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ORIGINAL ARTICLE

Development and validation of a real-time TaqMan PCR assay for the detection of betanodavirus in clinical specimens

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Abstract Betanodaviruses are the causal agents of viral encephalo-retinopathy, an infectious disease affecting more than 40 marine fish species, characterized by high morbidity and mortality. Because of its severe impact, robust diagnostic tools are required. The aim of this work was to develop and validate a real-time TaqMan PCR assay to detect betanodaviruses in clinical specimens by amplifying a conserved region of the RNA2 strand. The method proved to be specific and sensitive, being capable of detecting as low as 10 $TCID_{50}$/ml. For clinical validation, samples from 100 marine fish were collected during a natural outbreak of disease and tested by three distinct laboratory methods, namely real-time TaqMan PCR, RT-seminested PCR and virus isolation. The results indicated optimal agreement between tests. The assay that was developed is capable of detecting members of all of the betanodavirus genetic groups currently described and can be considered a valid alternative to the time-consuming and contamination-prone nested PCR.

Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), is a severe infectious disease affecting more than 40 species of marine fish worldwide and is characterized by abnormalities in movement, uncoordinated swimming, emaciation and heavy mortality, particularly during the larval and juvenile stages [3]. Typical lesions that have been reported include cellular vacuolation, necrosis and neuronal degeneration in the brain, spinal cord, retina and ganglia [15, 23]. The aetiological agent, belonging to the family *Nodaviridae*, genus *Betanodavirus*, consists of an icosahedral, non-enveloped virus with a diameter ranging from 20 to 34 nm. The genome is composed of a bisegmented, single-stranded, positive-sense RNA [22]. The RNA1 molecule (3.1 kb) encodes the RNA-dependent RNA polymerase of approximately 100 kDa, while the RNA2 (1.4 kb) contains a unique open reading frame (ORF) and encodes the structural capsid protein of 42 kDa [24]. Sommerset and Nerland [32] detected in cell cultures, but not in viral particles, the RNA3, a subgenomic transcript of RNA1. According to a phylogenetic analysis of the variable region (T4) of the viral capsid gene, Nishizawa et al. [26] divided betanodaviruses into four genotypes, later confirmed by Dalla Valle et al. [6]. These correspond to the species *Barfin flounder nervous necrosis virus* (BFNNV), *Redspotted grouper nervous necrosis virus* (RGNNV), *Striped jack nervous necrosis virus* (SJNNV) and *Tiger puffer nervous necrosis virus* (TPNNV). However, several Atlantic isolates grouped differently from the commonly recognized genetic clusters [12, 15, 19, 36], and therefore, the existence of an additional genotype should not be ruled out.

Betanodaviruses are the most important viral pathogens reported in cultured marine fish within the Mediterranean region [2]. Following an outbreak of VER it is very important to promptly contain the spread of the infection to other farms. The availability of specific and sensitive diagnostic methods represents the first step in detecting the causative agent and in screening and identifying infected fish, particularly broodfish that might transmit the infection to their offspring.

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Several analytical approaches, including electron microscopy, histology, IFAT, ELISA and virus isolation in cell culture are available [23], but the molecular-based techniques have shown higher sensitivity than these methods. In particular, several papers describe nested PCR-based assays for sensitive betanodavirus detection [5, 12, 13, 29, 35]. Despite the high sensitivity, nested-PCR protocols are contamination-prone and time-consuming and therefore not ideal for high-throughput diagnostic laboratories. Real-time PCR overcomes these disadvantages, offering robust results coupled with shorter processing and turnaround times. Currently, a few real-time TaqMan PCR-based diagnostic protocols are available in the literature, but their applicability appears to be restricted to Atlantic isolates [16, 20, 25] and so may be suboptimal for detecting strains circulating in the Mediterranean basin.

The aim of this study was to provide a comprehensive set of data on the development and laboratory and clinical validation of a real-time TaqMan PCR assay suitable for processing different sample matrices and capable of targeting the four known betanodavirus genotypes.

Materials and methods

Viral and bacterial strains

The viral strain 475/I98 was isolated from sea bass (*Dicentrarchus labrax*) during a VER outbreak occurring at a farm located on the Adriatic coast (genotype RGNNV, Table 1). The virus was propagated in SSN-1 cells [11] and used as a reference strain for standardizing and validating the assay. The viral titre, expressed as $TCID_{50}$/ml, was determined according to the endpoint titration procedure [21].

Additional betanodavirus strains isolated from different hosts and belonging to different genotypes were tested to assess the specificity and sensitivity of the method [7, 17, 18, 31, 37]. As recommended in the available guidelines [10, 28], specificity was also established using a panel of different microorganisms, including viral and bacterial isolates that are likely to be found in the marine environment. All of the pathogens tested are listed in Table 1.

Primers and probe design

Multiple sequence alignment including representative sequences that are publicly available in GenBank ($n = 87$) (http://www.ncbi.nlm.nih.gov) was performed using MEGA4 software [34]. The selected sequences were related to betanodavirus strains isolated from a wide range of species and geographic areas and belonged to different genotypes (Fig. 1). According to the alignment, in order to minimize mismatches, degenerate primers and probe were designed using the "Primer Express Software v2.0" (Applied Biosystems, Foster City, CA, USA) following standard procedures [8, 9]. Oligonucleotides were able to target and amplify a conserved 69-bp-long region of the viral genome, localized

Table 1 Fish pathogens tested to assess the sensitivity and specificity of the real-time TaqMan PCR assay for betanodavirus detection

Pathogen tested	Host fish or isolation matrix	Isolation year	Origin
Betanodavirus AH95Nor [37][a]	*Hippoglossus hippoglossus*	1995	Norway
Betanodavirus 475/I98 [7][a]	*Dicentrarchus labrax*	1998	Italy
Betanodavirus V26/I01[a]	*Dicentrarchus labrax*	1998	France
Betanodavirus 470/I00[a]	*Diplodus puntazzo*	2000	Italy
Betanodavirus 530/I00[b]	*Liza ramada*	2000	Italy
Betanodavirus 395/I01 [7][b]	*Epinephelus marginatus*	2001	Italy
Betanodavirus 334/I02[a]	*Solea solea*	2002	Italy
Betanodavirus 532/I02[b]	*Mullus barbatus*	2002	Italy
Betanodavirus 24/I05[a]	*Sparus aurata*	2005	Portugal
Betanodavirus SK-07 1324 [17][a]	*Gadus morhua*	2007	Norway
Betanodavirus SJNag93 (SJNNV) [18]	*Pseudocaranx dentex*	1993	Japan
Betanodavirus TPKag93 (TPNNV) [18]	*Takifugu rubripes*	1993	Japan
Betanodavirus SGWak97 (RGNNV) [18]	*Epinephelus septemfasciatus*	1997	Japan
Betanodavirus JFIWa98 (BFNNV) [18]	*Paralichthys olivaceus*	1998	Japan
VHSV 860/I94 [31]	*Scophthalmus maximus*	1994	Scotland
Aeromonas hydrophila (ATCC 7966)	Milk	*NA*	*NA*
Ph. damselae subsp. *damselae* (DSMZ 7482)	*Chromis punctipinnis*	1982	California
Ph. damselae subsp. *piscicida* (NCIMB 2058)	*Seriola queradiata*	1977	Japan
Vibrio alginolyticus (ATCC 17749)	Spoiled horse mackerel	1961	Japan
Vibrio anguillarum (ATCC 43305)	*Oncorhynchus mykiss*	1984	Denmark

NA not available

[a] Farmed

[b] Wild

in the RNA2 strand, encoding the coat protein (CP). The sequences of the primers and probe are as follows: RNA2 FOR 5′-CAACTGACARCGAHCACAC-3′, RNA2 REV 5′-CCCACCAYTTGGCVAC-3′, RNA2 probe 5’-6FAM-TYCARGCRACTCGTGGTGCVG-BHQ1-3’ (Fig. 1).

RNA extraction, cDNA synthesis and real-time TaqMan PCR

Total RNA was extracted from 100 µl of sample using the NucleoSpin® RNA II (Macherey–Nagel GmbH & Co., Düren, Germany) following the supplier's recommendations. RNA samples were kept at −80°C until use. cDNA was synthesized using random hexamers with the "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler 2.0 system and carried out in 20 µl with "LightCycler® TaqMan® Master" (Roche Diagnostics GmbH, Mannheim, Germany), 0.9 µM of each primer, 0.75 µM of probe and 5 µl of cDNA template. The thermal profile consisted of a 10-min incubation at 95°C followed by 45 cycles of 10 s denaturation at 95°C, 35 s annealing at 58°C and 1 s elongation at 72°C, followed by an additional 30-s cooling step at 40°C.

RT-seminested PCR

The performance of the newly developed real-time TaqMan PCR was compared with that of the RT-seminested PCR adopted at the OIE reference laboratory for encephalopathy and retinopathy (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy). The oligonucleotides used, which target a conserved region of viral RNA2 strand, are listed in Table 2. One-step RT–PCR was performed using a "Qiagen® OneStep RT–PCR Kit" (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Primers were used at a final concentration of 0.2 µM. The reaction consisted of a 30-min incubation at 54°C followed by a 2-min incubation at 94°C. This was followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C and 45 s elongation at 72°C; the reaction was terminated with a 10-min elongation at 72°C. Seminested PCR was carried out in a final volume of 50 µl using an "AmpliTaq® DNA Polymerase Kit" (Applied Biosystem, Foster City, CA, USA), using the reagents at the following concentrations: 1× GeneAmp PCR Buffer II, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer and 2.5 U AmpliTaq® DNA Polymerase. The thermal profile consisted of a 2-min incubation at 94°C followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 62°C and 30 s elongation at 72°C. The reaction was terminated by a 10-min elongation at 72°C. Thermocycling was conducted using the GeneAmp® PCR System 9700 (Applied Biosystem, Foster City, CA, USA).

Analytical sensitivity

The sensitivity and efficiency of the real-time TaqMan PCR were preliminarily assessed by creating a standard curve. The 230-bp-long RT-seminested PCR amplicon was cloned into the pCR®2.1 plasmid using a "TA Cloning® Kit" (Invitrogen, Carlsbad, CA, USA). The DNA was subsequently reverse-transcribed into synthetic RNA using a "MEGAshortscript™ Kit" according to the manufacturer's instructions (Ambion®, Applied Biosystem, Foster City, CA, USA). In vitro-transcribed RNA was then used as a template for making the standard curve. Subsequently, to establish whether different types of sample matrices could influence the analytical sensitivity of the assay, cell culture supernatants, brain homogenates and gonad homogenates were spiked with tenfold serial dilutions of titrated virus (strain 475/I98, Table 1). Briefly, brain and gonads were collected from healthy wild sea bass that were negative for betanodavirus by both the previously described RT-seminested PCR protocol and virus isolation in cell culture. Tissue (1 g) was weighed and homogenised with sterile quartz sand in 9 ml of Eagle Minimum Essential Medium with Earle's salts (Sigma–Aldrich®, Saint Louis, MO, USA) supplemented with 2% foetal calf serum and a mixture of antibiotics and antimycotics (10,000 U/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 µg/ml amphotericin B). Samples were then clarified by centrifugation. After virus spiking, total RNA was extracted from each dilution of virus and tested in triplicate by real-time TaqMan PCR and RT-seminested PCR. Simultaneously, viral isolation in SSN-1 cell cultures was attempted for each sample according to standard procedures [27].

Analytical specificity

The specificity of the method was assessed by testing the newly designed set of primers and probe on selected piscine bacteria and viral haemorrhagic septicaemia virus (VHSV) (Table 1). Furthermore, total RNA extracted from non-target microorganisms was combined with medium (10^5 $TCID_{50}$/ml) and low concentrations of the target virus (10^1 $TCID_{50}$/ml) to further investigate whether the presence of a co-infection could affect betanodavirus detection.

Intra-assay repeatability and inter-assay reproducibility

In order to determine the intra- and inter-assay variability, three viral concentrations (high: 10^7 $TCID_{50}$/ml, medium:

Fig. 1 Multiple sequence alignment of betanodavirus RNA2 nucleic sequences. GenBank accession numbers are listed. RNA2 FOR, RNA2 REV and RNA2 probe hybridize to positions 392–410, 445–460 and 422–442, respectively, of the RGNNV RNA2 sequence (GenBank accession number: DQ864760). Hybridization regions of the primers and the probe are highlighted in *grey*. Oligonucleotide sequences used in the real-time PCR are shown *underlined* on the *top line*. Degenerations are displayed in **bold**

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                      RNA2 FOR (5'→ 3')                   RNA2 PROBE (5'→ 3')    RNA2 REV (3'→5')
                      CAACTGACARCGAHCACAC                 TYCARGCRACTCGTGGTGCVG  CAVCGGTTYACCACCC

NC_004136  CTGCCTGATTCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACA
AY721615   .........C................................................................................
AB056572   .........C.............C...........T.....C.......................C...................TC.....
AF318942   ..........................................................................................
AM088776   ------...C.............C...........T.....C.......................C...................TC.....
AM085345   ------...C.........G...C.............AA.......G...........G...............................
AM085344   ------...C................................................................................
AM085343   ------...C................................................................................
AM085342   ------...C................................................................................
AM085341   ------...C................................................................................
AM085340   ------...C................................................................................
AM085339   ------...C..............................................G.................................
DQ864760   .........C................................................................................
DQ116038   .........C................................................................................
DQ116037   .........C................................................................................
DQ116036   .........C................................................................................
DQ116035   .........C................................................................................
AY690596   .........C.............C..................................................................
AY835642   .........C.............A..................................................................
AJ608266   ........CC.........G...C....................G..G...........G.....................TC....G
AJ698094   .........C.........G...C.............AA.......G...........G...............................
AJ698093   .........C................................................................................
AF245004   .........C.............C..................................................................
AY284974   .........C................................................................................
AY284973   .........C.............C..................................................................
AY284972   .........C................................................................................
AY284971   .........C.............C..................................................................
AY284970   .........C.........................................................................T......
AY284969   .........C.............C..................................................................
AY284968   .........C.............C..................................................................
AY284967   .........C.............C..................................................................
AY284966   .........C.........................................................................T......
AY284965   .........C.............C..................................................................
AY284964   .........C.............C..................................................................
AY284963   .........C.............C..................................................................
AY284962   .........C.............C..................................................................
AY600956   .........C................................................................................
AY620368   .........C................................................................................
AY620367   .........C................................................................................
AY510457   .........C................................................................................
AY324870   .........C................................................................................
AY140801   .........C................................................................................
AY140800   .........C.............C..................................................................
AY140799   .........C................................................................................
AY140798   .........C................................................................................
AY140797   .........C....................C...........................................................
AY140796   .........C................................................................................
AY140795   .........C................................................................................
AY140794   .........C................................................................................
AY140793   .........C.............C..................................................................
AF534998   .........C.............C..................................................................
AF283554   .........C.............C..................................................................
D38637     .........C.............C.............AA.......................................G..................
D38636     .........C................................................................................
D38527     .........C.....................................G..........................................
D30814     .........C.............C...........T.....C.......................C...................TC.....
AF499774   .........C................................................................................
AF281657   .........C................................................................................
AF445800   .........C.........G...C.............AA.A.....G...........G..............................T
AF245003   .........C................................................................................
AJ277811   ------...C................................................................................
AJ277810   ------...C..............................................G.................................
AJ277809   ------...C................................................................................
AJ277808   ------...C................................................................................
AJ277807   ------...C................................................................................
AJ277806   ------...C................................................................................
AJ277805   ------...C................................................................................
AJ277804   ------...C................................................................................
AJ277803   ------...C.....................................................................G..................
U39876     .........C.............C.......................G...........C..T....G............G.....G...
AF175520   .........C................................................................................
AF175519   .........C.............C...........T.....C.......................C...................TC.....
AF175518   .........C.............C..................................................................
AF175517   .........C................................................................................
AF175516   .........C................................................................................
AF175515   .........C................................................................................
AF175514   .........C................................................................................
AF175513   .........C................................................................................
AF175512   .........C................................................................................
AF175511   .........C.............C..................................................................
AF175510   .........C.......................................................................G.........
AF175509   .........C................................................................................
AB045980   .....C.........................................................................C.............C......
Y08700     .........C................................................................................
AJ245641   .........C.........G...C.............AA.......G.....C.....G...............................
D38635     .........C.........G...C.............AA.......G...........G...............................
AF160473   .........C.........G...C.............AA.......G...........G...............................
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10^5 $TCID_{50}$/ml and low: 2× limit of detection) from infected cell culture supernatants were tested in triplicate within the same run (intra-assay), on three different days (inter-assay) by two different operators. The coefficient of variation (CV) was calculated according to the Environmental Protection Agency protocol [9].

Diagnostic sensitivity and specificity: detection of betanodavirus in clinical specimens

To further evaluate the performance and the reliability of the method using clinical specimens, different organic matrices (including brain, eye, head, larvae, juveniles, sperm, eggs, faeces, bowel and intestinal content) were collected from suspected diseased and apparently healthy fish, both farmed and wild (Table 5). Specimens were sampled from May 2008 to April 2009 in a sea bass farm located on the Adriatic Sea coast and in close proximity to it. For molecular tests, approximately 1 g of sample was homogenised with a sterile pestle in 300 µl of lysis buffer from the NucleoSpin® RNA II kit (Macherey–Nagel GmbH & Co., Düren, Germany) and subsequently clarified by centrifugation. The supernatant was used for RNA extraction and tested both by real-time TaqMan PCR and RT-seminested PCR. The same samples were simultaneously processed for virus isolation in SSN-1 cell cultures according to the standard procedure [27]. Results of the three distinct laboratory tests were compared by using the Cohen's kappa statistic [1] calculated with the STATA 9.2 Statistics/Data Analysis software (StataCorp LP, TX, USA).

Results

Analytical sensitivity

The analytical sensitivity of the method was assessed by determining the limit of detection (LoD) as the lowest viral titre that can be detected by real-time TaqMan PCR in spiked cell culture supernatants, brains and gonads. The 475/I98 strain used for the validation tests was calculated to have a final titre of $10^{7.80}$ $TCID_{50}$/ml. The LoD of the method, including the RNA extraction procedure, was 10 TCID50/ml. The LoD of the new rRT–PCR protocol evaluated using in vitro-transcribed RNA was 3×10^2 copies/µl. The performance of the new method was compared with that of RT-seminested PCR and viral isolation by testing serial dilutions of the virus in different matrices. The results are summarised in Table 3. Both RT-seminested PCR and viral isolation were unable detect the virus at dilutions higher than $1{:}10^6$. The rRT–PCR protocol confirmed its higher sensitivity by detecting viral RNA up to a dilution of $1{:}10^8$ (Table 3).

The linearity and the efficiency of the reaction were determined by generating a standard curve in which serial dilutions of in vitro transcribed RNA were tested. Amplification plots and a standard curve are shown in Fig. 2a, b. The crossing point values (CP) of each dilution were plotted against the logarithm of the concentration expressed as RNA copy number, and this showed a linear relationship over 9 log dilutions, ranging from 3×10^2 to 3×10^{10} copies/µl. The amplification efficiency, E, determined using the equation $E = 10^{-1/\text{slope}}$ was optimal ($E = 2.033$).

Table 2 Primer sets used for the RT-seminested PCR for betanodavirus detection. Nucleotide positions correspond to GenBank accession number D38636

Method	Primer	Sequence (5′ → 3′)	Nucleotide position	Product size
One-step RT-PCR	VER1 (for)	GACTGGGACACGCTGCTAGA	114 → 133	349 bp
	VER2 (rev)	AGTCGAACACTCCAGCGACA	443 → 462	
Seminested-PCR	CP1 (for)	CAACTGACAACGATCACACCTTC	234 → 256	230 bp
	CP2 (rev)	CAATCGAACACTCCAGCGACA	443 → 463	

Table 3 Comparison of the sensitivity thresholds of real-time TaqMan PCR, RT-seminested PCR and viral isolation in SSN-1 cell culture

Matrix	Highest dilution yielding a positive result		
	Real-time TaqMan PCR	RT-seminested PCR	Virus isolation in SSN-1 cell culture
Cell culture supernatant	$1{:}10^8$	$1{:}10^6$	$1{:}10^6$
Brain	$1{:}10^8$	$1{:}10^6$	$1{:}10^6$
Gonads	$1{:}10^8$	$1{:}10^6$	$1{:}10^6$

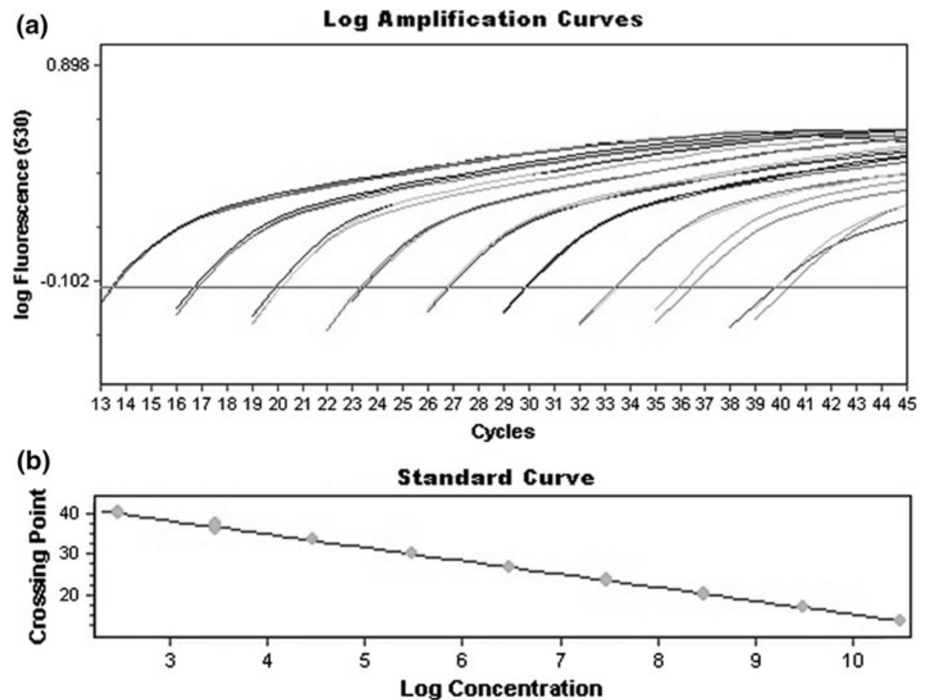

Fig. 2 **a**, **b** Analytical sensitivity of the real-time TaqMan PCR. **a** Amplification curves obtained by testing tenfold serial dilutions of quantitated synthetic RNA standards. The *x*-axis shows the number of PCR cycles. The *y*-axis shows the fluorescence values on a logarithmic scale. **b** Standard curve generated from the above data. The *x*-axis indicates the logarithm of the RNA concentration expressed in copy number. The *y*-axis indicates the CP value. The dynamic range of the method spanned 3×10^{10} and 3×10^{2} RNA2 copies/µl

Analytical specificity

The newly developed real-time TaqMan PCR was capable of detecting members of all of the betanodavirus genetic groups that have currently been described (Table 1). No positivity was observed when VHSV and the selected piscine bacteria were tested, except for a weak nonspecific fluorescence signal that was detected when testing *Vibrio alginolyticus.* This signal rose late in the reaction cycle with a high crossing-point value (CP 37.59).

Intra- and inter-assay variability

The coefficient of variation within-run and between-days was calculated as the percentage of the ratio of standard deviation and the average of the CP values obtained. The real-time TaqMan PCR demonstrated good repeatability and reproducibility, with the intra-assay CV ranging from 0.02 to 2.87% and the inter-assay CV ranging from 1.11 to 3.48% (Table 4).

Table 4 Intra-assay repeatability and inter-assay reproducibility

Virus titre	Intra-assay variability (CV%)	Inter-assay variability (CV%)
10^7 $TCID_{50}$/ml	$0.09 \leq CV \leq 2.87$	$1.11 \leq CV \leq 3.48$
10^5 $TCID_{50}$/ml	$0.02 \leq CV \leq 2.23$	$1.52 \leq CV \leq 2.27$
10^2 $TCID_{50}$/ml	$0.42 \leq CV \leq 0.92$	$2.11 \leq CV \leq 2.33$

Coefficient variation values are expressed as percentages (CV%)

Detection of betanodavirus in clinical specimens

In total, 100 animals were tested for betanodavirus using the three distinct analytical methods, and the results were compared. Thirty-five percent of the samples tested positive by real-time TaqMan PCR. Results obtained by RT-seminested PCR and by virus isolation in cell cultures showed good agreement (32 and 24% positivity, respectively) and confirmed that the newly developed assay was highly specific. Indeed, the concordance between the three tests was optimal, according to the Landis and Koch scale, and significant, with an overall Cohen's kappa statistic of 0.8565 and $p < 0.001$. All of the strains isolated from farmed and wild fish in cell culture belong to the RGNNV genotype according to the sequencing of RNA2 strand (data not shown). With few exceptions, data analysis revealed that high CP values ($\geq$31) are associated with negative results in cell culture isolation. On the other hand, all of the animals showing clinical signs were positive by real-time TaqMan PCR, RT-seminested PCR and virus isolation in SNN-1 cells, except for samples 425/6 and 425/7, which were negative by virological analysis. High fluorescence signals were detected in both samples, suggesting a good performance of the real-time TaqMan PCR for a range of organic matrices. Detailed results are showed in Table 5.

Table 5 List of clinical specimens sampled from May 2008 to April 2009 in the Adriatic sea

Sample	Host fish	Matrix	Analysis results		
			Real-time TaqMan PCR	RT-seminested PCR	Virus isolation in cell culture
316/1	*Dicentrarchus labrax*	Brain	–	–	–
316/2	*Dicentrarchus labrax*	Brain	–	–	–
316/3	*Dicentrarchus labrax*	Brain	–	–	–
316/4	*Dicentrarchus labrax*	Brain	–	–	–
316/5	*Dicentrarchus labrax*	Brain	–	–	–
316/6	*Dicentrarchus labrax*	Brain	–	–	–
316/7	*Dicentrarchus labrax*	Brain	–	–	–
316/8	*Dicentrarchus labrax*	Brain	–	–	–
316/9	*Dicentrarchus labrax*	Brain	–	–	–
316/10	*Dicentrarchus labrax*	Brain	–	–	–
316/11	*Dicentrarchus labrax*	Brain	–	–	–
316/12	*Dicentrarchus labrax*	Brain	–	–	–
316/13	*Dicentrarchus labrax*	Brain	–	–	–
316/14	*Dicentrarchus labrax*	Brain	–	–	–
327/1	*Dicentrarchus labrax*[a]	Brain	–	–	–
327/2	*Dicentrarchus labrax*[a]	Brain	–	–	–
327/3	*Diplodus puntazzo*[b]	Brain	–	–	–
327/4	*Sphyraena sphyraena*[b]	Brain	–	–	–
327/5	*Sarpa salpa*[b]	Brain	–	–	–
327/6	*Dicentrarchus labrax*[a]	Brain	–	–	–
327/7	*Dicentrarchus labrax*[a]	Brain	+ (29.93 CP)	+	+
327/8	*Dicentrarchus labrax*[a]	Brain	+ (32.36 CP)	+	–
327/9	*Dicentrarchus labrax*[b]	Brain	–	–	–
327/10	*Dicentrarchus labrax*[b]	Brain	+ (32.9 CP)	+	–
327/11	*Diplodus annularis*[b]	Brain	+ (34.36 CP)	+	–
327/12	*Diplodus annularis*[b]	Brain	–	–	–
352/1	*Dicentrarchus labrax*[a, c]	Brain	+ (11.88 CP)	+	+
352/2	*Trachurus picturatus*[b]	Brain	+ (29.01 CP)	+	+
352/3	*Boops boops*[b]	Brain	+ (32.58 CP)	–	–
352/4	*Sphyraena sphyraena*[b]	Brain	+ (26.01 CP)	+	+
352/5	*Sphyraena sphyraena*[b]	Brain	+ (33.51 CP)	–	–
352/6	*Sphyraena sphyraena*[b]	Brain	–	–	–
352/7	*Sparus aurata*[a]	Brain	–	–	–
352/8	*Sparus aurata*[a]	Brain	+ (31.35 CP)	+	–
352/9	*Dicentrarchus labrax*[a, c]	Brain	+ (11.57 CP)	+	+
352/10	*Dicentrarchus labrax*[a, c]	Brain	+ (9.91 CP)	+	+
425/1	*Seriola dumerili*[b]	Brain	–	–	–
425/2	*Seriola dumerili*[b]	Brain	–	–	–
425/3	*Dicentrarchus labrax*[a]	Brain	–	–	–
425/4	*Oblada melanura*[b]	Brain	–	–	–
425/5	*Scomber scombrus*[b]	Brain	–	–	–
425/6	*Dicentrarchus labrax*[a, c]	Intestinal content	+ (12.94 CP)	+	–
425/7	*Dicentrarchus labrax*[a, c]	Intestinal content	+ (10.66 CP)	+	–
425/9	*Boops boops*[b]	Brain	–	–	–
425/10	*Sparus aurata*[a]	Brain	+ (28.90 CP)	+	+
425/11	*Dicentrarchus labrax*[a, c]	Brain	+ (12.67 CP)	+	+
425/12	*Dicentrarchus labrax*[a, c]	Brain	+ (18.60 CP)	+	+

Table 5 continued

Sample	Host fish	Matrix	Analysis results		
			Real-time TaqMan PCR	RT-seminested PCR	Virus isolation in cell culture
549/1	*Diplodus sargus sargus*[b]	Brain	–	–	–
549/2	*Corvina nigra*[b]	Brain	–	–	–
549/3	*Seriola dumerili*[b]	Brain	–	–	–
549/4	*Dicentrarchus labrax*[a]	Eye	+ (15.51 CP)	+	+
549/5	*Dicentrarchus labrax*[a]	Faeces	–	–	–
549/6	*Dicentrarchus labrax*[a]	Brain	+ (18.90 CP)	+	+
549/7	*Dicentrarchus labrax*[a]	Brain	–	–	–
549/8	*Diplodus sp.*[b]	Brain	–	–	–
549/9	*Sphyraena sphyraena*[b]	Brain	–	–	–
549/10	*Dicentrarchus labrax*[a]	Brain	–	–	–
549/11	*Dicentrarchus labrax*[a]	Brain	+ (21.51 CP)	+	+
549/12	*Dicentrarchus labrax*[a]	Bowel	+ (26.89 CP)	+	+
549/13	*Dicentrarchus labrax*[a]	Brain	+ (15.71 CP)	+	+
549/14	*Seriola dumerili*[b]	Brain	–	–	–
549/15	*Seriola dumerili*[b]	Brain	–	–	–
549/17	*Dicentrarchus labrax*[a]	Bowel	–	–	–
596/1	*Trachurus picturatus*[b]	Brain	–	–	–
596/2	*Trachurus picturatus*[b]	Brain	–	–	–
596/3	*Trachurus picturatus*[b]	Brain	–	–	–
596/4	*Sardina pilchardus*[b]	Brain	–	–	–
596/5	*Sardina pilchardus*[b]	Brain	–	–	–
596/6	*Boops boops*[b]	Brain	–	–	–
596/7	*Boops boops*[b]	Brain	–	–	–
596/8	*Boops boops*[b]	Brain	–	–	–
596/10	*Dicentrarchus labrax*[a]	Brain	–	–	–
596/11	*Dicentrarchus labrax*[a]	Brain	–	–	–
596/12	*Dicentrarchus labrax*[a]	Brain	–	–	–
596/13	*Dicentrarchus labrax*[a]	Brain	+ (14.27 CP)	+	+
2/1	*Dicentrarchus labrax*[a]	Brain	–	–	–
2/2	*Dicentrarchus labrax*[a]	Brain	–	–	–
2/3	*Dicentrarchus labrax*[a]	Brain	–	–	–
2/4	*Dicentrarchus labrax*[a]	Brain	–	–	–
2/5	*Dicentrarchus labrax*[a]	Brain	+ (21.02 CP)	+	+
2/6	*Dicentrarchus labrax*[a]	Brain	+ (28.80 CP)	+	+
2/7	*Dicentrarchus labrax*[a]	Brain	–	–	–
69/4	*Sparus aurata*[a, c]	Juvenile	+ (35.30 CP)	+	+
69/5	*Sparus aurata*[a]	Brain, eye	+ (27.89 CP)	+	+
69/7	*Dicentrarchus labrax*[a]	Head	–	–	–
69/8	*Dicentrarchus labrax*[a]	Brain, eye	+ (33.33 CP)	+	+
69/9	*Dicentrarchus labrax*[a]	Brain, eye	+ (23.39 CP)	+	+
69/11	*Dicentrarchus labrax*[a]	Head	–	–	–
69/12	*Dicentrarchus labrax*[a]	Brain, eye	+ (14.08 CP)	+	+
114/1	*Dentex dentex*[a]	Sperm	–	–	–
114/2	*Sparus aurata*[a]	Sperm	–	–	–
114/3	*Sparus aurata*[a]	Eggs	–	–	–
114/4	*Sparus aurata*[a]	Eggs	+ (14.92 CP)	+	+
114/5	*Dicentrarchus labrax*[a]	Eggs	+ (32.56 CP)	–	–

Table 5 continued

Sample	Host fish	Matrix	Analysis results		
			Real-time TaqMan PCR	RT-seminested PCR	Virus isolation in cell culture
114/6	*Dicentrarchus labrax*[a]	Larvae	+ (34.58 CP)	+	–
114/8	*Dicentrarchus labrax*[a]	Brain, eye	+ (11.39 CP)	+	+
114/9	*Dicentrarchus labrax*[b]	Brain	–	–	–
114/12	*Dicentrarchus labrax*[a]	Brain, eye	+ (33.50 CP)	+	–
114/13	*Belone belone*[b]	Brain	–	–	–
114/14	*Dicentrarchus labrax*[a]	Brain	–	–	–

Fish species, matrices used and analysis results are reported. All of the samples were tested by real-time TaqMan PCR, RT-seminested PCR and virus isolation in SSN-1 cells

[a] Farmed animal

[b] Wild animal

[c] Animal showing clinical signs

Discussion

Viral encephalopathy and retinopathy is one of the most serious fish diseases, causing significant economic losses to the marine aquaculture industry. The availability of rapid, specific and sensitive diagnostic tools to correctly identify the disease and avoid its spread to farmed and wild populations is therefore necessary. The presence of betanodavirus in wild marine fish [13] and invertebrates [14] with no clinical signs, which can act as reservoirs of the disease, has been demonstrated previously. Indeed Peducasse et al. [30] and Castric et al. [4] suggested that cohabitation between healthy and diseased animals can be considered a risk factor for spread of infection. As betanodaviruses can persist in the aquatic environment for a long time, it has also been reported that the presence of the pathogen in seawater is responsible for contagion of larvae and fry in rearing units, confirming horizontal transmission to be critical for disease diffusion [25]. On the other hand, vertical transmission has been highly suspected because of the detection of viral particles in the gonads of infected spawners, even if viruses may not reside in the reproductive organs at all times, being more likely to be found there following stressful events [23].

Many conventional PCR-based diagnostic tools have been developed to promptly diagnose the disease, particularly RT nested-PCR assays, which have been demonstrated to be more sensitive than the single RT–PCR [35] and virus isolation in cell cultures. Despite good performance in terms of sensitivity, RT nested PCR is known to be time-consuming and contamination-prone. Real-time PCR avoids all of these disadvantages, since no post-amplification manipulation is required, and only a single amplification reaction is performed. In addition, in TaqMan-based real-time PCR, the specificity of the test is improved by the use of specific probes instead of fluorescent intercalants as in SYBR Green based real-time PCR. Korsnes et al. [20], Grove et al. [16] and Nerland et al. [25] developed three independent real-time TaqMan PCRs for detecting Atlantic halibut betanodavirus. As mentioned before, these tests may be suboptimal in detecting Mediterranean strains. Indeed, the protocol by Grove et al. [16] was applied to identify the Italian 475/I98 isolate, but no fluorescence signal was detected due to mismatches between the probe and the target region, as later confirmed by sequence analysis (data not shown). Starkey et al. [33] standardized a real-time nucleic-acid-sequence-based amplification (NASBA) protocol for the detection of piscine nodaviruses, but the method was unable to detect a strain isolated from a tiger puffer, *Takifugu rubripes* (genotype TPNNV). On the other hand, the SYBR Green I-based real-time PCR developed by Dalla Valle et al. [7] demonstrated high sensitivity and was capable of recognizing the four different betanodavirus genotypes, although the genetically distinct isolates of Atlantic origin were not tested. Furthermore, the specificity of the test was not assessed, and the method was not extensively validated using clinical specimens and different tissue matrices (e.g. reproductive organs). Nishizawa et al. [26], Grotmol et al. [15] and Dalla Valle et al. [6] have demonstrated previously that the capsid protein gene is sufficiently conserved among different strains of betanodaviruses; this evidence makes it the ideal target for primers and probe design. However, the introduction of degenerate positions in the set of oligonucleotides designed for this new assay was necessary to account for the genetic variability observed among the different strains. In fact, sequence alignments revealed relevant nucleotide differences in the genomes of betanodaviruses of Atlantic origin. Despite this genetic divergence, the laboratory validation demonstrated that the new assay was capable of recognizing all of the genetic groups currently described,

including betanodavirus isolates from Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*).

In the present study, the newly developed real-time TaqMan PCR was capable of detecting low concentrations of virus for each sample matrix tested. Indeed, when compared with the RT-seminested PCR and viral isolation in cell culture, the limit of detection of the real-time TaqMan PCR was 100 times lower. Validation tests demonstrated the possibility of efficiently detecting betanodavirus in complex tissue substrates such as gonads without affecting the sensitivity of the method, providing a good alternative to the analysis of brain tissue and therefore avoiding the need to sacrifice precious spawners. This new method showed high specificity with no cross-reactions with other microorganisms, except for a weak nonspecific fluorescent signal with a high CP value (>37.5) detected when testing *Vibrio alginolyticus*. Since the mean CP value corresponding to the LoD is ≤36, the diagnostic "cutoff" limit was set at 36 CP. The real-time TaqMan PCR showed good intra-assay repeatability as well as good inter-assay reproducibility, as demonstrated by the low CV% values obtained (Table 4). Importantly, 100 clinical samples collected during an outbreak of VER that occurred at a fish farm with floating cages were tested by real-time TaqMan PCR. This appears to be the first time that a PCR test for betanodavirus has been extensively validated on naturally exposed animals during an outbreak of disease. During the clinical phase of the validation process, samples were simultaneously analyzed by RT-seminested PCR and viral isolation in SSN-1 cells. The concordance between the three tests was optimal and statistically significant. As confirmed previously by Thiery et al. [35] and Gomez et al. [14], we observed that betanodavirus diagnosis by molecular detection is much more sensitive than viral isolation in cell culture. However, molecular assays can only detect the presence of the viral genome and do not provide information about the vitality of the virus or its infectious potential. Therefore, molecular diagnosis coupled with virus isolation provides the most complete overview of the clinical case under investigation. Betanodaviruses are neurotropic agents, and the brain represents the target organ for diagnostic tests. During the clinical phase of the validation, most of the specimens gathered were from nervous tissue (brain, eye, head, larvae and juveniles), but other sample matrices were also collected (10 samples, Table 5), consisting of intestinal contents, bowel, faeces, eggs and sperm. Of these matrix samples, 5 out of 10 gave positive results in real-time PCR, and 4 out of 5 were confirmed by at least one other independent test (RT nested PCR, $n = 4$ confirmed cases; virus isolation, $n = 2$ confirmed cases) indicating that the real-time TaqMan PCR is suitable for the analysis of complex substrates. This is in agreement with the results obtained in the laboratory on different tissue matrices.

The probe-based technology applied in the present study is faster and provides higher specificity when compared to SYBR Green chemistry, avoiding the melting analysis, which occasionally yields dubious results. The good performance in terms of sensitivity and repeatability associated with the optimal efficiency and linearity of the reaction makes this test suitable for quantitative assays in studies on pathogenesis and vaccine efficacy, both in experimentally infected and naturally exposed fish.

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