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**Development of new molecular methods for the diagnosis
and the study of viral diseases of fish**

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ABSTRACT

The increase in aquaculture operations worldwide has provided new opportunities for the transmission of aquatic viruses; the occurrence of viral diseases remains a significant limiting factor in aquaculture production and for the sustainability of biodiversity in the natural environment. Viruses that infect fish and cause disease are represented in 14 of the families listed for vertebrate viruses by the International Committee on the Taxonomy of Viruses. The viruses are the principal pathogens that are negatively impacting aquaculture both from an economic and sanitary point of view. For this reason, the ability to identify quickly the presence/absence of a pathogenic organism in fish would have significant advantages for the aquaculture systems. At present, the use of cultured eukaryotic cells in a controlled environment for virus propagation and isolation is widely accepted and is still considered the 'gold standard' for diagnosis of most viral diseases. The exponential growth of viruses in a cell culture system makes it a very sensitive method for virus detection if the cells used are of high susceptibility and quality, and if the cells are handled by experienced personnel. Furthermore, after the viral growth virus need to be identified and serological techniques have traditionally played this role. However, for correct diagnosis, several days of culture are required for cytopathic effects to be observed and subsequent virus identification. Molecular techniques have in recent years alongside the traditional ones, and in some cases, they have replaced traditional methods for the highest advantages in terms of speed and sensitivity of the diagnosis provided. Several molecular methods have found successful application in fish pathology both for confirmatory diagnosis of overt diseases and also for detection of asymptomatic infections. However, a lot of different variants occur among fish host species and virus strains and consequently specific methods need to be developed and optimized for each pathogen and often also for each host species.

For this reason this thesis focus on the molecular diagnosis of some relevant fish viral infections. The PhD dissertation consists of three chapters, the first chapter is a detailed

review entitled “Viral diseases of fish: molecular methods for diagnosis”. This work presents a complete description of the viruses that infect fish and cause disease taking an overview of viruses belonging to 14 different viral families and provides a relevant information regarding the most common methods and emerging technologies for the molecular diagnosis of viral diseases of fish. Moreover, the molecular methods currently available for diagnosis of viral infections in fish are reported and the advantages and disadvantages that they offer over conventional methods previously available are discussed.

The second and third chapters of the thesis consist of two experimental research for the set up of innovative techniques for the diagnosis of relevant fish diseases.

The second chapter is entitled “Development and application of a real time PCR assay for the detection and quantification of lymphocystis disease virus”. Lymphocystis disease virus (LCDV) is responsible of a chronic self-limiting disease affecting more than 125 teleosts. Viral isolation of LCDV is difficult, time consuming and often ineffective; the development of a rapid and specific tool to detect and quantify LCDV is desirable for both diagnosis and pathogenic study. In this study, a quantitative real-time PCR (qPCR) assay was developed using a Sybr Green based assay targeting a highly conserved region of MCP gene. Primers were designed on a multiple alignment, including all known LCDV genotypes. The viral DNA segment was cloned within a plasmid to generate a standard curve. Limit of detection was as low as 2.6 DNA copies/ μ l of plasmid and the qPCR was able to detect viral DNA from cell culture lysates and tissues 10 times lower than conventional PCR. Both gilthead seabream and olive flounder LCDV has been amplified and in silico assay showed that LCDV of all genotypes can be amplified. LCDV was detected in target and non-target tissues of both symptomatic and asymptomatic fish. The LCDV qPCR revealed to be highly sensitive, specific, reproducible and versatile to detect and quantify Lymphocystivirus and may be used also for asymptomatic carriers’ detection or pathogenesis study of different LCDV strains. The results of this study were recently published in *Journal virological methods* (2015).

The third chapter is entitled: “Development of a multiplex RT-PCR assay for simultaneous detection of the major viruses that affect rainbow trout (*Oncorhynchus mykiss*)”. In the last 10 years, rainbow trout (*Oncorhynchus mykiss*) has represented the second highest annually produced product in European aquaculture. The major viral diseases that affect rainbow trout are viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN) and sleeping disease (SD). In the presented study, we developed a multiplex RT-PCR (mRT-PCR) assay for the simultaneous detection of these four rainbow trout viruses in a single assay. The choice of primers was carried out based on the expected size of the fragments, the temperature and time required for the amplification, and the specificity for the target sequence. First, the method was optimized using reference strains of viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and sleeping disease virus (SDV) cultivated with permissive cell culture lines; subsequently, the method was used for the identification of these viral infections in rainbow trout samples. Twenty-two samples of rainbow trout, clinically suspected of having viruses were analyzed by the developed method to detect the presence of the four viruses, by directly analyzing the animal tissues. The mRT-PCR method was able to efficiently detect the viral RNA in infected cell culture supernatants and in tissue samples, highlighting the presence of single infections as well as co-infections in rainbow trout samples. VHSV/SDV and IHNV/SDV co-infections were demonstrated for the first time in rainbow trout. The mRT-PCR method was revealed to be an accurate and fast method to support traditional diagnostic techniques in the diagnosis of major viral diseases of rainbow trout.

CHAPTER I

VIRAL DISEASES OF FISH: MOLECULAR METHODS FOR DIAGNOSIS

1.1 Introduction

Over the past three decades, aquaculture has developed to become the fastest-growing food-producing sector in the world with 63.6 million tons production and 8.8 % annual growth rate. Driven by population growth, rising demand for seafood and a levelling of production from capture fisheries, the practice of farming aquatic animals has expanded rapidly to become a major global industry (FAO, 2012).

Farming of aquatic animals commonly involves displacement from their natural habitat to an environment that is new and sometimes stressful, the use of feeds that are sometimes live and often unnatural or artificial, and culture in stocking densities that are much higher than occur naturally. This has provided opportunities for exposure to new pathogens and conditions that can compromise defensive responses and facilitate pathogen replication and disease transmission (Walker and Mohan, 2009). Most importantly, the growth in aquaculture and increasing international trade in seafood has resulted in the rapid movement of aquatic animals and their products, with associated risks of the trans-boundary movement of pathogens (Walker and Winton, 2010).

The infectious diseases, represents the most limiting factor to aquaculture production because they increase the production cost due to the losses in dead fish, costs of treatments or decreased growth rate of diseased and convalescent fish. It is difficult to evaluate the real economic losses, due to the different factors related; however, it has been estimated that 10% of all cultured aquatic animals are lost as a result of infectious diseases (Blanco *et al.*, 2000). Among the causative agents of infectious diseases in aquaculture, the viruses are the principal pathogens that are negatively impacting aquaculture. Viral diseases have been difficult to control, due to the high susceptibility of aquatic animals at an early age, the lack of therapeutics, insufficient knowledge of the pathogenesis of viral infections and limited knowledge of natural resistance mechanisms in aquatic animals (Kibenge *et al.*, 2012).

Furthermore, aquaculture has been expanded, intensified, and diversified, based heavily on movements of live aquatic animals and animal products (broodstock, seed and feed). The world trade liberalization has accelerated the accidental spread and incursion of diseases into new populations and geographic regions.

Therefore, rapid detection and identification of pathogens is crucial to prevent viral transmission of disease and an effective disease management (Adam and Thompson, 2006).

Detection of aquatic animal viruses historically has been done by growth and isolation of viruses in living cell cultures appropriately chosen for the propagation of target virus. Subsequent virus identification through immunological or nucleotide procedures are then requested. The determination of a testing procedure is a complex decision involving factors of cost, timeliness, sensitivity, specificity, efficiency, and available host tissue and technology (Peters, 2004).

Many viral pathogens of animals are poorly characterized. To date, if a suspected new virus was identified and the virus could be cultured, morphology, physical characteristics, growth characteristics and antigenic nature were determined. However, this method of characterization is very time consuming and is limited to culturable viruses (in established cell lines or readily available primary cells). Usually, because of the time and expense, this characterization is limited to viruses that are associated with an important disease. However, a large portion of viruses is either unculturable, difficult to culture or are not associated with a disease of importance to justify in depth characterization or development of reliable serological reagents. Therefore, the development of broad spectrum diagnostic methods that obviate culture are needed as well as methods to bypass the cumbersome traditional methods of characterizing culturable viruses (Hanson *et al.*, 2006).

Last fifteen years, great advances took place in understanding the molecular biology of fish pathogens and their hosts. Molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish diseases and for the study of the epidemiology of viral, bacterial, and parasitic diseases. Detection of

nucleic acid molecules has demonstrated its usefulness for highlighting hardly culturable, non-culturable, and even dead microorganisms, generating appropriate novel or replacement technologies. The main advantages of molecular techniques are its higher sensitivity and specificity compared with other diagnostic methods such as serological assays and even culture methods in several cases, as well as its possibility to rapidly screen large numbers of samples during disease outbreaks (Cobo, 2012).

From an epidemiological point of view, the ability to screen rapidly numerous samples against pathogens are very important to prevent viral transmission of disease. Moreover, using nucleic acid as targets, molecular techniques can offer a tool for examining the relationships between genotypes of various pathogens, providing essential data for molecular epidemiology studies (Altinok and Kurt, 2003).

1.2 Fish Viruses

Viruses that infect finfish and cause disease are represented in 14 of the families listed for vertebrate viruses by the International Committee on the Taxonomy of Viruses (ICTV). The fish viruses containing DNA genomes are listed in the families *Iridoviridae*, *Adenoviridae*, and *Herpesviridae*, and those with RNA genomes are listed in the families *Picornaviridae*, *Birnaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Caliciviridae*, *Togaviridae*, *Nodaviridae*, *Retroviridae*, and *Coronaviridae* (Leong, 2008).

1.2.1 RNA Viruses of Fish

1.2.1.1 *Rhabdoviridae*

Fish rhabdoviruses are considered an important viral pathogen that affecting both wild and cultured fish throughout North America, Asia, and Europe (Kurath and Winton, 2008; Purcell, 2012). The first fish rhabdovirus was described in 1938 by Schaperclaus in European rainbow trout (*Oncorhynchus mykiss*). Since then, these viruses have been isolated, grown in tissue culture cells, and the genomes have been cloned and sequenced (Leong, 2008).

Members of this group are bullet-shaped, enveloped viruses and share a number of distinct features, including a simple negative-sense, single-stranded RNA (ssRNA) genome. The typical rhabdoviral genome encodes five basic structural proteins including the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large polymerase (L) protein (Fig. 1) (Bernard and Brémont, 1995). Three distinct genera of fish rhabdoviruses have been identified: *Novirhabdovirus* and *Perhabdovirus*, representatives of which have all been isolated from fish hosts, and *Vesiculovirus* (Whitfield *et al.*, 2011).

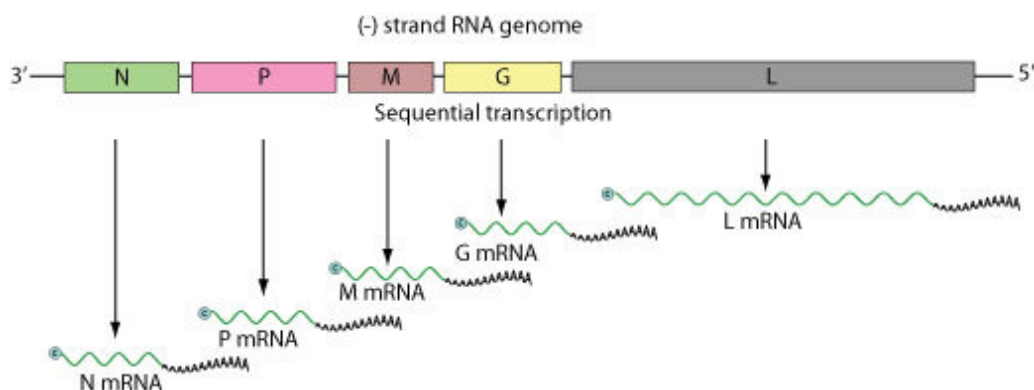


Figure 1. The typical rhabdoviral genome. Negative-stranded RNA linear genome, about 11-15 kb in size. Encodes for 5 to six proteins (http://viralzone.expasy.org/viralzone/all_by_species/76.html)

Members of the genus *Novirhabdovirus* are distinguished by the presence a sixth gene located in the genome between the G and the L genes (Fig. 2) encoding a non-structural ('non-virion' or NV) protein (Purcell, 2012). It has four important type species: *Infectious hematopoietic necrosis virus* (IHNV), *Viral haemorrhagic septicaemia virus* (VHSV), *Hirame rhabdovirus* (HIRVV) and *Snakehead rhabdovirus* (SHRV) (Gadd, 2013). Additional fish rhabdoviruses that are considered possible members of the *Novirhabdovirus* genus include eel viruses EEV-B12 and EEV-C26, but these require further genetic characterization to define their taxonomic status. The viruses in the four *Novirhabdovirus* species differ in several important aspects, including host and geographic range. Both IHNV and VHSV are globally important fish pathogens that are found in wild fish and cause significant disease burdens in fish reared in various aquaculture settings. IHNV has a relatively narrow host range restricted to cold-water salmon and trout fish, collectively referred to as 'Salmonids', in the families *Oncorhynchus* and *Salmo*. IHNV is endemic to western North America and it has been inadvertently spread to Europe and Asia by aquaculture related activities (Alonso *et al.*, 2003). In contrast, VHSV has extremely broad host range including 80 marine and freshwater fish species from diverse taxonomic families in Europe, North America and Asia. Although the major burden of VHSV in aquaculture is the disease in rainbow trout (*Oncorhynchus mykiss*) (Kurath and Winton, 2011). IHNV and VHSV are among the seven finfish viral species listed as 'notifiable' by the Aquatic Animal Health Code of the World Organization for Animal Health (OIE, 2014a) indicating their recognition as serious pathogen threats to global animal production systems. HIRVV affects hirame, the Japanese flounder (*Paralichthys olivaceus*), which is a highly prized food fish in Japan. Its host range includes ayu (*Pleuroglossus altivelis*) as well as salmonid fish, but it has been isolated outside of Asia (Leong, 2008). *Snakehead rhabdovirus* (SHRV), a rhabdovirus of warm-water fish, was isolated from a diseased snakehead fish (*Ophicephalus striatus*) during an epizootic outbreak in Thailand (Kasornchandra *et al.*, 1992).

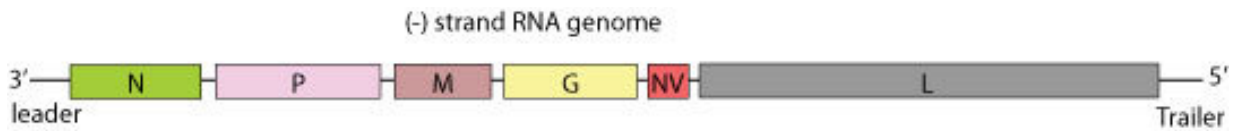


Figure 2. Novirhabdovirus genome. Negative-stranded RNA linear genome, about 11kb in size. Encodes for six proteins (http://viralzone.expasy.org/viralzone/all_by_species/76.html).

The genus *Vesiculovirus* comprises an ecologically diverse but genetically similar group of mammalian and fish rhabdoviruses. It currently contains the recognized species *Spring viremia of carp virus* (SVCV) as the type species and the tentative vesiculoviruses pike fry rhabdovirus (PFRV). However, a new genus has been proposed with the name *Sprivirus* and consist of both SVCV and PFRV (ICTV, 2013). *Spring viremia of carp virus* (SVCV) causes a highly contagious and serious disease of freshwater cyprinid fishes, generating significant economic and ecological impacts throughout the world (Phelps *et al.*, 2012). SVCV was first identified as the etiologic agent of an acute hemorrhagic disease in common carp (*Cyprinus carpio*) in Europe in 1972. Since then, the disease has been found in Asia, the Middle East, and most recently in South and North America (Leong, 2008). A growing number of fish viruses are related to viruses from the genus *Vesiculovirus*, such as the *Siniperca chuatsi rhabdovirus* (SCRV) and the *Starry flounder rhabdovirus* (SFRV) (Tao *et al.*, 2008; Talbi *et al.*, 2011). The *Scophthalmus maximus rhabdovirus* (SMRV), originally isolated from farmed turbot (*Scophthalmus maximus*) affected by lethal haemorrhagic disease in China, also has a phylogenetic relationship with the genus *Vesiculovirus*, but is genetically distinct from other rhabdoviruses (Zhang *et al.*, 2007; Zhu *et al.*, 2011).

A new genus of fish rhabdoviruses recognized since 2013 is *Perhabdovirus*. It has three species: *Perch rhabdovirus* (PRV), the type species, *Anguillid Rhabdovirus* (AngRV) and *Sea trout rhabdovirus* (STRV). Perhabdoviruses share morphological characteristics, genome organization and sequence similarities with vesiculoviruses and with viruses in the newly proposed genus *Sprivirus* (Gadd, 2013).

1.2.1.2 *Paramyxoviridae*

Members of *Paramyxoviridae* are causative agents of a number of diseases, with a host range that includes mammals, birds, reptiles and fish. The family constitutes a diverse group of enveloped viruses, which possess non-segmented, single stranded, negative sense RNA genomes (Lamb and Kolakofsky, 2001).

The first description of a paramyxovirus-like virus in fish was reported by Winton *et al.*, in 1985 during a routine health assessment of Chinook salmon juveniles in Oregon. This virus, now named *Pacific salmon paramyxovirus* (PSPV) grow slowly in established fish cell lines and have not been associated with disease in Salmonids (Batts *et al.*, 2008).

Miyazaki *et al.*, in 1989 reported another fish paramyxovirus that caused epidermal necrosis in juvenile black sea bream (*Acanthopargrus schlegeli*), this virus was identified in Japan by electron microscopy, but it was never cultured *in vitro* (Miyazaki *et al.*, 1989).

Atlantic salmon paramyxovirus (ASPV), a relatively recent addition to the family *Paramyxoviridae*, was first isolated in 1995, from Atlantic salmon (*Salmo salar*) suffering from proliferative gill inflammation (PGI) (Kvellestad *et al.*, 2003). PGI is a respiratory disease of Atlantic salmon, and has been associated with losses in Norwegian aquaculture since the 1980s, with an increase of outbreaks in the past years. The aetiology of the disease appears to be multifactorial. A primary causative agent has not yet been identified, but ASPV is associated with some cases (Kvellestad *et al.*, 2005). Recently, the *Atlantic salmon paramyxovirus* (ASPV) has been proposed as a species in the new genus *Aquaparamyxovirus* of the family *Paramyxoviridae* (Batts *et al.*, 2008).

1.2.1.3 *Orthomyxoviridae*

Infectious salmon anemia virus (ISAV) is the only fish *orthomyxovirus* that has been fully described to date. ISAV virions are pleiomorphic and enveloped with a diameter of 100–130 nm and 10–12 nm surface projections (Fig. 3).

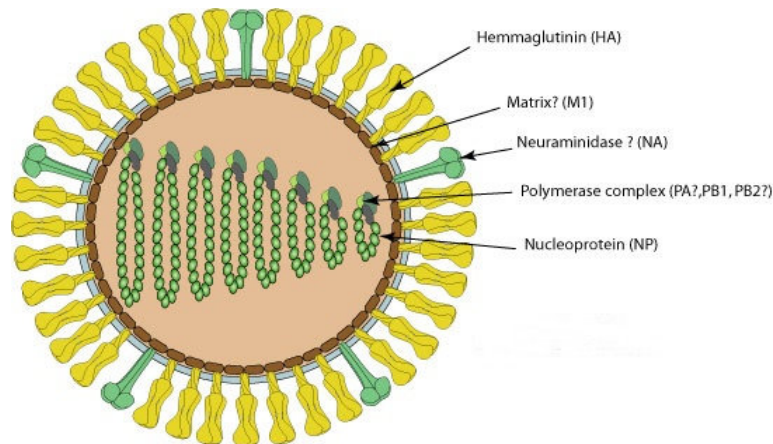


Figure 3. Structure of infectious salmon anemia virus (ISAV). The virions are enveloped and are 90-130 nm in diameter (http://viralzone.expasy.org/viralzone/all_by_species/223.html). .

The genome consists of eight single-stranded RNA segments: segment 1 encodes PB2, a component of the virion RNA polymerase; segment 2 encodes PB1; segment 3, the nucleocapsid protein NP; segment 4, the RNA polymerase PA; segment 5, acetylcholinesterase P3 or fusion protein; segment 6, hemagglutinin; segment 7, protein P4 and P5; and segment 8, proteins P6 and P7 (Fig. 4) (Leong, 2008).

Sequence analysis of these segments from different ISAV isolates consistently reveals two genotypes designated according to their geographic origin as European (Genotype I) and North American (GenotypeII) (Godoy *et al.*, 2008). A comparative sequence analysis of the PB1 gene of ISAV and other members of the *Orthomyxoviridae* led to its assignment as the type species of a new genus *Isavirus* (Leong, 2008).

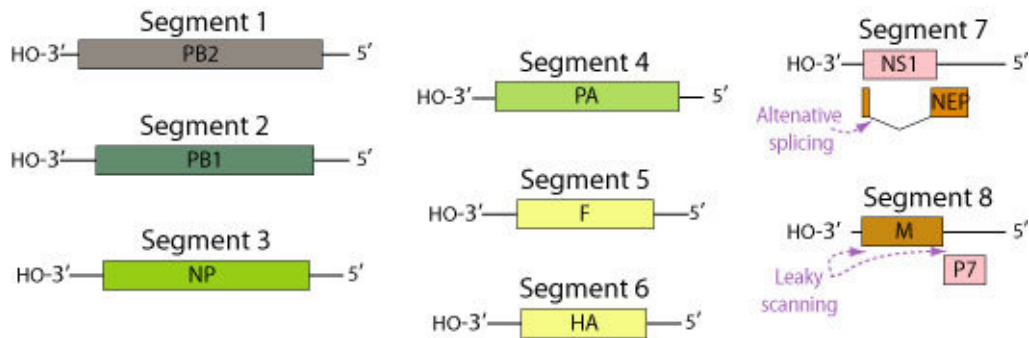


Figure 4. ISAV genome. Segmented ssRNA(-) linear genome, encapsidated by nucleoprotein (NP) Contains 8 segments coding for at least 8 proteins (http://viralzone.expasy.org/viralzone/all_by_species/76.html).

ISAV causes a highly lethal disease, which affected farmed Atlantic salmon displaying severe anemia, leucopenia, ascetic fluids, hemorrhagic liver necrosis, and petechiae of the viscera. The virus remains an emerging fish pathogen because of the asymptomatic infections in wild and farmed fish and the potential for emergence of new epizootic strains (Godoy *et al.*, 2014). Natural outbreaks of ISA have only been recorded in farmed Atlantic salmon, and in Coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge *et al.*, 2001). Subclinically infected feral Atlantic salmon and sea trout (*S. trutta*) have been identified (Kibenge *et al.*, 2004). Following experimental infection by bath immersion, ISAV has been detected by RT-PCR in rainbow trout (Biacchesi *et al.*, 2007), Atlantic herring (*Clupea harengus*) and Atlantic salmon (OIE, 2014b).

1.2.1.4 Picornaviridae

The first reported observation of picorna-like viruses in fish was made in 1988 from rainbow smelt (*Osmerus mordax*) in New Brunswick, Canada (Moore *et al.*, 1988). Since then, picornaviruses have been isolated from barramundi (*Lates*

calcarifer), turbot, sea bass (*Dicentrarchus labrax*), grass carp (*Ctenopharyngodon idella*), bluegill (*Lepomis macrochirus*), grouper (*Epinephelus tauvina*), Japanese parrotfish (*Oplegnathus fasciatus*), and salmonid fish. In most of these descriptions, the presumptive characterization of the etiologic agent as a picornavirus was based on growth in tissue culture cells and the observation of crystalline arrays in the cytoplasm of small virus particles with a size and morphology consistent with picornaviruses (Leong, 2008).

Members of the Picornaviridae are small (22–30 nm), non-enveloped viruses with icosahedral symmetry. Virions replicate cytoplasmically, form cytoplasmic inclusions and contain a single-strand of positive sense RNA (Iwanowicz *et al.*, 2000). A novel picornavirus was isolated from specimens of a diseased European eel (*Anguilla anguilla*) collected from Lake Constance in the Rhine River. This eel presented symptoms of haemorrhages at the head and the tail. EPV has a typical picornavirus genome layout, but its low similarity to known viral proteins suggests a novel species in the family *Picornaviridae* (Fichtner *et al.*, 2013). In many cases, sick fish infected with these viruses contain picorna-like virus particles in the brain and medulla and the victims display corkscrew-like swimming and eventually death (Leong, 2008).

1.2.1.5 *Nodaviridae*

All nodaviruses characterized from fish belong to the genus *Betanodavirus* which, together with the genus *Alphanodavirus* whose members infect insects, constitute the family *Nodaviridae*. *Betanodavirus* particles are small (20–30 nm), with an icosahedral shaped capsid and a naked single stranded RNA (positive sense) genome consisting of the two segments RNA1 and RNA2. The RNA1 (ca 3.1Kb) encodes the RNA-dependent RNA polymerase (RdRp) and the RNAi antagonist protein B2, whereas the RNA2 (ca 1.4 kb) encodes the capsid protein (Fig. 5) (Munday *et al.*, 1992).

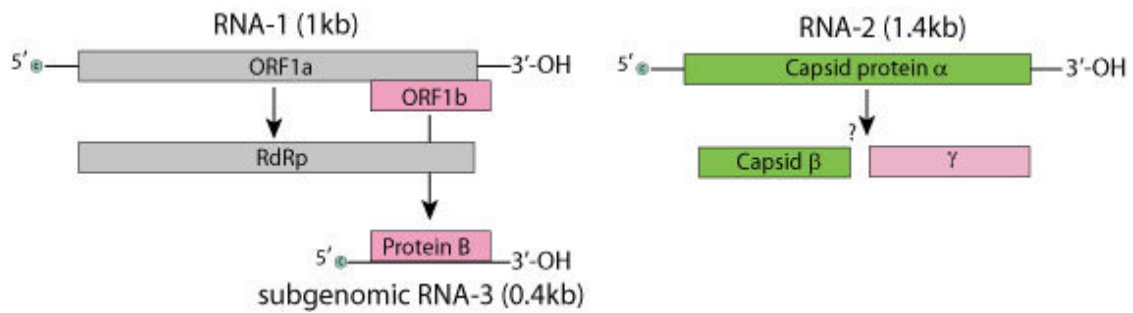


Figure 5. Segmented, bipartite linear, ssRNA(+) genome composed of RNA1=3.1 kb and RNA2=1.4 kb. Each genome segment 5' end is capped. The 3'end has no poly (A) tract (http://viralzone.expasy.org/all_by_species/614.html).

The betanodaviruses are the causative agents of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) in a variety of farmed marine fish. Yoshikoshi and Inoue (1990) were the first to report viral nervous necrosis (VNN) in hatchery-reared larvae and juveniles of Japanese parrotfish (*Oplegnathus fasciatus*), the disease was named VNN due to the clinical signs. To date, the disease has been reported in more than 50 fish species, mainly marine with the greatest impact being in striped jack (*Pseudocaranx dentex*), European sea bass (*Dicentrarchus labrax*), groupers, and flatfishes (Munday *et al.*, 2002; Sano *et al.*, 2011). The disease mainly affects the larval and juvenile stages. Brain, spinal cord and retina are considered the target organs in which the virus actively replicates causing extensive tissue vacuolization (Munday *et al.*, 2002). Genomic classifications of the betanodaviruses, traditionally, group these into four subtypes designated *Barfin flounder nervous necrosis virus* (BFNNV), *Striped jack nervous necrosis virus* (SJNNV), *Red-spotted grouper nervous necrosis virus* (RGNNV), and *Tiger puffer nervous necrosis virus* (TPNNV) (Nishizawa *et al.*, 1997). A novel subtype of nodavirus from turbot (*Psetta maxima*), was recently described (Johansen *et al.*, 2004) and is pending recognition as a fifth subtype within the betanodaviruses (Hodneland *et al.*, 2011).

Among the betanodavirus genotypes, the host range of TPNNV is limited to tiger puffer (*Takifugu rubripes*), whereas SJNNV which was initially isolated almost

exclusively in striped jack, have been detected in farm of sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) (Mori *et al.*, 1992; Munday *et al.*, 2002). The BFNNV genotype is regarded as the cold-water clade and several species from Northern-Europe, Atlantic coast of North America and Japan, have been shown to be susceptible to this genotype. Virus from this clade has been isolated from cold-water species such as Pacific cod (*Gadus microcephalus*) (Nishizawa *et al.*, 1997), winter flounder (*Pleuronectes americanus*) (Barker *et al.*, 2002), Atlantic cod (*Gadus morhua*) and Dover sole (*Solea solea*) (Starkey *et al.*, 2001), Atlantic halibut (*Hippoglossus Hippoglossus*) (Grotmol *et al.*, 1995) and haddock (*Melanogrammus aeglefinus*) (Johnson *et al.*, 2002). RGNNV genotype was found to have a broad host range causing disease in a wide variety of warm water fish species, particularly groupers and sea bass (Shetty *et al.*, 2012). Furthermore, the salinity seems to have low influence on the occurrence of the disease, some studies, in fact, have shown susceptibility of fish in freshwater (Hegde *et al.*, 2003) or fish reared in freshwater (Athanasopoulou *et al.*, 2003).

1.2.1.6 *Nidovirales*

The family *Coronaviridae* comprises two genera, *Coronavirus* and *Torovirus*, and is classified together with the families *Arteriviridae* and *Roniviridae* in the order *Nidovirales*. Members of the *Coronaviridae* share the common feature of pleomorphic, enveloped virions with diameters of 126–160 nm and prominent surface projections. The nucleocapsid is helical and contains a single molecule of linear, positive sense ssRNA (Leong, 2008).

Two nidoviruses have been detected in fish, the *White bream virus* (WBV) and the fathead minnow nidovirus (FHMNV). The first report of *White bream virus* (WBV) was recovered from a white bream (*Blicca bjoerkna* L.) collected during a routine examination of wild fish (Granzow *et al.*, 2001). A comprehensive analysis of the complete WBV genome (Schutze *et al.*, 2006) revealed that the virus was sufficiently

distinct to represent the type species (White bream virus) of a novel genus *Bafinivirus* within the subfamily *Torovirinae* of the family *Coronaviridae* (De Groot *et al.*, 2012). FHMNV has been isolated from both healthy and diseased fathead minnows (*Pimephales promelas*) in the midwestern portion of the United States. The isolate was initially characterized as a bacilliform virus, possibly belonging to the family *Rhabdoviridae*, although the virus had the uncharacteristic property of inducing syncytia in infected cell cultures (Iwanowicz & Goodwin, 2002). Based on phylogenetic analysis FHMNV appears to represent a second species in the genus *Bafinivirus* (Batts *et al.*, 2012).

1.2.1.7 *Togaviridae*

The family *Togaviridae* comprises the genera *Alphavirus* and *Rubivirus* among the vertebrate viruses. The genus *Alphavirus* includes pathogens of salmonids (McLoughlin & Graham, 2007) and marine mammals (La Linn *et al.*, 2001). These viruses have spherical virions, 70 nm in diameter, with a lipid envelope containing glycoprotein peplomers. The positive-sense ssRNA genome containing two ORFs: one occupying the 5' two-thirds of the genome and encoding four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4 or RdRp) and the other occupying the 3' one-third of the genome and encoding five structural proteins including the capsid protein and two or three envelope glycoproteins (Fig. 6) (Powers *et al.*, 2001; McLoughlin & Graham, 2007).

Salmonid alphaviruses (SAVs) are recognized as serious pathogens of farmed Atlantic salmon and rainbow trout in Europe (McLoughlin & Graham, 2007). Strains of salmonid alphavirus (SAV), identified to date have been assigned to six subtypes by phylogenetic studies (designated SAV subtypes 1–6) based on the analysis of partial E2 gene sequence data (Fringuelli *et al.*, 2008).

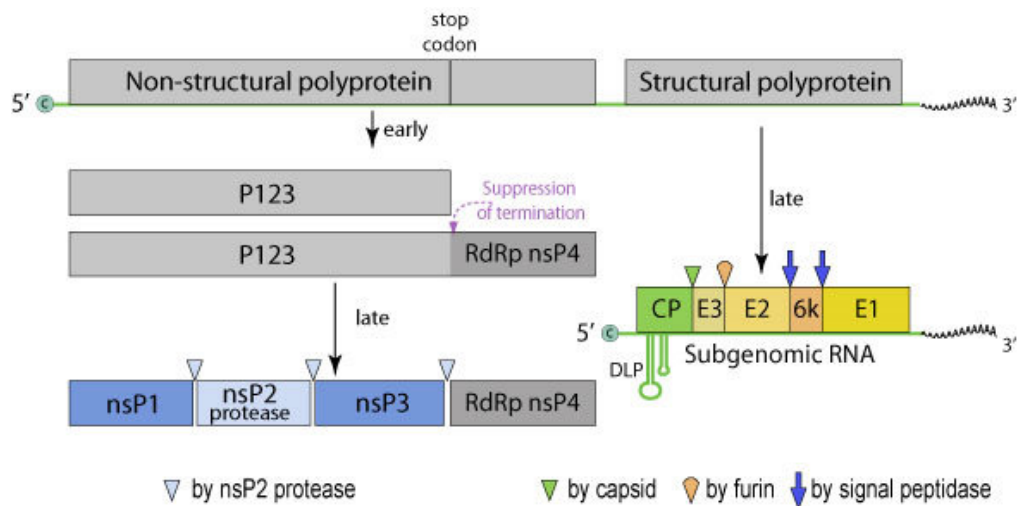


Figure 6. Alphavirus genome structure. Monopartite, linear, ssRNA (+) genome of 11-12 kb (http://viralzone.expasy.org/viralzone/all_by_species/625.html)

To date, all freshwater SAV strains collected from rainbow trout have belonged to SAV subtype 2 (Weston *et al.*, 2005; Fringuelli *et al.*, 2008). Prior to 2011, all genotyped strains of SAV reported from both Atlantic salmon and rainbow trout in Norway have belonged to SAV subtype 3 (Hodneland *et al.*, 2005; Karlsen *et al.*, 2006; Jansen *et al.*, 2010). Consequently, it has been assumed that all Norwegian SAV isolates belong to the SAV 3 cluster. However, in 2011, the detection of SAV subtype 2 like viruses in farmed marine Atlantic salmon was reported for the first time in Norway (Graham *et al.*, 2012). In contrast, viruses belonging to multiple subtypes (SAV 1, 2, 4, 5 and 6) have been reported from marine salmonid production in Ireland and Scotland (Fringuelli *et al.*, 2008; Graham *et al.*, 2012).

1.2.1.8 *Caliciviridae*

Viruses in the family *Caliciviridae* are known to infect a variety of marine species, including mammals, birds, fish, and invertebrates (Lang *et al.*, 2009). The family *Caliciviridae* is separated into five genera: *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* (ICTV, 2015). The vesiviruses include a group of viruses

characterized as marine caliciviruses, which was first isolated from California sea lions (*Zalophus californianus*) in 1972 (Smith *et al.*, 1973). Later, several species of pinnipeds and cetaceans (including seals, sea lions, walruses, whales, and dolphins), as well as an ocean fish, the opaleye perch (*Girella nigricans*), have been found to be susceptible to calicivirus infection (Smith *et al.*, 1980).

All the caliciviruses are nonenveloped with icosahedral symmetry. The genome consists of a linear, positive-sense ssRNA (Leong, 2008).

Recently has been identified a novel calicivirus in Atlantic salmon reared in Norway. The virus, named Atlantic salmon calicivirus (ASCV), has a high prevalence in farmed salmon and is found in fish suffering from several diseases and conditions and presumably also in healthy fish. Phylogenetic analysis based on the putative capsid encoding genome region did not cluster the virus with members of the marine *calicivirus* subgroup of the *Vesivirus* genus or any other virus of the known *calicivirus* genera or unclassified *calicivirus*, and may represent a new *calicivirus* genus (Mikalsen *et al.*, 2014)

1.2.1.9 *Retroviridae*

The family *Retroviridae* consists of two subfamilies, the *Orthoretrovirinae*, containing six genera, and the *Spumaretrovirinae*, containing only one genus. The piscine retroviruses constitute the genus *Epsilonretrovirus*, a genus established within the *Orthoretrovirinae* precisely to include the piscine retroviruses: *Walleye dermal sarcoma virus* (WDSV), *Walleye epidermal hyperplasia virus* type 1 (WEHV-1), *Walleye epidermal hyperplasia virus* type 2 (WEHV-2) (Leong, 2008). Two additional viruses, perch epidermal hyperplasia virus types 1 and 2 (PEHV-1, PEHV-2), are likely members of this group, but their sequences are incomplete. The exogenous piscine retroviruses, snakehead retrovirus (SnRV) and salmon swim bladder sarcoma-associated virus (SSSV) and the zebrafish endogenous retrovirus (ZFERV) have not

yet been assigned to a specific genus (Shen and Steiner, 2004; Rovnak and Quackenbush, 2010).

There are also numerous reports of C-type (retrovirus-like) particles of about 110–150 nm in epidermal papillomas of European smelt (*Osmerus eperlanus*) and in cells cultured from neurofibromas of damselfish (*Pomacentrus partitus*) (Leong, 2008). A retrovirus has also been suggested as the etiological agent of plasmacytoid leukemia in chinook salmon and was designated salmon leukemia virus (SLV) (Eaton and Kent, 1992).

Retroviruses from two proliferative skin lesions in walleye (*Sander vitreus*), walleye dermal sarcoma (WDS) and walleye epidermal hyperplasia (WEV), have been isolated and their sequence determined (Holzschu *et al.*, 1995; Rovnak *et al.*, 2007). These proliferative diseases were first reported by Walker in 1969 in walleye collected from Oneida Lake in New York State and have been reported to occur elsewhere in North America (Walker, 1969; Yamamoto *et al.*, 1985). The most interesting and defining feature of these proliferative diseases is their seasonal cycle (Bowser and Wooster, 1991). The highest incidence of disease occurs throughout the late fall until the spring spawning period at which time the lesions naturally regress. WEH lesions are broad, flat, translucent plaques of thickened epidermis that range in size from 2 to 3 mm up to 50 mm in diameter. WDS are cutaneous mesenchymal neoplasms that are randomly distributed on the body of the fish, arise from the superficial surface of the scales and range in size from 0.2-1.0 cm in diameter (Rovnak and Quackenbush, 2010). WDSV contained three additional open reading frames, ORF A, ORF B and ORF C. ORF A encodes a D-cyclin homolog (retroviral cyclin) that localizes in the nucleus of tumor cells in interchromatic granule clusters. ORF B directly interacts with the receptor for activated C kinase (RACK1) which leads to the activation of the protein kinase C signaling pathway. ORF C encodes a cytoplasmic protein that targets the mitochondria and is associated with apoptosis. It is expressed in regressing tumors when full-length viral RNA is synthesized. (Leong, 2008; Rovnak and Quackenbush 2010).

Perch epidermal hyperplasia virus type 1 and type 2 are retroviruses associated with hyperplastic lesions found in yellow perch (*Perca flavescens*). These lesions are similar to walleye epidermal hyperplasias and occur as thickened plaques on the fish's body (Lepa and Siwicki, 2011).

The snakehead retrovirus was first reported as a spontaneously productive infection of fish cell line SSN-1 derived from striped snakehead fish (*Ophicephalus striatus*). Examination by electron microscopy revealed C-type virus particles. Cell culture supernatants demonstrated high levels of RT activity and induced a cytopathic effect in the BF-2 cell line derived from bluegill fry (*Lepomis macrochirus*). All fish from which these cell lines were derived appeared clinically healthy (Frerichs *et al.*, 1991). Experimental infection of juvenile snakehead fish with SnRV showed no lesions in any of the infected fish (Frerichs *et al.*, 1993). The SnRV genome differs from the retroviruses of walleye because it has no ORF between the Unique region in the 5' LTR (U5) and the gag region (Leong, 2008).

The first outbreak of neoplastic disease involving the swim bladder of Atlantic salmon was observed in 1976, at a commercial fish farm in Scotland (Duncan, 1978). The affected fish were in poor physical condition, had swollen abdomens and presented multinodular masses on the external and internal surfaces of the swim bladder (Lepa and Siwicki, 2011). The swim bladder sarcoma virus (SSSV) provirus is 10.9 kbp in length with a simple gag, pro-pol, env gene arrangement similar to that of murine leukemia virus-like simple retroviruses. Phylogenetic analysis of pol sequences suggests that SSSV is most closely related to the sequenced zebrafish endogenous retrovirus (ZFERV) and that these viruses represent a new group of piscine retroviruses (Leong, 2008).

Zebrafish endogenous retrovirus (ZFERV) was originally isolated from the thymus of zebrafish (*Danio rerio*). The virus was detected in the sperm of different fish at the same genetic locus, indicating that it is an endogenous virus. The genome of ZFERV is 11.2 kb in length, and, like all retroviruses, contains three principal genetic domains (gag, pro-pol, env), flanked by LTRs. Gag and pro-pol genes are in the same

open reading frame. Phylogenetic analysis has shown that ZFERV is closest to murine leukemia virus (MLV)-related retroviruses and to walleye fish retroviruses, although the genome structure is more similar to MLV-related retroviruses (Shen and Steiner, 2004).

1.2.1.10 *Reoviridae*

Reoviruses that infect aquatic animals are grouped in the genus *Aquareovirus* in the family *Reoviridae* and are characterized by a nonenveloped double capsid shell, 11 segments of double-stranded RNA and seven structural proteins (VP1-VP7) which compose the viral particle including the outer capsid and central core (Fang *et al.*, 2005). Aquareoviruses were first isolated in the 1970s from North-American cyprinids and were initially referred to as ‘reoviruslike’ or ‘rotavirus-like’ aquatic viruses (Plumb *et al.*, 1979). They have subsequently been found in a wide variety of aquatic animals, including molluscs, finfish and crustaceans. Although these viruses are often isolated from apparently healthy individuals, they can also cause significant clinical signs and even severe disease (Fang *et al.*, 1989). Seven *Aquareovirus* species have been recognised by the International Committee for the Taxonomy of Viruses (ICTV) (*Aquareovirus A* to *Aquareovirus G*), although several other viruses have also been isolated, which may represent additional species (Attoui *et al.*, 2011). Aquareoviruses represent a serious threat to fish breeding and typical clinical signs of infection include haemorrhages, which can be severe (Fang *et al.*, 1989). Grass carp reovirus (GCRV) a strain of *Aquareovirus C*, causes an important disease in Asia, characterised by severe haemorrhage and up to 80% mortality in fingerling and yearling grass carp (Fang *et al.*, 1989). Striped bass reovirus (SBRV) included in the species *Aquareovirus A* was isolated from a moribund striped bass (*Morone saxatilis*) that was also infected with bacteria (Samal *et al.*, 1990). Golden shiner reovirus (GSRV) is a strain of *Aquareovirus C* that was originally isolated in 1977 from a moribund golden shiner bait fish (*Notemigonus crysoleucas*) (Plumb *et al.*, 1979). Even if GSRV is associated

with losses of bait fish in the USA, it is nearly identical to a Chinese isolate of GCRV (Attoui *et al.*, 2002). This virus is a significant pathogen of farmed grass carp and fathead minnows (*Pimephales promelas*) but has also been isolated from wild creek chub fish (*Semotilus atromaculatus*) in the USA (Goodwin *et al.*, 2006a). American grass carp reovirus (AGCRV) is a new member of the species *Aquareovirus G* isolated in the USA, from grass carp and golden shiner. This virus was implicated in a winter die-off of grass carp fingerlings on a commercial farm in Arkansas in the USA during 2005. Phylogenetic analyses indicate that golden ide reovirus (GIRV), isolated in Germany represents a second isolate of *Aquareovirus G* (Neukirch *et al.*, 1999).

1.2.1.11 Birnaviridae

The family *Birnaviridae* includes four genera: *Aquabirnavirus*, *Avibirnavirus*, *Blosnavirus* and *Entomobirnavirus* (ICTV, 2015).

Aquatic birnaviruses are the most abundant and diverse and are grouped in two separate genera: the *Aquabirnavirus* with the *Infectious pancreatic necrosis virus* (IPNV) and *Tellina virus* (TV) type species, and the genus *Blosnavirus* with the *Blotched snakehead virus* (BSNV) type specie (Da costa *et al.*, 2004).

Members of the genus *Aquabirnavirus* have nonenveloped, icosahedral capsids 60–70nm in diameter, and genomes composed of two segments, A and B, of dsRNA. Segment A encodes a polyprotein which is post-translationally cleaved to form three viral proteins VP2, VP3 and VP4, with VP2 epitopes being responsible for serotype specificity and the target for neutralizing antibodies. Segment B encodes VP1, an RNA-dependent RNA polymerase (Fig. 7) (Dobos *et al.*, 1995).

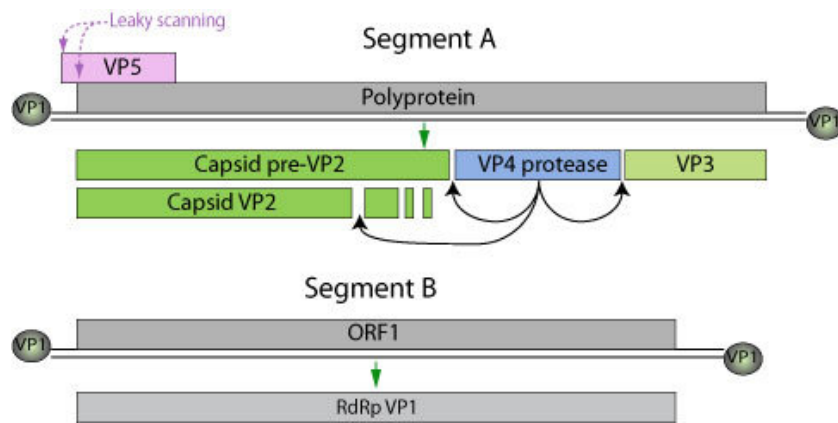


Figure 7. Aquabirnavirus genome structure. Segmented linear dsRNA genome with 2 segments (A,B) (http://viralzone.expasy.org/viralzone/all_by_species/571.html).

The type species *Infectious pancreatic necrosis virus* (IPNV) is the agent of infectious pancreatic necrosis (IPN), a highly contagious viral disease of salmonids, which occurs in all major salmon farming countries (Kibenge *et al.*, 2012). Mortality rates associated with disease outbreaks can be quite variable (5–100%) and it is probable that several host, viral and environmental factors influence the severity of the outbreak (Crane and Hyatt, 2011). Serological classification showed that the IPNV strains are divided into two serogroups (A and B). Within serogroup A, nine distinct serotypes were identified (Hill and Way, 1995; Dixon *et al.*, 2008), whose classification on the basis of deduced amino acid similarities of VP2 demonstrated that the strains clustered into 6 genogroups (Blake *et al.*, 2001) which tend to correlate with geographical and serological characteristics (Crane and Hyatt, 2011). Fish that are exposed to IPNV but either survive disease or do not develop clinical disease may become lifelong carriers of the virus, serving as IPNV reservoirs in populations and transmitting the virus vertically to progeny via the egg (McAllister *et al.*, 1987). IPNV has a wide variety of host species and persistent carriers among recovered hosts, for this reason it is difficult to eradicate, once established (Kibenge *et al.*, 2012).

1.2.2 DNA Viruses of Fish

1.2.2.1 *Iridoviridae*

The family *Iridoviridae* currently contains five genera: *Iridovirus*, *Lymphocystivirus*, *Ranavirus*, *Megalocystivirus* and *Chloriridovirus*. The genera *Lymphocystivirus*, *Ranavirus*, and *Megalocystivirus* contain all of the known iridoviruses that infect fish. Their common features are icosahedral virions, 120–350 nm in diameter that may acquire an envelope, and a viral genome consisting of one molecule of linear dsDNA of 100–303 kbp (Leong, 2008).

Lymphocystis disease virus (LCDV) is the etiological agent of this chronic and self-limited lymphocystis disease (LCD), described in over of 125 species of fish throughout the world (Noga, 2010). The lymphocystis disease occurs both in marine and freshwater fish and is generally associated with stress conditions related to farming, although it is commonly described also in free-living animals (Alonso *et al.*, 2005). Infected fish exhibit macroscopic and nodular lesions on the body surface because of rapid replication and subsequent inhibition of mitosis in the host's connective tissue cells. The hypertrophied cells, called lymphocysts, are common on skin, fins and occasionally around the mouth (Samalecos, 1986). Although this disease is rarely fatal, the infected fish are more prone to infection by other microorganisms, exhibit anaemia and may have a considerably reduced growth rate (Iwamoto *et al.*, 2002). Therefore, the appearance of this viral infection results in an important economic loss to the aquaculture industry because the diseased fish are not commercially viable. Based on the MCP gene sequence and pathogenicity of lymphocystiviruses, the viruses were divided into nine genotypes. Each genotype includes only one or a very limited number of fish host species: genotype I includes LCDV-1 isolated from the European flounder (*Platichthys flesus*), genotype II consists of Japanese flounder (*Paralichthys olivaceus*) isolates, genotype III of rockfish (*Sebastes schlegeli*) isolates, genotype IV of the sea bass (*Lateolabrax sp.*) and cobia

(*Rachycentron canadum*) isolates, genotype V of painted glass fish (*Pseudambassis baculis*) isolates, genotype VI of gourami (*Trichogaster leeri*, *T. trichopterus*) isolates, genotype VII of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), genotype VIII of largemouth bass (*Micropterus salmoides*) isolate and genotype IX of yellow perch strain (Hossain *et al.*, 2008; Cano *et al.*, 2010; Palmer *et al.*, 2012).

Ranaviruses and megalocytiviruses are recently emerged pathogens. All viruses included in these genera cause severe systemic disease, occur globally and affect several host species. In contrast, lymphocystiviruses cause superficial lesions and rarely cause the death of the fish (Whittington *et al.*, 2010). The ranavirus *Epizootic haematopoietic necrosis virus* (EHNV) from Australia was the first iridovirus to cause epizootic mortality in finfish (Langdon and Humphrey, 1987). The ranavirus *European catfish virus* has resulted in periodic high mortality epizootics among cultured European catfish including sheatfish, brown bullheads (*Ameiurus nebulosus*), and black bullheads (*Ameiurus melas*). Epizootics of Frog Virus 3-like viruses have been reported among cultured sleepy gobies (*Oxyeleotris marmoratus*) in Thailand (Prasankok *et al.*, 2002). There is still uncertainty surrounding the taxonomy of some putative ranaviruses such as *Singapore grouper iridovirus* (SGIV) and *Santee-Cooper ranavirus* (SCRV), both of which cause serious disease in fish. *Megalocytivirus* is divided into three major groups; *Infectious spleen and kidney necrosis virus* (ISKNV) which is reported to cause disease in numerous marine and freshwater fish species, red sea bream iridovirus (RSIVD) that mainly infects red sea bream (*Pagrus major*) and turbot reddish body iridovirus (TRBIV) that is reported to infect Asian flounder species (Subramaniam *et al.*, 2012).

1.2.2.2 Herpesviridae

Herpesviruses are large double-stranded DNA viruses that infect mammals, birds and fish. They have been classified into three separate families: *Herpesviridae*,

which, predominantly, includes pathogens of mammals, birds and reptiles; *Alloherpesviridae*, that consists predominantly pathogens of fish and amphibians; *Malacoherpesviridae*, which was identified in mollusk (oyster) (Davison *et al.*, 2009). Even though alloherpesviruses are distantly related to *Herpesviridae*, there are many similarities in the way they infect, replicate and persist in the host. The three main characteristics are a high level of host specificity, the apparent ability to intricately interact with the host defenses and the ability to establish long-term latency (Hanson *et al.*, 2011).

Herpesviruses are associated with disease outbreaks in over 14 species of fish. Alloherpesviruses that infect fish are grouped in three genus: *Ictalurivirus*, *Cyprinivirus* and *Salmonivirus* (ICTV, 2015). The genus *Cyprinivirus* includes three species of ciprinid herpesviruses (CyHV1, CyHV2 and CyHV3) and the *Anguillid Herpesvirus 1* (AngHV1). The genus *Ictalurivirus* contains *Ictalurid herpesvirus 1* and *Ictalurid herpesvirus 2* (IcHV1 and IcHV2) and *Acipenserid herpesvirus 2* (AciHV2). The genus *Salmonivirus* contains *Salmonid herpesvirus 1, 2 and 3* (SalHV1, SalHV2 and SalHV3) (Waltzek *et al.*, 2009; Doszpoly *et al.*, 2011). *Ictalurid herpesvirus 1* is also known as channel catfish virus (CCV) and has been isolated from channel catfish (*Ictalurus punctatus*) (Wolf and Darlington, 1971) and black bullhead (*Ameiurus melas*) (Alborali *et al.*, 1996). Clinical signs of CCV disease include erratic swimming, exophthalmia, distended abdomen and haemorrhage at the fin bases. Epizootics usually involve high mortality and occur sporadically on commercial fish farms in the southern United States during the summer months (Gray *et al.*, 1999). The DNA sequence of the entire CCV genome has been determined and the DNA consists of a 97 kbp unique long component (UL) encoding 65 open reading frames (ORF) bracketed by 18±5 kbp left and right direct repeats (DRL and DRR), each encoding 14 ORF (Davison, 1992). *Acipenserid herpesvirus 2* (AciHV-2), commonly named white sturgeon herpesvirus 2 (WSHV-2), is associated with the disease of farmed and wild white sturgeon, *Acipenser transmontanus*, in North America and Italy. (Hedrick *et al.*, 1991; Lepa and Siwicki, 2012).

The *Cyprinid herpes virus 2* (CyHV2) produces a systemic disease with lesions in hematopoietic tissue in common goldfish (*Carassius auratus*) (Goodwin *et al.*, 2006b). *Cyprinid herpes viruses 1* (CyHV1) is also known as carp herpesvirus, carp pox virus, and Herpesvirus cyprini. CyHV1 disease is most frequently characterized by mucoid to waxy epidermal growths on the skin of common and koi carp (*Cyprinus carpio*) (Hedrick *et al.*, 2000). CyHV3 disease occurs as epizootics in common and koi carp and has also been described as koi herpesvirus disease (KHVD). KHVD has been added to the list of notifiable diseases to the World Organisation of Animal Health (OIE) and it is also listed as a non-exotic disease in the recently enacted fish health regulations in the European Union (EU) (Directive 2006/88/EC). Epizootics involving mass mortality occur in spring and autumn, and carps of all ages are susceptible (Hedrick *et al.*, 2000). *Anguillid herpesvirus 1* (AngHV1) has been isolated from Japanese eel (*Anguilla japonica*) and European eel (*Anguilla Anguilla*) (Sano *et al.*, 1990; Van Beurden *et al.*, 2010)

The *Salmonid herpesvirus 1* (SalHV-1) was isolated on several occasions from a rainbow trout (*Oncorhynchus mykiss*) hatchery in the state of Washington in association with excessive mortality in young fish (Wolf *et al.*, 1978). *Salmonid herpesvirus 2* (SalHV-2) was isolated from *Oncorhynchus masou*, a landlocked Japanese form of Pacific salmon (Kimura *et al.*, 1981). *Salmonid herpesvirus 3* (SalHV-3) was originally found in cultured juvenile lake trout (*Salvelinus namaycush*) and it causes acute disease with mortality approaching 100%. (McAllister and Herman, 1989; Lepa and Siwicki, 2012).

1.2.2.3 Adenoviridae

Adenoviruses are linear, double-stranded DNA viruses, with a genome ranging from 26 to 45-kbp and an icosahedral capsid (Davison *et al.*, 2003). The family *Adenoviridae* comprises five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus* (Harrach *et al.*, 2011). The *Sturgeon ichtadenovirus*

A or white sturgeon adenovirus (WSAdV-1) is the single member of the genus *Ichtadenovirus* and up to now is the only known fish adenovirus (AdV) (Kovács *et al.*, 2003). The white sturgeon adenovirus (WSAdV-1) was found associated with infections of the mucosa of the alimentary tract among farmed juveniles of white sturgeon (*Acipenser transmontanus*) (Hedrick *et al.* 1991). Adenovirus-like particles have been associated with epidermal hyperplasia in cod (*Gadus morhua*) (Jensen & Bloch 1980) and in dab (*Limanda limanda*) (Bloch *et al.*, 1986).

1.3 Molecular tools for diagnosis of viral fish diseases

The rapid detection of pathogens in, both clinical and sub-clinical, infected fish is essential for effective health management in aquaculture. If pathogens can be detected and identified between harvesting and re-stocking or before a disease outbreak, then this can be extremely useful for effective outbreak disease control. Prompt action in the early stages of any disease problem can have an enormous impact on the scale of the outbreaks. Rapid diagnostic methods, therefore, provide powerful tools during emergency management (Adams and Thompson, 2008).

In recent years, great advances have taken place in understanding the molecular biology of fish pathogens and their hosts, and molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish diseases and for the study of the epidemiology of viral, bacterial, and parasitic diseases (Altinok and Kurt, 2003). The nucleic acid based molecular diagnosis techniques are fast, sensitive and highly accurate. Hence, these techniques efficiently diagnose pathogens from diseased and latent fish as well as aquatic environment that is monitored for ascertaining aquatic animal health status and disease surveillance (Biswas and Sakai, 2014).

1.3.1 Conventional Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is *in vitro* technique that consists of an exponential amplification of a DNA fragment, and its principle is based on the simulation of the mechanism of DNA replication *in vivo*. The PCR technique is based on the use of a thermostable DNA polymerase, which amplifies a specific region of the target. The polymerase activity is initiated by short, 15-30 base pair (bp) long oligonucleotides (primers), interacting with target fragment following the principle of Watson-Crick base pairing. It is a cycling reaction where each cycle contains three steps named denaturation, annealing and extension. The cycle repetition of these steps results in an exponential amplification, producing a vast amount of DNA at the end of the procedure (Fig. 8) (Heid *et al.*, 1996).

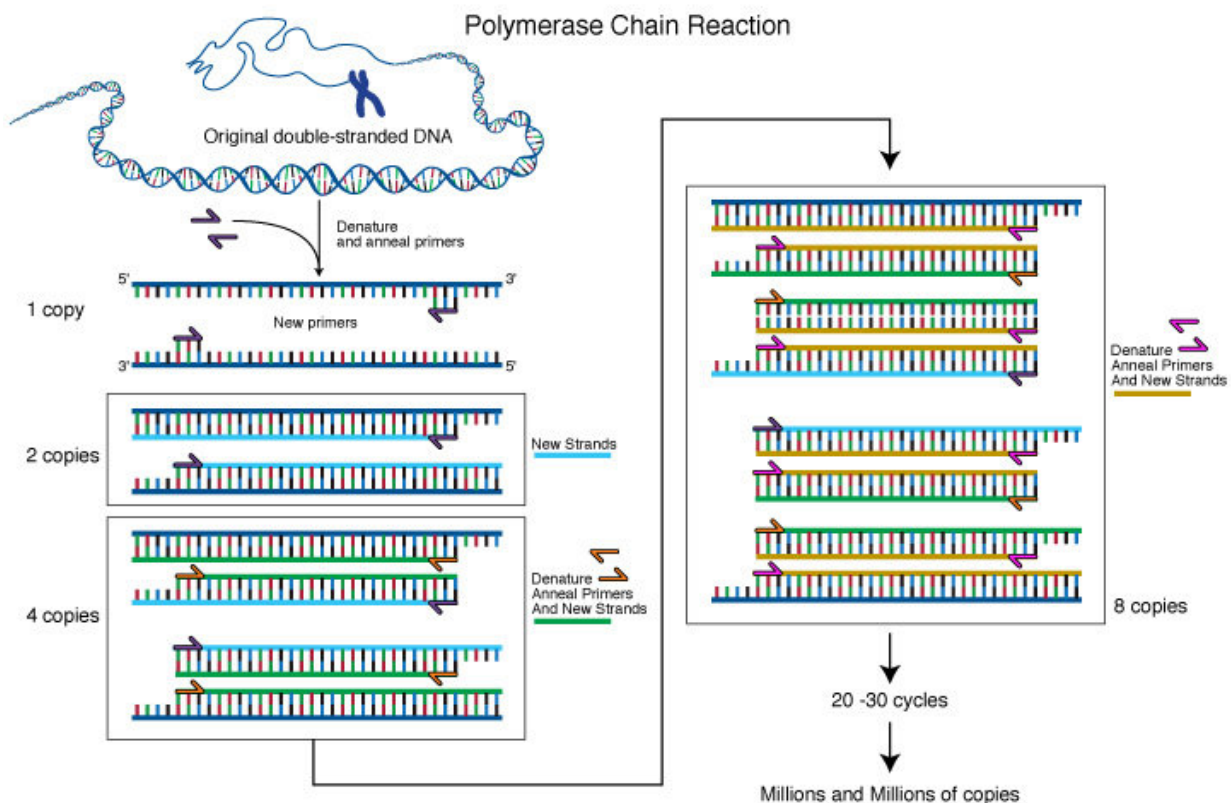


Figure 8. Basic principles of the PCR method (<http://petridishtalk.com/2010/12/05/the-polymerase-chain-reaction-a-microcosm/>).

As the reaction is going on, the used primers and dNTPs are incorporated into the newly synthesized DNA strands, which will compete with the primers on the later stages of the reaction. Finally, the reaction reaches a plateau phase and the amplification ceases or continues with very low efficiency. If the reaction consumes the available chemicals, it also stops the amplification (Kainz, 2000).

To identify the PCR product according to the specific length of the amplicon, gel electrophoresis is used and the DNA molecules can be visualized under UV light after staining with an intercalating dye.

The PCR is a fast and relatively simple technique that can detect a nucleic acid fragment and amplify it. PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected (OIE, 2012). However, the sensitivity of PCR could be also its major disadvantage since very small amounts of contaminating DNA (from a different sample) can also be amplified producing false-positive results. Moreover, the specificity of the generated PCR products may be altered by nonspecific binding of the primers to others similar sequences on the template DNA (Louie *et al.*, 2000).

The PCR techniques have had a rapid and tremendous progress in recent year. This technique have contributed to the identification and the characterization of several infectious agents that have great impact on human and animal health (Hernández-Rodríguez and Ramirez, 2012). To expand its utility in veterinary diagnostics and pathogen identification, PCR has been extensively modified over the years. This technique often in combination with other techniques has opened up numerous possibilities in epidemiological studies for the identification of individual strains and, in particular, the differentiation of closely related strains (Adams and Thompson, 2008).

In recent years, several conventional PCR tests have been developed and applied for the detection of the most important fish viruses (Table 1).

Specific PCR methods have developed for rapid detection and confirmation of koi herpesvirus in tissues of infected fish (Gilad *et al.*, 2002; Gray *et al.*, 2002). Successively, a robust and sensitive PCR assay based on primers selected from the defined DNA sequence of the TK gene was developed to improve the diagnosis of KHV infection. This assay compared to previously described PCR assays and to viral culture in diseased fish was shown to be the most sensitive method of diagnosis of KHV infection (Bercovier *et al.*, 2005).

The PCR assay described by Gould *et al.* (1995) provides a rapid method to detect EHNV in infected cell cultures and infected tissues from redfin perch (*Perca fluviatilis L.*), rainbow trout and barramundi.

Several conventional PCR assays have been developed to detect LCDV in several species (Hossain *et al.*, 2007; Kvitt *et al.*, 2008; Cano *et al.*, 2009). However, for detection of the virus in apparently healthy carriers, combining PCR with membrane hybridization has been recommended to increase the sensitivity of the assay (Cano *et al.*, 2007).

A fast and sensitive PCR assay has been described for detection of the red sea bream iridovirus (RSV) in infected red sea bream (Kurita *et al.*, 1998). This assay is recommended by the OIE (Office International des Epizooties) for diagnosis and surveillance of red sea bream iridoviral disease (RSIVD).

Table 1. Relevant DNA viruses of fish, targeted by PCR assays.

Virus	Reference
Koi herpesvirus (KHV)	Bercovier <i>et al.</i> , 2005
Oncorhynchus masou virus (OMV)	Aso <i>et al.</i> , 2001
Lymphocystis disease virus (LCDV)	Cano <i>et al.</i> , 2007
Red sea bream iridovirus (RSIV)	Kurita <i>et al.</i> , 1998
Epizootic haematopoietic necrosis virus (EHNV)	Gould <i>et al.</i> , 1995
Erythrocytic necrosis virus (ENV)	Emmenegger <i>et al.</i> , 2014

1.3.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR is one of the variants of the conventional PCR. The RT-PCR is also an *in vitro* procedure that combines the action of the reverse transcriptase enzyme with that of the DNA polymerase. Its main difference with conventional PCR is that this reaction starts from a RNA template extracted directly from the sample; the RNA is converted to a complementary DNA (cDNA). Subsequently, the newly synthesized cDNA is amplified using traditional PCR (Fig. 9) (Newton and Graham, 1994).

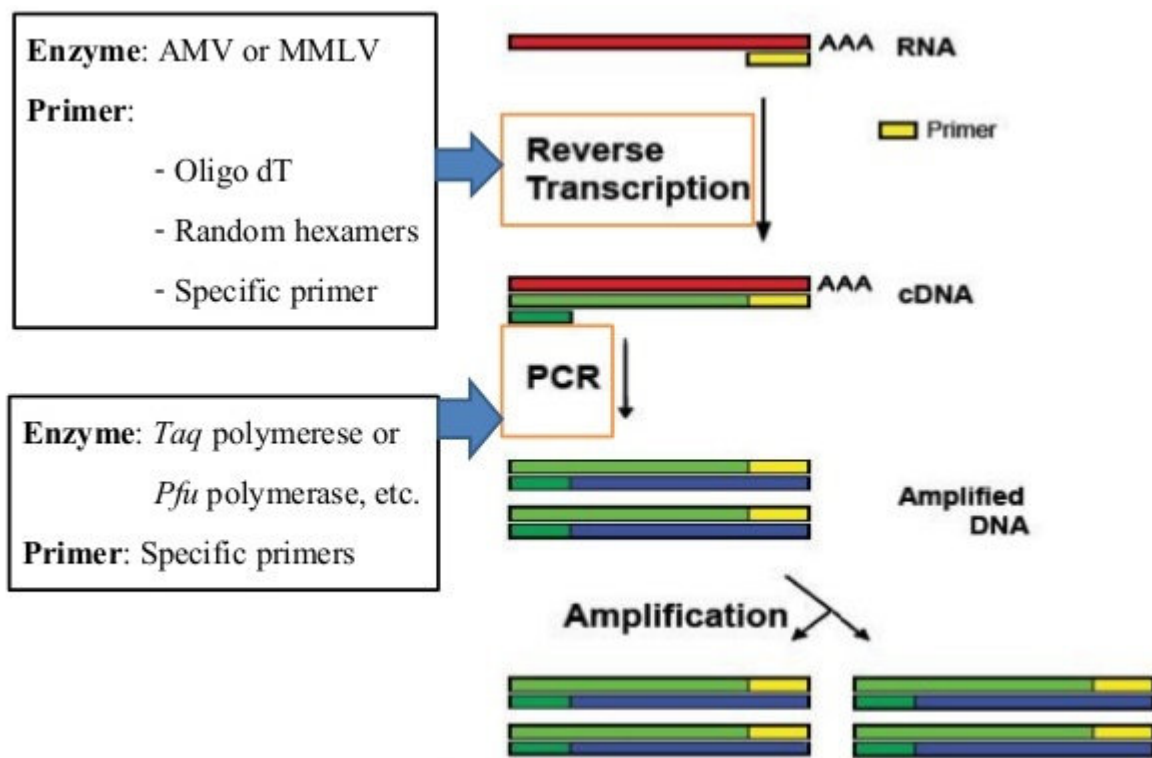


Figure 9. Flowchart of RT-PCR (Wikipedia, 2015)

http://en.wikipedia.org/wiki/Reverse_transcription_polymerase_chain_reaction

The Reverse Transcriptase (RT) reaction can be prepared with random primers, oligo(dT), or a gene-specific primer. The RT-PCR can be carried out either in two-step or one-step formats. In two-step RT-PCR, each step is performed under optimal conditions. cDNA synthesis is performed in a specific RT buffer, then one tenth of the

reaction is used for PCR. In one-step RT-PCR, reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both RT and PCR.

RT-PCR is a fast and sensitive method for detection of RNA viruses, but there are some disadvantages, such as the unstable nature of RNA, the risk of contamination. Furthermore, as in the case of PCR, this method cannot distinguish between infection and non-infectious virus (Bootland and leong, 1999).

RT-PCR is widely applied for the detection of many fish RNA viruses (Table 2). Reverse transcriptase-polymerase chain reactions (RT-PCR) assay were developed for the detection of *Viral haemorrhagic septicaemia virus* (VHSV) and *Infectious hematopoietic necrosis virus* (IHNV). With these techniques were possible to detect viral RNA in acutely and subacutely to chronically diseased fish as well as in asymptomatic VHS or IHN carrier fish (Miller *et al.*, 1998). RT-PCR has been used frequently to identify IHNV and is claimed to be more sensitive than traditional virus isolation (Bruchhof *et al.*, 1995; Miller *et al.*, 1998; Knusel *et al.*, 2007). The RT-PCR method reported by Snow *et al.* (2004) has proven capable of amplification of a wide range of VHSV genotypes and is recommended for use by the OIE.

The RT-PCR has been shown to be a very sensitive tool for the detection of IPNV in tissues and fish eggs of coho salmon. The use of this method could be an important tool for preventing the horizontal transmission of this virus (Lopez-Lastra *et al.*, 1994). Other studies have compared RT-PCR assay with traditional virus isolation and have revealed that RT-PCR is the most sensitive method for detection of IPNV. (Blake *et al.*, 1995; Taskdal *et al.*, 2001). A study to optimize and validate a RT-PCR for the detection of *Infectious pancreatic necrosis virus* (IPNV) was conducted by Kerr and Cunningham (2006). With this optimized technique was possible to detect all nine serotypes (A1-A9) of IPNV serogroup A.

Several RT-PCR methods were developed for detection of infectious salmon anaemia virus (ISAV). Amplification of parts of segment 8 provides a useful test for presence of the virus (Mjaaland *et al.*, 1997; Rimstad *et al.*, 1999). A RT-PCR was able

to detected ISAV in both serum and mucus of fish showing no clinical sign of ISAV or pathological lesions (Griffiths and Melville, 2000).

Table 2. Relevant RNA viruses of fish targeted by RT-PCR assays.

Virus	Reference
Infectious hematopoietic necrosis virus (IHNV)	Miller <i>et al.</i> , 1998
Viral hemorrhagic septicemia virus (VHSV)	Miller <i>et al.</i> , 1998
Infectious pancreatic necrosis virus (IPNV)	Saint-Jean <i>et al.</i> , 2001
Sleep diseases virus (SDV or SAV-2)	Fringuelli <i>et al.</i> , 2008
Infectious salmon anemia virus (ISAV)	Devold <i>et al.</i> , 2000
Spring viraemia of carp virus (SVCV)	Stone <i>et al.</i> , 2003
Viral encephalopathy and retinopathy virus (VERV)	Nishizawa <i>et al.</i> , 1994

1.3.3 Nested PCR and Semi-Nested PCR

Nested PCR is a variant of conventional PCR method that amplifies a target region of DNA with an outer primer pair in an initial reaction, followed by a second amplification step conducted using an internal primer pair (Mothershed and Whitney, 2006). This method increases the specificity and the sensitivity of the reaction since formation of the final product depends upon the bonding of two separate sets of primers and because two sets of amplification (each of the order of 25 cycles) are used (Kawasaki *et al.*, 1990). However, one of the major drawbacks with nested PCR is the risk of introducing contamination when a second PCR reaction is initiated using a portion of the mixture from the first reaction, and consequently it increases the risk of false-positive results. (Rolfs *et al.*, 1992).

Semi-nested PCR is basically the same technique, as the only difference is that one of the primers used in the second amplification is the same used in the first PCR amplification.

Both nested and semi-nested PCR therefore can increase the sensitivity as much as 1000 fold compared with conventional PCR (Kalland, 2009). Besides, the specificity is particularly enhanced because these techniques almost always eliminates any

spurious non-specific amplification products. This is because after the first round of PCR any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified (Querci *et al.*, 2006).

These techniques are also used to improve the sensitivity in the diagnosis of some viral diseases of fish. Gomez *et al.* (2004) reported an nested PCR technique able to detect betanodaviruses from apparently healthy cultured and wild marine fish.

Dalla Valle *et al.* (2000) reported that a nested PCR for *Dicentrarchus labrax* encephalitis virus (DIEV) was 10 to 100-fold more sensitive than the conventional RT-PCR and permitted to detect the virus in blood and sperm as well as in nervous and ovarian tissues. Similarly, Thiery *et al.* (1999) reported that the sensitivity for detection of the VERV in sea bass is greatly improved using a nested RT-PCR method.

A semi-nested RT-PCR was developed to detect the region encoding the glycoprotein gene of SVCV using specific primer sets (Stone *et al.*, 2003). This semi-nested PCR has been capable to detecting the virus at 10–50% tissue culture infective dose (TCID₅₀)/ml (Shivappa *et al.*, 2008). Compared with the others diagnostic methods for SVCV detection, the semi-nested RT-PCR is the most sensitive method (Shivappa *et al.*, 2008). The OIE (2014) recommends the use of this semi-nested RT-PCR for confirmatory identification of SVCV (OIE 2014).

1.3.4 Multiplex PCR

The Multiplex PCR technique is based on the use of multiple primer pairs to allow amplification of several templates within a single reaction (Belàk *et al.*, 2009). Multiplex PCR can detect several pathogens at one time with improvement of time and cost-efficiency of the diagnosis (Williams *et al.*, 1999). However, the use of multiple primers to target several templates may result in inefficient or preferential binding of some primers to their templates, while the degree of proximity of annealing

temperatures of different primers can negatively affect the outcome of the assay (Elnifro *et al.*, 2000). For these reason, multiplex PCR assay need an accurate optimization step to overcame all these troubleshoots. Furthermore, the assay can be difficult to standardise because annealing temperatures need to be established for each of the primer sets, the size of each amplicon must be sufficient to be able to produce distinct bands by gel electrophoresis, and the specificity and sensitivity need to be established for each pathogen (Adams and Thompson, 2008).

Nonetheless, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi and parasites (Altinok and Kurt, 2003). This techniche is also useful for detecting a species-specific target and a group-specific target in a single reaction (Mothershed and Whitney, 2006). For example, a multiplex PCR assay has been developed to identify all nine serotypes of IPNV serogroup A through the use of 3 pairs of primers (Barrera-Meija *et al.*, 2009).

Other examples show that multiplex PCR is widely used in the diagnosis of diseases of aquatic animals. Williams *et al.* (1999) reported the development of a multiplex RT-PCR assay for the detection of IPNV, IHNV and VHSV. The sensitivity levels of this multiplex RT-PCR assay have been comparable to those of direct isolation of virus in cell culture with dilutions of fish tissue homogenates. Liu *et al.* (2008), developed a new multiplex real-time RT-PCR to detect, identify and quantify three *Rhabdovirus* responsible for disease in fish SVCV, IHNV and VHSV, with multiple primer sets that demonstrated a high level of specificity (Liu *et al.*, 2008a).

1.3.5 Real-Time PCR

Real-time PCR technology is based on the detection of a fluorescent signal produced during the amplification of a DNA target. Rather than having to look at the amount of DNA target accumulated after a fixed number of cycles, real-time assays

determine the point in time during cycling when amplification of a PCR product is first detected. The PCR product is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise. That cycle number is referred to as the threshold cycle (Ct) (Bustin, 2005). During the exponential phase of the amplification, the amount of target amplified is proportional to the starting template and it is during these cycles that gene numbers are quantified using the Ct method. The Ct is reached when the accumulation of fluorescence (template) is significantly greater than the background level (Heid *et al.*, 1996). During the initial cycles, the fluorescence signal due to background noise is greater than that derived from the amplification of the target template. Once the Ct value is exceeded, the exponential accumulation of product can be measured. When the initial concentration of the target template is higher, the Ct will be reached at an earlier amplification cycle (Smith and Osborn, 2009).

Quantification of the initial target sequences of an unknown concentration is determined from the Ct values and can be described either in relative or in absolute terms. In relative quantification, changes in the unknown target are expressed relative to a coamplified steady state (typically a housekeeping) gene. This approach is commonly applied for studying of gene expression (Bustin, 2005). In absolute quantification protocols, the numbers of a target gene or transcript are determined on the basis of a standard curve generated from the amplification of the target gene present at a range of initial template concentrations, and then the Ct values for each template concentration are determined. Subsequently, a simple linear regression of these Ct values is plotted against the log of the initial copy number (Fig. 10). Quantification of the unknown target template is determined by comparison of the Ct values of the target template against the standard curve (Smith and Osborn, 2009).

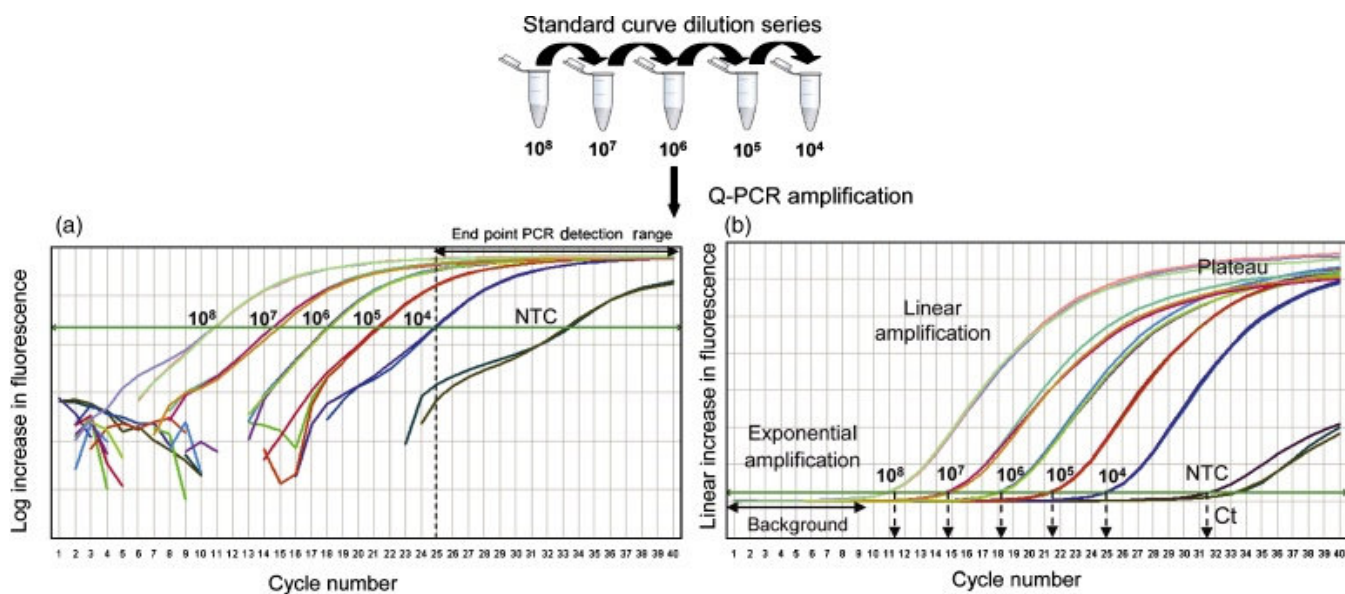


Figure 10. qPCR amplification from known concentrations of template DNA to construct standard curves for quantification of samples. (a) Log plot of the increase in fluorescence vs. cycle number of DNA standards; (b) Linear plot indicating the phases of a PCR amplification, the corresponding Ct values for each of the amplified standards.

In general, there are two different detection methods for real-time quantitative PCR (qPCR). One method relies on a fluorescent double-stranded DNA-binding dye (SYBR Green) and the other method relies on fluorescent resonance energy transfer (FRET) probes.

SYBR Green-based detection method rely on a class of DNA binding chemicals that emits fluorescence when intercalated into double stranded DNA (Fig. 11). As a result, the SYBR Green fluorescence increase proportionally to the increase in DNA amplification products. The primary disadvantage of the SYBR Green chemistry is that it may generate false positive signals because the SYBR Green binds to any double-stranded DNA, so, it can also bind to nonspecific double-stranded DNA sequences, including primer-dimers and nonspecific products. Anyway, this problem can be solved with the dissociation (melting) curve analysis. The melting curve should be carried out to confirm that the fluorescence signal is generated only from the target template and not from the formation of nonspecific PCR products (Giglio *et al.*, 2003)

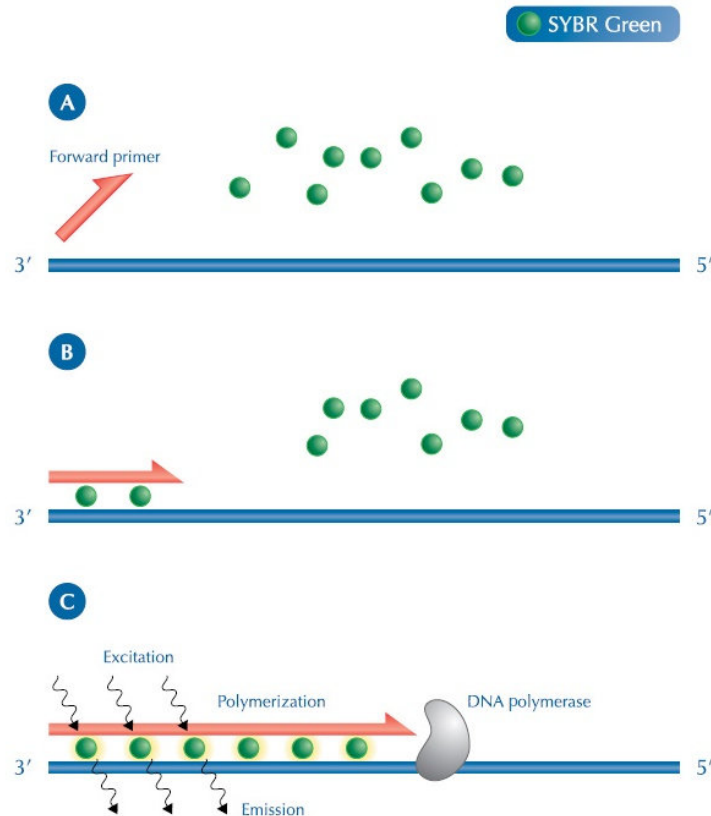


Figure 11. A) DNA is denatured and SYBR Green molecules are free in the reaction mix. B) Primers anneal and SYBR Green molecules bind to the dsDNA. C) DNA polymerase elongates the template and more SYBR Green molecules bind to the product formed resulting in exponential increase in the fluorescence level (<http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/>).

On the other hand, there are three different FRET probes: 5' nuclease probes (TaqMan), molecular beacons, and FRET hybridization probes.

The TaqMan probe is one of the most popular real-time PCR methods. TaqMan probes carry both a fluorescent dye and a quenching dye, and after the amplification step, the 5' nuclease activity of the Taq polymerase separates the fluorescent dye from the quencher, resulting in an increasing abundance of fluorescence after each PCR cycle (Fig. 12). TaqMan probes are, however, a more expensive option than using SYBR green chemistry and the former requires the presence of an additional conserved site within the short amplicon sequence to be present (Heid *et al.*, 1996).

Molecular beacons consist of a probe sequence embedded within two complementary 5-nucleotide-long arm sequences, and like the TaqMan probes, they carry both a fluorophore and a quencher. They are positioned at the ends of the probe, and in the absence of a target the probe exist as a hairpin structure, forcing the quencher near the fluorophore. When the probe hybridizes with a complementary target sequence, the fluorescent dye and the quencher are separated, so that a fluorescent signal is generated (Tyagi *et al.*, 1996).

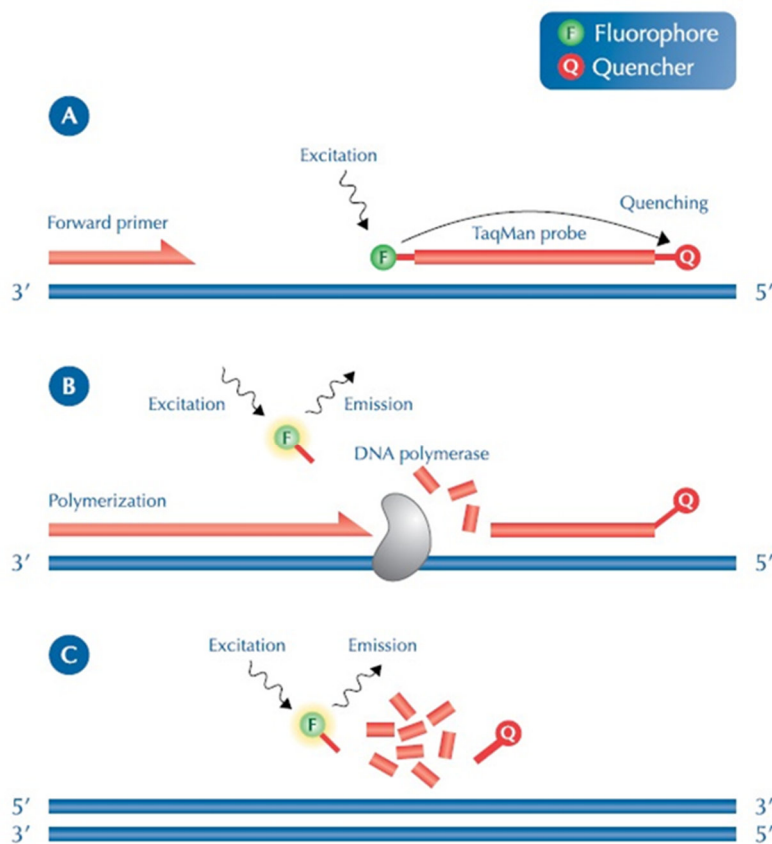


Figure 12. TaqMan Probe Chemistry. A) Primers and probe anneal to their target sequence. B) DNA polymerase extends the primer and encounters the probe, which is hydrolyzed from the 5' end releasing the fluorophore. The emission of the fluorophore is no longer quenched and increase in fluorescence can be detected. C) DNA polymerase hydrolyzes the whole probe from the template and completes strand elongation (<http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/>).

FRET hybridization probes consist of two DNA probes, each carrying a fluorophore. The upstream probe carries the fluorescent dye at the 3' end, and a second probe designed to hybridize downstream, carries an acceptor dye at its 5' end and is phosphorylated at its 3' end to prevent it from being used by Taq polymerase during PCR amplification. The fluorescence from the upstream probe is absorbed by the adjacent acceptor dye at the downstream probe when the probes are hybridized next to each other and the dye is excited and emits light with a third wave length which is detected (Ota *et al.*, 1998).

Its simplicity, specificity, and sensitivity, together with its potential for high throughput and the ongoing introduction of new chemistries, more reliable instrumentation, and improved protocols, has made real-time PCR the benchmark technology for the detection of DNA (Bustin, 2005).

Compared with the 'classical' single or nested PCR platforms, real-time PCR assays offer a number of important, well-known advantages:

- Faster and higher throughput assays;
- One-step amplification set-up that provides sensitivity and specificity close or equal to traditional nested PCR;
- Detection of amplified products measured online and in real-time through the lid, side or bottom of the reaction vessel without opening the system. Post-PCR handling of the amplicons/products is not required (i.e., the contamination risk posed by post-amplification products are minimized);
- The output is not only positive or negative, but enables a quantitative estimation of target or input nucleic acid in the sample;
- Hands-on time is greatly reduced, compared with traditional detection using agarose gels followed by intercalating staining (Belàk, 2009);

In recent years, real-time PCR has emerged as a powerful technique for the detection and quantification of several fish viruses such as *Infectious salmon anaemia virus* (ISAV) (Munir and Kibenge, 2004), betanodavirus (Dalla Valle *et al.*, 2005;

Grove *et al.*, 2006), salmonid alphavirus (Hodneland and Endresen, 2006), fish iridovirus (Wang *et al.*, 2006), *Anguillid herpesvirus 1* (AngHV1) (Van Beurden *et al.*, 2015), and Lymphocystis disease virus (LCDV) (Ciulli *et al.*, 2015).

The real-time PCR (qPCR) methods provide a tool to detect and quantify virus in tissue samples from the infected animal and the low detection limit of this technique has proven useful to detect virus in asymptomatic fish. Bowers *et al.* (2008) described a real-time PCR assay using the SYBR green I (fluorescent dye) to detect and quantify IPNV in pectoral fin, spleen, and head kidney tissue of IPNV-challenged rainbow trout. In this assay, primers designed to amplify the VP4 gene were used and the IPNV was reliably detected down to 10 RNA copies. This assay has been able to detect IPNV in challenged fish that did not show any clinical signs of infection, suggesting that it could be used to detect asymptomatic carriers.

A TaqMan assay using a fluorescent-labelled probes to N and G gene detected IHNV successfully in the kidney and brain of infected fish (100 copies/reaction) showing that this assay would be valuable for detecting IHNV carriers or reservoirs (Overturf *et al.*, 2001).

Both SYBR green and TaqMan assays have been reported for the detection of VHSV (Chico *et al.*, 2006; Matejusova *et al.*, 2008; Cutrin *et al.*, 2009). Matejusova *et al.* (2008) developed a rapid, accurate and sensitive quantitative real-time reverse transcriptase-PCR (qRT-PCR) that targeted a conserved region of the N gene of the VHSV. This assay has been demonstrated to be more sensitive than the conventional RT-PCR. Furthermore, this assay is able to detect all three European genotypes I, II and III of VHSV.

1.3.6 Loop-mediated isothermal amplification (LAMP)

A novel, rapid and sensitive technique named loop-mediated isothermal amplification (LAMP) has been developed by Notomi *et al.* (2000) and is capable of amplifying DNA or RNA under isothermal conditions with high specificity and

efficiency. LAMP employs a DNA polymerase with strand displacement activity (*Bst* DNA polymerase), along with two internal primers (FIP, BIP), and two outer primers (F3, B3) which recognize six different sequences in the DNA template, by incubating all the reagents in a single tube at a constant temperature, usually 63°C which is optimum for the activity of DNA polymerase (Fig. 13).

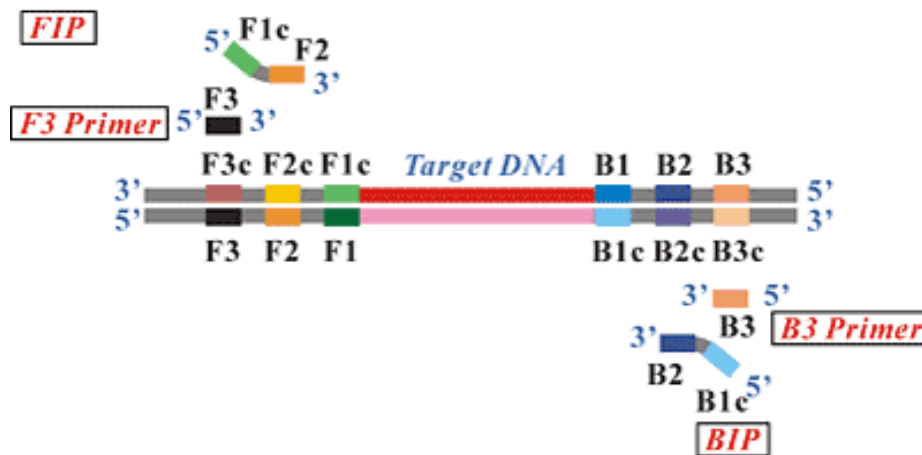


Figure 13. Design of the 4 types of primers (described in detail below) based on the following 6 distinct regions of the target gene (<http://loopamp.eiken.co.jp/e/lamp/primer.html>).

In particular, the mechanism of the reaction can be explained in three steps, an initial non-cyclic step, a cyclic amplification step, and an elongation step. The addition of a primer set that anneals at the loop structure in LAMP amplicons enhances specificity of the reaction and accelerates further the amplification time. In particular, using these specific primers, named loop-primers (LF, LB), the reaction time is reduced by half, making it a more efficient tool used in the practical applications of LAMP (Nagamine *et al.*, 2001; Fu *et al.*, 2011).

The LAMP is both sensitive as well as specific when compared with other DNA detection methods like conventional PCR. Large quantities of a targeted sequence (10^9 - 10^{10} copies) are produced in less than an hour. Furthermore, the high specificity is mainly due to hybridization of the four primers against six distinct sequences in the target DNA (Noren *et al.*, 2011). Moreover, this technique not require costly equipments, but only a water bath, which is commonly available in almost all

laboratories. Hence, the technique is also suitable in field condition (Notomi *et al.*, 2000). In addition, in this method the results can be seen directly by adding DNA binding dyes (SYBR green, HNB or Calcein) and electrophoresis is not needed (Parida *et al.*, 2008).

However, LAMP is less versatile than PCR techniques. LAMP is useful primarily as a diagnostic or detection technique, but is not useful for cloning or multiple other molecular biology applications enabled by PCR. Another disadvantage is that LAMP uses 4 (or 6) primers targeting 6 (or 8) regions within a fairly small segment of the genome, and therefore to design primer sets for LAMP is difficult. Free, open-source or commercial software packages are generally used to assist with LAMP primer design (Torres *et al.*, 2011) although the primer design constraints mean there is less freedom to choose the target site than with PCR.

Multiplexing approaches for LAMP are less developed. Because, the larger number of primers per target in LAMP increases the likelihood of primer-primer interactions for multiplexed target sets. Besides, the high sensitivity of the LAMP system makes it susceptible to false positives because of carry-over or cross-contamination (Savan *et al.*, 2005).

The LAMP reaction has been used successfully to detect fish viral infections and has been applied to the detection of several viruses (Table 3). This assay is suitable to be used in field conditions for the rapid detection of several pathogens in aquaculture animals, which allow expedited control and hygiene measures to be implemented to prevent spread of infection. (Biswas and Sakai, 2014).

The first LAMP-based detection was conducted for koi herpesvirus (KHV) identification by targeting the thymidine kinase (tk) gene (Gunimaladevi *et al.* 2004). The detection limit was found to be similar to that of the PCR assay made with the same primers. This rapid and efficient detection system could be a very feasible method to screen for KHV ornamental koi carps that are traded over various geographical areas.

A LAMP assay method has been described to offer a detection method for VHSV simpler than conventional RT-PCR. This assay targeted the G-protein sequence of

VHSV using a set of six primers. However, the detection limit of the RT-LAMP assay was found to be similar to a commonly used RT-PCR method and both methods detected VHSV RNA at a dilution of 10^{-6} (Soliman and El-Matbouli, 2006). A similar six-primer LAMP was applied to detect a double-stranded DNA virus, the Singapore grouper iridovirus (SGIV) which caused a large mortality in farmed grouper (Mao *et al.* 2008).

Table 3. Viruses identified by different LAMP methods from various fish species

Virus	Infected fish	Method used	Reference
Red seabream iridovirus	Red seabream	LAMP	Caipang <i>et al.</i> , 2004
Koi herpesvirus	Common carp	LAMP	Gunimaladevi <i>et al.</i> , 2004
Singapore grouper iridovirus (SGIV)	Singapore grouper	LAMP	Mao <i>et al.</i> , 2008
Turbot reddish body iridovirus (TRBIV)	Turbot	LAMP	Zhang <i>et al.</i> , 2009
Red-spotted grouper nervous necrosis virus (RGNNV)	Red-spotted grouper	LAMP	Xu <i>et al.</i> , 2010
Cyprinid herpesvirus-2	Goldfish and crucian carp (<i>Carassius carassius</i>)	LAMP	He <i>et al.</i> , 2013
Infectious hypodermal and hematopoietic necrosis virus (IHNV)	Rainbow trout	RT-LAMP	Gunimaladevi <i>et al.</i> , 2005
Viral hemorrhagic septicemia virus (VHSV)	Rainbow trout	RT-LAMP	Soliman and El-Matbouli, 2006
Spring viraemia of carp virus (SVCV)	Koi carp	RT-LAMP	Liu <i>et al.</i> , 2008b Shivappa <i>et al.</i> , 2008
Infectious pancreatic necrosis virus (IPNV)	Atlantic salmon	RT-LAMP	Soliman <i>et al.</i> , 2009
Nervous necrosis virus (NNV)	Grouper	RT-LAMP	Sung and Lu, 2009

Furthermore, Soliman *et al.* (2009) reported a sensitive and rapid reverse transcription LAMP (RT-LAMP) assay for IPNV detection. This assay uses six IPNV-specific primers designed to amplify VP4/VP3 junction area. This assay showed a considerably higher analytical sensitivity than RT-PCR because was able to detect approximately 0.0001fg the viral RNA.

1.3.7 Microarray Technology

The advancement in microarray technology created a breakthrough in diagnostics allowing many specific sequences to be analyzed simultaneously. Microarrays function in a number of different ways, but the common characteristic is that many specific pieces of nucleic acid can be identified through the use of complementary probes that make up the array (Belàk *et al.*, 2009). DNA microarrays offer a powerful platform for parallel analysis of gene transcription and expression on a genome-wide scale (Teng *et al.*, 2008). The ability to simultaneously screen for a large panel of pathogens in clinical samples, especially viruses, represent a major development in the diagnosis of infectious diseases. Furthermore, this technology could be especially useful in surveillance programs for detecting uncharacterized viral pathogens or highly variable virus strains in the same taxonomic genus or family (Dacheux *et al.*, 2010). The major disadvantage of this methodology is that microarrays instruments are expensive and of limited availability.

DNA microarray has been applied to the study of red sea bream iridovirus (RSIV). In this study, individual RSIV ORFs have been characterized at the transcriptional level and also classified into temporal kinetic classes by their dependence on de novo protein synthesis and viral DNA replication. The application of the microarray assay to the RSIV infection conducted in this study could help to understand the gene regulation strategies and the pathogenic mechanisms of RSIV and other iridovirus infections (Lua *et al.*, 2005).

By using a viral DNA microarray, the temporal gene expression of SGIV was characterized and the DNA microarray data were consistent with the results of real-time RT-PCR studies. This study reported important information on the processes of infection, pathogenesis and replication strategies of SGIV. The results of this study, offer important insights into the replication and pathogenesis of iridoviruses. (Chen *et al.* 2006).

Recently, Dacheux *et al.* (2010) reported the capacity for viral detection and identification of a newly described high-density resequencing microarray (RMA). The microarray RMA has been used for detection of multiple pathogen of the family *Rhabdoviridae* both previously identified or unknown. This technique have allowed an unprecedented phylogenetic analysis of 106 rhabdoviruses. Furthermore, this study suggested that this methodology may be used for the broad-spectrum surveillance and the broader-scale investigation of biodiversity in the viral world.

1.3.8 Nucleic acid sequence based amplification (NASBA)

NASBA is an isothermal transcription-based amplification method, particularly suitable for the detection and quantification of genomic, ribosomal, and messenger RNA (Sidoti *et al.*, 2013). In the NASBA procedure, target-specific amplification is achieved through oligonucleotide primers and the coordinated activity of 3 enzymes: reverse transcriptase, RNase H, and T7 RNA polymerase (Fig. 14) (Starkey *et al.*, 2004). During the reaction, a DNA intermediate is generated through a process that involves the hybridization of a primer to the RNA target. This primer (P1), which contains a T7 RNA polymerase promoter sequence, is then extended by AMV-RT to form a RNA-DNA hybrid.

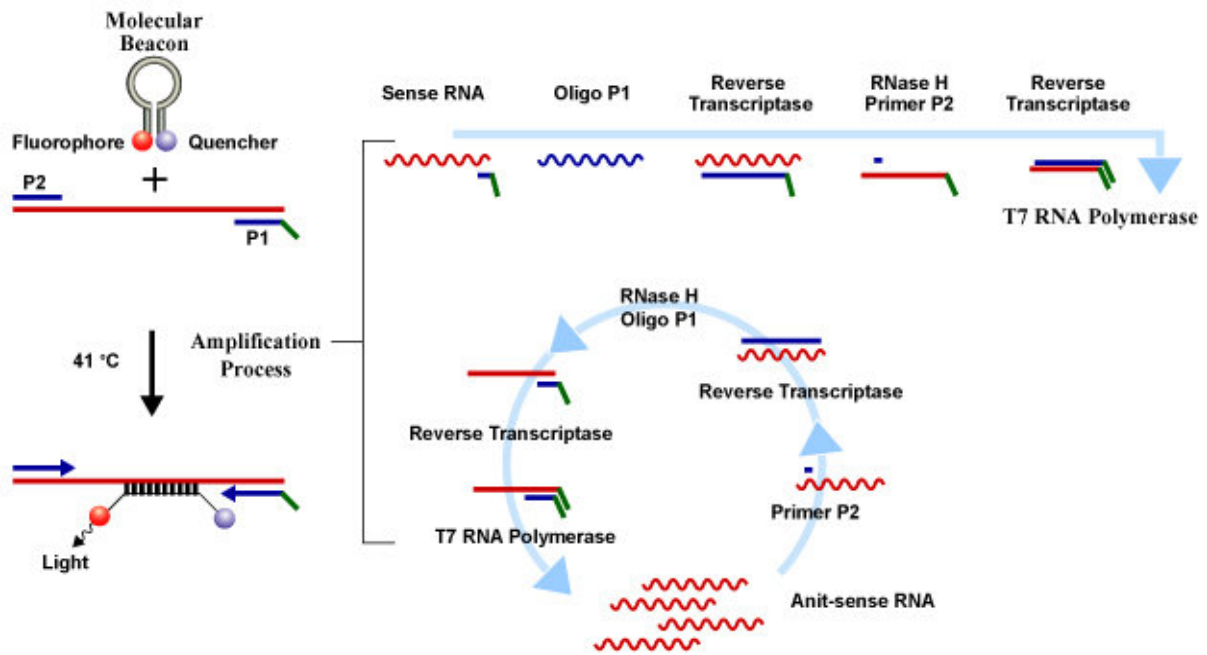


Figure 14. schematic representation of the NASBA amplification (http://www.premierbiosoft.com/tech_notes/NASBA.html).

The digestion of the RNA component of the hybrid by RNase H permits the binding of a second primer (P2) to the remaining DNA strand. The second primer is then extended by AMV-RT to form the double stranded DNA intermediate, which contains the T7-RNA polymerase promoter needed for transcription. Finally, the T7 RNA polymerase produces numerous RNA copies and once transcription is initiated, the resulting single-stranded RNA transcripts, which are anti-sense to the original RNA, can serve as a template to start a new amplification process (Wu *et al.*, 2001; Nugen *et al.*, 2009).

Real-time detection in NASBA can be performed using molecular beacons, which are incorporated directly into amplification reactions (Fig. 12) (Leone *et al.*, 1998). NASBA offers potential advantages compared to conventional RT-PCR. Basically, it is a continuous, isothermal process that does not require a thermocycler. Moreover, the optimal annealing temperature for primers does not have to be determined empirically. Furthermore, is a method based on the isothermal reaction occurring at a temperature of 41°C, and does not require denaturation; this mechanism prevents amplification of DNA genome in case of contamination, thus being very

selective for RNA target amplification. Another advantage is that no additional reverse transcriptase step is required, thus saving time and reducing the risk of contamination. However, the low temperature occurring in the reaction could be representing a risk factor for the specificity of the method. Therefore, a restriction of the NASBA method is probably that individual preparation of the chemical reagents mixture is difficult and commercial kits are expensive (Sidoti *et al.*, 2013).

Diagnostic procedures based on NASBA methodology have been described for some fish virus. Starkey *et al.* (2004) have developed a real-time NASBA procedure for detection of piscine nodaviruses, which have emerged as major pathogens of marine fish. Based on the detection of cell culture-derived nodavirus, and a synthetic RNA target, this real-time NASBA procedure has been approximately 100-fold more sensitive than single-tube RT-PCR.

A real-time NASBA assay has been also developed for detection of ISAV. This assay has been able to detect ISAV isolates from Scotland, Norway, and the Faroe Islands. However, the sensitivity of this assay is similar to that of a conventional RT-PCR (Starkey *et al.* 2006).

1.4 Conclusion

In conclusion, the development and the application of molecular techniques to fish disease diagnosis have provided numerous advantages in terms of efficiency of the diagnostic process. These techniques offer suitable tools of studying the etiological agents and their interaction with their hosts, and consequently concur largely to the control and the prevention of the diseases' spreading among farmed animal populations.

However, these reactions are strictly specific and peculiar to each viral species and often for individual genotypes and variants. For these reasons, it is essential to continue the research and the development of new techniques for fish viruses, to

provide effective and practical tools for research and detection of all pathogenic viruses to the scientific community and especially to the aquaculture sector.

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CHAPTER II

DEVELOPMENT AND APPLICATION OF A REAL TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF LYMPHOCYSTIS DISEASE VIRUS

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2.1 Abstract

Lymphocystis disease virus (LCDV) is responsible for a chronic self-limiting disease that affects more than 125 teleosts. Viral isolation of LCDV is difficult, time-consuming and often ineffective; the development of a rapid and specific tool to detect and quantify LCDV is desirable for both diagnosis and pathogenic studies. In this study, a quantitative real-time PCR (qPCR) assay was developed using a Sybr-Green-based assay targeting a highly conserved region of the MCP gene. Primers were designed on a multiple alignment that included all known LCDV genotypes. The viral DNA segment was cloned within a plasmid to generate a standard curve. The limit of detection was as low as 2.6 DNA copies/ μ l of plasmid and the qPCR was able to detect viral DNA from cell culture lysates and tissues at levels ten-times lower than conventional PCR. Both gilthead seabream and olive flounder LCDV has been amplified, and an in silico assay showed that LCDV of all genotypes can be amplified. LCDV was detected in target and non-target tissues of both diseased and asymptomatic fish. The LCDV qPCR assay developed in this study is highly sensitive, specific, reproducible and versatile for the detection and quantitation of Lymphocystivirus, and may also be used for asymptomatic carrier detection or pathogenesis studies of different LCDV strains.

Keywords:

Lymphocystis disease virus, real-time PCR, MPC gene, Genotyping, *Sparus aurata*, Carrier detection.

2.2. Introduction

Lymphocystis disease virus (LCDV) belongs to the family *Iridoviridae*, genus *Lymphocystivirus*, and is the causative agent of lymphocystis, a disease described in over 125 species of fish throughout the world (Noga, 2010). The lymphocystis occurs both in marine and freshwater fish and is generally associated with stress conditions related to farming, although it is also described in free-living animals (Alonso *et al.*, 2005). In the Mediterranean area, gilthead seabream (*Sparus aurata*) is the most affected species by lymphocystis, although other species, such as Senegalese sole (*Solea senegalensis*), are also affected (Cano *et al.*, 2010). Infection with *Lymphocystivirus* causes single or clustered tumour-like nodules that are localized to the skin, fins and tail (Woo *et al.*, 2002). Occasionally, infections in internal organs, such as the eyes, heart and spleen, have been reported (Wolf, 1988; Colorni and Diamant, 1995).

Although lymphocystis disease is frequently benign, it may be responsible for significant economic losses related to poor growth rate, non-marketability of fish with lesions and secondary bacterial infections resulting from the phenomenon of cannibalism (Bovo *et al.*, 1998; Alonso *et al.*, 2005). Despite some encouraging results of studies on vaccine candidates (Jang *et al.*, 2011), there are currently no available prophylactic or therapeutic tools for lymphocystis, and the development of rapid and specific diagnostic tools represents the most critical point to control the disease (Cano *et al.*, 2007). In particular, early detection of LCDV in asymptomatic fish is a critical resource in the control of the spread of the disease. Virus isolation requires homologous cell lines, but even under these conditions, it remains a time-consuming method (Cano *et al.*, 2007). Molecular methods are more rapid and sensitive, even compared with serological methods (Cano *et al.*, 2007). Several conventional PCR assays have been developed to detect LCDV in several species. However, for detection of the virus in apparently healthy carriers, combining PCR with membrane hybridization to increase the sensitivity of the assay has been recommended (Cano *et al.*, 2007). A real time PCR developed to detect and quantify LCDV in yellow perch (*Perca flavescens*) revealed

to be very useful to study the virus presence in symptomatic and asymptomatic fish (Palmer *et al.*, 2012). Nevertheless, molecular methods could be limited by the genetic variability among strains requiring the use of multiple primer pairs to detect LCDV in different fish species (Kitamura *et al.*, 2006; Palmer *et al.*, 2012). In fact, the qPCR assay developed for yellow perch LCDV was not applicable to gilthead seabream LCDV due to primer mismatches.

Two complete genome sequences are available for LCDV collected from different species and geographic areas: LCDV-1, isolated in Europe from European flounder (*Platichthys flesus*), and LCDV-C, isolated in China from Japanese flounder (*P. olivaceus*). Genome comparison showed a high diversity in term of size, organization and genetic identity between the two LCDV genomes (Zhang *et al.*, 2004). Furthermore, Lymphocystivirus can be classified into at least nine genotypes based on the major capsid protein (MCP) gene sequence, the main target used for phylogenetic studies of viruses of the family *Iridoviridae* (Cano *et al.*, 2010; Palmer *et al.*, 2012). Each genotype includes only one or a very limited number of fish host species: genotype I includes LCDV-1 isolated from the European flounder (*Platichthys flesus*); genotype II consists of Japanese flounder (*Paralichthys olivaceus*) isolates; genotype III of rockfish (*Sebastes schlegeli*) isolates; genotype IV of the seabass (*Lateolabrax sp.*) and cobia (*Rachycentron canadum*) isolates; genotype V of painted glass fish (*Pseudambassis baculis*) isolates; genotype VI of gourami (*Trichogaster leeri*, *T. trichopterus*) isolates; genotype VII of gilthead seabream (*S. aurata*) and Senegalese sole (*S. senegalensis*); genotype VIII of largemouth bass (*Micropterus salmoides*) isolate; and genotype IX of yellow perch strains (Hossain *et al.*, 2008; Cano *et al.*, 2010; Palmer *et al.*, 2012).

Recently, systemic and persistent infection was revealed in gilthead seabream and yellow perch and the development of a rapid and specific tool to detect and quantify LCDV is desirable for the great contribution that this method could bring to the knowledge of LCDV infection (Cano *et al.*, 2009; Palmer *et al.*, 2012).

This study aimed to genotype a Lymphocystivirus isolated from gilthead seabream of an Italian farm. Furthermore, the research aimed to develop a real-time PCR method for detection and quantitation of LCDV from cell culture lysates or directly from tissue. The application of the developed qPCR assay to diseased and recovered gilthead seabream revealed systemic and persistent infection as reported previously, thus adding a quantitative analysis.

2.3 Materials and methods

2.3.1 Fish samples

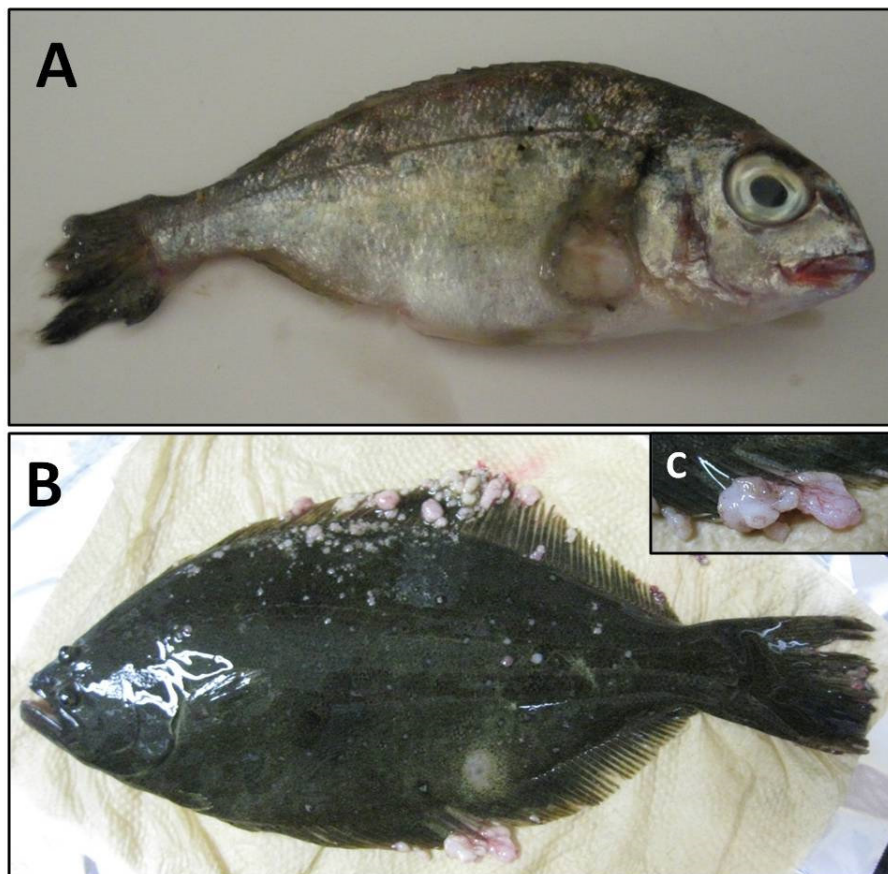


Figure 1. Gilthead seabream (A) and olive flounder (B) samples. (C) Detail of an oliveflounder lesion.

Gilthead seabream (*S. aurata*) samples were collected from an Italian farm in 2011. Diseased fish (samples SA42, SA43, SA64) were collected during a lymphocystis outbreak and showed typical whitish nodules on the opercula, fins and skin (Fig. 1A). A subject (SA68) showed only some hyperaemic areas and slight deformation of fins due to scarring events, so it was considered to be in a regressive phase of the disease, intermediate between the diseased and the recovered fish. Asymptomatic fish (SA76, SA77, SA78, SA79, SA80, SA81, SA82) were collected among recovered fish and did not show any signs of lymphocystis. An olive flounder (*P. olivaceus*) sample (PO6) was collected from a Korean farm in 2012 during a lymphocystis outbreak and showed whitish nodules, mainly on the fins (Fig. 1B, 1C).

2.3.2. Virological analysis

Nodules from two gilthead seabream (SA43 and SA64) were homogenized (10% w/v) in Leibovitz-15 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 1% L-glutamine (Gibco, Life Technologies) and 1% antibiotic–antimycotic solution (Gibco, Life Technologies). Centrifuged homogenates were decontaminated overnight with 1% antibiotic–antimycotic solution (Gibco, Life Technologies) and inoculated onto a BF-2 (Bluegill Fry) cell line monolayer. Cell cultures were maintained at 20°C for 3 weeks and examined daily using inverted microscopy for specific signs of virus growth (cytopathic effect) or other monolayer alterations. A mock-infected cell culture was also prepared as the negative control. Two serial subcultures were made for each sample by inoculating 500µl of infected cellular supernatant onto a 24-h-old BF-2 monolayer. After 1 h of absorption, the inoculum was removed to eliminate any viral residue from the previous passage. Second and third viral passages were done in duplicate.

2.3.3 DNA extraction

DNA was extracted from the cell culture supernatant inoculated with samples SA43 and SA64 or directly from the tissues of diseased (SA42, SA68, PO6) and asymptomatic fish (SA76, SA77, SA78, SA79, SA80, SA81, SA82). DNA was extracted from 200 µl cell culture supernatant using the Purelink Genomic DNA kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. DNA was extracted from about 50 mg of tissue using cetyl trimethylammonium bromide (C-TAB, Sigma, St. Louis, USA) and proteinase K (Sigma) digestion as described previously (Marengoni *et al.*, 2006). DNA was used soon after the extraction or stored at -20°C until use.

2.3.4 Conventional PCR, sequencing, cloning and standard preparation of DNA

Conventional PCR was performed on gilthead seabream LCDV using primers previously described (Table 1) targeting a portion of the DNA-dependent DNA polymerase gene (primers LF5/LR6) and a portion of the major capsid protein gene (primers LC1-F/LC1-R and LF7/LC1-R).

Tabela 1. Nucleotide sequences of the primers used in this study.

Name	Sequences 5'→3'	Target region	Amplified DNA product (base pair)	Reference
LF5	TTTGAATGGGAGGATCAC	DNA pol	405	Kvitt et al., 2008
LR6	TCCGTAAATGCTGTTAGC			
LC1-F	AGGTTCAAGCGTCACAAG	MCP	1356	Kitamura et al., 2006a
LC1-R	GGAAAACCCATTGATCCGT			
LF7	CGCGCTGCCTTATAATGA	MCP	789 with primer LC1-R	Kvitt et al., 2008
LCDV_qPCR_F1	AATGAAATAAGATTAACGTTTCAT	MCP	151 with primer LCDV_qPCR_R2 187 with primer LCDV_qPCR_R3	This study
LCDV_qPCR_R2	CGTTAGTGATCCATACTTG			
LCDV_qPCR_R3	TACCCATCAATCGACG TTC			

Conventional PCR with primers LF5/LR6 was used to detect LCDV in viral passages of BF-2 cell culture (sample SA43 and SA64).

DNA extracted directly from pathological tissues of the olive flounder (PO6) was amplified with primers LF7/LC1-R and sequenced to check the genotype of the olive flounder LCDV sample used in this study. Conventional PCR with primers LF7/LC1-R was performed with ten-fold serial dilutions of DNA extracted from gilthead seabream (SA42) and olive flounder (PO6) tissues to establish the limit of detection of this conventional PCR assay.

Gilthead seabream and olive flounder amplicons were purified with the High Pure PCR Product Purification kit (Roche, Mannheim, Germany) and then sequenced with the automatic sequencer, ABI Prism 310 (Applied Biosystems, Foster City, USA). Obtained sequences were compared to other sequences collected from GenBank using the ClustalW program implemented in BioEdit 7.0.5 (Hall, 1999). Nucleotide and amino acid identities were calculated among the Italian gilthead seabream LCDV (SA64) and the nine genotypes described in the literature using BioEdit. The gilthead seabream purified PCR product (1356 bp) obtained with LC1-F/LC1-R primers was then cloned into the pCR4/TOPO vector (Invitrogen). The resulting recombinant plasmid was purified with the Pure Link Quick Plasmid Miniprep kit (Invitrogen) and linearized using the restriction endonuclease Spe I (New England BioLabs, Ipswich, USA). The linearized plasmid was visualized and quantified by electrophoresis using Quantity One 1-D Analysis Software (Roche) by comparison with a 1-Kb DNA ladder (New England BioLabs).

2.3.5. Real-time qPCR assay

To develop a real-time PCR method to detect and quantify LCDV of all genotypes, primers were designed onto a multiple alignment (ClustalW method; Thomson *et al.*, 1994) of MCP gene sequences available from GenBank of LCDV

classified into nine genotypes (Fig. 2). Two forward primers and one reverse primer were designed to amplify two fragments of the MCP gene (Table 1). Real-time qPCR assays were performed using the 7300 ABI real-time PCR System (Applied Biosystems) and data were analysed using the associated software (Sequence Detection Software v2.05, Applied Biosystems). The real-time qPCR was optimized using ten-fold serial dilutions of linearized plasmid containing 2.6 to 2.6×10^9 copies of DNA/ μ l. The reaction mix contained 12.5 μ l GoTaq qPCR Master Mix 2X (Promega, Madison, USA), 0.08 μ M each primer, 0.25 μ l CXR reference dye, 2 μ l DNA and water to a final volume of 25 μ l. Cycling parameters consisted of an initial incubation at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 30 s, extension at 72°C for 30 s. The fluorescence was acquired during each extension phase. At the end of the amplification cycle, the mixture was subjected to a dissociation cycle (60–95°C) in order to verify the specificity of the PCR products obtained during amplification.

To assess the limit of detection of the real-time PCR assay, dilutions from 10^{-1} to 10^{-9} of the standard plasmid were tested in three technical repeats. The reproducibility of the qPCR assay with primers LCDV_qPCR_F1/R3 (LCDV F1/R3 qPCR) was determined using a standard method (Abdul-Careem *et al.*, 2006; Yun *et al.*, 2012). Six ten-fold dilutions of the DNA standard were tested in triplicate using 2 μ l DNA per reaction in three independent assays. To determine the intra-assay and inter-assay variation, the mean, standard deviation (SD) and coefficient of variation (CV) were calculated separately for each standard DNA dilution using threshold cycle (Ct) values. The intra- and inter-assay variability was assessed by the CV represented as the SD/mean expressed as a percentage.

The LCDV F1/R3 qPCR was applied to the viral DNA detection in pathological tissues and cell culture lysates. The limit of detection was tested on duplicates of ten-fold dilutions of DNA extracted directly from pathological tissues of both gilthead seabream (SA42) and olive flounder (PO6) samples.

LCDV_qPCR_F1
 AATGAAATAAGATTAACCTTTTCAT

G III	AY823414	TGCCTTACAATGAAATAAGATTAACCTTTTCATTTGAGAGA	626
G III	AB213004	TGCCTTACAATGAAATAAGATTAACCTTTTCATTTGAGAGA	612
G II	AB212997	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTGAGAGA	537
G II	AY849391	TGCCTTATAATGAAATAAGATCAACTTTTCATCTGAGAGA	636
G IV	EF103188	TGCCTTATAACGAAATAAGATTAACCTTTTCATCTGAGAGA	626
G IV	EF059992	TGCCTTATAACGAAATAAGATTAACCTTTTCATCTGAGAGA	612
G VII	GU320739	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTTAGAGA	593
G VII	GU320724	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTTAGAGA	593
G VII	GU320731	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTTAGAGA	491
G VII	EF184306	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTTAGAGA	593
G V	AB299163	TGCCTTATAATGAAATAAGATTAACCTTTTCATCTCAGAGA	607
G VIII	GU290550	TACCTTACAATGAAATAAGATTGACTTTTCATTTAAGGGA	593
G IX	GU939626	TACCTTATAATGAAATAAGATTAACCTTTTCATTTGAGAGA	612
G VI	AB299164	TACCTTATAATGAAATAAGATTAACCTTTTCATTTAAGAGA	581
G I	L63545	TTCTTATAACGAAATAAGATTAACCTTTTCATCTTAGAGA	544

* * * * *

← LCDV_qPCR_R2
 CAAGTATGGATCACTAACC

G III	AY823414	TGATTTAAAAGATGTACAAGTATGGATTACCAATGTAGTA	750
G III	AB213004	TGATTTAAAAGATGTACAAGTATGGATTACCAATGTAGTA	736
G II	AB212997	TGATTTAAAGGATGTGCAAGTATGGATTACTAATGTAGTA	661
G II	AY849391	TGATTTAAAGGATGTGCAAGTATGGATTACTAATGTAGTA	750
G IV	EF103188	TGATTTGAAGGATGTACAAGTATGGATTACCAACGTAGTA	750
G IV	EF059992	TGATTTGAAGGATGTACAAGTATGGATTACCAACGTAGTA	736
G VII	GU320739	TGATTTAAAAGACGTGCAAGTATGGATCACTAACGTGGTG	717
G VII	GU320724	TGATTTAAAAGACGTGCAAGTATGGATCACTAACGTGGTG	717
G VII	GU320731	TGATTTAAAAGACGTGCAAGTATGGATCACTAACGTGGTG	615
G VII	EF184306	TGATTTAAAAGACGTGCAAGTATGGATCACTAACGTAGTG	717
G V	AB299163	TGATTTAAAGGATGTACAAGTATGGATCAACCAATGTTGTA	731
G VII	GU290550	CGATCTAAAAGATGTTCAAGTATGGATTACTAACGTAGTT	717
G IX	GU939626	CGATCTAACCGACGTTCAAGTATGGATTACTAACGTAGTC	736
G VI	AB299164	TGATTTAAAAGATGTACAAGTATGGATTACTAACGTAGTA	731
G I	L63545	TGATTTAAAAGACGTTCAAGTATGGATTACTAATGCAGTG	694

* * * * *

← LCDV_qPCR_R3
 GAACGTCGATTGATGGGTA

G III	AY823414	GTAACCTAATGAAGAACGTCGATTGATGGGTA	790
G III	AB213004	GTAACCTAATGAAGAACGTCGATTGATGGGTA	776
G II	AB212997	GTAACCAATGAGGAACGTCGTTTAAATGGGTACAGTACCTA	701
G II	AY849391	GTAACCAATGAGGAACGTCGTTTAAATGGGTACAGTACCTA	790
G IV	EF103188	GTAACCTAACGAAGAACGTCGATTGATGGGTA	790
G IV	EF059992	GTAACCTAACGAAGAACGTCGATTGATGGGTA	776
G VII	GU320739	GTAACCTAACGAAGAACGTCGATTGATGGGTA	757
G VII	GU320724	GTGACCAACGAAGAACGTCGATTGATGGGTA	757
G VII	GU320731	GTGACCAACGAAGAACGTCGATTGATGGGTA	655
G VII	EF184306	GTAACCAACGAAGAACGTCGATTGATGGGTA	757
G V	AB299163	GTGACTAATGAAGAACGTCGATTGATGGGTA	771
G VIII	GU290550	GTAACCAACGAAGAACCGGACTAATGGGTACTGTACCTA	757
G IX	GU939626	GTAACCTAACGAAGAACGTCGATTGATGGGTATTACGCCTA	776
G VI	AB299164	GTAACCAATGAAGAACGTAGATTAAATGGGTACCGTACCTC	771
G I	L63545	GTTACCAATGAAGAACGCGGACTTATGGGTACCACTCCTC	734

* * * * *

Figure 2. Multiple alignment of Lymphocystivirus MCP fragment sequences. Primers position and mismatch are shown. Alignment was conducted with 35 sequences, but only that with mismatch are shown. The following sequences have been used: G-I: LCDV-1 (L63545; Tidona and Darai, 1997); G-II: LCDV-C (AY380826; Zhang *et al.*, 2004); JF00Kuma, JF03Yoshi, JF03GunNeA (AB212997, AB212998, AB213000; Kitamura *et al.*, 2006), JF, LCDV-K1 (AY849391, AY303804); G-III: RF03Yosu (AB213004; Kitamura *et al.*, 2006), RF (AY823414; Kim and Lee, 2007), KRF (AY849392); G-IV: SB98Yosu (AB247938) LCDV-RC (EF103188; Fu *et al.*, 2007), RC (EF059992); RC-Taiwan (EF378607); G-V: PGF05 (AB299163; Hossain *et al.*, 2008); G-VI: PG06 (AB299164; Hossain *et al.*, 2008); G-VII: SA3, SA5, SA6, SA8, SA9, SSE11, SA12, SA13, SA14, SA16, SA18, SA19, SSE20, SA22, SA23, SA24 (GU320724–GU320739; Cano *et al.*, 2010); SAEilat (EF184306; Kvitt *et al.*, 2008); G-VIII: Leetown NFH (GU290550; Cano *et al.*, 2010); G-IX: YP1 (GU939626; Palmer *et al.*, 2012).

The specificity of the LCDV qPCR was evaluated by performing the assay on RNA and DNA from European Catfish Virus (ECV), genus *Ranavirus*, family *Iridoviridae*, and Red Spotted Grouper Nervous Necrosis Virus (RGNNV), genus *Betanodavirus*, family *Nodaviridae*, and on uninfected BF-2 cells.

Viral load of viral passages in cell cultures of sample SA64 was quantified testing both repeats of the second and third passages. The reproducibility of the assay was also determined on viral DNA extracted from infected BF-2 cells using samples with high (SA64 first viral passage in cell culture) and low (SA64 second viral passage in cell culture) viral load. Intra-assay variation was determined with five repeats of the sample within a single run. Inter-assay variation was determined by testing the sample in three independent assays. To determine the intra-assay and inter-assay variation, the mean, SD and CV were calculated using the logarithm of DNA copies/ μ l. The intra- and inter-assay variability was assessed by the CV represented as the SD/mean expressed as a percentage.

2.3.6. Quantitation of LCDV load in diseased and asymptomatic fish samples

The real-time PCR assay was applied to detect viral DNA in several apparently healthy tissues (caudal fin, pectoral fin, eye, brain, spleen) of two diseased fish (SA42, SA68) and one asymptomatic fish (SA79). The two diseased fish analysed showed a different clinical stage: subject SA42 showed clearly visible whitish nodules on the opercula, fins and skin, while subject SA68 was in a regressive phase and showed only small residual lesions on the fins and skin. Furthermore, viral load was quantified in caudal fin samples collected from asymptomatic fish (SA76, SA77, SA78, SA79, SA80, SA81, SA82) to verify the presence of LCDV DNA in recovered fish and to test the ability of the LCDV F1/R3 qPCR assay to detect LCDV DNA in asymptomatic fish. Each run was conducted in triplicates of at least six ten-fold dilutions of the standard plasmid to generate a robust standard curve. A no-template control was

included in each run, and all samples were tested in two repeats. Mix and cycle were the same as used for the standard curve evaluation (see Section 2.3.5).

Data are expressed as the mean of the two repeats \pm SD and were analysed by two-way ANOVA followed by Bonferroni post hoc tests to determine differences between the LCDV loads of different fish and tissues (Prism version 4.0 software, GraphPad Software, San Diego, USA). The level for accepted statistical significance was $p < 0.05$.

2.4 Results

2.4.1. Virological analysis

BF-2 cell monolayers inoculated with tissue samples showed cell alterations after the first viral passage of both tested samples (SA43 and SA64). Same alterations were observed in the second viral passage inoculated with sample SA64, but not with sample SA43. No alterations were observed after the third viral passages of both tested samples. The alterations consisted mainly of increased cell mortality and mild vacuolization. Repeats of the second and third viral passages on cell cultures gave identical results. No alterations were observed in the cell control.

2.4.2. Conventional PCR, sequencing and genotyping

Conventional PCR was performed to obtain and characterize the DNA of the Italian gilthead seabream LCDV (SA64) and the olive flounder LCDV (PO6). A 405-bp fragment of the LCDV DNA-dependent DNA polymerase gene was obtained using LF5/LR6 primers, and a 1356-bp fragment of the LCDV MCP gene was obtained using LC1-F/LC1-R primers from DNA extracted from the Italian gilthead seabream. A 789-bp fragment was obtained for the LCDV MCP gene using LF7/LC1-R primers from

DNA extracted from the olive flounder tissue. No PCR products were obtained with LF5/LF6 and LC1-F/LC1-R primer pairs from the LCDV infected olive flounder tissue as expected due to the specificity of primers for the gilthead seabream strains (Kvitt et al., 2008). Alignment of LF5/LR6 primer sequences with the DNA-dependent DNA polymerase gene of LCDV-C from olive flounder (accession number: AY380826) confirmed a low complementarity (data not shown); furthermore, previous authors have described that primers LC1-F/LC1-R are not able to amplify the MCP gene of LCDV-C (Kitamura *et al.*, 2006).

Conventional PCR with primers LF5/LR6 was able to detect LCDV DNA of both SA43 and SA64 samples in the first and second viral passages of the BF-2 cell culture in two repeats for each sample, while no PCR products were obtained at the third viral passages of both repeats for SA43 and SA64 samples (sample SA64 is shown in Fig. 3).

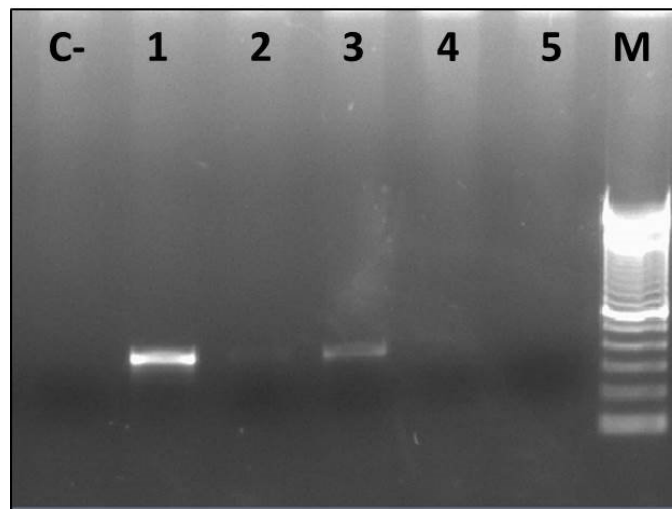


Figure 3. PCR with primers LF5-/LR6 of sample S64. C-, negative control. Line 1, first viral passage. Lanes 2–3, second viral passages (lane 2 first repeat, lane 3 second repeat). Lanes 4–5, third viral passages (lane 4 first repeat, lane 5 second repeat). M, 100bp molecular marker (Invitrogen).

The limit of detection of classical PCR primers LF7/LC1-R was established by testing ten-fold serial dilutions of DNA extracted from gilthead seabream and olive flounder tissues. LCDV was detected up to a dilution of 10^{-6} in the gilthead seabream sample, and to a dilution of 10^{-5} in the olive flounder sample (Fig. 4).

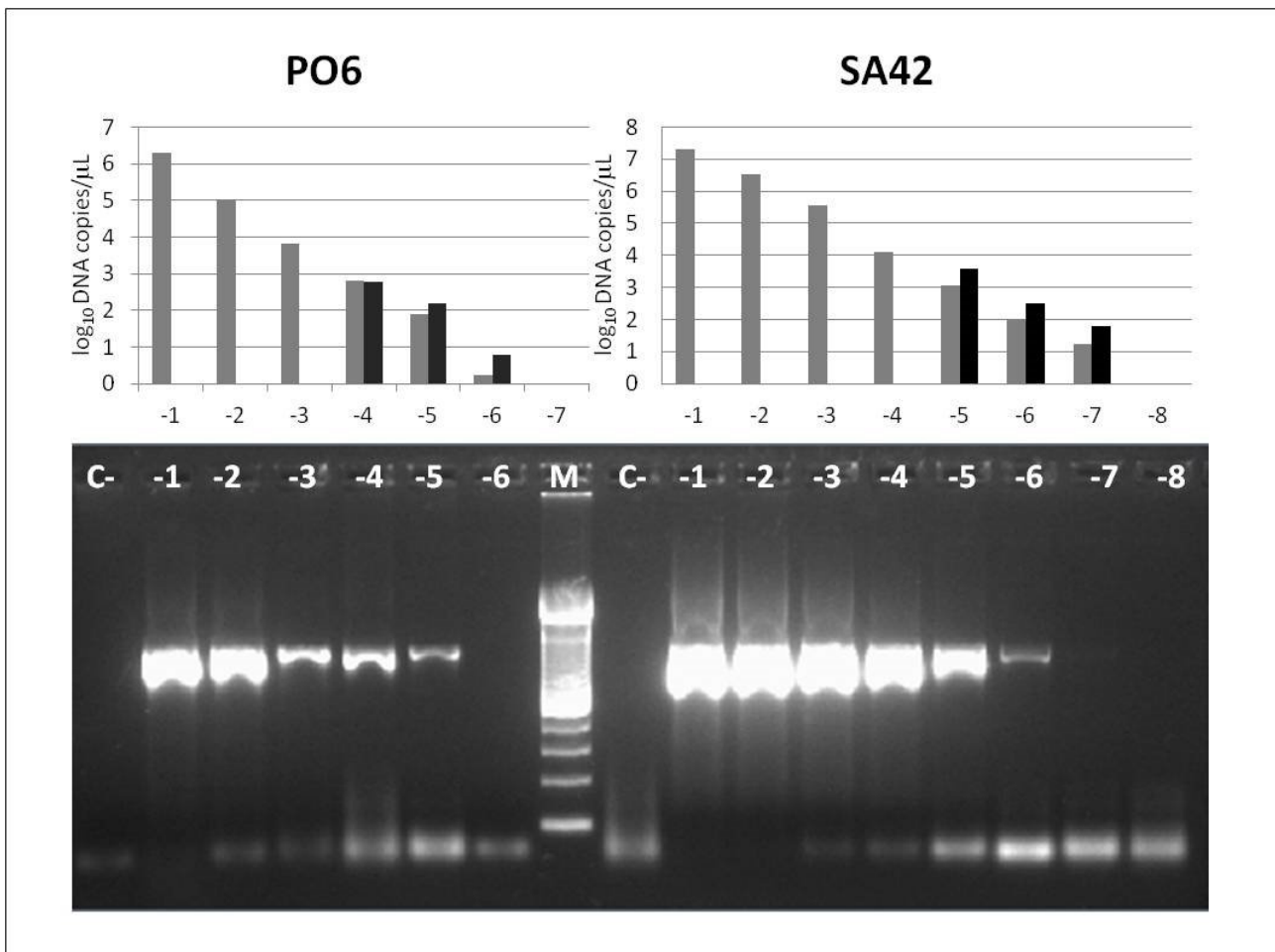


Figure 4. Limits of detection of real time PCR and conventional PCR with primers LF7/LC1-R. Graphics show viral DNA amounts detected by real time PCR. Dark columns show replicates. Lane C-, negative control. Lanes -1/-8 serial 10-fold dilutions of viral DNA (left: sample PO6, right: sample SA42). M, 100 bp molecular marker (Invitrogen).

Sequencing produced a 330-bp fragment of the DNA-dependent DNA polymerase gene and an 839-bp fragment of the MCP gene of the Italian gilthead seabream LCDV (SA64), whereas a 751-bp fragment of the MCP gene was obtained for the olive flounder LCDV (PO6). The sequences have been deposited in GenBank (NCBI) with accession numbers KP184510-KP184512. Amino acid sequences of the DNA-dependent DNA polymerase and MCP gene fragments were predicted using the BioEdit program.

Comparison of the DNA-dependent DNA polymerase gene fragment that encoded 110 amino acid residues with sequences collected from GenBank database showed 99%, 78% and 50% amino acid identity with the DNA polymerase protein of LCDV-SA-Eilat from gilthead seabream (EF375710), LCDV-C from olive flounder (NC_005902) and LCDV-1 from European flounder *P. flesus* (NC_001824), respectively.

The comparison of the MCP gene of the Italian gilthead seabream LCDV (SA64) with sequences of LCDV of the nine known genotypes showed nucleotide and deduced amino acid identities of 98.8–100% and 99.2–100%, respectively, with gilthead seabream LCDV isolated in Spain, Portugal, France, Tunisia and Israel, which have been classified as genotype VII (Cano *et al.*, 2010). Lower identity was shown with sequences of other genotypes (Table 2). A comparison between the MCP fragment of LCDV-SA64-Italy strain and 17 LCDV sequences classified within genotype VII available on the GenBank database showed that the Italian strain has a high nucleotide identity with gilthead seabream and Senegalese sole strains, regardless of geographic origin (Table 3). Furthermore, most of the nucleotide replacements among strains are non-coding. Moreover, some strains with lower nucleotide identity (SA3 and SA18) have a 100% amino acid identity (Table 3).

Table 2. Comparisons of nucleotide and deduced aminoacid sequences of the major capsid protein (MCP) gene fragment among the LCDV Italian stain (SA64) and genotypes of *Lymphocystivirus*. Genotypes are represented by the following strains (Genbank accession number and references): G-I: LCDV-1 (L63545; Tidona and Darai, 1997); G-II: LCDV-C (AY380826; Zhang *et al.*, 2004); JF00Kuma, JF03Yoshi, JF03GunNeA, (AB212997, AB212998, AB213000; Kitamura *et al.*, 2006a), JF, LCDV-K1 (AY849391, AY303804; Kim and Lee, 2007); G-III: RF03Yosu (AB213004; Kitamura *et al.*, 2006a), RF (AY823414; Kim and Lee, 2007), KRF (AY849392; Kim and Lee, 2007); G-IV: SB98Yosu (AB247938; Kitamura *et al.*, 2006b) LCDV-RC (EF103188; Fu *et al.*, 2007), RC (EF059992); RC-Taiwan (EF378607); G-V: PGF05 (AB299163; Hossain *et al.*, 2008); G-VI: PG06 (AB299164; Hossain *et al.*, 2008); G-VII: SA3, SA5, SA6, SA8, SA9, SSE11, SA12, SA13, SA14, SA16, SA18, SA19, SSE20, SA22, SA23, SA24 (GU320724-GU320739; Cano *et al.*, 2010); SAEilat (EF184306; Kvitt *et al.*, 2008); G-VIII: Leetown NFH (GU290550; Cano *et al.*, 2010); G-IX: YP1 (GU939626; Palmer *et al.*, 2012). Identity percentages of the most and least divergent isolates are shown.

		nucleotide identity									
		SA64	G-I	G-II	G-III	G-IV	G-V	G-VI	G-VII	G-VIII	G-IX
amino acid identity	SA64		78.9	85.1-85.3	84.5-84.7	87.0-87.1	89.2	83.3	98.8-100	85.2	83.5
	G-I	89.9		79.4-79.7	81.4-81.7	80.0-80.2	79	82.1	78.6-79.3	81.4	81.6
	G-II	95.6-96.4	88.8-89.2		86.8-87.3	90.3-91.2	86.5-86.7	86.0-86.4	84.8-85.5	84.2-84.5	82.5-82.8
	G-III	92.8-93.9	88.5-89.6	92.8-94.6		87.0-87.4	85.6-85.9	88.2-88.3	84.0-84.8	86.2-86.5	84.3-84.7
	G-IV	97.8-98.9	89.9-90.6	96.4-97.4	93.5-94.9		87.1-87.9	85.6-85.9	86.2-87.3	85.4-85.6	85.2-85.4
	G-V	97.8	89.9	95.3-96.0	92.4-93.5	96.7-97.8		84.8	88.5-89.2	84.8	83.7
	G-VI	92.1	88.8	92.8-93.5	93.5-93.9	92.4-93.1	92.4		83.1-83.7	87.3	85.5
	G-VII	99.2-100	89.2-89.9	95.3-96.4	92.1-93.9	97.1-98.9	97.1-97.8	91.3-92.1		84.8-85.4	83.0-83.5
	G-VIII	94.9	91.3	94.2-94.9	95.3-96.4	94.9-96.0	94.9	94.9	94.2-94.9		88.2
	G-IX	93.1	88.8	92.1-92.8	92.1-93.5	93.9-94.2	92.8	92.1	92.8-93.1	95.3	

Table 3. Pairwise comparisons of major capsid protein (MCP) gene fragment among the LCDV Italian strain (SA64) and 17 LCDV sequences classified within genotype VII and available on Genbank database. Genbank accession numbers and references: SA3: GU320724; SA5: GU320725; SA6: GU320726; SA8: GU320727; SA9: GU320728; SSE11: GU320729; SA12: GU320730; SA13: GU320731; SA14: GU320732; SA16: GU230733; SA18: GU320734; SA19: GU320735; SSE20: GU320736; SA22: GU320737; SA23: GU320738; SA24: GU320739 (Cano *et al.*, 2010); LCDV-SA-Eilat: EF184306 (Kvitt *et al.*, 2008).

		Nucleotide identity																	
		SA64	SA6	SA16	SA3	SA5	SA8	SA9	SA12	SA13	SA14	SA18	SA19	SA22	SA23	SA24	SSE11	SSE20	SA-Eilat
Amino acid identity	SA64		99.6	99.6	98.8	99.6	99.6	99.6	99.2	98.9	99.6	98.8	99.5	100	99.7	99.8	99.2	99.2	99.7
	SA6	100		100	99.1	100	100	100	99.6	89.9	99.8	99.1	99.8	99.6	81	99.5	99.6	99.6	99.6
	SA16	100	100		99.1	100	100	100	99.6	89.9	99.8	99.1	99.8	99.6	81	99.5	99.6	99.6	99.6
	SA3	100	100	100		99.1	99.1	99.1	98.8	89.7	99	100	99.2	98.8	80.3	98.6	98.8	98.8	98.8
	SA5	100	100	100	100		100	100	99.6	89.9	99.8	99.1	99.8	99.6	81	99.5	99.6	99.6	99.6
	SA8	100	100	100	100	100		100	99.6	89.9	99.8	99.1	99.8	99.6	81	99.5	99.6	99.6	99.6
	SA9	100	100	100	100	100	100		99.6	89.9	99.8	99.1	99.8	99.6	81	99.5	99.6	99.6	99.6
	SA12	99.6	99.6	99.6	99.6	99.6	99.6	99.6		89.8	99.5	98.8	99.5	99.2	80.9	99.1	99.5	99.2	99.2
	SA13	99.6	90.3	90.3	90.3	90.3	90.3	90.3	90.3		89.8	89.7	90.1	89.6	88.9	89.5	89.6	89.6	89.6
	SA14	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.2	89.9		99	99.7	99.6	81	99.5	99.5	99.5	99.6
	SA18	100	100	100	100	100	100	100	99.6	90.3	99.6		99.2	98.8	80.3	98.6	98.8	98.8	98.8
	SA19	100	100	100	100	100	100	100	99.6	90.3	99.6	100		99.5	80.9	99.4	99.5	99.5	99.5
	SA22	100	100	100	100	100	100	100	99.6	90.3	99.6	100	100		81.4	99.8	99.2	99.2	99.7
	SA23	99.6	81.3	81.3	81.3	81.3	81.3	81.3	81.3	89.3	81	81.3	81.3	81.3		81.5	80.9	80.6	81.1
	SA24	100	100	100	100	100	100	100	99.6	90.3	99.6	100	100	100	81.3		99.1	99.1	99.6
	SSE11	100	100	100	100	100	100	100	99.6	90.3	99.6	100	100	100	81.3	100		99.2	99.2
	SSE20	99.2	99.2	99.2	99.2	99.2	99.2	99.2	98.9	89.6	98.9	99.2	99.2	99.2	80.6	99.2	99.2		99.2
	SA-Eilat	100	100	100	100	100	100	100	99.6	90.3	99.6	100	100	100	81.3	100	100	99.2	

2.4.3. Standard curve and reproducibility assays

Serial ten-fold dilutions of plasmids were assayed in triplicate using real-time PCR with the designed primer pairs, and standard curves were generated based on Ct values. The linear range of the assay with primer pair LCDV_qPCR_F1/R3 (LCDV F1/R3 qPCR) was between 2.6×10^9 and 2.6 DNA copies/ μ l with an R2 value >0.99 and a reaction efficiency of 1.00 ± 0.20 (Fig. 5). PCR with the primer pair LCDV_qPCR_F1/R2 gave poorer outcomes, and so this primer pair was not used further.

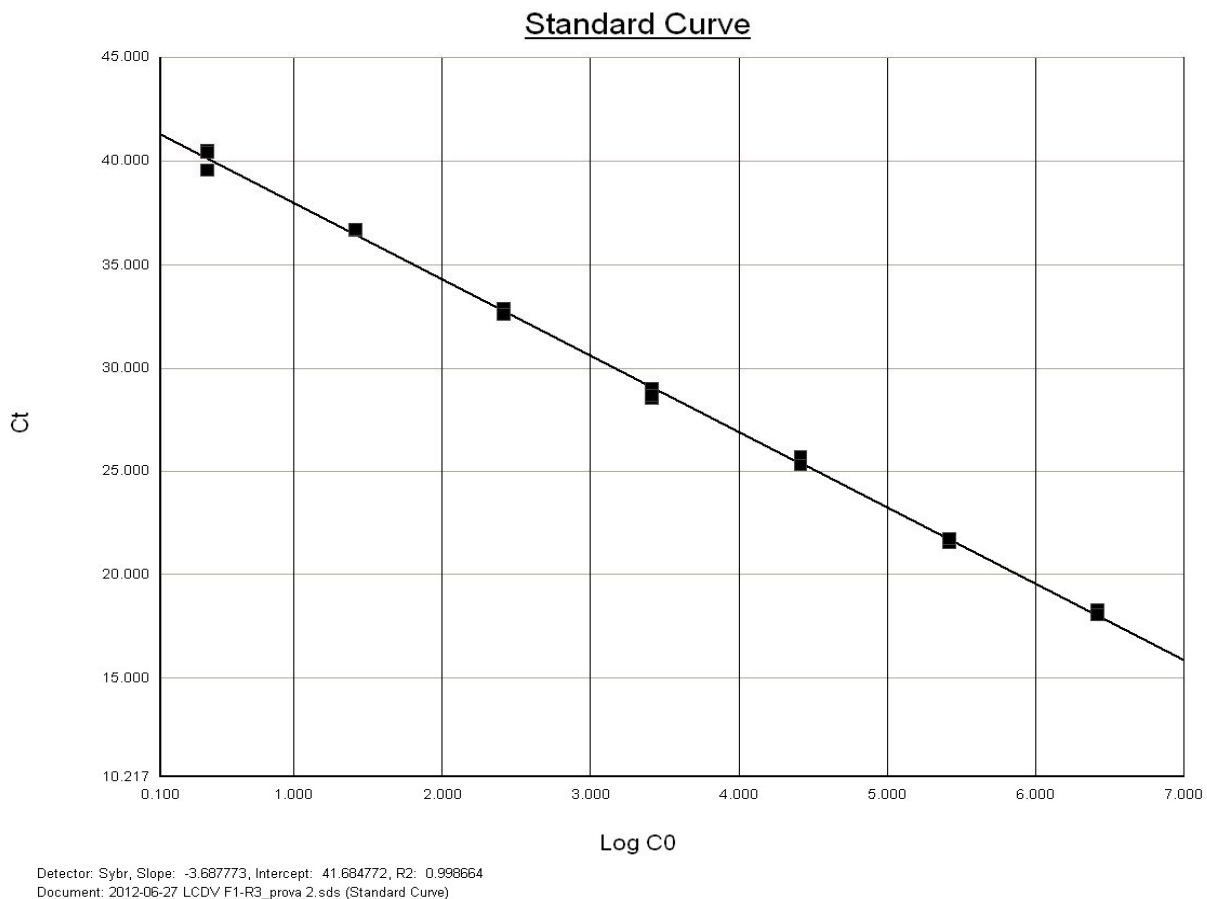


Figure 5. Standard curve for the LCDV F1/R3 qPCR obtained with 10-fold serial dilutions from 2.6×10^6 to 2.6 plasmid DNA copies/ μ l shows the linear relationship between Ct values and the dilutions of the plasmid DNAs of LCDV.

The limit of detection for LCDV F1/R3 qPCR was 2.6 DNA copies/ μ l. The mean \pm SD intra-assay variability of the DNA standard was 1.04% \pm 0.92%, whereas that of the inter-assay variability was 1.25% \pm 0.71%. Experimental intra- and inter-assay variability values are considered acceptable and are summarized in Table 4.

Table 4. Intra- and inter-assay reproducibility of the LCDV qPCR assay.

Variation	Dilution of standard	Crossing point		
		Mean Ct	S.D. ^a	CV (%) ^b
Intra-assay ^c	2.6x10 ⁵	19,08-19,26	0.04-0.25	0.23-1.31
	2.6x10 ⁴	22.71-23.32	0.04-0.32	0.19-1.40
	2.6x10 ³	26.95-27.37	0.10-0.28	0.37-1.03
	2.6x10 ²	31.34-31.73	0.04-0.47	0.11-1.48
	2.6x10	34.32-35.71	0.08-1.20	0.24-3.48
	2.6	38.70-40.38	0.11-1.09	0.29-2.77
Inter-assay	2.6x10 ⁵	19.18	0.09	0.49
	2.6x10 ⁴	22.97	0.31	1.36
	2.6x10 ³	27.14	0.21	0.78
	2.6x10 ²	31.48	0.22	0.70
	2.6x10	35.09	0.71	2.01
	2.6	39.50	0.84	2.14

^a Standard Deviation, ^b Coefficient of variation and ^c Minimum and maximum values obtained in 3 replicates are shown.

LCDV F1/R3 qPCR was able to detect and quantify viral DNA directly from infected cell culture lysates and from tissues. No PCR products were obtained for viruses, other than LCDV, such as ECV, RGNNV and for uninfected BF-2 cells. A dissociation curve with a single peak was obtained for the LCDV plasmid and samples (melting temperature, T_m = 81°C), but not for the uninfected BF-2 cells or viruses (ECV, RGNNV) other than LCDV (Fig. 6).

LCDV F1/R3 qPCR was able to detect and quantify viral DNA in all viral passages in the BF-2 cell cultures of the sample SA64, showing a higher sensitivity compared to conventional PCR with primers LF5/LF6. The samples were tested with multiple independent assays and the final result is shown as the mean of the individual results. Viral load quantitation showed a decreasing amount of viral DNA from the first

to the third passage (Fig. 7). Repeatability tests on samples with high and low viral load gave acceptable inter- and intra-CV values (Table 5).

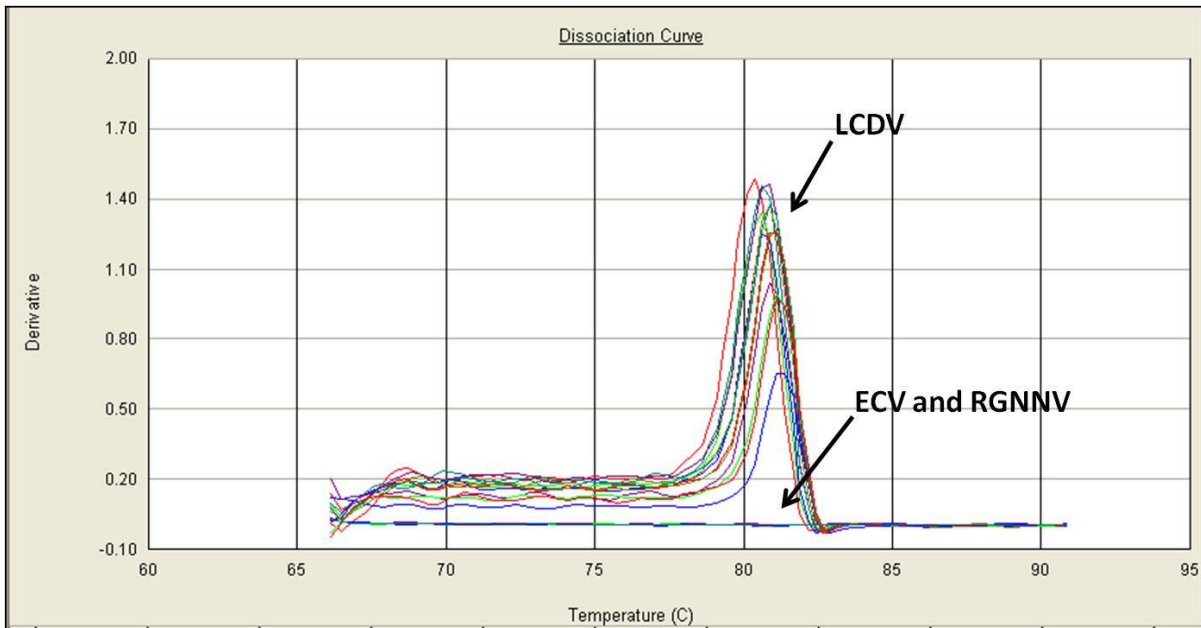


Figure 6. Dissociation curve of LCDV plasmid, positive samples and negative control (ECV and RGNNV).

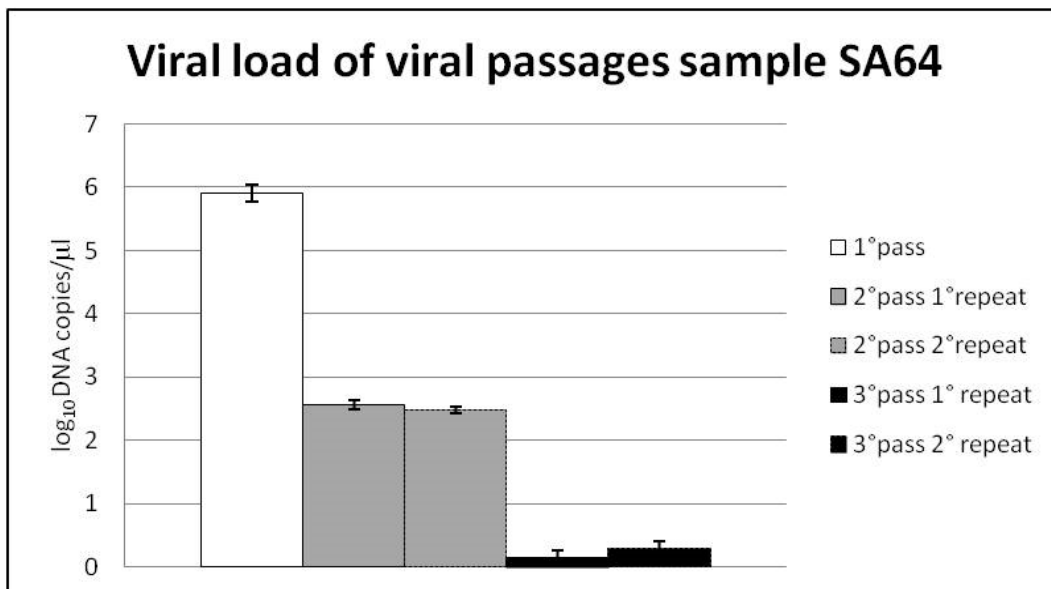


Figure 7. Viral load detected by real time PCR in viral passages of sample SA64.

Table 5. Intra-and inter-assay reproducibility of the amount of LCDV DNA concentration determined by real time PCR. Low: sample SA64 2° viral passage on cell culture, high: sample SA64 1° viral passage on cell culture.

Variation	DNA concentration	Crossing point			
		Mean ^a	log ₁₀ mean	log ₁₀ S.D. ^b	log ₁₀ CV % ^c
Intra-assay	Low	3.51x10 ²	2.54	0.06	2.37
	High	9.45x10 ⁵	5.90	0.31	5.26
Inter-assay	Low	3.67x10 ²	2.56	0.07	2.56
	High	8.35x10 ⁵	5.9	0.14	2.41

^a LCDV DNA is presented as target DNA copy/μl, ^b Standard Deviation, ^c Coefficient of variation.

The real-time PCR was able to detect and quantify the viral DNA in pathological tissues of both gilthead seabream (SA42) and olive flounder (PO6). Viral DNA was detected up to a dilution of 10⁻⁷ for sample SA42 and 10⁻⁶ for the sample PO6, showing a sensitivity ten-times higher than the conventional PCR with primers LF7/LC1-R (Fig. 4).

2.4.4. Viral load in diseased and asymptomatic fish

The application of real-time PCR to apparently healthy tissues from two diseased (SA42, SA68) and one asymptomatic (SA79) fish detected LCDV DNA in typical target tissues (caudal fin and pectoral fin) and in internal organs (eyes, brain and spleen). The tissues with the highest viral load were the pectoral and caudal fins in both diseased and asymptomatic fish. In diseased fish, viral load in fins was statistically higher ($p < 0.01$) than the viral load in all internal organs, whereas in the asymptomatic fish, viral load in fins was statistically higher than viral load in the eyes and spleen ($p < 0.05$), but not in the brain (Fig. 8). Subject SA42 showed a statistically higher viral load in the fins and eyes compared to SA68 and SA79 ($p < 0.05$), and subject SA68

showed a statistically higher viral load in fins and eyes compared to SA79 ($p < 0.05$) (Fig. 8).

The application of real-time PCR to the caudal fin samples taken from asymptomatic subjects showed the presence of viral DNA in all the tested subjects (Fig. 9). Viral loads detected in caudal fin samples of asymptomatic fish ($1.38 \times 10^6 \pm 2.25 \times 10^6$ DNA copies/ μ l) were significantly lower ($p < 0.05$) than those of diseased individuals (SA42: $3.1 \times 10^8 \pm 5.55 \times 10^6$ DNA copies/ μ l; SA68: $3.6 \times 10^7 \pm 3.64 \times 10^6$ DNA copies/ μ l) (Fig. 9).

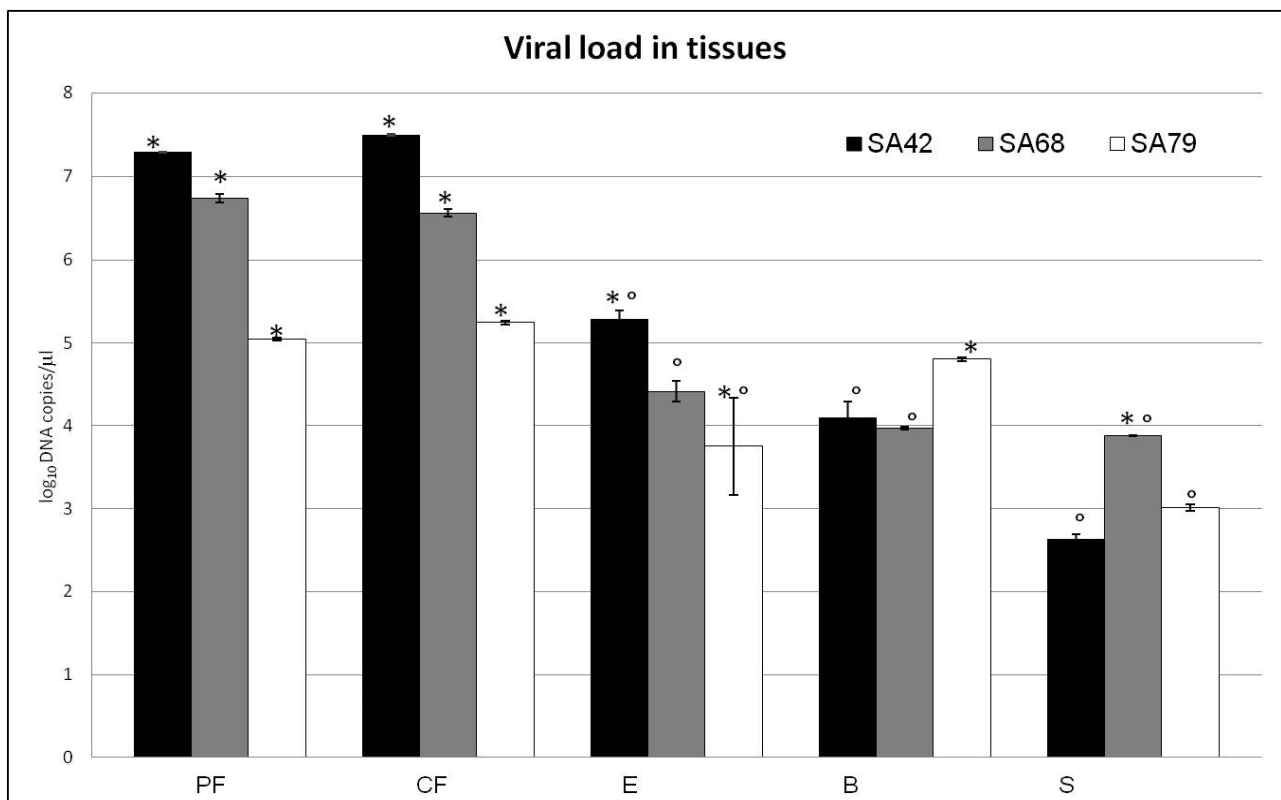


Figure 9. Viral load in caudal fin samples of diseased (SA42, SA68) and asymptomatic (SA76-SA81) fish. Columns with asterisk (*) show statistically significant differences ($p < 0.05$) between diseased and asymptomatic subjects.

2.5 Discussion

A thorough analysis of the MCP gene of LCDV isolates and the cytochrome b gene of host species showed that the LCDV strains and their host fish evolved independently (Yan *et al.*, 2011). However, field observations and experimental trials confirmed that the host range of each genotype is limited to one or a few species (Kitamura *et al.*, 2006; Hossain *et al.*, 2008; Cano *et al.*, 2010).

The LCDV detected in gilthead seabream have been shown to be a highly homogeneous group of viruses from a genetic point of view, with a high nucleotide and amino acid similarity of the MCP gene between the Italian strain (SA64) sequenced in this study and the viruses detected previously from the same species in Spain, Portugal, France, Israel and Tunisia, which have been classified as genotype VII (Cano *et al.*, 2010).

The ability of LCDV strains to replicate in vitro seems to depend on the source of the cell culture and each LCDV strain can be isolated only in limited and specific cell cultures, severely limiting the methods to study LCDV infection. In the present study, the BF-2 cell line was not able to allow efficient viral replication of gilthead seabream LCDV and viral DNA decreased drastically from one cell passage to another. The BF-2 cell line, however, has already been shown to be unable to yield infectious particles, and a defective assembly stage has been hypothesized (Walker and Hill, 1980). A cell line was set up from the fin tissue of gilthead seabream (SAF-1) and it was revealed to be suitable for isolation of LCDV from gilthead seabream in addition to being permissive for viruses other than Lymphocystivirus (Perez-Prieto *et al.*, 1999). Nevertheless, viral isolation and cultivation of Lymphocystivirus is still a difficult and time-consuming technique.

Several non-quantitative PCR assays have been developed successfully for Lymphocystivirus detection in several species, including gilthead seabream. Some of these assays have been set up to detect Lymphocystivirus from specific fish species and they have been tested against a limited number of LCDV genotypes (Kvitt *et al.*,

2008). However, some primer pairs failed to amplify LCDV that were classified into different genotypes, as in the case of JF-LCDV (genotype II) and RF-LCDV (genotype III) (Kitamura *et al.*, 2006).

In the present study, primers were designed on a multiple sequence alignment to minimize primer-template mismatches against all known LCDV genotypes. The PCR-based assay developed in this research gave equal sensitivity and efficiency in detecting and quantifying genome copies of two distantly related LCDV genotypes (VII and II), and was applied successfully to detect LCDV directly in pathological tissues of different species such as gilthead seabream and olive flounder. The unavailability of LCDV belonging to other genotypes made it impossible for us to test the assay against all the known genotypes; however, the alignment of the available GenBank sequences of target regions of the LCDV F1/R3 qPCR primers suggests that the primers will be adequate for the detection and quantitation of all LCDV genotypes. A further analysis of a wide range of strains using the developed method in this study will be able to confirm its suitability for the detection and quantitation of all genotypes.

The development of real-time PCR assays has been proposed recently in place of conventional PCR for advantages including being much less liable to cause carryover contamination, being quantitative and having high sensitivity (Palmer *et al.*, 2012). Palmer *et al.* (2012) developed a real-time PCR assay to detect and quantify LCDV in yellow perch, using primers specific for the LCDV genotype IX sequences. This technique has been revealed to be very useful for the study of LCDV in yellow perch, and viral load in symptomatic and asymptomatic fish (Palmer *et al.*, 2012).

High sensitivity is particularly important for detection of LCDV in asymptomatic carriers. The availability of methods to detect LCDV in carrier fish is a critical point in lymphocystis control. The application of a direct, rapid and sensitive assay for virus detection using samples collected without killing the animals, such as fin samples in the case of LCDV infection, may allow a proper screening of fish before they are introduced to a farm or before reproduction (Cano *et al.*, 2007).

Two methods are currently available to detect LCDV in asymptomatic carriers of gilthead seabream. One is based on serological analysis and the other is based on a PCR assay. However, both assays need additional steps to achieve a high sensitivity, such as a viral amplification step in cell culture and a subsequent viral concentration for the immunoblot technique and a slot-blot hybridization after the PCR assay, making these techniques more time consuming compared to real-time PCR (Cano *et al.*, 2006; Cano *et al.*, 2007).

The LCDV qPCR developed in this study revealed higher sensitivity compared to other conventional PCR assays in detecting LCDV both in cell culture lysates and directly in naturally infected tissues. In particular, the qPCR assay showed ten-times greater sensitivity than conventional PCR and it was able to detect 2.6 plasmid-DNA copies/ μ l.

Furthermore, the method detected successfully LCDV in caudal fin samples collected from asymptomatic fish, so the developed qPCR assay is a good candidate for in vivo healthy carrier detection through the screening of caudal fin samples. A previous study on yellow perch showed that real-time PCR assay is the method of choice to detect and quantify LCDV in asymptomatic fish (Palmer *et al.*, 2012).

The ability of real-time PCR to quantify, in addition to detecting, the virus allows several applications of this technique. In particular, pathogenesis studies could benefit from this opportunity, especially for viruses with low or no cultivability.

Important pathogenesis studies regarding lymphocystis were conducted recently revealing a systemic and persistent infection in gilthead seabream (Kvitt *et al.*, 2008; Cano *et al.*, 2009). In this context, the developed real-time PCR is a valuable complementary tool as has been noted by the authors of these studies (Cano *et al.*, 2009).

The application of the developed real-time PCR assay to diseased and asymptomatic fish demonstrated the suitability of the method for pathogenesis studies. Systemic infection was shown both in diseased and asymptomatic fish with the spread of the virus to internal organs, such as the spleen and brain, as well as to the eyes and

fins. In addition to the gilthead seabream, systemic infection was described previously for yellow perch (Palmer *et al.*, 2012), but LCDV was not detected in the internal organs of experimentally infected olive flounder (Hossain *et al.*, 2009). The use of a common diagnostic technique, such as the described real-time PCR, in pathogenesis investigations in different fish species may help to clarify the origin of such different pathogenesis pathways.

In this study, LCDV loads were correlated with the clinical stage in the pectoral fins, caudal fins and eyes. The highest values were found in the subject with nodules, intermediate values in tissues of fish with the regressive form and the lowest values in the recovered fish. Two orders of magnitude between viral load (pectoral and caudal fins) of the subject with typical LCD nodules and the asymptomatic subject were revealed even if tissues without lesions were used for both fish. However, the present study analysed only a very limited number of samples, whereas the analysis of a larger sample of subjects needs to be carried out to assess the correlation of viral load with different clinical stages.

No correlation between viral load and clinical stage was seen in internal organs such as brain and spleen. A previous investigation showed that viral copy number in the spleen and liver of symptomatic perch was four orders of magnitude higher than that in asymptomatic perch (Palmer *et al.*, 2012). Further studies will be able to answer whether some internal organs may be the sites of persistent infection due to active viral replication.

In summary, the developed LCDV qPCR was highly sensitive, specific and reproducible for the detection and quantitation of Lymphocystivirus, and it has proved to be suitable for various applications of considerable epidemiological and pathogenic interest for the study and the control of lymphocystis in different fish species.

2.6 Acknowledgements

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CHAPTER III

DEVELOPMENT OF A MULTIPLEX RT-PCR ASSAY FOR SIMULTANEOUS DETECTION OF THE MAJOR VIRUSES THAT AFFECT RAINBOW TROUT (*Oncorhynchus mykiss*)

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3.1 Abstract

The major viral diseases that affect rainbow trout (*Oncorhynchus mykiss*) are viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN) and sleeping disease (SD). In the presented study, we developed a multiplex RT-PCR (mRT-PCR) assay for the simultaneous detection of these four rainbow trout viruses in a single assay. The choice of primers was carried out based on the expected size of the fragments, the temperature and time required for the amplification, and the specificity for the target sequence. Firstly, the method was optimised using reference strains of *Viral haemorrhagic septicaemia virus* (VHSV), *Infectious haematopoietic necrosis virus* (IHNV), *Infectious pancreatic necrosis virus* (IPNV) and sleeping disease virus (SDV) cultivated with permissive cell culture lines; subsequently, the method was used for the identification of these viral infections in rainbow trout samples. Twenty-two samples of rainbow trout, clinically suspected of having viruses were analysed by the developed method to detect the presence of the four viruses, by directly analysing the animal tissues. The mRT-PCR method was able to efficiently detect the viral RNA in infected cell culture supernatants and in tissue samples, highlighting the presence of single infections as well as co-infections in rainbow trout samples. VHSV/SDV and IHNV/SDV co-infections were demonstrated for the first time in rainbow trout. The mRT-PCR method was revealed to be an accurate and fast method to support traditional diagnostic techniques in the diagnosis of major viral diseases of rainbow trout.

Keywords: *Infectious haematopoietic necrosis virus*, *Infectious pancreatic necrosis virus*, Multiplex RT-PCR, *Oncorhynchus mykiss*, Rainbow trout, Sleeping disease virus, *Viral haemorrhagic septicaemia virus*.

3.2 Introduction

In the last 10 years, rainbow trout (*Oncorhynchus mykiss*) has represented the second highest annually produced product in European aquaculture (Gomez-Casado *et al.*, 2011). The increase in aquaculture operations worldwide has provided new opportunities for the transmission of aquatic viruses. The occurrence of viral diseases remains a significant limiting factor in aquaculture production and for the sustainability of biodiversity in the natural environment (Crane and Hyatt 2011). Major viral diseases of trout are caused by viruses such as viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and sleeping disease virus (SDV).

VHSV and IHNV belong to the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Dietzgen *et al.*, 2012). Both of these viruses are associated with high mortality, especially in rainbow trout (Miller *et al.*, 1998; Skall *et al.*, 2005; Reichert *et al.*, 2013). Viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) represent two of the most economically important diseases in farmed salmonids worldwide (Bootland and Leong 1999) and both are notifiable in the European Community (Council Directive 2006/88/EC of 24 October 2006).

IPNV, a member of the family *Birnaviridae* and genus *Aquabirnavirus* (Delmas *et al.*, 2012), is a non-enveloped, double-stranded RNA virus which may induce mortalities as high as 90% in rainbow trout fry (Byrne *et al.*, 2008). IPNV is highly contagious and transmitted through a variety of routes. Adult fish can serve as asymptomatic carriers of the virus, eventually shedding the virus particles into the environment where other fish can become infected. For this reason, the development of sensitive detection methods is crucial for rapid management of infected stock (Bowers *et al.*, 2008).

SDV is responsible for sleeping disease (SD), an infectious disease of rainbow trout reared in fresh water and spread both in continental Europe and in the United Kingdom (Snow *et al.*, 2010). SDV was characterized as an *Alphavirus* of the family

Togaviridae (Powers *et al.*, 2012) and is now also known as Salmonid alphavirus subtype 2 (SAV 2) (McLoughlin and Graham 2007). Isolation of SDV from tissue is technically demanding and the major difficulty of the isolation process is the identification of virus-induced cytopathic effect, which normally requires several virus/cell passages to develop (Boscher *et al.*, 2006).

From an epizootiological perspective, it is significant that these causative agents are quickly identified and distinguished. Furthermore, determination of the origin of an outbreak may help to prevent further introductions (Bruchhof *et al.*, 1995). Use of cultured eukaryotic cells in a controlled environment for virus propagation and isolation is widely accepted and is still considered the 'gold standard' for diagnosis of most viral diseases (Cella *et al.*, 2013). The exponential growth of viruses in a cell culture system makes it a very sensitive method for virus detection if the cells used are of high susceptibility and quality, and if the cells are handled by experienced personnel. However, for correct diagnosis, several days of culture are required for cytopathic effects to be observed and subsequent virus identification (Jonstrup *et al.*, 2013).

Molecular techniques are extremely sensitive and provide rapid diagnosis and multiplexing options for the detection of several viruses at the same time (Cella *et al.*, 2013). Polymerase chain reaction (PCR)-based techniques, such as reverse transcription PCR (RT-PCR) and real-time RT-PCR (real time RT-PCR) have become important diagnostic tools for RNA virus detection (Ellis *et al.*, 2010; Calleja *et al.*, 2012).

In recent years, nucleic acid amplification techniques such as RT-PCR have been described and applied for the detection of several viruses, including VHSV, IHNV, IPNV and SDV because of their accuracy and high sensitivity (Graham *et al.*, 2006; Knusel *et al.*, 2007; Oryan *et al.*, 2012). However, the need to perform separate, individual PCR assays for each virus complicates the diagnostic process and increases costs (Williams *et al.*, 1999). Multiplex PCR assays have gained popularity because of their convenience in terms of cost and time (Giridharan *et al.*, 2005). The considerable advantages provided by these techniques make them attractive in the field of fish

pathology (Williams *et al.*, 1999; del Cerro *et al.*, 2002; Mata *et al.*, 2004; Altinok *et al.*, 2008; Altinok *et al.*, 2011).

The aim of the present study was to develop a multiplex RT-PCR (mRT-PCR) assay for the simultaneous detection of four rainbow trout viruses in a single assay. Once developed, the technique was applied for direct viral detection from tissues collected from animals suspected of having viruses. This technique enabled the detection of the presence of VHSV/SDV and IHNV/SDV co-infections, which until now has never been reported for rainbow trout.

3.3 Materials and methods

3.3.1 Virus titration

Viral haemorrhagic septicaemia virus (strain F1) and *Infectious hematopoietic necrosis virus* (strain 217-A) were inoculated onto a monolayer of EPC (epithelioma papulosum cyprinid) cells. *Infectious pancreatic necrosis virus* (strain Sp) and sleeping disease virus (strain F05-105) were inoculated onto a monolayer of CHSE-214 (chinook salmon embryo) cells. Titrations of the viruses were performed according to the end-point dilution method. Briefly, serial 1:10 dilutions of the viral solution were made and 100 µl of each dilution was pipetted onto an EPC or CHSE cell line, grown in a 96-well plate and incubated at 15 °C. After 7 days, each well was checked for cytopathic effects (CPE) using an inverted microscope. The 50% tissue culture infective dose (TCID₅₀/ml) was calculated using the method of Reed and Muench (1938), and the generated viruses were divided into volumes of 1 ml and conserved at -80 °C.

3.3.2 RNA extraction

Viral RNA was extracted from 100 µl of infected cell culture supernatant and was isolated according to the manufacturer's instructions with TRI REAGENT® (Molecular Research Center, Inc. Ohio, U.S.A.). The RNA pellet was resuspended in the 1X RNA-secure solution (Ambion, Inc. New York, U.S.A.) and then pre-heated to 60 °C for 20 min. The resuspended RNA was stored at -80 °C.

3.3.3 Primers

Four sets of primers that specifically amplified VHSV, IHNV, IPNV and SDV, respectively, were used (Table 1). Primers were selected from references or designed *ex novo* to obtain the optimal characteristics for use in the assay. The forward primer for IPNV, A4 F (5'-GTGGCCTATGAGAAGATGAC-3'), was designed from a conserved region within the VP2 gene of aquatic birnaviruses.

Table 1. Nucleotide sequences of the primers used in this study.

Virus	Primer Name	Sequence 5'-3'	Coding region	Product size (bp)	Reference
VHSV	VN-1F	GGGGACCCCAGACTGT	N	787	Knusel <i>et al.</i> , 2007
	VN-3R	TCTCTGTCACCTTGATCC			
IHNV	IN-1F	CAGAGACGGAGTATCGTCCC	N	310	Knusel <i>et al.</i> , 2007
	IN-2R	ACAACCTCAAGGACATTTTCC			
SDV	227 F	GACTGGCCTCCTTACGGGG	E1	227	Graham <i>et al.</i> , 2006
	227 R	TTACAACCGTGCGGTGCTGT			
IPNV	A4F	GTGGCCTATGAGAAGATGAC	VP-2	197	This study
	A2R	GACAGGATCATCTTGGCATAGT			Eissler <i>et al.</i> , 2011

The conserved region was identified from alignments of nucleotide sequences available in the GenBank database (Accession numbers: AF342727, AF343571, AF343572, AF343573, AY026345, AY026346, AY026347, AY026348, AF343570, AF342728, AY026482, AY026483, AY026484, AY026485, AF342729, AY026486, AY026487, AY026488, AY026489, AF342730, AF342731, AY026490, AF342733, AF342734, AF342735).

3.3.4 Optimization of the multiplex RT-PCR

First, the individual RT-PCR assays (singleplex) to detect each of the four viral pathogens (VHSV, IHNV, IPNV, SDV) were conducted under various conditions of annealing temperature, extension time and cycle quantity, in order to identify the optimal parameters for use in the multiple RT-PCR assay. Then, various primer concentrations were tested until we obtained an optimal product for each target virus.

The mRT-PCR assay was performed on RNA extracted from infected cell culture supernatants of each viral strain separately, and later on an RNA mixture of all four viruses. The negative control, which consisted of sterile water plus the reaction mix, and the positive control, which consisted of a mixture of RNA of the four fish viruses, were included in each mRT-PCR assay. Ten microlitres of amplified products were analysed by gel electrophoresis (100 V, 30 min).

3.3.5 Application of the mRT-PCR to clinical samples

The mRT-PCR assay was applied to detect viral RNA directly from 22 rainbow trout specimens, clinically suspected to have viruses. To rule out the amplification of non-specific products, specimens collected from two uninfected rainbow trout were also tested. All samples were also tested using a singleplex assay. Each sample consisted of a pool of about 100 mg of organs (kidney, spleen and heart); these tissues were collected from fish and homogenized. Total RNA was extracted with TRI

REAGENT® (Molecular Research Center Inc., Ohio, USA) according to the manufacturer's instructions. The RNA pellet was resuspended in 40 µl of 1X RNA-secure solution (Ambion, Inc., New York, USA), pre-heated to 60 °C for 20 min, and then stored at -80 °C.

3.4 Results and discussion

The infectious titers of the produced viral solutions were $10^{7.45}$ TCID₅₀/ml for VHSV, $10^{8.08}$ TCID₅₀/ml for IHNV, $10^{8.41}$ TCID₅₀/ml for IPNV and $10^{4.35}$ TCID₅₀/ml for SDV.

The optimisation procedure permitted us to obtain the following mRT-PCR protocol, which was used for virus detection in cell culture and fish tissues. The optimised reaction consisted of 15 µl of reaction mix containing 5 µl RNA, 7.5 µl 2X Reaction Mix SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA), 0.2 µM IHNV primer, 0.3 µM VHSV and SDV primers, 0.4 µM IPNV primer, and 0.5 µl Superscript III/Platinum Taq enzyme mix. The optimal thermal cycling conditions were 50 °C for 30 min, 95 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min.

The application of mRT-PCR assay to viral RNA extracted from infected cell culture supernatants yielded specific products for all four target templates both when tested separately and when the mRT-PCR was applied to the RNA mixture of the four viruses (Fig. 1). Nonspecific products were not obtained during amplification reactions. The following specific fragments of different sizes were obtained for the four target viruses: 787 bp for VHSV, 310 bp for IHNV, 227 bp for SDV and 197 bp for IPNV (Fig. 1).

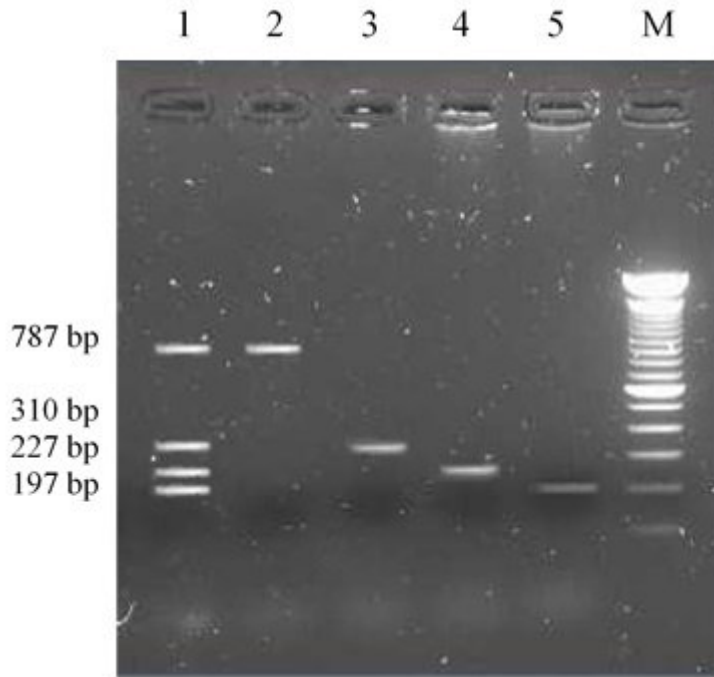


Figure 1. Fig. 1 Agarose gel showing results of multiplex RT-PCR assay from infected cell culture supernatants. Lane 1: simultaneous detection of VHSV, IHNV, SDV and IPNV. Lane 2: VHSV positive, 787 bp; Lane 3: IHNV positive, 310 bp; Lane 4: SDV positive, 227 bp; Lane 5: IPNV positive, 197 bp; Lane M: 100 bp molecular marker (Invitrogen).

The application of the mRT-PCR to RNA samples directly extracted from fish tissue yielded at least one PCR product for all clinically suspected samples. Each fragments' size permitted us to easily identify the virus infecting the sample. No amplification was obtained in tissue samples collected from uninfected fish.

Among the 22 tissue samples analysed by mRT-PCR, VHSV was detected in 16 samples, IHNV in 4 samples, and SDV in 5 samples. The viral RNA of IPNV was not detected in any of the tested samples. In two samples, the presence of both VHSV and SDV was shown, while in one sample both IHNV and SDV were detected (Fig. 2).

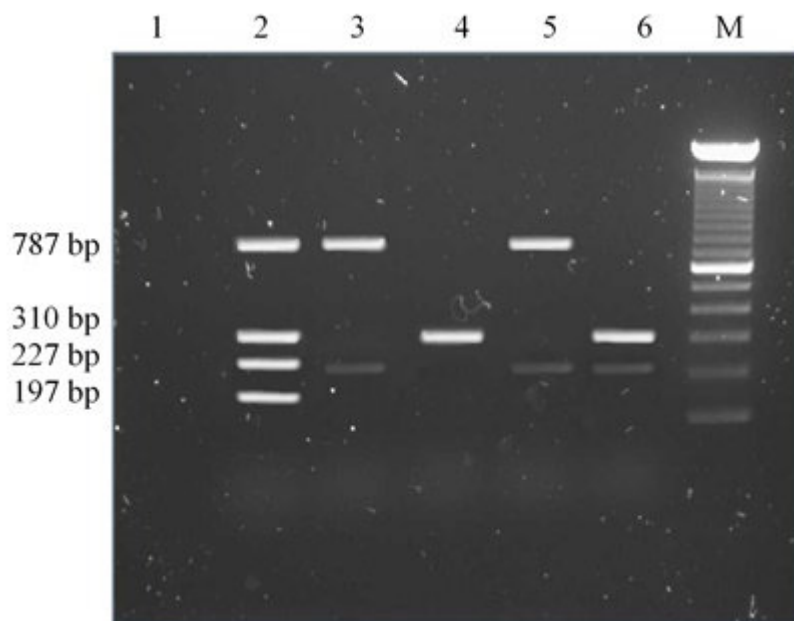


Figure 2. Agarose gel showing results of multiplex RT-PCR assay from tissues samples of rainbow trout. Lane 1: Negative control; Lane 2: Positive control; Lane 3: VHSV and SDV positive sample; Lane 4: IHNV positive sample; Lane 5: VHSV and SDV positive sample; Lane 6: IHNV and SDV positive sample; Lane M: 100 bp molecular marker (Invitrogen).

The availability of rapid, sensitive, and specific diagnostic methods for the detection of the major viral pathogens of farmed fish, such as VHSV, IHNV, IPNV and SDV is critical for preventing the spread of these pathogens in aquaculture. Several singleplex PCR assays have been developed for the detection and identification of fish pathogens (Graham *et al.*, 2006; Knusel *et al.*, 2007; Eissler *et al.*, 2011). However, a large number of individual PCR reactions would be necessary if single primer sets for each pathogen were used to screen a large number of clinical samples, resulting in a relatively costly and time-consuming process (Altinok *et al.*, 2011).

Compared to the single-target PCR techniques, the construction of a multiplex assay can be rather complex, considering the large number of primers required (Pestana *et al.*, 2010). In fact, the various primers might compete with each other, thus requiring a careful choice of primer sequences, annealing temperature and amplification time (Henegariu *et al.*, 1997). In the present study, multiple primer concentrations, timings and temperatures had to be tested, but finally, an m-PCR method was developed that

produced a single clear band for each target virus. The long and complicated optimisation procedure, however, could be used in multiple applications.

Multiplex PCR, in fact, potentially has considerable advantages in the laboratory that do not compromise test utility (Elnifro *et al.*, 2000). Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites (Elnifro *et al.*, 2000). The simultaneous detection of several pathogens with a multiplex PCR approach is relatively rapid and cost effective compared to standard virological assays. The multiplex assay has been shown to have wide application in diagnosing clinically suspected samples in the fields of both human and animal health (Giridharan *et al.*, 2005; Mengelle *et al.*, 2014). Moreover, it is arousing growing interest in the field of fish pathology (Williams *et al.*, 1999; del Cerro *et al.*, 2002; Mata *et al.*, 2004; Altinok *et al.*, 2008; Altinok *et al.*, 2011). The assay set up used in this study could be widely applicable in differential diagnosis in clinical cases characterized by non-specific signs, or in identifying viruses isolated in cell culture. In fact, more than one rainbow trout virus is able to grow in the same cell culture (Villas *et al.*, 1994; McLoughlin and Graham 2007).

Furthermore, the application of a multiplex assay can provide more information than originally sought, revealing asymptomatic infections and allowing more knowledge about the health status of the fish population being tested to be gained. This is particularly useful for new diseases that are very poorly investigated, especially if they have an insidious trend as in the case of sleeping disease (McLoughlin and Graham 2007). Despite not analysing asymptomatic fish, the samples collected were from fish and farms where no typical signs of sleeping disease were declared, so we suspect that the SDV presence, revealed by the mRT-PCR, was from asymptomatic forms of the disease. The presence of very weak positivity for SDV, obtained with the mRT-PCR, supports this hypothesis. Therefore, this assay could represent an important diagnostic tool to detect fish carrying the major pathogens in aquaculture, especially

when low prevalence is expected and large numbers of fish need to be analysed. It may be applicable for epidemiological and transmission studies and may contribute to efforts to control or eradicate infectious diseases (Altinok *et al.*, 2011). However, a wide application to asymptomatic carriers would be needed before using the mRT-PCR assay for surveillance purposes.

Traditional methods used for the virological examination of fish rely upon cell culture systems for isolation of the virus, and serum neutralisation or fluorescent antibody assays to identify the agent. Despite this, virus isolation using cell culture is actually one of the most sensitive methods for detecting viruses in symptomatic and asymptomatic fish (it is still considered the ‘gold standard’ method for diagnosis and surveillance programs). However, molecular methods offer some advantages over cell culture techniques, for example, in the detection of co-infections that require a more complex approach (Alonso *et al.*, 1999). Several studies have shown the occurrence of naturally occurring co-infections in rainbow trout and in particular, VHSV/IPNV and IHNV/IPNV co-infections have been described (LaPatra *et al.*, 1993; Vilas *et al.*, 1994). Furthermore, Alonso *et al.* (1999) demonstrated that IPNV interfered with IHNV replication in cell lines, decreasing the IHNV titer to undetectable levels. In such cases of co-infection, more sensitive detection methods for each virus are needed to avoid false negative results (Alonso *et al.*, 1999). The direct application of molecular techniques for the detection of viruses in tissue samples has been proved to have successful results (Knusel *et al.*, 2007); these techniques could also prove successful for co-infection analysis, because they reveal the presence of viral RNA directly in tissue without the use of cell culture.

The multiplex RT-PCR in this study was able to detect the presence of one or two viruses directly in rainbow trout tissues, showing that the technique can detect the presence of naturally occurring viral co-infections in rainbow trout. Specifically, in this study both VHSV/SDV and IHNV/SDV co-infections were detected. To the best of our knowledge, no reports about co-infection involving alphaviruses (SAV) have been

reported for rainbow trout, although IPNV/SAV co-infection was frequently reported in Norwegian Atlantic Salmon (Skotheim, 2009).

In conclusion, the results presented here demonstrate that the developed multiplex RT-PCR method was able to efficiently detect the viral RNA in infected cell culture supernatants and was directly able to detect viral RNA in tissue samples, highlighting the presence of single infections as well as co-infections in rainbow trout samples. This method can be useful for rapid diagnosis of major diseases in rainbow trout, thus helping to prevent disease outbreaks in rainbow trout farms, the most valuable fish industry in Italy. Furthermore, the method could be used to investigate simultaneously the spread of different diseases without largely increasing the cost of each survey.

Moreover, the conducted investigation has revealed the presence of VHSV/SDV and IHNV/SDV co-infections which, until now, have never been reported in rainbow trout. Further studies are necessary to clarify the epidemiological and pathological impact of these co-infections.

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FINAL CONSIDERATIONS

The present PhD thesis was aimed to investigate the use of molecular techniques for viral fish diseases. The first objective was to review current molecular techniques used for the study and the diagnosis of the major fish viral infections. Furthermore, the experimental section has the aim to propose two new molecular technologies for the detection and the study of fish viruses responsible of important diseases.

The increase in aquaculture operations worldwide has provided new opportunities for the transmission of aquatic viruses. The occurrence of viral diseases remains a significant limiting factor in aquaculture production and for the sustainability of biodiversity in the natural environment. The ability of the molecular techniques to identify quickly the presence/absence of a pathogenic organism in fish provide significant advantages for the control of infectious diseases in aquaculture systems. In addition, the sensitivity and high accuracy of molecular techniques offer suitable tools of studying the etiological agents and the interaction with their hosts. Consequently, these techniques contribute efficiently to the control and the prevention of the diseases, avoiding their spreading among farmed animal populations.

The literature reviewed in the first chapter showed that numerous and different viruses affect farmed fish populations. Multiple molecular techniques are reported for the diagnosis and the study of several of these viruses. Advantages, and in some case disadvantages, are reviewed for each method showing the great contribute that these techniques have provided for the study of fish viral infections.

The experimental approach of this PhD dissertation focuses on the development of the two innovative molecular techniques to diagnose and to study some important viral diseases of two fish species relevant to European aquaculture.

The technique developed and described in the second chapter was a quantitative real-time PCR (qPCR) assay for detection and quantification of lymphocystis disease virus (LCDV). It showed to be highly sensitive, specific, reproducible and versatile for the detection and quantitation of *Lymphocystivirus*. For these reasons, this technique

can find multiple application such as asymptomatic carrier detection or pathogenesis studies of different LCDV strains.

The multiplex RT-PCR assay, developed and described in the third chapter, was able to detect simultaneously VHSV, IHNV, IPNV and SDV RNA in tissue samples, showing the presence of single infections as well as co-infections in rainbow trout samples. This method was revealed a fast method to support traditional diagnostic techniques in the diagnosis of major viral diseases of rainbow trout.

Further investigations are recommended to provide new assays and improved the current techniques for the detection of fish viruses, as these will represent effective and practical tools for the study and the control of important viral fish diseases in the aquaculture industry