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Phylogenetic analysis of viral hemorrhagic septicemia virus from recent outbreaks in some of the farmed rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)

Fattahi F.[1]; Akhlaghi M.[1]; Mohammadi A.[2]; Soltanian S.[1]; Shahbazian N.[3]

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Abstract

During a fish disease outbreaks in 2015-2016 suspected to viral hemorrhagic septicemia virus (VHSV), moribund rainbow trout fries were obtained from fish farms. Samples of kidney, heart and spleen tissues were taken for examination. The full coding region of the G gene was amplified by RT-PCR reaction. Genotyping was carried out by phylogenetic analysis with reference sequences of four VHS genogroup. A phylogenetic tree was constructed with the maximum likelihood method and the Kumara 2- parameter substitution model by using MEGA software version 6.06. A maximum likelihood phylogenetic analysis reveals that all the Iranian VHS strains belonged to clade Ia-2 of genotype Ia. The intra- sequence comparison of Iranian VHS strains ranged from 99.1% to 100% (average was 99.55%). In this study, two strains (Chaharmahal and Bakhtiari (1) and Chaharmahal and Bakhtiari (3) showed 100% identity but 0.1% differences with Isfahan strain. The Iranian strains belonging to European genogroup are related (99% identity) to VHSV from recent Italian strains (VHSV/O.mykiss/I/TN/133/Apr10).

Keywords: VHSV, Phylogenetic, Rainbow trout, Iran

1-Aquatic Animal Health Unit, Clinical Studies Department, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

2-Virology Section, Pathobiology Department, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

3-Aquatic Animal Health and Diseases Management Department, Iranian Veterinary Organization, Tehran, Iran

*Corresponding author's Email: fatemehfattahi22@gmail.com

Introduction

Iran is situated in the Middle East between latitudes of 25° 00' n 39° 47'N and longitude of 44° 02' and 63° 02' E (FAO, 2016) .The total area of the country is 1 648 195 km^2 with 2 440 km coastline along the Persian Gulf and Oman Sea in the south, and ~~a~~ 740 km coastline in the north along the southern part of the Caspian Sea (Kalbassi *et al.,* 2013).

Iran is the largest producer of different types of fish species in the region, and is the world-leading producer of rainbow trout (*Oncorhynchus mykiss*) in fresh water at the world and Middle East (Kalbassi *et al.,* 2013; IFO, 2016). Rearing of rainbow trout takes place on the north, center, the northwestern and western parts of the country mostly in mountainous areas characterized by cool summers and cold winters (FAO, 2016). The cold water fish includes the rearing of rainbow trout on inland waters (IFO, 2016). Total production of Iran was 1093719 tons in 2016, and total aquaculture production was 459521 tons (IFO, 2016). In the 2016 the highest aquaculture production was belonged to warm water fish culture, 201097 tons and cold water fish culture (*O. mykiss*; 163325 tons), respectively. Iran growth rate of aquaculture is more than world average (IFO, 2016); the production of rainbow trout takes place in 30 provinces, which based on statistics of 2016. The most production was related to Chaharmahal and Bakhtiari province (22639 tons), Lorestan province (21460 tons), Mazandaran province (15427 tons), Kohgiluyeh and Boyer-Ahmad province (14700 tons) (IFO, 2016).

Viral hemorrhagic septicemia (VHS) is a serious disease causing great losses in rainbow trout (*O. mykiss)* farming (Sandlund *et al*., 2014).To date, VHSV has been isolated from more than 80 freshwater and marine fish species, many free-living anadromous from North American, Europe and Japanese waters and Korea, indicating a widespread occurrence of the virus in the marine environment of the northern hemisphere (Einer-Jensen *et al*., 2005; Kim and Faisal, 2010; Ito *et al*., 2012; Ahmadivand *et al.,* 2016). Viral hemorrhagic septicemia virus (VHSV) is an aquatic Rhabdovirus that is one of five viruses belonging to the genus *Novirhabdovirus*, family Rhabdoviridae and order Mononegavirales (Faisal *et al*., 2012; Garver *et al*., 2013). The virion is enveloped and contains a single-stranded, non-segmented linear, negative sense RNA genome. The whole genome is approximately11200 nucleotides in length and contains six genes, encoding a non-structural protein (NV) as well as the five structural proteins nucleocapsid- (N-), phosphor- (P-), matrix- (M-), glycol- (G-) and RNA polymerase (L-) protein (Schütze *et al*., 1999; Tordo *et al*., 2004; Kahns *et al*., 2012). Phylogenetic studies based on glycoprotein (G) and nucleoprotein (N) genes sequences have been revealed that VHSV clusters into four major genotypes (I, II, III, IV) and a number of sub lineages within genotype I and IV (Ia–Ie, IVa–IVb) (Snow *et al*., 1999; Einer-Jensen *et al*., 2004, 2005; Elsayed *et al*., 2006).

VHS has been identified with genotypes I–III predominantly occurring in Europe and genotype IV in North America and Asia (Snow *et al*., 1999; Nishizawa *et al*., 2002; Kim *et al.*, 2003; Einer-Jensen *et al*., 2004; Lumsden *et al*., 2007). Genotypes are correlated to geographic regions rather than host species but do not have any correlation to serotyping with neutralizing (G protein-specific) antibodies (Kahns *et al*., 2012; Einer-Jensen *et al*., 2014; Schönherz *et al*., 2016). Genotypes Ia, Ic and Id isolates from rainbow trout and genotypes Ib, Ie and II–IV isolates from marine species (Skall *et al*., 2005; Gadd *et al*., 2011).

VHS is being reported from Iran since last few years; Haghighi *et al.* (2008) reported the VHS outbreak in Iran occurred in rainbow trout cultured in Roodsar, Gilan province in the north of Iran in November 2005. Since 2013, outbreaks of VHS with high mortality have been reported from rainbow trout farms in several provinces and thus resulted in economic losses to the Iranian aquaculture industry (Absalanfard and Bokaie, 2016), the Iranian VHSV isolates were closely related to the 19 freshwater strains from Germany (Ghorani *et al*., 2016).

This study aimed to assess the phylogenetic of VHSV causing disease outbreak in cultured rainbow trout in four provinces of Iran, including Mazandaran, Chaharrmahal and Bakhtiari, Hamadan, Isfahan by analyzing the full-length glycoprotein gene sequence (1524 nt).

Materials and methods

Sample collection and Virus isolation

From December 2015 to July 2016, moribund rainbow trout fingerlings were obtained during outbreaks suspected to VHSV from fish farms in Iran. Outbreaks samples were collected from four provinces (Fig. 1). From each farm, 30 moribund fish were selected and transferred to central veterinary laboratory, Tehran, Iran. Samples of kidney, heart and spleen tissues were taken for examination (www.oie.int). Those samples have been used for the current research.

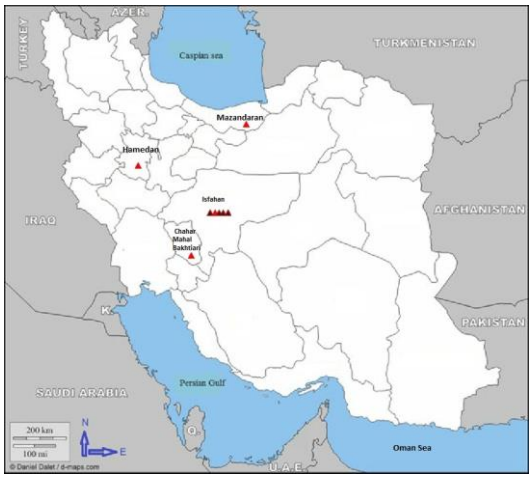

Figure 1: Sampling areas. The infected farms studied here are located in the northern, central, western and southwestern provinces of Iran.

RNA extraction and real-time RT-PCR

Total RNA extraction was performed on homogenized tissues from organ pools (600 µl), individual organ samples (20 mg), and from 350µl virus supernatant using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Purified RNA was eluted in 50 µl RNase-free water and stored at -80°C until use. Samples have also been detected by

real-time RT-PCR assay using Qiagen One Step RT-PCR kit, according to the instruction described by the www.oie.int.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

The full coding region of the G gene was amplified by RT-PCR reaction using Qiagen one-step RT-PCR kit. The primer pairs used are shown in Table 1. Products were not observed after the RT- PCR reaction using M466-F1, G784-R1 primer pairs. A pair of Primers FVH and RVH, were designed to amplifying a 530 bp region. Each reaction was performed in a total volume of 25 µl containing 2.5 µl RNA,1.5 µl 10 µM forward and reverse primers,5 µl 5xQiagen one Step RT-PC buffer,1 µl dNTP Mix ,1 µl Taq mix, 12.5 µl RNase-free water. Cycling conditions were 50°^{C} for 30 min, 95°^{C} for 15 min, followed by 35 cycles of 94°^{C} for 30 s, 56°^{C} for 30 s, 72°^{C} for 1 min, and a final extension at 72°^{C} for 10 min. Optimal annealing temperature for RT- PCR using primers (G330+/ G1243−) was established at 48°^{C}. Quantity and quality of the RT-PCRs were analyzed by 1 % agarose gel electrophoresis of 6µl PCR product and visualized under UV.

Table 1: Primers used for the RT-PCR amplification and sequencing of the VHSV full-length G gene.

Primer name	Sequence(5'−3')	Amplicon size(bp)	References
M466+ G784−	TTA GAC ATG GGA GTG TGA CTT TGC CAT TGT GAG CCC CCA	1063 bp	Garver *et al*., 2013
G330+ G1243−	ACT ACC TAC ACA GAG TGA C CAA TTT GTC CCC GAA TAT CAT	914 bp	Garver *et al*., 2013
G1124+ NV63−	G GGA GAG AAG CTG GTT GTG CTG TG GTC AGC ATC AAC TAC AA	550 bp	Garver *et al*., 2013
FVH RVH	AACCTCCTCTGTCCGACCT CCTTGCTTGCCTCGTCTG	530 bp	This study

Sequence and phylogenetic analysis

The PCR products from confirmed VHS samples were sequenced using the 3730/3730 xl automatic DNA Analyzers Sequencing (Applied Bio systems, Bioneer, Korea). Consensus sequences for each isolates were trimmed to 1524 nucleotides encompassing the entire G gene-coding region. Genotyping was carried out by phylogenetic analysis with reference sequences of four Geno groups. The reference sequences were obtained from GenBank database (NCBI). Nucleotide sequences were aligned using Multiple Sequence Alignment by CLUSTAL/W in Bio Edit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic tree was constructed with the maximum likelihood method and the Kumara 2-parameter substitution model by using MEGA software version 6.06 (Kumar *et al*., 2008). The assessment of the tree was confirmed by 1000 bootstrap sampling. Infectious hematopoietic

necrosis virus (IHNV) was determined by our GenBank Blast searches to be the closest known relative to VHSV and thus was used as an out-group .

Results

Nucleotide sequence accession numbers

Sequence analysis was confirmed VHS cases which were positive by RT-PCR. All VHSV G gene sequences generated here have been deposited on GenBank and assigned accession numbers MF925716-MF925721 (Table 2).

Table 2: Characteristics of the representative positive samples collected from Iran rainbow trout farms in this study.

Sample ID	Accession number	Sampling date	Province of origin
VHS -Chaharmahal (1)-Iran	MF925716	21 Dec 2015	Southwest
VHS- Isfahan- Iran	MF925717	13 April2016	Center
VHS -Chaharmahal (2)-Iran	MF925718	30 Jan 2016	Southwest
VHS –Hamadan- Iran	MF925719	20 Jul 2016	West
VHS- Chaharmahal (3)-Iran	MF925720	05 Mar 2016	Southwest
VHS -Mazandaran -Iran	MF925721	20 Jul 2016	North

Phylogenetic analysis

A maximum likelihood phylogenetic analysis reveals that all the Iranian VHS strains belonged to clade Ia-2 of genotype Ia (Fig. 2). All of the sequences were compared with the sequences previously have been submitted to GenBank from different parts of the world (Fig. 2). Geographical analysis showed that all VHS sequence reported in this study clustered together in a clade with the sequences previously published from Iran. The Iranian strains belonging to European genogroup are related (99% identity) to VHSV from recent Italian strains (VHSV/ *O. mykiss*/ I/ TN/ 133/ April10). It raises this probability that the source of all were the same strain probably from Iran or Italy (Fig. 3). The results documented the continued circulation of clade Ia-2 viruses throughout the North, Southwest, west and center of Iran (Figs 3, 4).The intra-sequence comparison of Iranian VHS strains ranged from 99.1% to 100% (average was 99.55%) (Fig. 4).

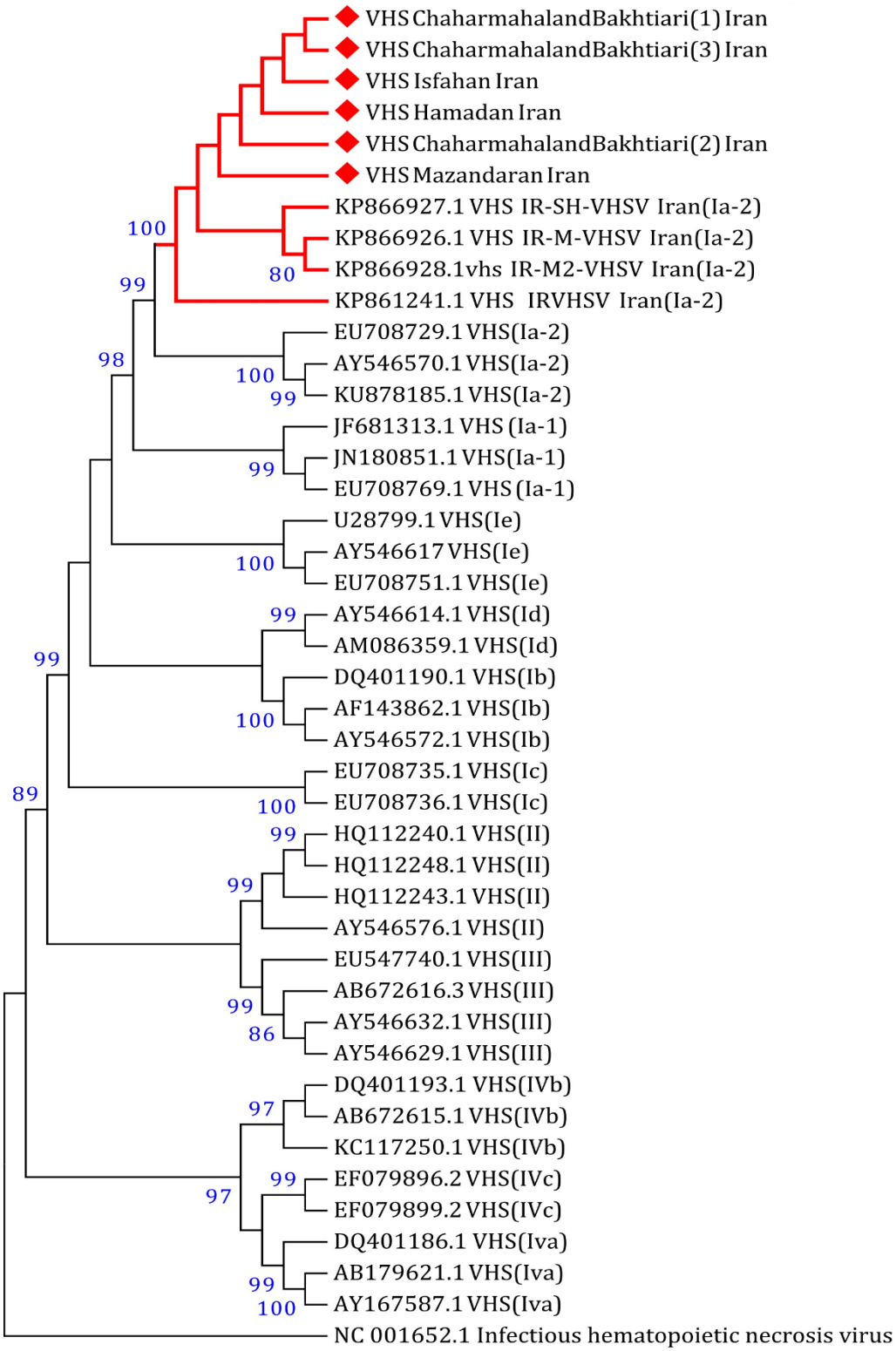

Figure 2: Phylogenetic tree of VHSV based on the complete G gene sequences of VHSV isolates from Iran compared to the reference sequences for each genotype. The Iranian sequences are belonged to clade Ia-2 of genotype Ia. Phylogenetic analysis was performed by MEGA software (version 6.06), with the Maximum likelihood method (ML) in Kimura-two parameter substitution model. Bootstrap value is 1,000 that shown on the trees. Bootstrap values below 70% were omitted. The previously reported VHS sequence from Iran was also included in the dataset. Iranian strains are shown by red rhombic. Infectious hematopoietic necrosis virus (IHNV) was used as an out-group.

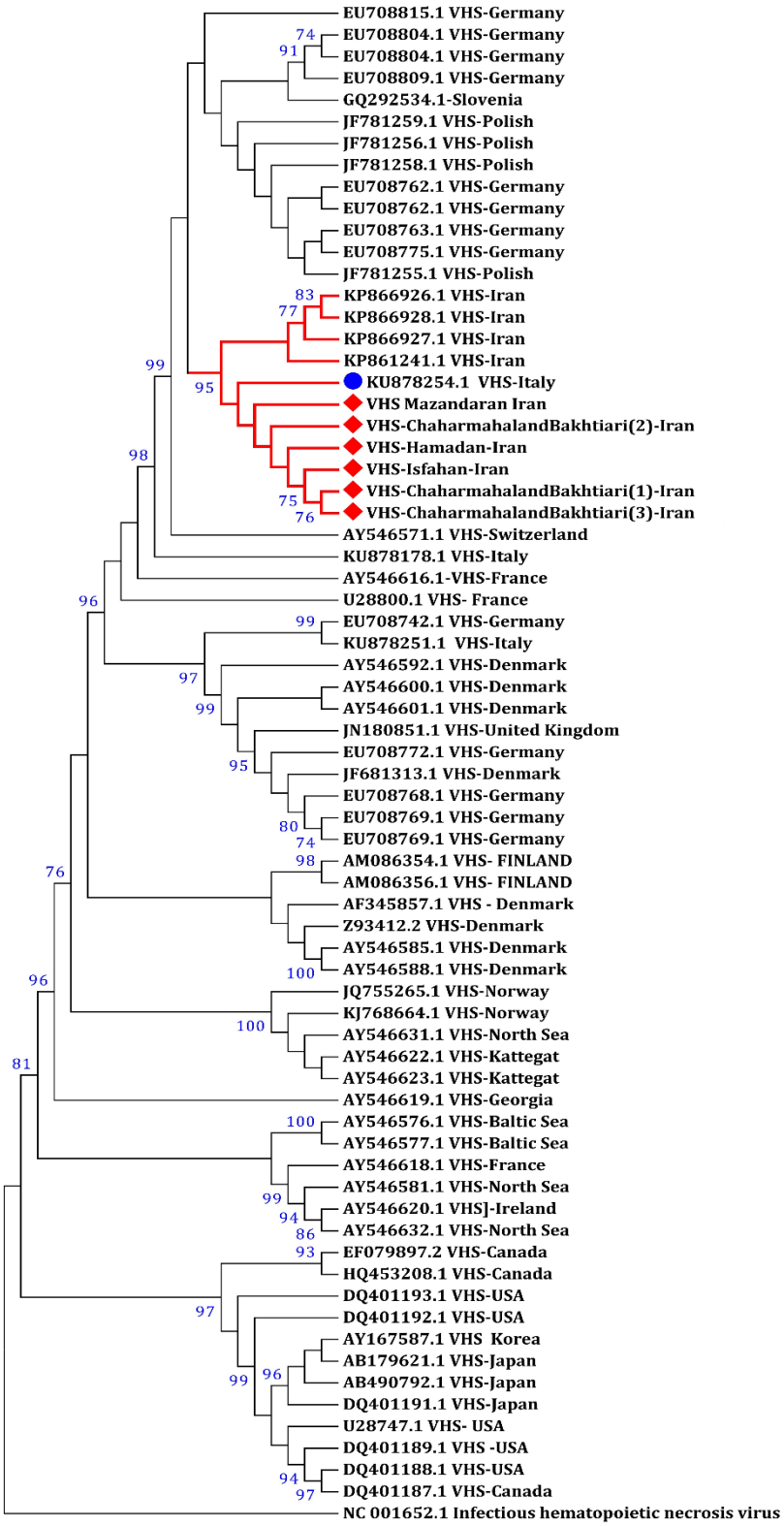

Figure 3: ML phylogenetic tree of VHSV based on the complete G gene. Analyses of Iranian VHSV isolates using the Kimura 2-parameter substitution model with 1000 bootstrap sampling in Molecular Using Evolutionary Genetics Analysis 6.06 software. Bootstrap values below 70% were omitted. Iranian strains are shown by red rhombic. Infectious hematopoietic necrosis virus (IHNV) was used as an out-group.

Seq->	MF9257	MF9257	MF9257	MF925719	MF9257	MF925721	KP861241.1	KP866926.1	KP866927.1	KP866928.1
MF925716	ID	0.999	0.994	0.995	1	0.995	0.996	0.996	0.991	0.991
MF925717	0.999	ID	0.995	0.996	0.999	0.996	0.996	0.996	0.992	0.992
MF925718	0.994	0.995	ID	0.992	0.994	0.992	0.993	0.993	0.988	0.988
MF925719	0.995	0.996	0.992	ID	0.995	0.993	0.994	0.994	0.989	0.989
MF925720	1	0.999	0.994	0.995	ID	0.995	0.996	0.996	0.991	0.991
MF925721	0.995	0.996	0.992	0.993	0.995	ID	0.995	0.994	0.989	0.989
KP861241.	0.996	0.996	0.993	0.994	0.996	0.995	ID	0.997	0.992	0.992
KP866926.	0.996	0.996	0.993	0.994	0.996	0.994	0.997	ID	0.994	0.995
KP866927.	0.991	0.992	0.988	0.989	0.991	0.989	0.992	0.994	ID	0.992
KP866928.	0.991	0.992	0.988	0.989	0.991	0.989	0.992	0.995	0.992	ID

Figure 4: Pairwise comparison between full-length VHSV G-gene nucleotide sequences, containing Iranian isolates.

Discussion

In the current research, we performed sequence analyses of the Iranian VHS based on the complete glycoprotein sequence. A previous study showed that the Iranian VHSV isolates were close related to the 19 freshwater strains from Germany (Ghorani *et al.*, 2016). However, geographical analysis showed that all VHS sequence reported in this study clustered together in a clade with the sequences previously published from Iran (Fig. 2). These data showed that the source of Iranian VHSV was similar in all regions from 2013 to 2016. Neighboring country Turkey has a problem with VHS; Nishizawa *et al.* (2006) reported Turkish VHSV isolates were classified into the sub lineages I-e of genotype I and were the most closely related to the GE-1.2 isolate. Turkish VHSV isolates having not introduced from European countries, it could be an indigenous type of VHSV distributing in the Black Sea environment. Notably, rainbow trout eggs have been introduced to Iran from several countries including the United Kingdom, Italy, Denmark, America, France, Poland, South African and Spain. Furthermore, illegal trade is also possible (Iranian Veterinary organization, 2017). Based on our sequence analysis, the isolate causing the Iranian outbreaks are very likely to have an Italian ancestor, as it is located in the Ia-2 clade (Fig. 3), it has been speculated that VHSV was introduced to Iranian trout farms through contaminated trout eggs from Italy (Fig. 3). The intra- sequence comparison of Iranian VHS strains ranged from 99.1% to 100% (average 99.55%). It shows that the source of them could be chain of transmission pathways. Our phylogenetic analysis has shown that the viruses were introduced by importation from Italy and were epidemic in Iran (Figs. 3, 4). The high mortality most frequently occurred

during the fall and early spring months (Iranian Veterinary organization, 2017) in periods of colder temperatures in Iran. Seasonal occurrence of this mortality shows a complex interaction of the host, pathogen and environment. Lower water temperatures during the fall and spring months, may reduce hosts' protective immune responses to VHSV infection, consequently leaving them more prone to the disease (Iranian Veterinary organization, 2017).

In this study, two strains Chaharmahal and Bakhtiari (1) and Chaharmahal and Bakhtiari (3) had showed 100% identity. One strain (VHS-Isfahan-Iran) had 0.1% differences with Chaharmahal and Bakhtiari (1), Chaharmahal, and Bakhtiari (3) (Fig. 4). This data showed that these farms situated in the sub basin zayandeh rood. However, we observed infection by identical viruses in farms with no connection via water; hence it is possible that the transmission of the disease occurred horizontally by mechanical vectors such as transmission of fry between farms or sharing of equipment or staff (Iranian Veterinary organization, 2017). Absalanfard Absalanfard and Bokaie (2016) showed that farms, which have authority health certificate by veterinary services as with this certificate, farms, adjust their management and biosecurity plan. In addition, transmission of the disease occurs horizontally by mechanical vectors such as rodents, anglers and birds moving between farms. So making fence can decrease the risk of introduction of virus to the farm. Ahmadivand *et al*. (2016) isolated the VHSV from trout farms in six provinces of Iran. Iranian Veterinary organization sanitation programs, such a surveillance, monitoring and eradication were efficient to prevent epidemics and successfully decreased VHSV incidence in Iran.

In conclusion, the result of our phylogenetic study shows that all the Iranian VHSV strains belongs to clade Ia-2 of genotype Ia and are related (99% identity) to VHSV from recent Italian strains (VHSV/O.mykiss/I/TN/133/Apr10).

The Figs. 3 and 4 show that although sanitation program is currently good enough to prevent nationwide epidemics and successfully decreased VHS incidence in Iran, illegal trade, transfer of fry between farms and sharing of equipment or staff in some areas continued circulation of viral agents in Iran. Our results documented the continued circulation of clade Ia-2 viruses throughout the North, Southwestern, west and center of Iran. This result ultimately assists the design of control strategies for VHS eradication.

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