6. Viral Encephalopathy and Retinopathy/Viral Nervous Necrosis (VER/VNN)

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6. Viral Encephalopathy and Retinopathy/Viral Nervous Necrosis (VER/VNN)

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6.1. Introduction

Among the disease outbreaks caused by infectious agents at animal farming facilities, those triggered by viruses are the most challenging to manage, and fish are no exceptions to this rule. Luckily, in the Mediterranean region, only a few viral diseases threaten the marine fish industry. Without any doubt, the most significant in terms of severity, economic impact and spread, is viral nervous necrosis (VNN, syn. viral encephalopathy and retinopathy - VER) which will be widely described in the following chapter. Another relevant disease, affecting mainly seabream (*Sparus aurata*), is lymphocystis disease (LCD) caused by a member of the Iridoviridae family, the LCDV. However, due to the transient nature of this disease and its limited impact on affected fish, it will not be included and described in the present diagnostic manual.

Viral agents other than NNV and LCDV have been described in marine fish species, such as birnaviruses, alphaviruses and aquareovirus, but they are reported only sporadically and their impact on marine aquaculture is unknown and presumably limited. More attention should be paid to exotic viruses, in particular to the red seabream iridovirus (RSIV) and related virusesbelonging to the Megalocytivirus genus, still undetected in the Mediterranean basin but a potential threat to aquaculture if introduced by the natural or anthropogenic route.

Fig. 6.1. a) Appearance of experimentally NNV infected European seabass. There are dead fish on the bottom of the tank, while live fish show impaired swimming capacity with some of them floating on the water surface with the typical "sickle" position; b) Hyperinsufflation of swim bladder in infected European sea bass; c) Different head lesions. From the left: mild congestion (left fish), congestion and erosion of the head skin (middle fish), necrosis of the nostril (right fish); d) hyperaemia of the brain in diseased seabass.

6.2. Aetiology of VNN

The causative agent of VER/VNN is the nervous necrosis virus (NNV), a *Betanodavirus* that belongs to the *Nodaviridae* family. Betanodaviruses are naked particles of 28 nm in diameter, which contain a segmented genome composed of two single-stranded, positive-sense RNA molecules named RNA1 and RNA2. These two segments encode the RNA-dependent RNA polymerase (RdRp) and the capsid protein (Cp), respectively. A subgenomic transcript (RNA3) synthesized from the RNA1 3' terminus during the acute phase of the disease produces a non-structural protein called B2 inhibiting the cell's RNA silencing mechanisms.

At present, *Betanodaviruses* are classified into four species, RGNNV, SJNNV, BFNNV, TPNNV and two types of reassortants, namely RGNNV/SJNNV and SJNNV/RGNNV. Three serotypes are described (A, B and C) partially correlating with the genotype. Reassortants fall in the same serotypes as their RNA1 parental species (Panzarin *et al*., 2016). Each genotype has a different optimum temperature range of replication, explaining the occurrence of outbreaks and geographical distribution (Costa and Thompson, 2016; Toffan *et al*., 2016; Doan *et al*., 2017). Barring the exception of BFNNV and TPNNV species, the other genotypes are all present in the Mediterranean Basin.

Betanodavirus replicates only in the brain, spinal cord and retina, where it causes the necrosis of the nervous cells, and consequently the typical abnormal swimming behaviour. Clinical signs include: apathy alternated to swirling and spinning movements, swim bladder hyperinflation, and blindness. Congestion and erosion of the head and nose, darkening and anorexia are also often present. Mortality can be as high as 100% in larvae, while in older fish is generally less severe. Stressors (i.e. feeding, sorting, netting, etc.) can increase the severity of the clinical signs and cumulative mortality. Survivors remain persistently infected and can transmit the disease to healthy fish for long time.

Table 6.1. NNV genotype serological classification and optimum growth temperature (*Modified from OIE Manual of Diagnostic Tests for Aquatic Animals and Panzarin et al., 2016)*

Genotype	Serotype	Optimum growth temperature
RGNNV	C	25-30°C
SJNNV	A	20-25°C
BFNNV	B	15-20°C
TPNNV	B	20°C
RGNNV/SJNNV	A	25-30°C
SJNNV/RGNNV	C	20-25°C

6.3. Host range

Among the fish species farmed in the Mediterranean basin, the European seabass (*Dicentrarchus labrax*) is certainly the most severely affected by VNN. European seabass is particularly susceptible to RGNNV, which is the most widely spread betanodaviral species in the Mediterranean countries. RGNNV is also highly virulent for groupers (*Epinephelus* spp.), and for this reason, it represents both an economic and ecological threat. It is noteworthy that in recent years, the emergence of the reassortant RGNNV/SJNNV has also caused recurrent outbreaks in larval stage gilthead seabream (Toffan *et al*., 2017), which was initially believed to be a species resistant to VNN. Flatfish (turbot, sole) are also very susceptible to NNV. Overall, betanodavirus has been detected in more than 160 fish species and several molluscs, both as susceptible host as well as carrier animals. Therefore, given the broad host range of

betanodavirus, all fish species can be considered susceptible to infection and monitoring and diagnosis should be performed accordingly.

6.4. Collection and shipment of fish samples for detection of NNV

Upon arrival at the laboratory, samples should be delivered in leak-proof containers and labelled properly. Please refer to Chapter 2.2. for specific instructions on biological substance shipping.

Specimens submitted to the laboratory may include:

- Fish carcasses (adult/market size specimens collected in a pool of 5; juveniles in pools of 10 specimens; pooled larvae at least 0.1 gr. of material).
- Organs (brains and/or eyes from adult/market size specimens collected in a pool of 5; brains and/or eyes from juveniles in pools of 10).
- Broodstock (always collected singly).
- Serum samples (collected singly).
- Live prey (Artemia and rotifer at least 0.1 g of material).
- Other (cell culture supernatant at least 0.5 ml, water samples and algae in suitable amounts depending on the scope).

The central nervous system (CNS) and the retina are the target organs for betanodavirus. Therefore, the analysis of tissue samples other than CNS (i.e. spleen, kidney, gills, blood etc.) is advisable for research purposes only.

When clinical signs and mortality are present, sampling of 5-10 diseased fish is sufficient to confirm the diagnosis of VNN (targeted surveillance). In the absence of a clinical outbreak, statistically significant numbers of fish should be collected (see Chapter 2.1.5.) in order to consider the batch as NNV-free (active surveillance). When looking for potential carriers, fish should be tested singly. On the other hand, the presence of betanodavirus should be excluded every time that an increase in mortality is observed, especially in larvae and juveniles (passive surveillance). As an additional biosecurity measure, it is recommended to test every new fish batch produced (in case of hatcheries) or introduced (from another farm), irrespective of species, origin and absence of mortality/clinical signs. Finally, because the NNV can be transmitted vertically, analysis of samples from broodstock might be required. Due to the low viral load in carrier fish, gonads and/or reproductive fluids should never be pooled. Furthermore, for broodstock, it would be advisable to combine molecular tests of reproductive fluids with serum samples for detection of antibodies (in those species where serological assays are available). Alternatively, mixed fertilized ova from the collection basket, produced by several fish in a broodstock tank, can also be analysed for preventive diagnosis. However, they are not considered a good target, mainly because of the difficulty in homogenizing this matrix and releasing the virus, which is present at very low concentrations. For a better and reliable diagnosis, it is recommended to test other target samples too (i.e. entire larvae, brains/eyes/heads) in order to increase the accuracy of the analytical result.

6.4.1. Sample preservation for viral isolation in cell culture

Specimens must be frozen (-20°C or lower) and subsequently shipped on dry ice. Alternatively, specimens must be placed in viral transport medium (VTM) and shipped frozen or refrigerated. VTM could be a cell culture medium with 10% fetal calf serum (FCS) and 1% antibiotics/antimycotics. The combination of 10,000 IU/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 µg/ml amphotericin B is commonly adopted, but other antibiotics/antimycotics of proven efficiency may be used as well.

6.4.2. Sample preservation for molecular diagnosis and typing

Specimens must be placed in sealed tubes with RNA stabilization solution (e.g. RNAlater®)[1] (1:5-w/v) and shipped at room temperature. Cell culture supernatants and tissue homogenates can be spotted in FTA®[2] cards and shipped at room temperature. Alternatively, refrigerated or frozen samples can also be used for molecular diagnosis, but the cold chain must be preserved during delivery.

6.4.3. Sample preservation for serological diagnosis

Anticoagulants should not be used when collecting blood. Sera (without blood clots) must be placed in sealed tubes and shipped frozen or refrigerated. Whole blood should never be sent directly to the laboratory because haemolysis of the red blood cells occurs releasing haemolysis products in the serum.

6.4.4. Samples preservation for histology and immunohistochemistry

Specimens should be placed in sealed containers with 4% buffered formalin and shipped at room temperature.

6.5. Diagnostic procedures for NNV

Since it is quite common that different betanodavirus species circulate in the same geographic region, the capability of detecting all viral species, as well as their correct identification is of utmost importance to provide accurate and reliable laboratory results. Therefore, as a first step in the diagnostic process, a molecular protocol capable of detecting all known betanodaviral species must be used. Real-time RT-PCR is preferable to conventional or nested PCR, due to better performances in terms of sensitivity, specificity and turn-around times. Upon positive results, virus isolation should be used as a confirmatory analysis, especially for the first detection in a certain region or in a new fish species. In these cases, genotyping of the NNV strain detected is also essential to gain information on viral phenotype, as different betanodaviruses show diverse pathogenicity, host tropism and temperature sensitivity.

[1] Invitrogen RNA*later*® Stabilization Solution is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. It minimizes the need to immediately process tissue samples or to freeze samples in liquid nitrogen for later processing. Tissue pieces can be harvested and submerged in RNA*later* solution for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation.

[2] FTA® Card, Whatman™ is a paper matrix laced with a proprietary mixture of chemicals that lyse cells and stabilize nucleic acids on contact for long term storage at room temperature.

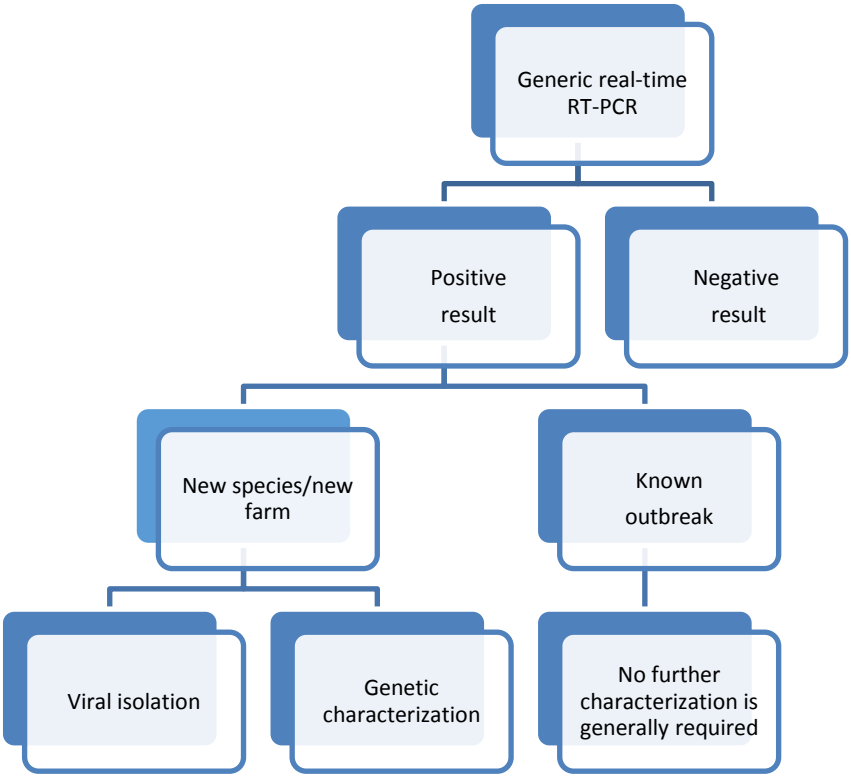

Fig. 6.2. Workflow for detection of NNV.

6.5.1. Preparation of samples for examination

Tissue samples collected from fish carcasses must be homogenized either by mechanical blender or with mortar and pestle, and subsequently resuspended in fresh VTM to a final dilution of 1:3 w/v. The homogenate is subsequently clarified by centrifugation at 2-5°C for 2 minutes at 2000-4000 x *g*. For molecular diagnosis, clarified supernatant can be directly processed, according to the commercial kit used for RNA purification. In the case of samples stored in RNA stabilization solution (e.g. RNAlater®), organs must be removed from the solution and processed as described above. For virus isolation, supernatant must be treated overnight at 4°C (or for 4 hours at 15°C) with 1% antibiotics/antimycotics. The combination of 10,000 IU/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 µg/ml amphotericin B is recommended, but other antibiotics/antimycotics of proven efficacy may be used as well. The antibiotic treatment aims at preventing bacterial contamination and makes filtration steps with membrane filters unnecessary. Betanodavirus, in fact, is a "sticky" virus and filtration may cause loss of viral particles and should be avoided.

6.5.2. Real-time RT-PCR

Total RNA can be isolated using commercial kits based on RNA affinity spin columns, according to the manufacturer's instructions. To preserve RNA integrity, it is recommended to store purified RNA for subsequent use at -80°C by adding RNAse Inhibitor. For molecular diagnosis, two generic protocols capable of detecting the four betanodavirus species are recommended.

These assays have been extensively validated and are used by many diagnostic laboratories (data from the 2nd International proficiency test for NNV). Adequate controls must be always included in every analytical session. The real-time PCR performances can vary depending on the reagents and the platform used. It is therefore recommended to adapt the protocols to the reagents and equipment available at the laboratory, verify the performances and establish a diagnostic cut-off.

6.5.2.1. One-step real-time PCR targeting RNA1 (Baud *et al.*, 2015):

Oligonucleotides

Oligonucleotide	Sequence 5' → 3'	Position in RNA1 (GenBank reference sequence JN189865)
oPVP154 (For)	TCCAAGCCGGTCCTAGTCAA	2717-2736
oPVP155 (Rev)	CACGAACGTKCGCATCTCGT	2865-2884
tqPVP16 (Probe)	Cy5-CGATCGATCAGCACCTSGTC-BHQ2	2772-2791

Reaction mix

Use QuantiTect Probe RT-PCR Kit (Qiagen)

Reagent	Final concentration/volume
oPVP154 (For)	600 nM
oPVP155 (Rev)	600 nM
tqPVP16 (Probe)	400 nM
2X RT-PCR Master Mix	1X
Enzyme mix	0,25 □l
RNA template	5 □l
DEPC Water	To 25 □l

Thermal profile

RT	Denaturation	Denaturation	Annealing/Extension
50°C	95°C	94°C	60°C
30 min	15 min	15 sec	60 sec
		40 cycles	

Technical performances

Analytical sensitivity: 100 copies of plasmid DNA with 95% confidence, $10^{2.5}$-$10^{2.85}$ $TCID_{50}$/ml.

Analytical specificity: capable of detecting RGNNV, SJNNV, BFNNV, TPNNV, RGNNV/SJNNV, SJNNV/RGNNV; negative results when testing VHSV, IHNV and IPNV.

Repeatability: 0,05-1,1% CV

Reproducibility: 0,43-1,78% CV

Robustness: 0,31-0,6% CV

6.5.2.2. Two-step real-time PCR targeting RNA2 (Panzarin *et al.*, 2010):

Oligonucleotides

Oligonucleotide	Sequence 5' → 3'	Position in RNA2 (GenBank reference sequence JN189992)
RNA2 FOR	CAACTGACARCGAHCACAC	418-436
RNA2 REV	CCCACCAYTTGGCVAC	471-486
RNA2 probe	6FAM-TYCARGCRACTCGTGGTGCVG-BHQ1	448-468

Reaction mix for RT
Use High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

Reagent	Final concentration/volume
10X RT Buffer	1X
10X RT Random Primers	1X
25X dNTP Mix	1X
MultiScribe Reverse Transcriptase 50U/µl	2,5U
RNA template	15 µl
DEPC Water	To 30 µl

Thermal profile for RT

Pre-incubation	RT
25°C	37°C
10 min	120 min

Reaction mix for real-time PCR

Use LightCycler TaqMan Master (Roche)

Reagent	Final concentration/volume
RNA2 FOR	900 nM
RNA2 REV	900 nM
RNA2 probe	750 nM
5X Reaction Mix	1X
cDNA template	5 µl
DEPC Water	To 20 µl

Thermal profile

Denaturation	Denaturation	Annealing	Extension
95°C	95°C	58°C	72°C
10 min	10 sec	35 sec	1 sec
	45 cycles		

Technical performances

Analytical sensitivity: 300 copies/µl of in vitro transcribed RNA, 10 $TCID_{50}$/ml

Analytical specificity: capable of detecting RGNNV, SJNNV, BFNNV (including AhNNV and AcNNV), TPNNV, RGNNV/SJNNV, SJNNV/RGNNV; negative results when testing *A. hydrophila*, *P. damselae* subsp. *damselae*, *V. anguillarum*, VHSV

Repeatability: CV 0.02-2.87%

Reproducibility: CV 1.1-3.48%

N.B: this assay can be adapted from two-step to one-step protocol, but a great loss of sensitivity may occur and therefore this modification is not advisable.

6.5.3. Sequencing

In order to genetically characterize betanodaviruses and identify possible re-assortment events, it is necessary to sequence both genomic segments.

The primers herein reported for partial sequencing allow performing a preliminary but informative characterization of the virus (Bovo *et al*., 2011). The protocol is intended for application on viral isolates, however, good results can also be obtained from diagnostic samples stored in an RNA stabilizing solution, and yielding less than 20-25 threshold cycle by real-time PCR.

Oligonucleotides

Target	Primer	Sequence 5' → 3'	Amplicon size (bp)	Position in the reference sequence (GenBank acc. no for RNA1: JN189865; GenBank acc. no for RNA2: JN189992)
RNA1	VNNV5	GTTGAGGATTATCGCCAACG	478	178-197
	VNNV8	CAGCAACACGGTAGTG		640-655
RNA1	For 521	ACGTGGACATGCATGAGTTG	630	521-540
	VNNV6	ACCGGCGAACAGTATCTGAC		1131-1150
RNA2	VNNV1	ACACTGGAGTTTGAAATTCA	605	342-361
	VNNV2	GTCTTGTTGAAGTTGTCCCA		927-946

Reaction mix

Use QIAGEN OneStep RT-PCR Kit (Qiagen)

Reagent	Final concentration/volume
Primer For	400 nM
Primer Rev	400 nM
dNTP Mix 10 mM each	0.4 mM
Qiagen OneStep RT-PCR Buffer 5X	1X
Qiagen OneStep RT-PCR Enzyme Mix	1 µl
RNA template	5 µl
DEPC Water	To 25 µl

Thermal profile

RT	Denaturation	Denaturation	Annealing	Extension	Final extension
50°C	95°C	94°C	55°C	72°C	72°C
30 min	15 min	40 sec	40 sec	70 sec	10 min
		40 cycles			

Other RT-PCR kits of proven efficacy and amplification profiles might be used as an alternative. Purity and size of PCR products must be evaluated by gel electrophoresis.

For a more complete characterization of betanodavirus strains, the complete sequence of RNA1 and RNA2 genetic segments should be obtained (protocol available upon request).

6.5.4. Virological examination

6.5.4.1. Cell cultures and media

The best cell line for NNV isolation is SSN-1 (Frerichs *et al.*, 1996). This cell line has been specifically designed to show clear cytopathic effects (CPE) when infected with betanodavirus. However also the E-11 cell line (Iwamoto *et al.*, 2000), actually a clone of SSN-1, can be adapted to this task. The latter is more stable and resistant therefore is easier to multiply compared to the progenitor cell line. However, if the virus is not present in a high amount, CPE can be transient in E-11 and therefore be difficult to see. For a diagnostic reason, no other cell line should be used. Both cell lines prefer L-15 medium (unless adapted to other cell culture medium) and primary plastic.

Cells have to be prepared 24h prior to infection and incubated with medium supplemented with 10% FBS and antibiotics at 25°C for the very first hours. Only when they are almost confluent can they be moved to the lower incubation temperature.

Multiplication ratio can vary from 1:2 to 1:4 according to the cell line.

Susceptibility of cells must be checked periodically (see chapter below).

6.5.4.2. Incubation and inoculation of cell monolayers

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 dilution of tissue material in the cell culture medium of 1:10–100.

Each 100 µl dilution should be inoculated into at least 2 cm^2 actively replicating cell culture monolayers. Both the normal and adsorption method may be used.

If the adsorption method is adopted, allow the inoculum to adsorb on the drained monolayers for 1 hour at 20°C. After the adsorption period, add the new medium without FBS supplement. If the normal method is adopted, the culture medium needs to be changed with a new one without FBS, before adding the inoculum.

Incubate at 20° (BFNNV – SJNNV - SJNNV/RGNNV) or at 25°C (RGNNV- RGNNV/SJNNV) according to the origin of the sample and the genotype expected (Panzarin 2014).

N.B: Optimum temperature for cold water betanodavirus is considered 15 °C, however, cells may suffer at this temperature.

Follow the course of infection by regular microscopic examination at ×40–100 magnification every 2-3 days for 10 days.

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

CPE in SSN-1 or E-11 cells is characterized by thin or rounded, refractive, granular cells with large vacuoles, and partial or complete disintegration of the monolayer.

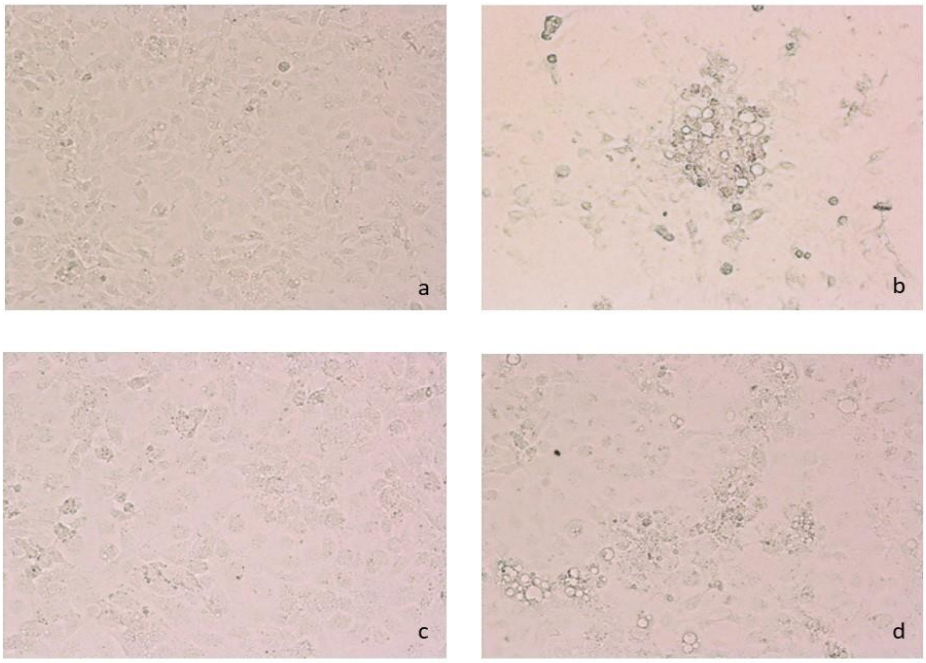

Fig. 6.3. a) Normal cell monolayer of SSN-1 and c) E-11 cell lines, respectively; b) Cytopathic effect of NNV at day 4 post-infection of SSN-1 and d) E-11 cell lines, respectively. Vacuolations are clearly visible. The observation made under light microscope Zeiss at 20 x

6.5.4.3. Subcultivation procedures

Collect aliquots (10%) of cell culture medium from all inoculated monolayers. Inoculate those aliquots constituting the primary culture into wells with the new cell monolayers, as described above (well-to-well subcultivation). Incubate and monitor as described above for a further 10 days.

If no CPE occurs during this period, collect an aliquot of cell culture supernatant and subject to generic real-time RT-PCR. If molecular analysis yields negative results the test may be considered negative.

If molecular analysis detects a positive signal a third passage can be performed in order to further multiply the virus and allow CPE to appear.

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

6.5.4.4. Procedure for titration to verify the susceptibility of the cell cultures to infection

A batch of NNV in low cell culture passage numbers should be used. This virus must be propagated in cell culture flasks of SSN-1 or E-11 cells as described before. At total CPE, the virus is harvested by centrifugation of cell culture supernatant at 2000 x g for 15 minutes and stored in 1 ml cryotubes. The virus shall be kept at -80 °C.

One week after freezing, three replicate vials with the virus are thawed under cold water and titrated on the cell lines. Titres are calculated according to the Reed and Muench formula. The average of the titre of these 3 vials is the reference value for cell susceptibility evaluation.

At least every six months, or if it is suspected that the susceptibility of a cell line has decreased, 1 new vial of the same NNV batch is thawed and titrated. Titration by endpoint dilution should include at least six replicates at each dilution step. The titre is then calculated as before and the value obtained is compared with the initial titre. If the titre decreases by a factor of 2 logs or more, compared with the initial titre, the cell line should no longer be used for surveillance purposes and a new cell line should be recovered from nitrogen or obtained by an approved source.

6.5.5. Virus identification

Virus identification can be performed with different laboratory techniques:

- Real-time RT-PCR/RT-PCR and if necessary sequencing
- Indirect fluorescent antibody test.

6.5.6. Indirect fluorescent antibody test

- Prepare monolayers of susceptible cells (E-11 or SSN-1) directly in 2 cm^2 wells of primary cell culture plastic plates or on cover-slips or chamber slides in order to achieve around 80-90% confluency, which is usually reached within 24 hours of incubation at 25°C.
- Inoculate 100µl of the viral suspensions to be identified using at least two tenfold dilutions.
- Incubate at 20°C or 25°C (according to the NNV strain) for 48–72 hours.
- Remove the culture medium and fix with cold 80% acetone for 10-30 minutes at room temperature.
- Rinse three times with PBS-Tween 0.05% (PBST).
- Allow the cell monolayers to air-dry.
- Add the cell monolayers with a drop (around 200-500 µl) of the primary antibody (i.e. rabbit anti-betanodavirus immune serum) and incubate for 30 minutes at 37°C in a humid chamber.[3]
- Rinse three times with PBST.
- Allow the cell monolayers to air-dry.

[3] N.B. The primary antibody must be diluted according to the manufacturer's instructions. Different antibodies according to the NNV serotype suspected or detected by molecular techniques should be used.

- Add the cell monolayers with a drop (around 200-500 µl) of the primary antibody with commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody) and incubate for 30 minutes at 37°C in a humid chamber.
- Rinse three times with PBST.
- Examine the treated cell monolayers directly on plates, or mount the cover-slips using 50% glycerol-PBS solution, prior to microscopic observation.
- All immunofluorence runs should include one positive and one negative control well.

Brilliant fluorescent cells scattered on the monolayer are visible in positive samples. The fluorescent signal is cytoplasmic with the unmarked nucleus clearly visible.

Always use negative control wells, where uninfected cells only are present.

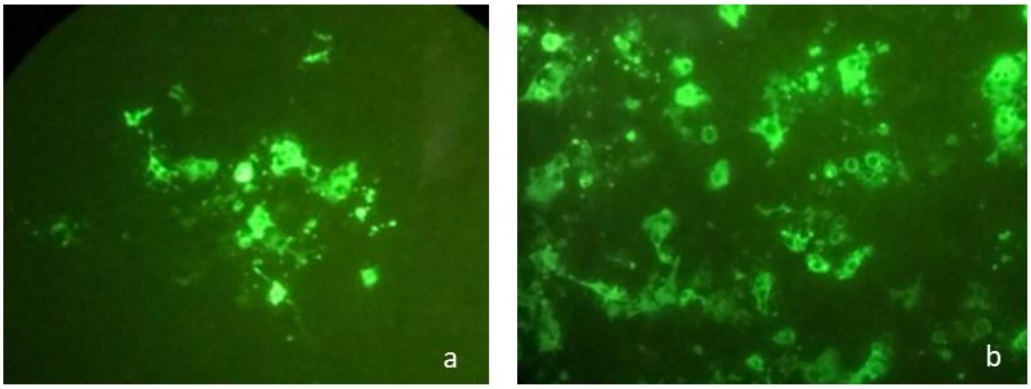

Fig. 6.4. Positive IF reaction on infected SSN-1 cell monolayer 3 days post-infection. a) Single positive focus, and b) spread positive cells. Observation made under fluorescence microscope Zeiss Axioskop at 20 x.

6.5.7. Histology

Buffered formalin-fixed samples should be processed according to standard histological techniques. Lesions occur only in the nervous tissues: retina, brain and spinal cord. Typical lesions are characterized by multiple intracytoplasmatic vacuolations appearing as empty areas of 5-10 µm in diameter, clearly separated from the surrounding areas, mainly present in the grey matter. Pyknosis, karyorrhexis, neuronal degeneration and inflammatory infiltration have been described in all the nervous tissues of the infected fish. Mild to severe congestions of the blood vessels evolving also to haemorrhages in the encephalic parenchyma and meninges are frequently observed. In the retina, vacuoles are generally evident in the outer and inner nuclear layers, as well as in the ganglion cell layer. Similarly, vacuoles can be observed in the optic nerve as well as in the spinal cord. These lesions are by far more prominent in diseased larvae and juveniles while in older symptomatic fish they are sometimes very rare and difficult to detect.

In clinically healthy fish, vacuoles in the brain and especially in the retina can be due to artefacts in particular processing problems; therefore it is always suggested to confirm the presence of the NNV antigen by IHC.

6.5.8. Immunohistochemistry IHC (avidin-biotin-peroxidase technique)

- Dewax sections (3 µm thick) with xylene (2 passages for 10 minutes each) and ethanol 100° (2 passages for 5 minutes each).
- Hydrate tissue sections in a decreasing ethanol series: 95°, 70°, 50°, distilled water (5 minutes each).
- Rinse with Tris Buffered Saline with Tween 20 (TBTS) for 5 minutes.
- Incubate sections with 0.1% trypsin (Porcine Trypsin, Sigma) in distilled water, for 30 minutes at 37°C in a humid chamber.
- Rinse twice with TBST for 5 minutes each time.
- Block endogenous peroxidase by incubating the slides for 10 minutes with anti peroxidase solution (Dako) at room temperature (RT).
- Rinse twice with TBST for 5 minutes each time.
- Incubate sections with ready to use normal horse serum (Vector Laboratories) for 20 minutes at RT.
- Rinse in TBTS for 5 minutes.
- Incubate sections with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) diluted in antibody diluent (Dako) for 60 minutes at RT[4].
- Rinse three times in TBST for 5 minutes each.
- Incubate with the secondary biotinylated antibodies (i.e. goat anti-rabbit immunoglobulins in 2.5% BSA) for 30 minutes at RT.
- Rinse two times in TBST for 5 minutes.
- Incubate with avidin/biotin-based peroxidase system (Vectastain ABC HRP - Vector Laboratories) for 30 minutes at RT.
- Rinse twice in TBST for 5 minutes.
- Incubate with chromogen substrate 3-amino-9-ethylcarbazole (AEC), prepared just before use, for 20 minutes at RT.
- Rinse with distilled water for 5 minutes.
- Counterstain with Harris' hematoxylin for 30 seconds.
- Mount sections in glycerol gelatine.
- All immunohistochemical runs should include one positive and one negative control section.

IHC should be always performed in case of NNV suspicion even in the absence of evident lesion. It has been described that immunoprecipitate can be visualized largely before the apparition of vacuoles or even in absence of them (Toffan *et al*., 2017; Valencia *et al.,* 2019; Mladineo, 2003). Positive samples will present red immunoprecipitates in nervous tissues mainly surrounding vacuoles but also following the shape of apparently normal neurons. Consider that pale diffuse red stain of tissues is not considered as specific immunolabelling (background) and depends on the quality and dilution of the antibodies used. Always use a negative control slide.

[4] N.B. The primary antibody must be diluted according to the manufacturer's instructions. Different antibodies according to the NNV serotype suspected or detected by molecular techniques should be used.

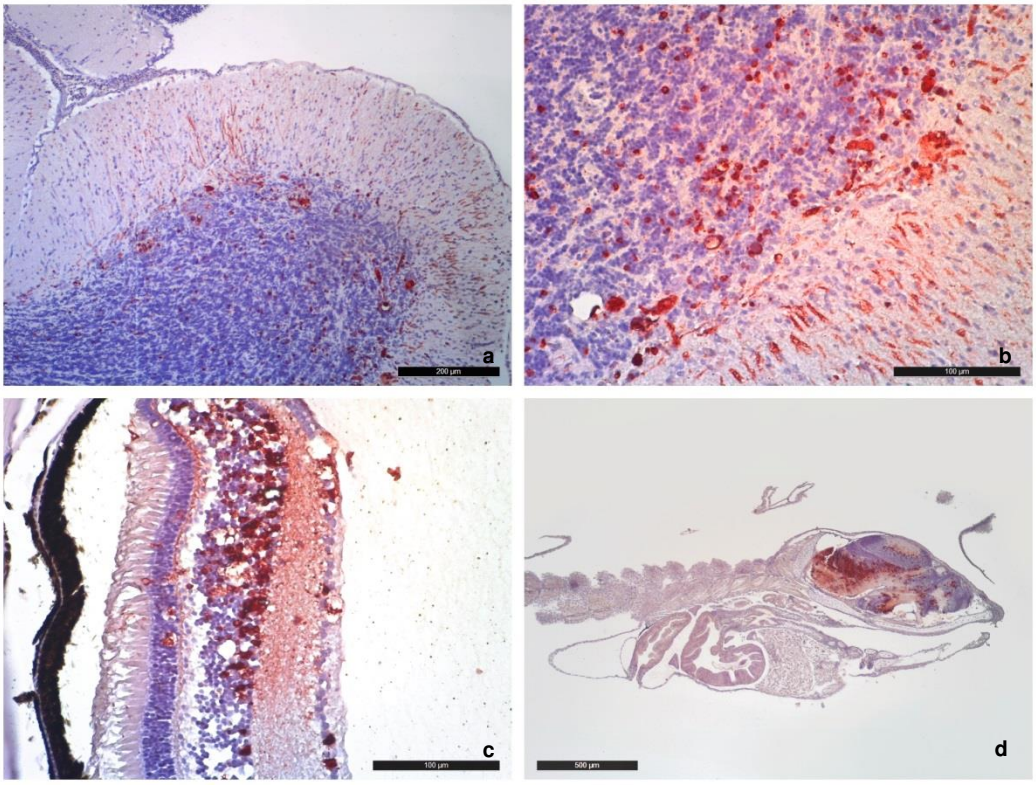

Fig. 6.5. a) and b) NNV infected seabass cerebellum showing strong immunoprecipitates in neurons soma and dendrites (10x and 25x); c) NNV infected seabass retina showing vacuolations in inner nuclear layer and in ganglion cell layer and spread immunoprecipitates mainly localized in inner and outer nuclear layer and in ganglion cell layer (25x); d) Widespread immunoprecipitates in the brain of 16 day post-hatching seabream larvae RGNNV/SJNNV infected.

6.5.9. Conservation of samples

Samples in RNA stabilization solutions can be stored indefinitely at -20°C.

Organs can be conserved at -80 C for months and isolated virus for many years. The viability of the virus decreases with time; however, betanodaviruses are quite resistant agents and therefore virus can be retrieved from positive samples after several years of storage.

Viral isolates can be stored indefinitely at -80°C.

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