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Multiplex PCR Assay for Detection of VHSV, IPNV and IHNV in Eyed Egg, Fry and Broodstock of Rainbow Trout, *Oncorhynchus mykiss*, Hatcheries in West Iran

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Infectious hematopoietic necrosis (IHN), viral hemorrhagic septicemia (VHS) and infectious pancreatic necrosis (IPN) are important viral diseases of salmonids especially rainbow trout leading to mass mortality among cultured fish. The aim of this study was molecular detection of IHNV, IPNV and VHSV in eyed egg, fries and broodstock of rainbow trout and comparison of their abundance among hatcheries in the western part of Iran. In this study, 300 eyed eggs, 150 fry and 6 broodstocks were studied in 15 hatcheries. All samples were examined by Reverse Transcriptase Polymerase Chain Reaction assay. The frequency of IHNV, IPNV and VHSV were 37.28, 46.96 and 8.19 percent, respectively as 20.89% of the eggs, 54.44% of fries and 17.14% of the broodstocks found to be infected by one of the viruses. The present study revealed that examined fish, both fry and broodstock, were simultaneously infected by all three kind viruses which is very important epidemiologically.

Key words: IHN, IPN, VHS, rainbow trout, RT-PCR.

VHSV and IHNV are members of the family *Rhabdoviridae* causing lethal diseases in several salmonid species. VHSV infects a wide range of marine, brackish and freshwater fish species mostly salmonidae (Einer-Jensen *et al*., 2004). Four major genogroups of VHS virus have been identified (Snow *et al*., 2004), Although the new strain from the Great lakes, identified as VHSV Genotype IVb, (Gagne *et al* ., 2007). Rainbow trout (*Oncorhynchus mykiss*) is currently listed as susceptible species to IHN and VHS viruses in the Directive 2006/88/EC (EFSA, 2008). Both diseases are considered as a "notifiable disease" by the OIE and they are also included in list II of the EU legislation (OIE, 2000). Also the IPN is considered as a "significant disease" by the OIE, and it is included in list III of the EU legislation (OIE, 2002). Aquatic birna viruses are characterized by bisegmented, double-strand RNA genome, the largest and most diverse group of viruses within the family Birnaviridae and include a variety of viruses from numerous species of fish and marine invertebrates (Wolf, 1988). The screening procedure is based mainly on virus isolation in cell cultures such as EPC (epithelioma papulossum of carp) and BF-2 (blue gill fin) for VHS and IHNV and CHSE-214 (from chinook salmon embryo) for IPNV fish lines .Confirmative identification may be achieved by use of immunological or molecular methods (Winton and Einer-Jensen, 2002; Dopazo and Barja, 2002). Outbreak of viral diseases in rainbow trout have

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been reported in Iran before (Fallahi *et al* 2006; Haghighi Khiabanian-asl *et al* 2007; Akhlaghi and Hosseini, 2007; Zargar *et al*., 2008 and Raissy *et al*., 2010 a,b). Among different cell culture, immunohishemical and molecular methods have been developed for identification of fish viruses in recent years, PCR is more successful in detecting viruses in fish tissue (Knüsel *et al*., 2007). RT-PCR is reported as applied method by many authors (Blake *et al* 1995; Rodríguez *et al* 1995; Miller *et al* 1998; Alonso *et al* 1999a; Barli Maganja *et al* 2002; Hope *et al* 2010; Knüsel *et al* 2007). The RT-PCR, seminested PCR and PCR-ELISA optimized for separate identification of fish viruses has been successful in detecting viruses in naturally occurring coinfections. The seminested PCR and PCR-ELISA was more sensitive than the cell culture method and was appropriate for the detection of low infective titres of IHNV in the presence of IPNV, when interference occurs (Barli-Maganja *et al* .,2002). In comparison between RT-PCR and traditional virus isolation on cell culture for detection of VHSV and IHNV, RT-PCR has been reported more sensitive than virus isolation (Knüsel *et al*, 2007). Millard *et al*. (2006) described a new method for molecular detection of IHNV and infectious salmon anemia virus (ISAV) by employing molecular padlock probe (MPP) technology combined with rolling circle amplification (RCA) and hyperbranching .In this method , viral RNA could be detected directly , either alone or in the presence of non- viral RNA from fish tissue.

In Iran, there has been an increase in the production and consumption of freshwater fish reared in aquaculture system in recent years, mainly rainbow trout (*Oncorhynchus mykiss*). Along with development of rainbow trout culture, occurrence of viral diseases such as IHN and IPN has been resulted in great economic losses in aquaculture trade of Iran. This study is aimed to identify three important viruses, IHNV, IPNV and VHSV, in eggs, fry and broodstock of rainbow trout by using Multiplex RT-PCR method.

MATERIALS AND METHODS

Sampling

Collection of fish and egg samples was accomplished during winter and spring 2011 from 15 hatcheries in the western part of Iran.

A total of 300 eggs, 150 fry and 10 stocks were randomly sampled and were transferred to the Biotechnology Research Laboratory, IAU, Shahrekord in cool box in a short time. Samples were kept in freezer (-70ºC) until molecular testing.

Preparation of samples

Every three eggs put into a 1.5 ml microtubes, they have completely crushed to the extent where a homogeneous mixture was obtained. The fry (less than 4 cm) crushed into the test tube and completely homogenized, then the amount of 100 mg was transferred into a micro tube. Also fish organs were homogenized into the test tubes and then 50 to 100 mg of them were entered into the micro tubes for RNA extraction.

Primers

The target gene and primer used to detection of each virus are indicated in table 1. All primers were synthesized by CinnaGen Co. (Tehran, Iran).

PCR amplification test

For multiplex RT-PCR, IPNV, IHNV, and VHSV viral RNA templates were reverse transcribed simultaneously, and PCR was performed with different combinations of multiple primer pairs for each virus described by Williams *et al*. (1999). This technique detects the VP2, N and G gens of aquatic birna viruses, IHNV and VHSV, respectively. The amplification reactions were performed in a 50-µl reaction mixture consisting of 4 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1.0% Triton X-100; Promega), 10 µl of reverse transcribed cDNA, 1.5 mM $MgCl_2$, 0.5 µM of each of the virulence gene-specific primers, 1.5 U of Taq polymerase (Sigma) .The amplifications were carried out in a Mastercycler gradient thermal cycler (Eppendorf) with 40 cycles of a initial denaturation at 94°C for 4 min , denaturation step at 94°C for 60 s, primer annealing at 60°C for 30 s and extension at 72°C for 90 s. Furthermore, the final extension step of 10 min at 72°C was performed. The negative control (no template DNA) was distilled water. The PCR products were detected by electrophoresis of 20 µL of each amplification mixture in 2% agarose gel in 1% Tris-acetate-EDTA buffer, after which the gel was stained with ethidium bromide (0.5 µg ml^{-1}). A UV transilluminator was used to visualize the bands, and results were recorded by photography.

RESULTS AND DISCUSSION

This study was carried out with the aim of molecular detection of IPNV, IHNV and VHSV in farmed rainbow trout located in West Iran. During this study, samples of eyed egg, fry and internal organs of rainbow trout broodstocks were investigated. Molecular detection of IHNV, IPNV and VHSV was done by Multiplex RT-PCR technique. Products of 206, 371 and 625 bp were obtained, as expected, from RT-PCR amplification of the IPNV, IHNV and VHSV genes (Figure 1). After PCR, the obtained bands were blasted with other sequences associated with IPN, IHN and VHS in the gene bank (NCBI, Gen Bank). Molecular diagnostic techniques such as PCR assays are increasingly used to detect and identify important viral fish pathogens including VHSV, IHNV, IPNV and ISAV (Blake *et al*., 1995 ;Williams *et al* ., 1999 ; Winton *et al* .,2002 ; Dopazo and Barja .,2002; Millard *et al* ., 2006; Knüsel *et al*., 2007; Hope *et al* .,2010).

The results revealed that the frequency of IHNV, IPNV and VHSV were 37.28, 46.96 and 8.19 percent, respectively. The percentage of total viral infection in the eyed eggs, fries and broodstocks were 20.89, 54.44 and 17.14, respectively. The abundance of IPNV was higher in eyed eggs and fry (11.17 % and 27.59 % of examined samples) while the abundance of IHNV was higher (5.96 %) in broodstocks than in eyed egg or fry (Table 2). One of the important results of the current study was proving the existence of all three viruses in the studied samples. Several reports of viral diseases have been accomplished in Iran and other countries in recent years. Raissy *et al*. (2010a) evaluated 30 fish farms in Chaharmahal va Bakhtyari Province ,Iran. They showed that 33.3% of all studied farms found to be infected by IHNV. Haghighi Khiabanian-asl *et al*. (2007) by using the immunohistochemistry and nested PCR assay, and Fallahi *et al* (2006) by indirect florescent antibody test have been identified the IHNV in some rainbow trout fish hatcheries in Iran. Other researchers also have been detected the IPNV in suspected rainbow trout farms in West Iran (Akhlaghi and Hosseini., 2007; Raissy *et al*., 2010b). Several new improved methods are applied for rapid diagnosis of viruses. These techniques have high effects on accurate identification of aquatic viruses. The one-step quantitative real-time polymerase chain reaction assay (qRT-PCR) is valuable for rapid and high-throughput prescreening of fish before confirmatory testing by cell culture or sequencing tissue-derived amplicons and especially in detecting infection in fish that do not show clinical signs of VHS(Hope *et al* 2010). Also A reverse transcription loop-mediated isothermal amplification (RTLAMP) protocol was developed for detection of IHNV RNA in rainbow trout. If the sensitivity of RT-LAMP and LAMP were compared with nested PCR, a10-fold higher sensitivity would be seen for the LAMP technique (Gunimaladevi *et al*., 2005).It was used as well as a rapid and highly sensitive diagnosis assay for IPNV which well lends itself to use in aquaculture health management and disease control (Soliman *et al*., 2009).

Table 1. Primer sequences used for PCR amplification and the expected amplicon sizes

Primer pairs	Sequences (5′ to 3′)	Target gene	PCR amplicon (bp)	Pathogen
WB2	CGTCTGGTTCAGATTCCACCTGTAGTG	VP2	206	Aquatic birnaviruces
WB1	CCGCAACTTACTTGAGATCCATTATGC			
IHN3	GTTCAACTTCAACGCCAACAGG	N	371	IHN
IHN4	TGAAGTACCCCACCCCGAGCATCC			
VHS3	CGGCCAGCTCAACTCAGGTGTCC	G	625	VHS
VHS4	CCAGGTCGGTCCTGATCCATTCTGTC			

Table 2. Frequency percent of detected viruses in different samples

VHS	IHN	IPN	Sample
0.74%	8.98%	11.17%	Eyed egg
4.47%	22.38%	27.59%	Fry
2.98%	5.96%	8.2%	Brood stock
8.19%	37.28%	46.96%	Total

Horizontal and vertical transmissions have been demonstrated in some of the viruses. In the horizontal way, the virus is shed in the faeces, urine and sexual fluids and in the vertical way, the virus is spread by egg-associated transmissions between fish populations. Control methods are currently based on the implementation of control programs, through the avoidance of the introduction of fertilized eggs. In recent years, there has been an increased effort to produce an efficient subunit or single virus protein vaccine or DNA vaccine using the available recombinant DNA

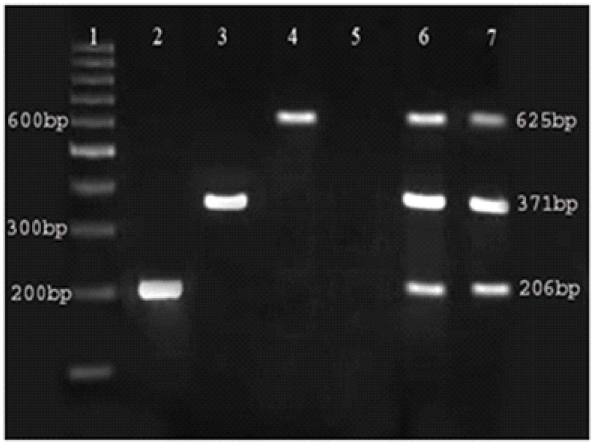

Fig. 1. Amplification products from infected rainbow trout using multiplex PCR assay for the detection of IPNV (206 bp) , IHNV(371bp)and VHSV(526bp). Lane 1, 100 bp DNA ladder; lanes 2,3,4 are IPNV , IHNVand VHSV positives, respectively . lane 5, negative control; lanes 6 and 7 are the simultaneous detection of viruses in samples

technology. However, at present none of these vaccines are licensed and commercialized to be used in Iran. In the field, IHNV immunity in rainbow trout is induced by challenge with live IHNV following pre-injection with either IPNV. Rainbow trout pre-infected with IPNV were protected from IHNV challenge 7 d later (relative percentage survival, RPS: 68.8%) (Kim *et al.,* 2009).

CONCLUSION

In the present study, we managed to detect and identify three major types of viral diseases by multiplex RT- PCR technique in western part of Iran. With regard to the high rate of IPNV and IHNV infections, regular health care and monitoring programs must be particularly considered. Thus, because of the increasing production of rainbow trout in Iran's inland waters and the important role of these viruses in pathogenicity and health risks for fish, the movement of infected fish or eggs to other regions of the country plays a critical role in spreading the disease. Consequently, fish health inspection programs should be developed to identify infected populations. The success of such fish health management programs depends on the rapid detection and identification of specific pathogens. Therefore, these rapid IHNV, IPNV and VHSV diagnostic tests will be a valuable tool for fish health management and monitoring programs.

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