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A Survey of Viral Hemorrhagic Septicemia (VHS) in Turkey

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Abstract

Existence of viral hemorrhagic septicemia virus (VHSV) has been recently reported in Turkey. A total of 5967 marine and freshwater fish were sampled to detect VHSV between 2007 and 2009 in the Black Sea region of Turkey. Of the 5967 fish, five fish tested positive by PCR reactions of tissue filtrates and supernatants of cell cultures showing CPE indicated by a specific 587 bp fragments from the glycoprotein (G) gene of VHSV. All the VHSV positive fish were turbots and none of the other fish species was VHSV positive. The nucleotide sequences among five isolates had shown 96% to 99% homology. In the phylogenetic study, all the new isolates were genogroup 1e. In this study, it was also discovered that an IPNV-VHSV mixed infected wild turbot.

Keywords: Viral haemorrhagic septicaemia virus, virus isolation, survey, Black Sea, phylogenetic analysis.

Türkiye'de Viral Hemorajik Septisemi (VHS) Taraması

Özet

Türkiye'de viral hemorajik septisemi virüsünün (VHSV) varlığı son yıllarda rapor edilmiştir. Bu çalışmanın amacı 2007-2009 yılları arasında, Karadeniz bölgesinde, tatlı su ve deniz balıklarında VHSV'nin varlığının araştırılmasıdır. Tarama bölgesinden 2009 yılının sonuna kadar 13 farklı deniz ve tatlı su balığı türünden toplam 5967 adet balık örneklenmiş, balık örneklerinden beş tanesi hem dokudan hem de CPE gösteren hücre kültürü süpernatantından, VHSV'nin glikoprotein geninin 587 bç'lik korunmuş bir bölgesine spesifik primerler kullanılarak yapılan PCR reaksiyonuyla pozitif bulunmuştur. Pozitif bulunan tüm örnekler yalnızca kalkan balıklarından alınmıştır. Bu beş izolatın nükleotit dizilimleri arasında %96 ila %98 homoloji olduğu görülmektedir. Yapılan filogenetik analizde tüm izolatların genogrup Ie içerisinde yer aldıkları tespit edilmiştir.

Anahtar Kelimeler: Viral hemorajik septisemi virüsü, virüs izolasyonu, tarama, Karadeniz, filogenetik analiz.

Introduction

Viral haemorrhagic septicemia (VHS) is the most serious viral disease of farmed rainbow trout (*Oncorhynchus mykiss*) in European countries (Jensen, 1965; Jensen *et al*., 1979) and it is listed as a notifiable disease by the World Organization for Animal Health's (OIE) (Anonymous 2010). The causative agent of VHS is a non-segmented, enveloped and single stranded RNA virus which is classified in Novirhabdovirus genus of the *Rhabdoviridae* family (Lenoir and Kinkelin, 1975; Bernard *et al*., 1990). The VHSV genome consists of approximately 11200 nucleotides and contains six genes in the order 3'-N-P-M-G-Nv-L-5', encoding a non-structural protein (Nv) with unknown function and five structural proteins: nucleocapsid- (N), phospho- (P), matrix- (M), glyco (G) and RNA polymerase (L) protein, respectively (Schutze *et al*., 1999).

Viral hemorrhagic septicemia virus was isolated for the first time in 1963 from freshwater cultured rainbow trout in Denmark (Jensen, 1963). It was only known to infect rainbow trout until the end of 1980's. Since then VHS was reported in various marine fishes in Europe (Mortensen *et al*., 1999; Schlotfeldt, 1991), including anadromous salmon (Winton *et al*., 1989) and marine fish in North America (Meyers and Winton, 1995; Smail, 1999). VHSV detected also in the Great Lakes region of North America (Elsayed *et al*., 2006) and North Atlantic coast of Canada (Gagne *et al*., 2007). It has been increasingly detected in a

wide range of wild marine species in Japan, Europe and North America (Hedrick *et al*., 2003; Mortensen *et al*., 1999; Takano *et al*., 2000). In 2004, the first outbreak of VHSV has occurred in a turbot (*Psetta maxima*) hatchery in Trabzon, Turkey. VHSV was found endemic (21.2%) on free living turbot spawners in a research which was carried out on turbot from Trabzon coastal area in 2005 (Nishizawa *et al*., 2006). However this studies provide limited information on the status of VHS in Turkey, because that was based on material which cultured turbot from Central Fisheries Research Institute (CFRI) and wild turbot caught from a limited area near the turbot culture unit. Therefore, in the present study, in order to evaluate the present situation, from autumn 2007 to spring 2009 a field survey of VHSV from wide range of marine and freshwater fish in Black Sea region of Turkey was carried out and multiple sequence comparison and then phylogenetic analysis on 400 bp highly conserved region of glycoprotein (G) were used to provide an insight into the overall genetic relationships of the VHSV isolates.

Materials and Methods

Sampling Periods and Sites

Spring and autumn (from autumn 2007 to spring 2009) were chosen as the sampling periods as outbreaks of VHS are most probable at these times (Wolf, 1988, Smail, 1999). VHSV is temperature sensitive, and the disease occurs at temperature changes below 15°C (Olesen, 1998; Skall *et al*., 2005). Surface water temperature of Black Sea ranges between 28.06±0.90°C (in Agust) and 8.80±1.15ºC (in February-March) (Aksungur *et al*., 2007). Private fish farms mainly produce rainbow trout and some of them also produces Black Sea trout (*Salmo trutta labrax*) for human consumption. There are several rainbow trout farms on each river and most facilities are small size (production between 5 and 100 tons a year) in especially eastern part of Black Sea region of Turkey (Bozoğlu *et al*., 2007). In the sampling periods eight sampling sites were chosen for marine fish. Those sites were chosen in collaboration with government fisheries authorities. Natural habitats were selected to include all major catchments throughout the Black Sea coast of Turkey. For the freshwater fish sampling 62 privet fish farms were selected considering the size of production, source of water and geographical substitutions. Map of sampling sites and detailed number of samples shown in Figure 1, Table 1 and Table 2.

Virus Isolation

According to fish size, pieces of heart, spleen, anterior kidney, and brain tissues where virus were abundant (Wolf, 1988, Smail, 1999), were pooled in a micro centrifuge tube for one to five fish. Each organ of adult turbot and rainbow trout broods was placed in one tube. The samples were kept on ice until further processing in the laboratory. In the laboratory, the organ pieces were homogenized using a lab homogenizer (Lab-GEN 7B, ColeParmer, USA). The homogenate was divided to three tubes, for reverse transcriptase-polymerase chain reaction, inoculation on tissue culture and additional purposes and stored at -80°C if the samples could not be processed further within 48 h.

Commission Decision of the European Communities cell culture protocol was performed (Comission of Decision, 2001). All the samples were inoculated onto BF-2 (bluegill fry caudal trunk) cell

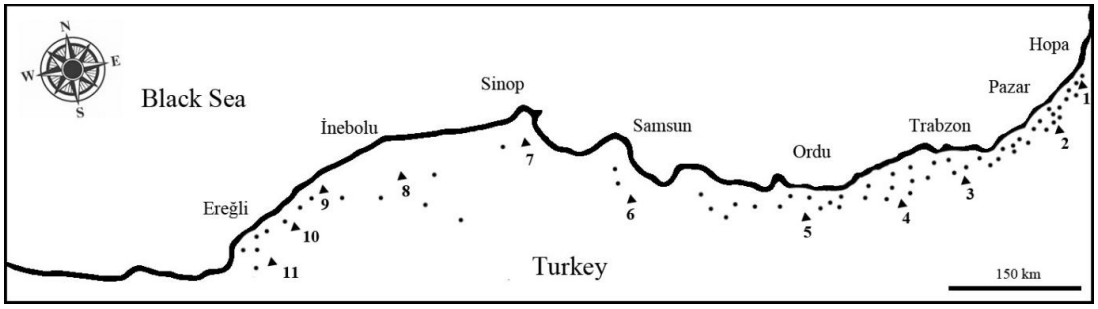

Figure 1. Map of marine and freshwater sampling sites located in eastern Black Sea. Numbered triangles indicate freshwater sampling cities.

Table 1. Detailed number of freshwater fish samples

Species	Sampling locations (listed in figure 1)											
	1	2	3	4	5	6	7	8	9	10	11	Total
Adult rainbow trout	96	168	117	237	193	50	15	82	34	31	66	1089
Juvenil rainbow trout	300	150	-	550	150	-	-	100	100	100	50	1500
Total	396	318	117	787	343	50	15	182	134	131	116	2589

Table 2. Detailed numbers of marine fish samples

Species	Sampling locations									
	Hopa	Pazar	Trabzon	Giresun	Ordu	Samsun	Sinop	İnebolu	Ereğli	Total
Bonito (*Sarda sarda*)	-	-	37	10	-	-	-	-	-	47
Anchovy (*Engraulis encrasicolus*)	112	143	350	55	-	89	34	75	47	905
Sea bream (*Sparus aurata*)	-	-	-	-	27	-	-	-	-	27
Sea bass (*Dicentrarchus labrax*)	-	-	-	-	36	-	-	-	-	36
Picarel (*Spicara smaris*)	-	201	160	20	-	17	-	-	-	398
Horse mackerel *(Trachurus trachurus*)	-	110	260	34	30	37	76	30	25	602
Red mullet (*Mullus barbatus batus*)	-	165	70	32	65	43	59	33	25	492
Witing (*Merlangius merlangus*)	95	90	182	78	23	74	105	28	39	714
Ray (*Raja clavata*)	-	-	58	-	-	-	-	-	-	58
Greater weever (*Trachinus draco*)	-	-	65	-	-	-	-	-	-	65
Corb (*Umbrina cirrosa*)	-	-	10	-	-	-	-	-	-	10
Turbot (*Psetta maxima*)	-	-	24	-	-	-	-	-	-	24
Total	207	709	1216	229	181	260	274	166	136	3378

line. The cell lines maintained in Eagle's MEM supplemented with 10% (v/v) fetal bovine serum, 150 IU penicillin G and 150 mg/ml streptomycin. Tissue homogenates were diluted 1:10 with Hanks' balanced salt medium and homogenized for 15 s with vortex and then supernatant was filtered aseptically through a 0.45-µm pore diameter membrane filter. The samples were inoculated in two wells of a 24-well plate with a 1:100 dilution, and one well with 1:1000 dilution of original material. Samples were incubated at 15°C and observed for appearance of cytopathic effect (CPE) for 2 weeks. All samples were subcultured and incubated for 2 weeks again. The supernatant of suspected wells showing CPE were passaged onto the same cell line and observed for 2 weeks more to confirm virus isolation.

RT-PCR Amplification and DNA sequencing

Viral RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription, extracted RNAs were heat denatured at 95°C for 5 min and then incubated at 42°C for 30 min in 10 µl of PCR buffer with reverse primer. RT-PCR amplification with primer set consists of VGsense (5'-CCA GCT CAA CTC AGG TGT CC-3') and VGanti (5'-GTC ACY GTG CAT GCC ATT GT-3'), targeting a 587- base region of the VHSV G gene (Nishizawa *et al*., 2006). After incubation at 99°C for 10 min, a targeted DNA was amplified in 50 µl of PCR buffer with a thermal cycler programmed for 1 cycle at 72°C for 10 min; 95°C for 2 min; 30 cycles, each consisting of 95°C for 40 s, 60°C for 40 s, and 72°C for 40 s; and a final hold step at 72°C for 5 min. The RT-PCRs were performed using a BioRad gradient thermal cycler. The amplified products were analyzed by 1.5% agarose gel electrophoresis, and visualized under UV irradiation after being stained with ethidium bromide. RT-PCR-amplified products were sequenced directly without cloning in order to minimize the bias associated with minor heterogeneities in the virus population. Purified PCR products were sequenced using an automated ABI PRISM 377XL DNA sequencer.

If cell culture showed CPE and it could not be confirmed as VHSV by RT-PCR, further analysis was performed for the detection of other possible fish viruses according to the manual of diagnostic tests for aquatic animals (Anonymus, 2006).

Nucleotide Sequence Analysis

Multiple sequence alignments of nucleotide sequences were performed with the CLUSTAL W program (Higgins and Sharp 1989). Phylogenetic analysis was performed using the MEGA4 software package, version 4.10. A starting tree was produced by the neighbourjoining algorithm with MEGA4 default parameters (Tamura, 2007).

Results

Virus Isolation

The total number of 5967 fish (3378 from the sea and 2589 from freshwater) sampled as detailed in Table 1 and 2 during the four sampling periods. Totally five VHSV isolations (ckc-1 to ckc-5) were achieved from CPE observed wells. All of the VHSV isolations were taken from turbot. Only one turbot showed gross signs that indicative of viral hemorrhagic septicemia pathology both externally and internally. Brain tissue of this fish tested positive for VHSV, hearth, spleen, head kidney and liver tissues were found positive for infectious pancreatic necrosis virus (IPNV) by further examinations (Figure 2).

RT-PCR Amplification and Nucleotide Sequence Analysis

Five viral isolates (ckc-1 to ckc-5) were

confirmed for VHSV by amplification of partial glycoprotein gene, targeting a 587 base highly conserved region of G gene (Figure 3). Sequencing of the partial glycoprotein (G) gene product was performed and all the sequences (ckc-1 to 5) were submitted to the Genbank (IDs: JF415086-JF415090). For the phylogenetic analysis some sequence data, which representing four genogroup, provided from Genbank. All the isolates appeared in class I-e of genotype I and exhibited >96% sequence identity at both the nucleotide and amino acid levels with the TR-Bs13/H15 and TR-SW13/G isolates which were isolated from cultured and wild turbot spawners in 2005. The relationship among the five Turkish isolates and 38 selected sequences is shown below in a phylogenetic tree (Figure 4).

Discussion

The aim of this study was to provide an overview on the status of viral hemorrhagic septicemia virus presence in Turkey. VHSV is temperature sensitive, and the related diseases occur at temperature changes below 15°C (Olesen, 1998, Skall *et al*., 2005). Surface water temperature of Black Sea changing between 28.06±0.90°C (in August) and 8.80°C±1.15°C (in February-March) (Aksungur *et al*., 2007). Spring and autumn were chosen as the sampling periods as outbreaks of viral fish diseases are most probable at these times (Wolf, 1988; Smail, 1999). The sampling study was started from autumn 2007 to spring 2009 and rivers which have farm on were chosen for freshwater sampling sites. At least 30 fish samples were taken per water source. A total of 62 private fish farms were visited to get samples. It was planned to take marine samples from eight sampling site according importance of catchments which are located in the most important region of Turkey for aquaculture activities. Totally 2589 freshwater and 3378 marine fish, including 13 different species, were sampled in sampling period.

Five new VHSV isolates (ckc-1 to ckc-5) can get from the samples which all the isolates from free living turbots. Geographically, no particular distribution pattern of sites with virus-positive fish was found. The only one fish showed gross signs of the disease. Prevalence of VHSV in free living turbots found (5/24) 20.83%. Although very high number of samples, the virus could not detected in any other marine species including whiting (*Merlangius merlangus*), and scad (*Trachurus trachurus*) which were known as susceptible species (Einer-Jensen, 2004).

Previous study on Black Sea turbot showed that the prevalence of VHS in free living turbot samples were 21.2% in 2005 (Nishizawa *et al*., 2006). The present prevalence of VHS in free living turbot samples has closely agrees the previous findings

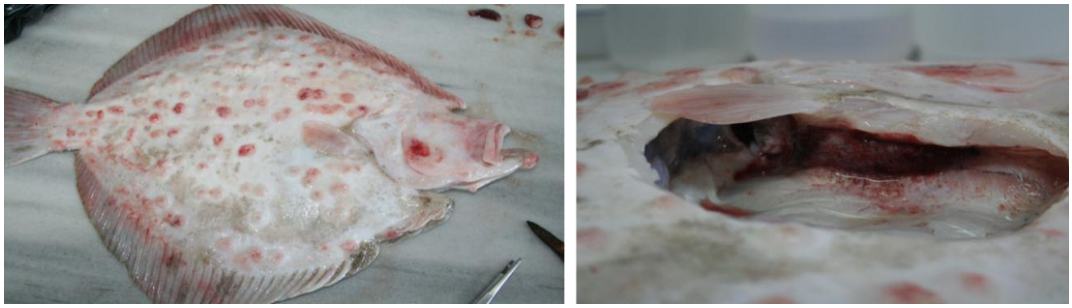

Figure 2. The wild turbot showed gross signs indicative of viral hemorrhagic septicemia pathology.

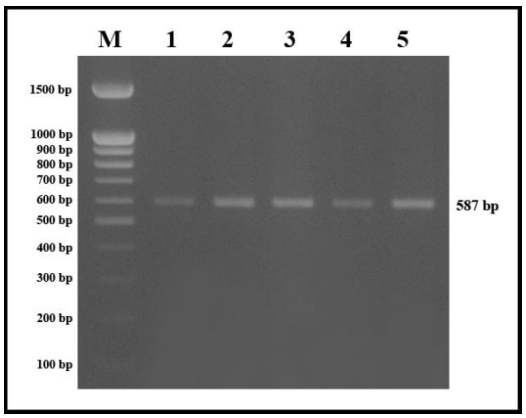

Figure 3. Agarose gel showing the product of RT-PCR assay for detection of virus in infected cell culture supernatants. Lines 1 to 5 idicate isolates ckc-1 to ckc-5 and line M indicates DNA marker.

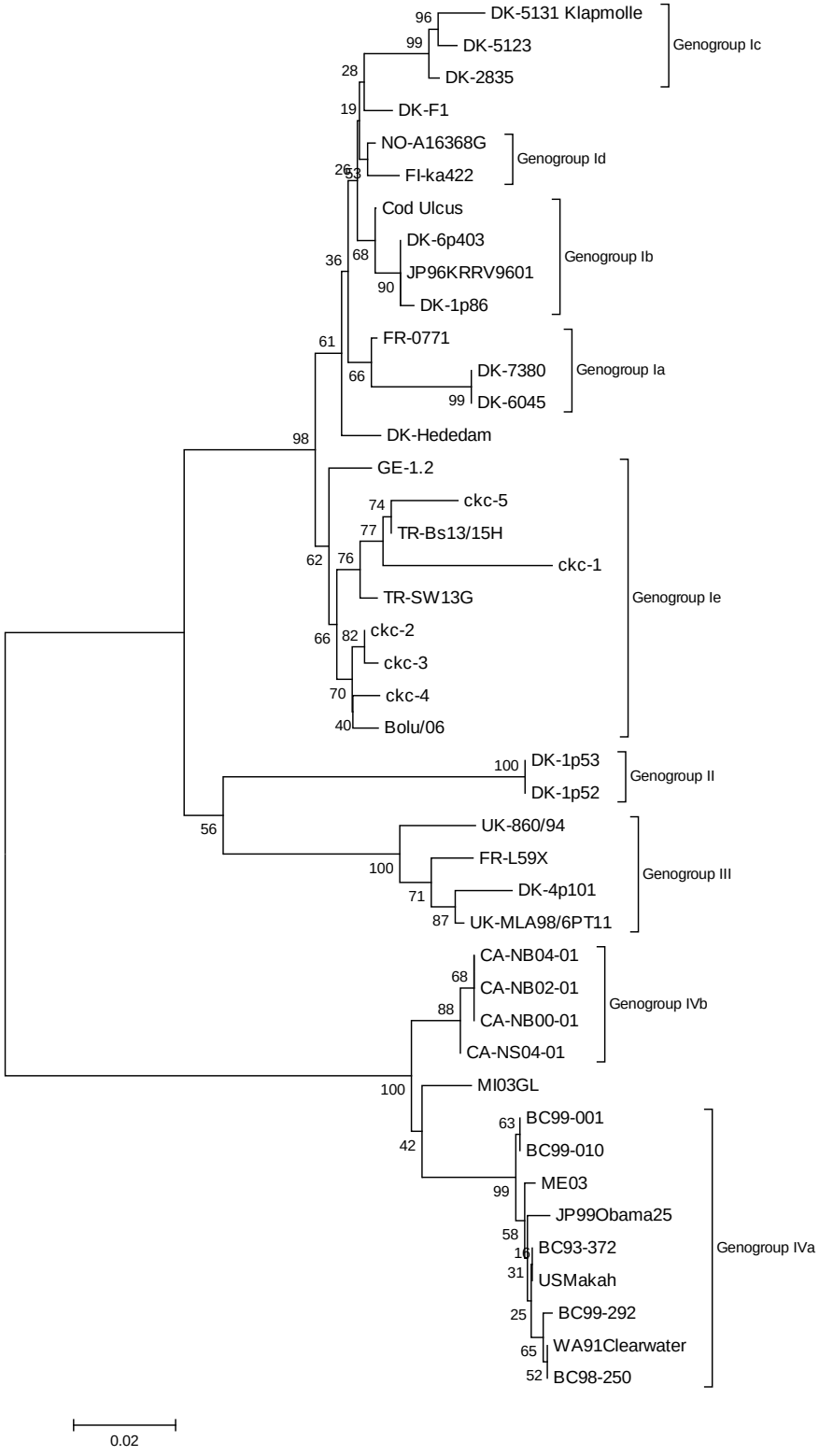

Figure 4. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.53547884 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Dopazo, 1994; Rzhetsky and Nei, 1992). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 43 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 394 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2007).

(Nishizawa *et al*., 2006). It is obvious that VHS is an endemic disease of free living turbots with prevalence of 20.83% in Black Sea. On the other hand one of the VHSV positive turbot found also infected with infectious pancreatic necrosis virus (IPNV). This mixed infected fish has IPNV in brain tissue and VHSV in heart and kidney tissue. It was studied that turbot is susceptible to IPNV (Mortensen, 1993). This is also the first detection of IPNV in wild marine fish in Black Sea.

The G protein has been shown to be the target molecule for neutralizing and protective antibodies (Bearzotti *et al*., 1999). The G gene sequence has also used previously to study genetic relationship of VHSV isolates (Einer-Jensen, 2004). Partial G gene nucleotide sequences of the representative five isolates, ckc-1 to ckc-5, were analyzed to evaluate the genetic relatedness among known VHSV isolates. As shown in Figure 1, the present radial tree of 39 VHSV isolates revealed four separate clades for genotypes I to IV; moreover, five minor clades for classes I-a to I-e were observed in genotype I, as previously identified (Einer-Jensen, 2004). All the isolates appeared in class I-e of genotype I and exhibited >96% sequence identity at both the nucleotide and amino acid levels with the TR-Bs13/H15 and TR-SW13/G isolates which were isolated from cultured and wild turbot spawners in 2005 (Nishizawa *et al*., 2006). The present findings indicate that all of the Turkish VHSV isolates are indigenous type of Black Sea environment.

Until 2004, there was no report on VHS in Turkey even though it is the biggest trout producer of Europe. The only one outbreak of VHSV reported until 2010 from a rainbow trout farm in Bolu province in 2006 (Kalaycı *et al*., 2006) while many outbreaks occurred in European countries (Anonymous, 2011). Surely lacking of report on VHSV doesn't prove the lacking of the disease in Turkey before 2004. Many rainbow trout farm producing larvae and transporting them to others in this region. Some of fishermen transport their fish from river aquaculture unit to marine cages in year. These activities make easy to spread diseases. Presence of viral hemorrhagic septicemia virus has already known in the Black Sea (Nishizawa *et al*., 2006). Although existence of the infection risks it is interesting that no virus isolation or detection of VHSV can be achieved from rainbow trout. The outbreak of VHS in Bolu proves there is a possibility that rainbow trout can be infected by VHS in Turkey, however in previous studies turbot isolates of VHSV were found not to infect rainbow trout fry. Phylogenetic analysis indicated that all Turkish isolates belong to the same genotype with high sequence homology, while in a previous study, turbot isolates of VHSV (TRBs13/H15 and TR-SW13/G) were found not pathogenic for rainbow trout fry (Nishizawa *et al*., 2006). To evaluate these confused results it needs to be further analysis on Turkish VHSV isolates.

According to fishermen, turbot stocks of Black Sea have indicatively reduced recent years; considering prevalance of VHS in turbot, VHS may be responsible of this reducing. The result brings some very important questions; Turkish VHSV isolates are how pathogenic for marine and freshwater fish species? May the disease transfect from turbot to trout in cages in the sea? To answer this question it need to be perform an infection trial with using the Turkish VHSV isolates.

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