

**Isolation and Identification of the Infectious Haematopoietic
Necrosis Virus (IHNV)-like Agent from Farmed Rainbow
Trout (*Oncorhynchus mykiss*) from Iran**

Fallahi, R.,¹ Soltani^{*2}, M., Kargar, R.,¹ Zorriehzahra, M.E.J.,³ Shchelkunov, I.,⁴
Hemmatzadeh, F.² and Nouri, A.¹

1. Razi Vaccine & Serum Research Institute; P.O.Box 11365-1558, Tehran, Iran

2. Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

3. Fish Health Unit, Iranian Fisheries Research Institute, Tehran, Iran

4. All-Russian Research Institute of Freshwater Fisheries, Rybnoye, Dmitrov Region,
Moscow, Russia

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Summary

The isolation of viral causative agent of fry trout mortality syndrome in farmed rainbow trout in Iran was discussed. Four hundred twenty samples were collected from six provinces for virological, serological and electron microscopic examinations from October 2001 till May 2002. No cytopathic effect (CPE) was observed in the cell cultures except for EPC and BF-2 cells in which signs of CPE appeared one day postinoculation during the second and third passages of two brooder ovarian fluids. Electron microscopic examination revealed the viral particles measured 67-73×128-182nm in the pelleted materials from the CPE positive in BF-2 and EPC cell lines. Indirect immunofluorescence antibody test were positive for some of smears obtained from kidney tissues of the larvae, the samples of ovarian fluids that showed CPE on EPC and BF-2 cell lines as well as CPE positive cell cultures. Using ELISA eight of all sera samples tested were positive with optical density ranged 0.316-2.268 compared to 0.06 for negative controls.

Key words: IHNV, rainbow trout, ELISA, electron microscopy, fluorescent antibody

* Author for correspondence E-mail: msoltani@chamran.ut.ac.ir

Introduction

Rainbow trout farms in Iran are situated mainly in the north, northwest, west and southwest of the country where the natural water supplies are of an adequate quality and quantity and suitable for trout farming. Water sources are directly used without any treatment either from adjacent springs or pumped from rivers. The returning waters to the rivers are untreated therefore allowing for easy entry and establishment of potentially important trout pathogens. If the amount of locally produced fry is insufficient to meet the production needs, eyed-eggs and fry are imported from the overseas such as England and Italy. Despite considerable losses during fry stage of up to one gram body weight have been experienced within the last several years (Soltani *et al* 2002), little efforts was undertaken so far to clarify the cause of such morbidities and mortalities. The mortality increased with the increase of stress, e.g. fish handling and transportation. The affected fish showed various gross signs including dark body coloration, exophthalmia, anorexia, spiral swimming, and haemorrhage at the base of fins and in the internal organs. Sometimes pseudofecal casts from the anus were observed. Generally, the level of mortality decreased after fish reached one gram body weight. Usually, bacteriological, mycological and parasitological examinations of the affected fish were negative, although some potentially pathogenic microorganisms such as *Flavobacterium/Flexibacterium* sp. and *Yersinia ruckeri*-like bacterium were occasionally found (Soltani & Rostami 1996, Soltani 1998, Soltani *et al* 1999, Soltani & Tarahomi 2002, Soltani *et al* 2002). Based on observable gross clinical signs, mortality pattern as well as serological findings by Akhlaghi (2001) a viral aetiology of the condition has been also suggested in addition to *Flavobacterium psychrophilum*.

Materials and Methods

Sample collection and processing. From October 2001 till May 2002 pools of four hundred twenty samples were collected from farmed rainbow trout in six Iranian provinces consisting of Tehran, Mazandaran, Gilan, Kohkiluyeh & Boyerahmad,

Fars and Markazi for virus isolation, electron microscopy and indirect fluorescent antibody test (IDFAT). Sera and ovarian fluids from broodstock fish, green eggs, eyed eggs, yolk sac fry (without yolk sac), whole viscera including kidney tissue from fry of less than one gram body weight and tissues of kidney, spleen and liver from fingerling fish were sampled. In some cases of distant hatcheries, the samples were collected in sterile screwed cap bottles containing Eagle's minimal essential medium, (EMEM) supplemented with 2% fetal bovine serum (FBS), 100IU/ml penicillin and 100µg/ml dihydrostreptomycin. The samples and affected fish were transported to virological laboratory alive or in crushed ice within 20h postsampling. In the laboratory they were homogenized with pestle, mortar and sterile sand, resuspended in EMEM and clarified by centrifugation at 4000g for 25min. Supernatants were diluted to 1:100 to 1:1000 (w/v) with EMEM supplemented with 10% FBS and 0.88g/L glutamine prior to inoculation of cell cultures.

Cell lines and virus isolation. Epithelioma papillosum cyprini (EPC), bluegill fry (BF-2), fathead minnow (FHM), rainbow trout gonad (RTG-2) and Chinook salmon embryo (CHSE-214) were used. All the five cell lines were grown in 25cm² cell culture flasks (Nunc) in minimal EMEM (pH7.2-7.4) with Earle's salts supplemented with 10% FBS, sodium bicarbonate (2.2g/L), L-glutamine (0.88g/L) and non-essential amino acids. Each sample was used for inoculation of EPC cells and one of the four other cell lines. After inoculation, EPC, FHM and BF-2 cells were incubated at 16-18°C and, others at 14-16°C. If CPE was observed, 0.1ml aliquots of cell culture fluids with scraped cells were used to inoculate fresh cell cultures. CPE-positive cell suspensions obtained after second or third passages were stored at -70°C. If no CPE was observed after 10-14 days postinoculation, three blind passages were done after one-two cycles of freezing (-70°C) and thawing of cell suspensions.

Electron microscopy (EM). The cultures of CPE-positive cells were collected, centrifuged at 6000g for 30min at 4°C and the supernatants were then

ultracentrifuged at 53000g at 4°C for 90min. The pellets were collected diluted in about 200µL phosphate buffer saline (PBS). applied onto EM grids and negatively stained with 2% phosphotungstic acid prior to examination with a Philips EM 400 electron microscope at accelerating voltage of 60 kV.

IDFAT. Smears were made of the CPE-positive cell cultures as well as of broodstock gonad fluids, and spleen and kidney from the larvae and fingerlings. The IDFAT was performed according to the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000). The smears were fixed with cold acetone, dried for at least 20min at room temperature and then were blocked with blocking buffer (pH7.2) (PBS containing 1% BSA and 0.05% Tween 80), incubated at 37°C for 30min and washed four times with PBS-Tween. A volume of 100µL of anti-IHNV rabbit antibody and/or anti-IHNV monoclonal antibody (CRL, Arhus, Denmark) diluted 1:20-1:40 was added to each smear. The smears were incubated at 37°C for 60min prior to four washings with PBS-Tween. A 100µL aliquot of fluorescein-isothiocyanate labeled with swine anti-rabbit immunoglobulin and/or rabbit anti-mouse immunoglobulin conjugates (Dako, Denmark) diluted 1:20 was added to each smear. After 60min incubation at 37°C, the smears were washed with PBS-Tween four times, mounted using 50% glycerol saline (pH 8.5) and examined in fluorescent microscope under ×200, ×400 or ×1000 magnifications. Negative and positive controls were provided using CPE negative cells and acetone fixed IHNV infected cell culture provided by Dr. Shchelkunov, respectively.

ELISA. Sera sampled obtained from 44 broodstock were used for a sandwich ELISA using anti-trout IgM monoclonal antibody (Aquatic Diagnostic Ltd, Product F11, Scotland). The test was performed according to procedure of the supplier. Absorbance values were immediately read with an ELISA microplate reader (Stat Fax, USA) at 450nm. Serial two-fold dilutions of both normal fish serum and PBS were included in the test as negative controls. Blank ELISA readings against wells filled with chromogen and stop solution were done as well. Results were considered positive if absorbance exceeded at least twice that of the negative control means.

Results

Virus isolation. No CPE was observed in the cell cultures except for EPC and BF-2 cells in which signs of CPE appeared one day post inoculation during the second and third passages of the ovarian fluids obtained from only two broodstocks. The CPE was characterized by darkened ovoid cells containing migrated chromatin to nucleus membrane, followed by rounding and sloughing of these cells to the surface (Figure 1a, b). By third day postinoculation the entire cell sheet became involved and the cells had all eventually detached from the substrate. Further passages in EPC and BF-2 cells at 16°C induced typical CPE one-day postinoculation.

EM. Virions were found in pelleted materials of the second passage in CPE positive BF-2 and EPC cells, which were inoculated with the broodstock fish ovarian fluids (Figure 2). The bullet shaped particles had diameter of 67-73nm and length of 128-182nm.

IDFAT. Results of IDFAT were positive for some of smears obtained from kidney tissues of the larvae as well as the samples of ovarian fluids that showed CPE on EPC and BF-2 cells (Figure 3). The smears from these samples were negative when tested by IDFAT for IPNV and VHSV (data not shown). Also, the smears obtained from the CPE positive cells were positive for IHNV only. Also, smears from CPE negative cells were negative for IHNV, IPNV and VHSV when tested in IDFAT.

ELISA. Eight of all sera samples tested were positive in ELISA showing an optical density 0.316-2.268 compared to 0.06 for negative controls. Two of these positive sera samples were obtained from the same broodstock fish whose ovarian fluids the virus was isolated from. These positive samples were obtained from trout hatcheries located in Tehran, Mazandaran and Kohkiluyeh & Boyerahmad provinces.

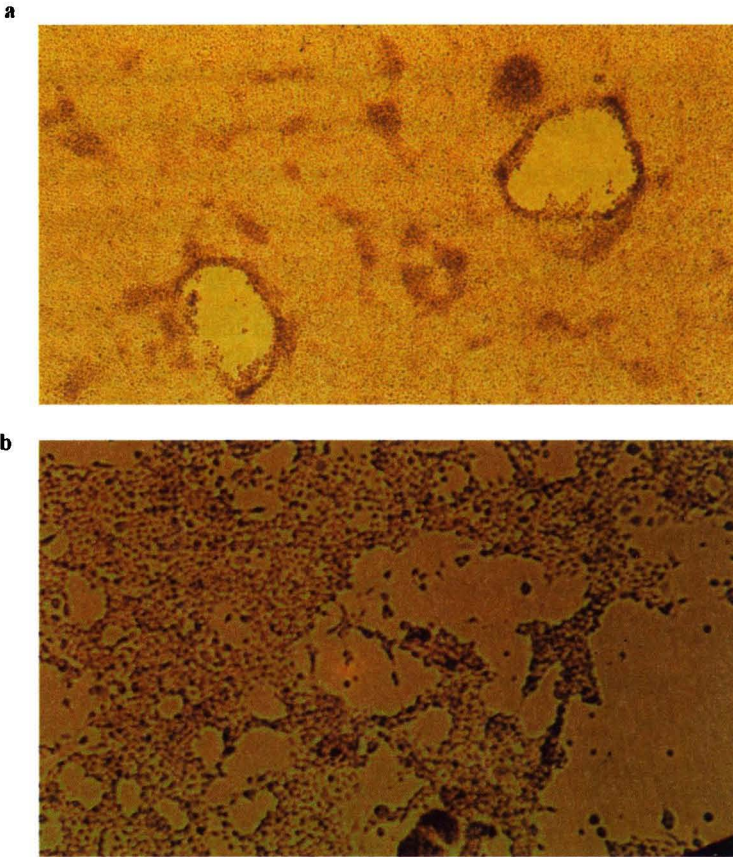


Figure 1. Cytopathic effect (CPE) of IHNV-like virus in third passage on EPC cells at 18°C. a) focal CPE at 24h and b) total CPE at 72h after inoculation (x25)

Discussion

This is the first report on isolation and identification of a viral agent in rainbow trout in Iran since 1957 when that fish was introduced for the first time. The origin of the virus is unknown to the moment. It is probable that the virus might be introduced with rainbow trout eyed-eggs imported from Europe during the last years. Although, this viral agent was isolated from two rainbow trout farms only, serological results of IDFAT indicate that several farms of different locations were infected with the virus. Some of these farms are supplied with spring water, while the others use river

water. Therefore, it is possible that IHNV has already spread through the water or with fish transfers to other farms of the country.



Figure 2. *Electron micrograph of IHNV-like virus in pelleted materials of EPC cells. The bullet shaped particles contain an outer membrane (x132000)*

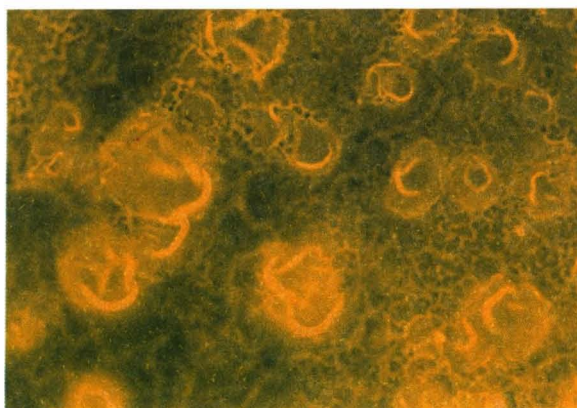


Figure 3. *IDFAT reaction on smear obtained from the ovarian fluids of broodstock rainbow trout (x200)*

To implement control measures and prevent disease outbreaks, it is important to evaluate how wide has the infection spread. To achieve this, screening of farmed fish populations is required. Unless disease control measures are implemented, IHNV has to be considered as a major potential threat to the Iranian rainbow trout culture. If the disease becomes wide spread, the larvae producers will have to introduce

increased number of fry to compensate the losses due to the disease. However, such considerations required adequate information on the *in vivo* pathogenicity of this isolated virus. The CPE induced by the virus is similar to that described in other reports for IHNV in EPC and BF-2 cells (Plumb 1999). The EM examination also showed a bullet shaped agent with a rhabdovirus-like morphology (Wolf 1988, Granzow *et al* 1997). Furthermore, the isolation of IHNV was confirmed in IDFAT (Lapatra *et al* 1989, Danton *et al* 1994). Results of ELISA test also showed the presence anti-IHNV antibodies in some of broodstock fish. Although, virus isolation, EM examination and serological data in this study provide adequate evidence for the existence of IHNV infection in Iranian farmed rainbow trout, further work is currently in progress to study additional aspects of the isolated agent and to assess its pathogenicity to fish under experimental conditions.

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