

First Record of Infectious Haematopoietic Necrosis Virus in Rainbow Trout Fry in Croatia

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Abstract

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The paper describes the first diagnosis of infectious haematopoietic necrosis virus (IHNV) in Croatia. The viral causative agent was detected in pooled organ samples from the imported rainbow trout fry on the fish farm. Reverse transcriptase - semi-nested polymerase chain reaction (RT- snPCR) was applied directly on the infected tissue for rapid virus detection. After isolation on cell cultures, IHNV isolate was characterised on the basis of the 303 nt region of the glycoprotein gene (Mid-G) sequence. Phylogenetic comparison to North American and European IHNV isolates revealed that this Croatian isolate belongs to the M genogroup, confirming the prediction of the M genogroup origin of European IHNV isolates. The introduction of the virus presents a threat of further spreading of the disease in Croatia, as the infected farm is in a direct contact with the open waters.

Rainbow trout, IHNV, RT-snPCR, molecular epidemiology

Infectious haematopoietic necrosis (IHN) is a world-wide disease problem in farmed rainbow trout and other cultured salmonid species. The disease is caused by a fish rhabdovirus, infectious haematopoietic necrosis virus (IHNV), which belongs to the genus *Novirhabdovirus* (Fauquet et al. 2001). IHN is typically characterized as an acute viremia with resulting haemorrhage and necrosis of major organ systems, although infected fish may not show clinical signs. Juvenile fish are more susceptible to the disease, and their mortality can reach over 90%, especially in hatcheries where the disease breaks out for the first time (Bootland and Leong 2003).

The transmission of IHNV between fish is primarily horizontal, and the major cause of a wide geographical spreading of the virus is the movement of infected eggs and/or fry. IHNV was first identified in western North America, but soon spread to Japan and Europe. Although IHNV has been detected in neighbouring countries, for example Italy in 1987 (Bovo et al. 1987), and Slovenia in 1993 (Jenčič et al. 2002), there were no reported cases in Croatia previously (Oraić and Zrnčić 2005). Fast IHNV spreading and virus establishment among carrier fish require a rapid and reliable diagnostic method. Reverse transcriptase-polymerase chain reaction (RT- PCR), which detects specific parts of the IHNV genome, has been described (Winton and Einer-Jensen 2002) and approved by OIE as a valuable confirmation method for the IHNV diagnosis (OIE 2003). Further molecular characterisation, based on the sequence comparison of the glycoprotein (G) and non-virion (NV) genes from the different IHNV isolates proved to be useful in understanding and explanation of IHNV spreading, evolution and origin (Troyer et al. 2000; Kurath et al. 2003; Troyer and Kurath 2003; Garver et al. 2003). Phylogenetic analysis of partial G gene sequences (Mid-G, 303 nucleotide region coding antigenic determinants) from the North American IHNV isolates revealed 3 major virus genogroups. Those groups are designated U, M and L for the upper, middle and lower portions of IHNV

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Table 1. IHNV isolates from North America and Europe with accession numbers of sequences used to draft the phylogenetic tree

IHNV Isolate Name	Accession Number	Genogroup	Location	IHNV Isolate Name	Accession Number	Genogroup	Location
CRO1	DQ323507	M	Europe - Croatia	193110	L40871	M	North America
1F	X89213	M	Europe - France	FF3	AF237984	M	North America
332	AY331657	M	Europe - Germany	FF4	AF237985	M	North America
13	AY331658	M	Europe - Germany	FF177	AF237991	M	North America
28	AY331659	M	Europe - Germany	FF84	AF237988	M	North America
8	AY331660	M	Europe - Germany	FF10	AF237986	M	North America
832	AY331661	M	Europe - Germany	FF191	AF237992	M	North America
D30	AY331662	M	Europe - Germany	FF86	AF237989	M	North America
42*	AY331663	M	Europe - Germany	FF2	AF237983	M	North America
62*	AY331664	M	Europe - Germany	FF114	AF237990	M	North America
V1100	AY331666	M	Europe - Germany	HO7	L40876.1	M	North America
LR-73	L40877	U	North America	Hg003	AY331680	M	North America
AK12	AY639795	U	North America	S56	AY331677	M	North America
R24	AY513062	U	North America	WRAC	L40882	M	North America
A40**	AY102268	U	North America	CST	L40875	M	North America
BC206**	AY639796	U	North America	FF31	AF237987	M	North America
WAC4	AY639797	U	North America	FF053	AY331678	M	North America
RB	U50401	U	North America	Hg205	AY331679	M	North America
R7	AY513068	U	North America	Col85	L40874	L	North America
R6	AY513070	U	North America	Col80	L40873	L	North America
W19	AY102269	U	North America	SRCV	L40881	L	North America
LR80	L40878	M	North America				

* IHNV isolates 42 and 62 have identical Mid-G sequences and are denoted as V4262 in the phylogenetic tree

** IHNV isolates A40 and BC206 with identical Mid-G sequences present branch AK40BC in the phylogenetic tree

geographical range in North America (Troyer et al. 2000). In a recent study Enzmann et al. (2005) showed that European IHNV isolates belong to the M genogroup and hypothesized monophyletic origin of European IHNV from the North American IHNV genogroup M ancestor.

In the current work, data on the first detection of IHNV in Croatia are presented. The object of the study was to apply RT-PCR directly on the diseased fish tissue to prove infection with IHNV in cultured rainbow trout fry imported from the neighbouring country to Croatia. Sequence analysis of the Mid-G region of Croatian IHNV isolate have been made for further comparison with North American and European isolates.

Materials and Methods

Sample collection

Our laboratory carries out continuous fish health inspection (twice a year) of the fish farm of interest. Thirty rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) fry randomly selected from each of the two farm tanks, with average weights and condition factors: 35.33 g/1.32 and 21.83 g/1.09 were caught by netting. Fish were delivered to our laboratory immediately upon their import from the neighbouring country of Slovenia. Detailed clinical observation was done prior to sample collection and virological examination. As rainbow trout fry were exported from a Slovenian fish farm with previous IHNV history, fish were highly suspect of containing IHNV. This was the reason for directly applying the RT-PCR assay as an initial diagnostic method for rapid IHNV testing.

RNA isolation and RT-PCR

Pools of different organs (spleen, heart, kidney and brain) were collected from fry and divided into 2 sample groups: A and B. Samples A (organ pools from every single fry) were immediately frozen at -80 °C prior to RNA extraction with TRI reagent (MRC, USA) following the manufacturer's instructions. Single step RT-PCR (Access, Promega) was carried out for the amplification of the gene fragment encoding G-protein as described previously (Barlić-Maganja et al. 2002). Additional semi-nested reaction was performed with HotStartTaq polymerase (Qiagen) according to manufacturer's instructions. Amplified products of both reactions were analysed by 1.7% agarose gel electrophoresis. Samples B were proceeded for cell line testing.

Cells and virus isolation

EPC (*epithelioma papulosum cyprini*) cells (Fijan et al. 1983) were used for the virus replication and isolation.

Samples B (6-fry pooled samples from the two tanks) were stored at 4 °C in minimal essential medium (MEM, Imunološki zavod, Zagreb) containing penicillin (800 u.i./ml), streptomycin (800 µg/ml), and Fungizone® (400 u.i./ml) (Gibco, BRL) during the night. Inoculation on the monolayer of EPC cells was done according to OIE procedure (OIE 2003). The infected medium supernatant fluid (250 µl) was proceeded for RNA extraction, semi-nested RT-PCR and gel electrophoresis as was described for the samples A. Non-infected cells were used as a negative control, and the reference strain IHNV 4008 was included in the experiment as a positive control.

Sequence analyses

After virus isolation on EPC cells, extracted RNA was used for Mid-G region amplification. RT-PCR was done using single step RT-PCR system (Access, Promega) and nested PCR with HotStartTaq polymerase (Qiagen). Primers and reaction conditions were described previously (Troyer and Kurath 2003; Garver et al. 2003). Nested PCR products were purified by a QIAquick gel extraction kit (Qiagen) and sequenced by an "ABI PRISM® 3100-Avant Genetic Analyzer" DNA sequencer (Applied Biosystems). Sequencing was repeated for the sequence confirmation. Mid-G sequence of Croatian IHNV isolate (designated CRO1) is stored at the GenBank under the accession number DQ323507. ClustalX (Thompson et al. 1997) was used for Mid-G sequences alignment of CRO1 and the representative North American and European IHNV isolates available at GenBank (Table 1). The Sacramento River Chinook Virus (SRCV) isolate of IHNV was used as an outgroup root (Nichol et al. 1995). Phylogenetic analysis was performed using PAUP* 4.0 beta version (Swofford 2002). Parsimony (heuristic search with tree-bisection-reconnection (TBR) branch-swapping option), uncorrected p-distance and neighbour-joining trees were constructed. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates.

Results and Discussion

Clinical observation

Haemorrhagic areas as the characteristic clinical signs of IHN were found by external observation only on the gills of examined fish. Fins were ragged, but there were no macroscopic changes on the visceral organs. Increased fish mortality was not recorded.

RT-PCR detection and virus isolation on the cells

For a fast IHNV diagnosis, RT-semi-nested-PCR method (Barlič-Maganja et al. 2002) was applied directly on the fish tissue. Characteristic RT-PCR products for the first and the second (semi-nested) reactions were obtained (682 pb and 549 pb) from every frozen sample analyzed from both tanks at the Croatian farm (B1 and B2), Fig. 1 (Plate xy).

Molecular identification of IHNV proved to be rapid and reliable, which is important to prevent further spreading of the disease (OIE 2003; Miller et al. 1998). Although much progress has been made in the development of a vaccine against IHNV (Lorenzen and La Patra 2005; Sommerset et al. 2005; Garver et al. 2005), there is no adequate one at the market. Thus, prevention of exposure to the causative agent and a fast diagnostic method are the only ways to control the virus.

Inoculated samples caused CPE on EPC cell cultures after 6 (Tank 1) and 4 (Tank 2) days post-inoculation. Collected supernatants from infected EPC were processed in RT-PCR and semi-nested reactions for the confirmation and products of expected size were obtained (Plate II, Fig. 1, B1 and B2 on EPC).

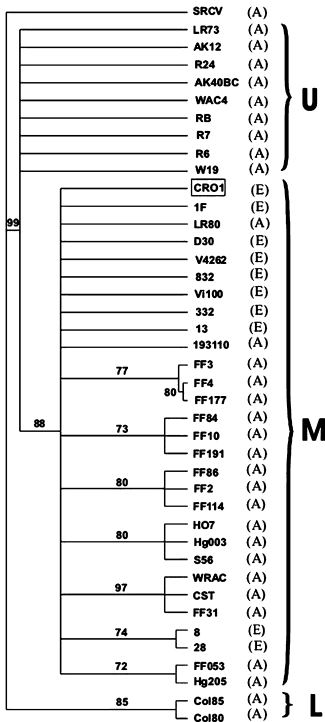


Fig. 2. Phylogenetic tree constructed from the Mid-G sequences of North American (A) and European (E) IHNV isolates using maximum parsimony method. Three genogroups denoted U, M and L for the upper, middle and lower portions of geographical distribution of IHNV in North America are shown. First Croatian isolate CRO1 is marked in the box. SRCV was used as an outgroup and nodes with bootstrap values < 70% were collapsed.

Sequence analysis

Nucleotide sequencing of Mid-G region of IHNV isolates from both tanks resulted in the identical sequence, accession No. DQ323507 at GenBank. We confirmed that CRO1 Mid-G is the most similar to the same region of European isolates, particularly to 1F, which represents first French IHNV outbreak (Baudin-Laurencin 1987; Enzmann et al. 2005). Nucleotide variation between CRO1 and F1 is 1.3% (4 nucleotide exchanges). CRO1 isolate varied from European isolates by 1.3 to 3.6% (4 to 11 nucleotide exchanges), and from North American isolates by 2 to 5.9% (6 to 18 nucleotide exchanges) in Mid-G region. Phylogenetic analysis of CRO1 and representative European and North American IHNV isolates revealed that CRO1 belongs to the M genogroup (Fig. 2). This result corresponds to the prediction that all European isolates belong to the M genogroup and originated from the M genogroup source. As IHNV in North America pre-dates its discovery in Europe, it is most likely that European IHNV originated from North America. Columbia River Basin with Hagerman Valley as an IHNV endemic focus in North America is of particular interest in searching for an M genogroup ancestor (Garver et al. 2003; Enzmann et al. 2005).

CRO1 Mid-G sequence fell on individual branch linked directly to the ancestral node of the M genogroup, like most of the other European isolates (Fig. 2), indicating no monophyletic origin. However, for detailed comparison of Croatian and European isolates, more comprehensive full G gene sequence analysis is necessary, because of short length and high similarity of Mid-G sequences between European IHNV isolates. EU regulations regarding fish transport in Europe are strict and the source of IHNV infection can be determined from the accompanying

health certificate. This data, together with molecular genotyping based on the sequence analysis should explain the mechanisms of virus spreading, epidemiology and evolution. The occurrence of IHNV in Croatia is a serious problem not just because of economic losses connected with a safe elimination of infected fish, but also due to the spreading of the virus. The first Croatian IHN outbreak has occurred in the farm that is directly linked to the open water of the river and represents a possible ecological threat in virus dissemination within wild freshwater fish populations. The results presented here provide a background for further investigations of the new IHNV outbreaks in Croatia.

První záchyt viru infekční hematopoetické nekrózy u plůdku pstruha duhového v Chorvatsku

Práce popisuje první záchyt viru infekční hematopoetické nekrózy (IHNV) v Chorvatsku. Původce byl detekován ve směsných vzorcích orgánů z importovaného plůdku pstruha duhového na rybí farmě. K rychlé detekci viru byla přímo v infikované tkáni provedena reverzní transkriptáza-semi-nested polymerázová řetězová reakce (RT-snPCR). Po izolaci na tkáňových kulturách byl izolát IHNV charakterizován na základě 303 nt oblasti sekvence glykoproteinového genu (Mid-G). Fylogenetickým srovnáním s izoláty HINV ze Severní Ameriky a Evropy bylo zjištěno, že tento chorvatský izolát patří do M genoskupiny. Potvrdil se předpoklad, že evropské izoláty IHNV pocházejí z M genoskupiny. Vzhledem k tomu, že zamořená farma je v přímém kontaktu s otevřenými vodami, představuje objevení viru hrozbu dalšího šíření onemocnění v Chorvatsku.

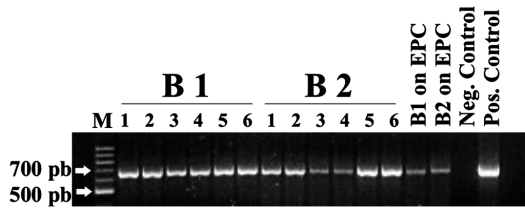
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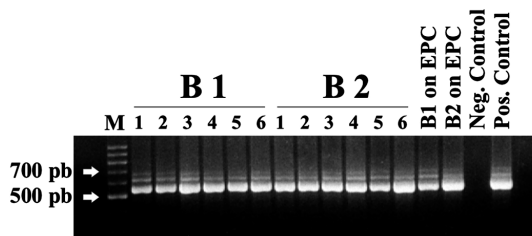
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(1)



(2)

Fig. 1. Agarose gel electrophoresis detection of IHNV in 6 frozen fish samples from tanks B1 and B2 (1-6) and in infected EPC cell lines by (1) RT-PCR and (2) semi-nested PCR. M – 100 bp DNA ladder.