CHAPTER 2.3.12.

VIRAL ENCEPHALOPATHY AND RETINOPATHY

1. Scope[1]

For the purpose of this chapter, viral encephalopathy and retinopathy (VER) otherwise known as viral nervous necrosis (VNN) is considered to be a serious disease of several marine fish species, characterised by significant losses associated to vacuolating lesions of the central nervous system and the retina.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The causative agent of VER or VNN was first identified as a new member of the family Nodaviridae following purification of brain tissues from affected striped jack larval, and the name striped jack nervous necrosis virus (SJNNV) was adopted (Mori et al., 1992). Subsequently other agents of VER/VNN were purified from some diseased fish species (Chi et al., 2001; Comps et al., 1994). The current taxonomy classifies the viruses into the genus Betanodavirus within the family Nodaviridae (Schneemann et al., 2005). Betanodaviruses are non-enveloped, spherical and approximately 25 nm in diameter. The genome consists of two molecules of positive-sense ssRNA: RNA1 (3.1 kb) encodes the replicase (110 kDa) and RNA2 (1.4 kb) encodes the coat protein (42 kDa). Complete nucleotide sequences of RNA1 and RNA2 were reported for SJNNV and others (Iwamoto et al., 2001; Iwamoto et al., 2004; Sommerset & Nerland, 2004; Tan et al., 2001). On the basis of the phylogenetic analysis of the T4 variable region of RNA1, betanodaviruses have been preliminarily clustered into four major genotypes, designated: SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type, and red-spotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa et al., 1997). The identified genotypes partially correlate with three different serotypes that have been identified by virus neutralisation with polyclonal antibodies, different host species and the in vitro optimum growth temperature (Iwamoto et al., 2000; Mori et al., 2003). Furthermore, an additional genotype including a turbot betanodavirus strain (TNV) has been proposed (Johansen et al., 2004). To avoid confusion regarding the taxonomy, a numerical nomenclature (cluster I, II, III and IV), independent from the host species origin, has been proposed by Thiéry et al., 2004. In Table 2.1., the official genotypes, the target host species and the optimal *in-vitro* growth temperature are reported (Iwamoto et al., 2000).

Table 2.1. *Betanodavirus genotypic and phenotypic variants*

Genotype	Serotype	Target host fish	Optimum growth temperature
SJNNV	A	Stripped jack	20–25°C
TPNNV	B	Tiger puffer	20°C
BFNNV	C	Cold-water fish: Atlantic halibut, Atlantic cod, flounders, etc.	15–20°C
RGNNV	C	Warm-water fish: Asian sea bass, European sea bass, groupers, etc.	25–30°C

2.1.2. Survival outside the host

Betanodaviruses are highly resistant in the aquatic environment and can survive for a long time in sea water at low temperatures (Frerichs et al., 2000) while at 25°C or higher, the survival rate is significantly affected. Contamination of the aquatic environment following the appearance of an outbreak is likely to persist during

1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2013. This disease is no longer listed by the OIE.

long periods and represent a source of infection for wild susceptible species. In frozen fish the virus may persist for long periods and it may represent a potential risk if raw fish is used for feeding (Mori et al., 2005). Outside the aquatic environment, betanodaviruses seem to lose their cytopathogenicity very easily. In drying conditions, >99% inactivation has been observed following a 7-day period at 21°C (Maltese & Bovo, 2007).

2.1.3. Stability of the agent (effective inactivation methods)

Common disinfectants such as sodium hypochlorite, iodine, hydrogen peroxide, and benzalkonium chloride are very useful for inactivating betanodaviruses while formalin shows a poor activity (Frerichs et al., 2000). Ozone has also been used to avoid or reduce virus contamination on egg shell surface (Grotmol & Totland, 2000) and virus contaminated water may be effectively sterilised by UV exposure (Frerichs et al., 2000).

2.1.4. Life cycle

The presence of reservoirs in the wild is very reasonably the original source of infection of farmed populations while the trade of infected juveniles represents the most common way to spread large amounts of virus particles in the environment. Little is known about the life cycle of betanodaviruses. Considering the results obtained from experimental infections performed by different authors, the virus most likely invades the host through the intestinal epithelium and peripheral nervous system, very soon reaching the central nervous tissues where it may induce the death of the host or remain for several years in survivors (Johansen et al., 2004). Dead decomposed fish may spread the virus in the environment reaching different biological vectors. Furthermore diseased fish may easily be cannibalised by predators who, besides the possibility of becoming infected, may spread the virus through contaminated faeces. Vertical transmission has been highly suspected in some species (Arimoto et al., 1992; Comps et al., 1994; Grotmol & Totland, 2000; Mushiake et al., 1994; Watanabe et al., 2000); in this case virus may reach developing gonads where it has been frequently detected (Dalla Valle et al., 2000; Mushiake et al., 1994; Nishizawa et al., 1996) and infect the eggs and seminal fluids (Nishizawa et al., 1994).

2.2. Host factors

2.2.1. Susceptible host species

To date, the disease has been reported in more than 50 fish species, mainly marine with the greatest impact being in striped jack, European sea bass (Dicentrarchus labrax), groupers, and flatfishes (Munday et al., 2002; Sano et al., 2011). A few outbreaks have also been documented in freshwater farms (Bovo et al., 2011; Chi et al., 2003). Fish species naturally affected by VER are listed in Table 2.2; other freshwater fish species developed clinical signs following experimental infection (Furusawa et al., 2007) suggesting the possibility of new hosts, particularly in the future when new species will be selected for aquaculture.

Table 2.2. *Fish species affected by VER/VNN*

Order	Family	Common name	Latin name
Acipenseriformes	Acipenseridae	Russian sturgeon	*Acipenser gueldenstaedti*
Anguilliformes	Anguillidae	European eel	*Anguilla anguilla*
Gonorynchiformes	Chanidae	milkfish	*Chanos chanos*
Siluriformes	Siluridae	Chinese catfish	*Parasilurus asotus*
		Australian catfish	*Tandanus tandanus*
Gadiformes	Gadidae	Atlantic cod	*Gadus morhua*
		haddock	*Melanogrammus aeglefinus*
Cyprinodontiformes	Poeciliidae	guppy	*Poecilia reticulata*
Perciformes	Acanthuridae	convict surgeonfish	*Acanthurus triostegus*
	Apogonidae	narrowstripe cardinalfish	*Apogon exostigma*
	Anarhichadidae	wolf fish	*Anarchichas minor*

Table 2.2. *Fish species affected by VER/VNN*

Order	Family	Common name	Latin name
Perciformes	Carangidae	striped jack	*Pseudocaranx dentex*
		purplish amberjack	*Seriola dumerili*
		yellow-wax pompano	*Trachinotus falcatus*
		snub nose pompano	*Trachinotus blochii*
	Centropomatidae	Asian sea bass	*Lates calcarifer*
		Japanese sea bass	*Lateolabrax japonicus*
	Cichlidae	Tilapia	*Oreochromis niloticus*
	Eleotridae	sleepy cod	*Oxyeleotris lineolata*
	Ephippidae	orbicular batfish	*Platax orbicularis*
	Latridae	striped trumpeter	*Latris lineata*
	Lutjanidae	crimson snapper	*Lutjanus erythropterus*
		mangrove red snapper	*Lutjanus argentimaculatus*
	Malacanthidae	Japanese or red tilefish	*Branchiostegus japonicus*
	Mugilidae	grey mullet	*Mugil cephalus*
		golden grey mullet	*Liza aurata*
		red mullet	*Mullus barbatus*
	Oplegnathidae	barred knifejaw or rock bream	*Oplegnathus fasciatus*
		spotted knifejaw	*Oplegnathus punctatus*
	Percichthydae	European sea bass	*Dicentrarchus labrax*
	Rachicentridae	cobia	*Rachycentron canadum*
	Sciaenidae	red drum	*Sciaenops ocellatus*
		shi drum	*Umbrina cirrosa*
		white weakfish	*Atractoscion nobilis*
	Scombridae	Pacific bluefin tuna	*Thunnus orientalis*
	Serranidae	red spotted grouper	*E. akaara*
		yellow grouper	*E. awoara*
		sevenband grouper	*E. septemfasciatus*
		blackspotted grouper	*E. fuscoguttatus*
		brown spotted grouper	*E. malabaricus*
		dusky grouper	*E. marginatus*
		kelp grouper	*E. moara*
		greasy grouper	*E. tauvina*
		dragon grouper	*E. lanceolatus*
		humpback grouper	*Chromileptes altivelis*
		white grouper	*Epinephelus aeneus*
		orange-spotted grouper	*E. coioides*

Table 2.2. *Fish species affected by VER/VNN*

Order	Family	Common name	Latin name
Perciformes	Sparidae	gilthead sea bream	*Sparus aurata*
	Cichlidae	Nile tilapia	*Oreochromis niloticus niloticus*
Tetraodontiformes	Monacantidae	thread-sale filefish	*Stephanolepis cirrhifer*
	Tetraodontidae	Japanese puffer fish	*Takifugu rubripes*
Pleuronectiformes	Pleuronectidae	barfin flounder	*Verasper moseri*
	Soleidae	common sole	*Solea solea*
	Scophthalmidae	turbot	*Psetta maxima*
		Japanese flounder	*Paralichthys olivaceus*
		Atlantic halibut	*Hippoglossus hippoglossus*
Scorpaeniformes	Sebastidae	white weakfish	*Sebastes oblongus*

For the source references, please contact the OIE Reference Laboratory.

2.2.2. Susceptible stages of the host

Although the disease mainly affects the larval and juvenile stages, serious mortalities have been also reported in market-size and adult fish, such as Atlantic halibut (*Hippoglossus hippoglossus*), sevenband grouper (*Epinephelus septemfasciatus*), and European sea bass.

2.2.3. Species or subpopulation predilection (probability of detection)

In infected farms the probability of detecting the causal agent is normally higher in juveniles than in older fish, while during spawning season the virus may be found in the gonads of broodstock (Dalla Valle et al., 2000; Mushiake et al., 1994). For this reason, surveillance programmes should include young fish as well as gonadal tissues, ovarian fluids and milt.

2.2.4. Target organs and infected tissue

Brain, spinal cord and retina are considered the target organs in which the virus actively replicates causing extensive tissue vacuolation. Intracytoplasmic inclusions have been described in the brain cells of European sea bass, Asian sea bass (*Lates calcarifer*), Japanese parrotfish (*Oplegnathus fasciatus*), and brown-spotted grouper (*Epinephelus malabaricus*) (Munday et al., 2002). The virus has been also detected in broodstock gonads (Dalla Valle et al., 2000; Mushiake et al., 1994; Nishizawa et al., 1996). In some species such as striped jack, European sea bass, barfin flounder (*Verasper moseri*), sevenband grouper and Atlantic halibut, brood fish are likely to be the most consistent virus reservoir and the most important source of infection for larvae and juvenile fish (Mushiake et al., 1994; Watanabe et al., 2000). Viruses may not replicate and reside in the reproductive organs at all times and are more likely to be found there after stressful conditions (Mushiake et al., 1994).

2.2.5. Persistent infection with lifelong carriers

In the wolfish (*Anarhichas minor*), experimentally infected by immersion, the virus has been detected in the brain at least up to 16 weeks post-exposure (Johansen et al., 2003). Following a natural outbreak in *Atlantic halibut*, the progression of the infection has been studied in survivors throughout a 1-year observation period (Johansen et al., 2002). The percentage of fish positive by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) remained high throughout the whole period and the virus was re-isolated from a subclinically infected fish at the end of the year, suggesting that fish surviving the infection may harbour the virus for a long period and may potentially transmit the infection to other fish. A cell line (BB) derived from the brain tissue of barramundi (*Lates calcarifer*) has been recently established and will offer a valid model to study viral infection and replication mechanisms both *in vivo* and *in vitro* (Chi et al., 2005).

2.2.6. Vectors

Considering that water is the most important abiotic vector, betanodaviruses can be easily spread, during a clinical outbreak, by one section of the farm to another directly through the water and by contaminating personnel, nets, boots and other equipment. Adequate biosecurity measures should be established, particularly inside the hatcheries (Mori et al., 1998). In open sea the transmission of the infection from one site to another is caused by tide, dominant currents, boats visiting different farms and wild migrating fish. Owing to the high resistance of the virus to acid conditions and at 37°C (Frerichs et al., 2000) ichthyophagous birds should be regarded as potential vectors. Furthermore because of the large volume of trade, particular attention should be addressed to molluscs originating from contaminated areas. The virus has been detected from sand worms belonging to the family Nereidae, genus Nereis collected close to an infected farm (Bovo et al., unpublished observations). The large international market existing as bite worms should be regarded as a further risk for spreading betanodaviruses from one region to another.

2.2.7. Known or suspected wild aquatic animal carriers

Though the role of wild carriers is still to be completely understood, data have been published on the detection of betanodaviruses from wild species in different regions (Barker et al., 2002; Ciulli et al., 2006; Gomez et al., 2004). It is still unclear if infected specimens should be considered just as a viral reservoir in which the pathogen may replicate without causing any mortality or if they should be considered susceptible animals. For this reason experimental infection trials are needed to better understand the significance of betanodavirus infection in wild fish.

2.3. Disease pattern

2.3.1. Transmission mechanisms

The disease may be reproduced in healthy fish by co-habitation, immersion, and injection (Munday et al., 2002). Horizontal transmission, frequently observed in the field, should be considered the most common way of disease transmission through contaminated water. Furthermore some evidence exists for vertical transmission from broodstock to offspring in striped jack, European sea bass, Asian sea bass, barfin flounder, Atlantic halibut, and sevenband grouper, as mentioned earlier (Comps et al., 1994; Mori et al., 1998; Mushiake et al., 1994; Watanabe et al., 2000). This fact is mainly reflected by the early occurrence of clinical disease and the detection of viral genetic material from mature gonads (Dalla Valle et al., 2000; Mushiake et al., 1994; Nishizawa et al., 1996); whether the virus is located inside or outside the eggs as a shell contaminant remains to be definitively proved. An additional possibility for transmission of the disease is represented by feeding broodstock with raw fish (Mori et al., 2005). There is also very strong evidence that ozonation of fertilised eggs from infected broodstock eliminates or reduces the infection rate in offspring (Mori et al., 1998), indicating vertical transmission may occur as egg-shell contamination, at least for some species.

2.3.2. Prevalence

Very few data are available on the prevalence of the disease. In Canada, certain populations of wild fish are suspected of acting as authentic natural reservoirs, in fact, the virus has been shown by PCR to be present in 0.23% of wild winter flounder (*Pleuronectes americanus*) (Barker et al., 2002). In Japan a representative sample of 30 species collected in two different bays confirmed that most farmed and wild fish tested positive (Gomez et al., 2004).

2.3.3. Geographical distribution

The disease has been officially reported from many regions. These include countries in South and East Asia (China [People's Rep. of], Chinese Taipei, India, Indonesia, Iran, Japan, Korea, Malaysia, Philippines, Thailand, Vietnam), Oceania (Australia, Tahiti), Mediterranean (France, Greece, Israel, Italy, Malta, Portugal, Spain, Tunisia), UK, Norway, Caribbean and North America (Canada, USA) (Munday et al., 2002). Furthermore suspicion of mortalities caused by betanodaviruses has been reported in wild groupers living along the Senegalese and Libyan coasts.

2.3.4. Mortality and morbidity

There are considerable variations in the age at which disease is first noted and the period over which mortality occurs (Munday et al., 2002). In general, the earlier the disease signs occur, the greater is the rate of mortality. In striped jack, mortalities are most frequently observed within 10 days after hatching while the earliest occurrence of disease is at day 2 post-hatching, resulting in almost complete loss of the larvae. In European

sea bass mortality is usually not seen until about 30 days post-hatching but outbreaks may occur even in market-size fish. The mortality rate is age-dependent. When larval stages are affected highest mortality, often reaching 100%, are observed while in juveniles and older fish lower losses have been generally reported.

2.3.5. Environmental factors

Water temperature is an important factor and the appearance of clinical signs can be significantly influenced by temperature.The effect of temperature is particularly well known in sea bass farming and the disease was preliminary identified as *Summer Disease*. The correlation between appearance of clinical signs and water temperature is partly supported by *in-vitro* studies (Iwamoto et al., 2000). Host specificity seems to exist in the RGNNV genotype to warm-water fish and in the BFNNV genotype to cold-water fish. In striped jack larvae infected by SJNNV (*in-vitro* optimum growth temperature: 20–25°C) no difference in mortality was noticed among different groups reared at different water temperatures ranging from 20°C to 26°C, while in seven-band grouper infected with RGNNV (*in-vitro* optimum growth temperature: 25–30°C) rearing water temperature (16–28°C) may influence the development of the disease. Higher mortality and earlier appearance of the disease were observed at higher temperatures, while water temperatures higher than 31°C inhibited the proliferation of RGNNV in humpback grouper (*Chromileptes altivelis*) (Yuasa et al., 2007). On the other hand, the infection of AHNV (BFNNV genotype, optimum: 15–20°C) in Atlantic halibut occurs at 6°C. Salinity does not seem to have any influence on the occurrence of the disease as outbreaks have been reported in freshwater species.

2.4. Control and prevention

Prevention of the disease may only be obtained avoiding the exposure of farmed population to the causative agents. Unfortunately the method is very difficult to apply in on-growing facilities while may be very useful in hatcheries provided they are using virus-free water and introducing juveniles obtained from virus-free broodstock.

2.4.1. Vaccination

Different studies have shown that immunisation using recombinant viral coat protein expressed in *Escherichia coli* or virus-like particles expressed in a baculovirus expression system or formalin-inactivated virus may be effective in controlling the disease (Tanaka et al., 2001; Thiéry et al., 2006; Yamashita et al., 2005). Recently, an inactivated RGNNV vaccine against VER of seven-band grouper was commercialised in Japan. One study showed that primary infection with an avirulent aquabirnavirus effectively suppressed secondary betanodavirus infection, suggesting the use of the aquabirnavirus as a potential immunomodulator (Yamashita et al., 2009).

2.4.2. Chemotherapy

No chemotherapy is available.

2.4.3. Immunostimulation

No data available.

2.4.4. Resistance breeding

No data available.

2.4.5. Restocking with resistant species

No data exist concerning the selection of resistant lineages within susceptible species.

2.4.6. Blocking agents

No data available.

2.4.7. Disinfection of eggs and larvae

Washing fertilised eggs in ozone-treated sea water or treatment of rearing water with ozone or chlorination seems to be effective in the control of the disease in larval production of striped jack, seven-band grouper, barfin flounder, and Atlantic halibut (Arimoto et al., 1992; Grotmol & Totland, 2000; Mori et al., 1998; Watanabe et al., 2000).

2.4.8. General husbandry practices

In addition to the general hygiene practices, such the UV treatment of water entering the hatchery, the adoption of sanitary barriers, regular fallowing and disinfection of tanks and biological filters, disinfection of facilities, utensils, avoidance of raw fish for feeding, it is important to reduce stress factors by improving the spawning-induction method, which includes providing adequate food for broodstock and decreasing the stocking density of larvae and juveniles (Mushiake et al., 1994). To avoid vertical transmission in hatcheries it has been proposed to test each brood fish by PCR methods carried out on gonadal biopsies and discard all positive specimens (Mori et al., 1998; Mushiake et al., 1994; Nishizawa et al., 1994); nevertheless on some occasions failure of the PCR method to detect the virus infection in selected spawners have been reported (Nishizawa et al., 1996).

An integrated control strategy, including the use of ELISA to test the level of specific antibody activity in each single brood fish, PCR carried out on sexual products and disinfection of embryonated eggs with ozonated water has been also proposed to control VER in barfin flounder (Watanabe et al., 2000).

Unfortunately the detection of specific antibodies by ELISA or neutralisation tests has not been sufficiently investigated and very little is known about the interpretation of serological results.

In the sea bass industry, it has been suggested that restocking of on-growing facilities located in infected areas should be performed during autumn when the number of clinical outbreaks is decreasing.

3. Sampling

3.1. Selection of individual specimens

Fish showing abnormal swimming behaviour, associated with loss of appetite and a progressive change in pigmentation, should be regarded as potentially infected and those specimens, in addition to moribund and freshly dead specimens, should be sampled for diagnostic purposes to confirm the suspicion. Whole fish are normally sent to the laboratory except in the case of very large specimen for which only head may be provided. In surveillance programmes the sampling of apparently healthy fish according to significant statistical sampling must be adopted.

3.2. Preservation of samples for submission

Waiting for submission to the laboratory, samples may be safely maintained at 4°C (2–3 days) or frozen at –20°C or –80°C (2–3 weeks).

3.3. Pooling of samples

Collective samples, representing 5–10 fish with clinical signs, may be accepted for diagnostic purposes. When looking for potential carriers single fish should be tested.

3.4. Best organs or tissues

Brain and eyes are the target tissues for diagnostic purposes. When larval or very young stages (<1 cm) are suspected the whole body may be processed. When fish length is between 1 and 6 cm the whole head including brain and eyes should be separated from the body and included in the sample. For larger fish only the brain and eyes should be collected.

Other organs than brain and eyes should be considered not suitable for diagnostic purposes. Nevertheless when brood fish has to be tested by non-invasive methods, eggs, ovary and seminal fluids and gonadal biopsies should be regarded as suitable samples, although only positive results should be viewed as conclusive.

3.5. Samples/tissues that are not suitable

Kidney, spleen, and heart, which are normally recommended for detection of several fish viral agents are not suitable for diagnosis of VER or VNN.

4. Diagnostic methods

For several years the 'Gold Standard' method to detect VER or VNN was isolation of viral agents in cell culture followed by immunological or molecular identification. Now several molecular tools characterised by high sensitivity have been

described but their definitive use needs further validation through inter-laboratory proficiency tests, or by equivalence testing with the Gold Standard.

4.1. Field diagnostic methods

4.1.1. Clinical signs

There are no external signs on the body surface and gills of infected fish except a progressive change in pigmentation described in a few species by different authors. In European sea bass cutaneous erosions in the mandibular and cranial areas, possibly due to traumatic origin caused by visual disturbance, have been occasionally reported.

4.1.2. Behavioural changes

Infected fish show a variety of erratic swimming behaviour patterns, such as spiralling, whirling or belly-up at rest (sometimes with inflation of the swim bladder), or lying down at the tank bottom or swim rapidly in circles or straight-ahead. Flatfish usually show less evident signs. Subjects may remain at length on the bottom bending their body with the head and tail raised. Loss of appetite has been frequently reported.

4.2. Clinical methods

4.2.1. Gross pathology

No macroscopic lesions have been associated to the infection except hyperinflation of the swim bladder, which has been frequently observed in different species, particularly during larval stages.

4.2.2. Clinical chemistry

No data applicable.

4.2.3. Microscopic pathology

The most common microscopical findings detected in different species consist of vacuolation and necrosis of nervous cells of the spinal cord, brain and/or retina. These lesions are by far more prominent in larvae and juveniles while in older symptomatic fish they are sometimes very rare and difficult to detect. The inflammatory process is usually very discreet and the presence of macrophages is possibly due to secondary infection.

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

Not applicable.

4.2.7. Electron microscopy/cytopathology

Subspherical viral particles, about 25 nm in diameter, can be visualised in brain, spinal cord and retina of heavily infected animals. The virions may appear either free in the cytoplasm or associated to the endoplasmic reticulum membranes, primarily in nerve cells astrocytes, oligodendrocytes, and microgliocytes. Nevertheless because of the limited analytical sensitivity the method is not a reliable diagnostic tool.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Reverse-transcription PCR (RT-PCR) is the most rapid and convenient method for diagnosing clinically infected fish while nested PCR or real-time PCR are useful tools for diagnosing subclinically infected fish, as carrier fish. In general, PCR-based methods applied to the diagnosis of viral diseases have as advantages

short processing time, rapidity of reporting and high sensitivity and specificity, and are therefore suitable tools for the rapid detection of betanodavirus in both clinically and subclinically infected fish. Nevertheless these methods need further validation through inter-laboratory proficiency tests. For this reason cell culture isolation followed by immunostaining or molecular identification still represents the 'Gold Standard' for diagnostic purposes.

Furthermore the virus has been identified by immunofluorescence (IF) performed on brain imprints or frozen sections and by immunohistochemistry (IHC) on brain and retina. IHC is the most appropriate test for the detection of virus in material fixed for histological analysis.

ELISA-based methods detecting betanodavirus antigens from target tissues have been published (Arimoto et al., 1992).

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

No data.

4.3.1.1.2. Smears

Brain imprints stained according to classical IF test have been reported as a suitable method for detection of betanodavirus infection. Because of its unknown sensitivity the method is recommended only in clinically affected fish.

4.3.1.1.2.1. Indirect fluorescent antibody test on brain imprints

This protocol has been advantageously applied for long time in the OIE Reference Laboratory; nevertheless the procedure has not been properly validated in terms of sensitivity and reproducibility. The method is recommended only in clinically affected fish.

i) Collect the brain from clinically affected fish (at least three specimens).

ii) Use blotting paper to remove any liquid excess.

iii) Make light imprints on the slide surface (five from each of the three specimens).

iv) Allow the imprints to air-dry.

v) Fix the imprints in absolute ethanol or cold acetone (–20°C) for 10 minutes.

vi) Rinse three times with 0.05% PBS/Tween 80 (PBST).

vii) Cover the imprints with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) and incubate for 30 minutes at 37°C in a humid chamber.

viii) Rinse three times with 0.05% PBST.

ix) Cover the imprints with a commercially available fluorescein isothiocyanate-conjugated secondary antibody (i.e. anti-rabbit Ig antibody) and incubate at 37°C for 30 minutes.

x) Rinse three times with PBST.

xi) Mount with a coverslip using glycerol saline, pH 8.5.

xii) Observe under IF microscope at 100–250×.

4.3.1.1.2.1.1. Interpretation of the results

4.3.1.1.2.1.1.1. Positive samples

Fluorescent cells containing green brilliant granules with a stardust appearance are easily detectable.

4.3.1.1.2.1.1.2. Negative samples

No fluorescent signal should be present.

4.3.1.1.3. Fixed sections

Specific detection of the causative agent may be achieved by indirect fluorescent antibody test (IFA) carried out on paraffin embedded or cryostatic tissues or by IHC carried out on paraffin embedded tissues

as reported from different authors (Johansen et al., 2003; Nguyen et al., 1997) using polyclonal or monoclonal antibodies.

4.3.1.1.3.1. Indirect fluorescent antibody test on paraffin sections

The following protocol is reported as an example but other IFA validated protocols may be used.

i) Cut 5 µm-thick- sections and transfer to polylisine-coated slides.

ii) Dry section overnight at 37°C.

iii) Deparaffinise with xylene 2 × 10 minutes with absolute ethanol 2 × 5 minutes.

iv) Rehydrate sections: 95°, 70°, 50° and distilled water.

v) Rinse with PBST.

vi) Cover the sections with 0.1% trypsin in PBS and incubate at 37°C for 30 minutes.

vii) Rinse three times with PBST.

viii) Cover the sections with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) for 30 minutes at 37°C in a humid chamber.

ix) Rinse three times with PBST.

x) Cover the sections for 30 minutes at 37°C with a commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody).

xi) Rinse three times with PBST.

xii) Mount with coverslip using glycerol saline, pH 8.5.

xiii) Observe under IF microscope and compare with positive and negative control.

4.3.1.1.3.1.1. Interpretation of the results

4.3.1.1.3.1.1.1. Positive samples

Specific brilliant fluorescence is observed in the cytoplasm of affected cells in brain and retina.

4.3.1.1.3.1.1.2. Negative samples

No fluorescent signal should be present in negative control.

4.3.1.1.3.2. Indirect fluorescent antibody test on cryostatic sections

The following protocol is reported as an example but other IFA validated protocols may be used. Because of the limited sensitivity the method is applicable only to clinically affected fish.

i) Collect brains or eyes from 2–3 clinically affected fish.

ii) Place the organs together in the same cryostatic sample support.

iii) Move the support to the cryostatic chamber at –20°C.

iv) When completely frozen, cut 5 µm-tick sections and transfer them to polylisine-coated slides.

v) Allow the sections to air-dry.

vi) Fix the sections with absolute ethanol or cold acetone (–20°C).

vii) Cover the sections with the primary antibody (i.e. rabbit anti-betanodavirus serum) for 30 minutes at 37°C in a humid chamber.

viii) Rinse three times with PBST.

ix) Cover the sections for 30 minutes at 37°C with a commercially available fluorescein isothiocyanate (i.e. conjugated anti-rabbit Ig antibody).

x) Rinse as before with PBST.

xi) Mount with a coverslip using glycerol saline, pH 8.5.

xii) Observe under IF microscope and compare with negative and positive controls.

4.3.1.1.3.2.1. Interpretation of results

4.3.1.1.3.2.1.1. Positive samples

Specific fluorescence is observed in the cytoplasm of the affected cells in brain or retina.

4.3.1.1.3.2.1.2. Negative samples

No fluorescent signal should be present in negative control.

4.3.1.1.3.3. Immunohistochemistry (avidin-biotin-peroxidase technique)

This protocol has been advantageously applied for a long time in the OIE Reference Laboratory and is given as an example; different IHC validated protocols may be adopted.

i) Dewax sections with xylene (2 × 10 minutes) and dehydrate with ethanol (2 × 5 minutes).

ii) Incubate in 3% H_2O_2 for 20 minutes at room temperature (RT), to block endogenous peroxidase.

iii) Hydrate tissue sections in a decreasing alcohol series 95°, 70°, 50°, distilled water (1 minute).

iv) Rinse with PBS (pH 7.3) at 37°C.

v) Incubate sections with 0.1% trypsin and 0.1% $CaCl_2$ + Tris buffer, for 30 minutes at 37°C.

vi) Rinse three times with PBS.

vii) Incubate sections with 5% BSA in PBS for 20 minutes at RT.

viii) Tap off BSA and wipe away the excess.

ix) Incubate sections with the primary antibody (i.e. rabbit anti-betanodavirus) diluted in 2.5% BSA for 60 minutes.

x) Rinse three times in Tris-buffer.

xi) Incubate with the secondary biotinilated serum (i.e. goat anti-rabbit immunoglobulins in 2.5% BSA for 20 minutes.

xii) Rinse three times in Tris-buffer.

xiii) Incubate with ABComplex/HRP (prepared just before use) for 20 minutes at RT.

xiv) Tap off ABComplex/HRP.

xv) Rinse three times with Tris-buffer.

xvi) Incubate with chromogen substrate AEC (3-amin-9-ethylcarbazole; prepared just before use) for 20 minutes at RT.

xvii) Rinse with distilled water for 5 minutes.

xviii) Counterstain with Harris haematoxylin for 30 seconds.

xix) Mount sections in glycerol gelatin.

xx) All immunohistochemical runs should include one positive and one negative control section.

4.3.1.1.3.3.1. Interpretation of results

4.3.1.1.3.3.1.1. Positive samples

Granular red deposits are detectable in tissues. Pale diffuse red stain of tissues is not considered as specific immunolabelling (background).

4.3.1.1.3.3.1.2. Negative samples

No immunolabelling detected.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture

The isolation of betanodaviruses in a small number of established fish cell lines is well documented. Two fish cell lines for isolating and propagating betanodaviruses are available for diagnostic and research purposes through the European Collection of Cell Cultures (ECACC): the SSN-1 cell line derived from

striped snakehead (Frerichs et al., 1996) and a cloned cell line (E-11) derived from SSN-1 itself (Iwamoto et al., 2000). Both these cells are useful for qualitative and quantitative analyses of all betanodaviruses. Further susceptible cell cultures (GF-1) have been developed and described (Chi et al., 1999) and may be used for research and diagnostic purposes provided sensitivity is regularly monitored. To verify the sensitivity of the cell cultures in use, titration of frozen reference virus must be performed at least every 6 months or whenever decreased cell susceptibility is suspected.

4.3.1.2.1.1. Inoculation of cell monolayers

For details on transportation, antibiotic treatment and virus extraction refers to the General Information chapter.

i) Homogenise samples with 1:5–1:10 volumes of Hanks' balanced salt solution (HBSS) or other equivalent medium, containing antibiotics to avoid bacterial contaminations.

NOTE: Gentamycin (1000 µg/ml) or penicillin (800 International Units [IU]/ml) and streptomycin (800 µg/ml) are suggested but different effective antibiotics may be used. The antifungal compounds Mycostatin or Fungizone may also be incorporated into the medium at a final concentration of 400 IU/ml. In addition 5% serum or albumen may be added.

The antibiotic treatment may be performed for 4 hours at 15°C or overnight at 4°C. The alternative of filtration through 0.22 µm membrane filter may be used instead.

ii) Inoculate the antibiotic treated tissue suspension at two different dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in a final dilutions of tissue material in cell culture medium of 1:50–100 and 1:500–1000, respectively.

NOTE: Each of 100 µl dilution should be inoculated into at least 2 cm^2 actively replicating cell cultures monolayers. Both the normal or the adsorption method may be used.

iii) In case the adsorption method is adopted, allow the inoculum to adsorb on the drained monolayers, for 1 hour at 20–25°C. After the adsorption period add the new medium supplemented with 5% FBS.

iv) If the normal method is adopted, the culture medium may be changed with a new one supplemented with 5% FBS, before to add the antibiotic treated suspension.

v) Incubate at 20–25°C according to the genotype expected.

NOTE: Optimal viral growth temperatures are different among the four genotypic variants: 25–30°C for RGNNV genotype, 20–25°C for SJNNV genotype, 20°C for TPNNV genotype, and 15–20°C for BFNNV genotype (Table 2.1.). For this reason the incubation temperature should be selected according to the genotypes present in the sampled area.

4.3.1.2.1.2. Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by regular microscopic examination at ×40–100 magnification for 10 days.

ii) If cytopathic effect (CPE) appears, identification procedures must be undertaken (see below).

NOTE: CPE in SSN-1 or E-11 cells is characterised by thin or rounded, refractile, granular cells with vacuoles, and partial or complete disintegration of the monolayer.

iii) If no CPE occurs after the primary incubation period (10 days), subcultivation must be performed on fresh cultures, using a similar cell growing area to that of the primary culture.

4.3.1.2.1.3. Subcultivation procedures

i) Collect aliquots (10%) of cell culture medium from all monolayers inoculated.

ii) Inoculate those aliquots constituting the primary culture into wells with the new cell monolayers, as described above (well-to-well subcultivation).

iii) Incubate and monitor as described above for further 10 days.

iv) If no CPE occurs during this period the test may be considered negative.

v) If CPE appears identification procedures must be undertaken (see below).

4.3.1.2.2. Antibody-based antigen detection methods

4.3.1.2.2.1. Indirect fluorescent antibody test

i) Prepare monolayers of susceptible cells directly in 2 cm^2 wells of cell culture plastic plates or on cover-slips or chamber slides in order to achieve around 70–80% confluency, which is usually reached within 24 hours of incubation at 25°C.

ii) Inoculate the virus suspensions to be identified using at least two tenfold dilutions.

iii) Incubate at 20°C or 25°C for 48–72 hours (See Section 4.3.1.2.1Cell culture).

iv) Remove the culture medium and fix with absolute ethanol or 80% cold acetone (–20°C) for 10–30 minutes.

v) Rinse three times with PBST.

vi) Allow the cell monolayers to air-dry.

vii) Treat the cell monolayers with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) for 30 minutes at 37°C in a humid chamber.

viii) Rinse three times with PBST.

ix) Cover the cell monolayers for 30 minutes at 37°C with commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody).

x) Rinse with PBST.

Examine the treated cell monolayers directly on plates, or mount the cover-slips using glycerol saline, pH 8.5, prior to microscopic observation.

4.3.1.2.2.1.1. Interpretation of the results

4.3.1.2.2.1.1.1. Positive samples

Brilliant fluorescent cells scattered on the monolayer.

4.3.1.2.2.1.1.2. Negative samples

No fluorescent signal should be detected.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Conventional PCR

The increasing number of available sequence data allowed the development of PCR-based diagnostic methods for betanodavirus detection, which can be optimised according to individual specificity needs. The F2-R3 primer set, targeting the T4 variable region of the RNA2 segment (Nishizawa et al., 1994), has been extensively used in several studies for both diagnostic and research purposes. However, these primers failed on some occasions because of sensitivity and specificity limits. Nishizawa et al., 1996observed that a very small amount of virus in spawners can escape PCR detection, yielding false negative results. On the other hand, Thiéry et al., 1999areported that the F2-R3 primer set was not capable of recognising the genome of a betanodavirus strain of Atlantic origin because of the genetic diversity of this virus. In agreement with this observation, Grotmol et al., 2000hypothesised that sequence variation among betanodaviruses might result in mismatches between oligonucleotides and their target region, therefore impairing the diagnostic power of the PCR. Since the publication of the F2-R3 primer set, several other PCR-based protocols have been developed to increase sensitivity and specificity of betanodavirus diagnostics, and some are reported in Table 4.1.Assays designed by Dalla Valle et al., 2000and Thiéry et al., 1999bwere tested on betanodavirus isolates of Mediterranean origin, while the protocol developed by Grotmol et al., 2000is suitable for detecting cold water strains.

Table 4.1. *Primer sets used for betanodavirus detection by conventional PCR*

Primer	Target	Sequence 5'– 3'	Amplicon size (bp)	Reference
VNNV1	RNA2	ACA-CTG-GAG-TTT-GAA-ATT-CA	605	Dalla Valle et al., 2000
VNNV2		GTC-TTG-TTG-AAG-TTG-TCC-CA		
VNNV3		ATT-GTG-CCC-CGC-AAA-CAC	255	
VNNV4		GAC-ACG-TTG-ACC-ACA-TCA-GT		
AH95-F1	RNA2	AGT-GCT-GTG-TCG-CTG-GAG-TG	341	Grotmol et al., 2000
AH95-R1		CGC-CCT-GTG-TGA-ATG-TTT-TG		
F2	RNA2	CGT-GTC-AGT-CAT-GTG-TCG-CT	about 430	Nishizawa et al., 1994
R3		CGA-GTC-AAC-ACG-GGT-GAA-GA		
F2	RNA2	CGT-GTC-AGT-CAT-GTG-TCG-CT	420	Thiéry et al., 1999b
R3		CGA-GTC-AAC-ACG-GGT-GAA-GA		
F'2		GTT-CCC-TGT-ACA-ACG-ATT-CC	294	
R'3		GGA-TTT-GAC-GGG-GCT-GCT-CA		

4.3.1.2.3.2. Real-time PCR

Generally speaking, real-time PCR methods can improve analytical performance in comparison with conventional PCR assays, providing reliable results with reduced sample handling. Grove et al., 2006developed and validated a real-time RT-PCR method for specifically detecting the Atlantic halibut nervous necrosis virus. The assay appeared to be optimal for the recognition of cold-water viruses but ineffective in targeting warm-water strains (e.g. RGNNV). Dalla Valle et al., 2005standardised a sensitive SYBR Green-based real-time PCR for fish nodaviruses based on two molecular targets (RNA1 and RNA2). The method was capable of detecting the four known genotypes and has been partially validated by using a RGNNV-type strain. Although the use of nonspecific double-stranded DNA dyes results in a considerable improvement of test sensitivity, melting analysis occasionally yields dubious results. It is known that probe-based chemistry is faster and provides higher specificity. Hick & Whittington, 2010optimised a TaqMan-based RT-qPCR (qR2T assay) for betanodavirus detection, which was extensively validated. Analytical and diagnostic sensitivity, repeatability and reproducibility were assessed. Assay specificity was also determined, but the protocol has not been tested on cold-water viral strains. To date, the TaqMan-based method developed by the OIE Reference Laboratory for VER or VNN, validated according to OIE Standards, appears to be suitable for detecting the four established genotypes as well as the Atlantic cod (*Gadus morhua*) and Atlantic halibut betanodaviruses (Panzarin et al., 2010). All primers and probe sets related to the cited literature are reported in Table 4.1.Below is the detailed protocol by Panzarin et al., 2010although other methods may be used.

4.3.1.2.3.2.1. RNA purification

Total RNA can be extracted from tissue homogenates diluted it 1:5 in Eagle minimum essential medium (the same homogenate can be used for virus isolation in cell cultures; for sample preparation see above) or infected cell monolayers, by using the NucleoSpin RNAII (Macherey-Nagel GmbH&Co.[2]) according to the manufacturer's recommendations. Alternative RNA isolation kits of proven performance can be used.

4.3.1.2.3.2.2. Reverse-transcription (RT) and cDNA synthesis

i) Prepare a reaction mix for the number of samples to be analysed. The 30 µl master mix for one reaction is made as follows (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems): 6.3 µl PCR-grade water; 3 µl 10× RT buffer; 1.2 µl 25× dNTP mix (100 mM); 3 µl 10× RT random primers; 1.5 µl MultiScribe™ reverse transcriptase (50 U/µl); 15 µl purified RNA.

2 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

ii) Place the tubes in the thermal cycler and apply the following conditions: 10 minutes pre-incubation at 25°C, 120 minutes reverse transcription at 37°C.

iii) Keep cDNA at –20°C until use.

4.3.1.2.3.2.3. Real-time TaqMan PCR

i) Prepare a reaction mix for the number of samples to be analysed. The 20 µl master mix for one reaction is MADE as follows (LightCycler® TaqMan® Master, Roche Diagnostics GmbH): 7.7 µl PCR-grade water; 0.9 µl primer RNA2 FOR (20 µM); 0.9 µl primer RNA2 REV (20 µM); 1.5 µl RNA2 probe (10 µM); 4 µl 5× Master Mix; 5 µl cDNA.

ii) Place the samples in the real-time platform and start the following thermal profile: 10-minutes incubation at 95°C followed by 45 cycles of 10 seconds denaturation at 95°C, 35 seconds annealing at 58°C and 1 second elongation at 72°C. Cycling conditions refer to the LightCycler 2.0 platform.

iii) Analyse data with the instrument-related software. The diagnostic cutoff limit is set at 36 CP (crossing point). In case of doubtful results (e.g. CP ≥ 36), repeat the analysis.

NOTE: Assay performances can vary depending on the conditions under which the protocol is carried out (for example, the thermal profile might need optimisation, depending on the platform in use). Of note, it is highly recommended to perform the protocol by Panzarin et al., 2010in two-steps, otherwise test sensitivity might be dramatically affected.

Table 4.2. *Primers/probe sets used for betanodavirus detection by Real-time PCR*

Primer	Target	Sequence 5' – 3'	Amplicon size (bp)	Reference
Q-RdRP-1	RNA1	GTG-TCC-GGA-GAG-GTT-AAG-GAT-G	273	Dalla Valle et al., 2005
Q-RdRP-2		CTT-GAA-TTG-ATC-AAC-GGT-GAA-CA		
Q-CP-1	RNA2	CAA-CTG-ACA-ACG-ATC-ACA-CCT-TC	230	
Q-CP-2		CAA-TCG-AAC-ACT-CCA-GCG-ACA		
P1	RNA2	GGT-ATG-TCG-AGA-ATC-GCC-C	194	Grove et al., 2006
P2		TAA-CCA-CCG-CCC-GTG-TT		
Probe		TTA-TCC-CAG-CTG-GCA-CCG-GC*		
qR2TF	RNA2	CTT-CCT-GCC-TGA-TCC-AAC-TG	93	Hick & Whittington, 2010
qR2TR		GTT-CTG-CTT-TCC-CAC-CAT-TTG		
R2probe2		CAA-CGA-CTG-CAC-CAC-GAG-TTG*		
RNA2 FOR	RNA2	CAA-CTG-ACA-RCG-AHC-ACA-C	69	Panzarin et al., 2010
RNA2 REV		CCC-ACC-AYT-TGG-CVA-C		
RNA2 probe		TYC-ARG-CRA-CTC-GTG-GTG-CVG*		

* Reporter dye, FAM; quencher, BHQ1

NOTE: with the exception of the above method, none of the protocols cited in paragraphs 4.3.1.2.3.1and 4.3.1.2.3.2has been officially validated through documented inter-laboratory proficiency tests. The protocol by Panzarin et al., 2012has been subjected to a ring trial involving five European laboratories.

4.3.1.2.3.3. Sequencing

Genome sequencing, besides being valuable for diagnosis confirmation, is also fundamental for epidemiological studies. Sequence analysis of the T4 variable region (Nishizawa et al., 1997) allows the correct assignment of genotype. However, the phylogenetic analysis based on the sequence of both genomic segments, appears to be more informative, and it is recommended in order to highlight possible reassortment events occurring between different betanodavirus genotypes and within genotype (Olveira et al., 2009; Panzarin et al., 2012; Toffolo et al., 2007).

4.3.1.2.4. Agent purification

Betanodaviruses may be easily purified by ultracentrifugation through cesium chloride gradients (Chi et al., 2001; Comps et al., 1994; Mori et al., 1992).

4.3.2. Serological methods

As there has been insufficient research, detection of specific antibodies has not been so far considered as a routine screening method for assessing the viral status of fish populations. Nevertheless evidence of specific antibodies has been reported by different authors. According to field observations an ELISA titre ≥ 1:40 is indicative of viral infection while values ≤ 1:10 is indicative of freedom from viral infection (Watanabe et al., 2000).

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of VER/VNN are listed in Table 5.1.The designations used in the table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. n.a = not applicable. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Betanodavirus surveillance, detection and diagnostic methods*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Larvae	Juveniles	Adults		
Histopathology	d	d	d	b	d
Histopathology followed by immnuostaining	d	d	d	b	d
Transmission EM	d	d	d	c	d
Isolation in cell culture followed by immunostaining or PCR	a	a	d	a	a
RT-PCR	b	b	b	a	a
RT-PCR followed by sequencing	d	d	d	b	a
Real-time PCR	a	a	a	a	a

EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from viral encephalopathy and retinopathy

Targeted surveillance should rely on regular monitoring of farms rearing susceptible species. If available larval stages and juveniles should preferably be sampled during the most suitable season taking in account the optimum temperature of the genotypes expected.

NOTE: At present no official or recommended procedures to test healthy populations have been issued to demonstrate freedom from the disease. Real-time PCR followed by virus isolation or conventional RT-PCR and sequence analysis in case of positive results should be considered the most suitable method for targeted betanodaviruses surveillance.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

VER or VNN shall be suspected if at least one of the following criteria is met:

i) Appearance of abnormal swimming behaviour in susceptible species;

ii) Typical histopathological lesions detected in a population of susceptible species;

iii) Typical CPE observed in cell cultures without identification of the causative agent;

iv) A single positive result from one of the diagnostic assays ranked as 'a' or 'b' in the *Presumptive diagnosis* column of Table 5.1.;

v) Transfer of live fish from an infected farm to another site;

vi) Existing of different epidemiological links between one infected farm and a second farm;

vii) Detection of specific antibody activity.

7.2. Definition of confirmed case

Any combination of at least two of the following two methods (with positive results):

i) A suspect case which has produced a typical CPE in cell culture with subsequent identification of the causative agent by either an antibody-based or molecular test.

ii) A second positive result from a different diagnostic assay ranked as 'a' in *Confirmatory diagnosis* column of Table 5.1.(except the combination of PCR + real-time PCR).

8. References

ARIMOTO M., MUSHIAKE K., MIZUTA Y., NAKAI T., MUROGA K. & FURUSAWA I. (1992). Detection of striped jack nervous necrosis virus (SJNNV) by enzyme-linked immunosorbent assay (ELISA). *Fish Pathol.*, **27**, 191–195.

BARKER D.E., MACKINNON A.M., BOSTON L., BURT M.D.B., CONE D.K., SPEARE D.J., GRIFFITHS S., COOK M., RITCHIE R. & OLIVIER G. (2002). First report of piscine nodavirus infecting wild winter flounder *Pleuronectes americanus* in Passamaquoddy Bay, New Brunswick, Canada. *Dis. Aquat. Org.*, **49**, 99–105.

BOVO G., GUSTINELLI A., QUAGLIO F., GOBBO F., PANZARIN V., FUSARO A., MUTINELLI F., CAFFARA M. & FIORAVANTI M.L. (2011). Viral encephalopathy and retinopathy outbreak in freshwater fish farmed in Italy. *Dis. Aquat. Org.*, **96**, 45–54.

CHI S.C., HU W.W. & LO B.L. (1999). Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV). *J. Fish Dis.*, **22**, 173–182.

CHI S.C., LO C.F. & LIN S.C. (2001). Characterization of grouper nervous necrosis virus. *J. Fish Dis.*, **24**, 3–13.

CHI S.C., SHIEH J.R. & LIN S.J. (2003). Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Dis. Aquat. Org.*, **55**, 221–228.

CHI S.C., WU Y.C. & CHENG T.M. (2005). Persistent infection of betanodavirus in a novel cell line derived from the brain tissue of barramundi *Lates calcarifer*. *Dis. Aquat. Org.*, **65**, 91–98.

CIULLI S., DI MARCO P., NATALE A., GALLETTI E., BATTIMANI M., SCAGLIARINI A., PUR PARI G., CANNELLA V., CASTIGLIONE F. & GUERCIO A. (2006). Detection and characterization of Betanodavirus in wild fish from Sicily, Italy. *Ittiopatologia*, **3**, 101–112.

COMPS M., PEPIN J.F. & BONAMI J.R. (1994). Purification and characterization of two fish encephalitis viruses (FEV) infecting *Lates calcarifer* and *Dicentrarchus labrax*. *Aquaculture*, **123**, 1–10.

DALLA VALLE L., TOFFOLO V., LAMPRECHT M., MALTESE C., BOVO G., BELVEDERE P. & COLOMBO L. (2005). Development of a sensitive and quantitative diagnostic assay for fish nervous necrosis virus based on two-target real-time PCR. *Vet. Microbiol.*, **110**, 167–179.

DALLA VALLE L., ZANELLA L., PATARNELLO P., PAOLUCCI L., BELVEDERE P. & COLOMBO L. (2000). Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. *J. Fish Dis.*, **23**, 321–327.

FRERICHS G.N., RODGER H.D. & PERIC Z. (1996). Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. *J. Gen. Virol.*, **77**, 2067–2071.

FRERICHS G.N., TWEEDIE A., STARKEY W.G. & RICHARDS R.H. (2000). Temperature, pH, and electrolyte sensitivity, and heat, UV and disinfectant inactivation of sea bass (*Dicentrarchus labrax*) neuropathy nodavirus. *Aquaculture*, **185**, 13–24.

FURUSAWA R., OKINAKA Y., UEMATSU K. & NAKAI T. (2007). Screening of freshwater fish species for their susceptibility to a betanodavirus. *Dis. Aquat. Org.*, **77**, 119–125.

GOMEZ D.K., SATO J., MUSHIAKE K., ISSHIKI T., OKINAKA Y. & NAKAI T. (2004). PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *J. Fish Dis.*, **27**, 603–608.

GROTMOL S., NERLAND A.H., BIERING E., TOTLAND G.K. & NISHIZAWA T. (2000). Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and design an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. *Dis. Aquat. Org.*, **39**, 79–88.

GROTMOL S. & TOTLAND G.K. (2000). Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. *Dis. Aquat. Org.*, **39**, 89–96.

GROVE S., FALLER R., SOLEIM K.B. & DANNEVIG B.H. (2006). Absolute quantitation of RNA by a competitive real-time RT-PCR method using piscine nodavirus as a model. *J. Virol. Methods*, **132**, 104–112.

HICK P. & WHITTINGTON R.J. (2010). Optimisation and validation of a real-time reverse transcriptase-polymerase chain reaction assay for detection of betanodavirus. *J. Virol. Methods*, **163**, 368–377.

IWAMOTO T., NAKAI T., MORI K., ARIMOTO M. & FURUSAWA I. (2000). Cloning of the fish cell line SSN-1 for piscine nodaviruses. *Dis. Aquat. Org.*, **43**, 81–89.

IWAMOTO T., MISE K., MORI K., ARIMOTO M., NAKAI T. & OKUNO T. (2001). Establishment of an infectious RNA transcription system for striped jack nervous necrosis virus, the type species of the betanodavirus. *J. Gen. Virol.*, **82**, 2653–2662.

IWAMOTO T., OKINAKA Y., MISE K., MORI K., ARIMOTO K., OKUNO T. & NAKAI T. (2004). Identification of host-specificity determinants in betanodaviruses by using reassortants between striped jack nervous necrosis virus and sevenband grouper nervous necrosis virus. *J. Virol.*, **78**, 1256–1262.

JOHANSEN R., RANHEIM T., HANSEN M.K., TAKSDAL T. & TOTLAND G.K. (2002). Pathological changes in juvenile Atlantic halibut *Hippoglossus hipoglossus* persistently infected with nodavirus. *Dis. Aquat. Org.*, **50**, 161–169.

JOHANSEN R., AMUNDSEN M., DANNEVIG B.H. & SOMMER A.I. (2003). Acute and persistent experimental nodavirus infection in spotted wolffish *Anarhichas minor*. *Dis. Aquat. Org.*, **57**, 35–41.

JOHANSEN R., ROVE S., SVENDSEN N.A.K., MODAHL I. & DANNEVIG B. (2004). A sequential study of pathological findigs in Atlantic halibut, *Hippoglossus hippoglossus*(L), throughout one year after an acute outbreak of viral encephalopathy and retinopathy. *J. Fish Dis.*, **27**, 327–341.

MALTESE C. & BOVO G. (2007). Viral encephalopathy and retinopathy. *Ittiopatologia*, **4**, 93–146.

MORI K., NAKAI T., MUROGA K., ARIMOTO M., MUSHIAKE K. & FURUSAWA I. (1992). Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology*, **187**, 368–371.

MORI K., MANGYOKU T., IWAMOTO T., ARIMOTO M., TANAKA S. & NAKAI T. (2003). Serological relationships among genotypic variants of betanodavirus. *Dis. Aquat. Org.*, **57**, 19–26.

MORI K., MUSHIAKE K. & ARIMOTO M. (1998). Control measures for viral nervous necrosis in striped jack. *Fish Pathol.*, **33**, 443–444.

MORI K., SUGAYA T., NISHIOCA T., GOMEZ D.K., FUJINAMY Y., OKA M., ARIMOTO M., OKINAKA Y. & NAKAI T. (2005). Detection of Betanodaviruses from feed fish used in marine aquaculture. *In*: 12th International Conference

Diseases of Fish and Shellfish, European Association of Fish Pathologists. Copenhagen (Denmark), 11–16 September 2005. Abstract O-142.

MUNDAY B.L., KWANG J. & MOODY N. (2002). Betanodavirus infections of teleost fish: a review. *J. Fish Dis.*, **25**, 127–142.

MUSHIAKE K., NISHIZAWA T., NAKAI T., FURUSAWA I. & MUROGA K. (1994). Control of VNN in striped jack: Selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). *Fish Pathol.*, **29**, 177–182.

NGUYEN H.D., MUSHIAKE K., NAKAI T. & MUROGA K. (1997). Tissue distribution of striped jack nervous necrosis virus (SJNNV) in adult striped jack. *Dis. Aquat. Org.*, **28**, 87–91.

NISHIZAWA T., MORI K., NAKAI T., FURUSAWA I. & MUROGA K. (1994). Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Dis. Aquat. Org.*, **18**, 103–107.

NISHIZAWA T., MUROGA K. & ARIMOTO M. (1996). Failure of the polymerase chain reaction (PCR) method to detect jack nervous necrosis virus (SJNNV) in striped jack *Pseudocaranx dentex* selected as spawners. *J. Aquat. Anim. Health*, **8**, 332–334.

NISHIZAWA T., FURUHASHI M., NAGAI T., NAKAI T. & MUROGA K. (1997). Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Appl. Environ. Microbiol.*, **63**, 1633–1636.

OLVEIRA J.G., SOUTO S., DOPAZO C.P., THIÉRY R., BARJA J.L. & BANDÍN I. (2009). Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. *J. Gen. Virol.*, **90**, 2940–2951.

PANZARIN V., FUSARO A., MONNE I., CAPPELLOZZA E., PATARNELLO P., BOVO G., CAPUA I., HOLMES E.C. & CATTOLI G. (2012). Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. *Infect. Genet. Evol.*, **12**, 63–70.

PANZARIN V., PATARNELLO P., MORI A., RAMPAZZO E., CAPPELLOZZA E., BOVO G. & CATTOLI G. (2010). Development and validation of a real-time TaqMan PCR assay for the detection of betanodavirus in clinical specimens. *Arch. Virol.*, **155**, 1193–1203.

SANO M., NAKAI T. & FIJAN N. (2011). Viral diseases and agents of warmwater fish.*In*: Fish Diseases and Disorders, Vol. 3: Viral, Bacterial and Fungal Infections, 2nd edition. Woo P.T.K & Bruno D.W., eds. CABI, London, UK, 166–244.

SCHNEEMANN A., BALL L.A., DELSERT C., JOHNSON J.E. & NISHIZAWA T. (2005). Family Nodaviridae.*In*: Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses. Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds. Elsevier Academic Press, London, UK, 865–872.

SOMMERSET I. & NERLAND A.H. (2004). Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). *Dis. Aquat. Org.*, **58**, 117–125.

TAN C., HUANG B., CHANG S.F., NGOH G.H., MUNDAY B.L., CHEN S.C. & KWANG J. (2001). Determination of the complete nucleotide sequence of RNA1 and RNA2 from greasy grouper (*Epinephelus tauvina*) nervous necrosis virus, Singapore strain. *J. Gen. Virol.*, **82**, 647–653.

TANAKA S., MORI K., ARIMOTO M., IWAMO T. & NAKAI T. (2001). Protective immunity of sevenband grouper, *Epinephelus septemfasciatus* Thunberg, against experimental viral nervous necrosis. *J. Fish Dis.*, **24**, 15–22.

THIÉRY R., ARNAULD C. & DELSERT C. (1999a). Two isolates of sea bass, *Dicentrarchus labrax* L., nervous necrosis virus with distinct genomes. *J. Fish Dis.*, **22**, 201–207.

THIÉRY R., RAIMOND J.C. & CASTRIC J. (1999b). Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. *Virus Res.*, **63**, 11–17.

THIÉRY R., COZIEN J., DE BOISSESON C., KERBART-BOSCHER S. & NEVAREZ L. (2004). Genomic classification of new betanodavirus isolates by phylogenetic analysis of the coat protein gene suggest a low host-fish species specificity. *J. Gen. Virol.*, **85**, 3079–3087.

THIÉRY R., COZIEN J., CABON J., LAMOUR F., BAUD M. & SCHNEEMANN A. (2006). Induction of a protective immune response against viral nervous necrosis in the European sea bass *Dicentrarchus labrax* by using betanodavirus virus-like particles. *J. Virol.*, **80**, 10201–10207.

TOFFOLO V., NEGRISOLO E., MALTESE C., BOVO G., BELVEDERE P., COLOMBO L. & DALLA VALLE L. (2007). Phylogeny of betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. *Mol. Phylogenet. Evol.*, **43**, 298–308.

WATANABE K., NISHIZAWA T. & YOSHIMIZU M. (2000). Selection of broodstock candidates of barfin flounder using an ELISA system with recombinant protein of barfin flounder nervous necrosis virus. *Dis. Aquat. Org.*, **41**, 219–223.

YAMASHITA Y., FUJITA Y., KAWAKAMI H. & NAKAI T. (2005). The efficacy of inactivated virus vaccine against viral nervous necrosis (VNN). *Fish Pathol.*, **40**, 15–21.

YAMASHITA Y., MORI K. & NAKAI T. (2009). Protection against viral nervous necrosis conferred by simultaneous inoculation of aquabirnavirus and inactivated betanodavirus in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg). *J. Fish Dis.*, **32**, 201–210.

YUASA K., ISTI KOESHARYANI & KETUT MAHARDIKA (2007). Effect of high water temperature on betanodavirus infection of fingerling humpback grouper *Cromileptes altivelis*. *Fish Pathol.*, **42**, 219–221.

*
* *

NB: There is an OIE Reference Laboratory for Viral encephalopathy and retinopathy
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on Viral encephalopathy and retinopathy