# Prevalence of Betanodavirus in Sea Bass (*Dicentrarchus Labrax*) for All Production Stages in Turkey

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**Original Article**

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## Abstract

Viral Nervous Necrosis (VNN) is one of the most important problems in sea bass aquaculture. Although there are many studies in the world on detection and molecular characterization of betanodaviruses causative agent of VNN, there are not enough studies related to prevalance to provide epidemiological maps. The purpose of this study was to investigate the betanodavirus prevalence in all bass hatcheries actively operating and in some farms in Turkey by RT-qPCR. A total of 2460 samples including fertilized egg, prelarva, postlarva, and fingerling were collected from 16 hatcheries as covering all production stages. A total of 600 sea bass were also collected from 20 farms. Betanodavirus was detected in a hatchery and a farm in fingerling sized sea bass and prevalances were found to be 6.25% and 5%, respectively. Virus isolation initially could not be achieved in E-11 but later SSN-1. Partial genome analysis of both RNA1 and RNA2 segments of the viruses, revealed that they were Redspotted grouper nervous necrosis virus genotype endemic in the Mediterranean basin. The absence of mortality related to VNN in the hatchery and farm, the healthy appearance of the sea bass, the low viral load detected and also output of retrospective epidemiological studies indicate that the infection is subclinical. Not detecting betanodavirus in other age groups where biosafety is implemented indicates that there is no active infection. In the light of these findings, it can be concluded that there are no circulating betanodavirus in hatcheries and the detected virus could be of sea-water origin.

## 1. Introduction

Viral Nervous Necrosis (VNN) is a serious viral disease that causes significant losses in many marine fish species associated with vacuolizations in the central nervous system and retina [1]. Many fish species with high economic importance have proven to be susceptible to VNN virus that have become a serious problem in aquaculture for the last thirty years [2, 3]. Causative agent of VNN is betanodavirus which belong to nodaviridae family [2]. Betanodaviruses have a non-enveloped, rounded morphology [4] and its genome consists of two segments, RNA1 and RNA2, which are single-stranded, positive polarity and contain three open reading frame-ORFs [4–6]. RNA1 (3.1 kb) is responsible for the synthesis of RNA-dependent RNA polymerase (RdRp) with a molecular weight of 110 kDa and carries all the information necessary for autonomous replication [7, 8]. RNA1 also plays a role in regulating the temperature sensitivity of the virus. RNA2 (1410 bp) is responsible for host tropism and immunoreactivity [4, 9–12]. RNA2 also contains the T4 multivariable region used in the classification of genotypes [13]. The third transcript, known as RNA3 (0.4 kb) originated from the RNA1 terminus during viral replication, is involved in the assembly of viral RNA in the host cell and encode non-structural B1 and B2 proteins. B2 (12 kDa) is an inhibitor of cell RNA silencing [8, 14, 15]. B1 protein has been suggested to be an antinecrotic death factor [16].

Betanodaviruses are grouped into four major genotypes based on phylogenetic analysis of the T4 variable region of the RNA2 segment including 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). A new genotype isolated from a turbot (*Scophtalmus maximus*) has been proposed as 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 species specific. Different genotypes have different host types and optimum *in vitro* growth temperatures [18–20].

Betanodaviruses are known to be influence on more than 120 species of farmed, wild fish and invertebrates from 30 different families belonging to 11 different order. Especially larvae and juvenile sized fish are sensitive to betanodaviruse and mortality of up to 100% occurs in these fish in epidemic situations [18]. Although mortality is dependent on age, different studies have identified both larval and market sizes of disease in different fish species [1]. VNN has been reported in many parts of the world except South America since the disease first emerged in 1985. RGNNV is the largest geographic cluster and is found in the entire Mediterranean basin, USA, French Polynesia, Asia and Australia [21–28].

There are many studies on the detection and molecular characterization of VNN virus in the world, however, there are not enough studies to construct epidemiological maps. The fact that VNN is not a notifiable disease in the OIE list or in the disease reporting legislation of the countries, it can not be reached to country prevalence and incidence information about the disease. 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% – 88.5% [25, 29–31]. The aim of this study is to investigate of betanodavirus prevalance based on epidemiological approach in all hatcheries and some farms in Turkey.

## 2. Materials And Methods

## 2.1. Sampling of hatcheries

All sea bass hatcheries registered and actively operating and some sea bass farms in Turkey were sampled in 2016–2017. Sixteen hatcheries and 20 farms were sampled. Sampled hatcheries were numbered from K-1 to K-16. A total of 2460 samples were collected from all production stages of all sea bass hatcheries. Each group consists of 10–30 fish, 15 groups of fertilized eggs (FE) from 8 hatcheries, 14 groups of prelarvae (preL) (0–5 days-old) from 9 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 9 hatcheries were sampled [32]. Water temperatures were measured as 15 ℃ in FE tanks, 15–16 ℃ in preL tanks, 16–20 ℃ in postL tanks, 16–22 ℃ in fry tanks, and 20–24 ℃ in fingerling tanks.

## 2.2. Sampling of farms

There are a total of 418 seabass farms in Turkey. Twenty farms were sampled according to calculation of 95% confidence interval, 100% sensitivity and 15% estimated prevalence [33]. Farms were numbered from Y-1 to Y-20. A total of 600 fish, including 30 fish from each farm, were collected by random sampling method. The length and weight of the fish were measured and divided into three groups as small, medium and large. The lengths and weights of the sea bass were 5.5–45 cm, 3.2–950 g, respectively.

## 2.3. Preparation of the samples

Homogenizates were obtained from whole body in FE, preL and postL, whole head in fry. Brain, spinal cord and eye were only obtained from fingerling and big size fishes. All samples were homogenized with a mortar, 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% foetal 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. Inoculums were stored at -80 °C until used.

## 2.4. 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 plate with SSN-1/E-11 cell lines were prepared using 1 ml Leibovitz-15 (L-15) (Gibco, USA, Ref: 11415-049) supplemented with 5% FCS and 1% antibiotic-antimycotic solution (Sigma, USA, Product Number: A5955) per well. Positive samples were inoculated onto 24 hours old cell and 85–90% confluency at 1/10 and 1/100 dilutions. Two wells at each plate were not inoculatated and were used as negative controls while two wells at each plate were inoculated with reference material and were used as positive controls. The plates were incubated at 25°C and were examined daily for cytopathic effect (CPE) during 10 days. Serial passages were made by freeze/thaw method after 10 days of inoculation [34, 35].

## 2.5. Molecular studies

Viral RNA extraction

Viral RNA extraction was performed using 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).

Quantitative Real Time RT-PCR

Quantitative Real Time RT-PCR (RT-qPCR) was performed using commercial extraction kit (Real Time Ready Virus Master, Roche, Cat. No. 05 992 877 01) and Real Time PCR device (Roche LyghtCycler ® 480 Multiwell Plate 96). Primers and probes [36] designed according to the T4 variable region of the RNA2 segment of the betanodavirus proposed by World Organisation for Animal Health [37] were used for amplification. Mastermix was prepared on ice according to kit manufacturer recommendations. Mastermix was placed in 96 well Real Time PCR plates Roche LightCycler® 480 Multiwell Plate 96, White, Germany, Ref: 04 729 692 001), then 15 µl of sample, positive control and negative control were added. Plate was centrifuged at 1500 g at 4 ºC for 2 minutes and RT-qPCR test was performed.

cDNA Syntesis, PCR ve Sequencing

cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit, Roche, Germany, No. 04 379 012 001) was performed from RNA extracts of positive samples. PCR was performed from cDNAs using commercial kit (FastStart High Fidelity PCR System, dNTPack Roche, Germany, Cat. No. 04 738 292 001) (Techne TC-412, United Kingdom) for partial sequencing Primers in PCR for partial genome sequence analysis were used recommended by Toffolo et al. [28] and Bovo et al. [38]. Partial sequencing of RNA1 and RNA2 segments was done by a commercial firm (Microsynt, Balgach, Switzerland). The correction and matching of the sequences were conducted with DNADynamo program (Blue Tractor Software Ltd., UK). The consensus nucleotide sequences obtained were compared and verified on the Basic Local Alignment Search Tool (BLAST) system in National Central for Biotechnology Information (NCBI) [39] For phylogenetic analysis, multiple sequence alignment of RNA1 and RNA2 partial nucleotide sequences of the isolates and reference sequences obtained from the GenBank (Supplemantary Information, Table.3) were performed with the ClustalW method using the MEGA6 program. The best Protein / DNA model was determined for both segments and the nucleotide similarity between sequences were determined. Phylogenetic trees were created with the Neighbor-Joining-NJ method using 1000 bootstrap values repetitions [40].

# 3. Results

## 3.1. Epidemiological datas

VNN disease suspicion was not detected in the anamnesis information obtained from sea bass hatcheries and farms. External and internal macroscopic pathological findings were not found during the preparation of the fish for the laboratory tests.

Investigation 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. Isolate was named as TR.VNN.01.02. In determining the betanodavirus prevalence of the country, the hatchery in which the virus was detected in any age group, regardless of the age groups of the sea bass in the hatchery, was evaluated as positive. In determining the epidemiological units, it was aimed to reveal the situation of betanodavirus countrywide, so the whole country was considered as a unit and hatcheries as a subunit. According to epidemiological approach, betanodavirus prevalence in Turkish hatcheries was calculated as 6.25% (1/16). The K-1 hatchery, detected betanodavirus, was sampled in May when the seawater temperature started to rise, and no virus was detected in postL and fry-sized fish collected in the same period. In the information obtained about the hatchery, it is stated that the facility is a closed system, biosecurity measures are applied, water supplied from the sea is filtered, ozone and UV sterilized, and older than 1.5-2 g fingerling are taken into the external system. It was learned that sea water that was not subjected was used in external system.

Investigation of betanodavirus in sea bass farms

Betanodavirus was detected in 1 of 20 farms (Y-20) in sea bass that were 3,2–10,4 g in weight and 6–10 cm in length. Isolate was named as TR.VNN.01.01. Betanodavirus prevalence in sea bass farms in Turkey was calculated as 5% (1/20).

Retrospective epidemiologic study

In order to better understand the country level epidemiology of betanodavirus, a retrospective epidemiological study was carried out in hatcheries/farms where the virus was detected and in related facilities. Investigating the other samples collected in the same period from the K-1 hatchery, no viruses were detected in the 15 day-old postL and 70 day-old fry. Monitoring study were carried out in the K-1 hatchery, preL and postL sea bass found in the hatchery during sampling were resampled (September 2017), however, the virus was not detected in these samples. It was determined that the hatchery in which the virus was detected did not have spawner, and the reared fish were obtained from 5–10 days-old preL in K-12 hatchery. In the epidemiological study carried out in the K-12 hatchery, it was determined that spawner, fertilized eggs and preL were reared. Betanodavirus was not detected in the fertilized egg and 2 day-old preL sampled from the K-12 hatchery in January 2017 and in December 2018. It was learned that there was no clinical symptom in terms of VNN disease and there was no abnormal mortality in both hatcheries. 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 a complete marine farm, it was an intermediate post-hatchery where fish to be sent from the hatchery to other farms were kept, there were no sea bass older than 3–4 months.

## 3.2. Determination of viral load

In the RT-qPCR test performed from direct tissue homogenisates of positive samples, Ct values and viral loads for TR.VNN.01.01 isolate were 32.19 and 3.10 x $10^2$ copies / µl, respectively; it was calculated as 31.56 and 4.82 x $10^2$ copies / µl for TR.VNN.01.02 isolate, respectively. RT-qPCR test was performed again after virus isolation in cell culture. Ct values and viral loads for TR.VNN.01.01 isolate were 20.96 and 7.29 x $10^5$ copies / µl, respectively; It was calculated as 24.87 and 4.83 x $10^4$ copies / µl for TR.VNN.01.02 isolate, respectively. Virus isolation in cell culture of field samples provided that increase the Ct value and viral load 7–12 and $10^2$-$10^3$ times, respectively.

## 3.3. Sequencing and phylogenetic analysis

Positive samples were found to be RGNNV genotype with phylogenetic analysis performed according to both RNA1 and RNA2 segments (Figs. 1 and 2). In the partial genome sequencing of the RNA1 segment of TR.VNN.01.01 and TR.VNN.01.02, 933 nt (between 192–1124 nt) and 937 nt (between 197–1133 nt), respectively, were obtained. In the partial genome sequencing of the RNA2 segment of TR.VNN.01.01 and TR.VNN.01.02, 569 nt (between 355–923 nt) and 567 nt (between 365–931 nt), respectively, were obtained. 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 analyzes. The nucleotide differences of the isolates obtained in the study and the reference isolates obtained from genbank were determined according to both RNA1 and RNA2 segments (Tables 1 and 2). The nucleotide similarities of the TR.VNN.01.01 and TR.VNN.01.02 were found to be 99.77% with respect to the RNA1 segment. The closest of the TR.VNN.01.01 isolate according to the partial sequences of the RNA1 segment was determined as betanodavirus isolated from sea bass in Cyprus with 99.21%. The subsequent similarities of TR.VNN.01.01 isolates were determined as betanodavirus isolated from three different freshwater fish in Italy and betanodavirus obtained from striped lahose in Greece, with 98.74% and 98.62% respectively (Table 1). The nucleotide similarities of the TR.VNN.01.01 and TR.VNN.01.02 were found to be 100% with respect to the RNA2 segment. The closest of the TR.VNN.01.01 isolate according to the partial sequences of the RNA2 segment was determined as betanodavirus isolated from sea bass in Cyprus with 99.58%. The subsequent similarities of TR.VNN.01.01 isolates were determined as betanodavirus obtained from striped lahose in Greece and betanodavirus isolated from three different freshwater fish in Italy with 99.37% and 99.15%, respectively (Table 2).

It was determined that betanodaviruses isolated from the hatchery and the farm were originated from the same geography and retrospective epidemiological studies showed that the farm and the hatchery were closely related, and the same relationship was also revealed by phylogenetic analysis. Partial nucleotide sequences of both the RNA1 and RNA2 segments of TR.VNN.01.01 and TR.VNN.01.02 were uploaded to GenBank, and GenBank accession numbers were assigned as MT451939 and MT451940 for RNA1 segment, MT451941 and MT451942 for RNA2 segment, respectively.

## 3.4. Virus Isolation

Positive samples were first inoculated onto E-11 cell culture, but no cytopatic effect (CPE) was observed. In addition, a total of 9 blind passages were performed by freeze-thawing in E-11 cell line and no CPE was observed in any passage. RT-qPCR test was performed at the end of each passage and no viral nucleic acid was detected. Positive samples were then inoculated onto SSN-1 cell line and CPE consist of vacuolation and lysis was observed after day 4 (Fig. 3). RT-qPCR test was performed after CPE observed, and the viral nucleic acid was detected. CPE positive SSN-1 cell culture supernatants were re-inoculated onto E-11 cell line and the virus was isolated in the E-11 cell line by ensuring the virus adaptation at the end of two passages.

## 4. Discussion

VNN is the biggest challenge in terms of the sustainability and development of aquaculture, and it poses a significant risk to aquaculture globally [3, 18]. VNN is endemic in the Mediterranean basin and numerous studies have demonstrated its occurrence in both farmed and wild fish [3, 41]. RGNNV is the most frequently detected genotype in the Mediterranean [18], however, the SJNNV genotype was also reported in the Iberian Peninsula in 2009 [27]. Reassortant RGNNV / SJNNV and SJNNV / RGNNV strains have only been reported in the Mediterranean [42].

Turkey has 42% of sea bass production in the world [43] and a strategic position in terms of sea bass production both in Europe and worldwide. The sea bass production in Turkey has high rate of 32.8% within all fish species [44]. The first detection of betanodavirus in Turkey was from sea bass in 2011 and were genotyped as RGNNV [45]. Subsequently, the RGNNV genotype betanodavirus was detected in sea bass and sea bream in the monitoring and screening studies conducted in 2012 and 2014 [46].

Since VNN is not a notifiable disease in animal diseases reporting systems such as OIE and ADNS, there are difficulties in evaluating the epidemiological situation of countries. However, it is seen that the VNN is endemic in the Mediterranean basin in the light of literatures. When the few studies conducted to determine epidemiological prevalence of betanodavirus are examined, it is seen that ranges from 0.23–88.5%. [25, 29–31, 47]. Here, we investigated

betanodavirus prevalence based on epidemiological approach in all hatcheries and some farms in Turkey. Betanodavirus prevalence in hatcheries and farms were found to be 6.25% and 5%, respectively.

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 environment and also to be highly resistant to external environment [48]. For this reason, larvae or fry-sized sea bass could be exposed to the virus through horizontal transmission in farm or hatchery used contaminated sea water [49]. It is seen that the water temperatures of the hatcheries in the study are lower than the optimum growth temperature of the RGNNV genotype. However, the main purpose of the study in hatcheries is to detect the vertical transmission of the virus that can be found persistently in the spawner and the virus that can be found in the environmental water, if any. The virus has not been detected in FE, preL, postL and fry sized fish, which supports the absence of persistent virus infection in spawners. When the decontamination methods of the water used in hatcheries are evaluated, it is thought that the facilities imlement biosecurity measures by using the filter and UV method and these help the water used to be free from virus. Viruses detected in hatcheries or farms were isolated from fish in units outside the closed system where sea water is used directly. It is thought that the detected viruses might be due to horizontal transmission from sea water where virus endemic region.

Betanodaviruses cause VNN disease in more than 120 fish species and with four generally accepted genotypes [3, 19, 20]. However, each genotype or isolates of the same genotype in different geographies show different pathogenicity on different fish species [50]. A certain amount of viral load should be present in the tissue to cause pathology [50–52]. In experimental infection in sea bass, it was shown that clinical signs did not occur up to $10^{6.55}$ $DKID_{50}$, 8.96 x $10^8$ copies for RNA2 and 1.21 x $10^7$ copies for RNA1 in brain samples. In addition, it was determined that those who remained alive and did not show clinical symptoms carried $10^{4.55}$ $DKID_{50}$, 1.29 x $10^7$ copies for RNA2, and 6.25 x $10^5$ copies for RNA1 [53]. The $DKID_{50}$ used in experimental infections appears to be $10^{4.55}$ [53] and $10^4$ [50]. Considering that the viral loads obtained in this study are between 3.10 and 4.82 x $10^2$ which is thought that the infectious loads are not sufficient. 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 in the farm or hatcheries.

In this study, samples found positive by RT-qPCR were primarily inoculated onto the E-11 cell line, but could not be isolated. Subsequently, isolation was provided in the SSN-1 cell line. In the 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 in the E-11 cell line. The researchers attributed this to the fact that positive samples either did not have sufficient virus titer to generate CPE, or that only virion or infective capsid may not be present despite having viral RNA [25]. Panzarin et al. [36] compared virus isolation in cell culture and RT-qPCR test and reported that although the method of inoculation was unknown, samples with ≥ 31 Ct value could not be isolated in SSN-1 cell line. A similar situation has been experienced in this study. All of the samples were collected from healthy appearance of the sea bass and the low Ct values of the positives were attributed to the low virus titer. The compatibility of field and laboratory data supports the hypothesis that there is no active infection in farms or hatcheries where the virus is detected, and this may be due to low viral load. However, in this study, as different from Sakamoto et al [25] SSN-1 cell line was used in addition to E-11 cell line and virus isolation was achieved. In the light of this experiences, although it has been reported that the E-11 cell line is more suitable for quantitative tests such as virus titration, it is thought that choosing the E-11 cell line for virus isolation in infections with low viral load may have negative consequences such as failure to isolation of the virus from cell line.

In conclusion, the presence and prevalence of betanodavirus was investigated in all age groups in both sea bass hatcheries and sea bass farms in Turkey. It was evaluated that there was no active infection in the juvenile sea bass and that they could be subclinically infected due to low viral load. It has been evaluated that biosafety measures prevent water-borne transmission of the virus, and it is suggested that biosafety measures implememented in hatcheries and farms should continue and seawater should not be used without sanitation. In addition, it is thought that the SSN-1 cell line should be used beside of E-11 cell line in the isolation of betanodavirus in subclinical infections. However, more and more specific studies are needed to determine the different cell culture adaptations of betanodaviruses detected as persistent or subclinical.

## Declarations

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**Data Availability Statement:**

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

**Conflict of Interest:**

Authors declare that have no conflict of interest.

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**Availability of data and material:**

The Republic of Turkey

**Code availability:**

Not applicable

**Author contributions:**

This study summaries the doctoral dissertation of the Murat KAPLAN. The study was supervised and coordinated by professor M. Taner KARAOĞLU. All authors read and approved the final manuscript.

**Ethics approval:**

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

**Consent to participate:**

Not applicable

**Consent for publication:**

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## Tables

Table 1

Nucleotid similarity according to partial RNA1 segment (%).

| İsolate | Nt similarity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TR.VNN.01.01 | 100,00 | | | | | | | | | |
| TR.VNN.01.02 | 99,77 | 100,00 | | | | | | | | |
| JN189816 | 99,21 | 98,97 | 100,00 | | | | | | | |
| JQ970429 | 98,74 | 98,50 | 99,32 | 100,00 | | | | | | |
| JQ970428 | 98,62 | 98,38 | 99,21 | 99,89 | 100,00 | | | | | |
| JQ970430 | 98,62 | 98,38 | 99,21 | 99,89 | 100,00 | 100,00 | | | | |
| JN189867 | 98,62 | 98,38 | 99,21 | 98,97 | 98,85 | 98,85 | 100,00 | | | |
| JN189805 | 98,62 | 98,38 | 99,21 | 98,97 | 98,85 | 98,85 | 99,09 | 100,00 | | |
| AM085317 | 73,14 | 73,42 | 73,42 | 72,96 | 72,76 | 72,76 | 73,89 | 74,08 | 100,00 | |
| AM085330 | 71,56 | 71,85 | 72,15 | 71,76 | 71,55 | 71,55 | 71,85 | 71,85 | 74,13 | 100,0 |
| AM085332 | 71,74 | 72,01 | 71,59 | 71,24 | 71,04 | 71,04 | 71,31 | 71,74 | 73,33 | 88,54 |
| | TR.VNN.01.01 | TR.VNN.01.02 | JN189816 | JQ970429 | JQ970428 | JQ970430 | JN189867 | JN189805 | AM085317 | AM08 |

Table 2

Nucleotid similarity according to partial RNA2 segment (%).

| İsolate | Nt similarity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TR.VNN.01.01 | 100,00 | | | | | | | | | |
| TR.VNN.01.02 | 100,00 | 100,00 | | | | | | | | |
| JN189968 | 99,58 | 99,58 | 100,00 | | | | | | | |
| JN189959 | 99,37 | 99,37 | 99,79 | 100,00 | | | | | | |
| JN189994 | 99,15 | 99,15 | 99,58 | 99,37 | 100,00 | | | | | |
| JN189966 | 99,15 | 99,15 | 99,58 | 99,37 | 100,00 | 100,00 | | | | |
| JN189958 | 99,15 | 99,15 | 99,58 | 99,37 | 100,00 | 100,00 | 100,00 | | | |
| JQ970432 | 98,94 | 98,94 | 99,37 | 99,16 | 98,94 | 98,94 | 98,94 | 100,00 | | |
| JN189920 | 65,47 | 65,47 | 65,47 | 64,97 | 65,96 | 65,96 | 65,96 | 64,39 | 100,00 | |
| AM085345 | 73,80 | 73,80 | 73,80 | 74,21 | 73,80 | 73,80 | 73,80 | 72,90 | 57,12 | 100,0 |
| D38637 | 65,39 | 65,39 | 65,39 | 64,91 | 65,87 | 65,87 | 65,87 | 66,29 | 72,70 | 64,88 |
| | TR.VNN.01.01 | TR.VNN.01.02 | JN189968 | JN189959 | JN189994 | JN189966 | JN189958 | JQ970432 | JN189920 | AM08 |

## Figures

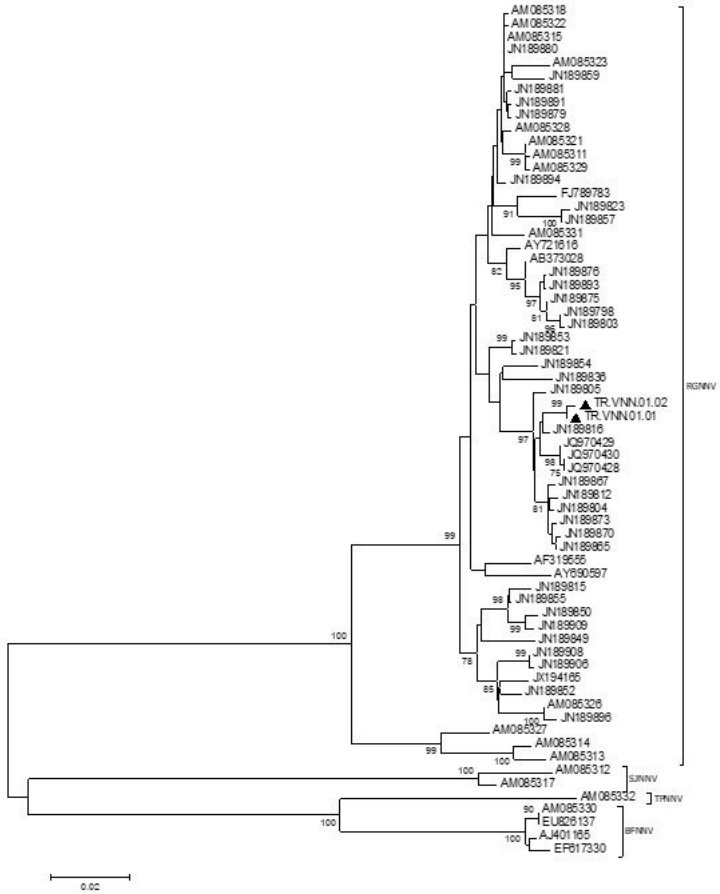


**Figure 1**

Phylogenetic tree according to partial genome of RNA1 segment. Phylogenetic tree was constructed based on anaylsis of 899 bp of RNA1 segment (Reference whole genom, Genbank accession number: FJ789783, was used beetwen 200-1099 nt) partial sequence of RNA1 segment using the NJ metod with TN93+G model. Confidence on tree contruction was assessed using 1000 bootstrap replicates. Only values >70% are indicated.

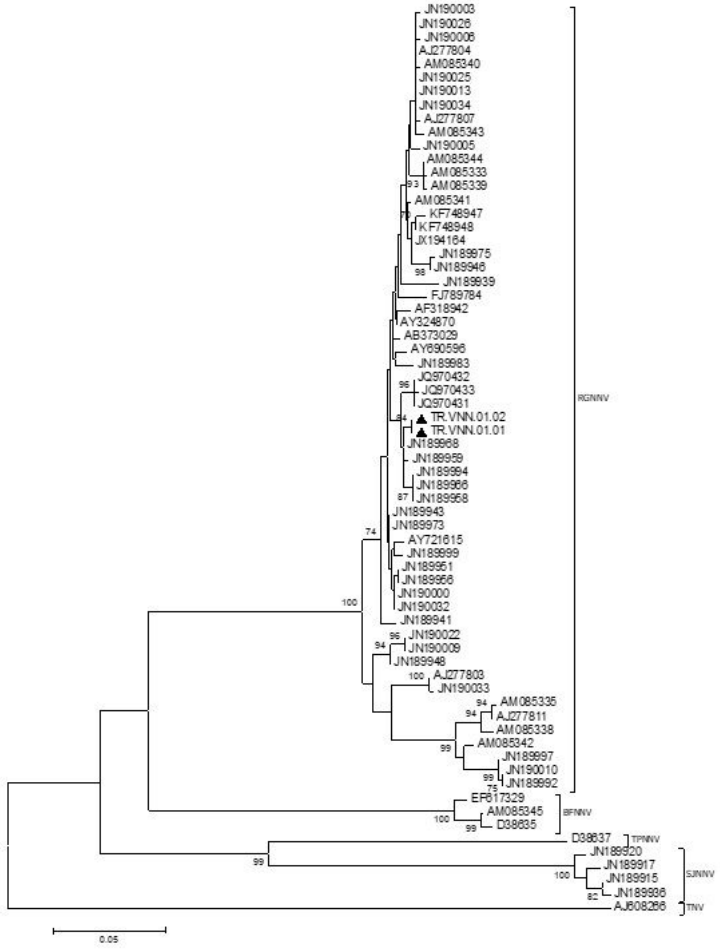


**Figure 2**

Phylogenetic tree according to partial genome of RNA2 segment. Phylogenetic tree was constructed based on anaylsis of 480 bp of RNA2 segment (Reference whole genom, Genbank accession number: FJ789784, was used beetwen 426-907 nt) partial sequence of RNA2 segment using the NJ metod with K2+G model. Confidence on tree contruction was assessed using 1000 bootstrap replicates. Only values >70% are indicated.

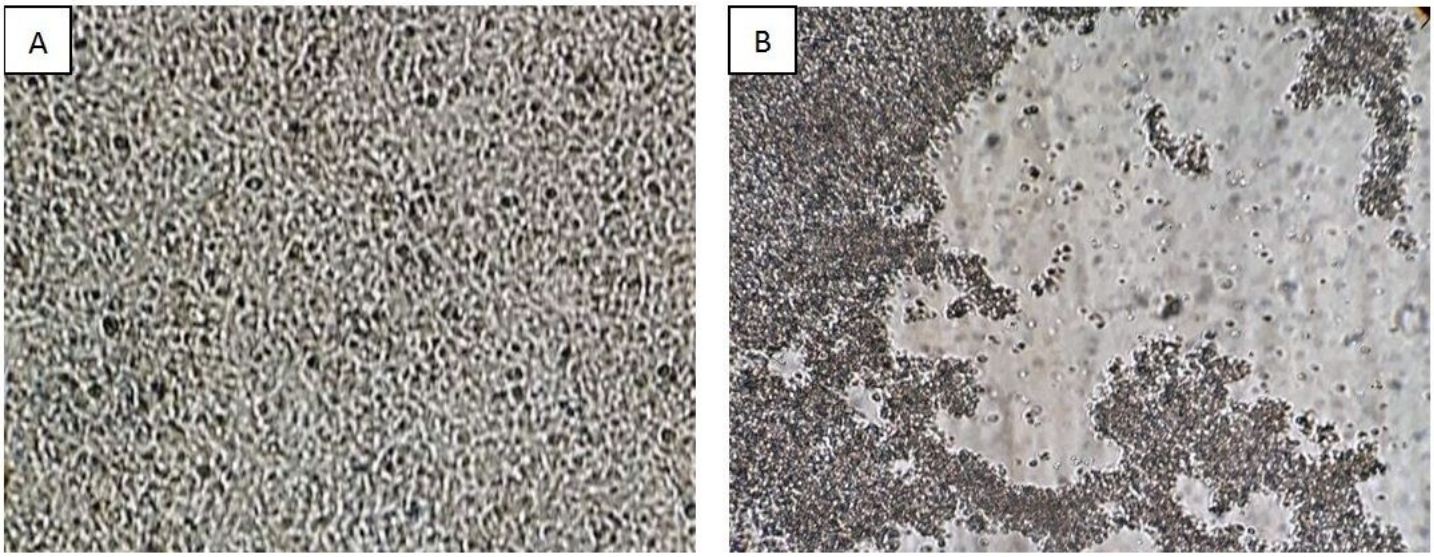


**Figure 3**

CPE of positive sample in SSN-1 cell line. A: Control, B: CPE of TR.VNN.01.02 isolate on day 7: vacuolization and lysis.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1Table.3.docx
- SequencesdatasVNN.fasta