

Infectious pancreatic necrosis in a recirculating rainbow trout (*Oncorhynchus mykiss*) culture system

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Abstract: During October 2009, a suspected outbreak of infectious pancreatic necrosis, with typical signs and high mortality, was observed in cultured rainbow trout (*Oncorhynchus mykiss*) fry (mean weight of 9 g) in a recirculating system in Fars Province, Iran. Tissue samples, including pyloric caeca as well as pancreatic tissues, liver, kidney, and spleen, were aseptically collected from 20 affected moribund fish for histopathological and molecular assays. Histopathologically, the exocrine pancreatic acinar cells exhibited the most notable pathology, including focal to extensive degeneration and coagulative necrosis with mononuclear inflammatory infiltrates. To confirm the diagnosis of clinical infectious pancreatic necrosis (IPN), a reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis were used. A 224-bp fragment of the IPN virus NS/VP3 (segment A) gene was amplified using cDNA prepared from infected pooled tissues of pancreas, liver, kidney, and spleen. The nucleotide sequence was most closely identified with the Sp serotype.

Key words: Infectious pancreatic necrosis, rainbow trout, recirculating system, histopathology, RT-PCR

Introduction

Aquatic birnaviruses with worldwide distribution are a problem in the freshwater rearing of salmonids and pose a significant risk of economic damage (1). Infectious pancreatic necrosis (IPN), a highly contagious viral disease of young salmonid fishes, is caused by the infectious pancreatic necrosis virus (IPNV) (2,3). IPNV is a bisegmented, double-strand RNA virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus*. The nonenveloped icosahedral capsid has a diameter of 60 nm and contains 2 genome segments, A and B (4,5). IPNV is known

to produce acute and systemic infections in farmed rainbow trout (*Oncorhynchus mykiss*), especially in fish under the age of 6 months (2) and, typically, those weighing 1-3 g (2,3). The disease has a wide geographical distribution, occurring in most major salmonid-farming countries of North and South America, Europe, and Asia (6).

Fish farming is a growing industry in Iran and the rainbow trout is one of the most promising new species for cultivation, both in flow-through and recirculating systems. Recirculating systems with a daily exchange rate of 10% or less are becoming more

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common, creating unique artificial environments for fish culture. These production systems can be located in regions where water resources are limited; where power, feed, or oxygen is relatively inexpensive and environmental impact can be minimized; or where populations, jobs, and local economies have declined. Unfortunately, favorable conditions occurring in recirculating systems may allow opportunistic microorganisms to cause disease (7).

IPNV was detected for the first time in Iran by Akhlaghi and Hosseini (8) using reverse transcription-polymerase chain reaction (RT-PCR). These results seem to confirm the earlier suspicion of the virulent nature of IPNV in rainbow trout flow-through system production (9). It is apparent that rapid detection and identification of specific pathogens can aid in the successful administration of fish health management programs (10) and consequently prevent economic losses due to viral diseases such as IPN. Therefore, the present investigation was undertaken to describe an outbreak of IPN in cultured rainbow trout in a recirculating culture system and to study the associated clinical signs.

Materials and methods

Fish sampling

During October 2009, a disease outbreak suspected of being IPN occurred in a private recirculating system fish farm in the province of Fars in southern Iran. The disease demonstrated a 12% mortality rate in cultured rainbow trout fry (mean weight of 9 g). The open-air fish farm used spring water with a temperature range of 12 to 14 °C and was equipped with 8 raceways featuring solids removal, biological filtering, and oxygenation systems. At the time of the outbreak, there were 25,000 fry in 5 raceways. The water recirculation rate was 350 L/min with an 8% water renewal rate every 24 h in the raceways. Fish were fed daily on a 4% body weight basis. A biomonitoring program was present throughout the system to record optional environmental measures. During our investigation, 120 moribund rainbow trout fry were collected, bearing visible signs of the disease.

Tissue samples from pyloric caeca, together with those from the pancreas, liver, kidney, and spleen of 20 affected moribund fish, were aseptically collected and each tissue sample was divided into 2 parts. The first portion of the tissue samples was used for histopathologic examination and immediately fixed in 10% neutral buffered formalin. The other portion was immediately preserved in a sterile Eppendorf microtube, frozen, and stored at -70 °C until RNA isolation could be performed. Fish were subjected to a detailed external and internal inspection prior to sampling. Direct smears from fish gills, skin, and fins were prepared for parasite examination, and sterile inoculations from fish kidneys were transferred into enriched media for bacterial isolation.

Histopathology

The fixed tissue specimens were dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin wax, divided into sections 5 µm thick, and stained with the hematoxylin and eosin (H&E) staining technique. All sections were examined using an ordinary light microscope.

RNA extraction

Viral genomic RNA was extracted from 50-100 mg of the mixed tissue samples using the guanidinium thiocyanate-phenol-chloroform method (RNX-Plus Isolation Kit, CinnaGen, Iran) according to the manufacturer's instructions. First, 1 mL of the RNX solution was added to homogenized pooled tissue samples and, after passing the lysate a few times through the pipette and mixing thoroughly by inversion, the samples were incubated at room temperature for 5 min. Next, 0.2 mL of chloroform was added; the contents were mixed, and the mixture was centrifuged at 12,000 × g (4 °C) for 15 min. The aqueous phase was recovered and transferred to a fresh tube. An equal volume of isopropanol was added and after being stored for 15 min on ice, the samples were centrifuged at 12,000 × g (4 °C) for an additional 15 min. Consequently, the supernatant was removed and the RNA pellet was washed by inversion with 1 mL of 75% cold ethanol and subsequent centrifugation for 8 min at 7500 × g at 4 °C. The RNA pellet was then dried at room temperature for 10-15 min, resuspended in 50-60 µL of nuclease-free water, and stored at -70 °C (8).

Oligonucleotide primers

The oligonucleotide primers used in this study were described by Taksdal et al. (11). The sequences of primers were as follows: DIAIPNF (5' ATC TGC GGT GTA GAC ATC AAA G) and DIAIPNR (5' TGC AGT TCC TCG TCC ATC CC). A 224-bp fragment of the NS/VP3 region of segment A of IPNV was expected to be amplified using these primers.

cDNA synthesis

Single-strand complementary DNA (cDNA) synthesis was performed using the RevertAid M-MuLV Reverse Transcriptase Kit (Fermentas) according to the manufacturer's instructions. Initially, 8 µL of RNA was mixed with 2 µL of 20 mM reverse primer and 2 µL of water, and was incubated at 65-70 °C for 10 min before being placed on ice for 5 min. At that point, 4 µL of reaction buffer, 1 µL of RiboLock RNase Inhibitor, and 2 µL of 10 mM dNTP mix were added to the initial solution. After incubation at 37 °C for 5 min, 1 µL of RevertAid M-MuLV reverse transcriptase was added to the reaction. The reaction was then incubated for 1 h at 42 °C. Finally, the cDNA was stored at -20 °C until the RT-PCR was performed (8).

RT-PCR

RT-PCR was carried out in a reaction volume of 25 µL containing 2.5 µL of PCR buffer (10×), 2 µL of MgCl₂ (50 mM), 0.5 µL of dNTPs (10 mM), 0.5 µL of each forward and reverse primer (20 mM), 4 µL of cDNA, 2.5 IU of Taq DNA polymerase (CinnaGen), and distilled water up to the final volume. The thermal program used for the RT-PCR was 94 °C for 2.5 min for an initial denaturing, followed by 35 cycles of 94 °C for 50 s, 60 °C for 45 s, and 72 °C for 50 s, with a final extension of 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis in TAE buffer. After the extraction of the PCR products from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer recommendations, the purified amplicons were cloned in vector pTZ57R/T and sequenced. The result of sequencing was analyzed using the DNASTAR program and compared with both the submitted GenBank sequences (ncbi.nlm.nih.gov) and the sequence of the previously isolated IPNV from Fars Province (8).

Results

Clinical signs and gross pathology

The moribund fish were lethargic, had poor appetite, showed erratic swimming behavior, and maintained a still position near the side of the raceway. Dark coloration of the skin, abdominal distention, mild to moderate exophthalmia, hemorrhages in ventral areas, and edema and swelling at the vent were typical macroscopic findings. At gross inspection, the internal organs were often found to be normal and fish did not show any infection with either parasites or bacteria.

Histopathology

Of the 20 moribund sampled fish on this farm, 18 showed typical histopathological changes, including focal to extensive degenerative changes and coagulative necrosis of different sizes in the exocrine pancreatic acinar cells (Figure 1a). These tissue changes were accompanied by mononuclear inflammatory infiltrates and severe hemorrhages (Figure 1b). Focal nuclear pyknosis, karyorrhexis, and karyolysis of the cells of the exocrine pancreatic acinar tissue were evident (Figure 1c). Inclusion bodies were not observed in the affected cells. Endocrine and most of the fatty peripancreatic tissues were normal. Focal necrosis, severe hemorrhages, and mild mononuclear inflammatory cell infiltration was observed in the liver parenchyma (Figures 1d and 1e). The mucosa of the intestine and the pyloric caeca in most instances was more or less intact. The convoluted tubules of the kidney showed degenerative changes and rarely tubular necrosis. Few melanomacrophages were infiltrated in the kidney parenchyma. Except for the presence of edema and hemosiderin granules, no other pathologic changes were evident in the parenchyma of the spleen.

PCR

A 224-bp fragment of the IPNV NS/VP3 gene was amplified using cDNA prepared from infected tissues from 13 fish of the 20 sampled fish. BLASTn comparison of the amplicon sequence against the nucleotide database indicated that the tissue was most closely identified with the Sp strain (98%-100%) but had only an 85.5% and 88% similarity to the nucleotide and amino acid sequence of the previously isolated IPN virus (Ab strain) found in Fars Province (Figure 2).

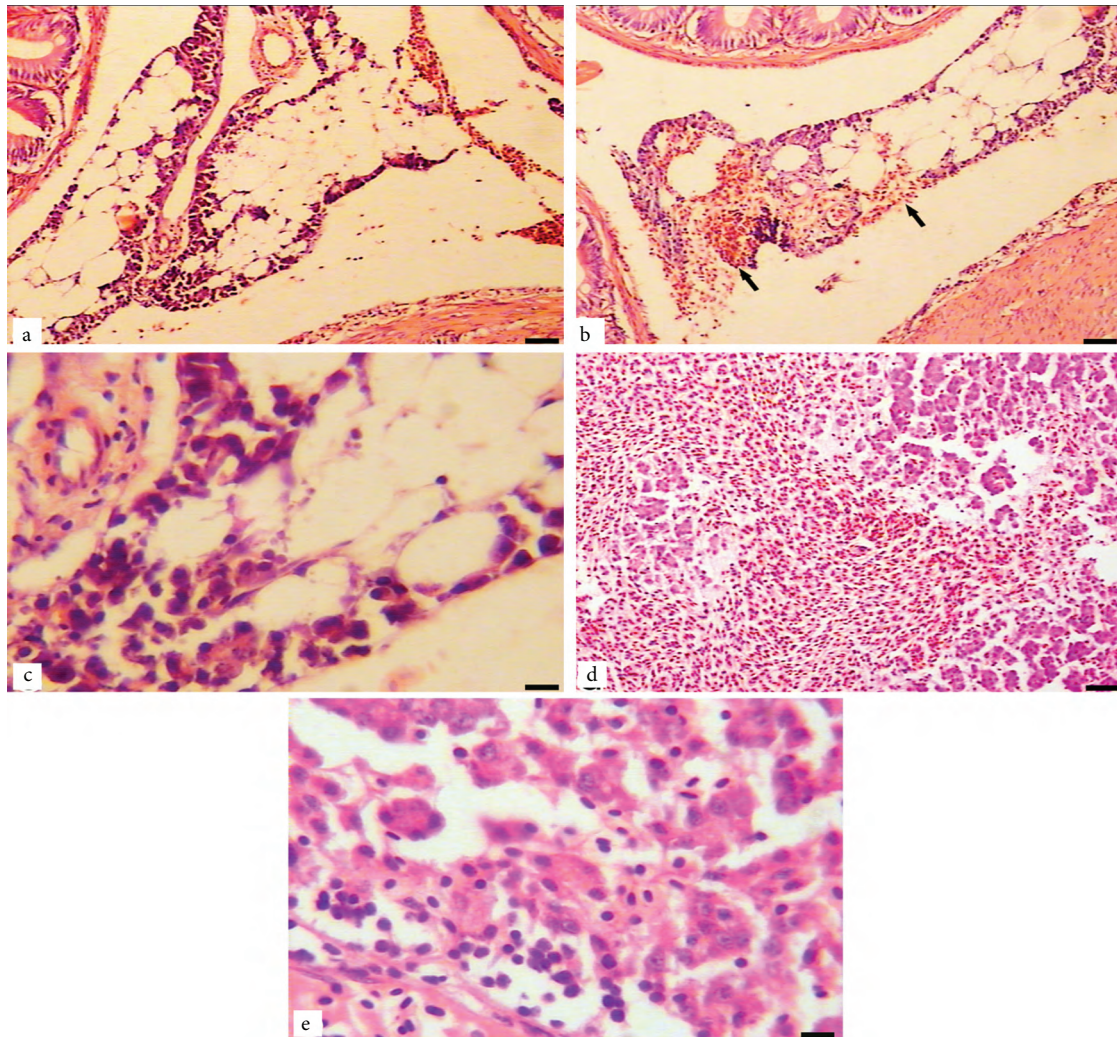


Figure 1. a) Extensive degenerative changes and coagulative necrosis in the exocrine pancreatic acinar cells of a rainbow trout fry. Endocrine and most of the fatty peripancreatic tissues are normal; H&E, scale bar = 83 μm . b) Pancreas of a rainbow trout fry showing marked lytic necrosis in pancreatic acinar cells with severe hemorrhages (arrow). The pancreatic tissue is infiltrated mildly with mononuclear cells; H&E, scale bar = 83 μm . c) Pancreas of a rainbow trout fry with extensive necrosis. Nuclear pyknosis, karyorrhexis, and karyolysis of the cells of the exocrine pancreatic acinar tissue are evident; H&E, scale bar = 21 μm . d) Liver of a rainbow trout fry showing lytic necrosis with severe hemorrhages. The hepatocytes of the liver parenchyma are destroyed; H&E, scale bar = 83 μm . e) Liver parenchyma of rainbow trout fry with mild mononuclear inflammatory cells infiltration; H&E, scale bar = 21 μm .

Discussion

This study describes the clinical signs, histopathology, and isolation of IPNV serotype Sp in rainbow trout cultured in a recirculating system as a new serotype in Iran. The clinical and pathological results of this study are consistent with previously described features of IPN reported from farmed

rainbow trout (12,13) and Atlantic salmon *Salmo salar* L. (14). IPNV replicates largely within pancreatic acinar cells and causes pyknosis, karyorrhexis, and karyolysis with a moderate inflammatory infiltrate in exocrine pancreas tissue (12,15). In this study, the pancreas exhibited the most notable pathology in 18 out of 20 moribund fish.



Figure 2. The alignment between nucleotide (A) and amino acid (B) sequences of NS/VP3 genes of 2 IPNV specimens isolated in 2007 (Ab serotype) and 2009 (Sp serotype). The underlined nucleotides and the highlights indicate the 5' end of the VP3 gene and protein differences.

As the most appropriate method for detecting birnaviruses, RT-PCR was carried out to confirm the diagnosis of clinical IPN (11,16-18). IPNV confirmation by RT-PCR shows that the marked lytic necrosis in pancreatic acinar cells and hepatocytes could be associated with IPNV replication in these cells. The results of the nucleotide sequence revealed that the present IPNV isolate is different from the IPNV isolate (Ab strain) previously reported in this region (8). This could be due to different sources of fry or eyed eggs. So far, IPNV histopathology and detection by RT-PCR has not been described with clinical signs and high mortality in rainbow trout larger than 3 g. The detection and confirmation of IPNV by RT-PCR in the present investigation and the comparison of the existing reports (8,19) show that the virus subsists in this region and potentially in neighboring countries such as Turkey. IPNV was probably introduced to this region through eyed eggs imported from different parts of the world during the past few years. In this study, 13 out of 18 moribund fish with notable pathological changes were RT-PCR-positive. The 5 false negative PCR results in the current investigation may be explained by the presence of PCR inhibitors that are coextracted from

fish tissues in combination with other unknown factors.

In the present study, mortality occurred in juvenile rainbow trout being reared at temperatures ranging from 12 to 14 °C. It must be noted that the fish were transferred to this farm after being reared to a weight of 5-6 g in a flow-through system with lower temperatures at another geographical location. The source of the infection is difficult to establish, but the presence and distribution of the virus within the region could be due to the exchange of fish between different hatcheries and production sites. In salmonids, potential transmission channels for IPN include infected broodstock, mammals, and fish-eating birds, as well as contaminated nets, containers, and other equipment (3). Stress and the method of transportation may enhance virus replication in persistently IPNV-infected fish (20,21). Important factors that may influence the distribution of viral diseases in fish include restocking measures, interspecies differences in susceptibility, and the specificities of the habitat or the farm production system (22). In the recirculating systems, stressful conditions such as suspended solids in culture tanks, biofilters, poor water quality, and high stocking

densities create a favorable environment for the reproduction of opportunistic microorganisms and may contribute to disease outbreaks (7,23). Ozmen et al. (24) concluded that recirculating systems may lead to oxidation stress affecting the antioxidant defense system and, ultimately, fish that are more sensitive to diseases and have a decreased capability to adapt to different water conditions. It seems that possible factors such as poor biosecurity, undesired transfer systems, changes of temperature, and a lack of water supply treatment could be responsible for the occurrence of this IPN outbreak in the fish with a previously asymptomatic infection.

To prevent the introduction of infectious diseases into a recirculating system, the best recommendation is to hatch eggs at the facility or buy fingerlings from a certified disease-free source. Additionally, newly arrived fingerlings should be quarantined before introduction into the system and reared in a pathogen-protected environment (7,23). The

presence of IPNV in salmonid farming shows that hygienic regulations and control policies such as testing the broodstock and destroying the infected fish and their reproductive materials should be concisely and accurately implemented to limit and control the spread of IPNV in this region. However, to improve the health management programs in this industry, active surveillance and collaboration between fish farmers and the relevant government fisheries authorities are required.

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