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Isolation of betanodavirus from farmed turbot *Psetta maxima* showing no signs of viral encephalopathy and retinopathy

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ABSTRACT

The isolation of a betanodavirus strain from juvenile turbot showing unspecific symptoms and very low mortality is reported. The presence of an IPNV-type virus was also revealed in the fish tissues, but only after nested-PCR, suggesting a very low viral load. In addition, different *Vibrio* species were isolated from some of the individuals. Both viral RNAs were sequenced and phylogenetic analysis indicated that the strain showed 99% identity with the RGNNV genotype. Experimental infections (via immersion and intraperitoneal route) were conducted in order to determine the susceptibility of turbot juveniles (2 and 5 g) to the NNV isolate at 15 and 18 °C (range of turbot rearing temperatures in our area). The results obtained indicated that although the viral isolate was able to replicate in the turbot tissues, it did not induce clinical disease in this fish species. These findings suggest that the existence of a reservoir of NNV-RGNNV type in wild fish in the area represents a low risk for the turbot farming industry.

1. Introduction

Viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) is a serious neuropathological condition causing important mortalities in the larvae and juveniles of an increasing number of fish species around the world. The etiological agents of the disease are viruses belonging to the genus *Betanodavirus* (family *Nodaviridae*). Betanodaviruses are non-enveloped virions with an icosahedral symmetry, about 25 nm in diameter and a viral genome consisting of two single-stranded (ssRNA) segments, the RNA1 and RNA2. The RNA1 (3.1 kb) encodes the viral replicase (Nagai and Nishizawa, 1999; Tan et al., 2001), whereas the RNA2 (1.4 kb) encodes the coat protein (Delsert et al., 1997; Nishizawa et al., 1994). In addition, a subgenomic RNA, termed RNA3, is transcribed from the 3′ end of RNA1 (Iwamoto et al., 2005; Sommerset and Nerland, 2004). On the basis of the comparison of a variable region of the coat protein gene, the betanodavirus are classified in four genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), red-spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) (Nishizawa et al., 1995). A fifth genotype, namely the turbot nervous necrosis virus (TNV) type was proposed after sequencing the PCR product obtained from turbot affected by a natural outbreak of VER in Norway (Johansen et al., 2004), but the virus was not isolated. Although no other reports have been published on VER infections in turbot so far, one of the first descriptions of nodavirus in fish, reported as encephalomyelitis, was made in this species (Bloch et al., 1991) as indicated by Munday et al. (2002) in their review. In addition, experimental infections have shown that this species is susceptible to nodavirus belonging to SJNNV and BFNNV genotypes (Husgard et al., 2001; Sommerset et al., 2003).

In recent years the detection of betanodavirus in apparently healthy wild fish (including turbot, but mainly flathead grey mullet, *Mugil cephalus*, blackspot seabream *Pagellus bogaraveo*, and white seabream, *Diplodus sargus*) has increased notably in our area, as reported by the Spanish *Junta Nacional Asesora de Cultivos Marinos* (JACUMAR) (http://www.magrama.gob.es/app/jacumar/planes_nacionales/Documentos/100_IF_GESAC_EPIDEMIOLOGIA_Anexo_I_GALICIA.pdf).

In the present study we report the isolation of a nodavirus strain from juvenile farmed turbot showing low mortalities and unspecific symptoms. Both viral RNAs were sequenced and phylogenetic analysis indicated that the strain showed 99% identity with RGNNV genotype. Experimental infections were performed in order to evaluate the susceptibility of turbot juveniles to this isolate.

2. Materials and methods

2.1. Fish analysis

Fourteen turbot (5 g average weight) showing swollen abdomen and mild levels of mortality (approximately 0.1% which lasted a month) were received in the facilities of the Instituto de Acuicultura

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(Universidad de Santiago de Compostela) at the end of December 2010 and subjected to virological analysis. Fish were sacrificed with a MS-222 overdose, necropsied under aseptic conditions and examined for internal lesions.

Spleen, kidney, and brain were aseptically collected from each fish. Brain samples were pooled and used for betanodavirus isolation, whereas pools of spleen and kidney were tested for the presence of viral haemorrhagic septicaemia (VHSV), infectious haematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). Tissue samples (brain and kidney/spleen) were mixed (1:10) with Earle's balanced salt solution (Hyclone Laboratories Inc.) supplemented with antibiotics (1000 UI ml^{-1} penicillin, 1000 μg ml^{-1} streptomycin, 500 μg ml^{-1} gentamycin and 10 μg ml^{-1} amphotericin B) and homogenised. After centrifugation of the homogenates at 2000 g for 20 min, the supernatants were transferred to new tubes, and incubated for 4 hours at 15 °C. Afterwards, the supernatans where inoculated (diluted at 10^{-1} and 10^{-2}) in duplicate onto 48-well plates of semi-confluent monolayers (around 80% confluence) of chinook salmon embryo (CHSE-214), bluegill fry (BF-2) and epithelioma papillosum cyprini (EPC), which where used for kidney/spleen samples, or E-11 (a clone of the cell line striped snakehead SSN-1), which was used for brain samples. CHSE-214, BF-2 and EPC were grown in Eagle's minimum essential medium (EMEM, Hyclone Laboratories Inc.) at 15, 20 and 20 °C, respectively, and E-11 was grown in L-15 (Gibco) at 25 °C. Growth media were supplemented with 10% foetal calf serum (FCS, BioWhittaker), 100 I.U. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. When monolayers were semiconfluent, the medium was substituted by fresh medium with 0 or 2% FCS for viral infection. All infected monolayers were incubated at 15 °C, and examined daily for the presence of cytopathic effect (CPE). After 15 d, positive and negative samples (cultures showing CPE or no-CPE, respectively) were subcultured by inoculating 0.1 ml of the scraped cell suspension onto new cultures. Subcultures were terminated after 15 d of incubation. Non-infected cells were used as controls.

2.1.1. RT-PCR and nested PCR detection

Total RNA was extracted from aliquots of the tissue samples using RNeasy Mini kit (Qiagen) following the manufacturer´s instructions. Primer pairs used for RT-PCR were Heppel F/R for IPNV (Heppell et al., 1992), cm3a and cm3b for VHSV (López-Vázquez et al., 2006), and F2-R3 (Nishizawa et al., 1994) for nodavirus. Complementary DNA (cDNA) synthesis was performed by mixing the viral RNA with 2.5 ng μl^{-1} of random primers (Promega) heating at 95 °C for 5 min and incubating at 4 °C for at least 1 min. Then a reverse transcription mixture containing Superscript III RT (Invitrogen) was added and incubated at 25 ° C for 10 min. The RT reaction was performed at 50 ° C for 50 min, followed by a 5 min/85 °C RT enzyme inactivation. The PCR reaction was performed using 4 μl of cDNA, 1.25 U of GoTaq® Flexi DNA polymerase (Promega) and 0.5 μM of the specific primer set. Following an initial 4 min denaturation step at 94 ° C, the mixture was subjected to 40 cycles of amplification (30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C) with a final extension of 10 min at 72 °C. The PCR products were analysed by electrophoresis on a 1.5% SeaKem® LE agarose gel (FMC Bioproducts).

The nested RT-PCR was performed using 3 μl from the RT-PCR reaction and the specific primers Heppel introF (5′-AAAGGCATGGGG CTGGAGAG-3′)/HeppelR for IPNV *cm3aintro* and *cm3bintro* for VHSV (López-Vázquez et al., 2006) and F21-R31 for nodavirus (Olveira et al., 2008), using the same protocol as described above.

2.2. Sequencing and phylogenetic analysis

The sequence of the coding regions of both RNA segments from the betanodavirus isolate was determined using the primer walking approach. A panel of 9 sets of primers (6 for RNA1 and 3 for RNA2) corresponding to overlapping regions of the betanodavirus genome (Olveira et al., 2009) were used.

Automated sequencing was performed using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter). Sequences of both segments were confirmed at least twice by sequencing upstream and downstream and, to solve any inconsistencies a second set of sequencing was performed. The sequences were edited using DNASTAR Lasergene® v.7.1 SeqMan II and EditSeq (DNASTAR). For comparative purposes, nucleotide sequences of reference strains of betanodavirus deposited in the GenBank were used (Table 1). All sequences were subjected to multiple sequence alignment using the DNASTAR Lasergene v.7.1 MegAlign program (DNASTAR). Following alignment, trees were constructed by Bayesian inference of phylogeny using BEAST 1.7.5 (Bayesian MCMC analysis of molecular sequences) (Drummond et al., 2012) and employing the GTR + G (RNA1) and HYK + G (RNA2) models. Three Markov chains were run for 10,000,000 generations and Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the 50,000 first generations. Sequences of an alphanodavirus – BBV [black beetle virus, accession no. NC_001411 (RNA1) and NC_002037 (RNA2)] – were used as an outgroup to produce rooted trees.

2.3. Experimental infection

2.3.1. Trial 1

Turbot (average weight 5 g) were obtained from a commercial fish farm. On arrival at the facilities of the University aquarium fish were divided into two groups (G1 and G2) and gradually acclimated to the desired temperature (15 or 18 °C, respectively) for 15 days. Water temperature was then maintained during the experimental infection. Prior to challenge, 20 fish from each group were examined for the presence of NNV, IPNV, IHNV and VHSV by inoculation in cell culture and RT-PCR.

Turbot were stocked at a density of 40 fish per tank in 100 l aquaria and exposed to virus at a concentration of 10^5 $TCID_{50}$ ml^{-1} in a total volume of 3 l for 3 h. As negative controls, two tanks (one per temperature) containing ten fish each were also set up using MEM with no virus. The experiment was terminated after 30 days. Fish were monitored daily for signs of disease and mortality. Brain and eye

Table 1
Reference strains used in the phylogenetic analysis.

Virus isolate (genotype)	Source	Country	Reference	GeneBank accession no.	
				RNA 1	RNA2
SJ93Nag (SJNNV)	Striped jack	Japan	Iwamoto et al. (2001)	AB056571	AB056572
SJNNV (SJNNV)	Striped jack	Japan	Nagai and Nishizawa (1999)	AB025018	–
SJ-G91 (SJNNV)	Striped jack	Japan	Nishizawa et al. (1995)	-	D30814
SGWak97 (RGNNV)	Red-spotted grouper	Japan	Iwamoto et al. (2004)	NC_008040	NC_008041
G9508KS (RGNNV)	Red-spotted grouper	Japan	Lee et al. (2002)	AY690597	AY690596
SpDl-IAusc1688.08 (RGNNV)	Sea bass	Spain	Olveira et al. (2009)	FJ803915	FJ829452
AH95NorA (BFNNV)	Atlantic halibut	Norway	Grotmol et al. (2000); Sommerset and Nerland (2004)	AJ401165	AJ245641
BF93Hok (BFNNV)	Barfin flounder	Japan	Nishizawa et al. (1995)	EU826137	EU826138
TPKag93 (TPNNV)	Tiger puffer	Japan	Nishizawa et al. (1995); Toffolo et al. (2007)	AM085332	D38637