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Review

Betanodavirus and VER Disease: A 30-year Research Review

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Received: 20 December 2019; Accepted: 4 February 2020; Published: 9 February 2020

Abstract: The outbreaks of viral encephalopathy and retinopathy (VER), caused by nervous necrosis virus (NNV), represent one of the main infectious threats for marine aquaculture worldwide. Since the first description of the disease at the end of the 1980s, a considerable amount of research has gone into understanding the mechanisms involved in fish infection, developing reliable diagnostic methods, and control measures, and several comprehensive reviews have been published to date. This review focuses on host–virus interaction and epidemiological aspects, comprising viral distribution and transmission as well as the continuously increasing host range (177 susceptible marine species and epizootic outbreaks reported in 62 of them), with special emphasis on genotypes and the effect of global warming on NNV infection, but also including the latest findings in the NNV life cycle and virulence as well as diagnostic methods and VER disease control.

Keywords: nervous necrosis virus (NNV); viral encephalopathy and retinopathy (VER); virus–host interaction; epizootiology; diagnostics; control

1. Introduction

Nervous necrosis virus (NNV) is the causative agent of viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN). The disease was first described at the end of the 1980s in Australia and in the Caribbean [1–3], and has since caused a great deal of mortalities and serious economic losses in a variety of reared marine fish species, but also in freshwater species worldwide.

The causative agent of VER was first described as a "picorna-like virus" [4,5], but after the characterization of a virus purified from diseased larval striped jack (*Pseudocaranx dentex)*, it was considered a new member of the family *Nodaviridae* [6]. This first piscine nodavirus was designated as striped jack nervous necrosis virus (SJNNV). Afterwards, piscine nodaviruses were also identified as the causative agents of VER outbreaks in other fish species, such as European and Asian seabass (*Dicenthrarchus labrax* and *Lates calcarifer*, respectively) [7]. The International Committee on taxonomy of viruses (ICTV) included the piscine nodaviruses within the genus *Betanodavirus* of the family *Nodaviridae* in their seventh report [8], grouping seven species, that were later reduced to the four recognized at present: red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and SJNNV in the eighth report [9]. The first isolation of a betanodavirus was obtained from diseased sea bass using the SSN-1 cell line from whole fry tissue of striped snakehead *Ophicephalus striatus* [10].

Over the last 30 years, numerous research articles on betanodaviruses and VER have been published and a considerable amount of knowledge on the disease and causative viruses is available at present. However, further research is needed in terms of virus–host interaction, viral transmission (infection routes, differences in host range among genotypes, viral stability in different environmental

conditions . . .), disease epidemiology (i.e., reservoirs, impact of global warming on the development and spread of the disease . . .) and infection control in fish farms. In this review, we present the latest findings related to the betanodavirus host range and distribution, with special emphasis on genotypes, host–virus interaction, and VER epidemiology, as well as diagnostics and potential control measures for the disease.

2. The Virus

2.1. Viral Structure

NNV is a small non-enveloped virus with a diameter of around 25–30 nm and a T = 3 icosahedral symmetry (180 copies of a single protein) [6]. The viral genome is composed of two single-stranded positive-sense RNA molecules known as RNA1 (1.01×10^6 Da) and RNA2 (0.49×10^6 Da), both co-packaged into a single virion (Figure 1). The 5′-ends of these RNAs are capped but their 3′-ends are not polyadenylated. The biggest segment, RNA 1, is composed of around 3100 nucleotides (nt) and contains an open reading frame (ORF) for the RNA-dependent RNA-polymerase (RdRp), also known as protein A. RNA2, the smallest segment (1410–1433 nt), codes for the capsid protein (CP) [6,11]. In addition, a subgenomic RNA, called RNA3 (371–378 nt), which is not packaged into virions, is synthetized from the 3′-end of RNA1 [12–14] and codes for two non-structural viral proteins: B1 and B2.

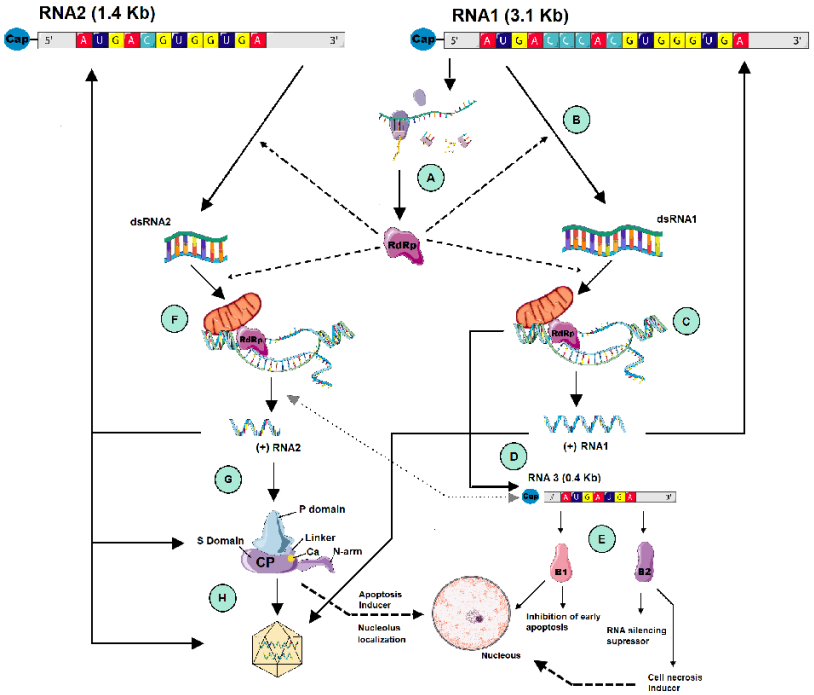

Figure 1. Schematic overview of the betanodavirus replication cycle: After entry, the viral bisegmented single stranded (+) RNA genome is released into the cytoplasm. Subsequently, host ribosomes translate the viral RNA1 into the viral RNA-dependent RNA polymerase (RdRp) (A). The RdRp is then used to copy the genomic (+) RNA1, synthetizing a (−) RNA strand and generating a dsRNA (B). The dsRNA is now used for replication/transcription into new RNA1 molecules (C), all this process takes place in association with outer mitochondrial membranes. Afterwards, a sub-genomic RNA, namely RNA3, is synthesized from the 3′ terminus of RNA1(D). RNA3 encodes -and is translated into- the two small proteins B1 and B2 (E) which show nuclear localization. In addition, RNA3, presumably like in alfanodavirus, also regulates RNA2 synthesis (F) and it is downregulated at the onset of RNA2 replication/transcription (dotted line). RNA2 translation yields the capsid protein (G) and, finally, nascent (+) RNA1 and (+) RNA2 molecules are packaged into progeny virions (H). Adapted from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

Protein A, one of the three non-structural proteins of the virus, has a molecular weight of 110 kDa and a variable size depending on the viral genotype: 983 amino acids (aa) in SJNNV, 982 aa in RGNNV and 981 aa in BFNNV [12,14,15].

The capsid protein (338 aa, except the CP of SJNNV, which is 2 aa longer), has a molecular weight of 37 kDa [11,15–17]. In Alphanodavirus, upon genome encapsidation, the precursor of the capsid protein, protein α, is auto-catalytically cleaved into proteins β and γ [18], generating the mature capsid. This mechanism was not observed in betanodavirus [11]. Instead, the capsid protein undergoes conformational changes which are important for its structure and functions. Intramolecular disulfide bondings between cysteines 187 and 201 [19] or cysteines 115 and 201 [20] have been shown to play a role in the assembly and thermal stability of the viral particles. The structure of the Grouper nervous necrosis virus (GNNV) CP has been disclosed [21] and consists of three different domains and a flexible linker region. The N-terminal arm (N-arm) is responsible for recruiting the RNA during encapsidation; the shell domain (S-domain), a conserved region that forms the cage for the encapsidated RNA and contains calcium-binding structures which seem to be essential for virus assembly [22], and the protrusion domain (P-domain) that includes the hypervariable region of the protein, is involved in the interaction with the host cell surface, and is also responsible for the trimerization of the protein. Besides these structural functions of the CP, it has also been reported to contribute to the modulation of the host cell life cycle during viral infection. A nucleolus localization signal has been identified in the N-terminal region (aa 23 to 31) of the protein, which is associated with cell cycle arrest [23]; at a later stage of infection, the accumulation of the CP triggers apoptosis by inducing a caspase-dependent cascade [24,25].

Although there has been some controversy about the existence of B1 [15,26], this protein, encoded by an ORF that matches the C-terminus of the protein A reading frame, has been demonstrated to play a role as an antinecrotic death factor, which reduces mitochondrial membrane potential (MMP) loss in grouper fin (GF-1) cells [27]. In addition, B1 has been localized in the cytoplasm of E-11 cells infected with an RGNNV strain at 24 hours post infection (hpi) and targeting the nucleus at 48 hpi in up to 95% of cells [28]. The nuclear localization of B1 was mediated by two arginine-rich nuclear targeting domains. This B1 nuclear localization causes cell cycle arrest, confirming its implication in the regulation of host cell survival at the early stages of viral infection in GF-1 cells.

The other ORF is in a +1 reading frame relative to protein A and encodes B2, which is required for the suppression of cellular RNA interference (RNAi) in infected cells [13,29]. B2 has also been identified as a necrotic death factor, which upregulates the expression of the proapoptotic gene Bax and induces MMP loss but not mitochondrial cytochrome c release [30,31]. As with B1, B2 is also localized in the cytoplasm at 24 hpi and in the nucleus at later stages of infection [29,32].

2.2. Viral Replication

The genome of positive-strand RNA viruses (+RNA viruses), like NNV, behaves like an mRNA within host cells to allow the expression of viral proteins which are first translated and then amplified by virus-encoded RdRps through negative-strand RNA intermediates. Progeny RNA serves as a template for additional rounds of replication and synthesis of viral proteins [18]. In the case of betanodaviruses, RNA replication is accompanied by the addition of cap structures to the 5′-ends of progeny RNA and the synthesis of a capped subgenomic RNA (sgRNA) derived from RNA1 [13].

RNA is replicated by protein A, which shows a domain spanning aa 582 to 808 and 585 to 819, in RGNNV and SJNNV respectively, containing six of the eight conserved motifs previously identified for RdRps of +RNA viruses [12,15]. Protein A catalyzes RNA synthesis in concert with mitochondrial membranes, mediating the formation of replication complexes with the mitochondria outer membrane (Figure 1). In the related genus *Alphanodavirus*, the N terminus of protein A functions both as a mitochondrial targeting signal and as a transmembrane domain for the tight association of the protein with cellular membranes [18]. Four transmembrane domains (TMDs) with a moderate level of hydrophobicity have been identified for two betanodaviruses, greasy grouper necrosis virus

(GGNNV) [33] and Atlantic Halibut Nodavirus (AHNV) [34]. These TMDs are located at positions 1–40, 225–246 for the AHNV, and at positions 153–173, 229–249 for the GGNNV and all but the TMD 153–173 were confirmed to contain mitochondrial targeting signals. In addition, nine amino acid signatures were identified in the sequences of these TMDs at positions 7, 19, 155, 223, 232, 235, 241, 251 and 254 after comparing different genotypes of betanodavirus [35], which could be related to the differences in the growth kinetics of the different genotypes [35]. Recently, one of these positions, amino acid 223, has been reported to probably be involved in NNV thermotolerance [36].

Regulation of the RNA replication depends on cis-acting elements at the 3'and 5' termini. However, these regulatory elements have not been demonstrated in betanodaviruses yet. In alphanodaviruses, the 3'terminal 50 nt of RNA2 contains a stem-loop structure (3´SL), which acts as a cis-acting replication signal capable of directing the replication of this segment [37]. A putative 3'SL structure has also been predicted in two betanodavirus strains, SJNNV and a reassortant RGNNV/SJNNV [38,39]. However, the role of the 3'SL in the regulation of RNA2 replication in betanodaviruses is not clear [39].

RNA replication in alphanodaviruses is also governed by internally located cis acting elements in both RNAs, and RNA 3 is involved in RNA 2 replication, acting as a trans-activactor of RNA2 and suffering a down-regulation at the onset of RNA2 synthesis [18]. Betanodavirus replication studies have suggested that RNA1 is expressed during the early stages of replication, with RNA2 expressed later [40], which would indicate that the betanodavirus cycle is organized into two phases as reported for the related genus alphanodavirus [18]: an early phase, where protein A molecules are synthetized up to a level that ensures the establishment of the replication complexes and a later phase in which capsid protein translation from RNA2 is up-regulated to enable virion packaging. In addition, the RNA1 copy number was significantly higher than that of RNA2 or of NNV infective particles [40]. The role of protein A, codified by RNA1, in the amplification of both genomic strands, would agree with these findings. RNA2 was also detected to a higher extent than the production of infective particles in the first 24 hpi. However, after that, RNA2 and viral infectivity did not differ significantly. These higher RNA2 levels could be due to the different roles of its encoded CP in cell division and apoptosis, as mentioned above.

The NNV replication cycle, as previously mentioned, involves the formation of a dsRNA replication intermediate that would immediately invoke the cellular RNAi response and the destruction of the viral RNA. However, as in its alphanodvirus counterparts, the B2 protein binds to and sequesters dsRNA to protect viral replication intermediates from the cellular RNAi antiviral machinery and allows RNA1 accumulation in the early phase of viral replication [29,41].

2.3. Taxonomic Classification

Based on a small variable sequence of RNA2, namely the T4 region, betanodaviruses have been traditionally classified into four genotypes, which correspond to the species recognised by the ICTV: RGNNV, BFNNV, TPNNV and SJNNV [42]. Three additional genotypes have been proposed, turbot nodavirus (TNV) [43], Atlantic cod nervous necrosis virus (ACNNV) [44] and Korean shellfish nervous necrosis virus (KSNNV) [45]. TNV has been widely accepted as the fifth genotype, although no isolates have been obtained yet; ACNNV, which clusters isolates from Atlantic cod (*Gadus morrhua*), haddock (*Melanogrammus aeglefinus*) and winter flounder (*Pseudopleuronectes americanus)*, has been included as a clade within the BFNNV genotype [46–50], and KSNNV is too recent to have been considered in the literature.

The isolation of nodavirus from other invertebrate hosts such as crustaceans, however, has lead to a reconsideration of the taxonomy of the *Nodaviridae* family and the proposal of a new genus—*Gammanodavirus* [51,52].

An alternative classification scheme of betanodaviruses was proposed by Thiéry et al. [53] which refers to betanodavirus genotypes as numbers (I, II, III and IV, corresponding to RGNNV, BFNNV, TPNNV and SJNNV, respectively) and establishes subgroups within the genotypes. This is the case of the three subgroups (a, b and c) recognized within genotype II (BFNNV), which reflect the genomic

differences between the Canadian Atlantic cod (IIa), the Barfin flounder (*Verasper moseri*) (IIb) and the Atlantic halibut (*Hippoglossus hippoglossus*)/French European sea bass (*Dicentrarchus labrax*) isolates (IIc) and the two subgroups within genotype IV (SJNNV), showing the differences between the strains isolated from Senegalese sole (*Solea senegalensis*) and gilthead sea bream (*Sparus aurata*) in the Iberian Peninsula (IVa) and those isolated in Japan (IVb) [53,54].

In recent years, the sequencing of both genomic segments has demonstrated the existence of natural reassortants between the RGNNV and SJNNV genotypes in Southern Europe. Although both combinations of genomic segments, SJNNV/RGNNV and RGNNV/SJNNV (RNA1/RNA2), have been observed in viral isolates obtained from fish, the second type has been detected more often [55–58].

Serological studies have demonstrated that the four genotypes can be grouped into three distinct serotypes [59,60]; however, a different correlation between serogroups and genotypes has been proposed. Thus, whereas according to Mori et al. [59] serogroup A would correspond to genotype SJNNV, group B to TPNNV and group C to RGNNV and BFNNV, Panzarin et al. [60] establish that serogroup B would include strains from genotypes BFNNV and TPNNV and that only RGNNV strains would be clustered in serogroup C.

2.4. Geographical Distribution and Host Range

Betanodaviruses are widely distributed (Figure 2), but the geographical distribution of the different genotypes seems to be related to their thermotolerance. As a result, the BFNNV genotype seems limited to cold-water fish in Japan and Northern areas of Europe and America (Figure 2, Tables 1–4). It has been mainly isolated from farmed fish, including Atlantic and Pacific cod (*G. macrocephalus*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut and barfin flounder; but it has also been detected in some wild species, such as Atlantic cod and different species of wrasse (ballan, corking and goldsinny wrasse, *Labrus bergylta, Symphodus melops* and *Ctenolabrus rupestri*) in Scandinavian coastal waters [47,50] and winter flounder (*Pseudopleuronectes americanus*) in Canada [61].

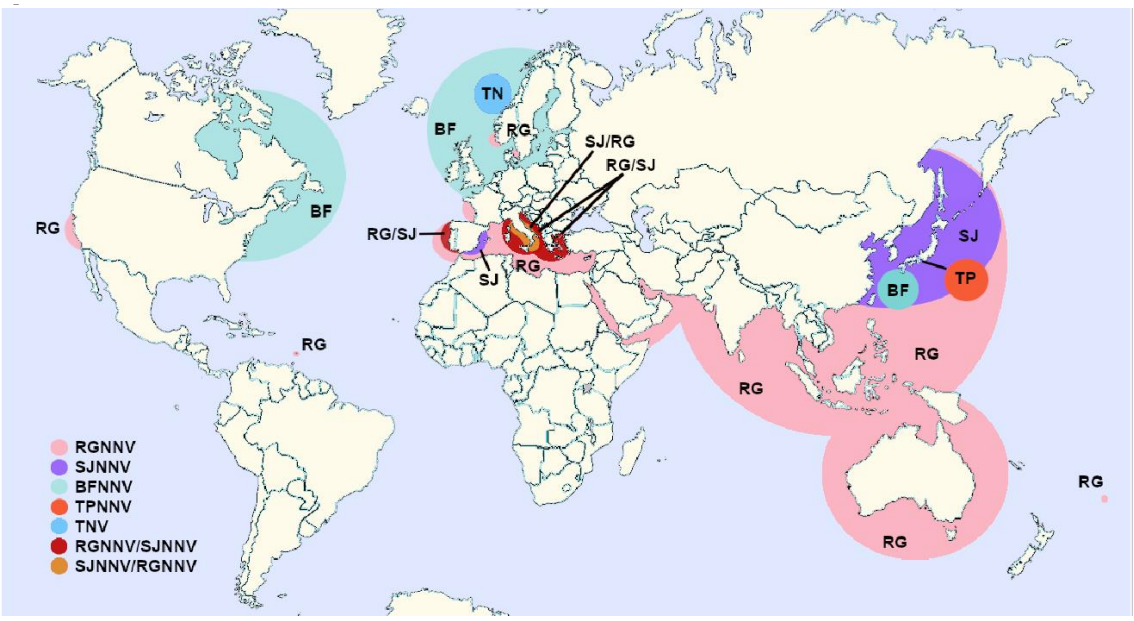

Figure 2. Distribution of Nervous necrosis virus (NNV) genotypes. Adapted from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

The RGNNV genotype, which affects tropical and temperate fish species, is the most widely distributed (Figure 2) and has the highest number of susceptible species (Tables 1, 2 and 4). In recent years, surveys conducted in different geographical areas have shown that RGNNV is widely distributed

not only in farmed but also among wild fish in the Mediterranean basin and along the coasts of Asia and Australia [46,62–72]. It has also been isolated from farmed white seabass (*Atractoscion nobilis*) in California [73]. In addition, it has recently been reported in wild ballan and corkwing wrasse inhabiting Scandinavian waters [50], which are usually associated to the BFNNV genotype. Finally, it is the only genotype associated to outbreaks in freshwater species in Europe, Asia and Australia (Table 3).

In contrast, the TPNNV genotype seems to be a minor variant because it has only been described in one species in Japan [42].

The SJNNV type, although for a long time considered limited to fish reared in Japanese waters, has also been detected in Senegalese sole and gilthead sea bream farmed on the Iberian Peninsula [54].

Finally, natural SJNNV/RGNNV reassortants have only been isolated from sea bass on the Italian coast [95], whereas the opposite form, RGNNV/SJNNV, is widespread in Southern Europe (Figure 2) and has been isolated from farmed European sea bass, sole (both common sole, *S. solea*, and Senegalese sole) and gilthead sea bream [55–57] and recently from wild Mediterranean horse mackerel (*Trachurus mediterraneus*) [58].

Table 1. Marine fish species affected in natural outbreaks by viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN).

Order	Family	Common Name	Latin Name	Status [a]	Genotype	References	Geographic Range
Centrarchiformes	*Latridae*	Striped trumpeteer	*Latris lineata*	F	ND [b]	[74]	Australia
	Oplegnathidae	Japanese parrotfish	*Oplegnathus fasciatus*	F	ND	[75]	Japan
		Spotted knifejaw	*O. punctatus*	F	RGNNV	[76,77]	Japan
		Australian bass	*Macquaria novemaculeata*	F	RGNNV	[46]	Australia
Gadiformes	*Gadidade*	Atlantic cod	*Gadus morhua*	F	BFNNV	[34,44,47,77,78]	Canada, USA, Norway, UK
		Pacific cod	*Gadus macrocephalus*	F	BFNNV	[59,79]	Japan, China
		Haddock	*Melanogrammus aeglefinus*	F	BFNNV	[44]	Canada, Atlantic USA
Gonorynchiformes	*Chanidae*	Milk fish	*Chanos chanos*	W	ND	[80]	India
Mugiliformes	*Mugilidae*	Flathead grey mullet	*Mugil cephalus*	F	RGNNV	[81]	Israel
		Golden mullet	*Liza auratus*	W	ND	[82]	Iran
		Leaping mullet	*L. saliens*	W	ND	[82]	Iran
Perciformes	*Centropomatidae*	Asian sea bass/Barramundi	*Lates calcarifer*	F	RGNNV	[48,68,81,83–86]	Taiwan, India, Singapore, Malaysia, Australia, Israel, China, Indonesia
		Japanese sea bass	*Lateolabrax japonicus*	F	ND	[87]	Japan
	Ephippidae	Orbicular batfish	*Platax orbicularis*	F	RGNNV	[88]	French Polynesia
	Carangidae	Striped Jack	*Pseudocaranx dentex*	F	SJNNV [c]	[6]	Japan
		Purplish amberjack	*Seriola dumerili*	F	ND	[76]	Japan
		Pompano	*Trachinotus blochii*	F	RGNNV	[89]	Malaysia
		Permit	*T. falcatus*	F	RGNNV	[83,84]	Taiwan
		Golden pompano	*T. ovatus*	F	RGNNV	[90]	China
	Lutjanidae	Firespot snapper	*Lutjanus erythropterus*	F	RGNNV	[83,91]	Taiwan, Malaysia
	Moronidae	European sea bass	*Dicentrarchus labrax*	F	RGNNV, SJ/RG [d]	[3,4,63,77,81,92–95]	Martinique, Spain, Portugal, Mediterranean
				W	RGNNV	[96]	Italy
	Pomacentridae	Clownfish,	*Amphiprion sebae Bleeker*	F	RGNNV	[97]	India
	Rachycentridae	Cobia	*Rachycentron canadum*	F	RGNNV	[84]	Taiwan
	Sciaenidae	Red drum	*Sciaenops ocellatus*	F	RGNNV	[81,98]	Korea, Israel
		Shi drum	*Umbrina cirrosa*	W/F	RGNNV	[77,95,99,100]	France, Italy, Adriatic Sea
		White seabass	*Atractoscion nobilis*	F	RGNNV	[73]	California (USA)
	Scombridae	Pacific bluefin tuna	*Thunnus orientalis*	F	RGNNV	[101]	Japan
	Serranidae	White grouper	*Epinephelus aeneus*	F	RGNNV	[81]	Israel
		Red spotted grouper	*E. akaara*	F	ND	[102,103]	Taiwan, Japan
		Yellow grouper	*E. awooara*	F	RGNNV	[104]	Taiwan, China

Table 1. *Cont.*

Order	Family	Common Name	Latin Name	Status [a]	Genotype	References	Geographic Range
		Duskytail grouper	*E. bleekeri*	F	RGNNV	[69]	China
		Orange-spotted grouper	*E. coioides*	F	RGNNV	[84,105]	Philippines, Taiwan
		Golden grouper	*E. costae*	W	RGNNV	[96,106]	Italy, Algeria
		Blackspotted grouper	*E. fuscogutatus*	F	RGNNV	[85,91,102]	Taiwan, Malaysia
		Dragon/Giant grouper	*E. lanceolatus*	F	RGNNV	[68,107,108]	China, Taiwan, Australia
		Malabar grouper	*E. malabaricus*	F	RGNNV	[107]	Taiwan
		Dusky grouper	*E. marginatus*	W	RGNNV	[96,106,109]	Italy, Algeria, Spain
		Kelp grouper	*E. moara*	F	ND	[110]	Japan
		Sevenband grouper	*E. septemfasciatus*	F	ND	[111,112]	Japan, Korea
		Greasy grouper	*E. tauvina*	F	RGNNV	[15,113,114]	Singapore
		Hybrid grouper	*E. fuscoguttatus x E. lanceolatus*	F	RGNNV	[115]	China, Indonesia
		Humpback grouper	*Chromileptes altivelis*	F	RGNNV	[46,83,84,116–118]	Taiwan, Indonesia, Malasia, Vietnam, Australia,
		Spottet coral grouper	*Plectropomus maculatus*	F	ND	[119]	Thailand
	Siganidae	Dusky sinefoot	*Siganus fuscescens*	F	RGNNV	[68]	China
	Sparidae	Gilthead seabream	*Sparus aurata*	F	RG/SJ [d]	[55,57,120]	Iberian Peninsula, Italy, Arabian Gulf
		Sobaity seabream	*Sparidentex hasta*	F	ND	[120]	Arabian Gulf
	Sparidae	White seabream	*Dioplodus sargus*	F	RGNNV	[95]	Italia, Adriatic sea
Pleuronectiformes	*Paralicthyidae*	Japanese flounder	*Paralichthys olivaceus*	F	RGNNV	[16,42,121]	Japan, Korea
	Pleuronectidae	Barfin flounder	*Verasper moseri*	F	BFNNV	[42,76]	Japan
		Atlantic halibut	*Hippoglossus hippoglossus*	F	BFNNV	[122–124]	Norway, United Kingdom
	Scophthalmidae	Turbot	*Scophthalmus maximus*	F	TNNV	[43]	Norway
	Soleidae	Dover sole	*Solea solea*	F	BFNNV	[77]	United Kingdom
		Senegalese sole	*S. senegalensis*	F	SJNNV, RG/SJ	[53,55]	Iberian Peninsula
Scorpaeniformes	*Sebastidae*	Oblong rockfish	*Sebastes oblongus*	F	RGNNV	[121]	Korea
Tetraodontiformes	*Monacanthidae*	Thread-sail filefish	*Stephanolepis cirrhifer*	F	ND	[125]	Thailand
	Tetraodontidae	Tiger puffer	*Takifugu rubripes*	F	TPNNV [c]	[110]	Japan

[a]. F, farmed, W, wild; [b], Not determined, [c]. Based on Nishizawa et al. [42], [d]. Reassortant RGNNV/SJNNV.

Table 2. Detection of nervous necrosis virus (NNV) in asymptomatic farmed fish species.

Order	Family	Common Name	Latin Name	Habitat	Geno-type	Detection	Reference	Geographic Range
Anguilliformes	*Muraenidae*	Blue ribbon eel	*Rhinomuraena quaesita*	Marine [a]	ND [b]	PCR	[126]	Korea/Indonesia [d]
Beryciformes	*Monocentridae*	Pinecone fish	*Monocentris japonica*	Marine [a]	ND	PCR	[126]	Korea/Japan [d]
Centrarchiformes	*Oplegnathidae*	Japanese parrotfish	*Oplegnathus fasciatus*	Marine	ND	PCR	[66,127]	Japan, Korea
Clupeiformes	*Engraulidae*	Japanese anchovy	*Engraulis japonicus*	Marine [a]	RGNNV	PCR	[126]	Korea/ Japan[d]
Gonorynchiformes	*Chanidae*	Milk fish	*Chanos chanos*	Marine/ freshwater [a]	ND	PCR	[126]	Korea/Japan [d]
Mugiliformes	*Mugilidae*	Flathead grey mullet	*Mugil cephalus*	Farmed	RGNNV	PCR	[95]	Italy, Adriatic sea
Perciformes	*Acanthuridae*	Yellow tang	*Zebrasoma flavescens*	Marine [a]	ND	PCR	[126]	Korea/Singapore [d]
	Carangidae	Look down fish	*Selene vomer*	Marine [a]	ND	PCR	[126]	Korea/N. America [d]
		Golden pompano	*Trachinotus ovatus*	Marine	RGNNV	PCR	[68]	China
	Lateolabracidae	Chinese seabass	*Lateolabrax sp*	Marine	RGNNV	PCR	[66]	Japan
		Japanese sea perch	*Lateolabrax japonicus*	Marine	RGNNV	PCR	[68]	China
	Moronidae	European sea bass	*Dicentrarchus labrax*	Marine	RGNNV	PCR, CC [c],	[128]	Italy
	Mullidae	Red mullet	*Mullus barbatus barbatus*	Marine	RGNNV	PCR, CC	[95]	Italy, Adriatic Sea
	Polycentridae	South American leaf fish	*Monocirrhus polyacanthus*	Freshwater [a]	RGNNV	PCR, CC	[126]	Korea/Amazon [d]
	Pomacentridae	Three spot damsel	*Dascyllus trimaculatus*	Marine [a]	RGNNV	PCR, CC	[126]	Korea/Singapore [d]
	Serranidae	Brown-spotted grouper	*E. chlorostigma*	Marine	ND	PCR	[120]	Arabian Gulf
		Giant grouper	*E. lanceolatus*	Marine	RGNNV	PCR	[68]	China
		Redspot grouper	*Epinephelus akaara*	Marine	RGNNV	PCR	[66]	Japan
		Yellow grouper	*E. awooara*	Marine	RGNNV	PCR	[68]	China
	Serrasalmidae	Red piranha	*Pygocentrus nattereri*	Freshwater [a]	RGNNV	PCR, CC	[126]	Korea/Amazon [d]
	Siganidae	Dusky sinefoot	*Siganus fuscescens*	Marine	RGNNV	PCR	[68]	China
	Sparidae	Gilthead seabream	*Sparus aurata*	Marine	ND, RGNNV	PCR	[63,95,129, 130]	France (Atlantic),Mediterranean
		Red seabream	*Pagrus major*	Marine	RGNNV	PCR	[66,131]	Japan, Korea
	Stromateidae	Silver pomfret	*Pampus argenteus*	Marine	RGNNV	PCR	[68]	China
Pleuronectiformes	*Paralicthyidae*	Japanese flounder	*Paralichthys olivaceus*	Marine	RGNNV	PCR	[66,127]	Japan, Korea
	Pleuronectidae	Marbled sole	*Pleuronectes yokohamae*	Marine	RGNNV	PCR	[66]	Japan
		Stone flounder	*Kareius bicoloratus*	Marine	RGNNV	PCR	[131]	Korea
	Sciaenidae	Red drum	*Sciaenops ocellatus*	Marine	RGNNV	PCR	[68]	China
	Scopthalmidae	Turbot	*Scophthalmus maximus*	Marine	RGNNV	PCR, CC	[132]	Spain

Table 2. *Cont.*

Order	Family	Common Name	Latin Name	Habitat	Geno-type	Detection	Reference	Geographic Range
Scorpaeniformes	*Sebastidae*	Black rockfish	*Sebastes inermis*	Marine	RGNNV	PCR	[66]	Japan
Scorpaeniformes		Oblong rockfish	*S. oblongus*	Marine	RGNNV	PCR	[66]	Japan
		Schlegel's black rockfish	*S. schlegelii*	Marine	RGNNV	PCR	[131]	Korea
	Sebastidae	Spotbelly rockfish	*S. pachycephalus*	Marine	RGNNV	PCR	[66]	Japan
	Synanceiidae	Devil stinger	*Inimicus japonicus*	Marine	RGNNV	PCR	[66]	Japan
Tetraodontiformes	*Monacanthidae*	Black scraper	*Thamnaconus modestus*	Marine	ND	PCR	[66]	Japan
	Tetraodontidae	Tiger puffer	*Takifugu rubripes*	Marine	RGNNV	PCR	[66]	Japan
Syngnathiformes	*Centriscidae*	Shrimp fish	*Aeoliscus strigatus*	Marine [a]	ND	PCR	[126]	Korea/Japan [c]

[a], ornamental fish; [b], Not determined; [c], isolation in cell culture; [d], place of detection/fish procedence.

Table 3. Freshwater fish species affected –in natural outbreaks- by viral encephalopathy and retinopathy (VER).

Order	Family	Common Name	Latin Name	Status	Genotype	Reference	Geographic Range
Acipenseriformes	*Acipenseridae*	Danube sturgeon	*Acipenser gueldenstaedtii*	Farmed	ND [a]	[133]	Greece
Anguilliformes	*Anguillidae*	European eel	*Anguilla anguilla*	Farmed	RGNNV	[83,84]	Taiwan
Cypriniformes	*Cyprinidae*	Goldfish	*Carassius auratus*	Ornamental	ND	[134]	India
		Rainbow sharkminnow	*Epalzeorhynchos frenatum*	Ornamental	ND	[134]	India
Cyprinodontiformes	*Poeciliidae*	Guppy	*Poecilia reticulata*	Farmed	RGNNV	[17]	Singapore
Perciformes	*Blenniidae*	Freshwater blenny	*Salaria flubiatilis*	Wild	RGNNV	[135]	Spain
	Centrarchidae	Largemouth black bass	*Micropterus salmoides*	Farmed	RGNNV	[136]	Italy
	Cichlidae	Tilapia	*Oreochromis niloticus*	Farmed	RGNNV	[49,137]	Europe, Indonesia
	Eleotridae	Sleepy cod	*Oxyeleotris lineolatus*	Farmed	RGNNV	[46]	Australia
	Moronidae	Hybrid striped bass	*Morone saxatilis* x *M. chrysops*	Farmed	RGNNV	[136]	Italy
	Percidae	Pike-perch	*Sander lucioperca*	Farmed	RGNNV	[136]	Italy
Siluriformes	*Plotosidae*	Catfish	*Tandanus tandanus*	Farmed	ND	[74]	Australia
	Siluridae	Chinese catfish	*Parasilurus asotus*	Farmed	RGNNV	[84]	Taiwan

[a], Not determined.

2.5. Viral Thermotolerance

The risk of infectious disease outbreaks in the aquatic environment depends on the interactions between host and pathogen, as well as environmental factors. Among these, temperature has a crucial effect on viruses hosted by fish, because these animals have virtually no capacity to maintain a difference between their body temperature and that of the environment. Temperature can modulate the ability of the fish to defend itself against infection but also the ability of the infectious agent to colonize the host fish. This effect is especially evident in betanodaviruses because betanodavirus genotypes show different optimal growth temperatures (15–20 °C for BFNNV, 20 °C for TPNNV, 20–25 °C for SJNNV and 25–30 °C for RGNNV) and therefore, natural infections can occur at different water temperatures depending on the viral type. Thus, whereas BFNNV has been reported to cause disease at temperatures as low as 4 to 15 °C [47,144], NNV outbreaks associated to RGNNV are linked to high water temperatures: from 23 to 30 °C (in sea bass) to 28 to 30 °C (in different grouper species) [144,145]. Experimental trials have also demonstrated the effect of temperature on NNV pathogenicity [145–150].

The temperature sensitivity of betanodaviruses seems to be regulated by RNA1 [35,151] and more specifically by the region encoding the amino acid residues 1–445 [151]. Experiments performed with a recombinant strain harboring six point mutations in this region confirmed its role in viral thermotolerance, and pointed to position 223 as a putative responsible for temperature regulation [36]. This study, however, showed that genomic regions other than 1–445 may be involved in NNV thermotolerance.

Global warming is causing serious modifications in aquatic environment parameters, including changes in dissolved oxygen and ocean carbon dioxide levels, salinity and temperature. It is well documented that changes experienced by ecosystems will affect the epidemiology of infectious diseases in animals, in the wild and under intensive farming conditions, including aquaculture [152–156]. The high number of wild asymptomatic species infected with NNV, mainly in Asian and Mediterranean countries [62,64,66–68,71,80,82] and the quick rise in the viral load and subsequent outbreak of mortalities observed in experimentally infected sole when water temperature increased from 16 to 22 °C [148], suggest that VER outbreaks could have dramatic effects on natural populations, as has already been reported for some endangered fish species in the Mediterranean [72,96,109]. On the other hand, it could be argued that ocean warming could decrease the pathologies associated with the BFNNV genotype. However, as indicated above, this genotype can cause disease in a range of temperatures from 4 to 15°C. Furthermore, the number of susceptible species to this genotype could also increase by the movement of some fish species to higher latitudes because of the warming of their natural habitats.

3. The Disease: Viral Encephalopathy and Retinopathy (VER) or Viral Nervous Necrosis (VNN)

Disease outbreaks have been reported mainly in early developmental stages (larval and juveniles), but significant mortalities have also been described in older fish [74]. Although clinical signs depend on the fish species, biological stage, phase of the disease and temperature, abnormal swimming behavior (spiral swimming, whirling, horizontal looping or darting) and loss of appetite are commonly observed among affected fish. Other signs include swim bladder hyperinflation and coloration abnormalities (pale or dark).

Histopathological analysis reveals extensive necrosis of the central nervous system (CNS), with extensive vacuolation and neural degeneration of the brain as well as vacuolation of the retina [5,94,157]. In addition to histological lesions in nervous tissues, hyperplasia with vacuolar degeneration of epithelial cells has been reported in the epithelial layer of the skin, gill operculum, and oral cavity in 1- and 2-day-old striped jack larvae [157]. There also seem to be histological differences according to fish size, because affected larval neural tissue showed a greater extent of vacuolation and in different brain areas (medulla oblongata and spinal cord) than adult fish, which showed lesions in peripheral layers of the molecular layer [158].

3.1. Routes of Infection and Spread through the Fish Body

Several portals of viral entry have been suggested, including epithelial cells covering the fish body and/or the fin [159], gills [159–161] and nasal and oral cavity [158–160,162].

The neurotropism of betanodaviruses has been repeatedly demonstrated (see reviews [74,144,163]) and viral replication seems to be almost entirely restricted to nerve tissue, preferentially brain and retina [157,162,164]. Histopathological studies have demonstrated the vacuolation of nerve cells in the olfactory lobe and cerebellum (Purkinje cell layer and the underlying granule cell layer) [10,82,119,120,125,135,161,165,166]. Megalocells and small nerve cell nuclei were also infected in the preoptic area, thalamus, medulla oblongata and spinal cord, whereas only a few small nerve cells were infected in the olfactory bulb and optic tectum [57,93,109,119,158,166–168].

To date, it has not been possible to identify the neuronal receptors involved in NNV entry and the available data on cell receptors comes from studies using cell lines. Viral entry is believed to occur through clathrin-mediated endocytosis [169] and interaction with different cell receptors has been proposed. Consequently, sialic acid seems to be involved in NNV binding to SNN-1 cells [170] and therefore also to E-11, which are a clone of SSN-1 [171]. In grouper fin cells (GF-1), the grouper heat shock cognate protein 70 (GHSC70) has been proposed as an NNV receptor or co-receptor protein [172] and in SB cells, receptors have been reported to probably be proteins located at lipid rafts or even specific lipids [169]. Recently, Nectin-4/PVRL4, belonging to the family of immunoglobulin-like cell adhesion molecules, has been identified as a potential cellular receptor for NNV in both seven-band grouper and transfected SSN-1 cells [173,174]. In the study performed with the seven-band grouper nectins, a possible interaction with NNV was predicted based on a docking simulation, and interaction with Nectin-4 was the most highly supported [173]. A subsequent assay performed on SSN-1 cells transfected with Nectin-4 indicated that the overexpression of this protein enhanced viral replication kinetics, whereas silencing reduced virus–cell interaction [174].

The C-terminal region of the NNV capsid protein, located in the P-domain [21], has been reported to be involved in host specificity [175,176] and positions 247 and 270 have been identified as putative receptor binding sites, because their substitution modifies the affinity of the virus for the neural receptors and affects the kinetics of the virus spreading in the brain [177].

Viral spreading is thought to be produced through the nervous system across the synaptic connections [146,178]. However, NNV has also been detected in blood samples of Senegalese sole, Atlantic cod and seven-band grouper [179–181], suggesting that the virus can also use the hematogenous route to spread throughout the fish body. Viral presence in non-neurological tissues (gills, fins, heart, anterior and posterior intestine, stomach, spleen, liver, kidney and gonads) [158,180,182–186] would support hematogenous spread. However, as all these organs are fully innervated, neural spread cannot be ruled out. Analyses of experimentally infected Atlantic cod suggest an initial viremia followed by neural spread [180]. Although in most cases NNV detection in non-neurologicaltissues has been performed using histological or molecular techniques, the presence of infective particles has been confirmed in gills, skin, fins and intestine of Senegalese sole [159] and testis of European sea bass and gilthead sea bream [187].

The dynamics of NNV infections seem to be fast; the RGNNV genome was detected in the brain of infected pompano at 4 hpi [188] and both genome and infective particles of a reassortant RGNNV/SJNNV strain were detected after 1 day post infection (dpi) in the brain of Senegalese sole [159]. However, differences related to genotypes and fish species can be observed because in Senegalese sole the RGNNV genome was not detected until 3 dpi, and the detection of infective particles took one more day (4 dpi) [159]. This fast replication also implies the fast development of disease signs in infected fish, which were observed as early as 2 dpi in intramuscularly injected juvenile European sea bass and bath infected Asian sea bass larvae [160,189]. In both cases, first mortalities were detected soon after the onset of clinical signs. In other intramuscular experimental infections of European sea bass, first disease signs were recorded after 4–5 dpi [164,185]. Clinical signs were also soon observed in bath infected Senegalese sole (3 dpi) [190] and slightly later in bath infected European sea bass (5

to 10 dpi) [145,147,160]. The onset of mortality was detected very soon in bath-infected striped jack larvae (3–4 dpi) [70] and at 5 dpi in Senegalese sole, reaching 100% mortality at 18 dpi [190].

3.2. Viral Transmission

Both horizontal and vertical transmission have been demonstrated in several fish species. Horizontal transmission, fish to fish or through the water body, has been reported in Asian sea bass or barramundi, European sea bass, gilthead sea bream, brown-marbled grouper (*Epinephelus fuscoguttatus*) and Senegalese sole [5,92,148,160,164,191–194]. Similarly, interspecies horizontal transmission has been observed between European sea bass and gilthead sea bream reared on the same farm [129] and in experimental trials between turbot and Atlantic cod [195] and between Asian seabass and brown-marbled grouper [194]. Viral shedding from gills and skin could be involved in this transmission, as reported in Senegalese sole [159]. The main factors affecting horizontal transmission in farming conditions are stocking density [196], and temperature [148,149]. Genotype also seems to be an important factor, as shown by different challenge experiments, i.e., reassortant RGNNV/SJNNV and SJNNV strains isolated from Senegalese sole caused low mortalities in European sea bass through bath challenges [145,190].

Invertebrates, mainly bivalve mollusks (mussel, clam and oyster) but also gastropods and some crustaceans (crab, shrimp and lobster species) (Table 5) can act as natural reservoirs and possible carriers of NNV and, therefore, play a role in viral transmission through the water column, which can be favored by NNV resistance to environmental conditions and long survival in sea water [10,197]. In addition, two crustacean species, brine shrimp (*Artemia salina*) and rotifer (*Brachionus plicatilis*), used as live food for marine fish larvae, have been demonstrated to be susceptible to NNV infection [198] and in the case of Artemia, capable of transmitting NNV to Senegalese sole larvae causing disease symptoms and high mortality [199].

Vertical transmission has been reported in striped jack [182,203], Japanese flounder (*Paralichthys olivaceus*), barfin flounder [204], Atlantic halibut [146], European and Asian sea bass [183,205]. Gonads have been demonstrated to be involved in viral shedding in striped jack, European sea bass and gilthead sea bream [182,187,203,205]. In striped jack and Senegalese sole, the intestine has also been reported to take part in viral release [159,182] which would result in the contamination of eggs [182].

Both ways of transmission are a serious concern for the fish farming industry. The detection of carriers among breeders, farmed population and fish to be introduced into aquaculture sites is at present one of the major strategies to control disease outbreaks [126,179,206–209].

Table 4. Detection of nervous necrosis virus (NNV) in asymptomatic wild fish species.

Order	Family	Common Name	Latin Name	Geno-type	Detection	Reference	Geographic Range
Anguilliformes	*Anguillidae*	European eel	*Anguilla anguilla*	SJNNV	PCR	[138]	Spain
	Congridae	Conger eel	*Rhynchocymba nystromi*	RGNNV	PCR	[68]	China
Atheriniformes	*Atherinidae*	Mediterranean sand smelt	*Atherina hepsetus*	RGNNV	qPCR [a]	[58]	Greece
		Ring-tailed cardinalfish	*A. aureus*	RGNNV	PCR	[68]	China
Aulopiformes	*Synodontidae*	Threadfin saury	*Saurida filamentosa*	RGNNV	PCR	[68]	China
		Brushtooth lizardfish	*S. lessepsianus*	RGNNV	PCR	[130]	Mediterranean
		Snakefish	*Trachinocephalus myops*	RGNNV	PCR	[68]	China
Bathrachoidiformes	*Bathrachoididae*	Lusitania toadfish	*Halobatrachus didactylus*	RGNNV	PCR, CC [b]	[64]	Spain
Beloniformes	*Belonidae*	Garpike	*Belone belone*	RGNNV	PCR, CC	[62]	Italy
	Exocoetidae	Mediterranean flyingfish	*Cheilopogon heterurus*	RGNNV	PCR	[58]	Greece
Centrarchiformes	*Latridae*	Striped trumpeter	*Latris lineata*	RGNNV	PCR	[46]	Australia
	Oplegnathidae	Japanese parrotfish	*Oplegnathus fasciatus*	RGNNV	PCR	[66]	Japan
Clupeiformes	*Clupeidae*	Pilchard	*Sardina pilchardus*	RGNNV	PCR, CC	[58,62,65]	Italy. Tunisia
		Round sardinella	*Sardinella aurita*	RGNNV	PCR	[65,130]	Mediterranean
		Sardine	*S. jussieui*	RGNNV	PCR	[68]	China
	Dussumieriidae	Slender rainbow sardine	*Dussumieria elopsoides*	RGNNV	PCR	[130]	Mediterranean
	Engraulidae	Commerson´s anchovy	*Tolephorus commersonnii*	RGNNV	PCR	[68]	China
		European anchovy	*Engraulis encrasicolus*	RGNNV	PCR	[58]	Greece
Gadiformes	*Gadidae*	Atlantic cod	*Gadus morhua*	BFNNV	PCR	[47]	Norway
		Poor cod	*Trisopterus minutus*	RGNNV	PCR, qPCR	[56,58]	Italy, Greece
		Pollock	*Pollachius pollachius*	ND	PCR	[47]	Norway
		Saithe	*P. virens*	ND	PCR	[47]	Norway
		Whiting	*Merlangius merlangus*	RGNNV	PCR	[62]	Italy
	Merluciidae	European hake	*Merlucius merlucius*	RGNNV	PCR, CC	[58,62]	Italy, Greece
	Macrouridae	Glasshead grenadier	*Hymenocephalus italicus*	ND	PCR	[139]	Italy
Heterodontiformes	*Heterodontidae*	Japanese bullhead shark	*Heterodontus japonicus*	RGNNV	PCR	[66]	Japan
Kurtiformes	*Apogonidae*	Red stoplight cardinalfish	*Apogon erythrinus*	RGNNV	PCR	[68]	China

Table 4. *Cont.*

Order	Family	Common Name	Latin Name	Geno-type	Detection	Reference	Geographic Range
Mugiliformes	*Mugilidae*	Flathead grey mullet	*Mugil cephalus*	RGNNV	PCR, CC	[58,62,66,69]	Japan, Italy, China, Greece
		Golden mullet	*Liza auratus*	RGNNV	PCR	[65]	Tunisia
		Thicklip grey mullet	*Chelon labrosus*	RGNNV, SJNNV	PCR	[64]	Spain
Notacanthiformes	*Notacanthidae*	Shorfin spiny eel	*Notacanthus bonaparte*	ND	PCR	[139]	Italy
Perciformes	*Carangidae*	Atlantic horse mackerel	*Trachurus thrachurus*	RGNNV	PCR, CC	[58,62,65]	Italy, Tunisia
		Japanese jack mackerel	*T. japonicus*	RGNNV, SJNNV	PCR, CC	[66–68,70,71]	Japan, China
		Mediterranean horse mackerel	*T. mediterraneus*	RGNNV, RG/SJ	PCR, CC	[58,62]	Italy, Greece
		Blue Jack mackerel	*T. picturatus*	ND	PCR, CC	[128]	Italy
		Bigeye trevalli	*Caranx oshimai*	RGNNV	PCR	[69]	China
		Greater amberjack	*Seriola dumerili*	RGNNV, SJNNV	PCR	[58,67]	Japan, Greece
		Japanese amberjack	*S. quinqueradiata*	SJNNV	PCR	[67]	Japan
		Indian threadfish	*Alectis indica*	RGNNV	PCR	[69]	China
		Japanese scad	*Decapterus maruadsi*	RGNNV	PCR	[66]	Japan
		Shrimp scad	*Caranx djedaba*	RGNNV	PCR	[69]	China
		Yellowstripe scad	*Selaroides leptolepis*	RGNNV	PCR	[68,69]	China
		Whitefin trevally	*Carangoides equula*	RGNNV, SJNNV	nPCR [c]	[67]	Japan
	Centrolophidae	Pacific rudderfish	*Psenopsis anomala*	RGNNV	PCR	[68]	China
	Centropomatidae	Asian sea bass/ Barramundi	*Lates calcarifer*	RGNNV	PCR	[48]	India
	Epigonidae	Cardinal fish	*Epigonus telescopus*	ND	PCR	[139]	Italy
	Gobiidae	Black goby	*Gobius niger*	RGNNV	PCR, CC	[62]	Italy
	Haemulidae	Trout sweetlips	*Plectorhynchus pictus*	RGNNV	PCR	[69]	China
	Kyphosidae	Stripey fish	*Microcanthus strigatus*	RGNNV	PCR	[66]	Japan
	Labridae	Ballan wrasse	*Labrus bergylta*	BFNNV, RGNNV	PCR	[50]	Sweden
		Corkwing wrasse	*Symphodus melops*	BFNNV, RGNNV	PCR	[50]	Norway

Table 4. *Cont.*

Order	Family	Common Name	Latin Name	Geno-type	Detection	Reference	Geographic Range
		Goldsinny wrasse	*Ctenolabrus rupestris*	BFNNV	PCR	[50]	Norway, Sweden
	Leiognathidae	Pugnose ponyfish	*Leigognathus insidiator*	RGNNV	PCR	[69]	China
		Berber ponyfish	*L. berbis*	RGNNV	PCR	[69]	China
		Slender ponyfish	*L. elongatus*	RGNNV	PCR	[68]	China
	Lutjanidae	Dory snapper	*Lutjanus fulviflamma*	RGNNV	PCR	[69]	China
		Humphead snapper	*L. sanguineus*	RGNNV	PCR	[69]	China
		John's snapper	*L. johni*	RGNNV	PCR	[69]	China
		Mangrove red snapper	*L. argentimaculatus*	RGNNV	PCR	[69]	China
		Russell's snapper	*L. russelli*	RGNNV	PCR	[69]	China
	Moronidae	European sea bass	*Dicentrarchus labrax*	ND	qPCR	[128]	Italy
	Mullidae	Red mullet	*Mullus barbatus barbatus*	RGNNV	PCR, CC	[62,64,130]	Mediterranean
		Surmullet	*M. surmuletus*	RGNNV	PCR, qPCR	[56,58,130]	Mediterranean
		Japanese goatfish	*Upeneus japonicus*	RGNNV	PCR	[68]	China
		Goldband goatfish	*U. moluccensis*	RGNNV	PCR	[130]	Mediterranean
	Nemipteridae	Japanese threadfin bream	*Nemipterus japonicus*	RGNNV	PCR	[68]	China
		Randall's threadfin bream	*N. randalli*	RGNNV	PCR	[130]	Mediterranean
	Percophidae	Ray finned fish	*Chrionema chlorotaenia*	RGNNV	PCR	[68]	China
	Pomacentridae	Heavenly damselfish	*Pomacentrus coelestis*	RGNNV	PCR	[66]	Japan
	Priacanthidae	Red bigeye	*Priacanthus macracanthus*	RGNNV	PCR	[68]	China
	Sciaenidae	Goatee croaker	*Umbrina russelli*	RGNNV	PCR	[69]	China
		Big head penah croaker	*Argyrosomus macrocephalus*	RGNNV	PCR	[68,69]	China
		Silver croaker	*A. argentatus*	RGNNV	PCR	[69]	China
		Meagre	*A. regius*	RGNNV, SJNNV	PCR	[140]	Spain
		Brown meagre	*Sciaena umbra*	RGNNV	PCR, qPCR	[58,141]	Italy, Greece
		Hoki	*Johnius belengerii*	RGNNV	PCR	[68]	China
		Shy drum	*Umbrina cirrosa*	RGNNV	PCR	[43]	Italy
	Scombridae	Atlantic mackerel	*Scomber scombrus*	ND	PCR	[47,58]	Norway, Greece
		Chub mackerel	*S. japonicus*	RGNNV, SJNNV	PCR	[67]	Japan
		Atlantic bluefin tuna	*Thunnus thynnus*	RGNNV	PCR, qPCR	[58,142]	Japan, Greece
	Serranidae	Areolate grouper	*Epinephelus. areolatus*	RGNNV	PCR	[69]	China
		Longfin grouper	*E. megachir*	RGNNV	PCR	[69]	China
		Longspine grouper	*E. fario*	RGNNV	PCR	[69]	China

Table 4. *Cont.*

Order	Family	Common Name	Latin Name	Geno-type	Detection	Reference	Geographic Range
		Dotted grouper	*E. epistictus*	RGNNV	PCR	[69]	China
		Honeycumb grouper	*E. merra*	RGNNV	PCR	[69]	China
		Kelp grouper	*E. moara*	RGNNV	PCR	[69]	China
		Rock grouper	*E. fasciatomaculatus*	RGNNV	PCR	[69]	China
	Siganidae	White-spotted spinefoot	*Siganus oramin*	RGNNV	PCR	[69]	China
	Sparidae	Bogue	*Boops boops*	RGNNV	qPCR	[58,128]	Italy, Greece
		Axillary seabream	*Pagellus acarne*	RGNNV	PCR, CC	[64]	Spain
		Black seabream	*Spondyliosoma cantharus*	RGNNV	PCR, CC	[64]	Spain
		Common two-banded seabream	*Diploidus vulgaris*	RGNNV	PCR, CC	[64]	Spain
		Sharpsnout seabream	*D. puntazzo*	RGNNV	PCR, qPCR	[58]	Greece
		Annular seabream	*D. annularis*	ND	qPCR	[198]	Italy
		Red porgy	*Pagrus pagrus*	RGNNV	PCR, qPCR	[58,143]	Spain, Greece
		Salema	*Sarpa salpa*	RGNNV	qPCR	[58]	Greece
		Picarel	*Spicara smaris*	RGNNV	qPCR	[58]	Greece
		Blotched picarel	*S. maena*	RGNNV	qPCR	[58]	Greece
		Gilthead seabream	*Sparus aurata*	RGNNV	PCR, qPCR	[58,95]	Italy, Greece
		Striped seabream	*Lithognathus mormyrus*	RGNNV	PCR	[130]	Mediterranean
	Sphyraenidae	European barracuda	*Sphiraena sphiraena*	RGNNV	qPCR, CC	[58,128]	Italy, Greece
	Terapontidae	Fourlined terapon	*Pelates quadrilineatus*	RGNNV	PCR	[69]	China
		Trumpeter perch	*P. quadrilineatus*	RGNNV	PCR	[68]	China
		Jarbua terapon	*Terapon jarbua*	RGNNV	PCR	[69]	China
	Zanclidae	Moorish idol	*Zanclus cornutus*	RGNNV	PCR	[66]	Japan
Pleuronectiformes	*Citharidae*	Branched ray flounder	*Citharoides macrolepidotus*	RGNNV	PCR	[68]	China
	Pleuronectidae	European plaice	*Pleuronectes platessa*	ND	PCR	[47]	Norway
		Winter flounder	*Pseudopleuronectes americanus*	BFNNV	PCR, CC	[61]	Canada
	Citharidae	Branched ray flounder	*Citharoides macrolepidotus*	RGNNV	PCR	[68]	China
Siluriformes	*Plotosidae*	Striped catfish eel	*Plotosus lineatus*	RGNNV	PCR	[66]	Japan
Scorpaeniformes	*Scorpaenidae*	Luna lionfish	*Pterois lunulata*	RGNNV	PCR	[66]	Japan
		Red scorpionfish	*Scorpaena scrofa*	RGNNV	qPCR	[58]	Greece

Table 4. *Cont.*

Order	Family	Common Name	Latin Name	Geno-type	Detection	Reference	Geographic Range
	Sebastidae	Black rockfish	*Sebastes inermis*	ND	PCR	[66]	Japan
		Schlegel's black rockfish	*S. schlegelii*	RGNNV	PCR	[66]	Japan
		Marbled rockfish	*Sebastiscus marmoratus*	RGNNV	PCR	[66]	Japan
	Triglidae	Gurnard	*Tigla lyra*	RGNNV	PCR	[62]	Italy
		Tub gurnard	*Chelidonichtys lucerna*	RGNNV	PCR, CC	[64]	Spain
	Triglidae	Spiny gurnard	*Lepidotrigla dieuzeidei*	RGNNV	qPCR	[58]	Greece
Syngnathiformes	*Fistuliridae*	Red cornetfish	*Fistularia villosa*	RGNNV	PCR	[68]	China
Trachichthyiformes	*Trachichthyidae*	Mediterranean slimehead	*Hoplostethus mediterraneus*	ND	PCR	[139]	Italy
Tetraodontiformes	*Diodontidae*	Freckled porcupine fish	*Diodon holocanthus*	RGNNV	PCR	[66]	Japan
	Monacanthidae	Threadsail filefish	*Stephanolepis cirrhifer*	RGNNV	PCR	[66]	Japan
		Black scraper	*Thamnaconus modestus*	RGNNV	PCR	[66]	Japan
	Tetraodontidae	Panther puffer	*Takifugu pardalis*	RGNNV	PCR	[66]	Japan

[a], real time-PCR; [b], isolation in cell culture; [c], nested-PCR.

Table 5. Detection of nervous necrosis virus (NNV) in shellfish.

Order	Family	Common Name	Latin Name	Genotype	Detection	References	Geografic Range
Arcida	*Arcidae*	Granular ark	*Tegillarca granosa*	RGNNV, BFNNV	PCR	[131]	Korea, Japan
Mytiloida	*Mytilidae*	Mussel	*Mytilus edulis*	RGNNV, BFNNV	PCR	[131]	Korea
		Mediterranean mussel	*M. galloprovincialis*	RGNNV	PCR	[58,200, 201]	Korea, Italy, Greece
Oegopsida	*Ommastrephidae*	Japanese common squid	*Todarodes pacificus*	RGNNV	PCR, CC [a]	[201]	Japan
Ostreoida	*Ostreidae*	European flat oyster	*Ostrea edulis*	RGNNV	qPCR [b]	[58]	Greece
		Pacific oyster	*Cassostrea gigas*	RGNNV, BFNNV	PCR	[131,200]	Korea, France
Octopoda	*Octopodidae*	Octopus	*Octopus vulgaris*	ND [c]	PCR, CC	[202]	Italy
Decapoda	*Palinuridae*	Spiny lobster	*Pamulirus versicolor*	ND	PCR	[126]	Japan
	Pandalidae	Southern humpback shrimp	*Pandalus hypsinotus*	RGNNV	PCR	[201]	Korea
	Penaeidae	Kuruma prawn	*Marsupenaeus japonicus*	RGNNV	PCR	[130]	Mediterranean
	Portunidae	Charybdid crab	*Charybdis bimaculata)*	RGNNV	PCR	[201]	Korea
		Blue crab	*Portunus pelagicus*	RGNNV	PCR	[130]	Mediterranean
Pectinida	*Pectinidae*	Scallop	*Patinopecten yessoensis*	RGNNV, BFNNV	PCR	[131]	China, Japan
Veneroida	*Veneridae*	Clam	*Ruditapes philipinarum*	RGNNV	PCR	[200]	Italy
		Common orient clam	*Meretrix lusoria*	RGNNV, BFNNV	PCR	[131]	Korea
		Chinese cyclina	*Cyclina sinensis*	BFNNV	PCR	[131]	China
		Manila clam	*Venerupis philippinarum*	RGNNV, BFNNV	PCR	[131]	Korea, China, Japan
		Venus clam	*Mercenaria mercenaria*	BFNNV	PCR	[131]	China
		Wrinkled venus clam	*Callista brevisiphonata*	BFNNV	PCR	[131]	China
		Warty venus	*Venus verrucosa*	RGNNV	qPCR	[58]	Greece
Neogastropoda	*Muricidae*	Red-mouthed rockshell	*Stramonita haemastoma*	RGNNV	qPCR	[58]	Greece

[a], isolation in cell culture; [b], real time-PCR; [c], Not determined.

3.3. Host Response

NNV infection in fish provokes a host immune response, which is not completely understood yet, but an excellent review on the subject has already been published [163]. Briefly, although fish resistance to viral infections is mediated by innate and adaptative response, the first one seems to play a relevant role [210]. Innate immunity represents the first antiviral defense and is mediated by interferon (IFN) and interferon-induced genes (ISGs).

To date, three types of IFN have been described in vertebrates (type I, II and III). Types I and II-IFN are present in fish [211,212] and both have been detected in individuals infected with betanodavirus [163]. I-IFN transcription has been reported to be up-regulated in sea bass [213] and grouper [212], II-IFN production increased in experimentally infected turbot [214], whereas both I and II IFN were induced in infected zebra fish [215]. IFN up-regulation has also been described in *in vitro* assays, in barramundi brain (BB), grouper brain (GB) and FHM cells [216–218]. The up-regulation of interferon regulatory factors (IRF) and ISGs has also been reported in different species: IRF1 in infected turbot, Mx in gilthead sea bream and European sea bass [213,219,220], ISG-12 in European sea bass [221] and ISG-15 also in European sea bass and Senegalese sole [220,222]. In addition, the induction of other pro-inflammatory cytokines has been analyzed; as a result, the increased expression of tumor necrosis factor α (TNFα) has been reported in gilthead sea bream and sea bass [219,221], Interleukin 1-β (IL-β) is up-regulated in gilthead sea bream [219] and IL-β together with IL-1 and IL-34 is over-expressed in golden pompano (*Trachinotus ovatus*) [223].

Regarding the cell immune response to betanodavirus infection, an increase in the expression of T-cell marker genes (TRCb, CD4-2, CD4, CD8a, CB8b, Lck, NCCRP-1 and ZAP-70) has been reported in infected Atlantic halibut, European sea bass and gilthead sea bream [213,214,224,225]. In addition, the proliferation of CD4-1-positive lymphocytes has been assessed by flow cytometry in infected olive flounder (*Paralichthys olivaceus*) [226].

In recent years, a great deal of progress has been made on high-throughput tools for sequencing the transcriptome (RNA-Seq), enabling genome-wide transcriptomic analysis and providing valuable information for understanding virus–host interactions [227]. Different transcriptomic analyses have been performed on both NNV-infected cells [228–233] and fish [234–238].

The transcriptome analyses of different cell lines susceptible to NNV have provided useful information about the immune response elicited against viral infection. In SSN-1 cells infected with a RGNNV strain, the down-regulation of 1138 genes and the up-regulation of 2073 involved in different pathways related to viral pathogenesis was observed. Subsequent analyses focusing on the apoptosis pathway showed an over-expression of Endonuclease G, which could be responsible for cellular apoptosis [230]. RNA-seq analyses of *D. labrax* brain (DLB-1) cells also infected with an RGNNV strain, showed the up-regulation of a high number of genes related to immunity, heat-shock proteins or apoptosis. Gene ontology enrichment revealed the down-regulation of transcripts related to the cytoskeleton and vesicle biology, suggesting that the failure of vesicle transport upon NNV infection could be a major mechanism behind the pathogenic effects on the fish nervous system [232]. In another study also performed with DLB-1 cells, the transcriptomic profiles obtained from European sea bass head-kidney leucocytes incubated with NNV infected and uninfected cells were very similar, supporting that cell-mediated cytotoxic activity in sea bass is not primed upon NNV infection [231]. Other cells used for transcriptome analysis were Asian seabass (*Lateolabrax japonicus*) epithelial cells (SB) [229], grouper kidney (GK) cells [228] and *Lateolabrax japonicus* brain cells (LJB) [233]. The assembly of the transcriptome of NNV-infected SB cells, as in the previously described cell lines, showed a strong induction of various genes relevant to innate immunity which were identified as receptor-transporting 3 (RTP3), Viperin, IRF3, IFN and two heat shock protein (HSP) family members (Hsp30 and 70) [229]. In the NNV-infected GK cells, 117 genes associated with protein processing in endoplasmic reticulum (ER) were identified. In addition, the tag-based digital gene expression (DGE) system revealed that ER stress response was clearly affected in NNV-infected GK cells. A further analysis revealed an interaction between the NNV capsid protein and the ER chaperone immunoglobulin heavy-chain binding protein

(BiP), suggesting that the capsid protein plays a role in the NNV-induced ER stress [228]. Finally, in LJB infected with an RGNNV strain, 1969 up-regulated genes and 9858 down-regulated genes involved in immune response pathways were identified. It was also observed that the p53 signaling pathway was involved in NNV infection and inhibited by RGNNV. The overexpression of *L. japonicus* p53 (Ljp53) significantly inhibited RGNNV replication and up-regulated the expression of apoptosis-related genes, suggesting that Ljp53 might promote cell apoptosis to repress virus replication [233].

Regarding transcriptome analysis in infected fish, several studies have been performed in grouper species. Thus, a significant up-regulation of antiviral proteins and NK-Lysin, a known antibacterial protein, was observed in the brain of NNV infected sevenband grouper (*E. septemfaciatus*). Furthermore, several chemokines, cathepsins and lepsins were also up-regulated [239]. Brain tissue was also analyzed in persistently infected Malabar grouper (*E. malabaricus*), showing that highly immune cell active signaling and surface receptor expression were triggered during persistent infection, as well as the interferon-induced response [236]. Therefore, although immune cell activity was high in brain tissue during persistent infection, this failed to eliminate all the viral particles from the infected host. Further examination of the impaired virus clearance pathway revealed the up-regulation of some genes involved in immune cell suppression, such as PDL1 and LAG3, which are considered critical markers for persistent and chronic infection [240,241]. A different study analyzed the transcriptome of kelp grouper (*E. moara*) immune tissues (liver, spleen and kidney) and although the expression of class I major histocompatibility complex (MHC) was significantly higher in three immune tissues of the diseased grouper, many immune related genes, including humoral immune molecules (such as antibodies), the cellular mediated cytotoxic molecules (such as perforin) and some adhesion related genes were down regulated [238].

In acute nodavirus infections, there must be a balance of induction and inhibition of immune responses [212], and nodaviruses must be able to evade the host's protective systems so that they can replicate and transmit progeny to other cells. However, it is worth mentioning that the immune response may contribute to disease signs and mortality, as shown in experimental infections in Senegalese sole performed with an RGNNV/SJNNV reassortant strain, highly virulent for this species, (wt), and an attenuated mutant strain showing two amino acid changes in the capsid, which caused a 40% mortality decrease [237]. In this study, a higher number of genes (633) were differentially expressed (DEGs) in animals infected with the highly virulent wt isolate, when compared with animals infected with the mutant strain (393). In addition, in the eye/brain samples the proportion between up-downregulated DEGs was 91% and 9% after infection with the wt isolate, whereas the proportion was completely inverted in fish infected with the mutant strain (11% and 89%). This result was corroborated in an experimental infection performed on sea bass with an RGNNV recombinant strain harboring the same mutations in the capsid protein [221] because low or no inflammatory induction (transcription of *mxA*, *isg 15* and *tnf alpha* genes) was observed in the brain of fish infected with the mutant strain, whereas a strong induction was observed in fish challenged with the wt isolate. Finally, a similar result was observed in primary cultures of grouper brain cells, where NNV infection may activate microglial proliferation and stimulate microglial secretion of interleukin (IL)-1b, which is a critical cytokine responsible for neuronal death [242].

On the other hand, it has been observed that some fish are resistant to infection with a certain genotype, but susceptible to infection with a different viral type, i.e., gilthead sea bream was long considered an asymptomatic carrier of RGNNV strains [129] and it has recently been reported to be highly susceptible to RGNNV/SJNNV reassortants [57]; in a similar way, an RGNNV isolate was also obtained from asymptomatic turbot and did not produce mortalities in experimental challenges [132], whereas this fish species undergoes high mortalities associated with TNV genotype [43]. However, to date, no studies have been performed to identify the factors involved in the susceptibility/resistance of these species to different viral types.

3.4. Disease Outbreaks

It is well known that a disease outbreak is influenced by three different parameters: the environment, the host and the pathogen [243]. Different environmental factors have been postulated as predisposing factors for VER outbreaks, including temperature, stocking density and stress [74]. As mentioned above, host response is being thoroughly studied, although the differential susceptibility to genotypes has not yet been analyzed. On the other hand, only a few reports have been focused on NNV virulence determinants. The fact that most NNV isolates obtained from farmed fish have caused high mortalities [5,72,92,97,102,137], and therefore can all be considered highly virulent strains, and the scarce number of isolates obtained from asymptomatic reared fish, which could be considered as avirulent, probably explains the low number of studies on NNV virulence. However, the increasing number of detections in farmed and wild asymptomatic fish [58,62–66,68,126,130,132,201] should also increase the number of isolates which could be used in the future for virulence studies.

Some recent reports have focused on the C-terminal region of the capsid protein, as it is involved in host cell recognition [175,176]. Studies performed with a reassortant RGNNV/SJNNV strain demonstrated that the substitution of amino acids 247 (Ser→Ala) and 270 (Ser→Asp) in the SJNNV-type capsid brought about a 40% reduction in virulence in sole [244] and reduced viral replication in sole neurons [177]. The modification of these positions in an RGNNV strain also resulted in a sharp decrease in mortality in infected sea bass [221], which confirms that these two positions are involved in NNV virulence, regardless of the viral genotype. Other studies have also indicated that substitutions in the 3'terminal end of RNA2 lead to an attenuation in virulence for Senegalese sole and a delayed replication in brain tissues which could be due to the interaction of RNA2 with host cellular proteins [39].

4. Epidemiology

4.1. VER Outbreaks and NNV Detections in Farmed Fish

Since the first descriptions in the 1990s, VER episodes have been constantly reported, mainly in marine fish reared in Asian, Australian and European waters [57,74,86,90,91,115,117,120,245]. The most important affected species include grouper, Asian seabass/barramundi, European sea bass, gilthead sea bream, Japanese and barfin flounder, Atlantic and Pacific cod and Atlantic halibut (Table 1). However, routine surveys conducted on farmed fish have revealed the existence of a great number of asymptomatic individuals which could act as carriers. Therefore, NNV asymptomatic carriers have been detected among farmed species that have been reported to undergo disease outbreaks, such as European sea bass, golden pompano, Japanese parrotfish (*Oplegnathus fasciatus*), Japanese flounder (*Paralichthys olivaceus*), different grouper species (*E. lanceolatus*, *E. akaara* and *E. awooara*), red drum (*Sciaenops ocellatus*), dusky sinefoot (*Siganus fuscescens*) and tiger puffer (*Takifugu rubripes*) [56,66,68,131]. In all these cases, except the detection in Japanese parrot fish, which was not typed, viruses causing non-clinical infection belonged to the RGNNV genotype. Furthermore, the RGNNV genotype, as described above, has been isolated from asymptomatic farmed fish which have suffered clinical infection caused by a different genotype, i.e., turbot [132] and gilthead sea bream [63]. The RGNNV genotype has also been detected by PCR in other reared fish species in Asian countries which have not suffered VER outbreaks to date [65–69] (Table 2).

All these reports suggest a high prevalence of the RGNNV genotype in farmed fish in Asian countries, but also in the Mediterranean basin, which can lead to disease outbreaks when temperature and/or fish density increases.

Finally, RGNNV strains have also caused most of the outbreaks in freshwater species. [17,46,49,84,135,137] (Table 3) and have been detected in ornamental fish, both marine and freshwater species (Table 2). It is worth noting the viral presence in two species native to the Amazon river, South American leaf fish (*Monocirrhus polyacanthus*) and red piranha (*Pygocentrus nattereri*) [126,246] because no reports of NNV have been carried out in South America, but the authors conclude that the infection most probably occurred in a Korean aquarium.

4.2. NNV in Wild Fish

In recent years VER outbreaks have also been reported in wild fish in different geographical areas (Table 1). Mortalities associated to NNV have been recorded in wild grouper *(E. costae* and *E. marginatus*) inhabiting European and African Mediterranean waters [72,96,106,109], in European sea bass also in the Mediterranean basin [96], in mullet (*Liza aurata* and *L. saliens*) in the Caspian sea [82] and in milky fish (*Chanos chanos*) in the Indian ocean [80]. In addition, different surveys, mainly in Asian and European waters, have reported the detection of NNV in a wide variety of asymptomatic fish belonging to more than 120 different species, 54 families and 19 orders [50,58,62–69,126,130,139,140] (Table 4). Around 90% of these detections have been genotyped and 86% have been clustered with the RGNNV genotype. SJNNV was also detected in 5.5% of those species, whereas BFNNV was only present in 1.8% of the RGNNV positive species (Table 4). All these data demonstrate the high prevalence of the RGNNV genotype among wild fish and the threat that it represents for these populations, some of them endangered ones [72]. The SJNNV genotype alone was detected in wild European eel in the Albufera lake (Spain) [138] and BFNNV in Atlantic cod and goldsinny wrasse [47,50], both in the Scandinavian peninsula. Similarly, a reassortant strain (RGNNV/SJNNV) has been detected in Mediterranean horse mackerel in Greece [58].

4.3. NNV in Invertebrates and other Marine Animals

NNV has been detected so far in 21 species of marine invertebrates belonging to 12 families and nine orders (Table 5). Most of these detections have been performed in bivalve mollusks, which can accumulate different particles, including viruses, from the surrounding water due their filter-feeding activity [200,247]. However, NNV has also been detected in cephalopods such as octopus and squid, [202] crustaceans [126,127,201] and gasteropods [58]. The genotype most frequently detected among invertebrates has been RGNNV, although BFNNV strains have also been reported in single detections or accompanying RGNNV strains in Asian waters. In addition, other mollusk isolates obtained in Asia have been clustered in a new proposed genotype, KSNNV.

Recently, the first betanodavirus isolation from a marine vertebrate other than fish has been reported from a loggerhead turtle (*Caretta caretta*) in Italy [248], widening the range of susceptible hosts for the virus. The animals did not show evidence of disease caused by NNV, which suggests their role as carriers.

5. Diagnostics

5.1. Cell Cultures

Isolation in cell culture represents a basic tool for a comprehensive study of any viral agent. Although different fish cell lines, including RTG-2, CHSE-214, BF2, SBL, FHM and EPC, were tested for susceptibility to NNV when the disease viral etiology was confirmed [4,6,11,111,249], the first successful isolation of a betanodavirus was not achieved until the SSN-1 cell line, established from whole fry tissue of striped snakehead *Ophicephalus striatus* [10] was used. Subsequently, the GF-1 cell-line derived from grouper *Epinephelus coioides*, E-11, a clonal line derived from SSN-1 cells, and SB derived from Asian sea bass, were also demonstrated to be useful for the isolation and proliferation of NNV [15,171,250]. It has been suggested that viral replication in these cell-lines and earlier failures in established cell lines may be due to the existence of a specific receptor for NNV [171].

However, in recent years the number of cell lines reported to be susceptible to NNV has increased substantially. Several of these cell lines have been derived from grouper; either from the brain tissue or the snout or the eye. GB cells, already mentioned, were obtained from the brain of yellow grouper (*E. awoara*), GBC1 and GB11 were derived from orange-spotted grouper (*E. coioides*) and *E. moara* brain (EMB) from kelp grouper; ELGSN was obtained from the snout of giant grouper (*E. lanceolatus*) and SIGE from the eye of orange-spotted grouper [104,251–254]. Other cell cultures derived from the brain of other fish species are: LJB, developed from sea perch (already mentioned), Chinese perch brain

(CPB) cells from mandarin fish (*Siniperca chuatsi*), *Trachinotus ovatus* brain (TOGB) cells from golden pompano and DLB-1, also previously mentioned, from European sea bass [232,255,256]. Moreover, OLHNI cells derived from the caudal fin of medaka (*Oryzias latipes*), *Trachinotus ovatus* head kidney (TOHK) and trachinotus ovatus kidney TOK from golden pompano, Japanese flounder skin (JFSK), SISK and SISS from the kidney and spleen, respectively, of Asian sea bass/barramundi [257–262] have also been reported to be suitable for NNV propagation. It should be noted that all these susceptibility assays have been performed only with RGNNV strains, probably because this is the predominant genotype in Asian countries, where all these cell lines have been developed.

Other cell lines derived from fish susceptible to NNV have been demonstrated to be persistently infected with the virus. This is the case of BB derived from the brain of Asian sea bass/barramundi and BMGB from brown marbled grouper brain, but they differ in the mechanism supporting viral persistence. Thus, whereas increased Mx expression was observed in BB cells [263], in BMGB persistence was not associated with Mx expression [264].

NNV replication has also been tested in mammalian cell lines. However, the temperature chosen was 28 °C, not 37 °C, for two reasons: this is the optimal temperature for viral replication (20–30 °C) [171] and because the NNV polymerase is not active at 37 °C [171,250]. Studies performed with human cells indicated that HeLa, 293T, and A549 cell lines only supported betanodavirus replication when transfection was performed [265]. However, Cos-1 cells, derived from African green monkey kidney, and DBT, a murine astrocytoma cell line, have been reported to show different degrees of susceptibility to betanodavirus strains. As a result, whereas in Cos-1 cells the titer of virus obtained was lower than that from infected fish cell lines [11], the RGNNV production in DBT cells was 10-fold-higher than in the fish cell line E-11 [266].

5.2. Diagnostic Procedures

Several diagnostic methods have been developed since the appearance of the disease. First diagnoses were based on the observation of abnormal swimming behavior and the typical histopathological lesions (vacuolation) in the brain and retina of infected fish [75,103]. However, histopathology was soon considered only appropriate for a presumptive diagnosis and confirmation by immunological methods, indirect fluorescent antibody (IFAT) or by immunoperoxidase staining was recommended [267]. Isolation in cell culture, achieved in 1996 [10], was a big breakthrough in VER diagnosis and for two decades it was considered the reference method to detect NNV infection, followed by immunological or molecular identification [245]. Although in recent years, as described in the previous epigraph, the number of cell lines reported to be susceptible to NNV infection has increased considerably, most isolations are still performed on SNN-1 or E-11 cells. However, this procedure is time-consuming and shows low sensitivity, which leads to false negatives, especially when fish with a low viral load are analyzed. In addition, a successful viral isolation is only achieved when brain tissues are used, making it necessary to kill the fish. Therefore, the use of molecular or serological techniques has gained importance over recent years [245].

Several PCR-based techniques (RT-PCR, nested PCR and RT-qPCR) targeted to one or both genomic segments have been reported [16,128,141,268–278]. Nishizawa et al. [268] reported the first RT-PCR protocol, based on the amplification of a 430 bp fragment of the T4 region, capable of detecting an SJNNV strain. This protocol later also proved to be useful for the detection of other NNV genotypes [16] and, as indicated in the epigraph on taxonomic classification, led to the present NNV classification and has been extensively used to type new isolates from different geographic origins [245]. However, some identification problems were observed due to the existence of genetic diversity [277,279] and new protocols were developed targeting more conserved regions of RNA2 or including a second amplification round [141,278,280]. Some of these PCR protocols have been used in non-lethal analyses (using blood, sperm or ovarian tissue) and although it was necessary to use nested PCR, detection levels were similar to those obtained in brain tissue [141,179]. Further developments of the PCR technique have led to a PCR method that allows RGNNV and SJNNV genotyping [273] and

to a nanoparticle-based paper lateral flow biosensor (LFB) for visual detection of RT-PCR products in biological samples using gold nanoparticles with a detection limit of 270 pg [281]. Lateral flow paper biosensors have demonstrated to be attractive analytical platform for detection of pathogens because they allow and accurate, rapid and sensitive diagnostics and are also appropriate for field analysis [281].

In recent years RT-PCR detection has given way to RT-qPCR assays and different protocols have been published, targeting one or both genomic segments [128,269–272,275,276]. In addition, a real time procedure combined with high resolution melting (HRM) has been proposed for NNV detection and genotyping [274]. RT-qPCR has also used for non-lethal detection, testing blood, gills and caudal fin samples [282].

NNV detection has also been accomplished through isothermal amplification methods which are aimed at viral detection with no special equipment [283–286]. These include nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and cross priming isothermal amplification coupled with lateral flow dipstick (CPA-LFD). NASBA showed a detection limit between 1.0 and 0.1 $TCID_{50}$ and between 10^3 and 10^2 copies of synthetic RNA transcript [283], LAMP showed a sensitivity 100-fold higher than that of nested PCR [284,286] and the detection limit of CPA-LFD was 10 RNA copies/µl comparable to that of RT-qPCR [285].

Serological analysis, mainly an enzyme linked immunosorbent assay (ELISA), has been used for NNV detection since the first disease outbreaks in eggs, larvae and brood stocks [203]. Since then, different ELISA protocols for fish antibodies and viral antigen detection have been reported [207,287–292].

However, ELISA is problematic for detecting fish antibodies due to its low reproducibility and high background optical density (OD) [293,294], caused by non-specific reactions between antibodies and NNV particles [295]. To solve this problem, a sandwich ELISA using immobilized fish sera has recently been reported [295]. In this ELISA protocol, NNV-specific antibodies could be indirectly detected by detecting NNV antigens captured by fish IgM immobilized onto ELISA plate wells. In addition, antiserum against fish IgM is not required, which means that NNV-specific antibodies are detectable from any fish species using only one antiserum against NNV [295].

The last modification of the ELISA technique reported for NNV detection is an enzyme linked apta-sorbent assay (ELASA) procedure, in which aptamers are used as substitutions of antibodies [296]. Unfortunately, the sensitivity of this procedure cannot be compared with that of the reported ELISA assays, because the detection limit was not provided in the same units.

Finally, in situ hybridization procedures have also been tested for NNV diagnosis, using cDNA-RNA or RNA-RNA probes [185,297]. cDNA-RNA hybridization was used to detect NNV in different organs of experimentally infected European sea bass [185] and RNA-RNA probes have been assayed using the brain tissue of sevenband grouper and also in vitro using SSN-1 cells [295].

Despite all the significant progress achieved in NNV detection over the last few years, recent advances linked to the use of nanotechnology, already reported in human and animal viruses [298–306], could provide a substantial improvement in these diagnostics in the near future. Different nanostructures are available at present, such as nanoparticles (NP), carbon nanotubes (CNT), dendrimers, and quantum dots (QDs) among others, and can be applied to the identification of nucleic acids, proteins and viral particles as well as antibodies [307,308]. To the best of our knowledge, nano-based diagnostic methods have only been used twice for the detection of fish viruses—the infectious pancreatic necrosis virus [309] and NNV [281]. The use of a nanoparticle-based LFB for the detection of NNV amplification products enables accurate and fast diagnostics under field conditions, although does not increase sensitivity compared to RT-qPCR assays.

5.3. Control of the Disease

Viral diseases are not easy to control once they have been introduced into an aquaculture system and, among them, NNV infections are especially difficult because of the high stability of the NNV particle in the environment [197]. Current control strategies have not been changed substantially

with respect to those included in previous reviews [163,245] and rely mainly on good husbandry practices, including biosecurity and sanitation. Briefly, the virus can be completely inactivated by means of chemical disinfectants such as sodium hypochlorite, calcium hypochloride, benzalkonium chloride, chloroquine and iodine [310,311] or by other chemical (ammonium chloride, ozone) or physical treatments (heat, ultra-violet light) [197,311]. The prevention of vertical transmission through the selection of NNV-free breeders is also commonly used. This selection can be performed by testing gonadal fluids, eggs and blood samples with ELISA or PCR-based techniques [128,179,205–207]. The vaccination of broodstock has also been reported as a promising method for avoiding vertical transmission [312].

Although at present the use of therapeutic measures is not extended in aquaculture facilities, a number of interesting studies have been performed to search for useful antiviral compounds to control NNV infections [313–319]. In a recent study, 1000 known drugs, 600 natural products and 400 bioactive components, have been tested for their activity against NNV in a cell viability-based screening assay [314]. This study indicated that proadifen hydrochloride, a cytochrome P 450 inhibitor, showed strong anti-NNV activity. The broad-spectrum antiviral drug Ribavirin has also been demonstrated to inhibit NNV replication, both in vitro and in vivo, in zebra fish larvae used as an experimental model [315,318]. The most recent reports have assessed the anti-NNV activity of chlorpromazine hydrochloride, an inhibitor of clathrin-mediated endocytosis [320] and of isoprinosine, a synthetic purine derivative with immunomodulatory and antiviral properties [321]. Chlorpromazine hydrochloride has shown successful inhibition of NNV infection in SISK cells [319] and isoprinosine was tested both on SSN-1 cells and zebra fish, showing an inhibitory effect of NNV infection. Besides, single-walled carbon nanotubes (SWCNTs) were used for drug delivery to improve anti-NNV activity [321]. Carbon nanotubes, but multiwalled ones, MWCNTs, were also used as a delivery system in a targeted CNS antiviral therapy. In this assay, zebra fish infected with pearl grouper nervous necrosis virus (PGNNV) was exposed to MWCNTs conjugated with polyethylenimine, ribavirin and PGNNV-specific nanobody. Results obtained indicated an obvious accumulation of the nanotubes in the brain of infected fish and a strong anti-PGNNV activity [322].

The use of different peptides, antimicrobial peptides (AMP) and affinity peptides (AFP) has also been explored for controlling NNV infections [313,317,323]. The AMP tested, epinecidin-1 and tilapia hepcidin1–5, have shown in vivo antiviral activity against NNV. Whereas treatment with hepcidin helped to reduce the viral load during infection, Epi-1 cleared the virus during and after the infection [313,323]. The anti-NNV AFPs specifically bound the virus, aggregating or disrupting the viral particles and inhibiting viral infection, by reducing the contact between the virus and cell surface [317].

The screening of potential anti-NNV compounds has also included the use of nucleic acid ligands, called aptamers [316]. These aptamers, with a high degree of affinity and specificity for many targets [324] and already used in viral research [325,326], have been demonstrated to bind to the NNV capsid protein and to reduce fish mortality.

6. Prevention

Due to the aforementioned difficulties of controlling VER, special emphasis has been made on vaccine development to prevent the disease. Different vaccine approaches have been looked into in recent years and several have been included in different reviews [163,245]; therefore, we will focus on the most recent ones. It is also worth mentioning that at present, two commercial inactivated (formalin-killed) vaccines against the RGNNV genotype, *Alpha ject micro®1Noda* (Pharmaq) and Icthiovac®*VNN* (Hipra) are available for sea bass vaccination in the Mediterranean market.

NNV inactivation has been demonstrated to be one of the most effective procedures to achieve a high degree of protection in different species including convict/seven-band grouper [327], orange-spotted grouper [225,312,328], potato grouper (*E. tukula*) [312], brown marbled grouper [329], giant grouper [330], Asian sea bass/barramundi [331] and Atlantic sea bass [289,332]. The inactivation

procedures included chemical (formalin, binary ethylenimine, BEI, and β-propiolactone) [225,289,312,327–329,331] and physical treatments (heat and UV) [289,332]. Regarding chemical treatments, Kai et al. [328] reported that the use of a BEI- inactivated vaccine resulted in greater protection (relative percent survival, RPS: 79%–95%) than a formalin-inactivated vaccine (RPS: 39%–43%). However, NNV inactivation with formalin was demonstrated to be more effective than inactivation with β-propiolactone [289]. A formalin-inactivated vaccine also provided a high level of protection in brown-marbled grouper (RPS: 86%–100%) [329]. In so far as physical treatments, although heat treatment did not elicit neutralizing antibodies against NNV [289], the UV-inactivated vaccine showed neutralizing activity and an RPS of 57.9% [332].

Another strategy for NNV vaccine development has been the use of subunit vaccines [163]. The latest studies include viral protein expression in recombinant yeast *Sacharomyces cerevissae* [333] or tobacco chloroplasts [334] as well as in *Escherichia coli* [335] and the use of a linear array epitope (LAE) technique [336]. The expression of the viral protein as virus-like particles (VLPs) has also been achieved in the yeast *Yarrowia lipolytica* [337]. The efficacy of recombinant proteins or VLPs has also been assessed in other studies [338–344] and the stability of the VLP vaccine after lyophilization has been demonstrated [345].

Protection conferred by recombinant-protein vaccines has been reported to be high, i.e., vaccinated giant grouper RPS values above 72% [336] and increasing up to more than 80% in Atlantic sea bass [340]. Recently, total protection has been claimed in vaccinated convict/seven band grouper and European sea bass [335,341].

Finally, the potential use of DNA vaccines to prevent VER outbreaks has been explored in turbot, grouper, European and Asian seabass [346–350]. The protection obtained was moderate in grouper and European sea bass (RPS: 43%–47% and 45%, respectively) [348,349], but considerably higher in Asian sea bass (RPS 77%) [350]. No protection was observed in turbot [346]. Whereas specific anti-NNV antibodies were only detected in grouper and Asian sea bass [347,349], the expression of cell-mediated cytotoxicity-related genes was observed in grouper and European sea bass [348,349], but it was not tested in Asian seabass [347].

All these vaccines have mainly been tested in larvae and juvenile fish, because these developmental stages are the most susceptible to the disease [74,163], but also in broodstock fish and the results obtained indicate that it may be a useful tool for preventing vertical transmission [312,331], as previously indicated.

The vaccine delivery methods tested include bath, intramuscular (i.m.) and intraperitoneal (i.p.) injection and oral vaccination. The bath method has mainly been used for inactivated vaccines, which have also been tested by i.m. or i.p. injection [289,328,329,331,332,349]. Injection has also been used for DNA vaccines [347,349] and subunit vaccines [335,336,338,340–342] and oral immunization strategies have been used either for inactivated, subunit or DNA vaccines [225,333–335,341,343,348,351,352]. The oral vaccine was given to the fish mixed with food [343] or by oral gavage [333,341] and encapsulated using either chemical compounds [225,348] or Artemia [353]. Furthermore, an oral inactivated vaccine has been demonstrated to confer protection in seven-band grouper after supplementation with capsaicin (a natural substance exerting an intestinal inflammatory reaction) [352].

Finally, a live vaccine has been tested in convict/seven band grouper [354]. Fish were i.m. injected with an NNV strain at $10^{4.3}$ $TCID_{50}$/ fish at 17 °C, which is not an appropriate temperature for VER development. Although 10.5% mortality was observed in the vaccinated fish, mortality recorded after the challenge with a homologous NNV strain was very low, yielding an RPS value of 95.8%. Attenuated vaccines have yet to be developed for NNV prevention, as reported for other fish viruses such as Cyprinid Herpesvirus 3 or Infectious hematopoietic necrosis virus [355,356]. However, recombinant NNV strains harboring mutations in the capsid protein and in the 3'NCR of RNA2 are promising candidates for attenuated vaccine development [39,177,221].

7. Conclusions

NNV infections are currently causing serious problems, mainly in the Mediterranean, Asian and Australian marine aquaculture industry. Over the last 30 years, the body of knowledge about NNV and VER disease has increased considerably; reliable and fast diagnostic techniques have been developed; epidemiological studies have shown the widespread distribution of NNV, especially of the RGNNV genotype, as well as the high number of susceptible and carrier fish species, which is constantly increasing. Additionally, transmission routes have been well demonstrated, including the role of invertebrates and wild fish. Finally, the considerable effort made to research prophylaxes should help to minimize fish farming losses in the short term. However, an increase in the epizootics in presently affected species is likely, as well as a greater risk of disease outbreaks in other farmed species not yet considered susceptible, because many aspects of virus biology and virulence mechanisms are not completely understood and because of global warming. Furthermore, increasing ocean temperatures may favor the number of reported VER episodes in wild species and contribute to the decline of some endangered species.

Author Contributions: I.B. & S.S. contributed equally to the preparation and writing of this revision. All authors have read and agreed to the published version of the manuscript.

Funding: Grants ED431C 2018/18 (Xunta de Galicia, Spain) and RTI2018-094687-B-C2 (Ministerio de Ciencia, Investigación y Universidades, Spain) co-funded by FEDER.

Acknowledgments: We would like to thank L. Pérez García-Estañ (Universidad Miguel Hernández, Elche, Spain) for the invitation to write this revision, C.P. Dopazo (Universidad de Santiago de Compostela) for his critical comments, which helped to improve the manuscript and J. Souto for his English corrections.

Conflicts of Interest: The authors declare no conflicts of interest.

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