

First isolation and identification of the Infectious Pancreatic Necrosis (IPN) virus from rainbow trout *Onchorhynchus mykiss* fingerlings farmed in Greece

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Abstract

During February 2000, two trout farms, situated in NW Greece, reported unusually high losses of hatchery reared rainbow trout fry and fingerlings. Live fish and chilled samples of diseased whole fish were delivered to a veterinary laboratory in Athens for diagnostic investigation. Infections with the fungus *Ichthyophonus hoferi* as well as the Gram negative bacterium *Aeromonas hydrophila* were diagnosed in some of the samples. However, the reported clinical signs, mortality levels, age and size of the fish and the necropsy findings suggested that IPNV might also be present. The presence of the virus was detected initially by means of a rapid ELISA test kit and later confirmed by virus isolation and identification from tissue and whole fish samples by an independent virology laboratory. In neutralisation tests the virus was found to relate more closely to the A2 (formerly Sp) serotype of aquatic birnaviruses suggesting that the original source of the virus is elsewhere in Europe. This is the first published report of IPN virus affecting farmed fish in Greece.

Introduction

Trout farms in Greece are mainly situated in the north of the country where the natural water supplies are of a good quality and favourable to aquaculture. Trout for the table are commonly raised in concrete walled raceways with a sand, gravel or concrete bottom. Water is sourced directly without any treatment either from adjacent springs, maintaining a constant temperature of around 15°C, or pumped from rivers with temperatures that vary seasonally from 14°C in the winter to 18°C in the summer. The water is returned to the river systems untreated and poor farming practices and hygiene measures allow for easy entry and establishment of trout pathogens. Most farms keep their own

broodstock and operate small hatcheries in order to cover their own needs for fry. When the quantity or quality of home produced fry is insufficient to meet production needs, eyed ova are imported, mainly from the US or Denmark.

Perennial disease problems that are responsible for considerable losses on most trout farms in Greece, comprise primarily Enteric Red Mouth (ERM) disease caused by the bacteria *Yersinia ruckeri* (Savvidis, 1991; Varvarigos, 1999) and Ichthyophonosis caused by the fungus *Ichthyophonus hoferi* (Varvarigos, 2000). A secondary pathogen of concern is cold water disease attributed to *Flexibacter psychrophilus* (Schlotfeldt and Alderman, 1995; Varvarigos, personal observation).

During February 2000 two trout farms in NW Greece reported mortality rates of 30%-55% among fry and fingerling rainbow trout in their hatcheries. Mortalities were seen to rise with the increase in fish handling and crowding during transfer between raceways and vaccination against ERM. A total of three disease outbreaks at the two farms were reported to the Vetcare laboratory in Athens and samples were submitted for diagnostic investigation. Although bacterial and fungal pathogens were observed in some of the fish, the gross clinical signs and mortality levels suggested an underlying viral aetiology.

Materials and Methods

Fish samples

Moribund diseased fish, ranging in size from 1g – 8g average wet weight, were collected from the farms and transported on ice to the Vetcare laboratory. Other fish were delivered live to the laboratory and observed in an aquarium for a few hours before undergoing post mortem. The internal organs were examined for the presence of gross lesions and then swab samples from the liver, spleen and kidney were taken for bacteriological examination and kidney tissue was excised for virological examination. Microscopic examinations were also made of fresh smears and squashes from liver, kidney, spleen and gill tissues from the fish.

Bacterial Cultures

Swab samples from the liver, spleen and kidney of fish received live at the Vetcare laboratory were plated onto Tryptone Soya Agar (TSA). The cultures were incubated at 25 °C for 36 hours. Colonies appearing were examined by Gram stain and biochemical reactions performed on API-20E test strips. The more

prominent isolates were tested for resistance to selected antibiotics.

Virological examination

Rapid detection of virus

Kidney tissue was sampled from a total of 30 trout fry from the three disease outbreaks and tested with a rapid IPNV test kit (Diagxotics Inc., 27 Cannon Rd., Wilton, CT 06897, USA). The kit is based on a modified antigen capture enzyme linked immunosorbent assay (ELISA) and tissue extracts were tested according to the manufacturer's instructions.

Virus isolation and identification

Pools of whole kidney tissue, from 6-8 fry, were transported, unfrozen, in viral transport medium (Glasgow modification of minimal essential medium (GMEM) supplemented with 10% new-born calf serum, 2 mM L-glutamine and 1% antibiotic + antimycotic solution (all Sigma)) to the CEFAS laboratory (Weymouth, UK). In addition, samples of 30 whole fry packed in dry ice were also submitted for virological examination which followed procedures recommended for the isolation and identification of IPNV in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 1997). Pools of viscera from each of five whole fry and the kidney tissue pools were homogenised with a pestle, mortar and sterile sand, re-suspended in transport medium and clarified by centrifugation at 2000 rcf for 20 minutes. Supernatants were then diluted to 1:100 and 1:1000 (w/v) with culture medium (GMEM supplemented with 10 % foetal bovine serum, 500 I.U./ml penicillin, 500 mg/ml of streptomycin and 2 mM L-glutamine) and inoculated onto monolayers of the established fish cells lines, BF-2 (Bluegill fry) and CHSE-214 (Chinook Salmon Embryo) in 12-well multidishes (Falcon). In-



Figure 1. trout fry about 1.5g suffering from IPNV. Dark colouration to posterior third (top fish) and slight swelling to head (bottom fish).

oculated monolayers were incubated at 15°C and examined daily for a cytopathic effect (cpe).

Virus neutralisation

Aquatic birnavirus strains, West Buxton, Sp (Spjarup), Ab (Abild), He (Hecht) and TV-2 were used to represent the A1, A2, A3, A4 and A5 reference serotypes, respectively. Rabbit antisera to purified preparations of these reference viruses had been produced previously as detailed in Hill and Way (1995). Neutralisation tests were carried out using 96- well cultures of BF-2 cells. Two-fold serial dilutions of each antiserum from 1/1000 – 1/2,560,000 were made in maintenance medium

(MM – GMEM supplemented with 2% foetal bovine serum) and mixed with an equal volume of virus diluted in MM to approximately 200 TCID₅₀ /ml. The mixtures were then incubated at 22°C and, after 1h, 100ml aliquots of each mixture were transferred to 4 wells of the 96 well culture plates. Control virus wells were also included containing equal volumes of the virus dilution mixed with MM. The neutralisation end point was taken as the last dilution where no cpe was observed after 5 days incubation at 15°C.

Results and Discussion

Gross clinical signs

Diseased fish were notably darker in colour and appeared weak and lethargic. Many fish were seen maintaining an almost still position in the water near the sides or bottom of the raceways and others showed uncoordinated spiral swimming. Exophthalmia and distention of the abdomen were evident but no hemorrhagic lesions, ulcers, or fin rot were observed. Closer inspection revealed that some fry showed darker coloration of the rear half or third of the body and small, but evident, swellings on the head (Fig. 1 and Fig. 2). These findings were more evident on the

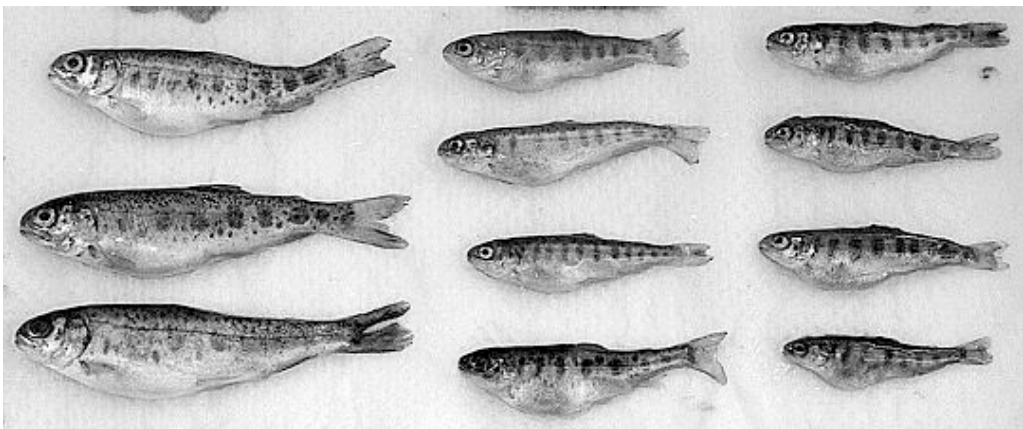


Figure 2. Trout fry from 2-7g with IPNV infection, showing abdominal distention and swellings to head.

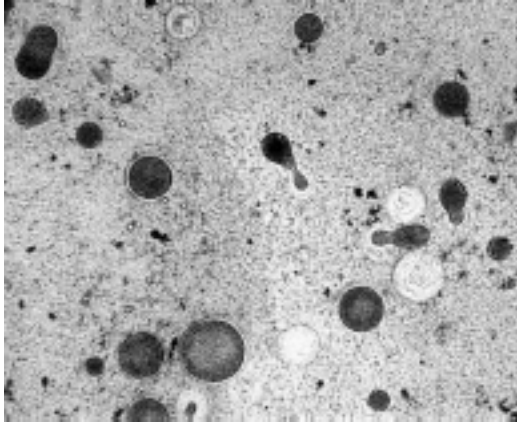


Figure 3. Fresh kidney tissue squash (unstained) from trout fry infected with ichthyophonosis as well as IPNV, showing double walled cysts of *I. hoferi* and germinating cysts (x100)

live specimens during observation in the aquarium and together with the distinct localized abdominal distention, raised suspicions about the possibility of IPNV infection (Post, 1987; Schlotfeldt and Alderman, 1995).

Internally, the alimentary tract was empty of food or faeces but moderately distended and filled with greyish mucus. The liver was dark and inflamed and the kidney and spleen were pale and swollen. Microscopic examination of tissue imprints and wet mounts from fry originating from one of the two farms revealed a widespread infection by the fungus *Ichthyophonus hoferi*. Large numbers of spherical quiescent cysts of varying sizes, with the characteristic thick double wall as well as maturing fungal cysts demonstrating distinct nuclei and active cysts showing plasmodial germination were observed on all slides (Fig. 3).

Bacterial Cultures

Examination of bacterial cultures showed growth of round, 1-2mm, pale colonies of Gram negative bacteria from one third of the fry sampled. These bacteria gave an API-20E profile of 3047165.

According to Austin and Austin (1999) this identifies *Aeromonas hydrophila*. This bacteria was found resistant *in vitro* to ampicillin, amoxycillin and potentiated sulfonamides, slightly sensitive to furazolidone, oxolinic acid and oxytetracycline and adequately sensitive to flumequine.

Virological examination

Kidney tissue sampled from diseased fry and fingerlings gave strong positive signals when tested with the rapid IPNV ELISA test kit. This identification of IPNV infection was then confirmed by isolation of the virus in cell culture. At the CEFAS laboratory, extracts from the sampled tissue and fry showed a cytopathic effect in CHSE-214 cells after 48h and in BF-2 cells at 72h post inoculation. Preliminary neutralisation tests using polyclonal rabbit antiserum raised against the West Buxton (A1 serotype) and Sp (A2 serotype) reference aquatic birnaviruses indicated that the Greek virus was more closely related to the A2 serotype than it was to the A1 serotype. The A2 serotype is the most prevalent of the European serotypes while A1 is the major North American serotype (Hill and Way, 1995).

More extensive neutralisation tests were then carried out which confirmed that the Greek IPNV isolate falls into the A2 serotype of the aquatic birnaviruses (Table 1).

This report represents the first confirmed isolation of IPNV virus from farmed rainbow trout in Greece. The only other record of the presence of IPNV disease in Greece was a personal communication from C. Carlson cited by Wolf (1988). However, this finding has not been confirmed in a published report. The IPNV infected fry populations almost certainly

Virus	Antiserum				
	A1	A2	A3	A4	A5
A1	1 280 000	20 000	<1 000	1 000	<1 000
A2	4 000	640 000	2 000	20 000	2 000
A3	8 000	2 000	1 280 000	1 000	20 000
A4	1 000	4 000	1 000	1 280 000	20 000
A5	4 000	1 000	40 000	<1 000	640 000
Greek	20 000	320 000	2 000	4 000	2 000

Table 1. Cross neutralisation titres of aquatic birnavirus reference serotypes and the IPN virus from Greece. Neutralisation end point was taken as the highest dilution where no CPE was observed at the end of the incubation period.

originated from imported, eyed ova. On one farm, adjacent raceways holding fry that were progeny of the farm's own broodstock were seen to suffer much lower rates of morbidity and mortality. The Greek IPNV isolate was found to belong to the A2 serotype of aquatic birnaviruses and the presence of this serotype, in combination with the records of introductions at the farm sites, strongly suggest that the origin of the imported ova is Denmark.

The two trout farms, where IPN has been verified, are situated at different locations. One is sourcing water directly from a spring, the other from an unconnected river system, hence, it is possible that IPN disease has already spread, with fish transfers, to other farms on other river systems in Greece. In order to implement control measures and prevent re-infections of IPN it is important to assess the magnitude of the disease spread. There will be a need to screen wild fish as well as the farmed populations in order to achieve this.

Unless disease control measures are implemented to prevent re-infections, IPN has to be considered in the future together with ERM and Ichthyophonosis as a potential major

cause of economic loss. If the disease is allowed to become widespread then Greek trout producers will have to introduce increasing numbers of fry each year in order to achieve their planned production.

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