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Genetic analysis of the complete G gene of viral hemorrhagic septicemia virus (VHSV) genotype Ie isolates from Turkey

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Abstract

Viral hemorrhagic septicemia virus (VHSV) is an enveloped non-segmented, single-stranded, negative-sense RNA virus that belongs to the *Novirhabdovirus* genus of the family *Rhabdoviridae*. This virus causes economically significant diseases in farmed rainbow trout, in Turkey, which is often associated with the transmission of pathogens from European resources. In this study, moribund rainbow trout (*Oncorhynchus mykiss)* samples were collected during an outbreak of VHSV in a rainbow trout fish farm in Bolu Province of Turkey in 2006. In addition, two VHSV strains were isolated from wild turbot (*Scophthalmus maximus*) in Trabzon Province of the Black Sea region of Turkey during a field survey. We have sequenced the full-length glycoprotein (G) gene of three VHSV isolates and compared them with 25 previously published gene sequences. Based on a complete gene nucleotide sequence, Turkish VHSV isolates were classified into class Ie of genotype I, which is closely related to GE-1.2 isolate (97.1-97.5% nucleotide identity and 98.2-98.4% amino acid identity) found in Georgia more than 30 years ago. These isolates could be an indigenous type of VHSV distributed in the Black Sea. On the other hand, Turkish isolates have 97.5-97.6% nucleotide identity and 98.8-99% amino acid identity with Finnish, Danish, and Norwegian isolates which are classified under Ib and Id. These results suggest that Turkish VHSV isolates may have orginated from Europe and co-circulated with indigenous strains which can threaten the aquaculture industry in Turkey.

Keywords: Genotype, Rainbow trout, Turbot, Turkey, VHSV

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Introduction

Viral hemorrhagic septicemia virus (VHSV) is an enveloped non-segmented, single-stranded, negative-sense RNA virus that belongs to the *Novirhabdovirus* genus of the family *Rhabdoviridae*. The virus genome encodes the nucleocapsid protein (N), polymerase associated phosphoprotein (P), matrix protein (M), surface glycoprotein (G) and viral polymerase (L) gene and a non-structural protein (NV) gene (Ammayappan and Vakharia, 2009; He *et al*., 2014; Kim *et al*., 2015). Viral hemorrhagic septicemia (VHS) is one of the most serious viral diseases of the aquaculture industry worldwide. VHSV causes disease not only in salmonids, but in many other marine species as well. The virus usually causes severe hemorrhages on the skin, the kidney and the liver, with mortality rates as high as 90% (Smail, 2000; Kalayci *et al*., 2006).

Phylogenetic analysis based on the nucleotide sequences of G gene of VHSV isolates from marine and freshwater fish revealed four genogroups (I–IV) and a number of sublineages (Ia–Ie, IVa–IVb). The genotypes appear to be more associated with geographical distribution rather than host species (Nishizawa *et al*., 2006; Einer-Jensen *et al*., 2004; Ghorani *et al*., 2016).

Until 2004, there was no report on VHS in Turkey even though it is the biggest trout producer of Europe. Only one outbreak of VHSV was reported in a rainbow trout farm in Bolu Province in 2006 (Kalayci *et al*., 2006), while many outbreaks occurred in European countries (Smail, 2000). Surely the lack of reports on VHSV does not prove the absence of the disease in Turkey before 2004. Many rainbow trout farms produce larvae and transport them to other farms. In addition, a significant number of rainbow trout is being transported from fresh water to marine ecosystems. These activities make it easy to spread diseases. The presence of viral hemorrhagic septicemia virus has already been known in the Black Sea (Nishizawa *et al*., 2006; Işıdan and Bolat, 2011). According to fishermen, turbot stocks of the Black Sea have indicatively reduced in recent years. Considering the prevalance of VHS in turbot, VHS may be responsible for this reducion. The result brings some very important questions. Are Turkish VHSV isolates pathogenic for marine and freshwater fish species? Could the disease be transmitted from turbot to trout in sea cages? To answer this question, one needs to perform a challange study using the Turkish VHSV isolates.

The partial and complete nucleotide sequences of VHSV have previously been determined (Einer-Jensen *et al*., 2004). In this study, we characterized the entire glycoprotein gene of the three Turkish VHSV isolates, Bolu/06 from rainbow trout, *Oncorhynchus mykiss* (Walbaum), detected from an outbreak in freshwater in Bolu province, ckc-4 and TR-WS13G from turbot, *Psetta*

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maxima (L.), caught from the Black Sea, Trabzon, Turkey in 2009. Although all of the VHSV-positive turbot fish exhibited no clinical signs of disease, affected rainbow trout fish exhibited congestion of internal organs; the inner wall of the swim bladder was thickened and contained numerous budding, fluid-filled vesicles. The Black Sea is a major sea in Turkey that has historically supported an economically and socially important sport fishery for many species of fish. VHSV has a very broad host-range, including numerous taxonomic families of fish. VHSV has been found in many different host species. It is a serious threat to all aquaculture species, including salmonids such as trout and salmon (Ammayappan and Vakharia, 2009). To understand the molecular characteristics of the Turkey VHSV strains, we thoroughly analyzed the entire glicoprotein gene sequences and compared them with other VHSV strains.

Materials and methods

The genomic RNAs of Turkish VHSV strains (Bolu/06, ckc-4 and TR-WS13G) were kindly provided by Dr. Gulnur Kalayci, Bornova Veteriner Control Institute, Izmir, Turkey and Hakan Isidan, Cumhuriyet University, Faculty of Veterinary Medicine, Sivas, Turkey and were used as a template. The consensus PCR primers were designed based on the available VHSV genome sequences (Genbank accession number AY546619) from the National Center for Biotechnology Information (NCBI). The complete glicoprotein genome sequences were aligned; highly conserved sequence segments identified, and used to design overlapping primers. The oligonucleotide primers used in this study, VHSVGF (5'-ATG GAA TGG AAT ACT TTT TTC TT-3') and VHSVGR (5'-TCA GAC CAT CGG GTT TCT GGA GAA-3'), are for amplification of a 1524-base region of the VHSV G gene. First strand synthesis was carried out in a tube containing 5 µL of RNA, which was denatured at 70°C for 10 min in the presence of DMSO (3 µL), 1 µL forward gene-specific primer, 1 µL of 25 mM dNTPs, and snap-cooled on ice for 1 min. The reaction mixture containing 2 µL of 10× RT buffer, 2 µL of 0.1 M DTT, 4 µL of 25 mM $MgCl_2$, 1 µL of Superscript III RT™, and 1 µL of RNase OUT™ was incubated at 50°C for 1 h. PCR amplifications were carried out using a *pfx*™ PCR kit (Invitrogen, CA), according to manufacturer's instructions. Briefly, the following mixture was used for PCR amplification: 3 µL of cDNA, 2 µL of primer mix; 5 µL of 10× PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KC1, 1% Triton X-100], 2 µL of 25 mM $MgCl_2$, 0.5 µL of *pfx* polymerase, and 37 µL of DEPC water, to make a final volume of 50 µL. The reaction was carried out in a thermal cycler (MJ Research Inc., Waltham, MA), using the following program: denaturation at 94°C for 30 sec; annealing for 30 sec at

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60°C; and extension at 68°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, CA).

The purified RT-PCR products were cloned into a pGEM®-T Easy vector vector (Promega, WI). Plasmid DNA from various clones was sequenced by dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystems, CA). The pair-wise nucleotide identity and comparative sequence analyses were conducted using Vector NTI Advance 11 software (Invitrogen, CA) and BLAST search from NCBI. Phylogenetic analyses were conducted using the Seaview 4 software (Tamura *et al.*, 2011). Construction of a phylogenetic tree was performed using the ClustalW multiple alignment algorithm and Neighbor-Joining method with 1000 bootstrap replicates.

Results

The complete G genome sequences of the Turkish VHSV strains were submitted to the GenBank (accession numbers KM972678-KM972680). In a comparison of sequences among Turkish VHSV isolates, 26 bases of nucleotide substitution and three residue of amino acid substitution (sequence identities of 98.2% at nucleotide and 99.4% amino acid levels) between Bolu/06 and TR-WS13G isolates; 14 bases of nucleotide substitution and three residues of amino acid substitution (sequence identities of 99% at nucleotide and 99.4% amino acid levels) between Bolu/06 and ckc-4 isolates; 24 bases of nucleotide substitution and one residue of amino acid substitution (sequence identities of 98.4% at nucleotide and 99.6% amino acid levels) between ckc-4 and TR-WS13G isolates, were observed. The complete G gene comparison of Turkish VHSV isolates with other VHSVs reveals a close relationship with strains from Finland, Norway, and Georgia, which were isolated from rainbow trout and Atlantic cod, respectively [FI-ka-66 (Finland), GE-1.2 (Georgia) (97.5%) and NO-A16368G (Norway) (97.6%)]. Other VHSV strains are only 86.2-97.4% identical to the Turkish VHSV isolates.

Discussion

The present radial tree based on the G gene open reading frame revealed the existence of four major clusters for genotypes I to IV. Moreover, five minor clusters for classes I-a to I-e were observed for genotype I, as described in previous studies (Einer-Jensen *et al*., 2005; Nishizawa *et al*., 2006; Pierce and Stepien, 2012; Gustafson *et al*., 2014). Genotype I-a represented European freshwater isolates from Austria, Denmark, France, and Switzerland; I-b included isolates originating from the Baltic Sea, Skagerrak, Kattegat, the North Sea, and the English Channel; I-c included Danish freshwater isolates; I-d was for Norwegian and Finnish isolates; and I-e included an isolate from Georgia. The

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Turkish isolates, Bolu/06, ckc-4 and TR-WS13G, appeared in class I-e of genotype I in the present tree (Fig. 1) and were the most closely related to the GE-1.2 isolate (AY546619), which was isolated from rainbow trout in Georgia in 1982 (Einer-Jensen *et al.*, 2004; Ghorani *et al*., 2016).

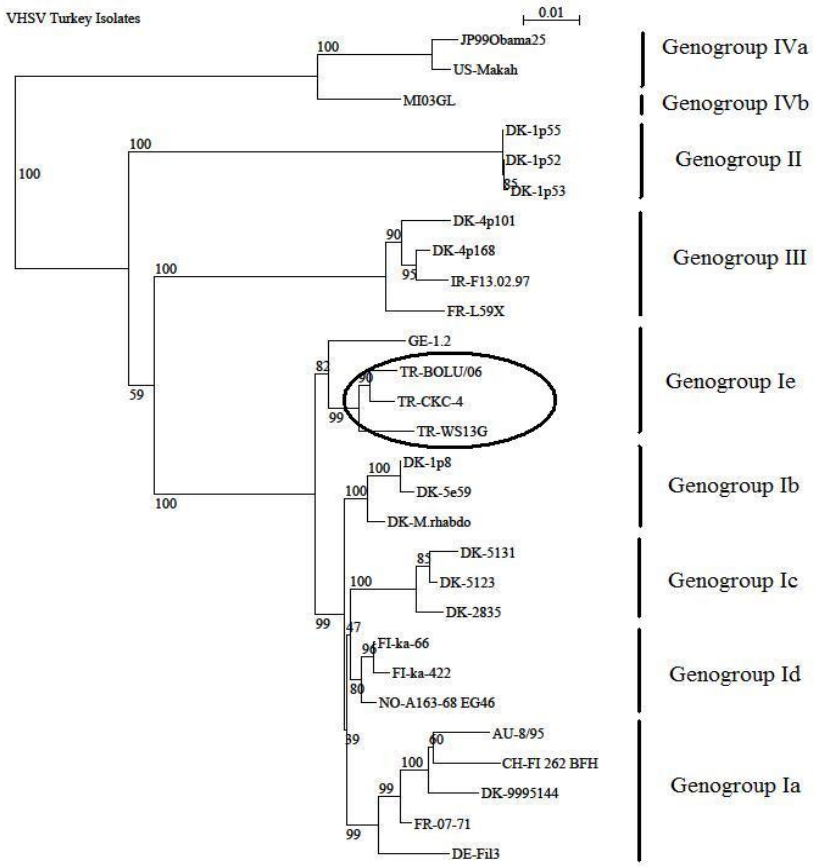


Figure 1: Radial tree based on nucleotide sequences of a complete G gene nucleotide sequence (nt 34 to 1557) among 25 worldwide isolates of VHSV. Isolates shown in circle originated from Turkey. Bootstrap values at 1,000 times construction are shown at major nodes. The scale bar is for a genetic distance marker (number of replacement nucleotides per site).

These isolates could be indigenous types of VHSV distributed in Black Sea. On the other hand, our isolates have 97.5-97.6% nucleotid identity and 98.8-99% amino acid identity with Finnish, Danish and Norwegian isolates which are classified under Ib and Id. These isolates may have originated from Europe. The Turkey salmonid industry was developed by importing breeding materials, a practice still in effect due to deficits in the national supply of roe. Importation of breeding materials is often associated with the transmission of pathogens (Reichert *et al.*, 2013). Turkey aquaculture industry

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has been importing breeding materials from European countires (Norway, Denmark, Spain, France etc.,). These results suggest that the Turkish VHSV isolates orginated from Europe and/or indigenous strains are co-circulating, which could threaten the aquaculture industry in Turkey.

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