

Isolation and Characterisation of a Variant Strain of Infectious Haematopoietic Necrosis (IHN) Virus

Dieter Fichtner¹, Sven Bergmann¹, Peter-Joachim Enzmann¹, Harald Granzow¹, Heike Schütze¹, Dieter Mock² and Johannes-Werner Schäfer²

¹ Federal Research Centre for Virus Diseases of Animals, Insel Riems and Tübingen, Germany.

² Landesanstalt für Ökologie, Fischgesundheitsdienst Nordrhein-Westfalen, Kirchhundem-Albaum, Germany.

Abstract

An unusual strain of IHNV was isolated after a clinical outbreak of the disease in rainbow trout fry. The causative agent tested negative for VHSV and IHNV by immunofluorescence performed by a regional diagnostic laboratory using commercially available monoclonal antibodies. The isolate, named DF04/99, was however identified as IHNV by EU-authorized diagnostic methods such as indirect immunofluorescence and virus neutralisation performed at the German National Reference Laboratory for Fish Diseases. Moreover, the virus was also identified as IHNV by immunoelectron microscopy and by reverse transcriptase-dependent polymerase chain reaction. This unusual isolate of IHNV differs in the amino acid sequence of the G- and NV-proteins from the common IHNV types RB, WRAC, and SRCV. A mutation in the G protein might be responsible for the missing reaction in immunofluorescence using standard commercially available MAbs. It is concluded that a continuous monitoring of the reaction pattern of MAbs with current isolates as well as the generation of new diagnostics which match the epizootiological situation are important tasks for a fast and reliable diagnosis of fish diseases.

Introduction

Viral Haemorrhagic Septicaemia (VHS) and Infectious Haematopoietic Necrosis (IHN) are diseases of high economic importance for fish farms of the member states of the European Union (EU). Assessment of the health status of fish stocks is based on inspection of fish production sites and subsequent laboratory examination of samples for the presence of VHS virus (VHSV) and IHN virus (IHNV) by authorised diagnostic methods according to the Fishing Epidemic Ordinance (anonymous,

1998) as well as the legislation of the EU (Anonymous, 1991, 1992, 1993).

In 1999 a fish farm producing rainbow trout fry suffered from a severe outbreak of a disease with signs characteristic of VHS and IHN. A cytopathogenic rhabdovirus which did not react with routinely used commercially available monoclonal antibodies (MAbs) against VHSV or IHNV was isolated by a regional diagnostic laboratory. This isolate was submitted for identification and further characterisation to the German National Reference Laboratory for Fish Diseases.

Virus strains	Isolated from	Origin	Reaction with anti-IHNV MAb Bio X in IFAT
IHNV K	<i>O. mykiss</i>	Round Butte Hatchery (RB), USA ¹	+
IHNV isolate 332	<i>O. mykiss</i>	Germany (Enzmann <i>et al.</i> , 1992)	+
IHNV isolate 4008	<i>O. mykiss</i>	Italy (Bovo <i>et al.</i> 1987) ²	+
IHNV SRCV	<i>O. tschawytscha</i>	Sacramento River Chinook Virus (SRCV), USA3	+
IHNV WRAC	<i>O. mykiss</i>	Western Regional Aquaculture Centre (WRAC), USA ³	+
IHNV RB-76	<i>O. mykiss</i> (Steelhead)	Round Butte Hatchery (RB), USA3	+
VHSV isolate Fi13	<i>O. mykiss</i>	Reference Laboratory Strain (Enzmann and Bruchhof, 1989)	-

Table 1. Reference strains and reaction with anti-IHNV MAb BIO X. ¹Obtained from P. de Kinkelin, Jouy-en-Josas, France. ²Obtained from N. J. Olesen, EU Reference Laboratory for fish Diseases, Aarhus, Denmark. ³Obtained from the "Marine Science Centre, Newport, Oregon, USA", for reference see Nichol *et al.*, 1995.

Materials and Methods

Cell cultures

The following cell lines were used for virus propagation: *Epithelioma papulosum cyprini* (EPC), fathead minnow (FHM), blue gill fry (BF-2) and rainbow trout gonad (RTG-2) (Fryer and Lannan, 1994). The cells were grown in 25 cm² cell culture flasks (Falcon, England) and in 24 or 96 well cell culture plates (Greiner, Germany; Costar, USA) at 20° and 26°C, respectively.

Viruses

Virus (registration number of German Reference Laboratory DF 04/99) was first isolated from homogenised organs (spleen, liver and kidney) of rainbow trout fry (6 pooled fish) at the beginning of clinical outbreak. Two months later a further isolate was obtained from brain of surviving rainbow trout. Virus strains and isolates used for reference are listed in Table 1.

Indirect immunofluorescence assay (IIFA)

The IIFA was performed according to the EC Commission Decision 96/240 (anonymous, 1992). Virus infected cells in 96 well cell culture plates (Costar, USA) were incubated at 15 °C for 48 h. Commercially available MAbs raised against VHSV and IHNV (BIO X, Belgium) and the anti-VHSV MAb ID8 from the German Reference Laboratory were diluted 1:20. The rabbit antisera F38 and F33 against VHSV and IHNV (Provided by EU-Reference Laboratory, Aarhus, Denmark) were diluted 1:50 and a rabbit hyperimmune serum raised against recombinant IHNV G protein (Schütze *et al.*, 1995) was used at a dilution of 1:800. The fluorescein-isothiocyanate labelled goat anti-mouse immunoglobulin conjugate (DAKO, Denmark) was diluted 1:100 and the fluorescein-isothiocyanate labelled goat anti-rabbit immunoglobulin serum (SIGMA, USA) 1:80.

Primer (-pair)	Used for	Sequence
VG1	RT	5' -ATGGAATGGAACACTTTTTTC-3'
IG1	RT	5' -ATGATCACCACTCCGCTCATT-3'
VG1-VD3	Diagnosis: 1. PCR-Reaction	5' -ATGGAATGGAACACTTTTTTC-3' 5' -TGTGATCATGGGTCCTGGTG-3'
IG1-ID3	Diagnosis: 1. PCR-Reaction	5' -ATGATCACCACTCCGCTCATT-3' 5' -GATTGGAGATTTTATCAACA-3'
VD5-VD3	nested-PCR	5' -TCCCGCTATCAGTCACCAG-3' 5' -TGTGATCATGGGTCCTGGTG-3'
ID4-ID3	nested-PCR	5' -CTCTGGACAAGCTCTCCAAGG-3' 5' -GATTGGAGATTTTATCAACA-3'
IG1-IG2	G-Gene Amplification	5' -ATGATCACCACTCCGCTCATT-3' 5' -CCGGTTTGCCAGGTGATACAT-3'
INV1-INV2	NV-Gene Amplification	5' -TGTATCACCTGGCAAACCGG-3' 5' -CAAGATCGAAGAAGTCCAT-3'

Table 2. Primers used for RT- and PCR-reactions. (Abbreviations: V: VHS; I: IHN; 1,4,5: sense primer; 2,3: antisense primer)

Virus neutralisation test (VNT)

The test was performed according to EC Commission Decision 96/240 (anonymous, 1992). The anti-VHSV serum F38 and the anti-IHNV serum F33 were diluted 1:25 and the polyclonal antibodies against the recombinant IHNV G protein was used at a working dilution of 1:50.

RT-PCR and sequence analysis

Total RNA was extracted from infected and uninfected cells by the use of the RNeasy kit as recommended by the supplier (Qiagen, Germany). Glycoprotein (G)- and Non-virion protein (NV)-genes from VHSV- and IHNV-strains and fragments thereof were amplified by RT-PCR using AMV Reverse Transcriptase (Promega, USA), and *Taq* Polymerase (Qiagen, Germany) as previously described (Miller et al., 1998). Primers used for reverse transcription and PCR are described in Table 2. Diagnostic tests were performed according to the protocols for RT-PCR demonstrated at the

“Workshop and Third Annual Meeting of EU National Reference Laboratories for Fish Diseases” (anonymous 1999a and b). PCR products were purified using Qiaquick Gel Extraction Kit (Qiagen, Germany). Cycle sequencing was done using the fluorescent dye deoxy-terminator cycle sequencing kit (Perkin Elmer). The products of the sequencing reaction were analysed by an ABI Prism DNA sequencer (Applied Biosystems).

Electron microscopy

Embedding of infected cell cultures for conventional electron microscopy was performed as previously described by Granzow et al.(1996). For immunoelectron microscopy (IEM) the monolayer cell cultures which showed a cytopathic effect were scraped off the plate and pelleted by low speed centrifugation. The resulting pellet was resuspended in 0.2ml PBS (Phosphat buffered saline, pH 7,2), and adsorbed to formvar coated 400 mesh copper grids, which had been carbon

Method	Diagnostics	Reaction
IIFT	anti IHNV mab BIO X	-
IIFT	anti-IHNV rabbit serum F33	+
IIFT	rabbit hyperimmune serum against recombinant IHNV G protein	+
VNT	anti-IHNV rabbit serum F33	+
VNT	rabbit hyperimmune serum against recombinant IHNV G protein	+
RT-PCR	IHNV primers (Table 2)	+
IEM	anti IHNV mab BIO X	-
IEM	anti-IHNV rabbit serum F33	+
IEM	rabbit hyperimmune serum against recombinant IHNV G protein	+

Table 3. Methods for investigation of isolate DF 04/99

stabilised and pretreated with glow discharge. They were then incubated with several antibodies, including the serum raised against recombinant IHNV-G-protein. After 5 washing steps the primary antibody was decorated by gold-tagged anti-species antibody (GAR₁₀ or GAM₁₀, British BioCell International, United Kingdom). Finally, the preparations were negatively stained with 2% phosphotungstic acid (PTA, pH 7.4) and examined with an electron microscope (EM 400 T, Philips, The Netherlands).

Animal experiments

Rainbow trout (mean weight about 10g) from a facility that are considered to be free of VHS and IHNV were used for experiments. A group of 20 fish was infected by immersion in 10⁴ TCID₅₀/ml of isolate DF04/99 in freshwater for 1 h. Thereafter the fish were held at 10°C. 19 non-infected fish were reared as control group under the same conditions. Virus reisolation from dead fish (organ pools of all dead fish each at day 7, 10, 11, 13, 14 and 15

post infection) and identification by IIFA was performed according to the EC Commission Decision 96/240 (anonymous, 1992).

Results

A cytopathogenic agent (DF04/99) was isolated after a clinical outbreak of disease in rainbow trout fry. The virus tested negative for VHSV and IHNV by immunofluorescence using commercial available anti-VHSV and -IHNV MAbs (BIO X). In the German Reference Laboratory the virus was however identified as IHNV by neutralisation, RT-PCR, immunoelectron microscopy and sequencing. In IIFA the virus could be identified as IHNV with the IHNV-antiserum F33 and with the polyclonal antiserum raised against recombinant G-protein of IHNV. The isolate DF04/99 was also neutralised by the same sera (Table 3).

Examination of DF04/99 by RT-PCR resulted in detection of the IHNV genome. By IEM with serum F33 labelled ribonucleoprotein

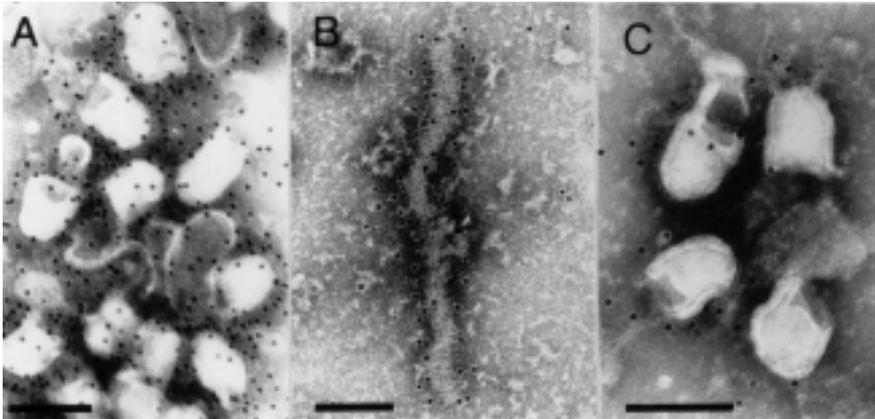


Figure 1. Immunogold labelling of the cell cultivated isolate DF04/99 with polyclonal anti-IHNV serum and anti-IHNV serum against recombinant glycoprotein G. Negative contrast with phosphotungstic acid, bar represents 150 nm. A) Virus particles labelled with polyclonal anti-IHNV serum F33. B) Rhabdovirus ribonucleoprotein aggregations labelled with the same serum. C) Labelling of virus particles with antibodies raised against recombinant IHNV glycoprotein.

structures as well as aggregations of virions could be detected (Figure 1 A, B). The IHNV G protein antiserum only labelled the envelope of virions (Figure 1 C). A sequence alignment with IHNV strain RB-76 as reference strain exhibited 11 amino acid changes in a fragment of the G-protein and 2 amino acid changes in the complete NV-protein. The amino acid sequence also differs from the strains SRCV and WRAC and from the German isolate "332" (Figures 2 and 3).

After experimental infection, strain DF04/99 induced a mortality of 100% in rainbow trout within 15 d p.i. (Figure 4). The fish showed external and internal signs of IHN. IHNV could be reisolated from pooled samples taken between post infection days 7 and 15 and identified by IIFA. The non-infected fish of control group did not show signs of disease.

Discussion

All IHNV isolates which were examined by the German Reference Laboratory since the first detection of IHNV in Germany in 1992 (Enzmann et al., 1992) and all tested reference strains reacted with the routinely used commercially available anti-IHNV MAb BIO X and reference sera (Table 1). DF04/99 is the first IHNV isolate which did not show reaction with this MAb. Identification of isolate DF04/99 as IHNV was performed by EU authorised diagnostic methods (anonymous, 1992) such as IIFT and VNT with polyclonal anti IHNV sera, including a rabbit hyperimmune serum raised against a recombinant IHNV G protein. The isolate was also identified as IHNV by RT-PCR and IEM (Table 3). The IHNV diagnosis was confirmed by the EU Reference Laboratory for Fish Diseases in Aarhus, Denmark.

An alignment of amino acid sequences deduced from nucleotide sequences from G- and NV-genes of the isolate DF04/99 (Figures 2

		10	20	30	40	50
RB	1	MITTPLILILITCGANSQTVKPD	TASESDQPTWSNPLFTYPEGCT	LDKLS		
WRAC	
SRCV		..I.....N.....
DF04/99	T.....H.....
RB	51	KVNASQLRCPRIFFDENRGLIAY	PTSIRSLSVGNLDGDIHTQGNH	IKVL		
WRAC	E.....
SRCV	A.....E.....
DF04/99	E.....
RB	101	YRTICSTGFFGGQTIKALVEMKL	STKEAGAYDTTTAAALYFPAPRC	QWY		
WRAC	
SRCV	
DF04/99	
RB	151	TDNVQNDLIFYTTQKSVLRDPY	TRDFLSDFIGGCKTKSPCQTH	SNVV		
WRAC	
SRCV	
DF04/99	
RB	201	WMGDAGIPACDSSQEIKGHLF	VDKISNRVVKATSYGHHPWGL	HRACMIEF		
WRAC	Q.....
SRCV	
DF04/99	Q.....
RB	251	CGKQWIRTDLGD LISVEYNSGAE	ILSFPKCEDKTVGMRGNLDD	FAYLDDL		
WRAC	
SRCV	
DF04/99	

Figure 2. Alignment of a fragment of the predicted G-proteins deduced from the IHNV RB, WRAC, SRCV, and DF04/99 gene sequences (Amino acids 1-300. Dots indicate identity to the RB sequence)

RB	1	MDHRDINTNM EALREVLRYK NKVAGHGFLF	DDGDLVWREE	DDATWRRLCD
WRAC	
SRCV		..P.....E.....G.....
INV332	I.....
DF04/99	G.....
RB	51	VVNALISSKR MQRVLYMDLS ITKGEHLLF	VDLQGTKNRL	HKEPRFRRLH
WRAC	
VSRVC	
INV332		..I.....
DF04/99	K.....
RB	101	ILIEDFLAYP R*		
WRAC	*
SRCV	L.....*
INV332	*
DF04/99	*

Figure 3. Alignment of the predicted NV-proteins deduced from the IHNV RB, WRAC, SRCV, 332 and DF04/99 gene sequences (Dots indicate identity to the RB sequence).

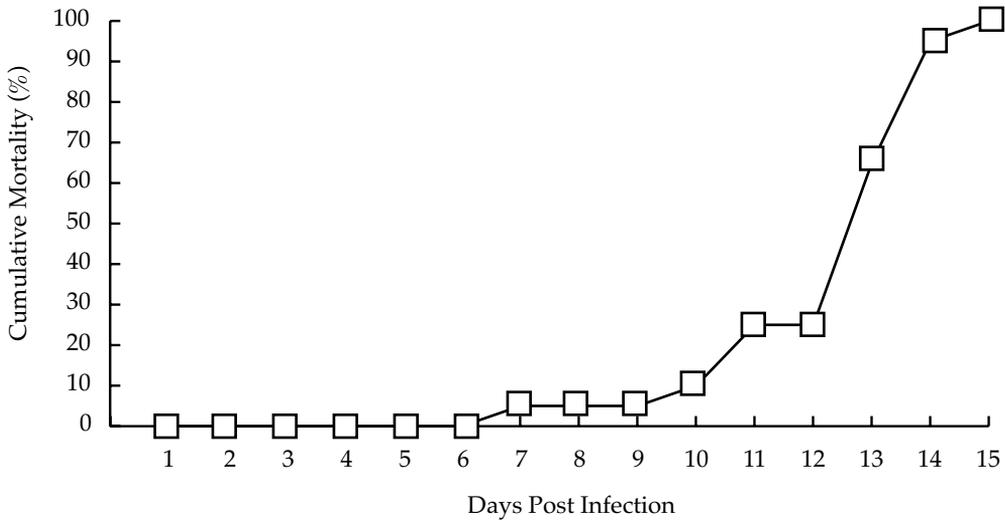


Figure 4. Cumulative Mortality of rainbow trout experimentally infected with isolate DF04/99. No mortality was observed in the control group.

and 3) shows that this unusual strain of IHNV differs from most common IHNV strains such as RB, WRAC, and SRCV. In the G-protein fragment (aa 1 – 300) analysed here, DF04/99 can be distinguished by 8 changes from all other strains examined. These changes might be responsible for the lack of reactivity in immunofluorescence with standard MAb (Table 3) directed against the glycoprotein G of IHNV. In addition, two mutations in the complete NV protein sequence were found compared with other IHNV strains including the reference strain of all hitherto in Germany isolated strains (strain 332). The virus revealed to be highly pathogenic in experimental infection (Figure 4).

In summary, we describe here for the first time an IHNV isolate which does not react in commonly used diagnostics. Similarly, a new variant of VHSV, designated as type "Wi", was isolated in Germany in 1994 which could not be diagnosed by commercially available MAb

(Fichtner et al., 1998). Therefore, it is concluded that a continuous analysis of the reaction pattern of MAbs with current isolates as well as the generation of new diagnostics which match the epizootiological situation are important tasks for a fast and reliable diagnosis of fish diseases.

References

Anonymous (1991) Council Directive 91/67/EEC of 28 January 1991 conceding the animal health conditions governing the placing on the market of aquaculture animals and products. OJ No L 46, 19.02.1991, p. 1, as last amended by directive 95/22/EC of 22 June 1995. OJ No L 243, 11.10.1995, p. 1.

Anonymous (1992) Commission Decision 92/532/EEG of 19 November 1992 laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases. OJ No L 337, 21 November 1992, p. 18, as last amended by Decision 96/240/EC of 5 February 1996. OJ No L 79, 29.3.1996, p. 19.

Anonymous (1993) Commission Directive 93/53/EEC of 24 June 1993 introducing minimum Community measures for the control of certain fish diseases. OJ No L 175, 19.7.1993, p.23.

Anonymous (1998) Verordnung zum Schutz gegen Süßwasserfisch-Seuchen, Muschelkrankheiten und zur Schaffung seuchenfreier Fischhaltungsbetriebe und Gebiete (Fischseuchen-Verordnung) vom 19. August 1998 (BGBl. I S. 2176).

Anonymous (1999a) 3. Riemser Seminar zur Diagnose virusbedingter Krankheiten der Fische, Insel Riems, Germany, 18.-19. Mai 1999, Seminarunterlagen.

Anonymous (1999b) Workshop and Third Annual Meeting of EU National Reference Laboratory for Fish Diseases, Aarhus, Denmark, June 9-11, 1999, Report.

Bovo, G., Giorgetti, G., Jørgensen, P. E. V. and Olesen, N. J. (1987) Infectious Haematopoietic Necrosis. First detection in Italy. Bull. Eur. Ass. Fish Path. 7: 117.

Enzmann P.-J. and B. Bruchhof (1989) Comparative studies on viral haemorrhagic septicaemia viruses and infectious haematopoietic necrosis virus. An attempt to demonstrate an immunological relationship. In: Fish Health Protection Strategies (Lillelund, K. and Rosenthal, H., Eds.) S. 107-120. Bundesministerium für Forschung und Technologie, Bonn.

Enzmann, P.-J., Dangschat, H., Feneis, B., Schmitt, D., Wizigmann, G. and Schlotfeldt, H.-J. (1992) Demonstration of IHN virus in Germany. Bull. Eur. Ass. Fish Pathol, 12: 185-188.

Fichtner, D., Bergmann, S., Enzmann, P.-J. and Granzow, H. (1998) Characterisation of Viral Haemorrhagic Septicaemia (VHS) virus-isolates. Bull. Eur. Ass. Fish. Pathol., 18: 56-61.

Fryer, J.L. and Lannan, C.N. (1994) Three decades of fish cell culture: a current listing of cell lines derived from fishes. Journal of Tissue Culture Methods 16, 87-94.

Granzow, H., Weiland, F., Fichtner, D. and Enzmann, P.-J. (1996): Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. J. Fish Dis., 20: 1-10.

Miller, T.A., Rapp, J., Wastlhuber, U., Hoffmann, R.W. and Enzmann, P.-J. (1998) Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. Dis. Aquat. Org. 34: 13-20.

Nichol, S.T., Rowe, J.E. and Winton, J.R. (1995) Molecular epizootiology and evolution of the glycoprotein and non-virion protein genes of infectious hematopoietic necrosis virus, a fish rhabdovirus. Virus Research 38: 159-173.

Schütze, H., Enzmann, P.-J., Kuchling, R., Mundt, E., Niemann, H and Mettenleiter, T.C. (1995): Complete genomic sequence of the fish rhabdovirus infectious haematopoietic necrosis virus. J. Gen. Virol. 76: 2519-2527.