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Viral encephalopathy and retinopathy in aquaculture: a review

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Abstract :

Viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), is a major devastating threat for aquatic animals. Betanodaviruses have been isolated in at least 70 aquatic animal species in marine and in freshwater environments throughout the world, with the notable exception of South America. In this review, the main features of betanodavirus, including its diversity, its distribution and its transmission modes in fish, are firstly presented. Then, the existing diagnosis and detection methods, as well as the different control procedures of this disease, are reviewed. Finally, the potential of selective breeding, including both conventional and genomic selection, as an opportunity to obtain resistant commercial populations, is examined.

Introduction

Although there is presently no strong evidence highlighting a possible raise of fish disease outbreaks due to climate change, increasing temperatures are expected to induce the spread of pathogens towards higher latitudes and to provoke negative impacts on fish physiology (Cochrane et al. 2009). Among others, the viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), is considered one of the most serious viral threats for almost all marine aquaculture fish species, and requires a special focus due to the fact that outbreaks mostly happen in warm conditions. This disease, detected in at least 70 cultured or wild marine and fresh water species, already caused serious economic losses in the aquaculture industry in the past decades, and we can anticipate larger impacts of this disease because of global warming.

No simple and effective procedures are available to treat this disease in fish. It is, therefore, important to develop tools and set up new approaches to limit the occurrence and impacts of VNN episodes in aquaculture farms.

To stress that need, we present here an extensive review about VNN disease in aquaculture, including the features of the virus, the available procedures to control this disease, and the potential of selective breeding and genomic selection for resistance to viral diseases, as a prospective way to prevent VNN disease in fish.

NERVOUS NECROSIS VIRUS

The causative agent of VNN, the Nervous Necrosis Virus, was classified as a member of the Nodaviridae family (Mori et al. 1992) which contains two genera: alphanodavirus and betanodavirus (Van Regenmortel et al., 2000). The species of the first genus were originally isolated from insects (figure 1), but appear to infect both vertebrates and invertebrates, and to cause the death of insect and mammalian hosts (Adachi et al. 2008). Betanodaviruses usually affect the nervous system of marine fish, leading to behavioral abnormalities and extreme high mortalities (Munday et al. 2002). In mammals, the pathogenicity of betanodaviruses is poorly reported, but mice have been demonstrated as non-susceptible, and human cells as not permeable to that genus (Adachi et al. 2008). Recently, a new emerging disease, the white tail disease (WTD) which affects the giant freshwater prawn and the whiteleg shrimp *Penaeus vannamei* has been demonstrated to be caused by the *Macrobrachium rosenbergii* nodavirus (MrNV). Sequence analysis of this virus suggests the existence of a new genus, gammanodavirus, infecting crustaceans (Qian et al. 2003; Senapin et al. 2012 - figure 1).

General morphology:

Betanodavirus virions were first described as non-enveloped, spherical in shape, and have icosahedral symmetry, with a diameter around 25nm and a capsid formed by 180 copies of a

single protein of 42 Kda (Mori et al. 1992). A similar virus of 20-34 nm in diameter was detected in infected Asian sea bass *Lates calcarifer* larvae, striped jack *Pseudocaranx dentex*, turbot *Scophthalmus maximus*, European sea bass *Dicentrarchus labrax* (Yoshikoshi & Inoue 1990; Glazebrook et al. 1990; Bloch et al. 1991; Munday et al. 1992) and many various fish species through the world were subsequently recorded to be infected by betanodaviruses (Munday et al. 2002; Shetty et al. 2012).

Molecular structure:

Betanodavirus contains a bi-segmented genome composed of two single-stranded, positive-sense RNA molecules (Mori et al. 1992). The sequence of RNA1 is about 3.1 kb, and includes an open reading frame (ORF) encoding a RNA-dependent RNA polymerase (RdRp) of 110 kDa catalyzing the replication of the virus, also named protein A (Nagai & Nishizawa 1999). The sequence of RNA2 (1.4 kb) encodes the capsid protein (37kDa) which may have a function in the induction of cell death (Guo et al. 2003). In addition, during the virus replication, a sub-genomic RNA (RNA3) is synthesized from the 3'-terminus of RNA1 (Ball & Johnson 1999). This RNA3 encodes two other nonstructural proteins, B1 (111 amino acids) and B2 (75 amino acids). Protein B1 displays anti-necrotic property enhancing the viability of viral host cell (Sommerset & Nerland 2004). Protein B2 is an inhibitor of host RNA silencing in either alphanodavirus or betanodavirus, but could also promote mitochondrial fragmentation and cell death induced by hydrogen peroxide production (Su et al. 2014).

Classification:

Betanodavirus was described for the first time from infected larval stripped jack. The name striped jack nervous necrosis virus (SJNNV) was consequently adopted (Mori et al. 1992). Subsequently other agents of VNN were isolated from diseased fish species (Munday et al. 2002). The first comparative studies between viral strains isolated from different marine fish species were done in the middle of the 1990s, where Nishizawa *et al.* reported the sequence of

SJNNV and four different fish Nodaviruses as well as four different insect Nodaviruses (Nishizawa et al. 1995). From a phylogenetic analysis of the RNA2 T4 variable region, betanodaviruses were classified into four different species designed as the SJNNV-type, the barfin flounder nervous necrosis virus (BFNNV)-type, the red-spotted grouper nervous necrosis virus (RGNNV)-type, and the tiger puffer nervous necrosis virus (TPNNV)-type (Nishizawa et al. 1997). These species partially correlate with three different serotypes determined from virus neutralization using polyclonal antibodies (serotype A for SJNNV species, B for TPNNV species and C for BFNNV and RGNNV species) (Morit et al. 2003). Each species corresponds to different host fish and different *in vitro* optimal growth temperatures (table 1). RGNNV is the most popular species because a variety of fish species, distributed in warm-water, are affected (optimal growth temperature of 25−30°C) (Asian sea bass, European sea bass, groupers…), whereas BFNNV is restricted to cold-water (15−20°C) marine fish species (Atlantic halibut *Hippoglossus hippoglossus*, Atlantic cod *Gadus morhua*, flounders…) and TPNNV infects a single species (Tiger puffer *Takifugu rubripes*) at an intermediate temperature (20°C). The SJNNV type was initially known to affect a few species cultured in Japan at 20-25°C (Iwamoto et al. 2000; Munday et al. 2002; Nishizawa et al. 1995; Toffan et al. 2016). However, it was also recently described in some fish species cultured in Southern Europe such as Senegalese sole *Solea senegalensis* in Spain, gilthead sea bream *Sparus aurata* and European sea bass in the Iberian Peninsula (Thiéry et al. 2004; Cutrín et al. 2007). This capacity to infect such warm water fish species is probably associated to reassortant RGNNV and SJNNV strains (Iwamoto et al. 2004; Toffolo et al. 2007; Panzarin et al. 2012; Toffan et al. 2016, see also Phylogenetic relationshipc paragraph). Phylogenetic analysis of betanodaviruses was also made based on the T2 region, which covers a larger RNA2 sequence than T4 (Chi et al. 2003; Johansen et al. 2004). This taxonomy has been used to genetically characterized new isolates in various fish species as well as in different areas

(Aspehaug et al. 1999; Starkey et al. 2000; Dalla Valle et al. 2001; Tan et al. 2001; Skliris et al. 2001; Johnson et al. 2002; Chi et al. 2003; Gagné et al. 2004; Sommerset & Nerland 2004; Thiéry et al. 2004; Johansen et al. 2004; Ransangan & Manin 2012; Vendramin et al. 2013). Because NNV is detected in many new species as well as new regions, description of new isolates and sequences are regularly published and could lead to evolution in the classification (table 1). For example, an additional genotype including a turbot betanodavirus strain (TNNV) was described in 2004. This species is currently awaiting classification (Johansen et al. 2004).

An alternative classification has been proposed (Thiéry et al. 2004). However, this numerical nomenclature (cluster I, II, III and IV), independent from the host species origin, is not extensively used because viruses from different clusters could infect a same host species, for example European sea bass (Thiéry et al. 1999) and the classification was not consistent with geographical areas (Dalla Valle et al. 2001; Thiéry et al. 2004; Cutrín et al. 2007).

Phylogenetic relationships:

Among the different species of betanodaviruses, amino acid sequences of RdRp protein and capsid protein share 87 to 99% and 77 to 100% of identity respectively (82 to 98% for the complete RNA1 nucleic sequence and 76 to 99% for the RNA2 segment (Okinaka & Nakai 2008). The topology of phylogenetic trees based on RNA1 and RNA2 distinguishes several clades, suggesting a high diversity despite relatively strong purifying selection on most codons (Panzarin et al. 2012). This important variability can be explain by a significant substitution rate but also by a re-assorting process specific to segmented viruses (Panzarin et al. 2012).

DISTRIBUTION AND TRANSMISSION

Distribution:

Viral encephalopathy and retinopathy is one of the most widespread viral disease of marine fish species cultured worldwide. A large number of species have been reported to be affected, especially larval and juvenile stages in which high mortalities were recorded (Munday et al. 2002; Shetty et al. 2012). Based on clinical signs, VNN disease has been documented since 1985 in Japanese parrotfish *Oplegnathus fasciatus* larvae and juveniles in Japan, while the pathogen was first observed in the brain of reared Japanese parrotfish (Yoshikoshi & Inoue 1990). Three years later, it was recorded in European sea bass produced in Martinique (West Indies, France) and French Mediterranean (Breuil et al. 1991). Since then, similar clinical signs with encephalitis associated with picorna-like viral particles were observed in the Asian sea bass *Lates calcarifer* cultured in Australia (Glazebrook et al. 1990; Munday et al. 2002), as well as in turbot *Scopthalmus maximus* (Bloch et al. 1991), red-spotted grouper *Epinephalus akaara* (Nishizawa et al. 1995), striped jack *Pseudocaranx dentex* (Mori et al. 1992), Japanese flounder *Paralichthys olivaceus* (Nishizawa et al. 1995), tiger puffer *Takifugu rublipes,* kelp grouper *Epinephelus moara* (Munday et al. 2002) and barfin flounder *Verasper moseri* in Japan (Nishizawa et al. 1995), and recently in golden grey mullet *Liza aurata* and leaping mullet *Liza saliens* in the Caspian Sea (Zorriehzahra et al. 2016).

Infections caused by NNV have been detected all around the world, with the notable exception of South America (Crane & Hyatt 2011; Shetty et al. 2012). It was the cause of mass mortality in Atlantic halibut in Norway and Scotland (Grotmol et al. 1997; Starkey et al. 2000) and in juvenile greasy grouper *Epinephelus tauvina* in Singapore (Hegde et al. 2002) and in groupers in Taiwan (Chi et al. 1997). Betanodaviruses have been the cause of high economical losses in aquaculture industry throughout the Mediterranean area. Mass

157 mortalities have been repeatedly recorded since 1991 on larvae and juvenile stages in
158 European sea bass in France (Breuil et al. 1991) as well as on grow-out size sea bass in
159 Greece, Italia and Tunisia (Le Breton et al. 1997; Bovo et al. 1999; Thiery et al. 2004;
160 Haddad-Boubaker et al. 2013). Grey mullet *Mugil cephalus*, red drum *Sciaenops ocellatus*,
161 and barramundi cultured in Israel were also reported to be affected by NNV (Ucko et al.
162 2004). Farmed Senagalese sole *Solea senegalensis* were reported as infected by RGNNV and
163 SJNNV in Spain (Thiery et al. 2004, Hodneland et al. 2011). More recently, RGNNV, SJNNV
164 genotypes and reassortant RGNNV/ SJNNV and SJNNV/RGNNV viruses have been reported
165 to infect several fish species (European sea bass, sea bream, Senegalese sole) in
166 Mediterranean Sea (Toffolo et al. 2007; Olveira et al. 2009; Hadda-Boubaker et al. 2013;
167 Panzarin et al. 2012; Toffan et al. 2016). A strain belonging to the RGNNV species caused
168 mass mortality in white sea bass *Atractoscion nobilis* reared in South California in 1999
169 (Curtis et al. 2001). NNV was also found in Atlantic cod and haddock *Melanogrammus*
170 *aeglefinus* juvenile stages on the Atlantic coast of North America (Johnson et al. 2002).
171 Furthermore, betanodaviruses do not only affect reared fish species, but have also been found
172 in a variety of wild fish species, as reported in table 2.

173 Regarding environment, although NNV is mostly known for infecting aquatic animals in
174 marine and brackish water, the reports of freshwater species infected by NNV have been
175 increasing (table 2). NNV infection was observed in freshwater eel and catfish aquaculture
176 systems in Taiwan (Chi et al. 2003) as well as in other freshwater species including sturgeon
177 *Acipenser gueldenstaedtii* (Athanassopoulou et al. 2004), tilapia *Oreochromis niloticus*
178 (Bigarré et al. 2009), largemouth bass *Micropterus salmoides*, pike-perch *Sander lucioperca*,
179 striped bass x white bass, *Morone saxatilis* x *Morone chrysops* (Bovo et al. 2011), guppy
180 *Poecilia reticulata* (Hegde et al. 2003), Australian catfish *Tandanus tandanus*, and sleepy cod
181 *Oxyeleotris lineolatus* (Munday et al. 2002). Zebrafish *Danio rerio* and goldfish *Carassius*

auratus were also found to be infected (Binesh 2013). Furthermore, the freshwater blenny *Salaria fluviatili*, which is an endangered species endemic to watersheds of the Mediterranean Basin, was also reported as affected by NNV (Vendramin et al. 2012). To date, the susceptibility of Mandarin fish *Siniperca chuatsi* to RGNNV, an important economical species in freshwater aquaculture in China, has been demonstrated (Tu et al. 2016). At present, at least 70 host species belonging to 32 families of 16 orders have been described as carriers of betanodavirus (table 2) and this disease is widely reported all over the world, with the exception of South America.

Transmission:

NNV is characterized by both vertical and horizontal transmission (Munday et al. 2002, see also figure 2). Vertical transmission was early described in a number of different fish species where betanodaviruses were detected in broodstock gonads or in early larval stages with typical symptomatic signs. It can occur from broodstock to larvae through germplasm, including the eggs or genital fluids as reported in striped jack, in barfin flounder or in European sea bass (Mushiake et al. 1994; Nishizawa et al. 1996; Mori et al. 1998; Watanabe et al. 2000; Dalla Valle et al. 2000; Breuil et al. 2002).

Horizontal transmission is a very difficult route to control because betanodavirus can easily spread during an outbreak via water but also rearing equipment (Mori et al. 1998; Watanabe et al. 1998). Horizontal transmission has been experimentally demonstrated by several routes: contact between healthy fish and diseased larvae (Arimoto et al. 1993), bathing fish in water containing betanodavirus-infected tissue homogenates (Arimoto et al. 1993; Tanaka et al. 1998; Grotmol et al. 1999), contamination using strains isolated from symptomatic fish (Koch postulate) (Thiéry et al. 1997; Peducasse et al. 1999) or contact of healthy fish with asymptomatic carriers (Skliris & Richards 1999; Breuil et al. 2002).

Once in the aquatic environment, betanodavirus can persist without host for a long time and can be spread widely by tide, aquatic transport means or migration of the wild hosts (Gomez et al. 2004; Gomez et al. 2008; Giacopello et al. 2013). As NNV was reported in sand worms belonging to the family *Nereidae* (Liu et al. 2006a) but also in crabs and mussels (Gomez et al. 2008), several studies are carried out to clarify the existence of non-fish carriers or vectors of NNV such as raw fish (trash fish), brine shrimp *Artemia salina* and mollusks used as feed for marine culture (Gomez et al. 2010; Costa & Thompson 2016). Commercial trade of aquatic animals should also be regarded as an important potential source of virus diffusion (Gomez et al. 2006).

DIAGNOSIS/DETECTION

First diagnostic approaches:

In the early 1990s, the structure of NNV was already clearly known but virus isolation using cell lines was not successful. Therefore, the method of VNN diagnostic relied on the observation of characteristic clinical signs. VNN is characterized by typical behavioral abnormalities (erratic swimming patterns such as spiraling or whirling, lying down at the tank bottom, rapid swimming, darker coloration…) associated to an impairment of the nervous system (figure 3) (Yoshikoshi & Inoue 1990; Breuil et al. 1991; Chi et al. 1997). Gross pathology examination frequently reveals a hyperinflation of the swim-bladder and hemorrhages on the brain tissue. The most common microscopical findings consist of vacuolation and necrosis of nervous cells of the spinal cord, brain and/or retina, particularly in larvae and juveniles stages. The infection is rarely accompanied by inflammatory processes. In presence of these typical signs, diagnosis must be confirmed by a laboratory test. Electronic microscopy allowed observation of virus particles free or membrane bound by endoplasmic reticulum in cells collected from infected organs (brain, retina) and revealed icosahedral, non-

enveloped viruses with a commonly reported diameter of 20-34 nm (Yoshikoshi & Inoue 1990; Glazebrook et al. 1990; Breuil et al. 1991; Bloch et al. 1991; Mori et al. 1992; Grotmol et al. 1997). Over two decades, the reference method to detect betanodavirus was isolation in permissive cell culture (striped snakehead cells SSN-1 or E11) followed by immunological (indirect fluorescent antibody test – IFAT, immunohistochemistry, enzyme-linked immunosorbent assay – ELISA; Nuñez-Ortiz et al. 2016) or molecular identification (RT-PCR, Nested RT-PCR, real time RT-PCR). However, cell culture is time consuming, requires a great experience, and some NNV strains are not always easy to detect because of a poor cultivability and/or the absence of induction of clear cytopathic effects. This is why molecular methods, particularly real-time RT-PCR, have been increasingly used (Munday et al. 2002; Shetty et al. 2012).

Direct molecular methods:

Numerous RT-PCR protocols have been described for the detection of VNN (table 3). The first RT-PCR published designed a set of primers (F2/R3) directed against 430 bp from the T4 variable region of the RNA2 segment of a SJNNV strain isolated from striped jack (Nishizawa et al. 1994). Later on, the same region was amplified from other isolates, such as red-spotted grouper (Nishizawa et al. 1995). This test, recommended by the World Organization for Animal Health (OIE) until 2006, was extensively used for routine diagnostic and genotyping of betanodavirus and led to the current classification (Nishizawa et al. 1995; Nishizawa et al. 1997). However, the sensitivity of this method is not only limited by a low viral load but also by the genetic diversity of the T4 region that leads to mismatches between the F2/R3 primers and their targets (Nishizawa et al. 1996; Thiéry et al. 1997; Dalla Valle et al. 2001). In some cases, it has been illustrated that betanodavirus in brain could be detected by immunohistochemistry whereas the same samples were negative by RT–PCR (Thiéry et al. 1997). In addition, low or false positive

as well as false negative results were reported in different fish species like striped jack, barfin flounder, European sea bass, shi drum *Umbrina cirrosa* and gilthead sea bream (Nishizawa et al. 1996; Mori et al. 1998; Thiéry et al. 1999; Watanabe et al. 2000; Dalla Valle et al. 2000). To improve the performance of this test and take into account genetic diversity reported in newly available sequences, further generations of tests were developed. Primers specific to more conserved region of the RNA2 or allowing to discriminate Mediterranean and Atlantic viral strains were published as well as Nested-PCR approaches allowing to improve the sensitivity by at least 100 times (Thiéry et al. 1999; Dalla Valle et al. 2000). More recently, Bigarré and colleagues designed a new set of primers in a highly conserved region (680 bp) named T6 in RNA2 which perfectly matches with a wide range of published sequences and detects at least three of the five described species namely RGNNV, SJNNV and BFNNV (Bigarré et al. 2010).

Since 2005, numerous real-time RT-PCR assays were developed to regularly adapt the primer sets and probes to newly published sequences (Dalla Valle et al. 2005; Fenner et al. 2006a; Panzarin et al. 2010; Hick & Whittington 2010; Hodneland et al. 2011; Baud et al. 2015). These Real-time RT-PCR assays, targeting RNA1 or RNA2, are now currently used for the diagnosis of betanodavirus because they are less time consuming than classical approaches and significantly decrease cross-contamination occurring during post amplification procedures (Hick & Whittington 2010; Hodneland et al. 2011). Recently, a one-step generic TaqMan® method targeting sequences found in a vast majority of known viral genotypes was validated and efficiently used to detect NNV in different geographic regions and host species (Panzarin et al. 2010; Baud et al. 2015), and an optimized loop-mediated isothermal amplification has been developed to detect NNV in *Epinephelus septemfasciatus* (Hwang et al. 2016). This last method showed improved sensitivity compared to PCR.

Detection of different NNV species coexisting in the same host is still complex and may require a combination of approaches (Lopez-Jimena et al. 2010). An ubiquitous assay detecting all species would be desirable, but because of the high genetic diversity of betanodavirus, selection of specific and wide spectrum primers allowing the detection of all possible variants still remains a big challenge (Hodneland et al. 2011).

Indirect serological methods:

Serological investigations have been developed for several viral fish diseases but only few of them are used for routine surveillance, despite the fact that diseases survivors often become latent carriers with significant antibody response. The major reasons for this are poor knowledge on the kinetics of the antibody response in fish at various water temperatures and lack of validation data. Nevertheless, several ELISA or serum neutralization tests described and improved over time proved their efficiency to detect antibodies specific to VNN (Watanabe et al. 1998; Huang et al. 2001; Fenner et al. 2006b; Scapigliati et al. 2010; Choi et al. 2014; Jaramillo et al. 2016b). For ELISA tests, the determination of the cut-off point is critical to make the distinction between virus free status and viral infection. These indirect methods are routinely used by several fish farms to regularly screen breeders. They have the advantage to be no-lethal and safe for fish, and allow a regular screening of the VNN serological status of a population at an individual level (Breuil & Romestand 1999; Watanabe et al. 2000; Breuil et al. 2002; Breuil et al. 2001; Jaramillo et al. 2016a; Nuñez-Ortiz et al. 2016).

CONTROL PROCEDURES

There are no simple and effective procedures to treat the viral disease in fish once established. Therefore, efforts were concentrated on the means and tools to prevent entry, diffusion and

302 persistence of the virus, mostly strict hygiene, vaccination and eradication of infected
303 populations (Gomez-Casado et al. 2011; Shetty et al. 2012).

304 In hatcheries, an important route of virus entry is infected asymptomatic breeders (Mushiake et
305 al. 1994; Watanabe et al. 1998). Although ozonation can seemingly prevent NNV transmission
306 from infected broodstock, it is not fully efficient because betanodavirus is not only present on
307 the surface of the eggs but also inside the eggs, and can also penetrate the egg via
308 spermatozoa (Kuo et al. 2012). A positive point is that vertical transmission can be controlled
309 effectively in hatcheries by combining detection via serological tests (ELISA) to detect anti-
310 VNN specific antibodies (in the blood serum of broodstock) or/and sensitive RT-PCR assays to
311 recognize viral RNA (in the eggs or genital fluids), combined with the elimination of positive
312 individuals (Mushiake et al. 1994; Breuil & Romestand 1999; Watanabe et al. 2000; Breuil et
313 al. 2002; Hodneland et al. 2011). Ozonation and ultra-violet light are also used to clean
314 fertilized eggs and control water quality during rearing larval and juvenile stages. Even if
315 treatment of larvae requires complicated procedures, these treatments appear effective to
316 prevent horizontal transmission (Arimoto et al. 1996; Watanabe et al. 1998; Grotmoll &
317 Totlandl 2000). Although betanodaviruses can be prevent effectively in hatchery based on
318 manage betanodavirus-free broodstock and disinfect hatchery water, the fish can be infected
319 betanodaviruses from the environment when they are cultured at grow-out stages.

320 Vaccination has been considered as an effective procedure for controlling VNN disease. A
321 number of vaccines made with inactivated NNV, virus-like particles (VLPs), recombinant C
322 protein and synthetic peptides from the C protein have been tested (Gomez-Casado et al.
323 2011). Recombinant betanodavirus coat proteins expressed in *Escherichia coli* was firstly
324 proposed in different fish species like sevenband grouper *Epinephelus septemfasciatus* and
325 humpback grouper *Cromileptes altivelis* (Tanaka et al. 2001; Yuasa et al. 2002), turbot and
326 Atlantic halibut (Húsgarð et al. 2001; Sommerset et al. 2005). More recent constructions

327 combined to artermia or *Vibrio anguillarum* induced significant levels of protection in larvae
328 of orange-spotted grouper *Epinephelus coioides* (Lin et al. 2007; Chen et al. 2011), and
329 enhanced virus-neutralizing antibody response was observed after immunisation at grow-out
330 stages with recombinant C protein (Sommerset et al. 2005). Virus-like particles have also
331 been developed to create a more effective procedure to control VNN disease (Thiéry et al.
332 2006; Liu et al. 2006b). To date, the efficiency of the pFNCPE42-DNA vaccine, which has
333 been developed using the capsid protein gene of an Indian isolate of fish nodavirus has been
334 illustrated in Asian sea bass with a high relative percent survival of 77.33% (Vimal et al.
335 2016). All these systems require to go through an injection. Consequently, they are only really
336 effective on grow-out size fish or to prevent vertical transmission in breeding, while the VNN
337 disease often occurs in early larval and juvenile stages at which the size of fish is too small to
338 allow vaccination by injection (Sommerset et al. 2005; Kai & Chi 2008; Brudeseth et al.
339 2013). A water-delivery strategy (immersion) could represent a more interesting way of
340 control (Kai & Chi 2008) but still needs to be improved.

341 The viral diversity of betanodavirus with at least four different species described is another
342 challenge to overcome for which DNA vaccines have numerous advantages compared to
343 traditional antigen vaccines (Gomez-Casado et al. 2011). However, no license has been
344 delivered to date for potential applications in commercial fish farms in some areas such as
345 Europe (Gomez-Casado et al. 2011; Brudeseth et al. 2013). The vaccine application is usually
346 expensive in fish and the protection generated often lasts for in short time because of the low
347 immune reactivity in early stages of life (Sommerset et al. 2005). For these disadvantages,
348 although a variety of vaccinations for NNV have been experienced (table 4), only one
349 inactivated RGNNV vaccine against NNV of sevenband grouper was commercialized in
350 Japan (Brudeseth et al. 2013). Nevertheless, work in progress to better understand the immune
351 mechanisms involved during NNV infection (Costa & Thompson 2016; Carballo et al. 2016;

Wu et al. 2016) will likely result in the near future in the improvement of the prophylactic strategies, like the use of preventive administration of interferons at the larval stage (Kuo et al. 2016) or of ribavirin as antiviral agent (Huang et al. 2016).

SELECTIVE BREEDING TO NNV RESISTANCE: PROSPECTIVE PROCEDURE

While selective breeding programs have been mostly targeting productivity traits like e.g. growth and carcass quality (Gjedrem & Thodesen 2005), disease resistance remains a major goal for breeding programs, as mortality caused by diseases is a major threat to aquaculture. Selecting fish with increased resistance to specific diseases seem to be feasible for most diseases (reviewed by Gjedrem 2015). Moreover, it provides cumulative and permanent improvement of resistance over generations at the population level, thus providing unique benefits when compared to other methods. Due to its cost however, the selective breeding strategy toward resistant cultured fish is particularly interesting when other prevention methods are inefficient. The use of resistant populations would not only reduce outbreaks, but also lower the cost of fish production (Ødegård et al. 2011; Yáñez et al. 2014a).

Disease resistance heritability in fish

Improving a trait by artificial selection basically requires sufficient genetic variation for this trait in the population. Genetic variation in disease resistance has been observed for many diseases, and most likely variation would be seen for all diseases (Bishop & Woolliams 2014). While heritability for resistance to viral diseases have been estimated in many species, it remains that most studies have been conducted in salmonids.

The heritability of resistance to viral diseases has been shown to be moderate to high in fish (table 5). In the first place, resistance to VHS virus (VHSV) was found highly heritable

375 (h^2=0.57-0.63) in rainbow trout (*Oncorhynchus mykiss*) when assessed by mortality (Dorson
376 et al. 1995; Henryon et al. 2005), while it was little heritable (h^2=0.11±0.10 and 0.13) when
377 resistance was assessed as the time until death following challenge (Henryon et al. 2002;
378 Henryon et al. 2005). Moderate to high heritabilities have been estimated for Infectious
379 Salmon Anaemia Virus (ISAV), ranging from 0.13 to 0.26 on the observable scale and from
380 0.19 to 0.40 on the liability scale (Gjøen et al. 1997; Ødegård et al. 2007a; Olesen et al., 2007;
381 Kjøglum et al. 2008; Gjerde et al. 2009), while the heritability of Infectious Pancreatic
382 Necrosis Virus (IPNV) resistance was also found moderate to high, ranging between 0.16 and
383 0.55 (Guy et al. 2006; Guy et al. 2009; Wetten et al. 2007; Kjøglum et al. 2008). Other viral
384 diseases in fish also display moderate to high heritability, such as resistance to salmon
385 pancreases disease virus (SPDV) in Atlantic salmon (*Salmo salar*) with a liability scale
386 estimate of 0.21±0.05 (Norris et al. 2008), and koi herpesvirus (KHV) resistance
387 (h^2=0.79±0.14) in common carp (*Cyprinus carpio*) (Ødegård et al. 2010a).

388 To date, a high heritability for NNV has been demonstrated, but only in Atlantic cod
389 (Ødegård et al. 2010b; Bangera et al. 2011; Bangera et al. 2013). Ødegård et al. (2010b)
390 compared the NNV resistance of three different groups of Atlantic cod including Norwegian
391 coastal cod (CC), Northeast Atlantic cod (NEAC) and their F1 crossbreds. They showned
392 that the highest survival was obsevered in CC (56%), followed by crosses (31%),
393 whereas the survival rate of NEAC was only 10%. The estimated heritability for NNV
394 resistance was high on the observed scale (0.43±0.07) and very high on the underlying scale
395 (0.75±0.11) (Ødegård et al. 2010b). Besides that, a high heritability for NNV resistance was
396 also recorded (0.68±0.14) by Bangera et al. (2011) who later on reported an extremely high
397 heritability (0.91 using a cure model) for NNV resistance in the same species (Bangera et al.,
398 2013). In addition, the genetic correlation between resistance to NNV and to a bacterial
399 disease (Vibriosis) was shown not to significantly differ from zero (Bangera et al. 2011). This

lack of correlation is similar to other studies in salmonids which estimated the genetic correlation between resistance against ISAV and furuculosis (Gjøen et al. 1997; Ødegård et al. 2007b; Kjøglum et al. 2008) or VHSV and enteric red-mouth disease as well as rainbow trout fry syndrome (Henryon et al. 2005).

The heritability of resistance to viral disease is moderate to high in almost exsisting studies, indicating viral disease resistance can be improve significantly based on selective breeding in farmed fish – and the prospects for NNV resistance are specially good, due to the high to very high heritability estimate (only in Atlantic cod for the moment).

Genetic Selection to Viral Disease Resistance in Fish

Following promising heritability estimates, experimental selective breeding for disease resistance has been undertaken and shown to be an effective solution to prevent the outbreak of viral diseases in farmed fish. In the end of the1980s, selective breeding for resistance to VHSV in rainbow trout was successfully tried in France, resulting in an improved resistance in the second generation, with 0 to 10% mortality, compared to 70 to 90% in the control group (Dorson et al. 1995). In Denmark, relatively VHSV-resistant broodstock were selected from a challenge test, and used to produce first and second generation gynogenetic offspring (Bishop & Woolliams 2014). Salmon commercial breeding programs have included resistance to furunculosis, ISAV and IPNV since 1993 in Norway (Gjøen et al. 1997; Moen et al. 2009; Yáñez et al. 2014). The effective of selective breeding for IPNV resistance in Atlantic salmon was illustrated by Storset et al. (2007), where the fish belonging to low and high resistant families were challenged in both freshwater and seawater and obtaining significant differences in mortalities, which ranged from 29-32% in high resistance families to 66-79% in low resistance families in both freshwater and seawater.

Quantitative Trait Loci mapping for resistance to viral diseases

Identifying portions of the genome called Quantitative Trait Loci (QTLs) linked to the disease resistance phenotype is expected to speed up the selection process by using Marker-Assisted selection (Massault et al. 2008; Bishop & Woolliams 2014).

Most of the QTLs identified for resistance to viral diseases in cultured fish have been identified in Salmonids, the most successful example being the IPNV resistance QTL. Three highly significant QTLs were first identified using microsatellite and AFLP markers in a backcross of rainbow trout strains displaying high and low resistance to IPNV, each explaining 13-15% of the phenotypic variance of the total phenotypic variance (Ozaki et al. 2001; Ozaki et al. 2007). For IPNV resistance in Atlantic salmon, even more significant QTLs have been identified (Houston et al. 2008; Houston et al. 2010; Moen et al. 2009; Gheyas et al. 2010), leading to a breakthrough with respect to the implementation of QTL in salmon breeding. A first QTL, producing a 75% difference in IPNV mortality between the alternative homozygotes, was mapped to linkage group 21 (LG21) (Houston et al. 2008). The same QTL was independently reported in 2009 in Norwegian population, where it explained 29% of the phenotypic variance (Moen et al. 2009). Gheyas et al. (2010) confirmed the resistance effect of the QTL from LG21 at the fry stage in freshwater, with a QTL heritability of 0.45 ± 0.07 on the liability scale and 0.25 ± 0.05 on the observed scale. In one family, 100% of the offspring homozygous for the susceptible QTL alleles died, whereas 100% of the offspring homozygous for the resistant QTL alleles survived (Gheyas et al. 2010).

QTLs for resistance to other viral diseases in Salmonids include QTLs for IHNV resistance (Palti et al. 1999; Palti et al. 2001; Miller et al. 2004; Rodriguez et al. 2004; Barroso et al. 2008), ISAV resistance (Moen et al. 2004; Moen et al. 2007), VHSV resistance (Verrier et al. 2013) and Salmonid Alphavirus (SAV) resistance (Gonen et al. 2015). Like for IPNV, the IHNV QTLs explained a high part of the phenotypic variance (up to 32.5% according to

448 Barroso et al. 2008), while it was more limited for the ISAV QTL (6% of the phenotypic
449 variance, Moen et al. 2007). In both cases, a significant association with MHC alleles was
450 later demonstrated (Palti et al. 2001; Miller et al. 2004 for IHNV; Kjøglum et al. 2006 for
451 ISAV).

452 About NNV resistance, five genome-wide significant QTLs, explaining 68% of the
453 phenotypic variance for resistance, detected based on 161 microsatellite markers in Atlantic
454 cod (Baranski et al. 2010), a very high amount, which can be paralleled to the very high
455 heritability of NNV resistance reported earlier. A later analysis with a 12K SNP array
456 confirmed both the high proportion of variance explained by genomic markers, and the
457 location of three of these QTLs (Yu et al. 2014). The latest QTLs related to NNV resistance
458 identified based on 146 microsatellite markers in Asian sea bass. In that study, Liu et al. 2016
459 detected multiple QTLs for NNV resistance and survival time. However, a few proportion of
460 the phenotypic variation were explained by those QTLs (2.2-4.1% for resistance and 2.2-3.3%
461 for survival time).

462 Taken altogether, these information about the QTLs for resistance to viral diseases in fish are
463 very promising for increasing the rate of resistance through selective breeding, especially as in
464 many cases QTLs seem to be of large effect, which gives good prospects to improve genetic
465 resistance in a relatively short term, by direct marker–assisted selection or by introgression of
466 QTLs from different populations (Bishop & Woolliams 2014). This possibility may especially
467 develop as SNP markers become more and more available and affordable, due to their
468 abundance and to fast technological developments, making both detection and selection of
469 QTLs more economically realistic.

Markers-assisted Selection (MAS) and Genomic Selection (GS) For Viral Disease in Fish

Breeding resistant fish based on survivors of challenge trials, although sometimes done, is generally undesirable due to the risk of vertical transmission of the pathogen. The usual way to overcome this limitation in conventional breeding is to perform sib selection. In sib selection, breeding candidates are kept in a pathogen-free environment, and selected using family-wise estimated breeding values obtained from the survival of fish from the same families challenged with the disease. Another possible way to select resistant fish without exposing them to the pathogen is the identification of relevant QTL and the application of molecular markers for Marker Assisted Selection (MAS), or the direct use of genotype data to perform Genomic Selection (GS). With both methods, fish are selected based only on their genotype, either at specific QTL-linked markers in the case of MAS, or at many markers, which may not all be linked to the resistance in the case of GS. This allows to avoid any contact between the breeding candidate and the pathogen. In terms of efficiency, the advantage of MAS compared to conventional selection is expected to be largest when the trait under selection has a low heritability – which is not generally the case for viral disease resistance in fish - or when the trait is not measured on the breeding candidates – which conversely is typically the case for disease resistance (Gjedrem 2015). With simulated traits and populations, the accuracy of selection was improved significantly by using MAS, compared to non-MAS in selective breeding in aquaculture (Sonesson 2007). Practical application of MAS in aquaculture breeding has been implemented for IPNV resistance Atlantic salmon in both Norwegian (Moen et al. 2009) and Scottish populations (Houston et al. 2010). Still, the limitation of MAS is that it requires prior knowledge of alleles that are associated with the traits of interest, which moreover have to be validated in the specific populations or even families under selection. Furthermore, MAS exploits only a limited part

494 of the genetic differences between individuals, as it does not exploit the polygenic background
495 variation, which may account for a large part of the genetic variance (Meuwissen et al. 2016).

496 An alternative approach for more polygenic traits is genomic selection. In this approach,
497 genetic markers are used to cover the whole genome so that all QTL, even non statistically
498 significant, are in linkage disequilibrium (LD) with at least one marker and selection is based
499 on genetic values predicted from all the markers (Meuwissen et al. 2001; Goddard & Hayes
500 2007; Meuwissen et al. 2016). The availability of high density SNP arrays in livestock and
501 now increasingly in aquaculture species is making both genomic selection and genome-wide
502 association studies (GWAS) feasible. GWAS approaches allow studies of the genetic
503 architecture of quantitative traits, while genomic selection will improve the accuracy of
504 selection in breeding programs (Houston et al. 2014). In terms of present realization of these
505 approaches, GWAS showed highly significant association of several SNPs with resistance to
506 IPNV, as well as population level linkage-disequilibrium in salmon commercial populations
507 (Houston et al. 2012). The implementation of such approaches is dependent on the
508 development of SNP genotyping arrays, which for the time being have mostly been developed
509 in salmonids, like a 130K array for farmed and wild Atlantic salmon in Scotland (Houston et
510 al. 2014), 160 K SNP markers were validated based on 200 K SNPs applied to different wild
511 and farmed populations of Atlantic salmon (Europe population, North America population
512 and Chile population) (Yáñez et al. 2014b), and a 57 K SNP chip which is now available for
513 rainbow trout (Palti et al. 2014). A 12K SNP array has been also developed in Atlantic cod,
514 containing markers distributed across all 23 chromosomes (Yu et al. 2014). It was already
515 used in a GWAS analysis for NNV resistance which revealed 29 genome-wide significant
516 SNPs for binary survival, and 36 genome-wide significant SNPs for number of days fish
517 survived, as well as high genomic heritabilities of 0.49 and 0.81 for the same traits,
518 respectively (Bangera et al. 2014). Identification of SNPs is being done in other species for

519 which NNV resistance is a key issue, such as European sea bass (Tine et al. 2014;
520 Palaiokostas et al. 2015) or Asian sea bass (Wang et al, 2015), which is promising for the
521 development of GWAS or GS for NNV resistance in those species.

522 CONCLUSION

523 Viral encephalopathy and retinopathy is widespread all over the world except in South
524 America. While many of the main marine species in aquaculture are affected by this disease,
525 no simple and effective procedures are available to treat it. Even though VNN can be prevented
526 in hatcheries based on efficient diagnostic methods to monitor the breeders and biosecurity
527 measures during hatchery rearing, this disease still occurs on grow-out sites. Vaccination may
528 be an efficient way to prevent disease occurrence, but because of the specific drawbacks of
529 present vaccination methods and the difficulty to efficiently protect early larval stages, this tool
530 is not fully effective in the case of VNN. Selective breeding has been demonstrated as an
531 effective solution to select resistant aquaculture populations for several diseases, and new
532 genomics based methods allow to foresee even higher efficiency of selective breeding for
533 disease resistance in the near future. However, to reach the expectations of a practical
534 genomic selection, more genetic resources and more advanced studies are required for the vast
535 majority of aquaculture species affected by NNV.

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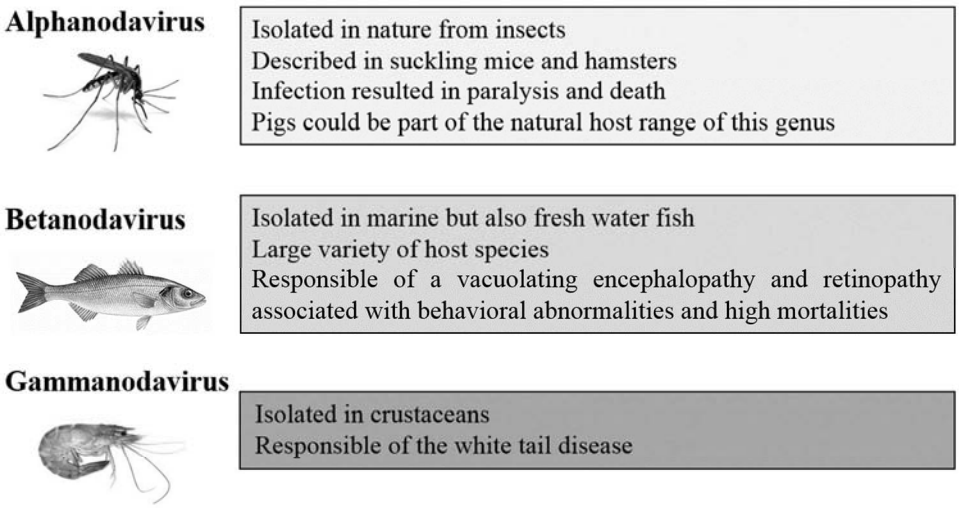

Figure 1: Three genera of Nodaviridae

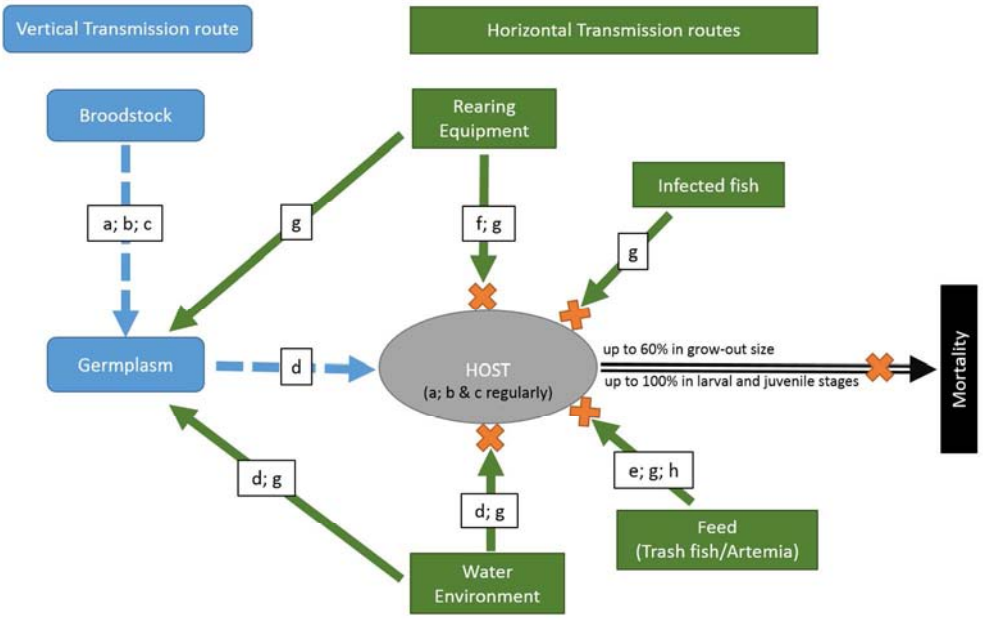

Figure 2: The different transmission routes of betanodaviruses and possible prevention modes. Blue discontinuous arrows represent vertical transmission routes; green arrows represent horizontal transmission routes; orange crosses display possible actions of genetics (by improving for fish natural barriers to infections or resistance/tolerance - see section "Selective breeding to NNV resistance: prospective procedure"); host represents either larvae/juvenile/grow-out size or broodstock; the possible prevention modes are: a: vaccination; b: serological diagnostic (ELISA) to screen and eliminate seropositive individuals; c: direct diagnostic (RT-qPCR) to screen and eliminate positive individuals or germplasm; d: ozone/UV/bleach water treatments; e: strict control of feed input to avoid NNV infected trash fish; f: unique equipment kit for each tank/pond/cage and adapted decontamination of equipment after use; g: Biosecurity measures during all production cycle; h: ozone treatment of artemia before feeding.

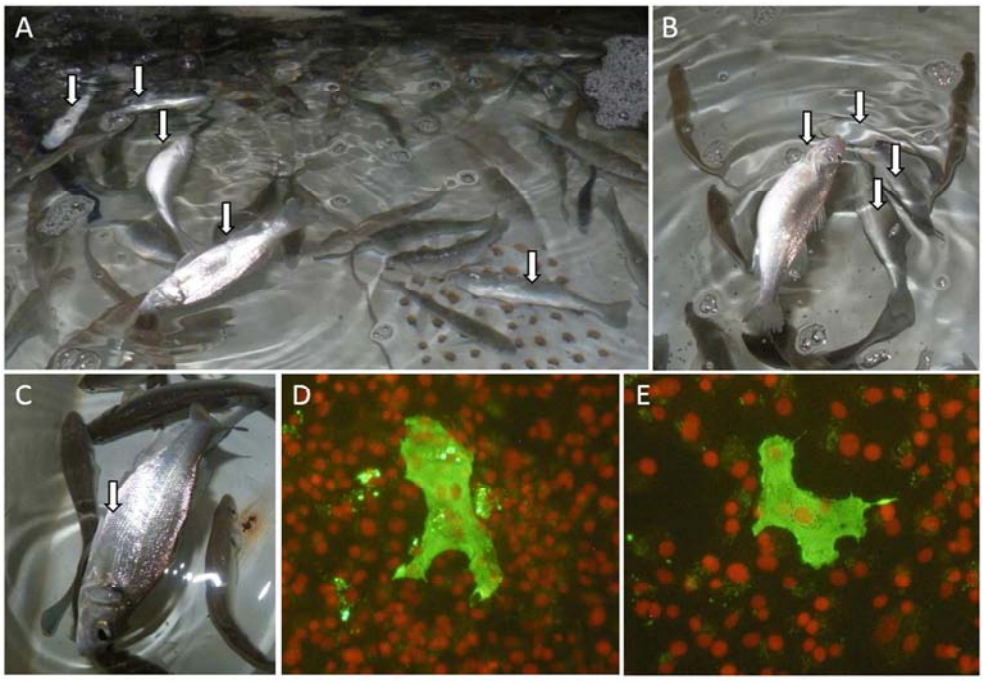

Figure 3: A, B & C. Typical clinical signs observed during experimental NNV infection in European sea bass (arrows show impacted fish). D & E. Positive immunofluorescence antibody test signal (in green) obtained for betanodaviruses on SSN1 cell line. Source: Anses, Ploufragan-Plouzané Laboratory, Viral diseases of fish Unit

Table 1: Species of the genus Betanodavirus

Species	GenBank accession no.	Optimal temperature forreplication	Serotype	Main hosts effected	Key Ref.
Species in the genus Betanodavivus					
Barfin flounder nervous necrosis virus – BFNNV-BF93Hok	RNA1 (EU826137 = NC_011063) RNA2 (EU826138 = NC011064)	15–20°C	C	Atlantic cod (***Gadus morhua***) Barfin flounder (***Verasper moseri***) Atlantic halibut (***Hippoglossus hippoglossus***)	Munday et al. 2002, Iwamoto et al. 2000, Morit et al. 2003, Vendramin et al. 2013
Redspotted grouper nervous necrosis virus – RGNNV -SGWak97	RNA1 (AY324869 = NC_008040) RNA2 (AY324870 = NC_008041)	25–30°C	C	Sevenband grouper (***Epinephelus septemfasciatus***) Redspotted grouper (***Epinephelus akaara***) Kelp grouper (***Epinephelus moara***) Orange spotted grouper (***Epinephelus coioides***) Dragon grouper (***Epinephelus lanceolatus***) Greasy grouper (***Epinephelus tauvina***) Humpback grouper (***Cromileptes altivelis***) Barramundi (***Lates calcarifer***) Japanese sea bass (***Lateolabrax japonicus***) European sea bass (***Dicentrarchus labrax***)	Munday et al. 2002, Iwamoto et al. 2000, Mori et al. 2003, Vendramin et al. 2013, Vendramin et al. 2014
Striped jack nervous necrosis virus – SJNNV-SJ93Nag	RNA1 (AB056571 = NC_003448) RNA2 (AB056572 = NC_003449)	20–25°C	A	Japanese striped jack (***Pseudocaranx dentex***) Gilthead sea bream (***Sparus aurata***) Senegalese sole (***Solea senegalensis***)	Nishizawa et al. 1997, Iwamoto et al. 2000, Mori et al. 2003, Thiéry et al. 2004, Vendramin et al. 2013 & 2014
Tiger puffer nervous necrosis virus – TPNNV-TPKag93	RNA1 (EU236148 = NC_013640) RNA2 (EU236149 = NC_013641)	20°C	B	Tiger puffer (***Takifugu rublipes***)	Iwamoto et al. 2000, Mori et al. 2003, Vendramin et al. 2013
Other genotypes which have not been approved as species					
Atlantic cod nervous necrosis virus - ACNNV	RNA1 (EF433472) RNA2 (EF433468)	15–20°C	C	Atlantic cod (***Gadus morhua***)	Nylund et al. 2008, Johnson et al. 2002
Atlantic halibut nodavirus – AHNV	RNA1 (AJ401165) RNA2 (AJ245641)	15–20°C	C	Atlantic halibut ***(Hippoglossus hippoglossus)***	Grotmoll & Totlandl 2000, Johnson et al. 2002, Sommerset & Nerland 2004
Dicentrarchus labrax encephalitis virus - DIEV	RNA2 (U39876)	25–30°C	C	Sea bass (***Dicentrarchus labrax***)	Dalla Valle et al. 2001 Johnson et al. 2002
Dragon grouper nervous necrosis virus - DGNNV	RNA1 (AY721616) RNA2 (AY721615)	25–30°C	C	Dragon grouper (***Epinephelus laceolatus***)	Panzarin et al. 2012 Johnson et al. 2002
Greasy grouper nervous necrosis virus - GGNNV	RNA1 (AF319555) RNA2 (AF318942)	25–30°C	C	Greasy grouper (*Epinephelustauvina*)	Tan et al. 2001, Johnson et al. 2002, Sommerset & Nerland 2004
Japanese flounder nervous necrosis virus - JFNNV	RNA1 (FJ748760) RNA2 (D38527)	25–30°C	C	Japanese flounder (***Paralichthys olivaceus***)	Panzarin et al. 2012, Johnson et al. (2002)
Lates calcarifer encephalitis virus - LcEF	RNA2 (AF175516)	25–30°C	C	Barramundi (***Lates calcarifer***)	Skliris et al. 2001
Malabaricus grouper nervous necrosis virus - MGNNV	RNA2 (AF245003)	25–30°C	C	Malabaricus grouper (***Epinephelus malabaricus***)	Johnson et al. 2002
Seabass nervous necrosis virus - SBNNV	RNA2 (Y08700)	20–25°C	A	Sea bass (***Dicentrarchus labrax***)	Thiéry et al. 2004
Solea senegalensis nervous necrosis virus - SSNNV	RNA1 (FJ803911) RNA2 (AJ698113)	20–25°C	A	Senegalese sole (***Solea senegalensis***)	Panzarin et al. 2012, Thiéry et al. 2004
Turbot nodavirus - TNV	RNA2 (AJ608266)	undefined	undefined	Turbot (***Scophthalmus maximus***)	Johansen et al. 2004
Marcrobrachium rosenbergii nodavirus - MrNV	RNA1 (AY231436) RNA2 (AY231437)	25–30°C	undefined	Giant freshwater prawn (***Macrobrachium rosenbergii***)	Senapin et al. 2012, Bonami & Sri Widada 2011
Peneaus vannamei nodavirus - PvNV	RNA1 (FJ751226) RNA2 (FJ751225)	25–30°C	undefined	Whiteleg shrimp (***Litopeneaus vannamei***)	Senapin et al. 2012, Tang et al. 2007

Table 2: Fish species influenced by VER/VNN

Host species					Species	Key ref.
Oder		Family	Common name	Latin name		
Marine species						
Farmed species	Decapoda	Penaeidae	Whiteleg shrimp	*Lipopenaeus vannamei*	PvNV	Tang et al. 2007
	Scorpaeniformes	Sebastidae	Black rockfish	*Sebastes inermis*	RGNNV	Gomez et al. 2004
			Oblong rockfish	*S. oblongus*		
			Spotbelly rockfish	*S. pachycephalus*		
	Pempheriformes	Lateolabracidae	Chinese seabass	*Lateolabrax sp.*		
	Perciformes	Sparidae	Red seabream	*Pagrus major*		
			Gilthead sea bream	*Sparus aurata*	SJNNV	Cutrín et al. 2007
		Oplegnathidae	Japanese parrotfish (Barred knifejaw)	*Oplegnathus fasciatus*	SJNNV	Yoshikoshi & Inoue 1990 Nishizawa et al. 1997
		Centropomatidae	Japanese sea bass	*Lateolabrax japonicus*	RGNNV	Mori et al. 2003
		Sciaenidae	White sea bass	*Atractoscion nobilis*	RGNNV	Curtis et al. 2001
		Percichthydae	European sea bass	*Dicentrarchus labrax*	RGNNV/SJNNV	Breuil et al. 1991 Thiéry et al. 2004
		Scombridae	Pacific bluefin tuna	*Thunnus orientalis*	RGNNV	Sugaya et al. 2009
		Rachicentridae	Cobia	*Rachycentron canadum*	RGNNV	Chi et al. 2003
		Carangidae	yellow-wax pompano	*Trachinotus falcatus*		
			Striped jack	*Pseudocaranx dentex*	SJNNV/TPNNV	Mori et al. 1992 Nishizawa et al. 1997
			Golden pompano	*Trachinotus blochii*	RGNNV	Ransangan et al. 2011
		Serranidae	Humpback grouper	*Cromileptes altivelis*	RGNNV	Yuasa et al. 2007
			Dragon grouper	*Epinephelus lanceolatus*	RGNNV	Lin et al. 2001
			Red-spotted grouper	*Epinephalus akaara*	RGNNV	Nishizawa et al. 1997
			Black spotted grouper	*Epinephelus fuscogutatus*	RGNNV	Chi et al. 1997
			Sevenband grouper	*Epinephelus septemfasciatus*	SJNNV	Fukuda et al. 1996
			Greasy grouper	*Epinephelus tauvina*	GGNNV	Hegde et al. 2002 Tan et al. 2001
			Orange-spotted grouper	*Epinephelus coioides*	RGNNV	Chi et al. 1999
			Brown-spotted grouper	*Epinephelus malabaricus*	RGNNV	Nishizawa et al. 1997
			Yellow grouper	*Epinephelus awoara*	RGNNV	Lai et al. 2001
			Kelp grouper	*Epinephelus moara*	undefined	Munday et al. 2002
	Tetraodontiformes	Tetraodontidae	Tiger puffer	*Takifugu rubripes*	TPNNV	Nishizawa et al. 1997
	Pleuronectiformes	Soleidae	Senegalese sole	*Solea senegalensis*	SJNNV	Thiéry et al. 2004
		Pleuronectidae	Barfin flounder	*Verasper moseri*	BFNNV	Nishizawa et al. 1995
			Atlantic halibut	*Hippoglossus hippoglossus*	BFNNV	Grotmol et al. 1997
		Paralichthyidae	Japanese flounder	*Paralichthys olivaceus*	SJNNV	Nishizawa et al. 1995
		Scophthalmidae	Turbot	*Scophthalmus maximus*	TNV	Johansen et al. 2004
	Perciformes	Centropomatidae	Barramundi/Asian sea bass	*Lates calcarifer*	RGNNV	Bloch et al. 1991
	Gadiformes	Gadidae	Pacific cod	*Gadus macrocephalus*	BFNNV	Mori et al. 2003
			Atlantic cod	*Gadus morhua*	BFNNV	Johnson et al. 2002
			Haddock	*Melanogrammus aeglefinus*	BFNNV	Gagné et al. 2004
Wild species	Perciformes	Epigonidae	Cardinal fish	*Epigonus telescopus*	undefined	Giacopello et al. 2013
		Serranidae	Wild dusky grouper	*Epinephelus marginatus*	RGNNV	Vendramin et al. 2013
			Wild golden grouper	*Epinephelus costae*		
		Sparidae	Bogue	*Boops boops* (L.)	RGNNV	Ciulli et al. 2007
		Mugilidae	Flathead grey mullet	*Mugil cephalus* (L.)		
			Golden grey mullet	*Liza aurata*	RGNNV	Zorriehzahra et al. 2016
			Leaping mullet	*Liza saliens*		
			Red mullet	*Mullus barbatus barbatus* (L.)	RGNNV	Ciulli et al. 2007
		Gobiidae	Black goby	*Gobius niger* (L.)		
		Carangidae	Horse mackerel	*Trachurus trachurus*		
			Japanese scad	*Decapterus maruadsi* (Temminck & Schlegel)	RGNNV	Gomez et al. 2004
	Lepisosteiformes	Lepisosteidae	Garpike (Longnose Gar)	*Lepisosteus osseus*	RGNNV	Ciulli et al. 2007
	Pleuronectiformes	Pleuronectidae	wild winter flounder	*Pleuronectes americanus*	BFNNV	Gagné et al. 2004
	Notacanthiformes	Notacanthidae	Shortfin spiny eel	*Notacanthus Bonaparte*	undefined	Giacopello et al. 2013
	Beryciformes	Trachichthyidae	Mediterranean slimehead	*Hoplostethus mediterraneus mediterraneus*		

	Gadiformes	Macrouridae	Glasshead grenadier	*Hymenocephalus italicus* (Giglioli)		
		Gadidae	Whiting	*Merlangi merlangus* (L.)	RGNNV	Ciulli et al. 2007
		Merlucciidae	European hake	*Merluccius merluc- cius* (L.)		
	Clupeiformes	Clupeidae	European pilchard	*Sardina pilchardus (Walbaum)*		
	Scorpaeniformes	Triglidae	Gurnard	*Chelidonichthys lucerna* (L.)		
		Sebastidae	Marbled rockfish	*Sebastiscus marmoratus* (Cuvier)	RGNNV	Gomez et al. 2004
	Tetraodontiformes	Monacantidae	Threadsail filefish	*Stephanolepis cirrhifer* (Temminck & Schlegel)		
			Black scraper	*Thamnaconus modestus* (Gunther)		
	Decapoda	Portunidae	Charybdid crab	*Charybdis bimaculata*	RGNNV	Gomez et al. 2008
		Pandalidae	Southern humpback shrimp	*Pandalus hypsinotus*		
	Mytiloida	Mytilidae	Mediterranean mussel	*Mytilus galloprovincialis*		
Freshwater species						
Farmed species	Acipenseriformes	Acipenseridae	Sturgeon	*Acipenser gueldenstaedi*	SJNNV	Athanassopoulou et al. 2004
	Anguilliformes	Anguillidae	European eels	*Anguilla anguilla*	RGNNV	Chi et al. (2003)
	Siluriformes	Siluridae	Chinese catfish	*Parasilurus asotus*		
			Australian catfish	*Tandanus tandanus*	undefined	Shetty et al. 2012
	Perciformes	Eleotridae	Sleepy cod	*Oxyeleotris lineolatus*	undefined	
		Centrarchidae	Largemouth black bass	*Micropterus salmoides* (Lacepede)	RGNNV	Bovo et al. 2011
		Percidae	Pike-perch	*Sander lucioperca*		
		Cichlidae	Tilapia	*Oreochromis niloticus*	RGNNV	Bigarré et al. 2009
	Decapoda	Palaemonidae	Giant freshwater prawn	*Macrobrachium rosenbergii*	MrNV	Bonami & Widada 2011
Ornamental/model fish species	Cyprinodontiformes	Poeciliidae	Guppy	*Poecilia reticulata*	RGNNV	Hegde et al. 2003
	Cypriniformes	Cyprinidae	Zebrafish	*Danio rerio*	RGNNV	Lu et al. 2008
			Goldfish	*Carassius auratus*	RGNNV	Binesh 2013
	Perciformes	Blenniidae	Freshwater blenny	*Salaria fluviatili*	RGNNV	Vendramin et al. 2012
	Beloniformes	Adrianichthyidae	Medaka	*Oryzias latipes*	RGNNV	Furusawa et al. 2006

Table 3: Primers/probes sets used for betanodavirus detection by RT-PCR

Primer/Probe	Target	GenBank accession number [§]	Sequence 5' - 3'	Position	Key Ref.
VNNV1 VNNV2 VNNV3 VNNV4	RNA2	AB056572	ACACTGGAGTTTGAAATTCA GTCTTGTTGAAGTTGTCCCA ATTGTGCCCCGCAAACAC GACACGTTGACCACATCAGT	343-362 953-934 366-383 620-601	Dalla Valle et al. 2000
AH95-F1 AH95-R1	RNA2	AJ245641	AGTGCTGTGTCGCTGGAGTG CGCCCTGTGTGAATGTTTTG	577-596 917-898	Grotmoll & Totlandl 2000
F2 R3	RNA2	AB056572	CGTGTCAGTCATGTGTCGCT CGAGTCAACACGGGTGAAGA	592-611 1017-998	Nishizawa et al. 1994
F'2 R'3	RNA2	Y08700	GTTCCCTGTACAACGATTCC GGATTTGACGGGGCTGCTCA	677-693 970-951	Thiéry et al. 1999
Q-CP-1 Q-CP-2	RNA2	D38636	CAACTGACAACGATCACACCTTC CAATCGAACACTCCAGCGACA	234-256 463-443	Dalla Valle et al. 2005
P1 P2 Probe	RNA2	AJ245641	GGTATGTCGAGAATCGCCC TAACCACCGCCCGTGTT TTATCCCAGCTGGCACCGGC*	141-159 351-335 183-202	Grove et al. 2006
qR2TF qR2TR R2probe2	RNA2	*LcNNV09_07* [†]	CTTCCTGCCTGATCCAACTG GTTCTGCTTTCCCACCATTTG CAACGACTGCACCACGAGTTG*	378-397 470-451 448-428	Hick & Whittington 2010
RNA2 FOR RNA2 REV probe	RNA2	DQ864760	CAACTGACARCGAHCACAC CCCACCAYTTGGCVAC TYCARGCRACTCGTGGTGCVG*	392-410 460-445 422-442	Panzarin et al. 2010
Nod1f Nod1r	RNA2	EF617335; AY744705; AF 175511; AB056572; AJ608266; D38637; D38635	TTCCAGCGATACGCTGTTGA CACCGCCCGTGTTTGC AAATTCAGCCAATGTGC*	322–341[d] 376–391[d] 356-372[d]	Hodneland et al. 2011
Nod2f Nod2r	RNA2	EF617335; AY744705; AF 175511; AB056572; AJ608266; D38637; D38635	CTGGGACACGCTGCTAGAATC TGGTCGTTGTCAGTTGGATCA AAATTCAGCCAATGTGC*	301–321[d] 414–434[d] 356-372[d]	Hodneland et al. 2011
RG-RNA2-F2: RG- RNA2-R2:	RNA2	D38636	CGTCCGCTGTCCATTGACTA CTGCAGGTGTGCCAGCATT	624–643 723–705	Lopez-Jimena et al. 2011
oPVP111 oPVP88	RNA2	AF245003 ; AF245004 ; AF281657 ; AF499774 ; AJ245641 ; AJ608266 ; D30814 ; U39876 ; EF433468 ; AY549548 ; EU236149	TCCTGCCTGAYCCAACTGAC TGGTCATCMACGATACGCAC	381-400[Þ] 1058-1039[Þ]	Bigarré et al. 2010
Q-RdRP-1 Q-RdRP-2	RNA1	D38636	GTGTCCGGAGAGGTTAAGGATG CTTGAATTGATCAACGGTGAACA	589-610 861-839	Dalla Valle et al. 2005
RG-RNA1-F: RG-RNA1-R:	RNA1	AY369136	GGCTCAGATCTGGTAATGTTTCAA CAAAGCCAAGGGAAGAAGCA	2144– 2167 2206–2187	Lopez-Jimena et al. 2011
oPVP154 oPVP155 Taqman-Probe	RNA1	AJ401165; EF617335; EU826137 ; AB025018 ; AB056571 ; AF319555 ; GQ402010 ; GQ402012 ; AY690597	TCCAAGCCGGTCCTAGTCAA CACGAACGTKCGCATCTCGT CGATCGATCAGCACCTSGTC*	2717-2736[¥] 2884-2865[¥]	Baud et al. 2015

[§]sequences from wich the primers or probes have been designed; *label position on probes; [†]the primers and probe design was achieved on an isolate obtained from a infected barramundi sampled but not reported in GenBank (Hick & Whittington, 2010); [d]the position of the primers and probe are based on SJNNV genome (AB056572); [Þ]the position of the primers and probe are based on BFNNV genome (AY549548); [¥]the position of the primers and probe are based on BFNNV genome (AJ401165).

Table 4: The different types of NNV vaccine tested in fish

Type of Vaccinations	Species	Method	Results/RPS	Key Ref.
Inactivated vaccines				
- BEI-inactivated HGNNV vaccine - Formalin-inactivated vaccines	Orange-spotted grouper *Epinephelus coioides* (early larval stage-40 dph with average body weight (BW) of 0.2 g and TBL of 2.4cm)	immersion	- RPS = 79% (BEI-inactivated NNV vaccines) - 39% (Formalin-inactivated NNV vac-cines)	Kai & Chi 2008
Formalin-inactivated vaccine (RGNNV)	Sevenband grouper *Epinephelus septemfasciatus* (juvenile-25.4 g)	injection	60% in fish groups immunized with $10^{7.5}$ $TCID_{50}$ per fish or higher doses.	Yamashita et al. 2009
BEI-inactivated HGNNV vaccine	Adult Orange-spotted grouper *Epinephelus coioides* (mean body weight of 1.35kg)	injection	High efficiency	Kai et al. 2010
Formalin-inactivated vaccine (RGNNV type)	Brown-marbled grouper *Epinephelus fuscogutattus* (5g)	injection	86 – 100%	Pakingking et al. 2010
Recombinant vaccines				
Recombinant capsid protein vaccine (Artemia-encapsulated recombinant *E. coli* expressing the NNV capsid protein gene)	Orange-spotted grouper *Epinephelus coioides* (Larvae-35dph)	oral	64.5%.	Lin et al. 2007
Recombinant capsid protein (*Vibrio anguillarum*-based oral vaccine)	Orange-spotted grouper *Epinephelus coioides* (fry)	oral	78.3%	Chen et al. 2011
Recombinant capsid protein (rT2 vaccine)	Turbot *Scophthalmus maximus* (weighing from 1 to 3 g (mean 1.8 g))	injection	82%	Húsgarð et al. 2001
Recombinant capsid protein vaccine (recAHNV-C) & vaccine plasmid (called pAHNV-C)	Turbot *Scophthalmus maximus* (Juvenile-mean weight 2.2 g)	injection	- 50%% in fish groups immunized with recAHNV-C (10 mg) + pAHNV-C (5 mg) - 57% in fish groups immunized with recAHNV-C (10 mg)	Sommerset et al. 2005
Recombinant protein vaccine-*E.coli* BL21 (DE3)	Sevenband grouper *Epinephelus septemfasciatus* (28g)	injection	88% in fish groups immunized with $10^{3.4}$ $TCID_{50}$ per fish	Tanaka et al. 2001
VLPs vaccines				
Virus-like particles (VLPs) of GNNV	- Dragon grouper *Epinephelus lanceolatus* (20g) - Malabar grouper *Epinephelus malabaricus* (20g)	injection	Significant efficiency	Liu et al. 2006b
Virus-like particles (VLPs) - MGNNV VLPs (trial 1) - SB2 VLPs (trial 2)	European sea bass *Dicentrarchus larbrax* - 66g - 22g	injection	 - 71.7 – 89.4% - 27.4 – 88.9%	Thiéry et al. 2006
DNA vaccines				
pFNCPE42-DNA vaccine	Asian sea bass *Lates calcarifer* (juvenile stage)	injection	77.33%	Vimal et al. 2016

Table 5: Recent heritability estimates of resistance to viral diseases in farmed fish species

Pathogen	Species (host)	Heritability: h^2 (±S.E.)		Notes	Key ref.
		Binary traits	Time until death		
VNNV	Atlantic cod (*Gadus morhua*)	h^2=0.75 (±0.11)		Threshold model (on the underlying scale)	Ødegård et al. 2010[b]
		h^2= 0.68 (±0.14)		Threshold model (on the underlying scale)	Bangera et al. 2011
		h^2= 0.91		CURE model	Bangera et al. 2013
VHSV	Rainbow trout (*Oncorhynchus mykiss*)	h^2= 0.63 (±0.26)		Linear model (angular transformation)	Dorson et al. 1995
			h^2= 0.13	On the logarithmic-time scale	Henryon et al. 2002
		h^2= 0.57	h^2= 0.11 (±0.10)	Survival, liability scale	Henryon et al. 2005
ISAV	Atlantic salmon (*Salmo salar*)		h^2= 0.13 (±0.03) (O.S.) h^2= 0.19 (U.S.)	Linear model (Observable scale)/ On the underlying liability scale	Gjøen et al. 1997
		h^2=0.24 (±0.03)		Threshold model using cross-sectional data	Olesen et al. 2007
		h^2=0.318(±0.022)		Threshold model (on the underlying scale)	Ødegård et al. 2007[a]
		h^2=0.319(±0.022)		Threshold model (on the underlying scale)	Ødegård et al. 2007[b]
		h^2=0.37		On the underlying liability scale	Kjøglum et al. 2008
		h^2=0.40 (±0.04)		On the underlying liability scale	Gjerde et al. 2009
IPNV	Atlantic salmon (*Salmo salar*)	h^2=0.43	h^2=0.16	transformed to the liability scale/Observed	Guy et al. 2006
		h^2= 0.31		Linear model (Observable scale)	Wetten et al. 2007
		h^2=0.55		On the underlying liability scale	Kjøglum et al. 2008
		h^2=0.38 (±0.017)		On the underlying liability scale	Guy et al. 2009
SPDV	Atlantic salmon (*Salmo salar*)		h^2= 0.21 (±0.005)	transformed to the liability scale/ Linear model (Observable scale)/	Norris et al. 2008
KHV	Common carp (*Cyprinus carpio*)	h^2=0.79 (±0.14)		On the underlying liability scale	Ødegård et al. 2010[a]