

Full Length Research Paper

Use of RT-PCR in diagnosis of infectious hematopoietic necrosis in rainbow trout hatcheries, Iran

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Distribution of Infectious Hematopoietic Necrosis (IHN) was studied in rainbow trout fish farms in Chaharmahal and Bakhtyari Province which is a major trout culture region in Iran. Overall 150 suspected rainbow trout fry with less than 3 g body weight from 30 fish farms in Chaharmahal and Bakhtyari Province, Iran, were studied and fishes of 10 hatcheries (33.3% of all studied farms) found to be infected with IHNV. Reverse transcriptase polymerase chain reaction (RT-PCR) was applied to the detection of infectious hematopoietic necrosis virus (IHNV). The product of 371 bp was amplified from the RNA extracts of infected organs of suspected fish. DNA sequencing of the PCR products demonstrated specificity of the amplification. The RT-PCR was found to be a simple, highly specific and sensitive method allowing diagnosis of IHN in a short time.

Key words: Reverse transcription-polymerase chain reaction (RT-PCR), IHN, rainbow trout.

INTRODUCTION

Infectious hematopoietic necrosis virus is an important fish rhabdovirus causing severe epizootics in salmonids such as rainbow trout (*Oncorhynchus mykiss*) and is lethal in many cases especially in young fish. The high economic cost of such losses has stimulated numerous studies, as a result of which salmonid viruses are better understood than other fish viruses. IHNV which was reported by Amend (1970) for the first time belongs to the family Rhabdoviridae and is an enveloped virion with a genome (~11 kb) consisting of a single molecule of linear, negative sense ssRNA which contains six genes (Murphy et al., 1995). The virus produces systemic infection with high mortality in farmed rainbow trout (*O. mykiss*) and in other species of salmonids such as cutthroat trout (*Salmo clarki*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), Pacific salmon (*Oncorhynchus tshawytscha*), chinook (*Oncorhynchus tshawytscha*), sockeye

(*Oncorhynchus nerka*), chum (*Oncorhynchus keta*), masou (*Oncorhynchus masou*), amago (*Oncorhynchus rhodurus*), and coho (*Oncorhynchus kisutch*). Experimental infections have been reported in species other than salmonids including pike fry, sea bream and turbot (Fernanda et al., 2004). IHNV is endemic and widely prevalent among populations of salmonids throughout much of its historic range along the west coast of North America. The virus has also become established with a high prevalence of infection in major trout growing regions of North America, Europe and Asia. IHNV was introduced to Iran through the movement of infected fish or eggs and now is responsible for high economic losses in hatchery-reared juvenile rainbow trout. At present, until vaccine technology improves, the only effective way to control the disease in aquaculture is to prevent exposure to the virus. Health checks are carried out regularly on trout and salmon reared in aquaculture in several countries (Eaton and Hulett, 1991; Winton, 1991; Olesen and Korsholm, 1997). Protocols include routine checks and diagnostic examinations to identify pathogens before

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fish are moved to another location.

There are excellent reports on the molecular biology of the virus (Kurath and Leong, 1985; Morzunov et al., 1995; Schutze et al., 1995; Hoffmann, 1995; Dhar, 2008) and the associated disease (Wolf, 1988). In this study RT-PCR was used for accurate detection of IHNV in fish farms and also to study the distribution of IHNV in trout farms in Chaharmahal and Bakhtyari Province.

MATERIALS AND METHODS

Sampling

Visits were made to the farms and suspected fish were collected and tested for IHNV. Overall 30 trout farms were studied during February 2009 to March 2010. Fish were dissected at the laboratory to remove kidney, spleen, liver and heart then the organs of each 5 fish were pooled for the PCR test. Samples were processed and assayed for viral infectivity within 3 h of collection. In routine diagnostic work 5 mg of organ (kidney, spleen, liver and heart) were used for isolation of viral RNA. To increase the likely-hood of detecting disease, fresh fish were preferred as samples over fish showing more advanced post-mortem changes and fish with pathological signs consistent with IHNV were preferred.

Primers

The specific primers were prepared according to Williams et al. (1999).

Nucleic acid extraction

RNA was extracted from samples using the RNeasy Total RNA Kit (Qiagen) according to the manufacturer's instructions.

Synthesis of cDNA

Synthesis of cDNA was carried out using moloney murine leukemia virus reverse transcriptase (MMLV-RT, Fermentas) and random hexamer primers (Fermentas). Reverse transcription of heat-denatured RNA (5 min at 70°C in 32 µl of reaction buffer for MMLV-RT in the presence of 0.1 mM of each dATP, dCTP, dGTP and dTTP) was performed after addition of 8 µl of reaction mixture (10 mM dithiothreitol, 0.4 µg of random hexamer, 5 U of RNase inhibitor (Fermentas) and 400 U of MMLV-RT) for 5 min at 22°C, 15 min at 37°C and 30 min at 42°C. After reverse transcription, the reactions were heated to 99°C for 5 min in order to inactivate MMLV-RT.

RT-PCR assay

Amplification of cDNA by PCR was carried out in a total volume of 50 µl in the reaction buffer for Taq DNA polymerase containing 1.25 U of Taq DNA polymerase (Fermentas), 100 pmol of each primer (Calcip F,R), 1 mM MgCl₂, 0.2 mM dNTP, and 4 µl of cDNA.

Amplification was performed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 1 min. After amplification, RT-PCR products were characterized by

1.5% agarose gel electrophoresis in Tris–borate–EDTA buffer.

RESULTS

A total number of 150 fry fish including 30 pools from 30 rainbow trout hatcheries were examined and fishes of 10 farms (33% of studied farms) found to be infected by IHNV.

A product of 371 bp was obtained, as expected, from RT-PCR amplification of the IHNV gene. Figure 1 shows PCR product as 371 bp paralleled by 1000 bp DNA ladder marker. Four regions of study, studied fish farms in each region and farms with IHNV are listed in Table 1.

DISCUSSION

Infectious hematopoietic necrosis is the most important viral disease of rainbow trout in many countries (Eaton and Hulett, 1991; St-Hilaire et al., 2002; Zargar et al., 2008). Depending upon the species, stock, and size of the fish, strain of the virus and environmental conditions such as temperature, an outbreak of IHNV may result in losses approaching 100%. Traditionally, the initial diagnosis of viral diseases affecting salmonids has relied upon epizootiological data such as species and age of fish, geographic location, water temperature as well as microbiological information (example, direct light or electron microscopic observation or isolation of the virus in cell culture). Isolation of a virus is typically followed by confirmatory identification using chemical and morphological criteria or by serological assays (Sanz and Coll, 1992; McCarthy, 2006). The viral examinations are conducted on stocks of trout and salmon include both routine health checks to monitor the status of the population and pathogen-free certification examinations which are required prior to moving fish from one location to another. These traditional methods are often time-consuming and expensive. In recent years, the techniques of molecular biology have provided new strategies for the rapid and sensitive detection of antigens, antibodies and nucleic acids. As these methods become more widely accepted, they will result in significant improvements in speed, precision, and sensitivity of the diagnosis of fish diseases. Methods used for the virological examination of fish rely upon cell culture systems for isolation of virus and serum neutralization or fluorescent antibody assays for identification of the agent. Beside the classic virological serology methods further tests such as PCR and DNA probe techniques are recommended for the identification and confirmation of fish viruses. There are many reports on molecular diagnosis of the virus by RT-PCR from

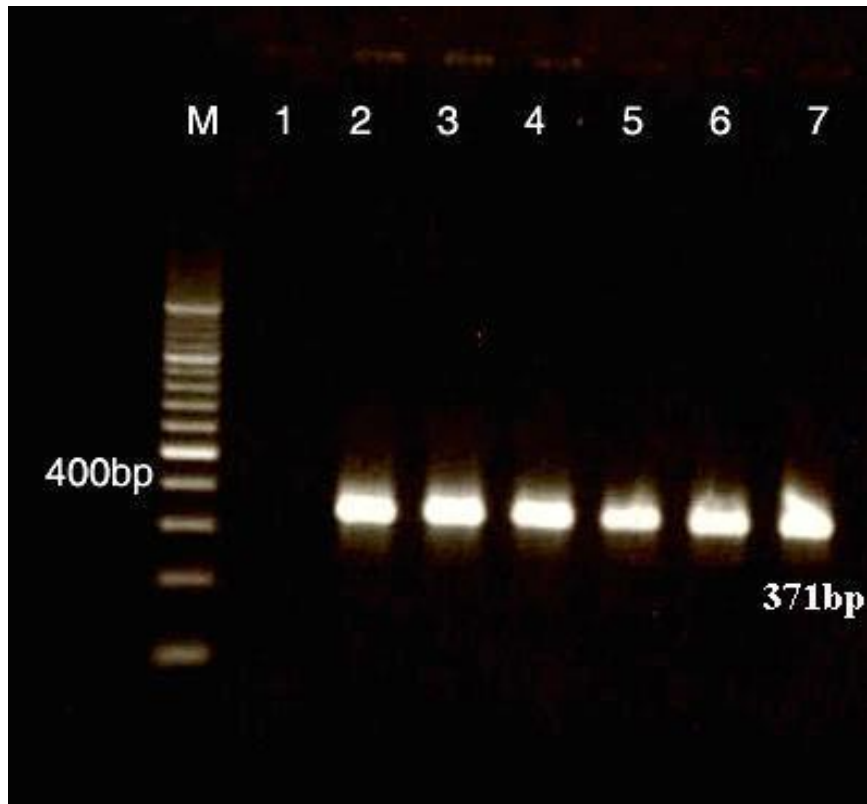


Figure 1. Typical agarose gel showing the detection and identification of IHNV in homogenates of tissue from infected fish. Lane 1, negative cell control lane 2, Positive control; lanes 3 to 7, samples; M, molecular size marker. The figure was prepared by using HP ScanJet II cx and Adobe Photoshop cs Ver 8.0.

Table 1. Studied fish farms in each sampling region, farms with IHNV and time of onset.

Region	Studied fish farms	Fish farms with IHNV	Time of IHN onset
Ardal	10	A	February 2010
		J	January 2010
		K	March 2010
		L	February 2010
		R	December 2009
Farsan	13	P	March 2010
		Q	March 2010
Shahrekord1		N	February 2010
Bazoft	6	M	February 2010
		O	February 2010

different countries (Kurath and Leong, 1985; Morzunov et al., 1995; Schutze et al., 1995). The virus has been also collected from hatchery-reared rainbow trout in Iran and

studied by both serological and molecular methods (Haghighi et al., 2007; Fallahi et al., 2006; Zargar, 2008). IHNV infection is responsible for great economic losses

in hatchery-reared rainbow trout in Iran in recent years. Therefore, prevention of infection plays a major role. Regular control measures to be taken throughout Europe, laying down sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases, have been recommended by a European Commission decision (96/240/EC) (Commission of the European Communities 1996). At present, the virus is diagnosed mostly by IFAT after conventional virus isolation in cell cultures. This method, although it is time consuming, works well in acute cases but it is extremely difficult to detect virus in subclinically or latently infected fish. IHNV identification is also possible by means of the neutralization test (NT) and enzyme-linked immunosorbent assay (ELISA). Zargar et al. (2008) found nested-RT-PCR a more reliable method for identification of the virus than IFAT. Haghighi et al. (2007) also found PCR more accurate than Immunohistochemical methods for diagnosis IHNV directly in fish tissues. Different authors have reported on PCR methods which detected IHNV in infected cell cultures (Arakawa et al., 1990; Bruchhof et al., 1995; St-Hilaire et al., 2002; Willimas et al., 1999). Miller et al. (1998) reported no difference in detecting the virus in organ samples and cultured cells. In this research Direct RNA isolated from organ samples was used for PCR operation. A RT-PCR assay was developed and applied to the detection of IHNV and study distribution of the virus in rainbow trout hatcheries in Chaharmahal and Bakhtyari Province. The very fast RNA extraction, reverse transcription and PCR permitted us to read the agarose gels within 5 to 6 h after whole fish arrived, which is of great importance when there is reason to believe that IHNV may be present. According to the results from 30 studied farms (with clinically IHN-positive fish), 10 farms were found to be IHN-positive by RT-PCR. The highest infection rate was found in Ardal region with 5 IHN-positive hatchery farms as half of studied hatcheries were infected by IHNV.

Similar results were found in previous studies as Fallahi et al. (2006) found 11 samples (11.11-%) to be positive from 99 studied samples in trout hatchery farms in Chaharmahal and Bakhtyari Province. Haghighi et al. (2007) reported 3 positive samples in Chaharmahal and Bakhtyari Province (33% of total samples) and totally 35 positive samples in Iran (35%). Zargar et al. (2008) found 27 IHN-positive hatchery farms from 30 studied farms in Chaharmahal and Bakhtyari Province. Regarding to the high rate of IHNV infection in hatchery farms in Chaharmahal and Bakhtyari Province, regular health care and monitoring programs must be particularly considered especially that the province is major trout culture region in Iran so 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, this rapid IHNV diagnostic test will be a valuable tool for fish health management and monitoring programs.

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