus isolation on BF-2 cell line by Wolf (1962). Although this disease is rarely fatal, fish showing the characteristic symptoms cannot be commercialized, causing important economic losses (Masoero *et al.* 1986). The aetiological agent of LCD is the lymphocystis disease virus (LCDV), belonging to *Lymphocystivirus* genus, which includes one species *Lymphocystis disease virus 1* (LCDV-1) originally isolated from the European flounder [*Platichthys flesus*

(Linnaeus 1758)] and European plaice [*Pleuronectes platessa* (Linnaeus 1758)]; in addition, three virus candidates are also included in this genus: LCDV-2, isolated from common dab [*Limanda limanda* (Linnaeus 1758)]; LCDV-C, isolated from Japanese flounder [*Paralichthys olivaceus* (Temminck & Schlegel 1846)]; and LCDV-RF, isolated from black rockfish [*Sebastes schlegelii* (Hilgendorf 1880)]. Lymphocystis disease virus-1 and related viruses are distinguished by host specificity, histopathology, viral protein profiles and DNA sequences (Jancovich *et al.* 2012).

One of the distinctive features of the *Iridoviridae* family is the presence of an MCP, which is the main structural component of the viral particles. MCP comprises 40–45% of the total viral polypeptides and has a molecular weight of approximately 50 kDa (Flügel 1985). The MCP gene has been recognized as a suitable target on which to perform phylogenetic studies of iridoviruses because it is relatively conserved within the family (Tidona *et al.* 1998). On the basis of the MCP gene sequence, nine genotypes of *Lymphocystivirus* have been proposed to date: LCDV-1 as genotype I; genotype II, consisting of Japanese flounder (LCDV-C) isolates; genotype III, which includes black rockfish (LCDV-RF) isolates; genotype IV, for cobia [*Rachycentron canadum* (Linnaeus 1766)] and Japanese sea bass [*Lateolabrax japonicus* (Cuvier 1828)] (LCDV-RC and LCDV-SB, respectively) isolates; genotype V includes painted glass fish [*Parambassis baculis* (Hamilton 1822)] (LCDV-CB) isolates; genotype VI for gourami [*Trichopodus leerii* (Bleeker 1852) and *T. trichopterus* (Pallas 1770)] (LCDV-TL) isolates; genotype VII includes gilthead sea bream [*Sparus aurata* (Linnaeus 1758)] and Senegalese sole [*Solea senegalensis* (Kaup 1858)] (LCDV-SA and LCDV-SSE, respectively) isolates; genotype VIII for a largemouth bass [*Micropterus salmoides* (Lacepède 1802)] isolate (strain Leetown NFH); and genotype IX, including an American yellow perch [*Perca flavescens* (Mitchill 1814)] isolate (Kitamura *et al.* 2006a,b; Hossain *et al.* 2008; Kvitt *et al.* 2008; Cano *et al.* 2010; Palmer *et al.* 2012). The genetic diversity of LCDV has been related to the host fish species (Kitamura *et al.* 2006a,b; Hossain *et al.* 2008). However, when studying the evolutionary relationship of LCDV and its hosts, Yan *et al.* (2011) did not obtain significant evidence of cospeciation between LCDV genotypes and their host fish species.

Viral characteristics

Virion structure

Lymphocystis disease viruses are large icosahedral viral particles that, depending on the host fish species, may vary in size from 120 to 340 nm in diameter (Tidona & Darai 1999; Paperna *et al.* 2001). The virus consists of a bilaminar capsid and a core that appears filamentous, displaying helicoidal symmetry (Madeley *et al.* 1978; Samalecos 1986; Heppell & Berthiaume 1992). The core is surrounded by a membranous structure that is clearly demonstrated in decaying virus (Smail & Munro 2001). Negative staining electron images of decaying viruses show that the outer electron-lucent layer of the capsid is composed of knobs, possibly attached to the inner capsid layer by a fringe of fibril-like external protrusions of 2.5 nm in length (Jancovich *et al.* 2012). The treatment of LCD virions with papain before staining revealed a capsomer lattice structure, presumably because the papain removed the outer capsid (Samalecos 1986).

The virions are heat labile and can be inactivated by ether, glycerol, 5-iododeoxyuridine and UV treatments (Wolf 1988; Iwamoto *et al.* 2002). Freezing–thawing cycles at −20°C may provoke a decrease in viral infectivity (Wolf 1962). In contrast, the virions show stability to pH 6–9 and are resistant to ultrasonic treatment (Walker & Hill 1980).

Chemical composition

Lymphocystis disease virions are composed of 42% proteins, 17% lipids and 1.6% nucleic acids, with sugars most likely representing a major portion of the remaining unidentified components (Robin *et al.* 1983). SDS-PAGE analysis revealed the presence of 33 structural polypeptides, ranging from 4 to 220 kDa, in LCDV-1 virions isolated directly from fish tumours (Flügel *et al.* 1982). However, purified virions obtained from other fish species showed a different electrophoretic pattern of 23–31 polypeptides ranging from 30 to 210 kDa (Robin *et al.* 1984; Garcia-Rosado *et al.* 2004). A common characteristic of all LCDV particles is the presence of an MCP of approximately 50 kDa composed of 459 amino acids, which represents up to 45% of the total protein content (Flügel *et al.* 1982; Robin *et al.* 1986; Heppell & Berthiaume 1992). The MCP is one of the antigenic proteins identified in LCDV that immuno-reacted with Japanese flounder antisera from diseased fish and also from formalin-inactivated LCDV-vaccinated fish (Jang *et al.* 2011). The enzymatic activities associated with purified virions include a viral-encoded ATP hydrolase, a protein kinase and a thymidine kinase (Flügel *et al.* 1982; Darai *et al.* 1983).

Several authors have reported the presence of carbohydrates in LCDV. Robin *et al.* (1986) showed the presence of 10 glycoproteins in highly purified virus particles of an LCDV strain originally isolated from largemouth bass. In addition, Garcia-Rosado *et al.* (2004) reported the existence of 8 glycoproteins, with molecular weights ranging from 76 to 210 kDa, in viral particles isolated from gilthead sea bream. Six of these glycoproteins presented a high content of mannose, and the other two contained a high

proportion of sialic acid and N-acetylglucosamine, respectively.

Although LCDV is a nonenveloped particle, it may contain 5–17% lipids that are readily digested by a treatment with phospholipase that has been described for other iridoviruses (Robin *et al.* 1983; Chinchar *et al.* 2005). These phospholipids constitute an internal lipid membrane that lies between the DNA core and the viral capsid. The origin of the internal lipid membrane is unclear. The composition of the internal lipid membrane suggests that this membrane is not derived from host membranes but is rather produced *de novo*. However, it has been suggested that the internal lipid membrane is derived from fragments of the endoplasmic reticulum and plays a key role in virion assembly (Jancovich *et al.* 2012).

The LCDV genome is a single linear double-stranded DNA molecule of 102.6 kbp for LCDV-1 and 186.2 kbp for LCDV-C (Jancovich *et al.* 2012). This genome is circularly permuted, terminally redundant and heavily methylated (22%), with a G+C content of 29.9% for LCDV-1 and 27.2% for LCDV-C (Darai *et al.* 1983; Wagner *et al.* 1985; Tidona & Darai 1997a; Jancovich *et al.* 2012). In addition, LCDV-1 DNA contains numerous short-direct, inverted and palindromic repetitive sequence elements (Schnitzler *et al.* 1987; Schnitzler & Darai 1989; Jancovich *et al.* 2012).

Genome organization

Complete DNA sequences of LCDV-1 and LCDV-C have been determined. The former encoded 195 potential open reading frames (ORFs), whereas LCDV-C possesses 240 potential ORFs (Tidona & Darai 1997b; Zhang *et al.* 2004; Jancovich *et al.* 2012). In LCDV-1, 108 largely nonoverlapping ORFs are likely to represent viral genes, and 38 show significant homology to proteins related to virus replication and transcription, such as DNA polymerase (ORF 135R), DNA polymerase processing factor (ORF 003L), DNA-dependent RNA polymerases (ORF 016L, ORF 025L and ORF 171R), DNA methyltransferase (ORF 005L), methyl-sensitive restriction endonuclease with specificity for CCGG target sites (ORF 178L), structure-specific endonuclease (ORF 191R), DNA-dependent ATPase (ORF 054R), DNA puff protein homologue (ORF 108L), proteins homologous to an early transcription factor subunit (ORF 132L), late promoter transactivator protein (ORF 032R), dsRNA-specific ribonuclease (ORF 137R), thymidine kinase (ORF 136R) and ribonucleoside-diphosphate reductases (ORF 027R and ORF 176L). In addition, other putative gene products showed significant homology to proteins involved in the virus–host interaction, including an insulin-like growth factor, a tumour necrosis factor receptor family, thioredoxin, cysteine proteinase, several protein kinases, a tissue differentiation factor, a collagen type IX homologue, β-hydroxy steroid dehydrogenase and ATPase, to name a few ORFs (010L, 022R, 035L, 036R, 043R, 047L, 063L, 080R, 088R, 093R, 094R, 095L, 122R, 125R, 128L, 153L, 158L and 167L, respectively) (Flügel *et al.* 1982; Koonin 1993; Müller *et al.* 1995; Tidona *et al.* 1996; Tidona & Darai 1997b; Sudthongkong *et al.* 2002; Essbauer *et al.* 2004; Kim & Lee 2007; Pontejo *et al.* 2013).

In the case of LCDV-C, Zhang *et al.* (2004) reported the presence of 240 potential ORFs and 176 nonoverlapping putative viral genes. A search of the GenBank database using the 176 individual putative genes revealed 103 homologues to the corresponding ORFs of LCDV-1 and 73 potential genes that were not found in LCDV-1 or in other iridoviruses. Among these 73 genes, eight genes contain coding sequences of conserved domains of cellular proteins, such as the caspase recruitment domain involved in apoptotic signalling (ORF 002L), thymidylate synthase (ORF 011L), the tumour necrosis factor receptor domain (ORF 016L), site-specific recombinase (ORF 047R), reverse transcriptase (ORF 051L), 7 transmembrane receptor (ORF 058L), the N-terminal domain of cell division protein 48 (ORF 209R) and collagen triple-helix repeat (ORF 216L). The remaining 67 novel genes do not show any significant homology with sequences in the public database.

Viral multiplication

The replication mechanism of LCDV has not been investigated, but a model for *Frog virus 3* (FV-3), a member of the genus *Ranavirus*, has been proposed (Chinchar *et al.* 2009; Jancovich *et al.* 2012). The cellular receptor(s) for FV3 is unknown but viral entry is achieved by clathrin-mediated endocytosis. In the case of LCDV-C, a 27.8-kDa protein associated with beta-actin in the plasma membrane of flounder gill cells has been identified as the virus receptor (Wang *et al.* 2011a). Following uncoating, viral cores enter the nucleus where first-stage DNA synthesis, and the synthesis of immediate early (IE) and delayed early (DE) viral transcripts, occurs. One or more virion-associated proteins act as transactivators and redirect host RNA polymerase II to synthesize IE and DE viral mRNAs using the methylated viral genome as a template. The gene products encoded by the IE and DE viral transcripts include both regulatory and catalytic proteins. The viral DNA polymerase catalyses the first round of viral DNA synthesis.

The newly synthesized viral DNA may serve as the template for additional rounds of DNA replication and early transcription, or it may be transported to the cytoplasm where the second stage of viral DNA synthesis occurs. In the cytoplasm, viral DNA is replicated as large, branched concatemers that are processed to mature DNA during DNA packaging. Viral DNA methylation also occurs in the cytoplasm of the host cell; although its precise role is

uncertain, it is hypothesised to protect viral DNA from endonucleolytic attack. The transcription of late (L) viral genes occurs in the cytoplasm, and full L gene transcription requires prior DNA synthesis. Homologues of the two largest subunits of RNA polymerase II are encoded by all iridoviruses. Whether this viral DNA-dependent RNA polymerase functions only in the cytoplasm to transcribe L viral genes or whether it also plays a role in continued early transcription has not yet been determined.

Virion formation occurs in the cytoplasm within morphologically distinct areas named viral assembly sites. Within these assembly sites, concatemeric viral DNA is packaged into virions by a 'headful' mechanism that results in the generation of circularly permuted and terminally redundant genomes, similar to those reported in the T-even Enterobacteria phages of the family *Myoviridae*. Following assembly, virions accumulate in the cytoplasm within large paracrystalline arrays or acquire an envelope by budding from the plasma membrane.

Epizootiology of LCDV

Lymphocystis disease has been described in more than 125 species of fish from both marine and freshwater environments (Anders 1989; Marcogliese *et al.* 2001; Paperna *et al.* 2001; Bunkley-Williams *et al.* 2002; Sheng *et al.* 2007a; Hossain *et al.* 2008; Xu *et al.* 2014; Huang *et al.* 2015). The affected species belongs to evolutionarily advanced orders of bony fish (teleosts), mainly including the families *Cichlidae, Osphronemidae, Centrarchidae, Gobiidae, Chaetodontidae, Pomacentridae, Sciaenidae, Serranidae* and *Pleuronectidae*. To date, LCD has not been reported in less-advanced fish orders, such as siluriformes, cyprinids and salmonids.

The disease is cosmopolitan, being widely distributed in all continents (Plumb 1993). In Europe, LCD is an endemic disease in the North Sea and Mediterranean zones, affecting both wild and cultured fish species, such as European flounder, common dab, European plaice, grey gurnard [*Eutrigla gurnardus* (Linnaeus 1758)], gilthead sea bream, black spot sea bream [*Pagellus bogaraveo* (Brünnich 1768)] and Senegalese sole (Paperna *et al.* 1982; Anders 1989; Basurco *et al.* 1990; Moate *et al.* 1992; Garcia-Rosado *et al.* 1999; Dethlefsen *et al.* 2000; Alonso *et al.* 2005). LCD is also a common fish disease in Asian aquaculture, particularly affecting Japanese flounder, black rockfish, cobia, Japanese sea bass, Japanese amberjack [*Seriola quinqueradiata* (Temmick & Schlegel 1845)], groupers [orange-spotted grouper, *Epinephelus coioides* (Hamilton 1822), brown-marbled grouper, *E. fuscoguttatus* (Forsskål 1775) and giant grouper, *E. lanceolatus* (Bloch 1790)], and red sea bream [*Pagrus major* (Temmick & Schlegel 1843)], as well as ornamental aquarium fish species (Matsusato 1975; Tanaka *et al.* 1984; Chen 1996; Park & Sohn 1996; Muroga 1997; Chun 1998; Xu *et al.* 2000, 2014; Zhang 2002; Xing *et al.* 2006; Hossain *et al.* 2008; Huang *et al.* 2015).

Only a few studies have been conducted on the fate of LCDV outside the host and whether it is able to remain viable for an extended period of time in water or sediments. However, it is classically assumed that viral transmission occurs through the skin and gills of fish by direct contact or by waterborne exposure (Wolf 1988; Bowser *et al.* 1999; Kvitt *et al.* 2008). Trauma of the skin via handling or netting, mating, parasitism and aggressive behaviour favour viral transmission among fish (Wolf 1988; Plumb 1993; Smail & Munro 2001). Sheng *et al.* (2007b) and Cano *et al.* (2009a) reported the possible transmission of LCDV by feeding in aquaculture facilities. The latter authors suggested that artemia nauplii might act as a reservoir of LCDV (Cano *et al.* 2009b). Later, these authors demonstrated two mechanisms of LCDV transmission to gilthead sea bream larvae: vertical transmission through eggs and horizontal transmission via LCDV-positive rotifers (Cano *et al.* 2013).

In aquaculture facilities, a high percentage of the fish population could be infected by LCDV, likely reflecting the ease of horizontal transmission, and viral infection incidences up to 70% have been described (Paperna *et al.* 1982; Sano 1988; Matsuoka 1995; Xing *et al.* 2006). The prevalence of LCD is affected by fish density, human manipulation, low salinity, water temperature, reduced oxygen conditions, nutritional deficiencies and chemical and biological water pollution (Paperna *et al.* 1982; Bowser *et al.* 1988, 1999; Berthiaume *et al.* 1993; Sindermann 1996; Vethaak & Jol 1996; Mellergaard & Nielsen 1997; Austin 1999; Grygiel 1999; Kitamura *et al.* 2007). Hossain *et al.* (2009) demonstrated the importance of temperature on the persistence of LCDV in Japanese flounder epidermal tissues. These authors found that lymphocystis cells appeared on the skin and fins at 35 days post-challenge at 20°C, but no clinical signs were observed in the fish reared at 10° and 30°C, although LCDV could be detected by PCR. They concluded that at low temperatures, LCDV is able to persist over a long period of time in the fish epidermis, producing a subclinical infection.

Disease features

The main characteristic of LCD is the appearance of small cream-coloured nodular lesions on the fish skin and fins (Colorni & Diamant 1995; Sarasquete *et al.* 1998). Each nodule consists of an LCDV-infected cell, named lymphocyst or lymphocystis cell, of up to 1 mm in diameter (Paperna *et al.* 1982). These hypertrophied cells may occur singly or grouped in raspberry-like clusters of tumour appearance. These cellular aggregates are usually whitish in

colour, but when they cover epithelial tissue that is rich in chromatophores, the chromatophores may render them greyish or darker (Wolf 1988; Smail & Munro 2001). In heavily affected fish, lymphocysts may cover the entire body, spreading from the gills to the fins (Paperna *et al.* 1982; Flügel 1985; Le Deuff & Renault 1993; Xing *et al.* 2006). Less frequently, they have also been described on eyes, causing exophthalmia, and internally over the mesenteries, peritoneum and several internal organs (Huizinga & Cosgrove 1973; Russell 1974; Dukes & Lawler 1975; Howse *et al.* 1977; Wolf 1988; Colorni & Diamant 1995; Xing *et al.* 2006).

Diseased fish show low growth rates, which may be caused by the anaemia generally associated with this disease (Nishida *et al.* 1998; Iwamoto *et al.* 2002). Mortalities are typically limited to those individuals whose swimming, breathing or feeding is severely impaired by particularly large and cumbersome growths of infected cells (Colorni & Padros 2011). In fish farms, LCD outbreaks may favour secondary bacterial infections, cannibalism and/or parasitic infestations, factors that may increase mortality rates (Williams *et al.* 2005; Colorni & Padros 2011; Dezfuli *et al.* 2012; Haddad-Boubaker *et al.* 2013).

Lymphocystis disease is a chronic and self-limiting disease that, depending on the host fish species and environmental conditions, may persist for a variable period of time (Williams 1996). Thus, the LCD-associated lesions may be evident for 1 year in cold-water fish, whereas they disappear after several weeks in warm-water species (Paperna *et al.* 1982; Gonzalez de Canales *et al.* 1996).

Lymphocystis disease virus infection has been described in bluegill [*Lepomis macrochirus* (Rafinesque 1819)] (Dunbar & Wolf 1966) and European plaice (Roberts 1976). Although the time course for the development and regression of lymphocysts is quite different in both fish species (28 days at 25°C in bluegill compared to 3 months at 10°C in plaice), certain definitive stages can be recognized:

1 Infected fibroblast-like cells cease dividing and increase their size. These hypertrophied cells show basophilic cytoplasms and develop prominent nuclei and nucleoli (Flügel 1985).
2 As the cell enlarges, cytoplasmic inclusions surrounded by halo-like clear areas become evident. Electron microscopy studies revealed that these areas are viral factories (Spitzer *et al.* 1982).
3 During maturation, a hyaline capsule becomes clearly demonstrable by haematoxylin–eosin staining (Peters & Schmidt 1995). In gilthead sea bream the hyaline capsule is composed of sulphate- and carboxyl-glycoproteins (Gonzalez de Canales *et al.* 1996; Sarasquete *et al.* 1998).
4 Finally, lymphocysts reach a degenerative phase in which nuclei and nucleoli appear condensed and poorly defined (Smail & Munro 2001). Inclusions remain near the periphery, and the hyaline capsule degenerates. Macrophages and phagocytic cells appear around the degenerated lymphocysts and may invade them. Lymphocysts collapse may cause *de novo* infection of adjacent fibroblasts. Viral shedding appears to occur only after lysis of the lymphocysts.

Viral pathogenesis and host immunity

Data about LCDV pathogenesis are limited and are mainly restricted to histopathological studies of skin lesions (Gonzalez de Canales *et al.* 1996; Sheng & Zhan 2004; Sheng *et al.* 2007b). More recently, immunohistochemistry (IHC), immunofluorescence (IF) or *in situ* hybridization (ISH) techniques have been used to study the course of the viral infection, as well as the LCDV tropism (Xing *et al.* 2006; Sheng *et al.* 2007b; Cano *et al.* 2009a, 2013). These latter authors applied the IHC and ISH techniques for LCDV detection in gilthead sea bream specimens from the same population. Lymphocystis disease virus was detected in all tested organs (skin/fin, gills, intestine, liver, spleen and kidney), both in diseased and recovered fish. Fibroblasts, hepatocytes and macrophages appear to be target cells for virus replication. These results indicate that the virus establishes a systemic infection in gilthead sea bream and persists in fish for an unknown period after the disappearance of clinical signs (Cano *et al.* 2009a).

The DNA microarray technology is a useful tool for studying viral gene expression and allows the analysis of complex transcriptional profiles of large DNA viruses. In addition, this technique enables the characterization of host gene expression in response to viral infections, providing a better understanding of the underlying pathogenic mechanisms. The process of lymphocyst formation has been investigated in experimentally infected Japanese flounder, determining in parallel the viral genome amount and the changes in fish gene expression (Iwakiri *et al.* 2014). The LCDV genome was first detected from the dorsal fins at 14 days post-infection (dpi), and the amount of viral genomes gradually increased in synchrony with lymphocyst development. The number of host genes that change their expression levels increased dramatically between 28 and 42 dpi. The results of the microarray analysis suggested that apoptosis inhibition, cell cycle arrest and alterations of collagen fibres may be implicated in lymphocyst formation in Japanese flounder fin cells.

The viral factors involved in lymphocyst formation are currently unknown. However, at least two viral gene products encoded by LCDV-C, a G protein-coupled receptor homologue (GPCR) and the thymidylate synthase (TS), can mediate cellular transformation *in vitro* when expressed in fish cell lines. GPCR inhibits cellular apoptosis in

transient transfected fathead minnow (FHN) and *epithelioma papulosum cyprini* (EPC) cells (Huang *et al.* 2007), whereas FHN cells constitutively expressing TS showed a transformed phenotype (Zhao *et al.* 2008).

Little is known about immunity to LCDV, but there is the suggestion that by replicating primarily in the skin, LCDV is shielded from an antiviral response until late in infection (Chinchar *et al.* 2009). The proliferation of macrophages and epithelioid cells around lymphocysts in the dermis has been described as an immune response in several fish species (Roberts 1976; Colorni & Diamant 1995; Sheng *et al.* 2007b). Recently, Dezfuli *et al.* (2012) demonstrated that piscidin 3-expressing acidophilic granulocytes, but not mast cells, are recruited and activated in the dermis of gilthead sea bream in response to LCDV infection. In addition, an enhanced phagocytic capability was observed in head kidney cells from American plaice [*Hippoglossoides platessoides* (Fabricius, 1780)] suffering LCD (Marcogliese *et al.* 2001).

In Japanese flounder, the humoral immune response was monitored by antibody titration in sera from apparently healthy, lymphocystis diseased and recovered fish from an aquaculture facility, and the recovered animals showed the highest ELISA absorbance values (Nishida *et al.* 1998). Lorenzen and Dixon (1991) reported an increase in seroprevalence and antibody titres against LCDV in wild European flounder populations. These findings suggest that fish can recover from LCD and develop acquired immunity.

There are also an increasing number of studies on the *in vivo* modulation of innate immunogenes expression after LCDV infection. These include the IRF-3 (interferon regulatory factor 3), IRF-7, IRF-8 and IRF-9 in Japanese flounder (Hu *et al.* 2010, 2011, 2013, 2014), and the STAT2 (signal transducer and activator of transcription2) and STAT3 in turbot (Wang *et al.* 2011b, 2013). All of them are upregulated shortly after infection, which demonstrates their role in the antiviral response of the host. The anti-LCDV activity of two of the gilthead sea bream Mx proteins (SauMx1 and SauMx2) has been demonstrated *in vitro* using three clonal populations of transfected CHSE-214, which stably express each of the SauMx proteins (Fernandez-Trujillo *et al.* 2013).

The recent identification of a single major genetic locus controlling susceptibility to LCDV infection in Japanese flounder opens the way to selective breeding programs designed to develop flounder populations that are highly resistant to LCD (Fuji *et al.* 2006, 2007). More recently, Hwang *et al.* (2011) demonstrated that TLR-2 (Toll-like receptor 2) mapped with the previously reported microsatellite marker that is associated with LCDV resistance, suggesting a relationship between host immune response and disease resistance.

Diagnostic methods

Although LCDV infections are generally benign and self-limiting, there are commercial concerns due to market rejection caused by the warty appearance of infected animals. For this reason, the development of rapid and sensitive diagnostic tools is very important for controlling the spread of this viral disease. Rapid diagnostic methods are required during the course of an outbreak, whereas highly sensitive methods are required to detect subclinical viral infections in carrier fish (Sanz & Coll 1992).

Classically, the diagnosis of LCDV has been based on the observation of disease symptoms. However, the development of rapid and specific diagnostic tools to control the viral dissemination in fish farms is highly advised because neither effective treatments nor commercially available vaccines currently exist. At present, the only feasible measures for disease prevention in aquaculture systems are general prophylactic practices, such as the exhaustive control of the fish to be introduced into the aquaculture system, the use of effective decontamination methods to prevent viral transmission from asymptomatic brood stock to larvae and the supply of virus–free live food (Anders 1989; Yoshimizu 2009).

Virus isolation in cell cultures

The official method for diagnosis of fish viruses, established by the World Organization for Animal Health (OIE), is based on virus isolation using susceptible cell lines and further confirmation by serological or molecular techniques (OIE 2014). Although this procedure is widely accepted and currently used to validate other diagnostic methods, it is time-consuming, and its effectiveness largely depends on the cell line used.

Lymphocystis disease virus is not readily propagated in cell culture and showed a narrow host cell range; therefore, virus isolation usually requires homologous cell lines. The first report of LCDV isolation was from bluegill using a fry cell line (BF-2), although these viruses can also propagate onto a largemouth bass cell line (LBF-1) (Wolf *et al.* 1966). Bejar *et al.* (1997) developed a fin-derived cell line from gilthead sea bream (SAF-1) that supports the replication of LCDV isolated from this fish species (Perez-Prieto *et al.* 1999), as well as from European flounder, common dab and European plaice (Alonso *et al.* 2007). Some LCDV gilthead sea bream isolates also replicate to a lesser extent on BF-2 cells (Garcia-Rosado *et al.* 1999; Alonso *et al.* 2007). The cytopathic effects (CPE) caused by LCDV on SAF-1 cells are similar to those reported on BF-2 cells, consisting in cellular rounding and the enlargement and presence of cytoplasmatic inclusions that become evident after 3–10 days of incubation (Garcia-Rosado *et al.* 1999).

Iwamoto *et al.* (2002) tested 39 fish cell lines to propagate LCDV isolated from Japanese flounder, and only a homologous cell line (HINAE) exhibited CPE after 6–9 days of incubation. Similarly, Zhang *et al.* (2003) tested 13 fish cell lines for isolation of LCDV, recording viral replication only on two of them (GCO and GCK cell lines) derived from the grass carp [*Ctenopharyngodon idella* (Valenciennes 1844)]. Nevertheless, CPE characteristics induced by LCDV-C in these cell lines are markedly different from those previously described. LCDV-C can also be propagated onto the FG-9307 cell line, derived from Japanese flounder gill tissue, but in this case, the virus induced apoptotic cell death (Hu *et al.* 2004).

In the years since 2010, several studies have been performed to obtain effective cell lines for LCDV *in vitro* propagation and vaccine development. Researchers at the Chinese Academy of Fisheries Sciences (Qingdao, China) have established at least seven cell lines that have proven to be susceptible to LCDV-C and turbot reddish body iridovirus (TRBIV). The cell lines derived from different fish species, such as turbot [*Scophthalmus maximus* (Linnaeus 1758)], half smooth tongue sole [*Cynoglossus semilaevis* (Günther 1873)], brown-marbled grouper and stone flounder [*Kareius bicoloratus* (Basilewsky 1855)] (Wei *et al.* 2009, 2010; Sha *et al.* 2010; Wang *et al.* 2010; Xu *et al.* 2011a; Zhang *et al.* 2011; Zheng *et al.* 2012).

Serological techniques

Serological techniques are usually performed for viral identification after virus isolation on cell culture, or, alternatively, they can be used as diagnostic tools for direct viral detection in fish tissues (Sanz & Coll 1992). These techniques can also be applied to determine the presence of antibodies against a particular pathogen in the serum of fish (Hattenberger-Baudouy *et al.* 1995; LaPatra 1996).

Several serological techniques, such as indirect immunofluorescence, flow cytometry and immunoblot, have been used for LCDV detection in cell culture (Garcia-Rosado *et al.* 2002; Cano *et al.* 2006). Among these, the immunoblot assay using an antiserum against a 60-kDa viral protein showed the highest sensitivity (32 ng mL^{-1} of purified LCDV), allowing the detection of viral antigens in SAF-1 cells inoculated with 10 $TCID_{50}$ per mL at 5 days post-inoculation (p.i.). Virus detection by CPE development in the same cells (recorded at 14 days p.i.) was only possible with inoculation titres above 10^2 $TCID_{50}$ per mL. This immunoblot assay also proved to be effective for LCDV diagnosis in gilthead sea bream tissue homogenates, from both diseased and asymptomatic fish, although a previous amplification step in cell culture was required for asymptomatic samples (Cano *et al.* 2006).

Cheng *et al.* (2006) developed a panel of five monoclonal antibodies (MAb) to LCDV that have been applied for LCDV diagnosis in Japanese flounder using enzyme-linked immunosorbent assay (ELISA) and immunoblot (Xing *et al.* 2006). More recently, these MAbs have been used for the development of two rapid detection tools: a gold immunochromatographic test strip (Sheng *et al.* 2012) and an antibody microarray (Sheng *et al.* 2013). The sensitivity of both methods (1 and 0.55 μg mL^{-1} of purified LCDV, respectively) makes them suitable for detecting LCDV antigens in asymptomatic fish, as has been demonstrated by these authors. Moreover, the test strip allows on-site detection of LCDV without requiring specialized equipment or personnel.

The date, the detection of fish antibodies against a virus has not been accepted as a routine screening method for assessing the viral status of fish populations because of gaps in the knowledge on the serological response of fish to virus infections (LaPatra 1996). However, antibody detection can be used at the population level as an indicator of previous exposure to the virus (Hattenberger-Baudouy *et al.* 1995). The ELISA technique has been used to determine both the seroprevalence of the LCDV, as well as the determination of specific antibody titre, in several wild European flounder populations (Lorenzen & Dixon 1991; Dixon *et al.* 1996) and in cultured Japanese flounder (Nishida *et al.* 1998).

PCR-based techniques

New sanitary regulations for fish have been implemented, which include sampling for the detection of asymptomatic LCDV carriers; therefore, it is necessary to develop molecular tools for diagnostic purposes (OIE 2014).

Polymerase chain reaction (PCR) is a rapid, sensitive and highly specific technique for detecting iridoviral infections (Mao *et al.* 1997; Grizzle *et al.* 2003). In the case of LCDV, several PCR techniques based on the sequences of MCP-coding genes have been developed in recent years. Using this technique, Cano *et al.* (2007) successfully detected LCDV from different marine fish species (European flounder, common dab, European plaice and gilthead sea bream) collected from both northern and southern Europe. PCR combined with blot hybridization was shown to be adequate for virus detection in tissue homogenates of asymptomatic gilthead sea bream carriers (Cano *et al.* 2007, 2009a). Similar PCR-based assays have been developed by other authors to detect LCDV in other fish species such as the Japanese flounder, black rockfish, turbot, redwing sea robin [*Lepidotrigla microptera* (Günther 1873)] and white-spotted puffer [*Arothrom hispidus* (Linnaeus 1758)] (Xing *et al.* 2006; Hossain *et al.* 2007; Sheng *et al.* 2007a; Zhan *et al.* 2010).

The aforementioned studies have demonstrated the applicability of the PCR-based methods to detect LCDV in asymptomatic carriers, but they do not provide quantitative results that can be useful in epidemiological and pathological studies. Based on competitive PCR technology, Zan *et al.* (2007) established a semiquantitative method for LCDV detection in Japanese flounder tissues. Real-time PCR is a powerful technique that has been used for the detection and quantification of several viral fish pathogens, including different iridoviruses (Wang *et al.* 2006; Pallister *et al.* 2007; Gias *et al.* 2011), showing better sensitivity than conventional PCR. Regarding LCDV, Palmer *et al.* (2012) developed a real-time PCR assay using fluorogenic primers, which proved to be reliable in the detection and quantification of subclinically infected yellow perch. More recently, a new real-time PCR assay has been developed and applied for viral quantification in diseased and asymptomatic gilthead sea bream (Ciulli *et al.* 2015).

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a technique in which DNA is quickly amplified under isothermal conditions with high specificity and sensitivity (Notomi *et al.* 2000). Loop-mediated isothermal amplification-mediated diagnosis has been successfully used for the detection of viral pathogens in the aquaculture industry, including several iridoviruses (Caipang *et al.* 2004; Mao *et al.* 2008; Zhang *et al.* 2009; Ding *et al.* 2010; Sung *et al.* 2010; Min *et al.* 2013). Li *et al.* (2010) developed and evaluated a LAMP assay for the rapid detection of LCDV from both diseased and apparently healthy Japanese founders. The assay was found to be very specific because no cross-reactivity was obtained using other iridoviruses, and its detection limit was similar to that of real-time quantitative PCR. Due to LAMP amplifies under isothermal conditions (between 63 and 65°C), a thermal cycler is not required. In addition, LAMP products can be detected visually using several fluorescent dyes that bind to dsDNA, such as SYBR Green, calcein or ethidium bromide, or using the formation of a white precipitate, magnesium pyrophosphate, as a by-product of the amplification reaction. Therefore, LAMP can be widely used for viral diagnosis, particularly in resource-limited settings.

Disease control and prevention

Viral disease prevention and control rely on the application of specific prophylactic measures (i.e. vaccination) or, alternatively, on the use of general control strategies, such as improved husbandry and water quality, better nutrition and lower stocking densities (OIE 2014).

Control of LCDV in intensive culture operations would demand scrupulous disinfection procedures at all stages of production, screening and quarantine of each fish lot to be introduced, and treatment of raw sea water used in the fish facility (Bowden *et al.* 1995). However, only a few studies have been performed on physical and chemical treatments against LCDV. Havikrishnan *et al.* (2010c) used a bath treatment with formalin, hydrogen peroxide and Jenoclean for LCDV-infected Japanese flounder. The authors concluded that these chemical agents enhanced the fish innate immune response and increased the fish resistance to the disease. However, these treatments cannot be systematically applied in aquaculture practice. For this reason, these authors evaluated the effect of herbal extracts and probiotics added to the fish diet in the course of LCDV infection in Japanese flounder, concluding that they act as immunostimulants that reduce the incidence of LCD (Havikrishnan *et al.* 2010a,b).

Although there is no commercial vaccine available for LCDV infection, both inactivated and genetically engineered vaccines targeting LCDV have been designed and evaluated in recent years. Formalin- and heat-inactivated LCDVs were used as vaccines and proved to have a protective effect in Japanese flounder (Yoshimizu & Iwamoto 2001; Xu *et al.* 2011b). Nevertheless, its use is hampered by the necessity to obtain large amounts of purified virus particles directly from diseased fish lesions.

DNA vaccination is based on the administration of plasmid DNA (pDNA) encoding a protective antigen, rather than the antigen itself. The subsequent expression of the antigen by cells in the vaccinated hosts triggers the host immune response. A single intramuscular injection of low amounts of DNA induces rapid and long protection in fish against economically important viruses affecting aquaculture production (Lorenzen & LaPatra 2005). Zheng *et al.* (2006) designed a DNA vaccine against LCDV composed of a plasmid containing a 0.6-kbp fragment of the MCP gene of LCDV-C. The expression of several immune-related genes significantly increased after vaccination, and specific anti-LCDV immunoglobulins were also detected in the sera of vaccinated fish (Zheng *et al.* 2010). In addition, this vaccine induced effective protection against LCD in Japanese flounder after intramuscular injection (Zheng *et al.* 2011).

Oral DNA-based immunotherapy is a new strategy for fish immunization in intensive culture. However, the rate of degradation of DNA vaccines by nucleases and acidic conditions in the fish gastrointestinal tract may reduce vaccine efficiency. To avoid this, the vaccine DNA can be delivered encapsulated in micro- or nanoparticles that prevent its degradation. Microspheres of alginate, chitosan and poly/DL-lactide-co-glycolide (PLGA) were tested by Tian *et al.* (2008a,b,c) for oral delivery of the LCDV pDNA

vaccine cited above. Following immunization, the authors detected transgene expression in several organs from fish vaccinated with encapsulated pDNA. The encapsulated vaccine also induced higher levels of antibodies compared to control fish vaccinated with naked pDNA. Later, Tian and Yu (2011) demonstrated a significant increase in resistance to LCDV infection after oral administration of the pDNA vaccine encapsulated into PLGA nanoparticles.

Remarks and future perspectives

Aquaculture now accounts for almost 50% of fish consumed by humans, and it continues to be a fast-growing animal-food-producing sector. However, the growth rates for aquaculture production have recently started slowing, reflecting the impacts of a wide range of factors, with biosecurity risks being the most significant. These biosecurity risks include the spread of transboundary aquatic animal diseases, risks derived from the use of veterinary medical products, biological invasion of species from human-assisted introductions and climate change consequences (Hine *et al.* 2012). To solve these problems, the FAO (2010) has proposed a regulatory instrument governing biosecurity, including the FAO/WHO Codex Alimentarius Commission (concerned with food safety), the World Organization for Animal Health (OIE) (concerned with animal life and health) and the International Plant Protection Convention (concerned with plant life and health).

The rise of novel systems of intensive aquaculture, increased global movement of aquatic animals and their products, and several sources of anthropogenic stress to aquatic ecosystems have led to the emergence of many new diseases of fish, including those of viral origin (Walker & Winton 2010). The case of LCDV is of great interest because these viruses become a threat to aquaculture practice and are the source of important economic losses. Several studies have been performed on this virus focussing both on its molecular biology and pathogenesis, but more studies are needed to understand the mechanisms of LCDV infection and to establish disease control measures. The development of rapid, sensitive and suitable on-site diagnostic methods will be essential for the early detection of LCDV at the fish farm. Gene microarray technology will advance our understanding of both LCDV pathogenesis and cultured fish immunity to this virus. However, the main focus should be on the control of LCD. Interfering RNA (iRNA)-based gene therapies appear to be a promising approach to silence viral gene expression and to inhibit viral transcription in fish. Finally, other approaches will be improving of disease resistance by selective breeding and/or transgenic fish production. This will require the identification of the genetic loci linked to LCD resistance in commercial fish species susceptible to this viral disease, as it has been previously carried out for Japanese flounder (Fuji *et al.* 2006, 2007).

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