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RESEARCH ARTICLE

Infectious Pancreatic Necrosis Virus (IPNV) in Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792): Molecular Detection and Histopathology

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ABSTRACT

This study aimed to investigate the presence of Infectious Pancreatic Necrosis Virus (IPNV) in samples collected from three rainbow trout farms in the Aegean Region of Türkiye, using the Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) method. A total of 60 moribund rainbow trout, weighing between 0.5 and 120g, were selected for the study between March and April 2023. Of these, 30 were analyzed using rRT-PCR, and 30 were subjected to histopathological examination. For the rRT-PCR analysis, whole fish weighing 10g or less were placed in sterile phosphate-buffered saline (PBS) containing antibiotics (10,000 IU/ml penicillin + 10 mg/ml streptomycin). Fish weighing over 20g, tissue samples from the pancreas, kidney, spleen, and liver were collected. For histopathological examination, the abdomens of smaller fish were cut open, and liver, pancreas, intestines, and gills of larger fish were fixed in 10% formalin solution for identification. Clinical and necropsy findings in the moribund fish included ascites, dark coloration, exophthalmos, skin lesions, eye hemorrhages, muscular atrophy, gill anemia, petechial hemorrhages in internal organs, kidney wasting, yellow exudate, liver hemorrhage, and splenomegaly. The rRT-PCR results indicated a 100% prevalence of IPNV in sampled fish from each of the three farms, in different weight categories. Histopathological examination revealed pathological changes in the pancreas, liver, stomach, intestines, and gills, with notable necrotic foci particular observed in the acinar cells of the pancreas, as well as the presence of inclusion bodies in liver. As a result, all samples taken from three fish farms located in the Aegean region of Türkiye were found to be positive. It is important to take the necessary biosecurity and prophylaxis measures against IPNV infection in rainbow trout farms.

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INTRODUCTION

Despite strategies aimed at the sustainable expansion of aquaculture, disease outbreaks continue to pose a significant challenge to this industry. Viral diseases, in particular, are critical concerns because there are currently no known cures, and they are associated with high mortality rates and substantial economic impacts. Infectious pancreatic necrosis virus (IPNV) causes severe diseases in aquaculture. The IPNV is known to cause disease in marine and freshwater fish. 63 species of molluscs, fish and crustaceans are affected by this virus (Fayaz *et al.,* 2023).

The IPNV was first reported by McGonigle in 1940 (Tapia *et al*., 2022). IPN was subsequently renamed "infectious pancreatic necrosis" because it is a disease that infects the pancreas. Because aquaculture expanded and fish transfers became more widespread, IPNV contamination increased, causing a higher incidence of the disease. It has been reported that IPNV infection has emerged in nearly all European countries, as well as in several countries in Asia and Africa (Tapia *et al.,* 2022). In Türkiye, IPNV was identified in 1993 using histopathological methods and scanning electron microscopy. Subsequent studies employing various methods have also reported the presence of IPNV (Candan, 2002; Ozan, 2010; Ogut and Altuntas , 2011; Kalaycı *et al.,* 2012; Gürçay *et al.,* 2013; Öztürk and Altınok, 2014; Tamer *et al.,* 2019; Tamer *et al.,* 2021).

One of the main characteristics of IPNV is resistance to various physicochemical conditions. Wolf *et al.* (2019) and Munro and Midtlyng (2011) have reported that the virus remains alive at very low pH levels, salinities ranging

from 0-40%. It has been suggested that the virus may reproduce effectively at temperatures ranging from 4 to 27.5°C (Dopazo, 2020).

The disease has been observed to have a particular effect on juvenile fish. Mortality of juvenile fish varies from 20-100% (Salgado-Miranda *et al.,* 2020). IPNV presents distinctive symptoms that can vary depending on the virus serotype, environmental conditions, host age and species (Dopazo, 2020). Symptoms of IPNV infection include; lethargy, darkening color, abnormal swimming, stagnation, accumulation near drainage points, swelling of the head area, exophthalmos, ascites, gill anemia, pseudofeces, internal organ hemorrhages, liver anemia, intestinal exudates, and petechial hemorrhages in the pyloric caeca (Noga, 2010; Eriksson-Kallio, 2022).

Histopathologically, IPNV infection is recognized by tissue damage in the liver (Lightner and Roberts and Pearson, 2005; Hillestad *et al.,* 2021) and vacuolar degeneration in pancreatic cells. In the gills, the virus may cause a separation of the respiratory epithelium from the blood capillaries and deformation of the gill lamellae (Zhu *et al.,* 2017). Inclusion bodies are also observable in tissues infected with IPNV (Eriksson-Kallio, 2022).

Cell culture remains the gold standard method for diagnosing IPNV (Munro and Midtlyng, 2006). However, this method has the drawback of requiring a time period of 10-14 days for virus isolation and identification. In contrast, PCR techniques have become a much faster and more practical alternative for identifying the virus (Godoy *et al.,* 2022). PCR can detect the virus even at concentrations below the detection limit of cell culture, which is particular useful when susceptible cells are unavailable or when the sample size is too small for cell culture (Benkaroun *et al.,* 2021). Additionally, histopathology plays a crucial role in supporting the diagnosis of the disease.

Although viral fish diseases result in significant losses in the aquaculture industry, there is currently no known cure. Therefore, effective protection measures are the only way to ensure disease-free farming. Rapid diagnostic methods are crucial for early disease detection, control, and understanding its epizootiology (Godoy *et al.,* 2022). Control can be achieved by identifying and monitoring the virus's sources and eliminating them. In this context, vaccination offers promising prospects for controlling IPNV infection. Several countries, including Canada, the United States, Norway, Chile, and the United Kingdom, have developed and commercialized vaccines for IPN (Pavelin *et al.,* 2021; Kan and Kubilay, 2023; Kumar *et al.,* 2024; Kan and Kubilay, 2024; Zhang *et al.,* 2025). However, in Türkiye, no commercial vaccines are available for controlling viral fish diseases, including IPN (Tamer *et al.,* 2021).

Although there are numerous molecular studies on IPNV infection, histopathological research remains relatively limited. This study aimed to diagnose IPN using both molecular and histopathological methods in rainbow trout farms located in the Aegean region of Türkiye.

MATERIALS AND METHODS

Fish sampling

Ethical approval: The study was approved by the Local Ethical Committee for Animal Experiments of the Eğirdir Fisheries Faculty, Isparta University of Applied Science with decision number 004, dated 05/01/2024.

In this study, fish samples were collected to detect IPNV from rainbow trout farms in the Eşen Stream in the Aegean Region of Türkiye. The sampling was conducted from three different trout farms between March and April 2023.

In the sampling, fish and sampling pools were selected randomly. Immediately after the sampled fish were taken, anamnesis information was taken, autopsy was performed and viral samples were taken. Temperature, oxygen and pH values of water in the sampled farms were measured and recorded (WTW Oxi 320 meter, YSI EcoSense EC300A, YSI EcoSense pH100A). Randomly 60 individuals (30 for rRT-PCR, 30 for histopathology) weighing 0.5-120g were selected from each farm. Fish weighing less than 20g were placed whole in sterile antibiotic (10,000 IU/ml penicillin+10 mg/ml streptomycin) phosphate-buffered saline (PBS) in sterile falcon tubes, and the pancreas, kidney, spleen and liver of fish weighing over 20g were removed.

For histopathological examination, the abdomen of small fish was cut, and the liver, kidney, spleen, lesion (if any), and pancreas of large fish were collected and preserved in 10% formalin solution and identified.

RNA extraction: The tissue samples were mixed and homogenized with PBS containing 1% antibiotic (Sigma-Aldrich, United Kingdom, Product No: P2272) at a ratio of 1/10 using a homogenizer. Then inocula were prepared. The prepared inoculum of each sample was divided into 2 ml cryovials (CRYO.S, greiner bio-one, Germany).

RNA extraction was performed using a commercial extraction kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, Germany) according to the procedure recommended by the manufacturer and an automatic extraction device (Roche MagNA Pure LC System).
Negative and positive controls were placed in each extraction plate to verify the process and detect possible cross-contamination. Reference IPN virus was used as a positive control, and PBS used in the material preparation phase was used as a negative control.

Real Time RT-PCR: In the Real Time RT-PCR (rRT-PCR) test, primers and probes designed according to the VP3 (internal protein) gene region of the IPN virus were used (Table 1). The TaqMan probe synthesized in the study was labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end (Bowers *et al.,*2008*,* Ørpetveit *et al.,* 2010).

Real-Time RT-PCR application to detect the nucleic acid of IPN virus, a commercial kit (Real Time Ready Virus Master, Roche, Germany) and a Real-Time PCR device of the same company (Roche LyghtCycler® 480 Multiwell Plate 96) were used. Mastermix was prepared according to the kit procedure.

After process, the plates were placed in the carousel of the PCR device. rRT-PCR analysis was performed by applying the reaction protocol given in Table 2 and the results were evaluated with the help of the computer connected to the device. Samples showing a fluorescent glow and a logarithmic increase on the computer screen connected to the Real-Time PCR device were considered positive.

Table 1: Probe and primer sequences used in the rRT- PCR method

Primer /Probe	Target Region	Nt Positions	5'-3' Sequence	Amplification Magnitude (bç)	Referance
VP3-F	VP3	2754–2774	CGACCGACATGAACAAAATCA		Ørpetveit *et al.* (2010)
VP3-R	(internal	2862–2842	AGTTGCAGCTGTATTCGCACA		
VP-3-Probe	protein)	2779–2805	FAM-5-TCTAGCCAACAGTGTGTACGG CCTCCC- 3-TAMRA	109	

Table 2: Protocol applied in Real Time RT-PCR test (temperature/time/ number of cycles)

RT	Convert to complementary DNA	53 °C 6 minute 50 °C 4 minute	1 Cycle
PCR	RT inhibition	95 °C 90 second	
	Pre-Denaturation	95 °C 5 second	
	Binding and synthesis	60 °C 30 second	45 Cycles
	Elongation	72 °C 5 second	

Histopathology: Fish samples collected from the farms were stored in a 10% buffered formaldehyde solution and identified for pathological examinations. For the formaldehyde solution to pass into the internal organs more quickly and for the detection process to be carried out more quickly, the abdominal cavity was opened by cutting the abdominal region with scissors from the ventral side to the front of the anus. Samples were taken from all internal organs, including the liver and pancreas, from fish samples fixed in formaldehyde and placed in tissue tracking cassettes. Samples whose detection phase was completed were placed in a fully automatic tissue tracking device (Leica ASP300S; Leica Microsystem, Nussloch, Germany) and taken to the tissue tracking phase. After tissue tracking, the samples were placed in metal cassettes and embedded in hot paraffin, completing this stage. Blocks were created from paraffin-embedded samples in a tissue embedding device (Leica Histocore Arcadia H) (Leica Microsystem, Nussloch, Germany). After cooling for several hours, 5µm-thick sections were taken from each of the blocks for histopathological examination using a fully automatic Leica 2155 rotary microtome (Leica Microsystem, Nussloch, Germany). Sections taken on slides were routinely stained with hematoxylin and eosin (HE) and examined under a light microscope. (Fayaz *et al.,* 2023; Mokhtar, 2021).

RESULTS

Clinical and autopsy findings: The sampling was done from rainbow trout grown in concrete ponds. Water temperature in the sampling ponds was measured as 13-14°C, dissolved oxygen amount was 8.5mg/l, pH was 8.5.

The external examination of the sampled fish revealed ascites, darkening of color, exophthalmos, skin lesions, eye bleeding, muscle melting, and anemia in the gills (Fig. 1).

At autopsy, petechial hemorrhages in internal organs, kidney melting, yellow exudate, liver hemorrhage, and splenomegaly were observed (Fig. 2). Fish tended to gather towards the pond's water outlet, showing erratic swimming and poor feeding activity. The mortality rate in 0.5-120g trout was found to be 60%.

Confirmation of IPNV by Real Time RT-PCR: The presence of the virus was confirmed by rRT-PCR for samples taken from each establishment (Fig. 3). When the crossing point (CP) values were examined, it was found that the CP value was between 32.15-37.86 in the 1st and 2nd farms, while it was 22.05 in the 3rd plant. A low value indicates a high viral load.

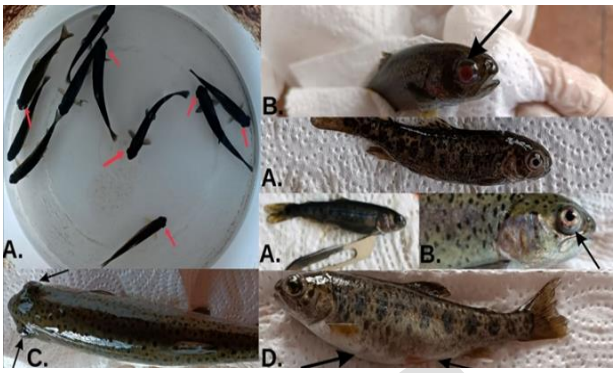

Fig. 1: Clinical appearance of fish samples suspected of IPNV. A. Darkening of color, exophthalmos (red arrow), B. Eye hemorrhage, C. Exophthalmos, D. Ascites.

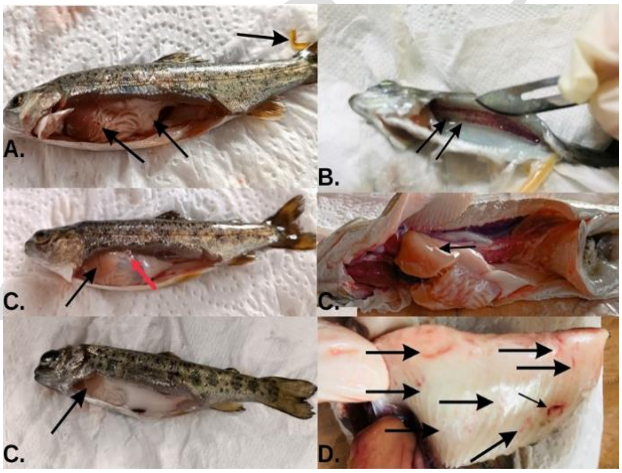

Fig. 2: Autopsy image of fish suspected of IPNV. A. Petechial hemorrhages in internal organs, yellow exudate B. Kidney lysis, C. Pale liver, hemorrhages in internal organs (red arrow), D. Petechial hemorrhages and hemorrhages in muscle.

Histopathological Findings: This study detected pathological changes in many organs, especially the pancreas and liver, in fish with IPN (Fig. 4.). Histopathological examination of the pancreas revealed widespread acinar cells and necrosis of endocrine cells in the islets of Langerhans (Fig. 4.). Microscopically, integrity disruption of exocrine glands was observed (Fig. 4.). Swelling and fragmentation were detected in both endocrine and exocrine epithelial cells. Cells have been found to be disconnected in many areas. The nuclei of the cells were observed to be necrotic and generally pyknotic. Inflammatory cell infiltrations of varying severity, mainly composed of lymphocytes, were detected around necrotic areas (Fig. 4.).

Numerous necrotic areas of varying sizes were noted in the livers (Fig. 4.). Inflammatory cell infiltrations of varying severity were observed around necrotic areas in the livers of many sick fish. Purple basophilic inclusion bodies were observed in the cytoplasm of some degenerated cells (Fig. 4.). Areas of bleeding were seen in both the liver and the pancreas.

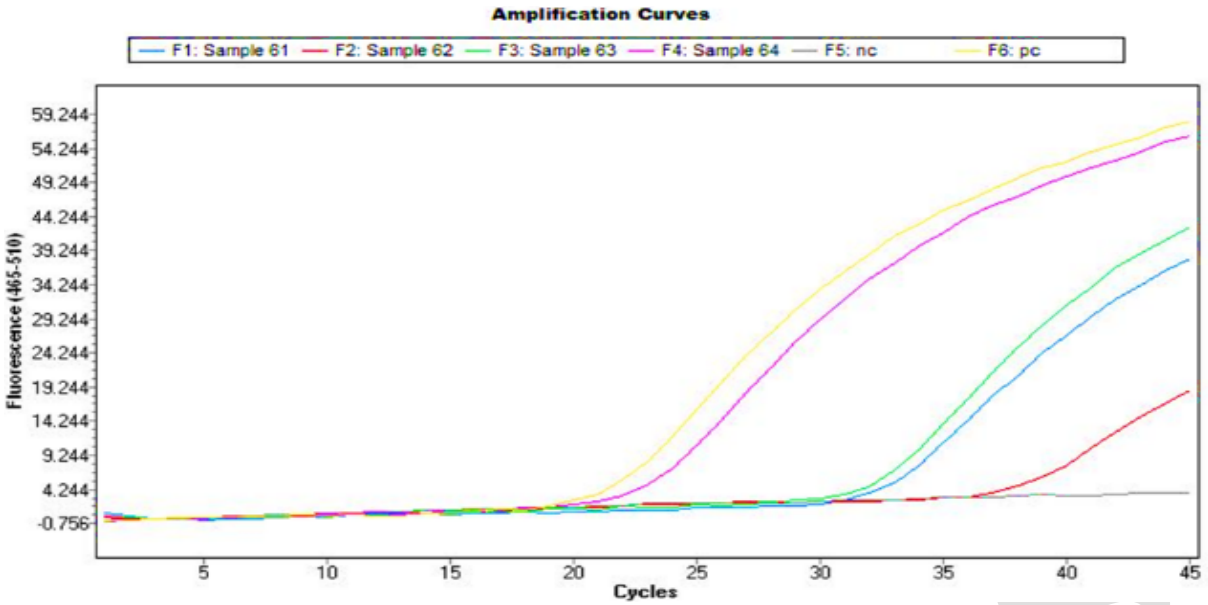

Fig. 3: Fluorescence curves of RNAs of fish samples. 1st Farm: Sample 61-62, 2nd Farm: Sample 63, 3rd Farm: Sample 64, nc: Negative control, pc: Positive control.

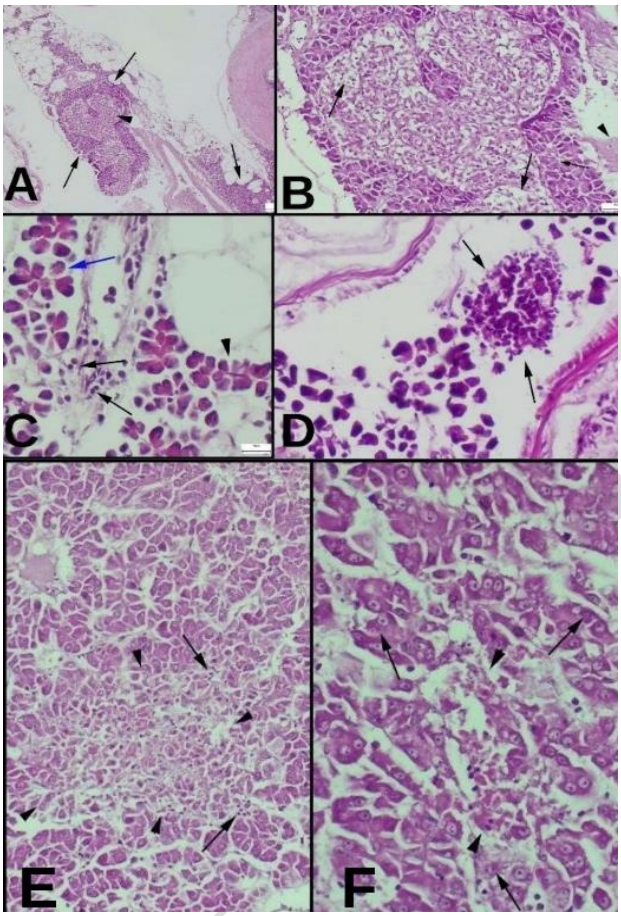

Fig. 4: A. View of the endocrine (arrowhead) and exocrine (arrows) portions of the pancreas, HE, Bar= 200µm. B. High magnification image of A, showing necrotic cells in the endocrine compartment (thick arrows), necrotic cells in the exocrine compartment (thin arrow) and edema in the peripancreatic tissue (arrowhead), HE, Bar= 50µm. C. Distortion in the appearance of the pancreatic gland acini (arrowhead), necrotic epithelial cells (blue arrow) and inflammatory cell infiltrates, HE, Bar= 20µm. D. Appearance of necrotic mass (arrows) in the pancreas, HE, Bar= 20µm. E. Appearance of necrotic area (arrowheads) in the liver and surrounding inflammatory cell infiltrates (arrows), HE, Bar= 50µm. F. Foci of necrosis in the liver (arrowheads) and basophilic inclusion bodies in some hepatocytes (arrows), HE, Bar= 20µm.

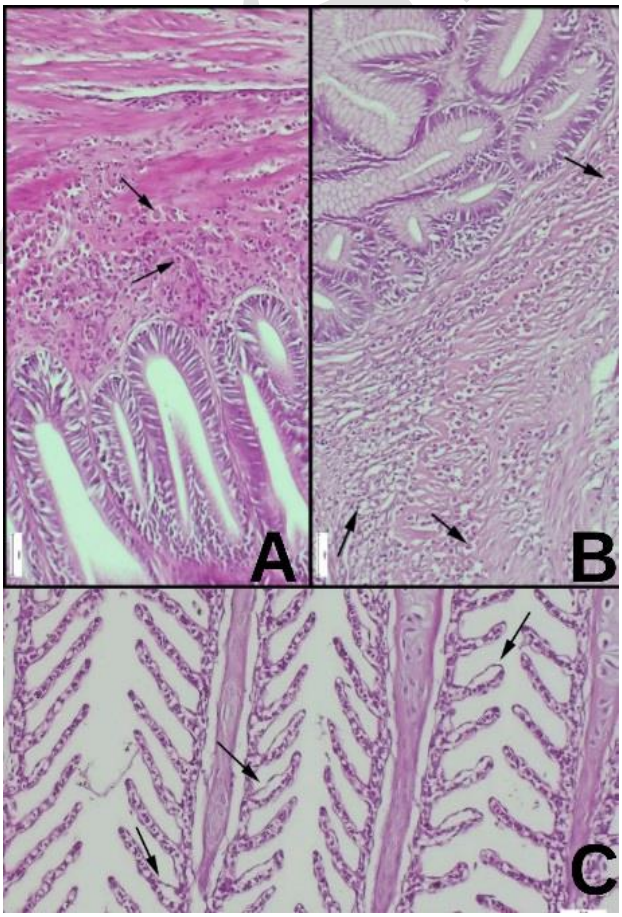

Fig. 5: A. Dense inflammatory cell infiltrations in the intestinal serosa (arrows), B. Cell infiltrations in the intestine, HE, Bar=50µm.C. Separation of epithelial cells in secondary lamellae in gills (arrows), HE, Bar= 50µm.

Inflammatory cell infiltrations were observed in the stomach and, more intensely, in the intestinal serosa (Fig. 5). Peritonitis was evident in many sick fish. Edema, mild inflammatory cell infiltration and separation of epithelial cells in secondary lamellae were detected in the gills.

DISCUSSION

After the emergence of IPNV in the 1940s, reports of the infection have been published in many parts of the world. IPNV infection was identified in cell culture by isolation, neutralization, immunofluorescence, immunoperoxidase, counterimmunoelectrophoresis, coagglutination, ELISA and PCR methods in rainbow trout (*O. mykiss*), (*Salmo salar*), *O. sutch, O. masu ishikawai, Salvelinus alpinus, S. trutta L., O. masou, Salvelinus leucomaenis, Hucho taimen* (Duan *et al.,* 2021; Benkaroun *et al.,* 2021), *Gadus morhua* (Romero-Brey *et al.,* 2009), *Reinhardtius hippoglossoides* (Romero-Brey *et al.,* 2009), *Scophthalmus maximus* (Cutrín *et al.,* 2004), *Sebastes mentella* (Romero-Brey *et al.,* 2009), *Esox lucius L.* (Blake *et al.,* 2001), *Carassius auratus* (Duan *et al.,* 2021).

IPNV infection in rainbow trout is a viral disease responsible for high mortality rates in juvenile trout. However, the detection of IPNV in large-sized trout, which has been increasingly farmed recently, supports the idea that the virus is an important pathogen that needs to be studied. In this study, IPNV was diagnosed in rainbow trout farms using molecular methods and the infection was confirmed histopathologically and pathological symptoms in the tissues were observed. This study is the first report in which IPNV was identified using molecular and histopathological methods from fish farms located on the Eşen Stream in Türkiye. The prior studies have reported that IPNV is common in Türkiye (Candan, 2002; Ögüt and Altuntaş, 2012; Kalayc *et al.,* 2012; Gürçay *et al.,* 2013; Öztürk and Altınok, 2014; Tamer *et al.,* 2019; Tamer *et al.,* 2021).

Clinical signs in the examined moribund fish were observed as exophthalmos, darkening of color, ascites, and melting muscle. Clinical symptoms were similar to those reported in the last studies about IPN (Eriksson-Kallio *et al.,* 2020; Dopazo, 2020). Autopsy findings included petechial hemorrhages in visceral organs, yellow exudate, splenomegaly, and anemia liver, being consistent with the findings of the former studies (Bruno *et al.,* 2013; Dopazo, 2020). Additionally, the abnormal turning swimming behavior was similar to previous studies (Rodriguez-Saint-Jean *et al.,* 2003).

In recent years, rRT-PCR has become a practical method for identifying viral diseases (Mackay *et al.,* 2002; Altinok and Kurt, 2003, Bowers *et al*., 2008). In this study, IPNV was detected by rRT-PCR in all sizes of individuals weighing 0.5-120g in three fish farms in our study area. According to rRT-PCR results, the viral load of the 3rd farm was found to be higher than other farms. The differences in viral load between farms may be related to the virulence and pathogenesis of the disease.

According to the findings of this study, it was concluded that the shared use of water resources by farms may be related to horizontal transmission through vector organisms. These findings support previous reports (Labrana *et al.,* 2008). Considering that eggs, fry, and broodstock are also transported between farms, it can be claimed that there is both horizontal and vertical contamination between farms.

In this study, typical histopathological findings of IPNV infection were obtained. The most prominent findings were necrotic foci, cell infiltration and inclusion bodies in the pancreas. Inclusion bodies are a major indicator of the disease (Mutoloki *et al.,* 2016). Wolf and Quimby made the pathological evaluation of the effect of IPNV infection on tissues, and pathological changes in the pancreas, kidneys and intestines and petechial hemorrhages in internal organs were reported (Tapia *et al.,* 2022). In this study, similar pathological changes were observed in the pancreas, kidney and intestines. According to the study conducted by Bruno *et al.,* (2013), necrosis was observed in the pancreatic exocrine gland in Atlantic salmon. In this study, similarly, intense necrotic cell findings were found in exocrine and endocrine cells (Fig. 5-7). The pathological changes reported in the study have been described in the scientific literature as being consistent with the formation of IPN (Munro and Midtlyng, 2011; Smail and Munro, 2012; Dopazo, 2020). In the study conducted by Zhu *et al.* (2017), separation of gill epithelial cells was observed, being similar to the findings of this study.

As a result, samples taken from rainbow trout farming enterprises in our study area were studied and it was found that juvenile and adult individuals in all enterprises were infected with IPNV. It was observed that all sampled individuals had clinical and pathological signs of the disease.

IPNV is more resistant than other viral fish pathogens and more resistant to different physical and chemical disinfectants. Since there is no known treatment for the pathogen in question, measures such as restricting egg and fish transportation processes, having certified imported eggs, conducting necessary examinations at the stage of entry into the country, improving biosecurity measures and separating infected fish from the population within the scope of quarantine measures should be implemented to prevent infection. In many European countries, including Türkiye, IPNV vaccines are not yet used commercially. Within the scope of prophylaxis measures, giving importance to vaccine studies in the future, developing and making commercial IPNV vaccines available and widespread use in our country are vital for the control of the disease. Likewise, epidemiological studies need to be developed to track the origin and source of IPNV using genotyping so that control strategies such as elimination of the contamination source can be implemented. In addition, annual and monthly surveillance studies should be continued in rainbow trout farming in Türkiye, and activities that increase the risk of IPNV emergence and spread should be identified and stopped.

Conclusions: In conclusion, all samples taken from three fish farms in the Aegean region of Türkiye were found to be positive using PCR and histopathological analysis. This disease causes serious mortality and symptoms in rainbow trout. Therefore, it is important to take the necessary biosecurity and prophylaxis measures against IPNV infection in rainbow trout farms.

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Data Availability: The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Ethical Approval Statement: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Isparta University of Applied Science (05.01.2024/No:004).

Authors contribution: Conceptualization: ŞK, AK; methodology: ŞK, AK; formal analysis and investigation: ŞK, AK, writing – original draft preparation: ŞK; Writing – review and editing: ŞK**,** AK.

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