Experimental infection with epizootic haematopoietic necrosis virus (EHNV) of rainbow trout (Oncorhynchus mykiss Walbaum) and European perch (Perca fluviatilis L.)

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Abstract

The aim of this study was the determination of the susceptibility of Polish farmed redfin perch (Perca fluviatilis L.) and rainbow trout (Oncorhynchus mykiss Walbaum) to experimental infection with haematopoietic necrosis virus (EHNV). A bath challenge model was tested at two temperature ranges: 13–15°C and 20–22°C. After 7 d, the first clinical signs and mortality were observed in fish kept at these temperatures. Significantly more mortality cases were reported in the redfin perch population, reaching a maximum of 24% compared with 12% in the rainbow trout group at 20–22°C. EHNV was reisolated from redfin perch and rainbow trout tissue in cell culture and the infection was confirmed by a molecular method and histopathology during the duration of the experiment. This study revealed that fish from Polish farms can be susceptible to EHNV even at lower temperatures.

Keywords: rainbow trout, redfin perch, epizootic haematopoietic necrosis virus, temperature, disease resistance.

Introduction

The epizootic haematopoietic necrosis virus (EHNV) is responsible for epizootic haematopoietic necrosis (EHN), a disease listed by EU Directive 2006/88/EC (4) and the World Organisation for Animal Health (OIE) (12). The virus was isolated for the first time from redfin perch (Perca fluviatilis) in Australia during an outbreak of the disease in fresh water, and then from rainbow trout (Oncorhynchus mykiss) (9, 10). This was due to the introduction of these species into an environment in which the virus became endemic. EHNV belongs to the genus Ranavirus in the family Iridoviridae. It is a cytoplasmic virus containing a large double-stranded DNA (dsDNA) genome, which is replicated in two stages: the main replication occurs in the nucleus and the second in the cytoplasm of the host cell (5).

Many ranaviruses have been isolated from sick or apparently healthy fish, amphibians, and reptiles (9). Some of these isolates are very similar on the serological and molecular levels (6). EHNV has never been isolated outside Australia, but similar systemic necrotising iridovirus syndromes have been reported in wild and farmed fish populations in Europe. These include bullhead catfish (Ictalurus melas) in France (European catfish virus – ECV) (14), sheatfish (Silurus glanis) in Germany (European sheatfish virus – ESV) (1), turbot (Scophthalmus maximus) in Denmark, and others in Finland (2). Other ranavirus isolates, such as pike perch iridovirus (PPIV) and short-finned eel ranavirus (SERV) have occasionally been isolated in symptomless fish from both freshwater and marine environments (16). Transmission of EHNV between susceptible hosts within a population is possible via water or ingestion of tissues from infected fish. Movement of infected trout fingerlings containing a low number of individuals with a subclinical infection was probably the most common means of spread of EHNV within the aquaculture industry (16).

The aim of the study was to determine the susceptibility of rainbow trout and redfin perch from Polish fish farms to EHNV. The study involved a bath infection model at two temperature ranges, reisolation
of the virus in cell culture, and confirmation of the results by PCR.

Material and Methods

Selection and fish husbandry. Rainbow trout fingerlings (70–90 g, 8–15 cm) and European perch fingerlings (60–80 g, 6–12 cm) comprising about 130 fish of each species were obtained from Polish fish farms of known health status. In these farms there was no history of EHNV infection. Before examination the fish were housed in glass aquaria. The fish were maintained at two temperatures: 11–13°C and 20–22°C. Control fish groups from the same fish farms were maintained under the same conditions. In the aquaria, water quality, temperature, and dissolved oxygen were monitored. The fish were acclimated, tested for viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), and spring viraemia of carp virus (SVCV) by RT-PCR according to the European Reference Laboratory for Fish Disease (EURL) protocol, and observed for three weeks before experimental infection.

Experimental challenge model. Experimental infection trials were conducted in aquaria which contained aerated water and had external filtration units. The EHNV isolate 86/8774 was used for the infection, which was originally isolated from clinically affected rainbow trout and was classified as reference virus after a proficiency test performed in 2009 (http://www.crl-fish.eu/) by the EURL. The virus was propagated in epithelioama papulosum cyprinid (EPC) cells, incubated at 21°C, and harvested at maximum cytopathic effect (CPE), about 4–5 days post inoculation (dpi). During the infection experiments, experimental fish were kept in 180 L aquaria. Each group of 50 rainbow trout and redfin perch fry were exposed by bath infection to 2 × 10⁸ TCID₅₀ mL⁻¹ of EHNV. Control fish were exposed to water containing cell culture medium without virus. From 0 to 30 dpi, when the experiment ended, fish were sacrificed and the kidney, liver, and spleen were collected and inoculated onto EPC cells for virus reisolation. Moribund fish were sacrificed and examined in the same way. In addition, 5–10 clinically healthy control and inoculated fish were euthanized and examined 0, 5, 10, 15, 20, 25, and 30 dpi.

Virus reisolation and confirmation by PCR. Tissue samples from a maximum of 10 fish were collected in one sterile tube containing at least 4 mL transport medium and representing one pooled sample. Next, the samples were completely homogenised and subsequently suspended in the transport medium. The final ratio of tissue material to transport medium was 1:10. The homogenate was centrifuged at 4 °C at 3000 × g for 15 min and the supernatant was collected. EPC cell lines were inoculated with the primary dilution and, in addition, a 1:10 and 1:100 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100, 1:1000, and 1:10 000 respectively. The cells were propagated in 24-well plates with Eagle’s MEM and a Tris buffer supplemented with a 10% FBS and standard concentrations of antibiotics 24 h before infection. The inoculated cells were incubated at 15°C and then were collected for virus identification when the CPE appeared, usually 4 to 7 d after inoculation.

Total DNA was extracted from the supernatant of the infected cells using the QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer’s instructions and examined by conventional PCR with MCP-2 primers (M-153 5’ ATGACCGTGCCCTCAC 3’ and M-154 5’ CCA-TCG-AGC-CGT-TCA-TGATG 3’) (12). The reaction was carried out using a GoTaq DNA polymerase (Promega, USA). A volume of 45 µL of the reaction mixture, containing 5 µL of extracted DNA and 20 µM of each primer, was subjected to the following thermal conditions: 94°C for 2 min, 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 60 s, and the final extension was performed at 72°C for 5 min. All PCRs were conducted with the use of a T3 Thermocycler (Biometra, Germany).

The amplified products were separated by electrophoresis in a 2% agarose gel with ethidium bromide in 1 × TBE buffer and visualised by UV transillumination using the EC3 Chemi HR 410 Imaging System (Ultra-Violet Products, UK). A 100 bp DNA ladder was used as a molecular size standard.

Histopathology. Pieces of gills and anterior kidney from infected fish with clinical signs and from control fish were excised and fixed in 10% neutral buffered formalin for at least 24 h. The tissues were processed routinely, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE).

Results

During the quarantine period, no clinical signs were observed and no significant pathogens were detected from randomly sampled fish. Infected rainbow trout were found dead or moribund, without other clinical signs of disease until the day of death. Moribund fish were dark, weak, showed lack of appetite, and swam with the symptoms of nervous system impairment. After the section, anaemic gills and internal organs were observed; a few fish had nodular livers and swelling of the spleen and kidney. The dead rainbow trout were observed only on 7 dpi at both temperatures. The mortality rate was 4% at 11–13°C and 12% at 20–22°C. In experimentally-infected redfin perch the most common and clearest clinical signs were observed from 7 to 12 dpi at both temperatures. The mortality rate was 16% at 11–13°C and 24% at 20–22°C. Higher temperature has a significantly positive
effect on the viral replication and infection which resulted in an increase in fish mortality and the occurrence of more numerous and pronounced clinical signs and pathological lesions.

A cytopathic effect on EPC cell monolayers inoculated with tissue homogenates from infected fish was observed in the first passage after 4 d. The CPE was observed in all collected samples from rainbow trout and redfin perch for the entire duration of the experiment. Although the fish did not always demonstrate clinical signs or die, EHNV was reisolated, which proves that the fish were asymptomatic carriers. Infected cell lines showed changes (ring-shaped cavities with a wreath of infected cells and vacuolation) characteristic for the cytopathic effect of ranavirus (Fig. 1). PCR with specific primers (MCP-153 and MCP-154) allowing amplification of a 625 bp region in the ranavirus MPC gene confirmed the results obtained.

Microscopic lesions were similar in both infected species and were therefore described together.

Gills. The gills showed hyperplasia in the respiratory cells resulting in fusion in the lamellae. Some gills showed severe destruction of the lamellae. Debris and necrotic cells were prominent in the vessel lumen in the gills of both species. Hypertrophy of epithelial cells was also observed. Gills had mild scattered focal or individual cell necrosis of pillar cells or other connective tissue cells within the filaments (Fig. 2).

Kidneys. The haematopoietic cells were depleted in dead fish. Many of the cells were necrotic and had a vacuolation of the nucleus. Interstitial nephritis with loss of haematopoietic cells and pyknosis of tubular epithelial cells were observed. Besides necrosis and degeneration, the tubular cells showed large, vesicular, hyperchromatic nuclei and dark basophilic cytoplasm. In redfin perch, discrete melanomacrophage centres were involved, and they occurred within an area of necrosis. (Fig. 3).

Fig. 1. Typical cytopathic effect induced by ranaviruses on epithelioma papulosum cyprinid cell culture EPC. (left- 48 h pi; right- 72 h pi; down- non-infected cells)

Fig. 2. Redfin perch (A) and rainbow trout (B) gills: necrotic cells and hyperplasia of epithelial cells. HE, 200×
Discussion

Ranaviruses are recognised as major pathogens, important economically and ecologically, as they are generally not host-specific and may result in outbreaks with a high mortality rate (18). The movement of animals out of their original geographical range brings together new hosts and potential pathogens, facilitates host-switching, and continues the cycle of disease emergence for a pathogen (13). The import of live ornamental fish is in large part responsible for spreading exotic diseases to EU aquaculture. Matbouli et al. (5) identified iridoviruses from different ornamental fish species from German pet shops. Some fish displayed skin lesions, such as skin erosion, while the others were apparently healthy. Identification of iridoviruses by PCR and product sequencing demonstrated high homology to the major capsid protein gene of other fish diseases caused by these viruses.

Redfin perch are extremely susceptible to EHNV infection because of pre-existing disease among other perch fish in Victoria in Australia, and they are not the first host of this virus (18). EHNV infection occurs mostly in summer when it develops very fast (10–11 d) at high temperatures (3, 9, 10, 18). At lower temperatures (12–18°C) virus incubation lasts for 28 d.

Rainbow trout show very low susceptibility to EHNV infection in the natural environment. Cases of the infection have been reported most often in young stages with total mortality of up to 4% at water temperature of 11–20°C (18, 20). Similarly to the redfin perch, the incubation period after intraperitoneal inoculation is 3–10 d at 19–21°C compared with 14–32 d at 8–10°C (19). Reddacliff et al. (15) reported that adult infected rainbow trout did not show clinical signs until the day of death and the fish died 3–10 d after inoculation only at 19–21°C of water temperature. In contrast, adult infected redfin perch showed clinical signs and gross lesions, and these fish died 10–11 d after inoculation at 19–21°C and 10–28 d after inoculation at 12–18°C.

This study revealed the fact that fish raised in Polish climatic conditions may be infected with EHNV and that this virus could occur in aquacultures in Poland and the EU.

Increased production in the fishing industry, more international distribution of stocking material, and uncontrolled distribution of ornamental fish may be risk factors for the translocation of EHNV. Therefore, procedures which regulate the import of live ornamental fish to the EU should be reviewed. Furthermore, monitoring programmes should be carried out in EU countries to ascertain the real situation of iridovirus infections in the EU.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regard care and use of laboratory animals (protocol no. 76/2010).

Acknowledgements: The authors would wish to thank Professor Jerzy Antychowicz for his helpful comments on the manuscript and the staff from the Department of Pathology, National Veterinary Research Institute, for excellent technical support with histopathological examinations.

References


