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ORIGINAL ARTICLE

Investigation of betanodavirus in sea bass (*Dicentrarchus labrax*) at all production stages in all hatcheries and on selected farms in Turkey

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Abstract

Viral nervous necrosis (VNN) is one of the most important problems in sea bass culture. Although there have been many studies on detection and molecular characterization of betanodavirus, the causative agent of VNN, there has been little focus on understanding its prevalence to create epidemiological maps. The purpose of this study was to investigate the prevalence of betanodavirus in active sea bass hatcheries and on selected farms in Turkey by RT-qPCR. A total of 2460 samples, including fertilized eggs, prelarvae, postlarvae, fry, and fingerlings, were collected from 16 hatcheries to cover all production stages. A total of 600 sea bass were also collected from 20 farms. Betanodavirus was detected in one hatchery (1/16) in fingerling-sized sea bass, and the prevalence of betanodavirus at the hatchery level was calculated to be 6.25%. Betanodavirus was also detected on one farm (1/20) in fingerling-sized sea bass, and the prevalence of betanodavirus at the farm level was calculated to be 5%. Virus isolation initially could not be achieved in E-11 cells, but later, SSN-1 cells were used successfully. Partial genome sequence analysis of the RNA1 and RNA2 segments of the viruses revealed that they were of the red-spotted grouper nervous necrosis virus genotype, which is endemic in the Mediterranean basin. The absence of mortality related to VNN in the hatcheries and on the farms, the healthy appearance of the sea bass, the low viral load detected, and the results of retrospective epidemiological studies indicated that the infection was subclinical. Not detecting betanodavirus in other age groups where biosecurity was implemented indicates that there was no active infection. In light of these findings, it can be concluded that there was no betanodavirus circulating in hatcheries, and the virus might have been of seawater origin.

Introduction

Viral nervous necrosis (VNN) is a serious viral disease that causes significant losses in many marine fish species. It is associated with vacuolizations in the central nervous system and retina [1]. Many fish species with high economic importance have proven to be susceptible to betanodavirus infection, resulting in VNN becoming a serious problem in aquaculture over the last 30 years [2, 3]. The causative agent of VNN is betanodavirus, which belongs to family *Nodaviridae* [2]. Betanodaviruses have non-enveloped, rounded virions [4], and the genome consists of two segments, RNA1 and RNA2, which are positive-sense single-stranded molecules, and they contain three open reading frame (ORFs), two on RNA1 and one on RNA 2 [4–6]. RNA1 (3.1 kb) encodes an RNA-dependent RNA polymerase (RdRp) with a molecular weight of 110 kDa and carries all of the information necessary for autonomous replication [7, 8]. RNA1 also plays a role in regulating the temperature dependence of the virus. RNA2 (1.4 kb) encodes a capsid protein that is responsible for host tropism and immunoreactivity [4, 9–12]. RNA2 also contains the T4 multivariable region, which is used in the classification of genotypes [13]. A third transcript, known as RNA3 (0.4 kb), originates from the RNA1 terminus during viral replication. RNA3 is involved in the synthesis of viral RNA in the host cell and encodes the non-structural proteins B1 and B2. B2 (12 kDa) is an inhibitor of cell RNA silencing [8, 14, 15], and the B1 protein has been suggested to be an anti-necrotic-death factor [16].

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Betanodaviruses are grouped into four major genotypes based on phylogenetic analysis of the T4 variable region of the RNA2 segment. These include striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), and red-spotted grouper nervous necrosis virus (RGNNV) [53]. An isolate from a turbot (*Scophthalmus maximus*) was proposed to be a new genotype (turbot nervous necrosis virus [TNV]) by Johansen et al. [17]. However, these genotypes tend to be associated with a certain range of water temperatures rather than specific host species. Different genotypes have different host types and optimal *in vitro* growth temperatures [18–20].

Betanodaviruses are known to infect more than 120 species of farmed and wild fish and invertebrates from 30 different families belonging to 11 orders. Larvae and juvenile fish are especially sensitive to betanodavirus infection, and mortality up to 100% occurs in these fish in epidemic situations [18]. Although mortality is dependent on age, different studies have identified outbreaks of disease in both larval and market-sized populations in some fish species. Outbreaks have also been reported in market-sized Atlantic halibut, seven-band groupers (*Epinephelus septemfasciatus*), and sea bass [1]. VNN outbreaks are mostly seen in larvae and juvenile fish, and age is a major risk factor for susceptibility to disease [54]. Survivors can be subclinically infected for a long time [54, 60], and while subclinical infections are mostly asymptomatic, mortality can reach 60% in some circumstances, and typical VNN symptoms can be observed up to subadult ages [41, 61]. Since the disease first emerged in 1985, VNN has been reported throughout the world except South America. RGNNV is the largest geographic cluster and is found in the entire Mediterranean basin, USA, French Polynesia, Asia, and Australia [21–28].

There have been many studies on the detection and molecular characterization of betanodaviruses, but there are not enough studies to construct epidemiological maps. The fact that VNN is not a notifiable disease on the OIE (World Organisation for Animal Health) list or in the disease-reporting legislation of endemic countries makes it difficult to determine its prevalence within a country or the incidence rates within a population. However, in a small number of prevalence studies conducted in farmed or wild populations in different parts of the world, betanodavirus positivity was found at rates varying between 0.23% and 100% [25, 29–31, 54–56].

The aim of this study was to investigate the prevalence of betanodavirus using an epidemiological approach focused on all production stages in active sea bass hatcheries and on selected farms in Turkey and to perform phylogenetic analysis on any betanodavirus isolates obtained. A detailed survey was conducted to investigate the prevalence of betanodavirus to determine which production stage has the greatest risk with respect to the epidemiology of the virus. Retrospective and prospective studies were also carried out after initial sampling to better understand the relationships between viruses from different areas.

Materials and methods

Sampling of hatcheries

There were 21 sea bass hatcheries in Turkey according to the official records of 2016. Production cycles and whether the hatcheries were active or not were determined by official correspondence. Sampling plans were developed with the knowledge that 16 of the 21 hatcheries were in production (Table 1). All active sea bass hatcheries operating in Turkey were sampled in 2016-2017. Sampled hatcheries were numbered from K-1 to K-16. Sample sizes collected from hatcheries and farms were determined based on a 95% confidence interval, 10% estimated prevalence, and 100% sensitivity calculation [33]. Thirty samples were collected from hatcheries at each stage of production. A total of 2460 samples were collected from all production stages from all sea bass hatcheries in groups consisting of 10-30 fish. There were a total of 15 groups of fertilized eggs (FE) from eight hatcheries, 14 groups of prelarvae (preL) (0-5 days old) from nine hatcheries, 22 groups of postlarvae (postL) (5-40 days old) from 13 hatcheries, 43 groups of fry (40-80 days old) from 13 hatcheries, and 41 groups of fingerlings (80-120 days old) from nine hatcheries (Fig. 1) [32]. Water temperature was measured as 15 °C in FE tanks, 15-16 °C in preL tanks, 16-20 °C in postL tanks, 16-22 °C in fry tanks, and 20-24 °C in fingerling tanks (Table 2).

In determining the betanodavirus prevalence of the country, the hatchery in which the virus was detected, regardless of the age groups of the sea bass in the hatchery, was considered positive. In determining epidemiological units, since the aim was to assess the betanodavirus situation countrywide, the whole country was considered a unit, and the hatcheries were considered subunits.

Sampling of farms

There were a total of 418 seabass farms in Turkey. Twenty farms were selected randomly using an Excel programme for sampling. Thirty samples were collected from each farm based on a calculation of the 95% confidence interval, 100% sensitivity, and 10% estimated prevalence [33] totalling 600 fish collected across all selected farms. Farms were numbered from Y-1 to Y-20. Samples were collected from different batches representing all available age groups as much as possible. However, sample size were same in all batches. For example, if the farm had three different batches, 10 samples

Table 1 Dates of sampling at hatcheries

Hatchery code	Fertilized eggs	Prelarvae (0-5 days)	Postlarvae (5-40 days)	Fry (40-80 days)	Fingerling (80-120 days)
K-1	-	-	8-10 May 2017	8-10 May 2017	8-10 May 2017
K-2	-	21 December 2016	21 December 2016	02 August 2017	-
K-3	-	31 May 2017	31 May 2017	31 May 2017	-
K-4	-	-	31 May 2017	31 May 2017	31 May 2017
K-5	-				02 August 2017
K-6	13 February 2017	13 February 2017	13 February 2017	28 March 2017	28 March 2017
K-7	28 March 2017	-	28 March 2017	28 March 2017	-
K-8	13 February 2017	13 February 2017	13 February 2017	13 February 2017	13 February 2017
K-9	-	-	28 March 2017	28 March 2017	28 March 2017
K-10	-	-	March 2017	-	-
K-11	4 January 2017	4 January 2017	-	-	-
K-12	16 March 2017	16 March 2017	16 March 2017	16 March 2017	-
K-13	3 January 2017	3 January 2017	3 January 2017	7 March 2017	7 March 2017
K-14	-	-	-	24 April 2017	24 April 2017
K-15	13 December 2016	13 December 2016	12 April 2017	12 April 2017	-
K-16	15 February 2017	15 February 2017	15 February 2017	31 July 2017	31 July 2017

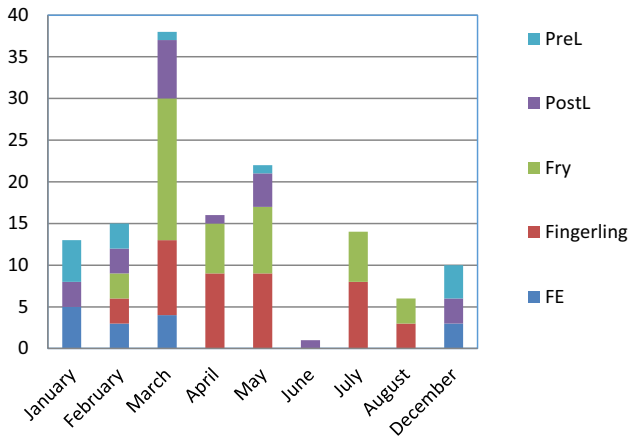

Fig. 1 Distribution of sea bass sampled from hatcheries for betanodavirus detection by month. FE, fertilized egg; PreL, prelarvae; PostL, postlarvae. The number of samples in the pool were as follows: egg, >30 individuals; PreL and PostL, 10-30 individuals; fry and fingerling, 10 individuals

were collected from each. If the farm had six batches, five samples were collected from each. Fish were collected from cages using fishing rods or strainers, according to size. The length and weight of the each fish were measured. The length and weight of the sea bass were 5.5-45 cm and 3.2-950 g, respectively.

A questionnaire was used during the sampling of hatcheries and farms. Geographical location, the length of time they had been in business, other farms or hatcheries they were cooperating with, disease background, mortality observations, and current water temperature at the time of sampling were all written on the form.

Retrospective epidemiological study

In order to better understand the prevalence of betanodavirus in the country, a retrospective epidemiological study was carried out in hatcheries and on farms where the virus was detected, and also in related facilities. The study noted the rearing status of the fish, the stage at which the virus was active, and possible sources of contamination. For this

Table 2 Information about samples collected from hatcheries for detection of betanodavirus in Turkey in 2016-2017

Production stage	Age (days)	Water temperature (°C)	Number of hatcheries[a] sampled	Number of groups	Tissue type
FE	0	15	8	15	Pooled eggs
PreL	0-5	15-16	9	14	Pooled preL
PostL	5-40	16-20	13	22	Pooled postL
Fry	40-80	16-22	13	43	Head
Fingerling	80-120	20-24	9	41	Brain and eyes

[a]Sampling was carried out according to the production type of the hatchery

purpose, resampling was carried out from the hatcheries and farms as described above.

Preparation of the samples

Homogenates were obtained from whole-body samples in FE, preL, and postL; whole head in fry; and brain, spinal cord and eye from fingerlings and larger fish. All samples were homogenized using a mortar and pestle and sterile sand (sea sand, Merck, Germany, CAS-No: 14808-60-7) and suspended in Eagle's minimal essential medium (EMEM, Sigma-Aldrich, United Kingdom, product no M4655) supplemented with 2% fetal calf serum (Biochrom, Germany) and 1% antibiotic-antimycotic solution (Sigma-Aldrich, USA) at a ratio of 1:5 (w/v). The homogenates were clarified by centrifugation at 4000 *g* for 15 min at +4 °C and percolated with 0.45-µm filtrate (Sartorius, USA) for use in molecular and virological studies. Inocula were stored at -80 °C until used.

Virus isolation

Striped snakehead fish fry (SSN-1) and E-11 (cloned from SSN-1) cell lines were used for virus isolation. Twenty-four-well cell culture plates with SSN-1 or E-11 cells were prepared using 1 ml of Leibovitz-15 (L-15) medium (Gibco, USA, Ref: 11415-049) supplemented with 5% fetal calf serum and 1% antibiotic-antimycotic solution (Sigma, USA, product number A5955) per well. Positive samples were inoculated onto 24-hour-old cell cultures at 85-90% confluency at 1:10 and 1:100 dilutions. Two wells in each plate were not inoculated and used as negative controls, while two wells in each plate were inoculated with reference positive controls. The plates were incubated at 25 °C and were examined daily for cytopathic effect (CPE) for 10 days. Serial passages were made by the freeze/thaw method after 10 days of inoculation [34, 35].

Molecular studies

Viral RNA extraction

Viral RNA was extracted using a commercial extraction kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, Germany, product no. 03038505001) and an automatic extraction device (Roche MagNA Pure LC System, Germany). Two hundred microliters of clarified homogenate was used for the extraction process.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (RT-qPCR) was performed using a commercial kit (Real Time Ready Virus Master, Roche, cat. no. 05 992 877 01) and a real-time PCR device (Roche LightCycler 480 Multiwell Plate 96). Primers and probes [36] designed based on the T4 variable region of the RNA2 segment of betanodavirus were used for amplification as proposed by OIE [37]. The master mix was prepared on ice according to the recommendations of the kit manufacturer with 7.6 µL of H_2O, 0.4 µM F primer, 0.4 µM R primer, 0.4 µM probe, 4 µL of 5x buffer, and 0.4 µL of enzyme for each sample. Fifteen µL of master mix was placed in each well of a 96-well real-time PCR plate (Roche LightCycler® 480 Multiwell Plate 96, White, Germany, Ref: 04 729 692 001), followed by 5 µL of sample. Positive and negative controls were included. The total volume was 20 µL. The plate was centrifuged at 1500 *g* at 4 °C for 2 minutes, and the RT-qPCR test was performed. The cycling conditions were as follows: a single cycle of 10 minutes at 50 °C for reverse transcription, followed by 65 seconds at 95 °C and 45 cycles of 10 seconds at 95 °C, 30 seconds at 54 °C, and 1 second at 72 °C. RT-qPCR test was optimised before testing field samples. The RT-qPCR optimisation values were as follows: efficiency, 98%; sensitivity, 100%; specificity, 100%; slope, -3.373; R^2, 0.98. In each run, 5 µL of plasmid pTZ57R (Thermo Scientific, USA) containing the T4 variable region of the RNA2 segment (2.82×10^1 to 2.82×10^6 copies/mL) was used as a standard for quantitation.

cDNA synthesis, PCR, and sequencing

cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit, Roche, Germany, no. 04 379 012 001) was performed using RNA extracts from positive samples. The cDNA was then amplified by PCR using a commercial kit (FastStart High Fidelity PCR System, dNTPack Roche, Germany, cat. no. 04 738 292 001) (Techne TC-412, United Kingdom) for partial sequencing. PCR primers for partial genome sequence analysis were used as recommended by Toffolo et al. [28] and Bovo et al. [38], and partial sequencing of RNA1 and RNA2 segments was performed commercially (Microsynt, Balgach, Switzerland). Sequences were corrected and matched using the DNADynamo program (Blue Tractor Software Ltd., UK). The consensus nucleotide sequences obtained were compared and verified using the Basic Local Alignment Search Tool (BLAST) system at the National Central for Biotechnology Information (NCBI) [39]. For phylogenetic analysis, a multiple sequence alignment of RNA1 and RNA2 partial nucleotide sequences of the isolates and reference sequences obtained from the GenBank database (Supplementary Table S6) were performed by the Clustal W method using the MEGA6 program. The best protein/DNA model for both segments and the percent nucleotide sequence identity between sequences were determined. Phylogenetic trees were created by the neighbor-joining (NJ) method using 1000 bootstrap repetitions [40].

Results

Epidemiological observations

VNN was not suspected in the sea bass hatcheries or farms during the sampling process in 2016 and 2017. External and internal macroscopic pathological findings were not found during the preparation of the fish for the laboratory tests.

Detection of betanodavirus in sea bass hatcheries

Betanodavirus was detected in 1 of 16 hatcheries (K-1) in fingerling-sized sea bass that were 115 days old, 2.5-3.74 g in weight, and 6.5-7 cm in length. The isolate was named TR.VNN.01.02. Three pools, each consisting of 10 fingerling-sized sea bass collected from the K-1 hatchery, were found to be positive for betanodavirus. Using an epidemiological approach, the betanodavirus prevalence in Turkish hatcheries was calculated to be 6.25% (1/16). In the K-1 hatchery, betanodavirus was detected in May when the seawater temperature started to rise. No virus was detected in postL and fry fish collected from the K-1 hatchery during the same period. The facility was a closed system with biosecurity measures, and the seawater was filtered and ozone- and UV-sterilized. Once fingerlings exceeded 1.5-2 g, they were taken to an external system in which the seawater did not undergo the same treatment.

Investigation of betanodavirus on sea bass farms

Betanodavirus was detected on one of 20 farms (Y-20) in sea bass that were 3.2-10.4 g in weight and 6-10 cm in length. The isolate was named TR.VNN.01.01. The betanodavirus prevalence on sea bass farms in Turkey was calculated to be 5% (1/20). Randomly selected sea bass farms for investigation of betanodavirus infection were located on the Aegean and Mediterranean coasts. The Aegean coast has approximately 85% of the total sea bass farms of Turkey, whereas the Mediterranean coast has only 5%. The locations of the sampled farms, the sizes of the fish, and the water temperature are shown in Table 3. The sampling was conducted to include an almost equal number of fish from all available age groups from the farms. The water temperature during the sampling period was measured to be 24-26 °C in the Aegean Sea and 28-30 °C in the Mediterranean Sea, which are suitable temperatures for betanodavirus replication.

Retrospective epidemiological study

When testing other samples collected from the K-1 hatchery in the same period, no viruses were detected in 15-day-old postL and 70-day-old fry. A monitoring study was carried out in the K-1 hatchery, resampling the preL and postL sea bass (September 2017); however, no virus was detected in these samples. The K-1 hatchery did not have a spawning program, and the reared fish were obtained from the K-12 hatchery as 5- to 10-day-old preL sea bass. In the epidemiological study carried out in the K-12 hatchery, it was determined that spawners, fertilized eggs, and preL were reared. Betanodavirus was not detected in fertilized eggs or 2-day-old preL sampled from the K-12 hatchery in January 2017 and in December 2018. No clinical signs of VNN disease and no abnormal mortality were observed in either hatchery. It was determined that the Y-20 farm was associated with the K-1 hatchery, and the fingerling-sized sea bass that came out of this hatchery came to the Y-20 farm. Later, it was determined that the Y-20 farm was not an entirely marine farm; it was an intermediate post-hatchery where fish were sent from the hatchery to be distributed to other farms, and there were no sea bass older than 3-4 months.

Determination of viral load

In the RT-qPCR test performed on direct tissue homogenisates of positive samples, the Ct value and viral load for the TR.VNN.01.01 isolate were 32.19 and 3.10×10^2 copies/µL, respectively. For the TR.VNN.01.02 isolate, these values were 31.56 and 4.82×10^2 copies/µL, respectively. The RT-qPCR test was performed again after virus isolation in cell culture. The Ct value and viral load for the TR.VNN.01.01 isolate were then 20.96 and 7.29×10^5 copies/µL, respectively, and for the TR.VNN.01.02 isolate, they were 24.87 and 4.83×10^4 copies/µL, respectively. Virus isolation in cell culture of field samples resulted in an increase in the Ct value and viral load of 7-12 and 10^2-10^3 times, respectively.

Sequencing and phylogenetic analysis

Positive samples were found to contain the RGNNV genotype when phylogenetic analysis was performed using sequences of both the RNA1 and RNA2 segments (Figs. 2 and 3). Partial sequences of 933 nt (nt 192-1124) and 937 nt (nt 197-1133) were obtained for the RNA1 segment of TR.VNN.01.01 and TR.VNN.01.02, respectively, and for RNA2 of TR.VNN.01.01 and TR.VNN.01.02, partial sequences of 569 nt (nt 355-923) and 567 nt (nt 365-931) were obtained. These sequences were uploaded to the GenBank database, and were assigned the accession numbers MT451939 and MT451940 for the RNA1 segment and MT451941 and MT451942 for the RNA2 segment. Shorter regions with a length of 899 nt for the RNA1 segment and 480 nt for the RNA2 segment were used in order to compare more isolates in phylogenetic

Table 3 Information about samples collected from sea bass farms for detection of betanodavirus in Turkey in 2016-2017

Location	Farm code	Number of samples[a]	Weight (g)	Length (cm)	Sampling date	Water temperature (°C)
Aegean Sea	Y-1	10	850-950	40-45	29.09.2016	25
Aegean Sea	Y-1	20	300-600	30-35	29.09.2016	
Aegean Sea	Y-2	10	400-600	30-35	23.08.2016	27
Aegean Sea	Y-2	10	200-300	25-30	23.08.2016	
Aegean Sea	Y-2	10	30-50	10-15	23.08.2016	
Aegean Sea	Y-3	20	200-350	25-30	23.08.2016	26
Aegean Sea	Y-3	10	30-50	10-15	23.08.2016	
Aegean Sea	Y-4	20	200-300	25-30	23.08.2016	27
Aegean Sea	Y-4	10	30-50	10-15	23.08.2016	
Aegean Sea	Y-5	30	30-50	10-15	20.09.2016	24
Aegean Sea	Y-6	10	300-400	30-35	23.08.2016	26
Aegean Sea	Y-6	20	30-50	10-15	23.08.2016	
Aegean Sea	Y-7	10	600-850	35-40	23.08.2016	27
Aegean Sea	Y-7	10	250-350	25-30	23.08.2016	
Aegean Sea	Y-7	10	30-50	10-15	23.08.2016	
Aegean Sea	Y-8	20	300-600	30-35	23.08.2016	27
Aegean Sea	Y-8	10	40-80	15-20	23.08.2016	
Aegean Sea	Y-9	10	600-850	35-40	23.08.2016	26
Aegean Sea	Y-9	10	250-350	25-30	23.08.2016	
Aegean Sea	Y-9	10	40-80	15-20	23.08.2016	
Mediterranean Sea	Y-10	20	200-400	30-35	24.08.2016	30
Mediterranean Sea	Y-10	10	150-200	20-25	24.08.2016	
Aegean Sea	Y-11	30	600-850	35-40	05.09.2016	25
Aegean Sea	Y-12	30	250-350	25-30	05.09.2016	25
Aegean Sea	Y-13	30	80-100	15-20	05.09.2016	25
Aegean Sea	Y-14	10	400-600	35-40	06.09.2016	25
Aegean Sea	Y-14	10	200-250	20-25	06.09.2016	
Aegean Sea	Y-14	10	30-50	10-15	06.09.2016	
Mediterranean Sea	Y-15	30	20-40	10-15	20.09.2016	28
Aegean Sea	Y-16	20	200-400	25-30	07.09.2016	26
Aegean Sea	Y-16	10	30-50	10-15	07.09.2016	
Aegean Sea	Y-17	10	250-350	25-30	07.09.2016	25
Aegean Sea	Y-17	10	150-200	20-25	07.09.2016	
Aegean Sea	Y-17	10	20-30	10-15	07.09.2016	
Aegean Sea	Y-18	15	300-400	30-35	07.09.2016	25
Aegean Sea	Y-18	15	30-50	15-20	07.09.2016	
Aegean Sea	Y-19	15	250-350	25-30	27.09.2016	24
Aegean Sea	Y-19	15	300-400	30-35	27.09.2016	
Mediterranean Sea	Y-20	30	3.2-10.4	6-10	20.09.2016	29

[a]Thirty samples were collected from each farm. Samples were divided according to rearing stage. Samples were collected to represent almost all rearing stages found on the farms

analysis. Nucleotide differences between the isolates obtained in this study and reference isolates obtained from GenBank were observed in both RNA segments (Tables 4 and 5). TR.VNN.01.01 and TR.VNN.01.02 showed 99.77% sequence identity in the RNA1 segment. The closest relative of the TR.VNN.01.01 isolate based on partial sequences of the RNA1 segment was a betanodavirus isolated from sea bass in Cyprus, with

99.21% nucleotide sequence identity over 899 bases. The TR.VNN.01.01 isolate was found to be similar to isolates from three different freshwater fish in Italy and a betanodavirus obtained from striped lahose in Greece, with 98.74% and 98.62% identity, respectively (Table 4). The nucleotide sequences from the RNA2 segment of TR.VNN.01.01 and TR.VNN.01.02 were found to be 100% identical. The closest relative of the TR.VNN.01.01 isolate based on partial sequences of the RNA2 segment were a betanodavirus isolated from sea bass in Cyprus, with 99.58% nucleotide sequence identity over 480 bases, followed by a betanodavirus obtained from striped lahose in Greece and a betanodavirus isolated from three different freshwater fish in Italy, with 99.37% and 99.15% identity, respectively (Table 5).

Virus isolation

Positive samples were first inoculated onto E-11 cell culture, but no cytopathic effect (CPE) was observed. In addition, a total of nine blind passages were performed by freeze-thawing in the E-11 cell line, but no CPE was observed in any passage. RT-qPCR tests were performed at the end of each passage, and no viral nucleic acid was detected. Positive samples were then inoculated onto the SSN-1 cell line and CPE consist of vacuolation and lysis was observed after day 4. An RT-qPCR test was performed after CPE was observed, and viral nucleic acid was detected. CPE-positive SSN-1 cell culture supernatants were re-inoculated onto the E-11 cell line, and the virus was isolated in the E-11 cell line by ensuring virus adaptation at the end of two passages.

Discussion

VNN is the biggest challenge to the sustainability and development of aquaculture, and it poses a significant risk to aquaculture globally [3, 18]. Turkey produces 42% of the world's sea bass [42]. It is in a strategic position in terms of sea bass production both in Europe and worldwide, and sea bass represent 32.8% of all fish species reared in Turkey [43]. There is a lack of studies on betanodavirus in Turkey despite its importance as a sea bass pathogen. Here, we investigated betanodavirus at all production stages in active sea bass hatcheries and on select farms to gain a better understanding of the epidemiology of the virus. The first survey of its kind in Turkey was to investigate the presence or absence of betanodavirus conducted in 29 sea bass and sea bream farms and hatcheries in 2010 and 2011, where the virus was not detected [57]. The first detection of betanodavirus in Turkey was from sea bass during an outbreak on a farm on the Mediterranean coast in 2011, and this virus was genotyped as RGNNV [44]. Subsequently, the RGNNV genotype betanodavirus was detected in sea bass and sea bream in monitoring and screening studies conducted in 2012 and 2014 in the Aegean Region in the west of Turkey [45]. In another monitoring study, betanodavirus was first detected in the Black Sea on a sea bass farm in 2016, but the virus was not detected again in subsequent screening studies. The researchers suggested that the virus might have originated from the Mediterranean region as a result of the transfer of juvenile fish to the farm [58]. The few studies performed in the past were either regional or the investigation of clinical infections. In the light of these findings, it was necessary to investigate the presence of betanodavirus in Turkey more broadly because of the country's high sea bass production capacity. We tested all active sea bass hatcheries and selected farms in Turkey and obtained samples from all stages of production from eggs to market-sized fish.

In this study, betanodavirus was detected in only one of the 16 hatcheries, in fingerling-sized fish that were 115 days old, 2.5-3.74 g in weight, and 6.5-7 cm in length. The virus was not detected in FE, preL, postL, or fry-sized fish, which suggests the absence of persistent virus infection in spawners. All of the units, with the exception of the fingerling unit, were closed systems that implemented strict biosecurity measures. When the methods used to decontaminate the water in the hatcheries were evaluated, it was concluded that they were adequate to eliminate virus. Previously, Hick et. al. investigated betanodavirus infections in a barramundi hatchery to determine the stage of growth at which infection and virus transmission occurred. They found that a failure in biosecurity measures could result in outbreaks and that age was a risk factor for betanodavirus infection [54]. Thus, biosecurity measures are strongly recommended to prevent the spread of betanodavirus infections [18, 54, 59]. In this study, viruses detected in hatcheries or on farms were isolated from fish in units outside the closed system where seawater was used directly. It is likely that the viruses were acquired through horizontal transmission from seawater, where the virus is endemic to the region. Because, betanodavirus was not detected in any of the units containing FE, preL, postL, and fry, nor in any hatchery where biosecurity measures were implemented, these results suggest that spawners might be free from betanodavirus. However, this was not confirmed in the present study, as spawners were not tested virologically or serologically.

It has been reported that water temperature affects the activity of the RdRp, which is necessary for the replication of the virus, and that different optimum temperatures are required for the epidemiology of different genotypes [31]. It is known that betanodaviruses are highly resistant to both freshwater and seawater environments and also to the external environment [47]. For this reason, larvae or fry-sized sea bass could be exposed to the virus through horizontal transmission on farms or in hatcheries using

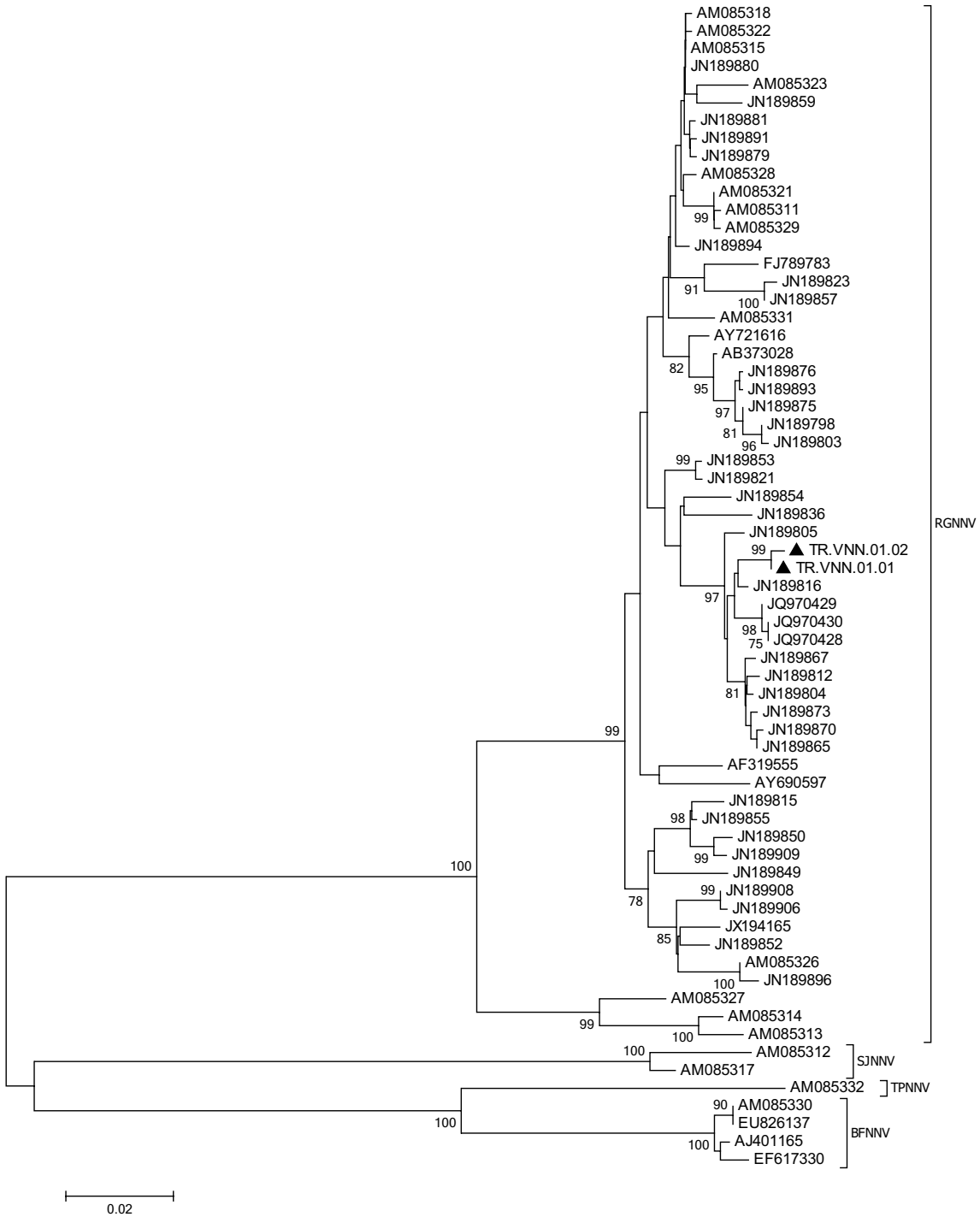

Fig. 2 Phylogenetic tree based on partial sequences of the RNA1 segment. The tree was constructed based on anaylsis of 899 bp of the RNA1 segment (nt 200-1099 of GenBank accession number FJ789783) by the NJ method with the TN93+G model and 1000 bootstrap replicates. Bootstrap values >70% are shown.

contaminated seawater [48]. Water temperatures for the hatcheries in the study were lower than the optimum growth temperature of the RGNNV genotype. However, the main purpose of the study in hatcheries was to detect the vertical transmission of the virus that could be found persistently in spawners and any virus that could be found in the environmental water.

It is known that age is major risk factor for betanodavirus infection. Although VNN outbreaks can occur in market-sized fish, it is accepted that larvae or juvenile sea bass are

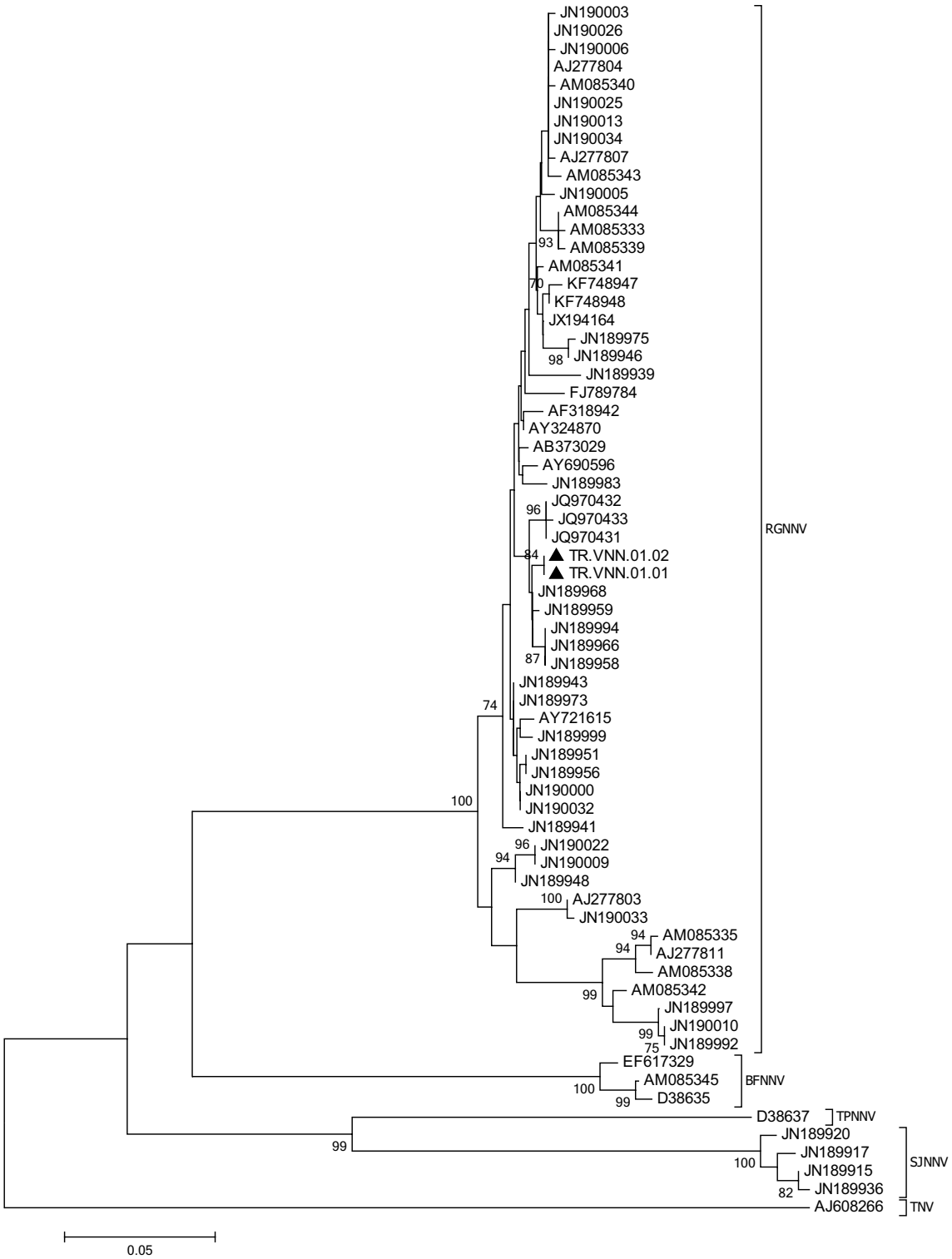

Fig. 3 Phylogenetic tree based on partial sequences of the RNA2 segment. The tree was constructed based on anaylsis of 480 bp of RNA2 segment (nt 426-907 of GenBank accession number FJ789784) by the NJ method withthe K2+G model and 1000 bootstrap replicates. Bootstrap values >70% are shown.

Table 4 Nucleotide sequence identity of partial RNA1 segments (%)*

İsolate	% Identity											Country
TR.VNN.01.01	100.00											Turkey
TR.VNN.01.02	99.77	100,00										Turkey
JN189816	99.21	98.97	100.00									Cyprus
JQ970429	98.74	98.50	99.32	100.00								Italy
JQ970428	98.62	98.38	99.21	99.89	100.00							Italy
JQ970430	98.62	98.38	99.21	99.89	100.00	100.00						Italy
JN189867	98.62	98.38	99.21	98.97	98.85	98.85	100.00					Italy
JN189805	98.62	98.38	99.21	98.97	98.85	98.85	99.09	100.00				Greece
AM085317	73.14	73.42	73.42	72.96	72.76	72.76	73.89	74.08	100.00			SJNNV
AM085330	71.56	71.85	72.15	71.76	71.55	71.55	71.85	71.85	74.13	100.00		BFNNV
AM085332	71.74	72.01	71.59	71.24	71.04	71.04	71.31	71.74	73.33	88.54	100.00	TPNNV
	TR.VNN.01.01	TR.VNN.01.02	JN189816	JQ970429	JQ970428	JQ970430	JN189867	JN189805	AM085317	AM085330	AM085332	

*Only the six most closely related RGNNV isolates and other genotypes of betanodavirus were used for comparison

Table 5 Nucleotide sequence identity of partial RNA2 segments (%)*

İsolate	% Identity											Country
TR.VNN.01.01	100.00											Turkey
TR.VNN.01.02	100.00	100.00										Turkey
JN189968	99.58	99.58	100.00									Cyprus
JN189959	99.37	99.37	99.79	100.00								Greece
JN189994	99.15	99.15	99.58	99.37	100.00							Italy
JN189966	99.15	99.15	99.58	99.37	100.00	100.00						Italy
JN189958	99.15	99.15	99.58	99.37	100.00	100.00	100.00					Italy
JQ970432	98.94	98.94	99.37	99.16	98.94	98.94	98.94	100.00				Italy
JN189920	65.47	65.47	65.47	64.97	65.96	65.96	65.96	64.39	100.00			SJNNV
AM085345	73.80	73.80	73.80	74.21	73.80	73.80	73.80	72.90	57.12	100.00		BFNNV
D38637	65.39	65.39	65.39	64.91	65.87	65.87	65.87	66.29	72.70	64.88	100.00	TPNNV
	TR.VNN.01.01	TR.VNN.01.02	JN189968	JN189959	JN189994	JN189966	JN189958	JQ970432	JN189920	AM085345	D38637	

*Only the six most closely related RGNNV isolates and other genotypes of betanodavirus were used for comparison

more susceptible to betanodavirus infection. This epidemiological aspect has been shown in several studies [1, 18, 54, 59, 60]. Although larval and juvenile fish are more susceptible to VNN disease, subclinical infection may develop in individuals that survived the disease or were infected after the susceptible period [54, 59, 60, 62]. Jaramillo et al. showed that clinical signs occur up to 5 weeks of age in Asian sea bass [62]. Toffan et al. reported that, in sea bream experimentally infected with an RGNNV/SJNNV reassortant, clinical signs did not occur after 21 days of age and that juveniles at 75 days of age did not show clinical signs after experimental infection and were subclinically infected with the virus for about 1 year [60]. Hick et al. reported that the virus causes clinical signs in Asian sea bass until 27 days of age, after which subclinical infection occurs, and it continues for up to 18 months, but the infection resolves and the prevalence decreases over time [54]. Subclinical infection is mostly asymptomatic, but in some cases, it has been shown that it could cause epidemics in fish older than juveniles, as the virus is reactivated by the effect of environmental changes [41, 61, 63, 64]. In this study, both viruses, isolated from a hatchery and a farm, originated from fingerling-sized sea bass. The conditions in the hatchery are already known, and if the virus is endemic in the region and seawater is used without any biosecurity measures, it may not be surprising to detect the virus due to horizontal contamination, since larvae and juvenile-sized fish are already grown. The ages of the fish collected in the farm survey were representative of almost all stages, and the weight range was from 3.2 g to 950 g (Table 3). Samples were collected from different locations with different water temperature and on different dates, but betanodavirus was detected in the smallest-sized sea bass collected in the farm survey. This supports the earlier hypothesis that age is an important factor in susceptibility to betanodavirus infection.

Since VNN is not a notifiable disease in animal disease reporting systems such as OIE and the Animal Disease Notification System (ADNS), it is difficult to evaluate the epidemiological situation in individual countries. However, a review of previous reports reveals that VNN is endemic in the Mediterranean basin. When the few studies conducted to determine epidemiological prevalence of betanodavirus are examined, it can be seen that it ranges from 0.23% to 100% [25, 29–31, 46, 54–56]. Here, we investigated the prevalence of betanodavirus in all hatcheries and on selected farms in Turkey using an epidemiological approach. The prevalence of betanodavirus in hatcheries and on farms was found to be 6.25% and 5%, respectively. It is very difficult to compare the results of this study with other results because of differences such as location, target population, framework of the study, sample size, sensitivity, specificity, and whether the study was epidemiologically based. However, evaluating the marginal benefit provided by this study, all of the hatcheries were investigated, and the positivity rate was 6.25%. All production stages in the hatcheries were included in the study, and fingerling-sized sea bass were observed to be more susceptible in facilities lacking biosecurity measures. The geographical distribution of the farms sampled in the study was more homogeneous than that of the hatcheries, considering their location on the Mediterranean or Aegean coast. Fifteen of the 16 hatcheries and 85% of the farms in Turkey were located in the Aegean Sea. Water temperatures rise earlier and decrease later in the Mediterranean than in the Aegean Sea and Black Sea, conferring a longer period for betanodavirus replication. During sampling of farms in this study, the water temperatures were measured to be 24-27 °C in the Aegean and 28-30 °C in the Mediterranean. These temperatures are conducive to replication of the RGNNV genotype. Although 17 of the 20 farms sampled were located in the Aegean, no virus was detected on any of them. Although only three farms on the Mediterranean coast were sampled in this study, one of them was found positive. Previous studies have demonstrated that betanodavirus infection in both farmed and wild fish is endemic in the Mediterranean basin [3, 41], and RGNNV is the most frequently detected genotype in the Mediterranean [18]. It is therefore necessary to continue screening and monitoring studies on betanodavirus epidemiology in farmed and wild fish in the Mediterranean.

Viral loads of the betanodaviruses detected in this study were low. Betanodaviruses cause VNN disease in more than 120 fish species, and there are four generally accepted genotypes [3, 19, 20]. However, isolates of the same genotype from different locations have been shown to have different pathogenicity in different fish species [49]. It has been observed that a certain viral load in the tissue is necessary to cause pathology [49–51]. In experimental infection in sea bass, it was shown that clinical signs did not occur up to $10^{6.55}$ $TCID_{50}$, 8.96×10^8 copies of RNA2, and 1.21×10^7 copies of RNA1 in brain samples. It was determined that those that remained alive and did not show clinical signs carried $10^{4.55}$ $TCID_{50}$, 1.29×10^7 copies of RNA2, and 6.25×10^5 copies of RNA1 [52]. The $TCID_{50}$ used in experimental infections appears to be $10^{4.55}$ [52] and 10^4 [49]. The viral loads observed in this study were between 3.10×10^2 and 4.82×10^2, which is thought to be insufficient to cause disease. This hypothesis is supported by the absence of any disease in the fish from which the two isolates were obtained and the absence of VNN-related mortality at the farms or hatcheries.

In this study, samples found positive by RT-qPCR were used to inoculate E-11 cells, but no virus was isolated. Subsequently, isolation was accomplished using the SSN-1 cell line. In a study conducted by Sakamoto et al. [25] for the detection of betanodavirus in wild marine species, samples found positive by nested PCR could not be isolated using E-11 cells. The researchers attributed this to the fact that

positive samples either did not have a sufficient virus titer to generate CPE or that no infectious virions were present despite detection of viral RNA [25]. Panzarin et al. [36] compared the rates of virus isolation in cell culture and positive RT-qPCR test results and reported that although the method of inoculation was unknown, inoculation with samples with a Ct value ≥ 31 did not result in growth in the SSN-1 cell line. In this study, all of the samples were collected from healthy-looking sea bass and the low Ct values of the positive samples were attributed to a low virus titer. The compatibility of field and laboratory data supports the hypothesis that there was no active infection on the farms or in the hatcheries where the virus was detected, and this may be due to a low viral load. However, in contrast to the study of Sakamoto et al. [25], we used the SSN-1 cell line in addition to the E-11 cell line, and virus isolation was achieved. Thus, although it has been reported that the E-11 cell line is more suitable for quantitative tests such as virus titration [19], it appears to be unsuitable for virus isolation when the viral load is low.

In conclusion, the prevalence of betanodavirus was investigated in all age groups in sea bass hatcheries and on sea bass farms in Turkey and found to be 6.25% and 5%, respectively. Betanodavirus was detected in juvenile fish, as reported previously, and more care should therefore be taken to prevent betanodavirus infection in juvenile fish. It was concluded that there was no active infection in the juvenile sea bass and that they might have been subclinically infected, since the viral load was low and no clinical signs were observed. It has been suggested that biosecurity measures prevent water-borne transmission of the virus, and untreated seawater should not be used in hatcheries or on farms. Finally, the SSN-1 cell line should be used in addition to the E-11 cell line for isolation of betanodavirus in cases of subclinical infection. However, more-specific studies are needed to determine the different cell culture adaptations of betanodaviruses detected in persistent or subclinical infections.

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Data availability statement All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Availability of data and material The datasets generated for this study can be found in the NCBI database (MT451939 MT451940 for the RNA1 segment and MT451941 and MT451942 for the RNA2 segment of the detected betanodaviruses).

Ethical approval This study was conducted with permission from the Ethics Committee on Animal Experiments of İzmir/Bornova Veterinary Control Institute (permission date, 22.12.2015; permission number, 28).

Consent for publication The manuscript was approved and officially approved by the Ministry of Agriculture and Forestry (permission date, 11.12.2015; permission number, 55016929-774-38909).

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